

A new *Alternaria* species from grapevine in China

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Abstract Twelve *Alternaria* strains were isolated from pedicels and rachis from different parts of grapevines in China. In a phylogenetic analysis, nine showed a close relationship with *Alternaria longipes*, and are described as a new species, *A. viniferae* sp. nov., but the other four clustered with *A. alternata* and *A. arborescens* within the *alternata* species-group. The new species can clearly be separated from other related small-spored *Alternaria* species based on sequences of portions of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and *Alternaria* major allergen (Alt a 1) genes as well as by distinctive morphology.

Keywords Pathogen · Small-spored group · *Vitis yunnanensis*

Introduction

In the *Alternaria* genus, about 640 names have been included (www.indexfungorum.org) and, of these, nearly 300 taxa are accepted (Simmons 2007). They are a cosmopolitan fungal group that includes saprophytic, endophytic and pathogenic

species, widely distributed in soil and organic matter in decomposition (Pavón et al. 2012; Polizzotto et al. 2012). Among them, *Alternaria alternata* has been recorded as causing diseases of more than 380 host species of plant (Bernadovičová and Ivanová 2011). Sain et al. (2009) reported that it caused a new leaf spot disease of *Calotropis gigantea* in Rajasthan, India. Interestingly, Musetti et al. (2005) reported that *A. alternata* produces metabolites which can inhibit sporulation of *Plasmopara viticola* (the grapevine downy mildew pathogen).

Molecular delimitation of *Alternaria* and related genera, including *Crivellia*, *Embellisia*, *Nimbya*, *Sinomyces*, *Ulocladium*, and *Undifilum* with *Stemphylium*, as the sister taxon has been accomplished by sequencing several genes including the internal transcribed spacer (ITS) region, the mitochondrial small subunit (mtSSU), and the protein coding genes glyceraldehydes-3-phosphate dehydrogenase (*gpd*) and *Alternaria* major allergen (Alt a 1) (Hong et al. 2005; Pryor and Bigelow 2003; Zhang et al. 2009; Runa et al. 2009; Tóth et al. 2011; Wang et al. 2011).

We examined 12 *Alternaria* strains isolated from grapevines collected in 2010–2011 in China. Morphological observations and phylogenetic analysis of combined *gpd* and Alt a 1 gene regions of these isolates showed that all belonged to the *alternata* species-group, but one taxon with nine isolates, which did not fit any known species of this genus, is thus described here as a new species.

Materials and methods

Isolation

Pedicels and rachis were collected from grapevines in 11 locations within four provinces of China (Beijing, Gansu, Shandong, Sichuan). Samples were placed in clean plastic bags, taken to the laboratory and stored at 4 °C until examined. The plant parts (pedicels and rachis) were scrubbed under running water to remove surface debris, dissected into

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Table 1 Strains used in phylogenetic analyses and their corresponding GenBank accession numbers

Species	Sources	GenBank accession	
		<i>gpd</i>	Alt a 1
<i>A. tenuissima</i>	E.G.S. 34–015	AY278809	AY563302
<i>A. tagetica</i>	E.G.S. 44–044	AY562407	AY563297
<i>A. dauci</i>	ATCC 36613	AY278803	AY563292
<i>A. solani</i>	ATCC 58177	AY278807	AY563299
<i>A. porri</i>	ATCC 58175	AY278806	AY563296
<i>A. crassa</i>	DDG Acr1	AY278804	AY563293
<i>A. pseudorostrata</i>	E.G.S. 42–060	AY562406	AY563295
<i>A. longipes</i>	E.G.S. 30–033	AY278811	AY563304
<i>A. viniferae</i>	HGUP 5003	KJ802460	KF913634
	HGUP 5005	KJ802463	KF913639
	HGUP 5006	KJ802464	KF913633
	HGUP 5007	KJ802465	KF913632
	HGUP 5008	KJ778162	KF913631
	HGUP 5014	KJ802461	KF913637
	HGUP 5016	KJ802467	KF913630
	HGUP 5019	KJ802470	KF913638
	HGUP 5020	KJ802469	KF913640
<i>A. alternata</i>	E.G.S. 34–016	AY278808	AY563301
	HGUP 5009	KJ802466	KF913635
	HGUP 5012	KJ802462	KF913636
	HGUP 5018	KJ802468	KF900224
<i>A. arborescens</i>	E.G.S. 39–128	AY278810	AY563303
<i>A. cinerariae</i>	E.G.S. 33–169	AY562413	AY563308
<i>A. sonchi</i>	E.G.S. 46–051	AY562412	AY563307
<i>A. cheiranthi</i>	E.G.S. 41–188	AY278802	AY563290
<i>A. radicina</i>	ATCC 96831	AY278797	AY563286
<i>A. carontiincultae</i>	E.G.S. 26–010	AY278798	AY563287
<i>A. petroselini</i>	E.G.S. 09–159	AY278799	AY563288
<i>A. smyrnii</i>	E.G.S. 37–093	AY278801	AY563289
<i>A. brassicicola</i>	EEB 2232	AY278813	AY563311
<i>A. mimicula</i>	E.G.S. 01–056	AY562415	AY563310
<i>A. cetera</i>	E.G.S. 41–072	AY562398	AY563278
<i>A. photistica</i>	E.G.S. 35–172	AY562402	AY563282
<i>A. oregonensis</i>	E.G.S. 29–194	AY562403	AY563283
<i>A. metachromatica</i>	E.G.S. 38–132	AY562404	AY563285
<i>A. conjuncta</i>	E.G.S. 37–139	AY562401	AY563281
<i>A. ethzedia</i>	E.G.S. 37–143	AY278795	AY563284
<i>A. argyranthemii</i>	E.G.S. 43–033	AY562400	AY563280
<i>A. blumeae</i>	E.G.S. 40–149	AY562405	AY563291
<i>A. brassicae</i>	BMP 0322	AY562414	AY563309
<i>A. brassicicola</i>	EEB 2232	AY278813	AY563311
<i>A. capsici</i>	E.G.S. 45–075	AY562408	AY563298
<i>A. cucumerina</i>	BMP 0188	AY562409	AY563300
<i>A. dumosa</i>	E.G.S. 45–007	AY562410	AY563305
<i>A. eryngii</i>	E.G.S. 41–005	AY562416	AY563313
<i>A. euphorbiicola</i>	E.G.S. 42–049	AY562417	AY563314
<i>A. japonica</i>	ATCC 13618	AY278814	AY563312
<i>A. macrospora</i>	DDG Am1	AY278805	AY563294

ATCC American type culture collection, Manassas; BMP B.M. Pryor, Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA; DGG D.G. Gilchrist, Department of Plant Pathology, University of California, Davis, CA 95616, USA; EEB E.E. Butler, Department of Plant Pathology, University of California, Davis, CA 95616, USA; E.G.S. E.G. Simmons, Mycological Services, Crawfordsville, IN 47933, USA; HGUP Herbarium of the Department of Plant Pathology, Agricultural College, Guizhou University, China

Table 1 (continued)

Species	Sources	GenBank accession	
		<i>gpd</i>	Alt a 1
<i>A. mimicula</i>	E.G.S. 01–056	AY562415	AY563310
<i>A. mouchaccae</i>	E.G.S. 31–061	AY562399	AY563279
<i>A. photistica</i>	E.G.S. 35–172	AY562402	AY563282
<i>P. herbarum</i>	ATCC11681	AY278823	AY563277

small segments (approximately 1 cm length), and surface-sterilized by sequential immersion in 70 % ethanol for 30 s and then in 14 % hypochlorite for 30 s. The segments were rinsed in sterilized water for 1 min. Surface-sterilized segments (4 segments/plate) were placed on potato dextrose agar

(PDA; 20 g, white potato boiled and filtered, 20 g dextrose, 20 g agar, 1 L distilled water) supplemented with 5 ppm streptomycin to inhibit bacterial growth. Three plates were used for pedicels and rachis of grapevine plant. The plates were incubated at 25 °C for 5–7 days. Single spore isolation

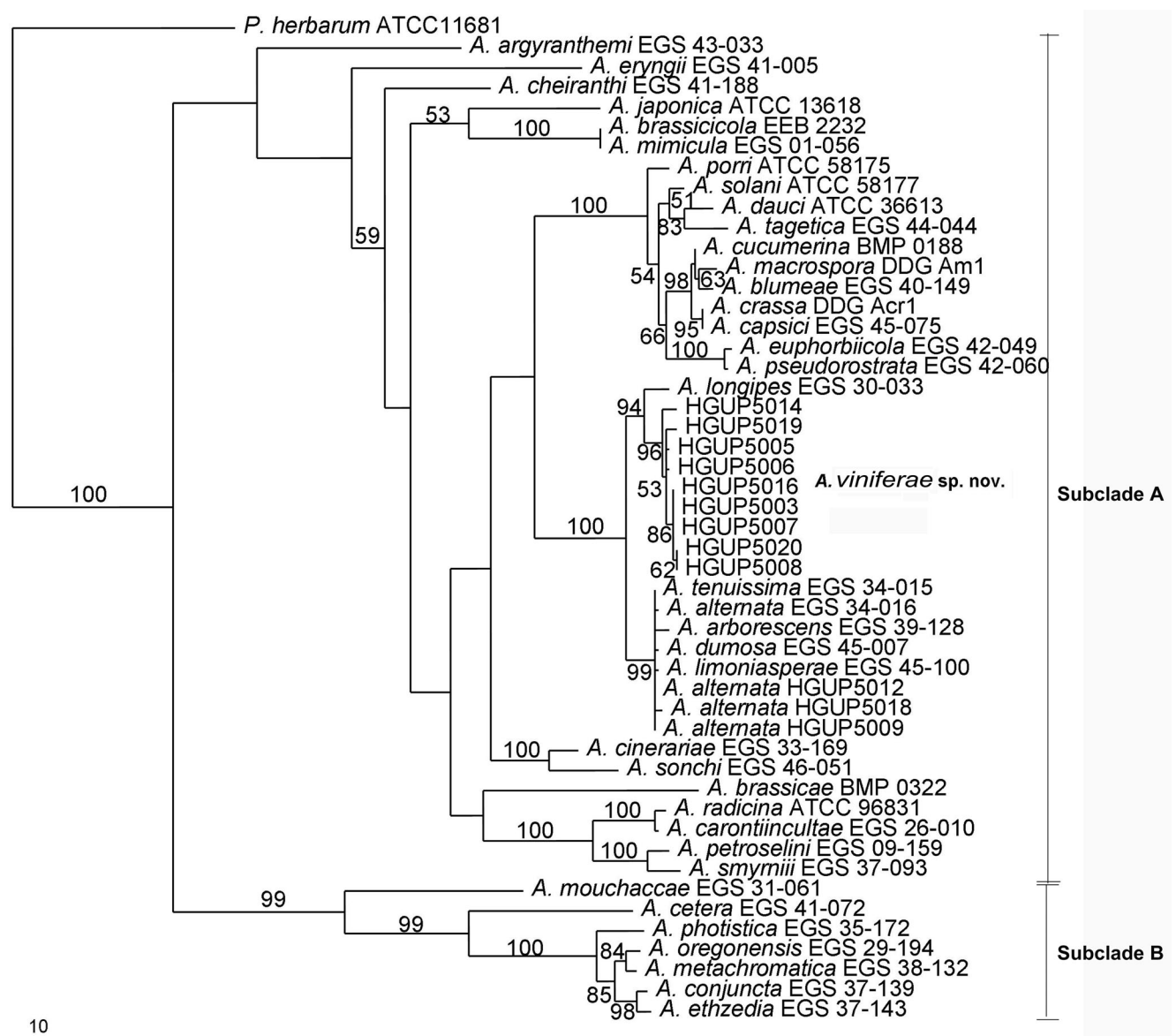


Fig 1 Phylogenetic tree generated from parsimonious analysis based on combined Alt a 1 and *gpd* sequences. Bootstrap values ≥ 50 % are indicated above the branches. The tree is rooted with *Pleospora herbarum* (ATCC 11681)

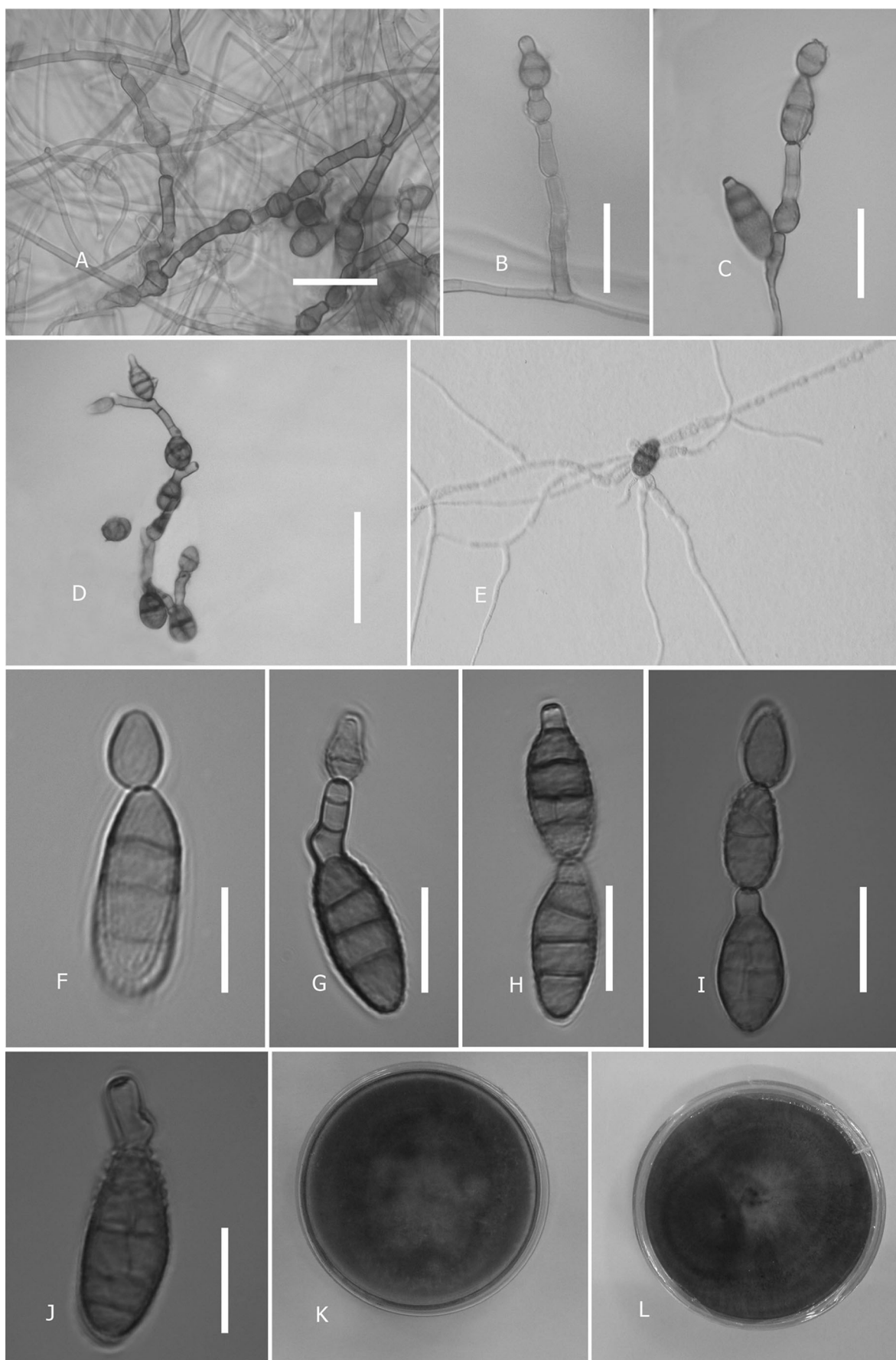


Fig 2 *Alternaria viniferae* (Holotype HGUPd5019) on PCA. **a** Conidia and conidiophores in low power lens (10×); **b–d** developing conidia from conidiophores; **e** germinating conidium; **f–j** conidia; **k, l** two-week-old cultures on PCA: **k** upper of colony, **l** reverse of colony. Scale bars (**a–b**) 20 µm, (**d**) 40 µm, (**f–j**) 10 µm

followed the method of Chomnunti et al. (2011). Single conidia from necrotic tissue fragments incubated in high humidity, were selected under a stereomicroscope and transferred to PDA and potato carrot agar (PCA; 20 g, white potato boiled and filtered, 20 g white carrot boiled and filtered, 20 g agar, 1 L distilled water) plates at 25 °C for 5–7 days with under a 16/8-h cool-white fluorescent-UV light/dark cycle (Simmons and Roberts 1993; Simmons 2007). A Nikon 80i microscope (Nikon, Japan) was employed for micro-morphological examinations. The ex-living culture is maintained at Herbarium of Guizhou University, Plant Pathology (HGUP), and CBS-KNAW Fungal Biodiversity Center (CBS).

DNA extraction, amplification and DNA sequencing

Genomic DNA was extracted from colonies grown on PDA, using the Fungal gDNA Kit GD2416 (Biomiga, CA, USA) and following the manufacturer's instructions. The primer *gpdF/gpdR* (Berbee et al. 1999) was used for *gpd* amplification, and amplification of *Alt a 1* gene was conducted using primers *Alt-for/Alt-rev* (Hong et al. 2005). Amplification reactions were performed in a Bio-RAD PTC-200 thermocycler in a 25-µl reaction mixture using the following final concentrations or total amounts: 5 ng DNA, 1× PCR buffer (20 mM Tris/HCl pH 8.4, 50 mM KCl), 1 µM of each primer, 2.5 mM MgCl₂, 0.25 mM of each dNTP, 0.5 unit of Taq polymerase. The PCR amplified DNA fragments were fractionated in 1 % agarose gels in 0.5× TBE buffer, and DNA was visualized by ethidium bromide staining and UV illumination. Sequencing was performed with an ABI PRISM 3730 DNA autosequencer using either dRhodamine terminator or Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

Preliminary nucleotide sequence alignments were constructed using Clustal X1.81 (Thompson et al. 1997). Phylogenetic analysis of combined ITS sequence and *gpd* sequence was computed using MP analysis in PAUP* (Swofford 2000). In the MP analyses, trees were inferred using heuristic search option with tree bisection reconnection (TBR) branch swapping and 1000 random sequence additions, maxtrees were 5000, branches of zero length were collapsed and all parsimonious trees were saved. Measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI), and

rescaled consistence index (RC). Bootstrap analyses (Hillis and Bull 1993) were based on 1000 replications. *Pleospora herbarum* (ATCC 11681) was selected as outgroup. The aligned sequence matrix contained *gpd+Alt a 1* data of 49 *Alternaria* isolates downloaded from GenBank (Table 1).

Results

Phylogenetic analysis

The aligned sequence matrix contained *gpd+Alt a 1* data of 49 *Alternaria* isolates downloaded from GenBank. In the combined tree, 602 characters are constant, 107 variable characters are parsimony-uninformative, and 308 characters are parsimony-informative. The 1017 characters included in the parsimony analyses yielded two most parsimonious trees (TL=1,68, CI=0.559, RI=0.803, RC=0.449, HI=0.441), one of which is presented (Fig. 1).

All 49 *Alternaria* isolates (Fig. 1) clustered together with a high bootstrap support (100 % BS). The *Alternaria* group was further divided into two subclades (A <50 %, B 99 %). Our 12 isolates were all placed in subclade A as members of *alternata* species-group supported by 77 % bootstrap value. Nine isolates (HGUP 5003, 5005, 5006, 5007, 5008, 5014, 5016,

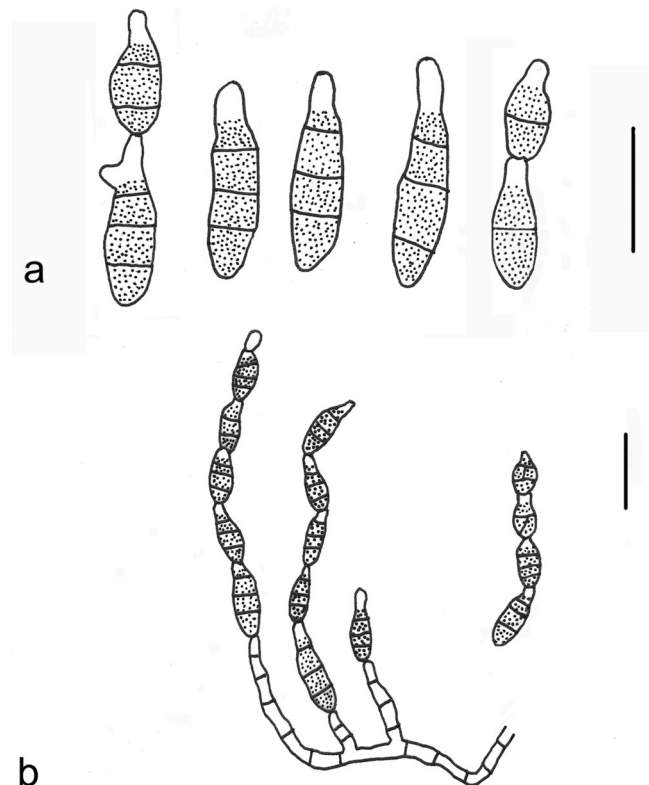


Fig 3 *Alternaria viniferae* (Holotype HGUPd5019) on PCA. **a** Conidia. **b** Conidia and conidiophores; Scale bars 20 µm

5019, 5020) showed a very close relationship with *A. longipes* (E.G.S. 30–033), with 100 % bootstrap support, and should be looked upon as one independent species. The three isolates (HGUP5009, 5012, 5018) clustered together with *A. alternata* (E.G.S. 34–016), *A. arborescens* (E.G.S. 39–128), *A. tenuissima* (E.G.S. 34–015), *A. dumosa* (E.G.S. 45–007) and *A. limoniasperae* E.G.S. 45–100 supported by a high BS (99 %). Although we believe our nine isolates, which are similar to *A. longipes*, belong to one species, below the species level they still possess some phylogenetic divergence (Fig. 1). For instance, HGUP 5014 shows a relative divergent relationship with other isolates which obtained 96 % BS.

Taxonomy

Alternaria viniferae Yong Wang bis, Y.Y. Than, K.D. Hyde, X.H. Li, sp. nov. Figs. 2 and 3.

Mycobank MB 804574

ETYMOLOGY In reference to the host from which the fungus was isolated.

Colonies on PCA fast growing, circular, with abundant grayish aerial mycelium, occasionally forming pinkish aerial mycelium towards the margin in younger colonies, with conspicuous concentric zonations of growth and sporulation and, attaining a diameter of 8.5–9 cm in 14 days at 25 °C. Hyphae subhyaline, branched, smooth, septate, 3.5–4 µm wide. Conidiophores solitary, simple, straight or flexuous, with a single or up to three terminal conidiogenous loci, up to 300 µm long, 3–5 µm wide, with a terminal scar, dark brown, pale at the tips, multi-septate. Conidiogenous cells tretric, initially terminal and later becoming intercalary through sympodial growth. Conidia solitary or in a chain of 2–5 or more, straight, narrow-ellipsoid to obclavate, tapering to a secondary conidiophore,

dull pale tan to dilute brown, with smooth outer wall, sometimes becoming verrucose at maturity, 28–40×6–12 µm including secondary conidiophores (false beak) when present, usually with 2–3 transverse and 0–1 longitudinal septa. The secondary conidiophores with 2–4 perforate geniculations, are 8 µm or less long, 2–3 µm wide.

Holotype China, isolated from grape pedicel, Hongxiang village, Gansu Province, December 2009, X.H. Li, HGUP5019 (dried culture HGUPd5019), ex-type HGUP5019, and CBS 136064, additional cultures examined: isolated from grape pedicel, Beijing, September 2011, X.H. Li, HGUP5014; isolated from grape rachis, Shandong Province, September 2011, X.H. Li, HGUP5005; isolated from grape rachis, Beijing September 2011, X.H. Li, HGUP5006; isolated from grape pedicel, Beijing, June 2010, X.H. Li, HGUP5016; isolated from grape rachis, Chengdu city, Sichuan Province, June 2011, X.H. Li, HGUP5003; ibid. HGUP5020; ibid., HGUP5008.

Discussion

The phylogenetic analysis indicated that the new species, *A. viniferae*, belongs to *alternata* species-group, all of which produce relatively short conidia 20–50 µm long (Simmons 1995, 1999a, b). The *alternata* species-group includes *Alternaria alternata*, *A. longipes*, *A. limoniasperae*, *A. dumosa*, *A. tenuissima*, and *A. arborescens* (Hong et al. 2005; Lawrence et al. 2012). In a phylogenetic study, Lawrence et al. (2012) found that *A. longipes* formed a branch independent from four other species (*Alternaria alternata*, *A. limoniasperae*, *A. dumosa*, *A. tenuissima*, and *A. arborescens*). Our nine isolates of *A. viniferae* clustered together with *A. longipes* with a moderate BS (63 %). The

Table 2 A morphological comparison of *A. grapsis* and other species in *alternata* species-group

Species	Conidia				Conidiophores (µm)
	Shape	Size (µm)	Septation	Ornamentation	
<i>A. grapsis</i>	Narrow-ellipsoid to obclavate	28–40×6–12	3–4 transverse, 0–1 longitudinal	Smooth	300 long, 3–5 wide
<i>A. alternata</i>	Elliptical	25–30 (–40)×5–9	4–7 transverse, a few or no longitudinal	Densely punctulate in juvenile conidia, becoming granular to variously verrucose as conidia mature	
<i>A. longipes</i>	Narrow-ovoid or ellipsoid	12–35 (–48)×5–12 (–14)	3–8 transverse, 0 or only 1 (–2) in 1–2 cells of narrower conidia or a few more in broader conidia	Smooth or punctate	60 long
<i>A. tenuissima</i>	Ovoid or obclavate	32–45×11–13	1–6 transverse septa and 0–2 longitudinal septa	Obscurely, to conspicuously punctulate	

other four isolates of *Alternaria* that we isolated belong to *A. alternata* sensu stricto. Conidia of *A. viniferae* have 3–4 transverse septa, whereas those of *A. longipes* have 3–8 transverse septa and *A. alternata* has 4–7 transverse septa. Conidia of *A. viniferae* are smooth, which differs from *A. limoniasperae* with punctulate to punctuate conidia, *A. tenuissima* with obscurely to conspicuously punctulate conidia, *A. dumosa* with punctulate to strongly punctate overall and *A. arborescens* with prominently punctate and verrucose conidia. A detailed comparison between *A. viniferae* and members of *alternata* species-group is provided in Table 2.

Compared with other *Alternaria* pathogens in grapevine, for example *A. vitis* and *A. viticola*, the conidia of *A. viniferae* are obviously smaller than that of *A. vitis* (40–60×12–14 µm) and *A. viticola* (25–45×10–12 µm) (Simmons 2007). In GenBank, there are only ITS sequences (JX418362 and JX418362) of these two pathogens, but not authoritative sequences. We compared our sequences with them, and they were different (data not shown). Thus, we confirm the novelty of *A. viniferae*.

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