Molecular and Morphological Assessment of *Septoria* Species Associated with Ornamental Plants in Yunnan Province, China

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Abstract: The Karst landform is the main geographic characteristic in South China. Such areas are rich in vegetation and especially suitable for growth of shrubs and herbaceous plants. In this study, 11 *Septoria* strains were obtained from different plants’ leaves collected in the Kunming Botanical Garden, Yunnan Province, China. Based on single-gene and multi-gene analyses of five gene loci (*lefl*, *rpb2*, *tub2*, ITS, and *LSU*) and four gene regions (without *LSU*), these strains were found to belong to three independent phylogenetic lineages representing five species, including four novel taxa, and one new record for China. Five single gene trees were also provided to evaluate the effectiveness of each gene for discriminating the species, as a result of which *tub2* was found to have the most suitable DNA barcode for rapid identification. Morphological descriptions, illustrations, and comparisons are provided for a more comprehensive assessment. Genealogical Concordance Phylogenetic Species Recognition (GCPSR) with a pairwise homoplasy index (PHI) test was used to evaluate the conclusions of the phylogenetic analyses.

Keywords: GCPSR; molecular assessment; new taxa; *Septoria*

1. Introduction

*Septoria* Sacc., established by Saccardo in 1884, belongs to the Mycosphaerellaceae family of fungi and accommodates around 1000 species [1,2], although only 200 species have been confirmed by molecular data [2]. Many of these species cause leaf spot diseases of numerous cultivated and wild plants [3]. According to its morphology at the primary generic level, *Septoria* includes coelomycetous asexual morphs, which produce pycnidial conidiomata having holoblastic, hyaline, smooth, filiform-to-cylindrical multi-septate conidia [4–9]. On the basis of a polyphasic approach to taxon delimitation, Verkley et al. [3] pointed out that septoria-like fungi preserved in CBS were in fact distributed over three main clades and introduced a novel genus: *Caryophylloseptoria* Verkley, Quaedvlieg and Crous. Quaedvlieg et al. [10] re-defined *Septoria* as having pycnidial to acervular conidiomata and hyaline conidiophores that give rise to conidiogenous cells that proliferate both sympodially and percurrently to form hyaline, filiform conidia with transverse eusepta. Crous et al. [11] introduced *Aceruleoseptoria* on account of its black, erumpent conidiomata, and the old name *Septoria capensis* G. Winter was transferred to this genus [12]. More DNA sequence data are necessary to support the morphological characters in this species identification [10].

In this study, 11 *Septoria* strains were obtained from different ornamental plants in a South China Karst region. Morphological comparisons, phylogenetic analyses based...
on five gene loci, DNA base-pair differences, and GCPSR evaluation confirmed that they formed three phylogenetic lineages representing five *Septoria* species comprising four novel species and one new Chinese record.

### 2. Materials and Methods

#### 2.1. Fungus Collection and Isolation

The isolates included in this study were collected from the Kunming Botanical Garden, Yunnan Province, China, in 2018. Pure cultures were obtained by single-spore isolations following the methods of surface sterilization and incubation of specimens [13]. After 24 h of incubation, germinated conidia were transferred to the new potato-dextrose agar (PDA) medium and incubated at 25 °C. The holotype specimens were deposited in the Herbarium of the Department of Plant Pathology, Agricultural College, Guizhou University (HGUP). The type cultures were deposited in the Culture Collection at the Department of Plant Pathology, Agriculture College, Guizhou University, China (GUCC), and the Mae Fah Luang University Culture Collection (MFLUCC) in Thailand (Table 1).

| Species                | Isolate No. | GenBank Accession No. |
|------------------------|-------------|-----------------------|
|                         | tef1 | tab2 | rpb2 | LSU | ITS |
| Cercospora beticola    | CBS 124.31 | KF253246 | KF252780 | KF252304 | KF251802 | KF251298 |
| Septoria aegopodina    | CBS 123741 | KF253282 | KF252807 | – | KF251838 | KF251334 |
| S. anthrisci           | CBS 109020 | KF253286 | KF252811 | KF252340 | KF251843 | KF251339 |
| S. anthurii            | CBS 346.58 | KF253288 | KF252813 | KF252342 | KF251845 | KF251341 |
| S. apiicola            | CBS 400.54 | KF253292 | KF252817 | KF252346 | KF251849 | KF251345 |
| S. astericola          | CBS 128593 | KF253294 | KF252819 | KF252348 | KF251851 | KF251347 |
| S. astragali           | CBS 109116 | KF253298 | KF252823 | KF252352 | KF251855 | KF251351 |
| S. atropurpurea        | CBS 348.58 | KF253299 | KF252824 | KF252353 | KF251856 | KF251352 |
| S. bothriospermi       | CBS 128599 | KF253301 | KF252826 | KF252355 | KF251858 | KF251354 |
| S. bupleuricola        | CBS 128603 | KF253303 | KF252828 | KF252357 | KF251860 | KF251356 |
| S. calendulae          | CBS 349.58 | KF253304 | KF252829 | KF252358 | KF251861 | KF251357 |
| S. callistephi         | CBS 128590 | KF253305 | KF252830 | KF252359 | KF251862 | KF251358 |
| S. campanulare         | CBS 128604 | KF253308 | KF252833 | KF252362 | KF251865 | KF251361 |
| S. carvi               | KML 1833  | –     | –     | –     | –     | KX453687 |
| S. cerastii            | CBS 128612 | KF253311 | KF252836 | KF252365 | KF251868 | KF251364 |
| S. cf. agrimoniicola   | CBS 128602 | KF253284 | KF252809 | KF252338 | KF251841 | KF251337 |
| S. cf. rubi            | CBS 128646 | KF253314 | KF252839 | KF252368 | KF251871 | KF251367 |
| S. cf. sonchi          | CBS 128757 | KF253500 | KF253020 | KF252546 | KF252057 | KF251552 |
| S. cf. stachydicola    | CBS 128662 | KF253513 | KF253034 | KF252599 | KF252071 | KF251566 |
| S. chamaecisti         | CBS 350.58 | KF253318 | KF252843 | KF252372 | KF251875 | KF251371 |
| S. chelidonii          | CBS 128607 | KF253319 | KF252844 | KF252373 | KF251876 | KF251372 |
| S. chromolaena         | CBS 113373| KF253321 | KF252846 | KF252375 | KF251878 | KF251374 |
| S. chrysanthemella     | CBS 128716 | KF253325 | KF252850 | KF252379 | KF251882 | KF251378 |
| S. cirsi              | CBS 128621 | KF253328 | KF252853 | KF252382 | KF251885 | KF251381 |
| S. citri               | CBS 315.37 | KF253465 | –     | KF252511 | KF252021 | KF251516 |
| S. citricola           | CBS 356.36| KF253329 | KF252854 | KF252383 | KF251886 | KF251382 |
| S. clematidis          | CBS 108983 | KF253330 | KF252855 | KF252384 | KF251887 | KF251383 |
| Species                  | Isolate No. | GenBank Accession No. |
|-------------------------|-------------|-----------------------|
|                         |             | tef1                  |
|                         |             | tub2                  |
|                         |             | rpb2                  |
|                         |             | LSU                   |
|                         |             | ITS                   |
|                         |             |                      |
| S. codonopsidis         | CBS 128620  | KF253333              |
|                         |             | KF252858              |
|                         |             | KF252387              |
|                         |             | KF251890              |
|                         |             | KF251386              |
| S. convolvuli           | CBS 128627  | KF253336              |
|                         |             | KF252861              |
|                         |             | KF252390              |
|                         |             | KF251893              |
|                         |             | KF251389              |
| S. coprosmae            | CBS 113391  | KF253255              |
|                         |             | KF252787              |
|                         |             | KF252313              |
|                         |             | KF251812              |
|                         |             | KF251308              |
| S. crepis              | CBS 128619  | KF253338              |
|                         |             | KF252863              |
|                         |             | KF252392              |
|                         |             | KF251895              |
|                         |             | KF251391              |
| S. cretae              | CBS 135095 T| –                     |
|                         |             | KF252720              |
|                         |             | –                     |
|                         |             | KF251736              |
|                         |             | KF251233              |
| S. cruciatum            | CBS 123747  | KF253340              |
|                         |             | KF252865              |
|                         |             | KF252394              |
|                         |             | KF251897              |
|                         |             | KF251393              |
| S. cucubali             | CBS 102386  | KF253344              |
|                         |             | KF252869              |
|                         |             | KF252398              |
|                         |             | KF251901              |
|                         |             | KF251397              |
| S. cucurbitearum        | CBS 178.77  | KF253346              |
|                         |             | –                     |
|                         |             | KF252400              |
|                         |             | KF251903              |
|                         |             | KF251399              |
| S. dearnessii           | CBS 128624  | KF253347              |
|                         |             | KF252871              |
|                         |             | KF252401              |
|                         |             | KF251904              |
|                         |             | KF251400              |
| S. digitalis            | CBS 391.63  | KF253349              |
|                         |             | KF252873              |
|                         |             | KF252403              |
|                         |             | KF251906              |
|                         |             | KF251402              |
| S. dispori              | GUCC 2127.1 T| MT996515              |
|                         |             | MT984348              |
|                         |             | MT993632              |
|                         |             | MT985366              |
|                         |             | MT974584              |
| S. dispori              | GUCC 2164.3 | MT996523              |
|                         |             | MT984357              |
|                         |             | MT993641              |
|                         |             | MT985375              |
|                         |             | MT974593              |
| S. dispori              | GUCC 2164.4 | MT996524              |
|                         |             | MT984358              |
|                         |             | MT993642              |
|                         |             | MT985376              |
|                         |             | MT974594              |
| S. dispori              | GUCC 2127.4 | MT996517              |
|                         |             | MT984350              |
|                         |             | MT993634              |
|                         |             | MT985368              |
|                         |             | MT974586              |
| S. dolichospora         | CBS 129152  | KF253350              |
|                         |             | KF252874              |
|                         |             | –                     |
|                         |             | KF251907              |
|                         |             | KF251403              |
| S. dysentericae         | CBS 131892  | KF253353              |
|                         |             | KF252877              |
|                         |             | KF252406              |
|                         |             | KF251910              |
|                         |             | KF251406              |
| S. eckmanii             | CBS 113612  | KF253355              |
|                         |             | KF252879              |
|                         |             | –                     |
|                         |             | KF251912              |
|                         |             | KF251408              |
| S. epambrosiae          | CBS 128629  | KF253356              |
|                         |             | KF252880              |
|                         |             | KF252407              |
|                         |             | KF251913              |
|                         |             | KF251409              |
| S. epilobii             | CBS 109084 T| KF253358              |
|                         |             | KF252882              |
|                         |             | KF252409              |
|                         |             | KF251915              |
|                         |             | KF251411              |
| S. erigerontis          | CBS 109094  | KF253360              |
|                         |             | KF252884              |
|                         |             | KF252411              |
|                         |             | KF251917              |
|                         |             | KF251413              |
| S. eucalyptorum         | CBS 118505 T| KF253365              |
|                         |             | KF252889              |
|                         |             | KF252415              |
|                         |             | KF251921              |
|                         |             | KF251417              |
| S. exotica              | CBS 163.78  | KF253366              |
|                         |             | KF252890              |
|                         |             | KF252416              |
|                         |             | KF251922              |
|                         |             | KF251418              |
| S. galeopsidis          | CBS 102411 T| KF253372              |
|                         |             | KF252896              |
|                         |             | KF252422              |
|                         |             | KF251928              |
|                         |             | KF251424              |
| S. gentianae            | CBS 128633  | KF253374              |
|                         |             | KF252898              |
|                         |             | KF252424              |
|                         |             | KF251930              |
|                         |             | KF251426              |
| S. gerberae             | CBS 410.61  | KF253468              |
|                         |             | KF252988              |
|                         |             | KF252514              |
|                         |             | KF252024              |
|                         |             | KF251519              |
| S. glycinicola          | CBS 336.53  | KF253377              |
|                         |             | KF252901              |
|                         |             | –                     |
|                         |             | KF251933              |
|                         |             | KF251429              |
| S. limonum              | CBS 419.51  | KF253407              |
|                         |             | KF252931              |
|                         |             | KF252456              |
|                         |             | KF251963              |
|                         |             | KF251459              |
| S. linicola             | CBS 316.37  | KF253408              |
|                         |             | KF252932              |
|                         |             | KF252457              |
|                         |             | KF251964              |
|                         |             | KF251460              |
| Species          | Isolate No. | GenBank Accession No. |
|------------------|-------------|-----------------------|
|                  | tef1        | tub2                  | rpb2 | LSU | ITS |
| *S. lobeliae*    | CBS 113392  | KF253460              | KF252981 | KF252507 | KF252016 | KF251511 |
| *S. longipes*    | GUCC 2131.1 | –                     | MT984351 | MT993635 | MT983569 | MT974587 |
| *S. lycocotoni*  | CBS 109089  | KF253409              | KF252933 | KF252458 | KF251965 | KF251461 |
| *S. lycopersici* | CBS 128654  | KF253410              | KF252934 | KF252459 | KF251966 | KF251462 |
| *S. lycopicola*  | CBS 128651  | KF253412              | KF252936 | KF252461 | KF251968 | KF251464 |
| *S. lysimachiae* | CBS 102315  | KF253413              | KF252937 | KF252462 | KF251969 | KF251465 |
| *S. malagutii*   | CBS 106.80  | KF253418              | –     | KF252467 | KF251974 | KF251470 |
| *S. matriariae*  | CBS 109001  | KF253420              | KF252943 | KF252469 | KF251976 | KF251472 |
| *S. mazi*        | CBS 128755  | KF253422              | KF252945 | KF252471 | KF251978 | KF251474 |
| *S. melissae*    | CBS 109097  | KF253423              | KF252946 | KF252472 | KF251979 | KF251475 |
| *S. menthae*     | CBS 404.34  | KF253424              | KF252947 | –      | KF251980 | KF251476 |
| *S. napelli*     | CBS 109105  | KF253426              | KF252949 | KF252474 | KF251982 | KF251478 |
| *S. obesa*       | CBS 128623  | KF253429              | KF252952 | KF252477 | KF251985 | KF251481 |
| *S. oenanthicola*| CBS 128649  | KF253433              | KF252954 | KF252239 | KF251737 | KF251234 |
| *S. oenanthis*   | CBS 128667  | KF253432              | KF252953 | –      | KF251989 | KF251485 |
| *S. orchidearium*| CBS 128631  | KF253434              | KF252955 | KF252482 | KF251990 | KF251486 |
| *S. pachyspora*  | CBS 128652  | KF253437              | KF252958 | KF252485 | KF251993 | KF251488 |
| *S. paridis*     | CBS 109111  | KF253438              | KF252959 | KF252486 | KF251994 | KF251489 |
| *S. passiflorica*| CBS 102701  | KF253442              | KF252963 | KF252490 | KF251998 | KF251493 |
| *S. perillae*    | CBS 128655  | KF253444              | KF252965 | KF252491 | KF252000 | KF251495 |
| *S. petroselini* | CBS 182.44  | KF253446              | KF252967 | KF252493 | KF252002 | KF251497 |
| *S. phlogis*     | CBS 128663  | KF253448              | KF252969 | KF252495 | KF252004 | KF251499 |
| *S. pileicola*   | GUCC 2131.3 | MT996519              | MT984353 | MT993637 | MT985371 | MT974589 |
| *S. pileicola*   | GUCC 2131.4 | MT996520              | MT984354 | MT993638 | MT985372 | MT974590 |
| *S. polygonorum* | CBS 109834  | KF253453              | KF252974 | KF252500 | KF252009 | KF251504 |
| *S. posoniensis* | CBS 128645  | KF253456              | KF252977 | KF252503 | KF252012 | KF251507 |
| *S. protearum*   | CBS 778.97  | KF253472              | KF252992 | KF252517 | KF252028 | KF251523 |
| *S. protearum*   | GUCC 2127.3 | MT996516              | MT984349 | MT993633 | MT985367 | MT974585 |
| *S. pseudonapelli*| CBS 128664  | KF253475              | KF252995 | KF252520 | KF252031 | KF251526 |
| *S. putrida*     | CBS 109088  | KF253477              | KF252997 | KF252522 | KF252033 | KF251528 |
| *S. rumicum*     | CBS 503.76  | KF253478              | KF252998 | KF252523 | KF252034 | KF251529 |
| *S. saccardoi*   | CBS 128756  | KF253479              | KF252999 | KF252524 | KF252035 | KF251530 |
| *S. sanguisorbigena* | GUCC 2131.2 | MT996518              | MT984352 | MT993636 | MT985370 | MT974588 |
| *S. sanguisorbigena* | GUCC 2164.1 | MT996521              | MT984355 | MT993639 | MT985373 | MT974591 |
| *S. sanguisorbigena* | GUCC 2164.2 | MT996522              | MT984356 | MT993640 | MT985374 | MT974592 |
| *S. scabiosicola*| CBS 109093  | KF253487              | KF253007 | KF252532 | KF252043 | KF251538 |
| *S. seneciosis*  | CBS 102366  | KF253492              | KF253012 | KF252538 | KF252049 | KF251544 |
| *S. siegesbeckiae* | CBS 128659  | KF253494              | KF253014 | KF252540 | KF252051 | KF251546 |
| *S. sii*         | CBS 102370  | KF253497              | KF253017 | KF252543 | KF252054 | KF251549 |
| *S. sisyrrchii*  | CBS 112096  | KF253499              | KF253019 | KF252545 | KF252056 | KF251551 |
Table 1. Cont.

| Species             | Isolate No. | GenBank Accession No. | tef1    | tub2    | rpb2    | LSU     | ITS     |
|---------------------|-------------|-----------------------|---------|---------|---------|---------|---------|
| S. stachydicola     | CBS 128668  | KF253512              | KF253033| KF252558| KF252070| KF251565|
| S. stachydidis      | CBS 109127  | KF253517              | KF253038| KF252563| KF252075| KF251570|
| S. stellariae       | CBS 102376  | KF253521              | KF253042| KF252567| KF252079| KF251574|
| S. taraxaci         | CBS 567.75  | KF253524              | KF253045| KF252570| KF252082| KF251577|
| S. tinctoriae       | CBS 129154  | KF253525              | KF253046| KF252571| KF252083| KF251578|
| S. tormentillae     | CBS 128647  | KF253527              | KF253048| KF252573| KF252085| KF251580|
| S. urticae          | CBS 102375\textsuperscript{T} | KF253530          | KF253051| KF252576| KF252088| KF251583|
| S. verbasciicola    | CBS 102401  | KF253531              | KF253052| KF252577| KF252089| KF251584|
| S. verbena          | CBS 113438  | KF253532              | KF253053| KF252578| KF252090| KF251585|
| S. villarsiae       | CBS 514.78  | KF253534              | KF253055| KF252580| KF252092| KF251587|
| S. violae-palustris | CBS 128644  | KF253537              | KF253058| KF252583| KF252095| KF251590|

Ex-type isolates are labeled with "\textsuperscript{T}".

2.2. Morphological Studies

Morphological characters were recorded from cultures that had been incubated for 2 to 3 weeks. For light microscopy, the relevant structures were mounted in Shear’s liquid, distilled water or lactic acid and examined with an Olympus BX53 microscope. Measurements of 30 conidia and other structures were made at a magnification of 1000× [14]. Taxonomic information of the new taxa was submitted to the MycoBank database (www.mycobank.org, accessed on 24 March 2021).

2.3. DNA Extraction, Amplification (PCR), and Sequencing

Methods outlined in [15] were followed for DNA extraction, amplification (PCR), sequencing, and phylogenetic analysis. Fresh fungal mycelia of strains were harvested using a sterile scalpel, and the genomic DNA was isolated using A BIOMIGA Fungus Genomic DNA Extraction Kit (GD2416) according to the manufacturer’s protocol. The DNA was amplified in a 25 \( \mu \)L reaction volume containing 2.5 \( \mu \)L 10\times PCR buffer, 1 \( \mu \)L of each primer (10 \( \mu \)M), 1 \( \mu \)L template DNA, 0.25 \( \mu \)L Taq DNA polymerase (Promega, Madison, WI, USA), and 18.5 \( \mu \)L ddH\textsubscript{2}O. Five gene regions—loci \( \beta \)-tubulin (\( \text{tub2} \)), internal transcribed spacer (ITS), Translation elongation factor 1-alpha (\( \text{tef1} \)), 28S nrDNA (\( \text{LSU} \)), and RNA polymerase II second largest subunit (\( \text{rpb2} \))—were targeted for Polymerase Chain Reaction (PCR) amplification and subsequent sequencing. The primers used and amplification conditions of the genes are listed in Table 2. The DNA sequences were submitted to GenBank and their accession numbers are provided in Table 1. The generated sequences for each locus and the reference sequences of ex-type or representative sequences of Septoria species downloaded from GenBank (Table 1) were aligned with the online version of MAFFT v. 7,307 [16,17].
Table 2. Primers, primer sequences, and thermal cycling program for PCR amplification.

| Locus | Primer | Primer Sequence 5’ to 3’                        | Annealing Temperature (°C) | Direction | Reference |
|-------|--------|--------------------------------------------------|-----------------------------|-----------|-----------|
| tef1  | EF-728F| CATCGAGAAGTTCCGAGAAGG                           | 52                          | Forward   | [18]      |
|       | EF-2   | GGAATACACGATSATCATGTT                           |                             | Reverse   | [19]      |
| tub2  | T1     | AACATGCAGGTGAGATGTAAGT                         | 52                          | Forward   | [20]      |
|       | β-Sandy-R | GCRCNGVGACRATCTTTGTT                 |                             | Reverse   | [21]      |
| rpb2  | tRPB2-5F| GAYGAYMGWGATCAYTYGG                            | 49                          | Forward   | [22]      |
|       | tRPB2-414R | ACMANNCCCGARTGNGWRTTRG                  |                             | Reverse   | [23]      |
| LSU   | LSU1Fd | GRATCAGGTAGGRATACCCG                          | 52                          | Forward   | [24]      |
|       | LR5    | TCCTGAGGCAACTTCG                               |                             | Reverse   | [25]      |
| ITS   | IT55   | GGAAGTAAAAGTCTGGAACAGG                         | 52                          | Forward   | [26]      |
|       | ITS4   | TCCTGCCTATTGATATG                             |                             | Reverse   | [26]      |

2.4. Phylogenetic Analyses

The alignments were checked and manually improved where necessary using MEGA v. 5 [27]. Phylogenetic analyses were performed by maximum parsimony (MP), maximum likelihood (ML), and Bayesian methods for individual and combined locus datasets. Ambiguous regions were excluded from the analyses and gaps were treated as missing data. Maximum parsimony analysis was performed in PAUP v. 4.0b10 [28] using the heuristic search option with 100 random taxon additions and tree bisection and re-connection (TBR) as the branch-swapping algorithm with Maxtrees = 5000. Branches of zero length were collapsed and all multiple, and equally most parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1000 bootstrap replications [29]. Other measures calculated included tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC).

The resulting PHYLIP file was used to generate the ML tree on the CIPRES Science Gateway [30] using RAxML-HPC2 black box with 1000 bootstrap replicates and GTRGAMMA as the nucleotide substitution model. Bayesian analyses were launched with random starting trees for 10,000,000 generations. The heat parameter was set at 0.15 and trees were saved every 1000 generations until the average standard deviation of split frequencies reached 0.01 (stop value). Burn-in was set to 25% after which the likelihood values were considered to be stationary. All resulting trees were visualized with FigTree v. 1.4.3 (Institute of Evolutionary Biology, University of Edinburgh, UK) [31].

2.5. Genealogical Concordance Phylogenetic Species Recognition Analysis

The Genealogical Concordance Phylogenetic Species Recognition (GCPSR) concept with a pairwise homoplasy index (PHI) test was used to analyze the new species, their species boundaries, and their most closely related taxa as described by Quaedvlieg et al. [32]. The recombination level within phylogenetically closely related species was determined with the PHI test performed using SplitsTree4 [33,34]. The concatenated datasets (tef1, rpb2, tub2, ITS, and LSU) were used. The relationships between different taxa were visualized in splits graphs with both the Log-Det transformation and splits decomposition options. A pairwise homoplasy index below a 0.05 threshold (Fw < 0.05) indicated the presence of significant recombination in the dataset.

3. Results

3.1. Phylogenetic Analyses

Eleven Septoria strains isolated from different plant hosts were sequenced. PCR products of 450–536 bp (tef1), 440–453 bp (tub2), 458–524 bp (ITS), 799–863 bp (LSU), and 718–1083 bp (rpb2) were obtained. By alignment with the single-gene region and
then in combination in the order of tef1, rpb2, tub2, ITS, and LSU with Cercospora beticola (CBS 124.31), 2434 characters were obtained: tef1, 1–479; rpb2, 480–824; tub2, 825–1149; ITS, 1159–163; and LSU, 1636–2434. Among these characters, 1672 were constant, while 195 variable characters were parsimony-uninformative and 567 were parsimony informative. The parameters of the MP phylogenetic trees are shown in Table 3, and the procedure yielded a single most parsimonious tree (Figure 1). Similar topologies were obtained by MP, ML, and Bayesian methods. In the Septoria phylogenetic tree (Figure 1), all Septoria isolates were grouped together, but only the BI support was high (BPP = 1), while the three major clades received greater statistical support (Branch 1: ML/BI = 98%/0.99; Branches 2: MP/ML/BI = 88%/87%/0.99; Branch 3: MP/ML/BI = 88%/80%/1.00). Six strains (GUCC 2131.1, GUCC 2131.2, GUCC 2131.3, GUCC 2131.4, GUCC 2164.1, and GUCC 2164.2) were grouped in the clade that included S. posoniensis and S. exotica (MP: 95%, ML: 92% and BPP: 0.94) in Branch 1. In this group, five strains (GUCC 2131.2, GUCC 2131.3, GUCC 2131.4, GUCC 2164.1 and GUCC 2164.2) formed an independent branch adjacent to GUCC 2131.1 and S. posoniensis (MP: 76%, ML: 86%, and BPP: 0.95), but these five strains were split into two sub-branches: one containing GUCC 2131.2, GUCC2164.1, and GUCC2164.2, and the other containing GUCC 2131.3 and GUCC2131.4, with good support (MP: 75%; BPP: 1.00). Strain GUCC 2127.3 was aligned to the branch that included S. chamaecisti, S. citri, S. citricola, S. protearum, and S. limonum with high statistical support (MP: 98%, ML: 100% and BPP: 1) but small phylogenetic distances. Strains GUCC 2164.3, GUCC 2164.4, GUCC 2127.1, and GUCC 2127.4 formed a strongly supported group (MP: 95%; ML: 100%; BPP: 1.00) closely related to S. coprosmae and S. verbenae with good support values (MP: 85%; BPP: 0.96). In Branch 2, four strains clustered in a clade in which GUCC 2127.1, GUCC2164.3 and GUCC2164.4 formed a sub-group, were very close to GUCC 2127.4, supported by high statistical values (MP: 95%, ML: 100%, and BPP: 1).

Table 3. Parameters for MP analyses.

| Locus         | Total Characters | Number of Parsimony-Informative Characters | TL  | CI   | RI   | HI   | RC  |
|---------------|------------------|------------------------------------------|-----|------|------|------|-----|
| ITS           | 486              | 43                                       | 176 | 0.642| 0.76 | 0.358| 0.488|
| LSU           | 799              | 31                                       | 112 | 0.625| 0.863| 0.375| 0.539|
| rpb2          | 345              | 18                                       | 780 | 0.273| 0.746| 0.727| 0.204|
| tef1          | 469              | 231                                      | 1498| 0.379| 0.707| 0.621| 0.268|
| tub2          | 325              | 165                                      | 1221| 0.326| 0.774| 0.674| 0.252|
| tef1 + rpb2 + tub2 + ITS | 1625 | 548                                  | 3927| 0.328| 0.716| 0.672| 0.235|
| tef1 + rpb2 + tub2 + ITS + LSU | 2434 | 567                          | 4075| 0.330| 0.720| 0.670| 0.238|

We also compared the DNA base-pair differences in five different loci between our strains and related species (Supplementary Table S1). This revealed that the LSU gene region was too conserved for species-level identification, and the ITS had little value, but tef1, tub2, and rpb2 provided more than 80% of the DNA base-pair differences (Supplementary Table S1). We also built a phylogenetic tree based on four loci, excluding the LSU region (Figure 2), using the parameters for MP analysis in Table 3. The topology showed highly similar placements of our strains in the Septoria in Figure 1; however, in Figure 2 only two branches were formed and all members of Branch 3 were integrated with Branch 1. To evaluate the distinctive effectiveness of different DNA markers, five single gene trees were constructed (Supplementary Figures S1–S5) and all MP parameters were as indicated in Table 3. Through comparison, we found that only tub2 and tef1 included more parsimonious characters (50.7% and 49.2%), and the sequence of tub2 was shorter than that of tef1. Moreover, the topology originating from the tub2 gene region was more similar to Figure 2.
Figure 1. Maximum Parsimony (MP) topology of *Septoria* generated from a combination of *tef1*, *rpb2*, *tub2*, ITS, and *LSU* sequences. *Cercospora beticola* (CBS 124.31) was used as outgroup taxon. MP and ML above 50% and BPP above 0.90 were placed close to topological nodes and separated by ‘/’, otherwise were labeled with ‘-’.
Figure 2. Maximum parsimony (MP) topology of *Septoria* generated from a combination of *tef1*, *rpb2*, *tub2*, and ITS sequences. *Cercospora beticola* (CBS 124.31) was used as an outgroup taxon. MP and ML above 50% and BPP above 0.90 were placed close to topological nodes and separated by “/”; otherwise, they were labeled with “-“.
3.2. Genealogical Concordance Phylogenetic Species Recognition

In order to determine evolutionary independence, the GCPSR concept was applied to the GUCC 2164.2, GUCC 2131.4, GUCC 2131.1, and related taxa S. chrysanthemella (CBS 128716), S. exotica (CBS 163.78), and S. posoniensis (CBS 128645). A pairwise homoplasy index (PHI or Fw) less than 0.05 provided evidence of the presence of significant recombination within a dataset. According to the GCPSR analysis, our dataset showed PHI of 0.116, indicating no significant genetic recombination among our strains and related taxa. Hence, it was concluded that these taxa were significantly different from each other.

For GUCC 2164.3 and GUCC 2127.4 and related species S. coprosmae (CBS 113391) and S. verbenae (CBS 113438), the pairwise homoplasy index (PHI or Fw) was $1.173 \times 10^{-8}$, which provided evidence for the presence of significant recombination within a dataset. The four strains could belong to a single species.

3.3. Taxonomy

(1) Septoria sanguisorbigena Y.Y. An & Yong Wang bis, sp. nov. (Figure 3)

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Figure 3. Septoria sanguisorbigena (GUCC 2164.2) (a) Leaf spot symptoms on the host. (b) Colony on PDA culture. (c) Conidiomata formed on PDA culture. (d) Section through a conidioma. (e) Peridium. (f–k) Conidiogenous cells, (l,m) Conidia. Scale bars: (c) = 25 µm, (d) = 50 µm, (e) = 20 µm, (f–j) = 10 µm, (k) = 5 µm, (l,m) = 10 µm.

Etymology: The name refers to the plant host, from which the fungus was collected.
Description in vitro: Colonies: on PDA 15–25 mm diameter after 2 weeks with a undulating even margin, restricted, irregularly pustulate; the surface almost black with low and finely felted diffuse, grey-to-white aerial mycelium. Conidiomata: pycnidial, epiphyllous, immersed, subglobose to globose, black, 120–250 µm diameter; ostiolum central, circular, initially 25–35 µm wide, later becoming more irregular and up to 100 µm wide, conidiomatal wall 20–40 µm thick, composed of an outer layer of angular-to-irregular cells mostly 4.5–10 µm diameter with pale to orange-brown walls and an inner layer of isodiametric, hyaline cells 7–20 µm diameter. Conidiogenous cells: hyaline, discrete, holoblastic, sympodially or percurrently proliferating, ampulliform, 4.5–8 × 1.5–2.5 µm (avg. = 5.6 ± 2 µm, n = 30). Conidia: hyaline, filiform, straight to somewhat flexuous, the upper cell tapered into obtuse apex, relatively wide truncated base, (1–)3–5–(7) septate, not or only indistinctly constricted at the septa, contents granular or with minute oil-droplets around the septa and at the ends, 12.5–30 × 0.6–2 µm (avg. = 20.5 × 1.3 µm, n = 30). Sexual morph unknown.

Type: CHINA, Yunnan Province, Kunming Botanical Garden, from leaves of Sanguisorba officinalis L., February 2018, Y.Y. An (HGUP 2164.2, holotype); ex-type culture GUCC 2164.2; isotype culture MFLUCC 20-0185.

Other material examined: CHINA, Yunnan Province, Kunming Botanical Garden, from leaves of Sanguisorba officinalis, February 2018, Y.Y. An (HGUP 2164.2); from leaves of Pilea cadierei Gagnep. & Guillaumin, February 2018, Y.Y. An (HGUP 2131.2).

Notes: Phylogenetic analyses confirmed that three strains (GUCC 2131.2, GUCC 2164.1, and GUCC 2164.2) had a close relationship with S. chrysanthemella, S. exotica, S. longipes, S. pileicola, and S. posoniensis and this was supported by credible statistic values of the MP and ML methods (Figure 1). However, the independent branch only included those strains with high support values (MP: 95%, ML: 90%, and BPP: 0.99) adjacent to S. pileicola with moderate MP bootstrap but 1.00 BPP support. The new species had narrower conidia (0.6–2 µm) with 3–5 septa than those of S. pileicola (1.5–3.5 µm) with only 1–2 septa. In addition, this new taxon had obviously smaller conidia (12.5–30 × 0.6–2 µm) than S. chrysanthemella (34–66 × 2.5–3 µm) and S. longipes (17–46.5 × 1.5–2.5 µm) [35]. Septoria posoniensis and S. exotica have longer conidia, which was different to the new species [33,34]. DNA base differences indicated these three strains had nearly the identical sequence data (only two different bases on ITS region), but on protein-coding genes possessed more differences to distinguish them from related species (Supplementary Table S1). GCPSR test also provided a powerful proof to clarify them as different species.

(2) Septoria pileicola Y.Y. An & Yong Wang bis sp. nov. (Figure 4)

MycoBank MB 839126

Etymology: The name refers to the plant host from which the fungus was collected.

Description in vitro: Colonies: on PDA up to 10–15 mm diameter, with an even, glabrous, colourless margin in 2 weeks. Mycelium: greenish grey to dark slate-blue, immersed, throughout covered by well-developed, tufty whitish-grey aerial mycelium that later attains a reddish haze; reverse black, but margin paler; in the central part of the colony numerous pycnidia develop, releasing pale vinaceous to rosy-buff conidial ball. Conidiomata: pycnidial, epiphyllous but sometimes also visible from the underside of the lesion, one to a few in each leaf spot, subglobose to globose, brown to black, usually fully immersed, 80–120 µm diam. Ostiolum: central, initially circular and 15–30 µm wide, later becoming more irregular and up to 45 µm wide, surrounding cells concolorous to pale brown. Conidiogenous cells: hyaline, discrete, doliform, or narrowly to broadly ampulliform, holoblastic, with a relatively narrow elongated neck, proliferating percurrently several times with distinct annellations, often also sympodially after a few percurrent proliferations, 5.5–12 × 2–3.5 µm. Conidia: cylindrical or filiform-cylindrical, straight to slightly curved, narrowly to broadly rounded at the apex, narrowing slightly or more distinctly to a truncate base, (0–)1–2-septate, not or slightly constricted around the septa, hyaline, contents with a few minute oil-droplets and granular material in each cell in the rehydrated state, 8.5–30 × 1.5–3.5 µm. Sexual morph unknown.
Figure 4. *Septoria pileicola* (GUCC 2131.4): (a) Leaf spot symptoms on the host. (b) Colonies on PDA culture. (c,d) Conidiomata on PDA culture. (e,f) Section though conidioma. (g–k) Conidiogenous cells. (l,m) conidia. Scale bars: (c,d) = 125 µm, (e–g) = 10 µm, (h–m) = 10 µm.

Type: CHINA, Yunnan Province, Kunming Botanical Garden, from leaves of *Pilea cadierei* Gagnep. & Guillaumin, February 2018, Y.Y. An (HGUP 2131.4, holotype); ex-type culture GUCC 2131.4; isotype culture MFLUCC 20-0184.

Other material examined: CHINA, Yunnan Province, Kunming Botanical Garden, from leaves of *Pilea cadierei*, February 2018, Y.Y. An (HGUP 2131.3).

Note: Phylogenetic analyses based on five gene regions showed that *Septoria pileicola* strains GUCC 2131.3 and GUCC 2131.4 are closely related to *S. chrysanthemella*, *S. exotica*, *S. longipes*, *S. posoniensis*, and *S. sanguisorbigena* (Figure 1), but formed a subclade with *S. sanguisorbigena*. After morphological comparisons, we found that *Septoria pileicola* can be distinguished from *S. sanguisorbigena* by its wider conidia, and from *S. posoniensis* and *S. exotica* by its shorter conidia with obviously fewer septa [36,37]. For *S. chrysanthemella* and *S. longipes*, the species had apparently shorter conidia [35]. The two strains of *Septoria pileicola* had nearly the identical sequences (only one different ITS base pair); however, the **tub2** gene provided enough base distinction to separate it from related species (Supplementary Table S1) according to the guidelines of Jeewon and Hyde [38]. The PHI value was 0.116 (>0.05), indicating no significant genetic recombination among *S. pileicola,*
S. sanguisorbigena, S. chrysanthemella, S. exotica, and S. posoniensis. Thus, they should belong to different species [39].

(3) Septoria longipes Y.Y. An & Yong Wang bis sp. nov. (Figure 5)

MycoBank MB 839127

Etymology: The name refers to the long conidia of this species.

Description in vitro: Colonies: on PDA 11–15 mm diameter, with an even, light brown to dark-brown margin in 2 weeks; immersed mycelium grey to dark slate-blue in the center, black near the margin. Aerial mycelium: well-developed, white to snow white, covering the colony surface. Conidiomata: pycnidial, numerous, mostly epiphyllous, semi-immersed, black, mostly 80–200 µm diameter, with a central, first narrow, later wider opening, releasing pale white cirrhi of conidia. Conidiomatal wall: one or two layers of brown-walled, angular cells, lined by a layer of hyaline cells. Conidiogenous cells: hyaline, discrete, holoblastic, sympodially or percurrently proliferating, ampulliform, 8–16 × 1.5–5.5 µm. Conidia: filiform to filiform-cylindrical, straight, flexuous or curved, attenuated gradually to the narrowly truncate base, (0–)3–5(–8)-septate, 17–46.5 × 1.5–2.5 µm. Sexual morph unknown.

Figure 5. Septoria longipes (GUCC 2131.1) (a) Leaf spot symptoms on the host. (b) Colony on PDA. (c) Conidiomata on PDA culture. (d–g) Conidiophores, Conidiogenous cells and conidia. (h–j) Conidia. Scale bars: (c) = 20 µm. (d–j) = 10 µm.

Type: CHINA, Yunnan Province, Kunming Botanical Garden, from leaves of Pilea cadierei Gagnep. & Guillaumin, February 2018, Y.Y. An (HGUP 2131.1, holotype); ex-type culture GUCC 2131.1)

Notes: Only one strain (GUCC 2131.1) of this taxon was available. It clustered with S. posoniensis supported by MP (70%) and Bayesian (0.93) analyses and is closely related to S. chrysanthemella, S. exotica, S. pileicola, and S. sanguisorbigena. Morphological comparisons indicated that GUCC 2131.1 differed from S. posoniensis by conidia by more septa, and from S. chrysanthemella (4–10 × 5–6 µm) by larger conidiogenous cells (8–16 × 1.5–5.5 µm) [35,36]. This species produced longer conidia than S. pileicola and
It was confirmed that two protein-coding genes, except for *tef1*, provided enough base distinction with related species (Supplementary Table S1). GCPSR test also supported them as different species.

(4) *Septoria dispori* Y.Y. An & Yong Wang bis sp. nov. (Figure 6)

Mycobank MB 839128

Etymology: The name refers to the plant host from which the fungus was collected.

Description in vitro: Colonies: on PDA 2.0–3.5 mm diameter, with an even to slightly ruffled, glabrous, dull yellow margin in 2 weeks, spreading, remaining almost plane, immersed mycelium yellowish brown to brown; aerial mycelium well-developed, goose feather flocculent on the surface of the colony; numerous conidiomatal initials developing at the surface, mature ones releasing cirrhi of conidia that first are milky white, later salmon, sometimes merging to form slimy masses covering areas of the colony surface. Conidigenous cells: hyaline, broadly or elongated ampulliform, normally with a distinct neck, hyaline, holoblastic, proliferating percurrently, annellations indistinct, 10–15 × 1.5–2.5 μm. Conidia: cylindrical to filiform-cylindrical, slightly to strongly curved, rarely somewhat flexuous, narrowly rounded to pointed at the apex, attenuated gradually or more abruptly towards a narrowly truncate base, 3–5–8-septate, later with secondary septa dividing the cells, sometimes breaking up into smaller fragments in the cirrhus, not or slightly constricted around the septa, hyaline, 14–41.5 × 1.5–2.5 μm. Sexual morph unknown.

Type: CHINA, Yunnan Province, Botanical Garden of Kunming country, from leaves of *Disporum bodinieri* (Levl. et Vaniot.) Wang et Y. C. Tang, February 2018, Y.Y. An (HGUP 2127.1, holotype); ex-type culture GUCC 2127.1.
Other material examined: CHINA, Yunnan Province, Kunming Botanical Garden, from leaves of *Disporum bodinieri*, February 2018, Y.Y. An (HGUP 2127.4); from leaves of *Sanguisorba officinalis* L., February 2018, Y.Y. An (HGUP 2164.3 and HGUP 2164.4).

Note: Four strains (GUCC 2127.1, GUCC 2127.4, GUCC 2164.3, and GUCC 2164.4) of *Septoria dispori* clustered together with high statistical support (MP: 95%, ML: 100%, BPP: 1.00) adjacent to *S. coprosmae* and *S. verbenae*. Thus, we consider these four strains to be a single species. *Septoria coprosmae* produced spermatogonia of an *Asteromella*-state, but this species did not [40]. Conidia of *S. verbenae* possessed fewer septa than those of *Septoria dispori* [41]. GUCC 2127.4 showed some phylogenetic distance from the other three strains, however DNA base comparison (Supplementary Table S1) revealed only 11 bases that had *tub2* differences. The PHT test confirmed significant recombination between strains GUCC 2164.4 and GUCC 2164.3 and they were morphologically similar. Thus by combining the above evidence, we established the four strains as a new taxon.

(5) *Septoria protearum* Viljoen & Crous, in Swart, Crous, Denman & Palm, S. Afr. J. Bot. 64(2): 144 (1998) (Figure 7)

![Figure 7. Septoria protearum (GUCC 2127.3) (a. Leaf spot symptoms on the host. (b,c) Colony on PDA. (b) From above; c. from below). (d) Mycelium. (e) Conidiophores. (f–l) Conidiogenous cells and conidia. (m–q) Conidia. Scale bars: (d) = 125 µm. (e–h) = 10 µm. (i) = 5 µm. (j–q) = 10 µm.](image)

Description in vitro: **Colonies**: on PDA 15–25 mm with an even, glabrous white margin in 2 weeks, plane spreading, immersed. **Mycelium**: pink, lacking aerial hypha. **Conidiomata** developing after 1 week, mostly immersed and releasing whitish conidial slime. **Conidiogenous cells**: hyaline, cylindrical, broadly to narrowly ampulliform, with a distinct neck of variable length, holoblastic, with several distinct percurrent proliferations, more rarely also sympodial after a sequence of percurrent proliferations of the same cell, 5–10(–13.5) × 2.5–3(–3.5) µm. **Conidia**: filiform, straight, more often irregularly
curved, 0–4 septate, not or only inconspicuously constricted around the septa, hyaline, 16–25 × 2.5–3.5 µm. Sexual morph unknown.

Material examined: CHINA, Yunnan Province, Kunming Botanical Garden, from leaves of *Disporum bodinieri* (Levl. et Vaniot.) Wang et Y. C. Tang, February 2018, Y.Y. An (HGUP 2127.3), living culture GUCC 2127.3.

Notes: DNA base comparison (Supplementary Table S1), revealed that sequences of strain GUCC 2127.3 were identical to the ex-type strain of *S. protearum* (CBS 778.97) in four gene regions. Conidial shape and size range of *S. protearum* (12–22 × 1.5–2 µm) were similar to the present strain [42]. The number of conidial septa of the two strains was also the same (0–4 septa). Thus, we conclude that GUCC 2127.3 is *S. protearum*.

4. Discussion

Verkley et al. [3] pointed out that for the identification of the *Septoria* species, morphological description must be integrated with sequences analyses. Quaedvlieg et al. [10] treated species in *Septoria* within a modern taxonomic framework and pointed out that *Septoria* spp. formed a well-defined phylogenetic clade. Regarding morphology, the species concept was to produce pycnidial, ostiolate conidiomata; conidiophores reduced to conidiogenous cells that proliferate sympodially; and hyaline, filiform conidia with transverse eusepta that fit the original concept of [4]. We followed this system and applied morphological and phylogenetic approaches to the present study. After comparing the topologies of five single-gene and two multi-gene trees (Figures 1 and 2 and Supplementary Figures S1–S5), we showed that *Septoria* forms two branches (Branch 1 and Branch 2), mainly because only the phylogenetic trees based on the LSU region and five DNA fragments (including LSU) supported three branches, whereas the conserved LSU sequences included the least parsimonious characters (31/799) (Table 3). In morphology, all species in Branch 3 produced filiform or fusiform, sub-straight to slightly curved conidia mainly with 3 septa, which was not a unique characteristic. Thus, we proposed exclusion of the LSU region for multi-gene analyses of *Septoria* at the species level, but always as the primary DNA barcode with more parsimonious characters (43/486), the ITS fragment was conserved in the present phylogenetic analysis.

The *S. protearum* complex accommodated eight members: *S. citri, S. citicola, S. chamaecistii, S. gerberae, S. hederae, S. lobelia, S. limonum*, and *S. protearum*, according to Verkley et al. [3]. Apart from *S. protearum*, the other species were old names without ex-type cultures, and thus no sequences were available. The base comparison of DNA sequences originated from Verkley et al. [3], who indicated that among these eight species there were only approximately 10 base-pair differences on the *rpb2* fragment (434 characters) of *S. gerberae, S. hederae*, and *S. lobelia* compared to the other five species, while for the other four gene regions, their sequences were nearly identical (≤1 base difference) (Supplementary Table S1). On the other hand, in the literature these seven species are depicted only by simple descriptions often without drawings or photographs, which does not strongly support them as different taxa. Comparing with the sequences from Verkley et al. [3] and in the absence of type materials, we were more willing to believe that they belonged to the same species, *S. protearum*.

Our 11 strains isolated from *Disporum bodinieri, Pilea cadierei, Sanguisorba officinalis* all from the Botanical Garden of Kunming county represented five *Septoria* species and included four novel species supported by morphology and phylogeny. *Septoria sanguisorbigena* was obtained from two plant hosts (*Sanguisorba officinalis* and *P. cadierei*), and *S. dispori* was also on two hosts (*D. bodinieri* and *Sanguisorba officinalis*). *Septoria pileicola* and *S. longipes* were only discovered on one host (*P. cadierei*). Our *S. protearum* strain was on *D. bodinieri*. Verkley et al. [3] recalled that trans-family host jumping must be a major force driving the evolution of *Septoria*. Our results support this hypothesis as we found the same species on different hosts. However, our findings revealed that the *Septoria* species did not show any host specialization, which differs from the view of Verkley et al. [3].
5. Conclusions

In this study, our 11 *Septoria* strains represented five species including four novel taxa, and one new record for China by morphological comparison and multi-gene analyses. The *Septoria* species are pathogens often causing leaf spot diseases of many plant hosts worldwide [10]. Based on previous studies, relatively sufficient reference sequences are available for rapid identification of *Septoria* pathogens. By comparing the parsimonious-informative characters of different DNA fragments (Supplementary Table S1), we showed that either *tef1* or *tub2* is suitable as a secondary DNA barcode, and that the latter was more discriminating than the former. Moreover, the DNA fragment of *tub2* (≈300 bp) was shorter than that of *tef1* (≈450 bp) with a high PCR amplification success rate. Consequently, a standardized approach including morphological characters and phylogenetic analysis is needed for the correct and precise identification of *Septoria* isolates.

**Supplementary Materials:** The following are available online at [https://www.mdpi.com/article/10.3390/jof7060483/s1](https://www.mdpi.com/article/10.3390/jof7060483/s1).

**Figure S1:** The phylogenetic tree based on ITS region. **Figure S2:** The phylogenetic tree based on LSU region. **Figure S3:** The phylogenetic tree based on *rpb2* region. **Figure S4:** The phylogenetic tree based on *tef1* region. **Figure S5:** The phylogenetic tree based on *tub2* region. *Cercospora beticola* (CBS 124.31) was used as outgroup taxon. MP and ML above 50% and BPP above 0.90 were placed close to topological nodes and separated by “/”, otherwise were labeled with “–”. Taxa from this study are highlighted in green. Table S1: DNA base difference between our *Septoria* strains and related species.

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