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Research Papers

Heterosporicola beijingense sp. nov. (*Leptosphaeriaceae, Pleosporales*) associated with *Chenopodium quinoa* leaf spots

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Summary. A coelomycetous fungus with hyaline, aseptate, oblong to ellipsoidal conidia was isolated from living *Chenopodium quinoa* leaves with leaf spots, in Beijing, China. Maximum likelihood and Bayesian analyses of a combined LSU, SSU, ITS and TEF sequence dataset confirmed its placement in *Heterosporicola* in *Leptosphaeriaceae*. The new taxon resembles other *Heterosporicola* species, but is phylogenetically distinct, and is introduced as a new species. *Heterosporicola beijingense* sp. nov. is compared with other *Heterosporicola* species, and comprehensive descriptions and micrographs are provided.

Key words. DNA analyses, morpho-molecular taxonomy, pathogens.

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INTRODUCTION

Quinoa (*Chenopodium quinoa*) is a dicotyledonous pseudocereal (Vega-Galvez *et al.,* 2010) which maintains high productivity in low fertility soils and under conditions of water shortage and high salinity (Tapia *et al.,* 1997). Quinoa seeds are rich in nutrients, with high protein content including all

nine essential amino acids with high concentrations of histidine, lysine and methionine. The seeds also lack gluten and contain high amounts of several minerals (including calcium, magnesium and iron), and health-promoting compounds such as flavonoids (Dini *et al.,* 1992; Wright *et al.,* 2002). Quinoa was first introduced to Shanxi Province (China) in 2011 and transferred rapidly to other provinces, including Gansu, Jilin, Sichuan and Qinghai (Li *et al.,* 2017). With the increase in quinoa crops, the number of pests and diseases of quinoa have also increased (Li *et al.,* 2017). Among them, fungal diseases are responsible for significant production losses (Lee *et al.,* 2019). Brown stalk rot, downy mildew, gray mould, leaf spot and root rot are the major fungal diseases that affect quinoa production in China and worldwide (Valencia-Chamorro, 2003; Testen *et al.,* 2013; Li *et al.,* 2017).

Vilca (1972) described *Ascochyta hyalospora* from leaf spots of quinoa (Boerema *et al.,* 1977). The first symptoms are light spots of indefinite area on the quinoa leaves (Boerema *et al.,* 1977; Alandia *et al.,* 1979; Li *et al.,* 2017), and with time pycnidia can be observed, and the leaves become dry and fall (Li *et al.,* 2017). Leaf spots have become a rapidly increasing fungal disease in the cultivation of quinoa in China (Wang *et al.,* 2014).

Heterosporicola (as *Heterospora*) was initially considered as a section of *Phoma* by Boerema (1977). De Gruyter *et al*. (2013) raised *Heterospora* to generic rank to accommodate two species (*H. chenopodii* and *H. dimorphospora*) of *Phoma* sect. *Heterospora* that clustered in *Leptosphaeriaceae*. The current name *Heterosporicola* was proposed to accommodate *Heterospora* by Wijayawardene *et al*. (2018) as the remaining species of *Phoma* sect. *Heterospora* clustered in the family *Didymellaceae* (Aveskamp *et al.,* 2010). The sexual morph of *Heterospora* is presently undetermined (De Gruyter *et al.,* 2013 Ariyawansa *et al.,* 2015; Wijayawardene *et al.,* 2017; Hyde *et al.,* 2018).

The present study introduces a novel potential plant pathogenic *Heterosporicola* species, associated with quinoa leaf spots occurring in Mentougou and Yanging districts (Beijing) in China. A multigene-based phylogram is also presented to infer phylogenetic relationships of this species.

MATERIALS AND METHODS

Sample collections, examination and isolation

Symptomatic quinoa leaves were collected from six fields in Beijing Mentougou and Yanging districts in August and July 2018. Leaf samples placed in Zip-lock plastic bags were brought to the laboratory and incubated at room temperature (25°C). An attempt was made to obtain axenic cultures following the single spore isolation method (Chomnunti *et al*., 2014) onto potato dextrose agar (PDA) and malt extract agar (MEA). A tissue isolation method was also used in attempts to isolate fungi from diseased leaves. Small leaf pieces (0.5 × 0.5 cm) were surface sterilized (Schulz *et al*., 1993) to eliminate epiphytic fungi, and were then incubated on PDA or MEA. Three replicates from each sample were maintained.

Digital images of fruiting structures were captured with a Canon 450D digital camera fitted to a Nikon ECLIPSE 80i compound microscope. Squash mount preparations were made from conidiomata near symptomatic leaf areas. Measurements of fungus structures were made using the Tarosoft (R) Image Frame Work program, and the images used for figures were processed with Adobe Photoshop CS3 Extended v. 10.0 (Adobe®). Herbarium specimens of the new species were deposited in the Mae Fah Luang University Herbarium (MFLU) and the Beijing Academy of Agricultural and Forestry Sciences (JZB), China. Faces of fungi and Index Fungorum numbers were registered according to Jayasiri *et al*. (2015) and Index Fungorum (2020). The new species was established following the guidelines of Jeewon and Hyde (2016).

DNA extraction, PCR amplifications and sequencing

A DNA extraction kit (E.Z.N.A.® Forensic DNA kit, D3591-01, Omega Bio-Tek) was used to extract DNA from fresh fruiting bodies from fungal isolates, following the manufacturer's instructions (as conidia did not germinate on any of the media used). Extracted DNA was used for PCR reactions with the following ingredients: each amplification reaction contained 0.125 µL of 5 units μL^{-1} Ex-Taq DNA polymerase (TaKaRa), 2.5 µL of 10 × PCR buffer, 2 µL of 2 mM $MgCl_2$, 2.5 µL of 2 mM dNTPs, 1 µL of 0.2–1.0 µM primer, <500 ng DNA template, and was adjusted with double-distilled water to a total volume of 25 µL. PCR amplification and sequencing was performed of the ITS gene region using the primer pair ITS5 and ITS4 (Carbone and Kohn, 1999). The LSU, SSU and TEF gene regions were amplified and sequenced, respectively, using the primer pairs LR0R/LR5 (Vilgalys and Hester, 1990), NS1/NS4 (White *et al.,* 1990) and EF1-983F/EF1-2218R (Rehner and Buckley, 2005). The amplification profiles for all four gene regions were as follows: an initial denaturing step for 2 min at 94°C, followed by 35 amplification cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s and exten-

sion at 72°C for 90 s, and a final extension step of 72°C for 10 min (Brahmanage *et al.,* 2019). Purification and sequencing of PCR products were carried out using the above-mentioned PCR primers at Bio-med Biotech Company (Beijing, China). Sequences were checked for ambiguity, assembled and deposited in GenBank.

Phylogenetic analysis

Sequence data were compared by BLAST searches in the GenBank database at the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/nucleotide/). Initial BLAST similarity indices showed that the isolates were very similar to *Heterosporicola*. *Heterosporicola* strains were compared with other related sequences of *Leptosphaeriaceae*, following procedures of Dayarathne *et al*. (2015) and Tennakoon *et al*. (2017) (Table 1). Sequences were aligned with MAFFT v. 7.0 (Kuraku *et al.,* 2013), combined using Bioedit 7 (Hall, 1999) and refined manually. Phylogenetic trees were generated using maximum likelihood (ML) and Bayesian inference (BI). The ML trees were generated with RAxML-HPC2 on XSEDE (v. 8.2.8) (Stamatakis, 2014) in the CIPRES Science Gateway platform (Miller *et al.,* 2010), using the GTR+I+G model of evolution. Bayesian analyses were performed for both individual and combined datasets using MrBayes v. 3.0b4 (Ronquist and Huelsenbeck, 2003). Nucleotide substitution models were determined with MrModeltest v. 2.2 (Nylander, 2004). A dirichlet state frequency was predicted for all four data partitions and GTR+I+G was the best model. The heating parameter was set to 0.2 and trees were saved every 1,000 generations (Ronquist *et al.,* 2012). Posterior probabilities (PP) (Rannala *et al.,* 1998; Zhaxybayeva and Gogarten, 2002) were defined by the Bayesian Markov Chain Monte Carlo (BMCMC) sampling method in MrBayes v. 3.0b4 (Huelsenbeck and Ronquist, 2001). The resulting trees were viewed with FigTree v.1.4.0 (Rambaut, 2009) and the final layout was done using Microsoft PowerPoint (2016).

RESULTS AND DISCUSSION

Symptoms

Numerous yellowish brown to reddish brown circular spots with lighter surrounding tissues were observed on affected quinoa leaves at the initial stage of disease development. Whirls of black conidiomata and shot holes on the leaves also were observed at later stages (Figure 1).

Phylogenetic analysis

The combined LSU, SSU, ITS, and TEF sequence dataset belonging to *Leptosphaeriaceae*, with *Phoma herbarum* (CBS 615.75) as the outgroup taxon, comprised 36 taxa with 2,436 nucleotide characters. RAxML analysis of the combined dataset yielded a best tree (Figure 2) with a final ML optimization likelihood value of -8815.833844. The matrix had 438 distinct alignment patterns, with 20.53% undetermined characters or gaps. Estimated base frequencies were; A = 0.244796, C = 0.219925, G = 0.271915, T = 0.263364. Substitution rates were AC = 1.726279, AG = 2.859320, AT = 2.066497, CG = 0.464869, CT = 6.764103, and GT = 1.000000. The gamma distribution shape parameter α = 0.171569. Phylogenetic trees obtained from ML and BI were similar in topology. Phylogenetic results indicated that isolates of *Heterosporicola beijingense* clustered together in a subclade with strong support (100% ML, 1.00 PP), and closely related to *H. chenopodii* and *H. dimorphospora* (Figure 2).

Taxonomy

Heterosporicola beijingense Brahmanage & K.D. Hyde, ***sp. nov.*** **Figure 2**

Index Fungorum: IF 557214, *Facesoffungi number*: FoF 07325

Etymology: Name refers to the geographical region Beijing, China, where the species was first found.

Holotype: JZB3400001

Saprobic or *pathogenic* on leaves of quinoa (*Chenopodium quinoa*). *Leaf spots* on quinoa leaves irregular, necrotic, with conidiomata arranged in several whorls. Sexual morph: undetermined. Asexual morph: Coelomycetous. *Conidiomata* 200–600 µm wide, pycnidia immersed to semi-immersed, globose to subglobose, black and each with an inconspicuous ostiole. *Conidiomatal wall* 15–60 µm wide, composed of 3–5 layers of cells of *textura angularis*, pale yellowish brown. *Conidiogenous cells* 4–8 × 4–6 µm ($\bar{x}$ = 6 × 5 µm, n = 20), phialidic, subglobose to short conical. *Microconidia* 3.8–4.4 × 1.4–2.1 µm ($\bar{x}$ = 4.2 × 1.8 µm, n = 30) hyaline, aseptate, oblong to ellipsoidal with two to many guttules. *Macroconidia* not observed.

Material examined: CHINA, Beijing, Mentougou, on living leaves of *Chenopodium quinoa* (*Amaranthaceae*), July 2018, Rashika S. Brahmanage, LC41 (JZB3400001, holotype), *ibid*., LC43 (JZB3400002), *ibid*., Yanging district, on living leaves of *Chenopodium quinoa* (*Amaranthaceae*), July 2018, Rashika S. Brahmanage, LC44

Table 1. Taxa used in this study and their GenBank accession numbers for SSU, LSU, ITS and TEF DNA sequence data. Type strains are indicated with ^{T} and newly generated sequences are in bold.

Taxa	Strain number	GenBank accessions			
		ITS	SSU	LSU	TEF
Alloleptosphaeria italica^{T}	MFLUCC 14-0934	KT454722[a]	-	KT454714	-
Alternariaster bidentis^{T}	CBS 134021	NR159551	-	KC609341	-
Alternariaster helianthi^{T}	CBS 327.69	KC609335	KC584627	KC584369	-
Alternariaster centaureae-diffusae^{T}	MFLUCC 14-0992	KT454724	KT454731	KT454716	-
Alternariaster trigonosporus^{T}	MFLU 15-2237	NR159558	-	KY674858	-
Heterosporicola chenopodii^{T}	CBS 448.68	FJ427023	EU754088	EU754187	-
Heterosporicola chenopodii	CBS 115.96	JF740227	-	EU754188	GU349077
Heterosporicola dimorphospora	CBS 345.78	JF740203	-	GU238069	-
Heterosporicola dimorphospora	CBS 165.78	JF740204	JF740098	JF740281	-
Heterosporicola beijingense	**JZB3400001**	**MN733734**	**MN733738**	**MN737597**	**MN786372**
Heterosporicola beijingense	**JZB3400002**	**MN733735**	**MN733739**	**MN737598**	**MN786373**
Heterosporicola beijingense	**JZB3400003**	**MN733736**	**MN733740**	**MN737599**	**MN786374**
Heterosporicola beijingense	**JZB3400004**	**MN733737**	**MN733741**	**MN737600**	**MN786375**
Leptosphaeria slovacica	CBS 389.80	JF740247	JF740101	JF740315	-
Leptosphaeria doliolum^{T}	CBS 505.75	JF740205	NG062778	GU301827	GU349069
Leptosphaeria doliolum	MFLUCC 15-1875	KT454727	-	KT454734	-
Leptosphaeria ebuli^{T}	MFLUCC 14-0828	NR155323	KP753954	KP744488	-
Leptosphaeria conoidea	CBS 616.75	MH860957	JF740099	MH872726	-
Neoleptosphaeria rubefaciens^{T}	CBS 223.77	JF740243	-	JF740312	-
Neoleptosphaeria jonesii^{T}	MFLUCC 16-1442	NR152375	NG063625	KY211870	KY211872
Paraleptosphaeria macrospora	CBS 114198	JF740238	-	JF740305	-
Paraleptosphaeria nitschkei^{T}	CBS 306.51	JF740239	-	JF740308	-
Paraleptosphaeria rubi^{T}	MFLUCC 14-0211	KT454726	KT454733	KT454718	-
Paraphoma radicina	CBS 111.79	NR156556	EU754092	EU754191	KF253130
Plenodomus pimpinellae	CBS 101637	JF740240	-	JF740309	-
Plenodomus guttulatus	MFLUCC 151876	KT454721	KT454729	KT454713	-
Plenodomus salviae	MFLUCC 130219	KT454725	KT454732	KT454717	-
Pseudoleptosphaeria etheridgei^{T}	CBS 125980	NR111620	-	MH875320	-
Sphaerellopsis macroconidiale	CBS 658.78	KP170659	-	KP170727	KP170684
Sphaerellopsis hakeae	CPC 29566	NR155859	-	KY173555	-
Sphaerellopsis paraphysata	CPC 21841	NR137956	-	-	-
Subplenodomus valerianae	CBS 630.68	JF740251	KY554199	GU238150	-
Subplenodomus violicola^{T}	CBS 306.68	FJ427083	GU238231	GU238156	-
Subplenodomus galicola^{T}	MFLU 15-1863	NR154454	-	KY554199	-

CBS: Centraalbureau voor Schimmelcultures, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; JZB: Beijing Academy of Agricultural and Forestry Sciences; MFLU: Mae Fah Luang University Herbarium; MFLUCC: Mae Fah Luang University Culture Collection
[a] Sequence data from Dayarathne *et al.* (2015) and Tennakoon *et al.* (2017).

(JZB3400003), *ibid.*, LC45 (JZB3400004). Living cultures are not available.

Notes: *Heterosporicola beijingense* isolated from *Chenopodium quinoa* resembles *H. chenopodii* and *H. dimorphospora* (Van der Aa and van Kesteren, 1979, de Gruyter *et al.*, 2013). To support establishment of the new taxon as per the guidelines of Jeewon and Hyde (2016), we examined the nucleotide differences within the ITS and TEF regions. ITS base pair differences between *H. beijingense* and *H. chenopodii* were 6.5% (36 out of 550bp), and 6.9% (38 out of 550 bp) between *H. beijinense* and *H. dimorphospora*. The TEF base pair difference between *H. beijingense* and *H. chenopodii* was 2.3% (23 out of 895bp), but there were no TEF data generated from *H. dimorphospora* for comparison.

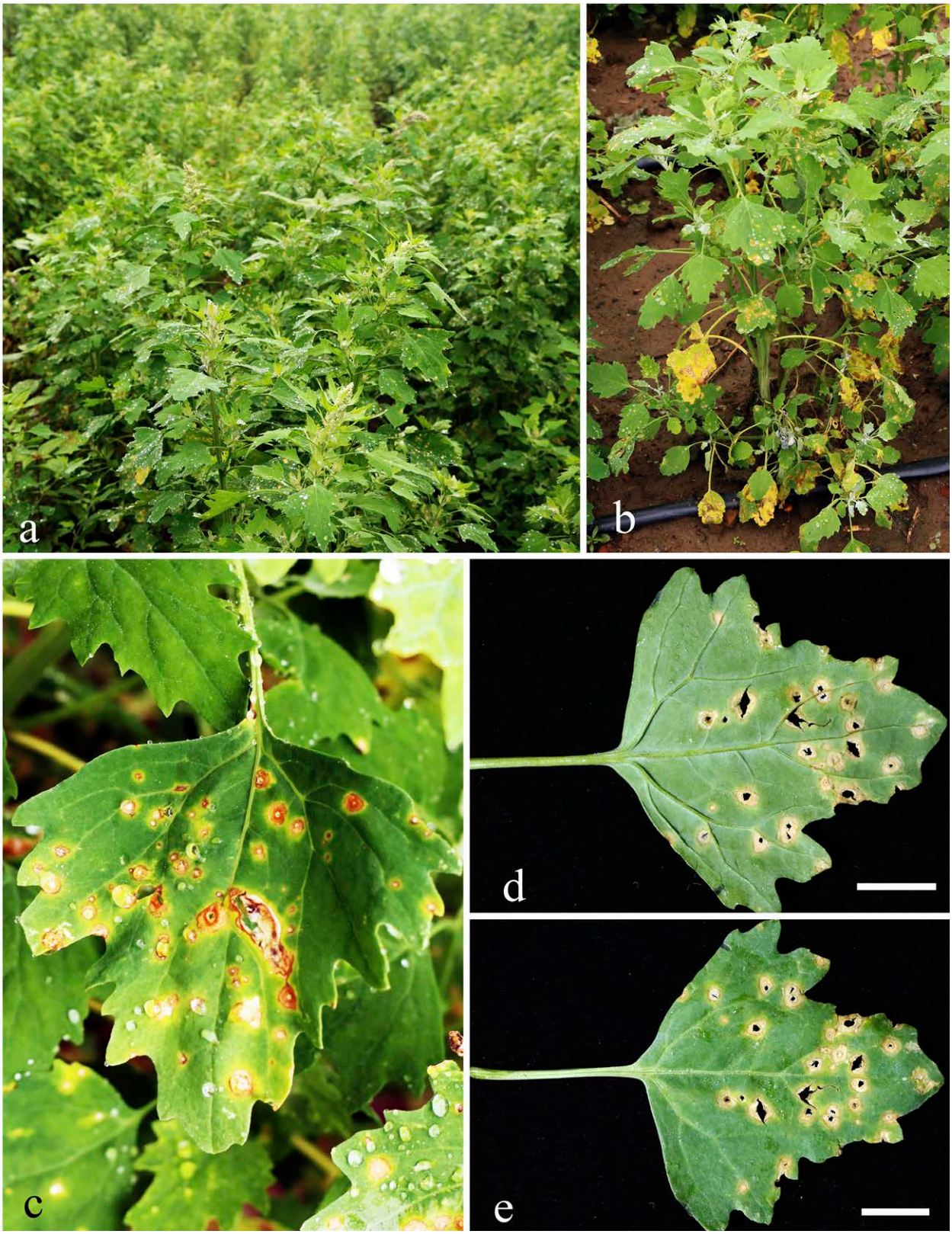

Figure 1. *Chenopodium quinoa* leaf spots. a–b, Diseased plants in the field. c, Closeup of a diseased plant. d–e, Closeup of a diseased leaf (e, upper surface, d, lower surface). Scale bars: = 1 cm.

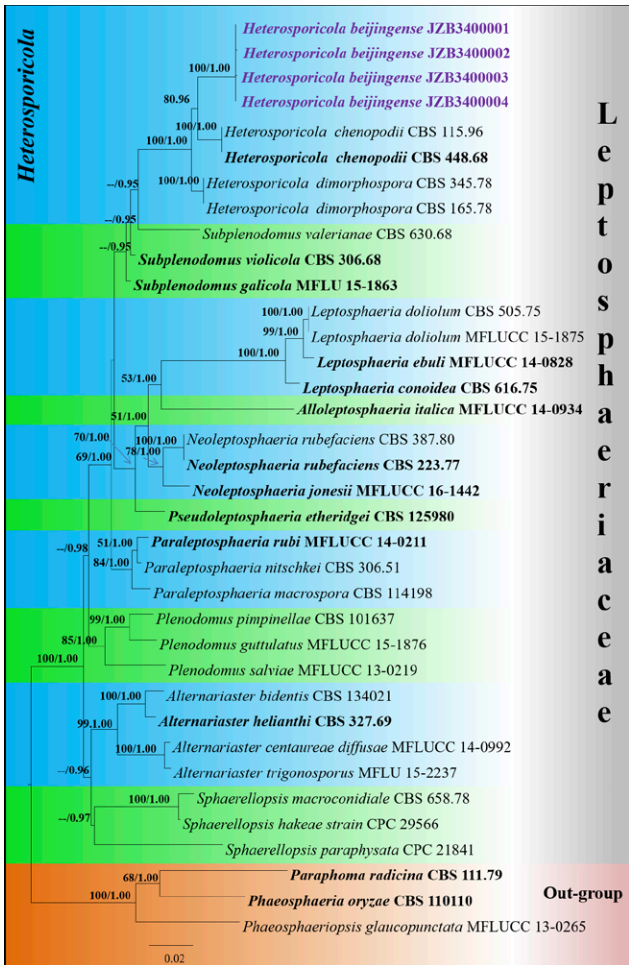

Figure 2. Maximum Likelihood tree generated by RAxML based on combined LSU, SSU, ITS and TEF sequence data from taxa of *Leptosphaeriaceae*. Bootstrap support values for ML ≥65% and Bayesian posterior probabilities >0.95 are given above each branch. Newly generated strains are in blue bold and ex-type sequences are in bold black.

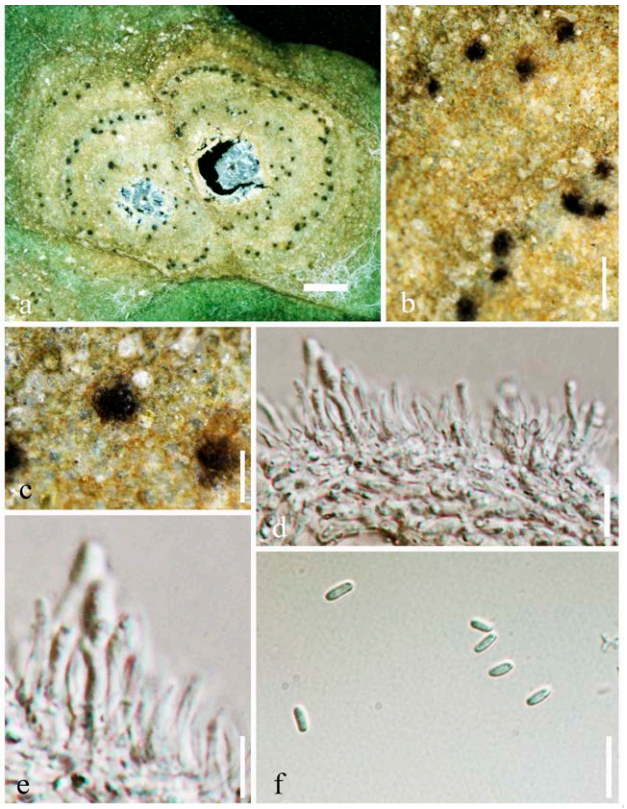

Figure 3. *Heterosporicola beijingense* on Quinoa leaves (JZB3400001 holotype). a–c, Pycnidia on leaf surface. d–e, Conidial contacts. f, Conidia. Scale bars: a = 100 µm, b = 500 µm, c = 200 µm, d-f = 10 µm.

DISCUSSION

Heterosporicola species have been reported as pathogens on *Chenopodium* species (Boerema, 1997; De Gruyter *et al.*, 2013 Alves *et al.*, 2013). Among two previously reported *Heterosporicola* species, *H. dimorphospora* is a parasite on species of *Chenopodium* in North- and South-America (van der Aa and van Kesteren, 1979). In some parts of South America, this fungus causes eye-shaped stem lesions on *Chenopodium quinoa* (van der Aa and van Kesteren, 1979). *Heterosporicola chenopodii* (= *Phoma variospora*) is a very common pathogen on species of *Chenopodium* in Europe. On account of the septate macroconidia *in vivo, H. chenopodii* is sometimes confused with *Ascochyta caulina*. *Heterosporicola* is closely related to *Subplenodomus*. No sexual morph is known for *Heterosporicola* (De Gruyter *et al.*, 2013).

The new species reported here, *H. beijingense*, produces "bird eye"-like yellowish brown to reddish brown spots in characteristic circular arrangements on living *Chenopodium quinoa* leaves. However, *Heterosporicola chenopodii* produces pale yellowish brown or whitish leaf spots with narrow purplish-brown borders mostly on *C. album*, while *H. dimorphospora* formed pale brown leaf spots or eye-shaped lesions on stems especially on *C. quinoa* (Boerema *et al.*, 1997).

Morphological differences between *H. beijingense*, *H. chenopodii* and *H. dimorphospora* are described in Table 2, and it is clear that these three species differ from one another in conidiomata, conidiogenous cell and conidium dimensions. The other two *Heterosporicola* species produce two types of conidia (macro- and micro-conidia). However, we did not observe macroconidia in *H. beijingense*.

We could not obtain axenic cultures for this species on PDA and MEA or oatmeal agar (OA), either by single spore isolation or tissue isolation methods. However, *H. chenopodii* and *H. dimorphospora* are known from cul-

Table 2. Morphological comparison of *Heterosporicola* species.

Species name	Size (μm)				References
	Conidiomata width	Conidiomatal wall	Conidiogenous cells	Conidia	
H. chenopodii	100–550 wide	6–14	4–10	Macroconidia 15–20(–27) × (3–)3.5–4.5, Microconidia 3–6 × 1.2–1.7	van der Aa and van Kesteren (1979)
H. dimorphospora	80–200 seldom up to 300 wide	10–25	3–8	Macroconidia 16.2–22.5(25) × 3.8– 4.5(7) Microconidia 4.2–5.0 × 2–2.5	van der Aa and van Kesteren (1979)
H. beijingense	200–600 wide	15–60	4–8 × 4–6	Macroconidia not observed Microconidia 3.8–4.4 × 1.4–2.1	This study

tures on different media including PDA and OA (Boerema *et al.*, 1997). *Heterosporicola beijingense* may have specific growth requirements for macroconidium production.

This study focused on identifying fungal species associated with leaf spots on quinoa, and confirmation of their identity. Data were not collected to estimate disease severity, incidence and pathogenicity of *Heterosporicola beijingense*. Pathogenicity experiments, disease severity and incidence evaluations with appropriate field trials are recommended to confirm the pathogenicity of this species.

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