Alternaria species in section Alternaria associated with Iris plants in China

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Plants of the Iris genus have been widely cultivated because of their medicinal, ornamental, and economic values. It commonly suffers from Alternaria leaf spot or blight disease leading to considerable losses for their commercial values. During an investigation of 14 provinces or municipalities of China from 2014 to 2022, a total of 122 Alternaria strains in section Alternaria were obtained from diseased leaves of Iris spp.. Among them, 12 representative strains were selected and identified based on morphological characterization and multi-locus phylogenetic analysis, which encompassed the internal transcribed spacer of rDNA region (ITS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), translation elongation factor 1 alpha (TEF1), RNA polymerase second largest subunit (RPB2), Alternaria major allergen gene (Alt a 1), an anonymous gene region (OPA10-2), and endopolygalacturonase gene (EndoPG). The strains comprised two known species of A. alternata and A. iridicola, and two new species of A. setosae and A. tectorum, which were described and illustrated here. Their pathogenicity evaluated on Iris setosa indicated that all the strains could induce typical Alternaria leaf spot or blight symptoms. The results showed that the virulence was variable among those four species, from which A. tectorum sp. nov. was the most virulent one, followed by A. setosae sp. nov., A. iridicola and A. alternata.

KEYWORDS
Alternaria, Iris, morphology, phylogenetic analysis, pathogenicity

Introduction

The Iris Linn. is the largest genus in the Iridaceae containing about 300 species in worldwide (Xiang et al., 2020). It is one of the most extensively cultivated plants in modern landscaping because it is a great ground cover material for urban greening (Zhao et al., 2000; Clair, 2005; Li et al., 2020). In China, the plants have been widely grown as a year-round ornamental with various large and colorful flowers (Yang et al., 2017). Some Iris species are rich in beneficial chemicals, served as effective pharmaceutical ingredient to treat various diseases including cancer, inflammation, bacterial and viral infections (Tahara et al., 1991; Huang, 2004; Meng et al., 2016). In addition, Iris plants are also utilized in the
Materials and methods

Sampling and isolation

During a large-scale investigation on Alternaria species in sect. Alternaria associated with Iris spp. in China (Figure 1), diseased leaf samples were collected from 14 provinces or municipalities (Table 1). For the isolation of Alternaria, the samples were cut into small pieces, placed on moist filter papers in Petri dishes and incubated at 25°C for the sporulation (Liu et al., 2019). The samples were observed using a stereomicroscope. The single spore was picked by a sterile glass needle and inoculated onto potato dextrose agar (PDA: Difco, Montreal, Canada). A total of 122 strains were obtained and kept into test-tube slants stored at 4°C in the Fungal Herbarium of Yangtze University (YZU), Jiangzhou, Hubei, China. Twelve representative strains (Table 2) representing different species were selected for the present study after pre-morphological, pre-phylogenetic, and pre-pathogenicity assays.

Morphology

The Alternaria strains were cultured on PDA at 25°C for 7 days in darkness to determine the cultural features. To examine the conidial morphology, fresh mycelia were grown on potato carrot agar (PCA) and V8 juice agar (VRA) media, then incubated at 22°C with a light period of 8 h light/16 h dark (Simmons, 2007). After 7 days, morphological characteristics of sporulation patterns, conidiophores and conidia were visualized and photographed with a Nikon Eclipse Ni-U microscope system (Nikon, Japan). Fifty randomly selected conidia for each strain were observed and measured to determine the morphology.

DNA extraction and PCR amplification

Genomic DNA was extracted from mycelium scraped from the surface of 5-day-old colonies on PDA using the CTAB method described in Watanabe et al. (2010). Seven gene regions including internal transcribed spacer of rDNA region (ITS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), translation elongation factor 1 alpha (TEF1), RNA polymerase second largest subunit (RPB2), Alternaria major allergen gene (Alt a 1), an anonymous gene region (OPA10-2), and endopolygalacturonase (EndoPG) were used for phylogenetic analysis. The PCR amplifications were performed with primer pairs of ITS5/ITS4 (White et al., 1990), gpd1/gpd2 (Berbee et al., 1999), EF1-728F/EF1-986R (Carbone and Kohn, 1999), RPB2-5F/RPB2-7cR (Liu et al., 1999), Alt-for/Alt-rev (Hong et al., 2005), OPA10-2L/OPA10-2R (Andrew et al., 2009) and PG3/PG2b (Andrew et al., 2009), respectively. A 25-μL PCR mixture contained 21 μl 1.1× Taq PCR Star Mix (TSINGKE, Beijing, China), 2 μl template DNA, and 1 μl of each primer was performed in a BIO-RAD T100 thermo cycler. The amplified

peroxide and cosmetic industries considering the fragrance reasonability (Wang et al., 2010).

During the cultivation of Iris spp., the plants suffer from various diseases induced by fungal, bacterial, viral, and nematode pathogens (Umesh et al., 2010; Lu et al., 2016; Wang et al., 2020; Wang et al., 2022). Pathogenic fungal species have been commonly found in connection with the genera of Alternaria, Botrytis, Colletotrichum, Fusarium, Harzia and Puccinia (Bobev, 2009; Liu et al., 2016; Schultes et al., 2017; Choi et al., 2019; Wang et al., 2022). Leaf spot and blight diseases caused by Alternaria species in sect. Alternaria is prevalent on Iris plants worldwide presenting the typical symptoms of brown spot with a yellow halo or blighted leaf, which greatly reduce their ornamental values. Alternaria triticola in China, Japan, and USA (Zhang, 2003; Simmons, 2007; Nishikawa and Nakashima, 2020), A. iridiustralis in Australia, China, and New Zealand (Simmons, 2007; Luo et al., 2018), A. tenuissima in China (Zhang, 2003; Sun et al., 2019; Li et al., 2020) have been reported as fungal pathogens on Iris plants.

The Alternaria is a ubiquitous fungus comprising many destructive plant pathogens, which can result in economic losses on a large number of significant agronomic crops and ornamentals (Thomma, 2003; Kahl et al., 2015). There has been a longtime controversy on the classification criteria of Alternaria (Lawrence et al., 2016). Simmons proposed a reasonable standard for the morphological taxonomy of Alternaria species based on sporulation patterns and conidial morphology, by which around 280 species were described and summarized into two sections of large-spored and small-spored of Alternaria (Simmons, 2007). Since the 20th century, molecular taxonomic structure has been developed and applied to identify Alternaria species with various gene fragments (Pryor and Gilbertson, 2000; Hong et al., 2005; Lawrence et al., 2012, 2013; Woudenberg et al., 2013, 2014; He et al., 2021). Recently, the multi-locus phylogenetic analysis plays an important role in assisting Alternaria classification (Woudenberg et al., 2015; Zheng et al., 2015; Sofie et al., 2017; Cheng et al., 2022). It separates Alternaria into 29 sections and 7 monotypic lineages (Woudenberg et al., 2013; Marin-Felix et al., 2019; Bessadat et al., 2020; Gannibal et al., 2022; Huang et al., 2022). Besides, in combination with morphology and molecular approaches is commonly used to determine the Alternaria up to species level (Lawrence et al., 2016; Gannibal, 2019; Bessadat et al., 2021; Zhao et al., 2022).

One of the large Alternaria sections, sect. Alternaria contains most of the small-spored Alternaria species with concatenated conidia including about 60 morphological or host-specific species (Simmons, 2007; Woudenberg et al., 2015), which are frequently encountered on the disease leaf samples of Iris plants. To know the species population associated with Iris plants in China, a large-scale sample collection and fungal isolation had been conducted from 2014 to 2022, from which a total of 122 strains of sect. Alternaria were obtained. To comprehend their species levels, this study aims to identify the species using morphological traits and multi-locus phylogenetic analysis. In addition, their variety of virulence is assessed on Iris setosa.

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Materials and methods

PCR mixture contained 21 μl 1.1× Taq PCR Star Mix (TSINGKE, Beijing, China), 2 μl template DNA, and 1 μl of each primer was performed in a BIO-RAD T100 thermo cycler. The amplified
program for PCR amplifications of the seven gene regions was referenced from Woudenberg et al. (2015). Successful amplified products were purified and sequenced by TSINGKE company (Beijing, China). The obtained sequences for each gene were deposited in GenBank\(^1\) with the accession numbers indicated in Table 2.

### Phylogenetic analysis

The ITS, GAPDH, TEF1, RPB2, Alt a 1, OPA10-2 and EndoPG gene sequences were launched for BLAST\(^1\) searches in NCBI.\(^2\) Their relevant sequences were retrieved from GenBank database and referenced from Woudenberg et al. (2015; Table 2). *Alternaria alternantherae* (CBS 124392) from *Alternaria* section of *Alternantherae* was used as outgroup taxon (Table 2). Sequences were aligned and edited by the program of PHYDIT v3.2 (Chun, 1995). The seven gene sequences were concatenated and edited manually in MEGA v.7.0.26 (Kumar et al., 2016). Phylogenetic trees were constructed based on Bayesian inference (BI) analysis using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003), and maximum likelihood (ML) method using RAxML v.7.2.8 (Stamatakis, 2006). The best-fit model for the data was calculated by the Akaike Information Criterion (AIC) using MrModelTest v. 2.3 (Posada and Crandall, 1998). The Bayesian analysis (MrBayes v. 3.2.1; Ronquist et al., 2012) of two simultaneous Markov Chain Monte Carlo (MCMC) chains were run from random trees for 10,000,000 generations and sampled every 100 th generations. The first 25% of the samples were discarded as the burn-in and the run was automatically stopped when the average standard deviation of

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1. https://www.ncbi.nlm.nih.gov/
2. www.ncbi.nlm.nih.gov
| Species/Strain | Host/Substrate | Country | GenBank accession number |
|---------------|----------------|---------|-------------------------|
| **A. alstroemeriae** | | | |
| CBS 118808 R | Alistroemeria sp. | Australia | KP124296, KP124153, KP125071, KP124764, KP123845, KP123993, KP124601 |
| CBS 118809 T | Alistroemeria sp. | Australia | KP124297, KP124154, KP125072, KP124765, KP123845, KP123994, KP124602 |
| **A. alternantherae** | | | |
| CBS 124392 | Solenococcus melongena | China | KC584179, KC584096, KC584633, KC584374, KP123846 |
| **A. alternata** | | | |
| CBS 916.96 T | Arachis hypogaea | India | AF347031, AY278808, KC584634, KC584375, AY563301, JQ811978 |
| CBS 102604 | Minnesoa tangelo | Israel | KP124334, AF562410, KP125110, KP124802, AY563305, KP124035 |
| CBS 102602 | Minnesoa tangelo | Turkey | KP124332, KP124187, KP125108, KP124800, KP123881, AY295023 |
| CBS 102599 | Minnesoa tangelo | Turkey | KP124330, KP124185, KP125106, KP124798, KP123879, KP124032 |
| CBS 102595 | Citrus jamhiri | USA | FJ664676, AY562411, KC584666, KC584408, AY563306, KP124029 |
| CBS 127672 | Astragalus bisulcatus | USA | KP124382, KP124234, KP125160, KP124852, KP123930, KP124086 |
| CBS 103.33 | Soil | Egypt | KP124302, KP124159, KP125077, KP124770, KP123852, KP123999 |
| CBS 102.47 | Citrus sinensis | USA | KP124303, KP124160, KP125079, KP124772, KP123854, KP124001 |
| CBS 117.44 | Godetsia sp. | Denmark | KP124303, KP124160, KP125079, KP124772, KP123854, KP124001 |
| CBS 102596 | Citrus jamhiri | USA | KP124328, KP124183, KP125104, KP124796, KP123877, KP124030 |
| CBS 918.96 | Dianthus chinensis | UK | AF347032, AY278809, KC584693, KC584435, AY563302, KP124026 |
| CBS 121455 | Broussonetia papyrifera | China | KP124368, KP124220, KP125146, KP124838, KP123916, KP124072 |
| CBS 121547 | Pyrus breitneri | China | KP124372, KP124224, KP125150, KP124842, KP123920, KP124076 |
| CBS 127671 | Stanleya pinnata | USA | KP124381, KP124233, KP125159, KP124851, KP123929, KP124085 |
| CBS 106.34 | Linum usitatissimum | Unknown | Y17071, JQ646308, KP125078, KP124771, KP123853, KP124000 |
| CBS 121336 | Allium sp. | USA | KJ862254, KJ862255, KP125141, KP124833, KP862259, KP124067 |
| CBS 119543 | Citrus paradisi | USA | KP124363, KP124215, KP125139, KP124831, KP123911, KP124065 |
| CBS 195.86 | Euphorbia esula | Canada | KP124317, KP124173, KP125093, KP124785, JQ646398, KP124017 |
| CBS 119399 | Minnesoa tangelo | USA | KP124361, JQ646328, KP125137, KP124829, KP123910, KP124063 |
| CBS 479.90 | Citrus sudachi | Japan | KP124319, KP124174, KP125095, KP124787, KP123870, KP124019 |
| CBS 121348 | Platycodon grandiflorus | China | KP124356, KP124219, KP125144, KP124836, KP123915, KP124070 |
| CBS 595.93 | Pyrus pyrifolia | Japan | KP124320, KP124175, KP125096, KP124788, JQ646399, KP124020 |
| CBS 118818 | Vaccinium sp. | USA | KP124359, KP124213, KP125135, KP124827, KP123908, KP124061 |
| CBS 118814 | Solanum lycopersicum | USA | KP124357, KP124211, KP125133, KP124825, KP123906, KP124059 |
| CBS 118815 | Solanum lycopersicum | USA | KP124358, KP124212, KP125134, KP124826, KP123907, KP124060 |
| CBS 118811 | Brassica oleracea | USA | KP124356, KP124210, KP125132, KP124824, KP123904, KP124057 |
| CBS 118812 | Daucus carota | USA | KC584193, KC584112, KC584652, KC584393, KP123905, KP124058 |
| CBS 119408 | Euphorbia esula | USA | KP124362, JQ646326, KP125138, KP124830, JQ646410, KP124064 |
| CBS 540.94 | Nicotiana tabacum | USA | AY278835, AY278811, KC584667, KC584409, AY563304, KP124147 |
| CBS 121333 | Nicotiana tabacum | USA | KP124444, KP124293, KP125223, KP124914, KP123990, KP124150 |
| CBS 104.32 | Geopirrion sp. | Zimbabwe | KP124430, JQ646312, KP125209, KP124900, JQ646395, KP124135 |

(Continued)
| Species/Strain | Host/Substrate | Country | GenBank accession number |
|---------------|----------------|---------|------------------------|
| **CBS 102601** | Minneola tangelo | Colombia | KP124433, KP124282, KP125212, KP124903, KP123979, KP124138, KP124749 |
| **CBS 102597** | Minneola tangelo | USA | KP124432, KP124281, KP125211, KP124902, KP123978, KP124137, KP124748 |
| **YZU 171270** | Iris tectorum | China | OP341610, OP352298, OP374451, OP352286, OP293709, OP293721, OP352274 |
| **YZU 171499** | Iris tectorum | China | OP341534, OP352295, OP374448, OP352283, OP293708, OP293719, OP352271 |
| **YZU 181050** | Iris tectorum | China | OP341604, OP352297, OP374450, OP352285, OP293707, OP293720, OP352273 |
| **YZU 181280** | Iris tectorum | China | OP341598, OP352296, OP374449, OP352284, OP293706, OP293718, OP352272 |
| **A. arborescens** | Peat soil | Switzerland | KP124392, KP124244, KP125170, KP124862, KP123940, KP124096, KP124705 |
| **CBS 119545** | Senecio skirrhodon | New Zealand | KP124409, KP124260, KP125180, KP124879, KP123956, KP124112, KP124722 |
| **CBS 119544** | Avena sativa | New Zealand | KP124408, JQ646321, KP125186, KP124878, KP123955, KP124106, KP124716 |
| **CBS 112749** | Malus domestica | South Africa | KP124402, KP124254, KP125180, KP124872, KP123949, KP124106, KP124713 |
| **CBS 105.24** | Solanum tuberosum | Unknown | KP124393, KP124245, KP125171, KP124863, KP123941, KP124097, KP124706 |
| **CBS 109730** | Solanum lycopersicum | USA | KP124399, KP124275, KP125175, KP124867, JQ46390, KP124101, KP124710 |
| **CBS 105.49** | Contaminant blood culture | Italy | KP124396, KP124248, KP125174, KP124866, KP123944, KP124100, KP124709 |
| **A. betae-kenyensis** | Beta vulgaris var. cicla | Kenya | KP124419, KP124270, KP125197, KP124888, KP123966, KP124123, KP124733 |
| **A. burnsii** | Human sputum | India | KP124425, KP124275, KP125203, KP124894, KP123972, KP124129, KP124739 |
| **CBS 108.27** | Gomphrena globosa | Unknown | KC584236, KC584162, KC584727, KC584468, KP123850, KP123997, KP124605 |
| **CBS 107.38 T** | Cuminum cyminum | India | KP124420, JQ46305, KP125198, KP124889, KP123967, KP124124, KP124734 |
| **CBS 118816** | Rhizophora mucronata | India | KP124423, KP124273, KP125201, KP124892, KP123970, KP124127, KP124737 |
| **CBS 118817** | Tinospora cordifolia | India | KP124424, KP124274, KP125202, KP124893, KP123971, KP124128, KP124738 |
| **A. citricancri** | Citrus paradisi | USA | KP124363, KP124215, KP125139, KP124831, KP123911, KP124065, KP124674 |
| **A. echthornae** | Eichhornia crassipes | India | KC146356, KP124276, KP125204, KP124895, KP123973, KP124130, KP124740 |
| **CBS 119778 R** | Eichhornia crassipes | Indonesia | KP124426, KP124277, KP125205, KP124896, KP123973, KP124131, KP124741 |
| **A. gaisen** | Gossypium sp. | Zimbabwe | KP124432, KP124281, KP125211, KP124902, KP123978, KP124137, KP124748 |
| **CBS 102597** | Minneola tangelo | USA | KP124432, KP124281, KP125211, KP124902, KP123978, KP124137, KP124748 |
| **CBS 102601** | Minneola tangelo | Colombia | KP124433, KP124282, KP125212, KP124903, KP123979, KP124138, KP124749 |

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split frequencies reached below 0.01. The phylogram was plotted and edited in Figtree v.1.3.1 (Rambaut and Drummond, 2010). The branch support for the ML analysis was assessed with 1,000 replicates.

Pathogenicity tests

*Iris setosa* was commonly cultivated as an ornamental or medicinal plant in China. The plants were purchased from the local market and transplanted into clean pots with organic sterile soil grown in greenhouse under a light period (12 h) at 25 °C for 2 months. The test strains were cultured on PDA at 25 °C for 5 days, and a diameter of 6 mm disc was removed from the edge of colonies. It was inoculated on wounded living leaves of *I. setosa* helped with a fine sterile needle (eight punctures). Clean PDA discs were used as controls. To ensure an accuracy of pathogenicity assessment, a consistent treatment method was adopted for each assay. For each strain, two plants were inoculated with four sites, which were replicated for three times. To confirm the Koch’s law, the pathogen was re-isolated from the diseased symptoms and compared with the original strains based on morphology. The disease incidence was recorded, and the lesion size (LS = the maximum lesion length) was measured after 7 days. The LS values were the mean value of three replicates ± standard deviation. The least significant difference test ($p < 0.05$) was analyzed by IBM SPSS Statistics 23.

Results

Phylogenetic analysis

On the basis of BLAST searches, all 122 *Alternaria* strains were belonged to sect. *Alternaria*. Phylogenetic analyses using the combined dataset of the seven gene sequences (ITS,
GAPDH, TEF1, RPB2, *Alt a 1*, OPA10-2 and *EndoPG* were conducted including 79 strains of sect. *Alternaria* (Table 2), which comprised 3,340 characters (470 from ITS, 534 from GAPDH, 240 from TEF1, 603 from RPB2, 429 from *Alt a 1*, 622 from OPA10-2, 442 from *EndoPG*). The resulted topologies of BI and ML analysis were similar to each other, and the ML tree was shown in Figure 2 for basal phylogeny. It showed that strains of YZU 171270, YZU 171499, YZU 181050 and YZU 181280 fell into four different subclades of *A. alternata*. Strains of YZU 161051, YZU 161186, YZU 161119 and YZU 191090 were grouped well together with *A. iridicola* supported by values of 1.0/100% (PP/BS). Two strains of YZU 191101 and YZU 191076 fell into a distinct single lineage (PP/BS = 1.0/99%) close to *A. gaisen* supported by the values of 1.0/100% (PP/BS), which could be considered as a new species. The remaining two strains of YZU 161050 and YZU 161052 also formed an individual branch with high PP/BS values of 1.0/100% representing a new species in the phylogram, sister to *A. iridicola* (PP/BS = 0.99/98%). Two single lineage (PP/BS = 1.0/99%) close to *A. iridicola* strains associated with *Iris* spp. were described in this study.

### Morphology

According to morphological traits, the present *Alternaria* strains associated with *Iris* spp. were *A. alternata* (Figure 3) and *A. iridicola* (Figure 4) and two new species of sect. *Alternaria* (Figures 5, 6 and Table 3). The two new species were illustrated and described in this study.

### Taxonomy

*Alternaria setosae* Y.N. GOU & J.X. Deng, sp. nov. Figure 5

**MycoBank No.:** 845326

**Etymology:** In reference to the pathogenic host species name, *Iris setosa*.

**Typification:** China, Fujian Province, Fuzhou City, from leaf spot of *Iris japonica*, 15 April 2019, J.X. Deng, (YZU-H9004, holotype), by culture YZU 191101.

**Description:** Colonies on PDA circular, light cottony and white to off-white in the center, villiform with white at the edge, reverse dark brown at centers, with scattering shape, 83.4–84.8 mm in diam., at 25°C for 7 days. On PCA, conidiophores arising from substrate or lateral of aerial hyphae, straight or curved, smooth-walled, septate, pale brown; (12.5–)14–50 × 3–4 μm (av.: 24.5 × 4 μm); conidia 4–12 units per chain, medium yellow-brown, almost smooth to almost smooth-walled, short to long-ovoid or ellipsoid, (12–)14–45 × 7–14 μm (av.: 31.5 × 10.5 μm). 1–6 transverse septa. 0–2–(−3) longitudinal septa. On V8A, conidiophores straight or curved, smooth-walled, septate, 18–43 (−50.5) × 3.5–5 μm (av.: 31 × 4 μm), conidia 4–11 units in a chain, medium yellow-brown, almost smooth to conspicuously elliptoid overall, 20–43 × 7–12.5 μm (av.: 30 × 9.5 μm), 2–7 transverse septa, 0–2−(−3) longitudinal septa.

**Notes:** Phylogenetic analysis reveals that the species is sister to *A. gaisen* on the basis of a combined dataset of ITS, GAPDH, TEF1, RPB2, *Alt a 1*, OPA10-2 and *EndoPG* gene regions. After a nucleotide pairwise comparison with *A. gaisen* in those seven regions, there are 1/470 bp, 1/534 bp, 3/240 bp, 3/603 bp, 0/429 bp, 16/622 bp and 0/442 bp site differences, respectively. Morphologically, this species can be differentiated by producing smaller conidia with less transverse septa (Table 3). It also readily distinct to *A. tectorum* sp. nov. and *A. iridicola* in sporulation pattern and conidial size (Table 3).

*Alternaria tectorum* Y.N. GOU & J.X. Deng, sp. nov. Figure 6

**MycoBank No.:** 845325

**Etymology:** In reference to the host species name, *Iris tectorum*.

**Typification:** China, Hubei Province, Jingzhou City, from leaf spot of *Iris tectorum*, 21 May 2016, J.X. Deng, (YZU-H0025, holotype), by culture YZU 161050.

**Description:** Colonies on PDA circular, cottony with dense hyphae, off-white, reverse buff in the center, with white margin,
FIGURE 3
Morphology of the four strains of A. alternata. (A) Colony phenotype (on PDA for 7 days at 25°C); (B,C) Sporulation patterns (on PCA at 22°C); (D) Conidia Bars: B,C = 50 μm; D = 25 μm.

FIGURE 4
Morphology of Alternaria iridicola. (A) Colony phenotype (on PDA for 7 days at 25°C); (B,C) Sporulation patterns (on PCA at 22°C); (D) Conidia Bars: B,C = 50 μm; D = 25 μm.
50–52 mm in diam., at 25°C for 7 days. On PCA, conidiophores arising from substrate, straight to slightly curved, septate, pale to dark brown, 32–58.5 (−62.5) × 3.5–4.8 μm (av.: 45.5 × 4 μm); conidia 1–6 in a chain, solitary or straight in chains with few branches, broad-ovoid or broad-ellipsoid, smooth-walled, 13–38.5 × 5.5–13 μm (av.: 23.5 × 9 μm), 0–6 transverse septa, 0–2 (−3) longitudinal septa, On V8A, conidiophores straight to slightly curved, septate, pale to dark brown, 31.5–54.5 × 3.5–4.75 μm (av.: 41.5 × 4 μm). conidia 2–7 per chain, pale to medium yellowish brown, smooth walled, broad-ovoid or broad-ellipsoid, 13.5–37.5 × 5.5–13.5 (av.: 26 × 9.5 μm), 1–7 transverse septa, 0–2 (−3) longitudinal septa.

Notes: Phylogenetic analysis shows that the species (13–38.5 × 5.5–13 μm in body size) falls in an individual clade with high PP/BS values of 1.0/100% representing a new species in the phylogram, sister to A. iridicola (47–87 × 15–27 μm in size), but it can be significantly distinguished by the conidial size and shape (Table 3). Comparing with A. iridicola, the present species contains 1/442 bp, 16/603 bp and 37/622 bp nucleotide differences in EndoPG, RPB2 and OPA10-2 gene sequences, respectively.

Pathogenicity tests

Pathogenicity tests inoculated with those 12 Alternaria strains for 7 days, the results showed that all strains were 100% pathogenic to Iris setosa (Table 4; Figure 7). When inoculated with mycelium discs, the small spot was normally appeared after 4 days on the living leaves. Then it expanded into nearly round to long elliptic, dark brown necrotic lesions coupling with a narrow yellow halo around the periphery of the spot. Besides, the necrotic part of spot is easy to rupture and form perforation. For some strains (A. tectorum), the spots spread quickly until most of the leaves becoming withered causing blight. The symptoms were identical to those in the fields (Figure 1). The virulence among the species was variable (Table 4). Strains of A. tectorum sp. nov. showed the most virulent to I. setosa with the lesion size (LS) around 48 to 49 mm, followed by A. setosae sp. nov. (LS around 26–27), then A. iridicola and A. alternata. There were no symptoms on the control leaves. To verify Koch’s rules, the re-isolation of the causal pathogen was performed, which was consistent with the inoculated strains.
Discussion

The sect. Alternaria is found containing 17 species and a species complex of A. arborescens (AASC; Woudenberg et al., 2015; Li et al., 2022). After a large-scale sample collection of Iris plants in China, two known species (A. alternata and A. iridicola) and two novel species in sect. Alternaria were detected in this study. In addition, the two new species also identified and described as A. setosae sp. nov. and A. tectorum sp. nov. based on the morphological characteristics and multi-locus phylogenetic analyses.

Alternaria alternata is one kind of small-spored Alternaria, which taxonomy is controversial due to the similar conidial morphology (Zhang, 2003; Simmons, 2007). Later, it is classified as one species based on multi-gene phylogenetic analyses, encompassing 35 morphospecies identified by Simmons (2007) which could not reliably distinguish those species (Woudenberg et al., 2015). Meanwhile, it resulted in confusion to the related taxonomic works on their classification. Morphologically, strain YZU 171270 grouped in a sub-clade containing the type strain of A. tenuissima can sporulate with long straight conidial chains without branch. The results agreed with Simmons (2007) for the descriptions of A. alternata and A. tenuissima. In China, A. tenuissima has been reported on I. tectorum in Qingdao city (Sun et al., 2019) and I. ensata in Anqing city (Li et al., 2020), whose sporulation patterns are not provided. Although sporulation phenotype cannot accurately reflect the evolutionary relationship, it still is of great value for subgroup classification of A. alternata. Besides, the present strains falling into different clade or subclade (Figure 2) are highly variable (Figure 3). For example, both strains YZU 181050 and YZU 181280 are similar in sporulation, but strain YZU 181280 is characterized by comprising larger conidia with blunted beak up to 71.5 μm long (Figure 3). The morphological boundary of A. alternata has not been clearly defined yet and how to classify them accurately needs further study (Aung et al., 2020).

Alternaria iridicola has been recognized as a large-spored Alternaria (ca. 60–125 × 17–33 μm in size) comprising broadly obclavate conidia with a blunt conical or sturdy cylindric apex, which is a host specific species to Iris plants (Simmons, 2007). Gannibal and Lawrence (2018) considered that A. iridicola shares...
traits with both sect. *Panax* and *Porri* of *Alternaria*, which was not easily distinguished in terms of section affiliation. Latterly, this species is the only large-spored *Alternaria* in small-spored sect. *Alternaria* according to multi-locus phylogenetic analysis (Nishikawa and Nakashima, 2020).

Nishikawa and Nakashima (2020) took experiments to assess the host range of *A. iridicola*, for which *I. laevigata* and *I. hollandica* were proved host related, except *I. ensata*. The species was firstly reported causing leaf spot disease on *I. tectorum* from Xinxiang city of China in 2015 (Zhai et al., 2015). In this study, *I. japonica* is a new host for *A. iridicola* in China, which also can infect *I. setosa*.

In this study, two new species of *A. setosae* and *A. tectorum* are grouped with *A. gaisen* and *A. iridicola* in a clade, sister to *A. alstroemeriae*. The morphological comparisons are listed in Table 3. Compared with *A. gaisen* (30–45 (−55) × 13–15 (−18) μm with 5–8 septa), *A. setosae* sp. nov. is different by producing smaller conidia (14–45 × 7–14 μm) and less transverse septa (1–6; Simmons, 2007). In comparison with *A. iridicola*, *A. tectorum* sp. nov. is an obviously a small-spored species (Simmons, 2007). The results increase two members for sect. *Alternaria*. In the previous reports, *A. iridiaustralis* has been reported on *Iris* spp. in Australia and New Zealand (Simmons, 2007) and on *Iris ensata* as causal foliar pathogen in China (Luo et al., 2018), which is characterized by broad-ovoid or broad-ellipsoid and long ellipsoid conidia, obviously not like the present four species.

| Species/Strain | Shape | Conidia | Conidia per chain | References |
|---------------|-------|---------|-------------------|------------|
| *A. alternata* | Ovoid, ellipsoid, or subsphearo or with short beak | 7–30 (−40) × 5–12 | 3–5 (−30) | 1–7 | 4–20 | Simmons (2007) |
| ZYU 171270    | Almost slender ellipsoid or ovoid, sometimes with short beak | 10–36 × 6–23 | 4–17.5 | 1–5 | 3–8 | This study |
| ZYU 171499    | Ovoid or ellipsoid with cylindric apex | 14–51 (−66.5) × 7.5–18.5 | – | 1–6 | 4–8 | This study |
| ZYU 181050    | Ovoid, or ellipsoid or with blunted beak | 11.5–40 × 4–7.5 | 5.5–26 | 3–6 | 5–10 | This study |
| ZYU 181280    | Ellipsoid, ovoid, almost with long beak | 22–76.5 (−84.5) × 7.5–16 | 6–67.5 (−71.5) | 1–9 | 3–8 | This study |
| *A. gaisen*   | Short to long ovoid or ellipsoid | 30–45 (−55) × 13–15 (−18) | – | 5–8 | 3–9 | Simmons (2007) |
| *A. iridiaustralis* | Broad-ovoid or broad-ellipsoid and long ellipsoid | 30–50 × 13–24 | – | 3–4 | 1–2 | Simmons (2007) |
| *A. iridicola* | Broadly obclavate with a blunt conical or sturdy cylindric apex | 60–125 × 17–33 | 20–70 | 5–9 | 3–9 | Simmons (2007) |
| *A. setosae* sp. nov. | Obclavate or long ovoid, sometimes cylindrical with columnar beak | 36–74 × 3–26 | 10–74 | 1–8 | 4–5 | This study |
| *A. tectorum* sp. nov. | Short to long ovoid or ellipsoid | (12–) 14–45 × 7–14 | – | 1–6 | 4–12 | This study |
| *A. setosae* sp. nov. | Broad ovoid or broad ellipsoid | 13–38.5 × 5.5–13 | – | 1–6 | 2–9 | This study |
During the isolation of *Alternaria* from *Iris* plants, *A. alternata* like species are frequently observed on moisten cultured leaf tissues, such as *A. alternata*, *A. iridicola*, *A. setosae* sp. nov. and *A. tectorum* sp. nov. Consequently, their virulence is assessed on wounded leaves of *I. setosa*, and considerable variation is observed among the species in the pathogenicity tests, from which *A. tectorum* sp. nov. shows the most pathogenic, followed by *A. setosae* sp. nov., *A. iridicola*, and finally *A. alternata*. The pathogenic mechanism comprises mechanical penetration, secreting degradation enzymes, metabolites, and toxins, which causes plant diseases (Meng et al., 2009). *Iris* plants are near to the ground growing, which suffers vulnerable damage caused by raining splashing, animals, and so on. When there are small wounds on *Iris* leaves, it is easier to attack by pathogens, which could significantly lower their economic and ornamental values. On the other hand, mycelia discs of the four species were inoculated on unwounded living leaves of *Iris setosa* for 14 days (data not shown). *Alternaria tectorum* sp. nov. (LS around 20 mm) and *A. iridicola* (LS 6 to 18 mm) can penetrate the leaves and induce symptoms. While *A. alternata* and *A. setosae* sp. nov. fail to penetrate and cause infection. The present results will provide experimental evidence and reference for the prevention and control of *Iris* leaf spot caused by *Alternaria alternata* like species.

### Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

### Author contributions

JD, SA, and YG did the study design. JD, SA, CH, and AH collected the diseased leaf samples. YG and SA performed the

### Table 4 Disease incidence and lesion size on *Iris setosa* induced by the present *Alternaria* species.

| Species         | Strain     | Disease incidence (%) | Lesion (mm)  |
|-----------------|------------|------------------------|--------------|
| *A. tectorum* sp. nov. | YZU 161050 | 100 ± 0 a | 48.97 ± 0.65 a |
|                 | YZU 161052 | 100 ± 0 a | 48.28 ± 0.36 a |
| *A. setosae* sp. nov. | YZU 191101 | 100 ± 0 a | 26.87 ± 0.31 b |
|                 | YZU 191076 | 100 ± 0 a | 26.76 ± 0.25 b |
| *A. iridicola*   | YZU 161051 | 100 ± 0 a | 24.15 ± 0.33 bc|
|                 | YZU 191090 | 100 ± 0 a | 23.55 ± 0.22 bc|
|                 | YZU 161119 | 100 ± 0 a | 22.48 ± 0.54 c |
|                 | YZU 161186 | 100 ± 0 a | 22.51 ± 0.60 c |
| *A. alternata*   | YZU 171270 | 100 ± 0 a | 12.39 ± 0.63 d |
|                 | YZU 181050 | 100 ± 0 a | 11.77 ± 0.59 d |
|                 | YZU 181280 | 100 ± 0 a | 10.22 ± 0.40 d |
|                 | YZU 171499 | 100 ± 0 a | 10.08 ± 0.28 d |

Disease incidence (DI) was evaluated by counting the percentage of diseased leaves. Lesion size (LS = the maximum lesion length) values are the mean value of three replicates ± standard deviation. Values followed by different lowercase letters within a column are significantly different according to the least significant difference test (P < 0.05) using IBM SPSS Statistics 23.

**FIGURE 7**

 Pathogenicity of the present *Alternaria* species on *Iris setosa*.
experiments and analyzed the data. YG wrote the manuscript. JD and YG contributed to manuscript revision, read, and approved the submitted version.

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