Cercosporoid leaf pathogens from whorled milkweed and spineless safflower in California

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Abstract: Two cercosporoid species are respectively described from Mexican whorled milkweed (Asclepias fascicularis) and spineless safflower (Carthamus tinctorius) from California. Passalora californica represents a new pathogen on Asclepias fascicularis, while Ramularia cynarae is confirmed on Carthamus tinctorius and Cynara cardunculus (Asteraceae), and an epitype designated. Pathogenicity is also established for both pathogens based on Koch’s postulate.

Key words: ITS, Passalora, Ramularia, systematics

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INTRODUCTION

During the course of routine collecting of cercosporoid fungi on different host plants in California, two species were collected on respectively Mexican whorled milkweed (Asclepias fascicularis) and spineless safflower (Carthamus tinctorius).

Asclepias fascicularis is an erect, native perennial plant that grows upright and can reach 1 m tall. The plant grows in grasslands, pastures, roadside ditches, stream banks, and on the borders of cultivated fields. Mexican whorled milkweed contains potent neurotoxins and is often responsible for the poisoning of livestock that eat the plant. In 2010 in coastal Santa Clara County, California, milkweed plants were affected by an undescribed leaf spot disease. Initial symptoms consisted of grey-green, irregularly shaped patches on leaves. Patches expanded and turned brown as disease progressed. For severely affected leaves, most of the laminar portion of the leaf could be diseased; such leaves turned brown, twisted and curled, and dried up. Dark green to black fungal growth was consistently observed on both the adaxial and abaxial sides of the spots. Hyaline conidiophores emerged as fascicles from leaf stomata, bearing chains of hyaline conidia.

The aims of the present study were to firstly identify the pathogens associated with the leaf spot diseases on the two respective hosts, and secondly to establish pathogenicity, thereby confirming Koch’s postulates.

MATERIALS AND METHODS

Isolates

Single conidial colonies were established from sporulating conidiomata on Petri dishes containing 2 % malt extract agar (MEA; Crous et al. 2009) and incubated at 25 °C under continuous near-ultraviolet light to promote sporulation. Reference strains are maintained in the CBS-KNAW Fungal Biodiversity Centre (CBS) Utrecht, The Netherlands.

DNA isolation, amplification and phylogenetic analysis

Genomic DNA was isolated from fungal mycelium grown on MEA, using the UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Solana Beach, CA, USA) according to the manufacturer’s protocols. The primers V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & White sporulation was visible on both the adaxial and abaxial sides of the spots. Hyaline conidiophores emerged as fascicles from leaf stomata, bearing chains of hyaline conidia.

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MATERIALS AND METHODS

Isolates

Single conidial colonies were established from sporulating conidiomata on Petri dishes containing 2 % malt extract agar (MEA; Crous et al. 2009) as described earlier (Crous et al. 1991). Colonies were sub-cultured onto potato-dextrose agar (PDA), oatmeal agar (OA), synthetic nutrient-poor agar (SNA), and MEA (Crous et al. 2009), and incubated at 25 °C under continuous near-ultraviolet light to promote sporulation. Reference strains are maintained in the CBS-KNAW Fungal Biodiversity Centre (CBS) Utrecht, The Netherlands.

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Control plants were sprayed with sterile distilled water and then maintained in a greenhouse (24 to 26 ºC). Leaves of control plants were gently rubbed with sterile cotton and then handled in the same way. Sequences derived in this study were lodged at GenBank, the alignment in TreeBASE (<treebase.org/treebase/index.html>), and taxonomic novelties in MycoBank (<MycoBank.org>; Crous et al. 2004).

**Pathogenicity**

Two different approaches were followed to confirm Koch’s postulates on the respective host plants. To demonstrate pathogenicity on *Asclepias fascicularis*, diseased leaves having ample sporulation were gently rubbed against leaves of healthy potted milkweed plants. Inoculated plants, placed on top of pans containing water, were enclosed in clear plastic bags for 48 h and then maintained in a greenhouse (24 to 26 ºC). Leaves of control plants were gently rubbed with sterile cotton and then handled in the same way.

For pathogenicity tests on *Carthamus tinctorius*, a suspension of mycelial fragments was prepared. The sporeless mycelial growth from agar cultures was removed, placed in water, and then macerated with a polystyrene homogeniser (Brinkmann, New York). The resulting suspension was filtered through cheesecloth and then sprayed onto potted spineless safflower plants. Inoculated plants, placed on top of pans containing water, were enclosed in clear plastic bags for 48 h and then maintained in a greenhouse (24 to 26 ºC). Leaves of control plants were gently rubbed with sterile distilled water and then handled in the same way.

**Morphology**

Morphological descriptions are based on preparations made from host material in clear lactic acid, with 30 measurements determined per structure, using a Zeiss Axioscope 2 microscope with differential interference contrast (DIC) illumination. Colony characters and pigment production were determined per structure, using a Zeiss Axioscope 2 microscope with differential interference contrast (DIC) illumination. Colony characters and pigment production were rated according to the colour charts of Rayner (1970).

**RESULTS AND DISCUSSION**

**Phylogeny**

Approximately 1700 bases, spanning the ITS and LSU regions, were obtained from the sequenced cultures. Only the ITS sequences were used in the phylogenetic analyses. The manually adjusted ITS alignment contained 34 taxa (including the *Cladosporium brunehii* outgroup sequence) and, of the 509 characters (including alignment gaps) used in the phylogenetic analysis, 96 were parsimony-informative, 99 were variable and parsimony-uninformative, and 314 were constant. Only the first 1 000 equally most parsimonious trees were retained from the heuristic search, the first of which is shown in Fig. 1 (TL = 353, CI = 0.756, RI = 0.879, RC = 0.665). The phylogenetic tree of the ITS region (Fig. 1) shows that the obtained sequences cluster in the *Mycosphaerellaceae*, specifically in the clades called “Clade 3, *Ramularia*” (Crous et al. 2009c) and close relatives to “Clade 7, *Dothistroma*” (Crous et al. 2009c).

**Pathogenicity**

Fourteen days after inoculation of *Asclepias fascicularis*, symptoms similar to those seen in the field appeared on inoculated milkweed leaves and sporulation was observed 6 to 8 d later. The fungal sporulation was examined and found to be morphologically the same as the originally described fungus. Control plants did not develop the leaf spot disease. The experiment was repeated and the results were the same. Ten days after inoculation of *Carthamus tinctorius*, symptoms similar to those seen in the field appeared on inoculated plants and sporulation was observed 5 to 7 d later. The fungal sporulation was examined and found to be morphologically the same as the originally described fungus. Water-treated control plants did not develop the leaf spot disease. The experiment was repeated and the results were the same.

**Taxonomy**

*Passalora californica* S.T. Koike & Crous, *sp. nov.*

MycoBank MB517865

(Fig. 2)

**Etymology:** Named after the state in which it was collected, California, USA.

*Passalorae clavatae var. hansenii* similis, sed conidiis angustioribus, (32–)55–95(–180) × (4–)5–6 µm, diametro maximo in medio cellulae basalis.

**Typus:** USA: California: Santa Clara County, on leaves of *Asclepias fascicularis*, 19 July 2010, S.T. Koike (CBS H-20512 – holotype; cultures ex-holotype CPC 18389 = CBS 128857, CPC 18391 (GenBank accession numbers: ITS HQ728115 and HQ728116, for CPC 18389 and 18391, respectively).

Leaf spots irregular, frequently covering the breadth and length of the leaf, black due to profuse sporulation. *Conidiomata* amphiogenous, sporodochial, arising from stromata; stroma globose, brown, 30–100 µm wide, 10–30 µm high, giving rise to conidiophores. *Conidiophores* in dense sporodochia, brown, verruculose, frequently reduced to conidiogenous cells or with one supporting cell, 15–25 × 3–8 µm, subcylindrical, mostly straight, at times once geniculate-sinuous; under moist
conditions on underside of leaf developing further, sporodochia consisting of a mixture of short, stubby, and longer flexuous conidiophores, straight, subcylindrical, sometimes geniculate-sinuous at apex, up to 100 µm long, and 4–5 µm wide. Conidiogenous cells on reduced conidiophores terminal, integrated, brown, verrucose, 10–15 × 4–6 µm; scars apical and lateral, thickened, darkened, refractive, 1–1.5 µm diam; conidiogenous cells on elongated conidiophores terminal, subcylindrical, straight or geniculate-sinuous, medium brown, 15–35 × 4–5 µm. Conidia solitary, obconically truncate with prominent tapers in basal cell; hilum 2 µm diam, darkened, thickened, refractive, (32–)55–95(–180) × (4–)5–6 µm, (1–)3–5(–9)-septate; conidia formed on PDA

Fig. 1. The first of 1 000 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the LSU sequence alignment. The scale bar shows 10 changes and bootstrap support values > 75 % from 1 000 replicates are shown at the nodes. Novel sequences generated in this study are shown in bold and the two treated species in coloured boxes. The tree was rooted to Cladosporium bruhnei (GenBank EF679337).
and MEA became up to 300 µm long, but retained the same conidial width and characteristic obconically truncate basal cell with prominent taper.

Culture characteristics: Colonies erumpent, irregular, with sparse aerial mycelium and feathery margins, reaching up to 4 mm on all media tested after 2 wk at 25 ºC. On MEA surface umber, reverse chestnut; on PDA surface grey-olivaceous, reverse fuscous-black; on SNA surface rust, with red crystals forming in agar, reverse umber; on OA surface grey-olivaceous, with red pigment diffusing into agar.

Notes: Several species of Passalora have been described from Asclepias that need to be compared to P. californica. Passalora clavata var. clavata has olivaceous to olivaceous-brown, 1–6-septate conidia, 20–80 × (3–)4–6(–7) µm, thus smaller than those of P. californica. Passalora clavata var. hansenii (on Asclepias syrica, California), is similar to P. californica in having 1–14-septate conidia, 15–100(–180) × (4–)5–8(–10) µm, but differs in having conidia that are wider (4–)5–8(–10) µm, being widest in the second or third basal cell, not in the middle of the basal cell as in P. californica, which has a prominently abrupt basal taper (basal cell abruptly obconic, versus long obconically truncate basal region in P. clavata var. clavata and P. clavata var. hansenii; Braun & Mel'nik 1997). Other species on this host include P. elaeochroma, which has strongly geniculate-sinuous conidiogenous cells and loosely fasciculate, frequently branched conidiophores (conidia 20–90 × 4–6.5 µm, 1–7-septate), and P. venturioides, which has secondary hyphae with solitary conidiophores, diffuse leaf spots, and shorter conidia, 20–80(–100) × 4–7 µm, 1–10-septate (Braun & Mel'nik 1997). The two cultures of P. californica sequenced in this study differed with one nucleotide in their second internal transcribed spacer region and were distantly related to, amongst others, Mycosphaerella arachidis (GenBank EF157739) and Phaeoramularia dissiliens (GenBank AF222835) (Fig. 1).

Ramularia cynarae Sacc., Michelia 1: 536 (1879).

Synonyms: See Braun (1998).

(Fig. 3)
Leaf spots amphigenous, medium brown, with pale brown centre, subcircular to somewhat ellipsoid, up to 14 mm diam. Mycelium internal and external, consisting of hyaline, smooth, branched, septate hyphae, 1.5–2 µm wide. Conidiophores fasciculate, amphigenous, subcylindrical in loose fascicles, arising from brown stomata, erect, geniculate-sinuous, 0–4-septate, hyaline, smooth, 60–120 × 2–3 µm. Conidiogenous cells terminal, unbranched, smooth, hyaline, subcylindrical, 20–50 × 2–3 µm; scars terminal and lateral, thickened, darkened and somewhat refractive, 1 µm diam. Conidia hyaline, smooth, with obconically truncate ends.

Ramoconidia subcylindrical to obclavate, (15–)20–25(–45) × (3–)3.5–4 µm, 1(–3)-septate, with 1–3 subdenticulate hila that are thickened, darkened and somewhat refractive, 1–1.5 µm diam. Intercalary conidia aseptate, ellipsoid-ovoid, (7–)9–11(–15) × (3.5–)4 µm, 0(–1)-septate; terminal conidia aseptate, ellipsoid-ovoid, apex rounded to obconically truncate, base obconically truncate, (5–)7–9(–10) × 3–4 µm; hila thickened, darkened, somewhat refractive, 1 µm diam. When sporulating on SNA, ramoconidia were (12–)17–22(–32) × (2.5–)3–4 µm, intercalary conidia (7–)8–10(–13) × 4–5(–6) µm, and terminal conidia 7–9 × 4–5 µm.

Fig. 3. Ramularia cynarae (CBS H-20513). A. Leaf spots on artichoke (Cynara cardunculus). B. Conidiophore fascicles on leaf surface. C–F. Branched conidial chains in vivo. G–K. Conidiophores and branched conidial chains on SNA. Bars = 10 µm.
Culture characteristics: Colonies erumpent, spreading, surface folded, with sparse to moderate aerial mycelium, and smooth, lobate margins, reaching 15–20 mm after 2 wk at 25 °C. On MEA surface smoke-grey, reverse iron-grey; on PDA surface pale olive-grey in middle, olive-grey in outer region, iron-grey underneath; on OA white in middle (due to profuse sporulation), olive-grey in outer region.

Specimens examined: France: Saintes: Brunaud, on Cynara scolymus, herb. Saccardo in PAD, holotype. USA: California: Santa Clara County, Morgan Hill, on leaves of Carthamus tinctorius, 19 Oct. 2010, S.T. Koike (CBS H-20513, cultures CPC 18725, 18726 = CBS 128779 (GenBank accession number: HQ728118); Monterey County, Castroville, on leaves of Cynara cardunculus, 10 Aug. 2010, S.T. Koike (CBS H-20514 – epitypus hic designatus; cultures ex-epitype CPC 18427, 18426 = CBS 128912; GenBank accession number: HQ728117).

Notes: Based on a recent revision of the genus Ramularia (Braun 1998), two species were accepted on Carthamus, namely R. cynarae and R. cercosporelloides (a nom. nov. for Cercosporella carthami, see Braun 1998). Ramularia cercosporelloides is distinct in having obovoid (–subclavate), cylindrical conidia, that are longer and wider, (15–)20–45(–7) µm, than the range observed in the present collections, conidia being (5–)10–35 × (1.5–)2–5(–7) µm) (Braun 1998). Isolates obtained from Cynara in the present study were found to be identical in morphology and on their ITS DNA sequences to isolates from Carthamus, supporting the hypothesis that R. cynarae is a wide-range pathogen on Asteraceae, rather than a species complex (Braun 1998). A species of Ramularia identified as R. carthami has previously been reported in California on agronomic safflower grown for oil production in northern, inland counties in 2005 (Hostert et al. 2006). Given our present knowledge of the Ramularia complex occurring on Asteraceae, this record will have to be re-confirmed, as it may represent R. cynarae.

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