Mycoscience (2009) 50:179–189
DOI 10.1007/s10267-008-0471-z

FULL PAPER

Daisuke Kurose · Harry C. Evans · Djamila H. Djeddour
Paul F. Cannon · Naruto Furuya · Kenichi Tsuchiya

Systematics of *Mycosphaerella* species associated with the invasive weed *Fallopia japonica*, including the potential biological control agent *M. polygoni-cuspidati*

Received: July 22, 2008 / Accepted: December 3, 2008

Abstract Native to Japan, *Fallopia japonica*, most frequently referred to as Japanese knotweed, is a highly problematic invasive weed, particularly in the UK and North America. During surveys for natural enemies of this plant in Japan, two species of *Mycosphaerella* were collected. One of these was identified as *M. polygoni-cuspidati*, and is redescribed and neotypified. Causing a damaging leaf spot disease of *F. japonica* throughout its natural range in Japan, it is absent from the host's exotic range. The restriction of *M. polygoni-cuspidati* to *F. japonica* in its center of origin, together with its severe impact on host fitness, indicates that this is a coevolved natural enemy with high potential as a classical biological control agent for the long-term management of this ecologically and economically important weed. In the field, the fungus has a reduced life cycle, with only spermogonia and pseudothecia (ascomata) being formed. Ascospores are the primary source of infection, and studies show that the mycelium from in vitro cultures is also infective and hyphae penetrate mainly via the stomata. A further, undescribed species of *Mycosphaerella* co-occurs with *M. polygoni-cuspidati*, here proposed as the new species *M. shimabarensis*. Both species have been studied using cultural, morphological and molecular phylogenetic methods.

Key words Classical biological control · Fungal life cycle · Japanese knotweed · Molecular systematics · New species

Introduction

Fallopia japonica (Houtt.) Ronse Decr., commonly known as Japanese knotweed in the UK and "itadori" in Japan, is a dioecious, rhizomatous, perennial herb belonging to the family Polygonaceae. It is also cited in the literature as *Reynoutria japonica* Houtt. (Beerling 1991) or, more frequently, as *Polygonum cuspidatum* Sieb. & Zucc. (Kitamura and Murata 1961; Makino 1961, 2000; Zhou et al. 2003). In Japan, this species occurs from sea level to mountain tops where it is a pioneering colonizer of disturbed volcanic habitats, as well as of riparian ecosystems (Zhou et al. 2003; Kurose et al. 2006). It can also be found in urban and agricultural situations; along roadsides and railways; on embankments in rice fields and in orchards; but it is only rarely described as invasive or troublesome (Makino 2000; Kurose et al. 2006).

Several distinct varieties have now been recognized (Bailey and Conolly 2000), but it is only *F. japonica* var. *japonica* – with a natural range restricted to southern Honshu, Kyushu and Shikoku Islands – that has become weedy and invasive (Kurose et al. 2006). This invasiveness has become especially problematic in Europe and North America, imposing a significant financial burden on urban developers and on land, water, and transport managers alike, as well as posing a threat to biodiversity. Shoots from the tenacious rhizomes can break through gravel, tarmac, and even concrete (Beerling 1991), while the dense foliage (up to 2–3 m in height) can outcompete most native herbs, particularly in riparian habitats (Child and Wade 2000). In such situations, flooding risks are increased as the monocultures die back in winter, exposing river banks to erosion. Japanese knotweed has been described as the most feared plant in the British Isles (Mabey 1998), and an official study by the UK Governments Department for Environment, Food and Rural Affairs (DEFRA) has estimated that it would cost upward of £1.5 billion to bring this weed under control in the UK alone (Kurose et al. 2006). In Germany, annual costs for knotweed control and subsequent restoration of waterways and watercourses have been put at almost €30 million; yearly control along only 1% of the total railway system has been calculated at €2.4million (Reinhardt et al. 2003). It is not surprising, therefore, that alternative and more sustainable or long-term methods for management of this weed are now being considered.

D. Kurose · N. Furuya · K. Tsuchiya (✉)
Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
Tel. +81-92-642-2834; Fax +81-92-642-2834
e-mail: kentsuch@agr.kyushu-u.ac.jp

H.C. Evans · D.H. Djeddour · P.F. Cannon
CABI Europe-UK Egham, Egham, Surrey, UK

This article focuses on redescription and illustration of *Mycosphaerella* species causing leaf spots on and collected from *F. japonica* throughout its natural range in Japan, which forms part of a collaborative research program to evaluate natural enemies as classical biological control agents for management of this invasive alien weed in the UK.

Materials and methods

Collection and isolation of materials

Surveys were undertaken in Japan from 2000–2007 throughout the natural range of Japanese knotweed and its close relatives on the islands of Honshu, Kyushu, and Shikoku, concentrating on Kyushu, from where the original clonal biotype of *F. japonica* var. *japonica* was introduced into Europe (Bailey and Conolly 2000). Leaf samples with disease symptoms and freshly fallen leaves were dried in a plant press for 3–5 days, with daily changes of paper, and then transferred to wax packets. Initially, all material was hand-carried or sent under license to high containment quarantine facilities at CABI Europe (UK, Ascot), in which all the experimental work was undertaken. Later, material was also retained at Kyushu University for processing.

Leaf spots were examined with a binocular microscope and hand sections made to determine the spore stages present. For direct isolation, leaf pieces (approximately 1 cm^2) were excised with a scalpel from the periphery of the lesions, immersed in 5% sodium hypochlorite for 5 min, washed three times in sterile distilled water (SDW), and transferred to Petri dishes (5-cm diameter) containing either tap water agar (TWA), malt extract agar (MEA), or potato carrot agar (PCA). All media recipes can be found in Waller et al. (2001). When mature ascomata were identified, leaf pieces were excised as above, immersed in SDW overnight, and then attached with white Vaseline to the lower surface of a Petri dish lid, face downward over TWA, and placed in an incubator at 20°–22°C to await ascospore discharge. Blocks of agar containing ascospores were selected, using a binocular microscope and a hypodermic needle, and transferred to PCA. Single-spore isolations were also made using a dilution and streaking method (Waller et al. 2001).

Inoculation

Young leaves of the UK clonal biotype of Japanese knotweed were inoculated by removing discharged ascospores from TWA dishes with a fine paint brush, mixing these with SDW containing 0.1% Tween 80, and then brushing the suspension onto both leaf surfaces. Inoculated plants were placed in a customized dew chamber (Mercia Scientific, Birmingham, UK) set at 20°C, without light for 48 h. In later infectivity tests, the ascospore inoculum was replaced by suspensions containing finely chopped mycelium excised from actively growing PCA cultures.

All plants were maintained in individual, ventilated, clear plastic boxes within a controlled temperature (CT) room set at 20°C and with 12-h dark/12-h light regimen.

Phenotypic characterization

For microscopic examination, fruiting bodies were mounted in lactophenol cotton blue or lactofuschin. Line drawings were made with a drawing tube linked to a Nikon Optiphot-2 and images taken with a Nikon Coolpix digital camera.

For cultural characteristics, four isolates were compared. Plugs of agar (5 mm diameter) were removed with a cork borer from actively growing cultures and transferred to 5-cm-diameter Petri dishes containing PCA, and incubated at 15°, 20°, 22.5°, 25°, and 30°C with either a 12-h light/dark regimen or in complete darkness. Colony diameter was measured at 4 weeks after inoculation, with ten replicates per treatment. To determine if mature ascomata could be produced in vitro, selected isolates were also grown at a range of temperatures on other media – cornmeal agar (CMA), oatmeal agar (OA), potato dextrose agar (PDA), and vegetable juice agar (V8) – which, according to the literature, had been successful with related *Mycosphaerella* spp. (Snyder 1946; Goetz et al. 1993).

Genotypic characterization

DNA isolation and PCR amplification

Mycelia grown on PCA at 20°C were harvested after 1 month. Genomic DNA was extracted from mycelia with the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the supplier's protocols. The internal transcribed spacer (ITS) region including the 5.8S rDNA were amplified as a single fragment using the polymerase chain reaction (PCR) with *Taq* DNA polymerase and with the primer pairs ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). Optimal conditions for PCR were determined with a 50-µl reaction volume containing 5.0 µl 10× *Taq* buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl and 20% Triton X-100), 1.25 U *Taq* polymerase, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1.0 µl DNA template solution, and 0.2 µM each primer. Amplification of the desired fragment was performed with a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 5 min initial denaturation at 96°C, 36 cycles of denaturation for 45 s at 94°C, annealing for 30 s at 50°C, and extension for 1.5 min at 72°C; the reaction was terminated after a final extension at 72°C for 8 s.

DNA sequencing and phylogenetic analysis

PCR products were purified using the QIAquick PCR purification kit (Qiagen), following the manufacturer's protocol and prepared for sequencing using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Bio-

Table 1. List of the strains used in the molecular phylogenetic analysis with sequence accession numbers

Fungal name	Isolate no.[a]	Host plant	Country of origin	GenBank accession no.
Chaetothyriales sp.	IMI 401915	*Fallopia japonica*	Japan	**AB434914**[b]
Davidiella macrospora	IMI 147030	*Iris* sp.	United Kingdom	**AB435067**
D. tassiana	ATCC 66670	CCA-treated Douglas-fir pole	USA	AY251078
Dissoconium aciculare	STE-U 1534	*Medicago* sp., associated with *Erysiphe* sp.	Germany	AF173308
Mycosphaerella africana	CBS 680.95	*Eucalyptus viminalis*	South Africa	AY626981
M. arachidis	–[c]	*Arachis hypogaea*	–	AF297224
M. aurantia	CBS 110500	*Eu. globulus*	Australia	AY725531
M. berkeleyi	IMI 169888	*Ar. hypogaea*	Trinidad and Tobago	**AB435066**
M. brassicicola	CBS 174.88	*Brassica oleracea*	Germany	EU167607
M. capsellae	CPC 11677	*Draba nemorosa* var. *hebecarpa*	South Korea	DQ303091
M. communis	CBS 110747	*Eu. nitens*	South Africa	AY725535
M. delegatensis	IMI 362252	*Eu. camaldulensis*	Ethiopia	**AB435070**
M. fragariae	CBS 298.34	*Fragaria* sp.	Netherlands	AY152596
M. graminicola	IMI 190859	x *Triticale*[d]	Ethiopia	**AB435068**
M. graminicola	CBS 100330	*Triticum aestivum*	Netherlands	AY152601
M. hedericola	CBS 441.86	*Hedera helix*	France	AY490772
M. lateralis	CPC 11732	*Eu. globulus*	Spain	DQ302974
M. mori	IMI 356555	*Morus alba*	India	**AB435069**
M. polygoni-cuspidati	IMI 393527	*F. japonica*	Japan	**AB434907**
M. polygoni-cuspidati	IMI 395027	*F. japonica*	Japan	**AB434908**
M. polygoni-cuspidati	IMI 401910	*F. japonica*	Japan	**AB434909**
M. polygoni-cuspidati	IMI 401913	*F. japonica*	Japan	**AB434910**
M. polygoni-cuspidati	IMI 395028	*F. japonica*	Japan	**AB434911**
M. polygoni-cuspidati	IMI 401912	*F. japonica*	Japan	**AB434912**
M. pseudoendophytica	CMW 9098	*Eucalyptus* sp.	South Africa	AF468874
M. punctiformis	CBS 113265	*Quercus robur*	Netherlands	EU167569
M. pyri	CBS 100.86	*Pyrus communis*	Netherlands	EU167606
M. rubella	CBS 288.49	*Angelica sylvestris*	–	AY490767
M. shimabarensis	IMI 401914	*F. japonica*	Japan	**AB434913**
M. sumatrensis	CPC 11178	*Eucalyptus* sp.	Indonesia	DQ303050

[a] IMI, International Mycological Institute, CABI Europe-UK Egham, Bakeham Lane, Egham, UK; ATCC, American Type Culture Collection, Virginia, USA; STE-U, culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CPC, culture collection of Pedro Crous, housed at CBS; CMW, culture collection of Mike Wingfield, housed at FABI, Pretoria, South Africa

[b] Accession numbers in bold denote sequences obtained as part of this study

[c] Not known

[d] A hybrid of wheat (*Triticum* sp.) and rye (*Secale* sp.)

systems, Foster City, CA, USA) with the same primers used for PCR amplification under the following conditions: 1 cycle of 96°C for 1 min, 25 cycles of 96°C for 10 s, 53°C for 5 s, 60°C for 4 min. Cycle sequencing reaction products were finally purified using a BigDye XTerminator Purification Kit (Applied Biosystems), and then sequenced using an ABI 3730xl DNA Analyzer (Applied Biosystems).

Sequence alignments were generated using programs in Clustal W (Thompson et al. 1994). The aligned sequences were analyzed by the neighbor-joining (NJ) method (Saitou and Nei 1987), using MEGA4.0 (Tamura et al. 2007). Distances were computed using a two-parameter distance method (Kimura 1980) and were then used to construct a NJ phylogenetic tree using MEGA4.0. Bootstrap NJ analyses were carried out with 1000 replicates.

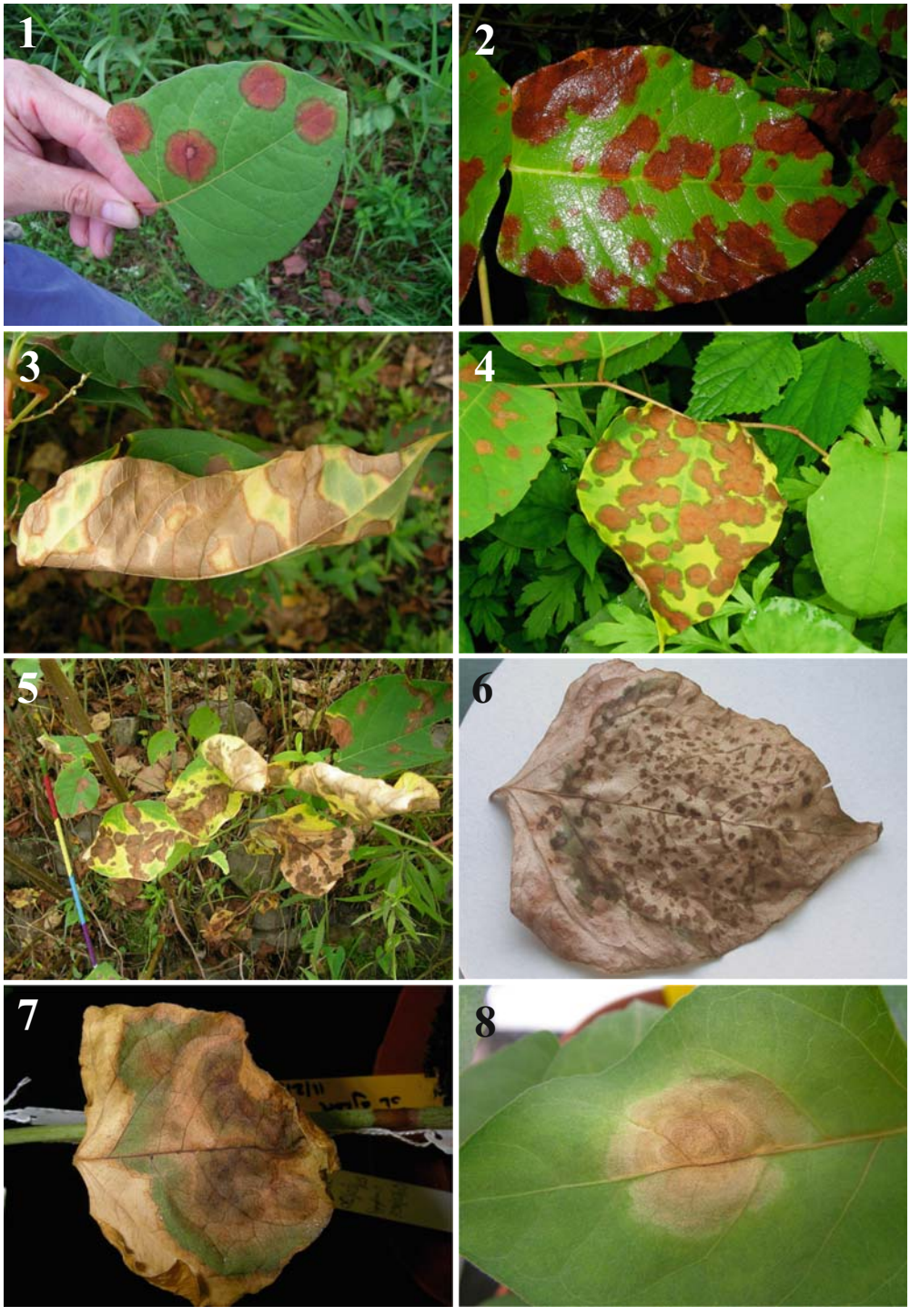
Sequence data determined in this article were deposited in DDBJ (DNA Data Bank of Japan, National Institute of Genetics, Mishima, Shizuoka, Japan) and GenBank (Table 1). For comparison with other species, sequence data of 17 isolates were obtained from GenBank. The alignment has been sent to TreeBASE.

Results and discussion

Surveys

Leaf spot caused by *Mycosphaerella polygoni-cuspidati* Hara (1918) proved to be common and invariably damaging wherever *F. japonica* was found during the surveys in Japan.

Lesions on the upper leaf surface were chestnut brown and circular, surrounded by a darker, water-soaked halo (Fig. 1); elongated and coalescing in older infections (Fig. 2); lesions on the lower surface were pale brown with a prominent grayish-brown halo and whitened veins (Fig. 3). Heavy infection resulted in widespread leaf necrosis (Fig. 4), leaf distortion, and premature defoliation (Fig. 5). The most common fructifications observed were black, immersed to semi-erumpent spermogonia, usually solitary, scattered or densely gregarious, sometimes in concentric rings within the lesion and around it in the still-green leaf tissues, on both sides of the leaf but predominantly on the upper leaf



Figs. 1–5. Disease symptoms of *Fallopia japonica* infected with *Mycosphaerella polygoni-cuspidati* in the field. **1** Circular, chestnut-brown lesion, with darker water-soaked halo (taken at Mt. Hiko on 5 July 2005). **2** More elongated and coalescing older lesions (taken at Mt. Hiko on 7 July 2005). **3** Lesion reverse exposed to show pale brown coloring with grayish halo and whitened veins (taken at Mt. Hiko on 5 July 2005). **4** Heavy infection and pronounced necrosis (taken at Mt. Hiko on 30 June 2003). **5** Distorted leaves with premature senescence (taken at Omura on 7 July 2005)
Figs. 6–8. Symptoms on *Fallopia japonica* following artificial inoculation with cultured mycelium of *Mycosphaerella polygoni-cuspidati*. **6** Heavy infection showing "hypersensitive" reaction with discrete spotting. **7** Leaf distortion and chlorosis 2 months after inoculation. **8** Development of spermogonia in lesion

surface. Ascomata or pseudothecia were encountered less frequently, appearing larger and more erumpent than the spermogonia; rarely grouped in ascostromata, and most were immature. A high proportion of the fructifications, on both attached and fallen leaves, were eaten out and appeared to be targeted at an early stage in their development by fungivorous arthropods.

The localities in which mature ascospores were positively identified are listed in Table 2. The hosts involved were all *F. japonica* var. *japonica*. However, similar leaf spots were also collected on *F. japonica* var. *compacta* (Hook. f.) J.P. Bailey and *F. japonica* var. *uzenensis* (Honda) Yonek. & H. Ohashi from high-altitude sites in Honshu Island.

Another species of *Mycosphaerella* was found on freshly fallen leaf samples of native *F. japonica* plants, which had smaller asci and ascospores and which grew more rapidly in culture. This species is described below as a new species, *M. shimabarensis*. In addition, a black yeast-like anamorphic fungus with curved filiform conidia was recovered from

Table 2. Data on herbarium collections of *Mycosphaerella polygoni-cuspidati*[a] from *Fallopia japonica* var. *japonica* in Japan

IMI no.	Locality	Altitude (m)	Collection date
393531	Chigouchi, Hiroshima	650	5 Sep. 2000
401908	Yamaguchi River, Yamaguchi	110	5 Sep. 2000
401911	Abu River, Yamaguchi	380	5 Sep. 2000
401964	Mt. Fugen-dake, Nagasaki	900	4 Oct. 2003
401965	Mt. Aso, Kumamoto	680	14 Jul. 2004
401970	Omura, Nagasaki	250	17 Jul. 2004
401966	Mt. Ichifusa, Miyazaki	500	20 Jul. 2004
401967	Takeo, Saga	45	17 Jul. 2004
401968	Mt. Hiko, Fukuoka	488	1 Sep. 2004
393527	Kami-Naka, Kochi	390	3 Jul. 2005
393528	Omura, Nagasaki	250	7 Jul. 2005
401910	Yamaguchi, Yamaguchi	90	10 Jul. 2005
393529[b]	Mt. Hiko, Fukuoka	488	5 Jul. 2005
395027	Nishinagano, Hiroshima	180	10 Jul. 2005
401913	Aso-Kuju National Park, Oita	910	5 Jul. 2005
401972	Shimabara, Nagasaki	350	8 Jul. 2005
395028	Omura, Nagasaki	250	10 Aug. 2005
401969	Mt. Hiko, Fukuoka	488	28 Sep. 2005
395029	Mt. Hiko, Fukuoka	488	24 Aug. 2006
401912	Mt. Hiko, Fukuoka	488	26 Sep. 2007

[a] Confirmed by presence of mature ascomata
[b] Neotype designated in this paper

cultures derived from one collection of *M. polygoni-cuspidati*, which was originally thought to be its anamorph. However, molecular analysis showed that the fungus was unrelated to *Mycosphaerella*, with probable affinities to the *Chaetothyriales*, and it may be a parasite of the *Mycosphaerella*. It was designated as an outgroup in the phylogenetic tree.

Pathogenicity of *Mycosphaerella polygoni-cuspidati*

Ascospores began to be discharged 10–12 h after the preconditioned leaf samples were placed in the dew chamber. In most cases, however, more prolonged incubation was necessary before spore shadows appeared on the agar surface. Maturation of the ascomata was successional as spores continued to be produced for up to 5 days. Such "conditioned" leaf samples were air-dried, stored, and reused as a source of inoculum. Leaves inoculated with ascospore suspensions did not show symptoms up to 15 days after inoculation. The average time to symptom expression (incubation time) was 16–20 days, although this varied considerably between and within isolates. For example, in three successive inoculations with strain IMI 393527, undertaken in July, August and September 2005, the incubation times were 36, 27 and 16 days respectively. Symptoms first appeared in the form of discrete, chocolate-brown spots. In heavily-infected leaves, the spots developed slowly and remained small (Fig. 6), often followed by premature senescence; this is interpreted as a hypersensitive reaction caused by overinoculation. Symptom expression was essentially similar to that observed in the field, often accompanied by leaf distortion and general chlorosis (Fig. 7). Spermogonial production was highly variable, but could be enhanced by placing infected plants in a dew chamber for several days (Fig. 8). Mature ascomata were never observed.

Spermatia failed to germinate on TWA. Mycelium from cultures of IMI 401965, IMI 395028, IMI 395027, and IMI 401969 proved to be infective, with the incubation period from inoculation to symptom expression being 16, 21–37, 21, and 15 days, respectively. Severe leaf spotting and heavy leaf fall were features of the mycelial inoculations.

Cultural characteristics

Cultures from ascospores of most isolates of *M. polygoni-cuspidati* developed slowly, measuring 12–19 mm in diameter after 4 weeks on MEA at 20°–22°C, and were grayish brown with a black reverse. Groups of spermogonia, initially white but becoming dark brown to black, developed after 1–2 months, but production was highly variable both within and between isolates. Droplets containing spermatia were produced by a small percentage of the spermogonia, but ascomata were never observed on any of the media tested during the study.

Mycosphaerella shimabarensis had different colony characteristics to the isolates of *M. polygoni-cuspidati*, being faster growing (measuring 23–25 mm after 4 weeks) and greenish gray (Fig. 9A,C). This difference was also reflected in the controlled temperature experiment on PCA where *M. shimabarensis* grew significantly faster at temperatures between 15°C and 25°C, although growth was severely curtailed at the higher temperature (30°C) compared to the other isolates (Fig. 9A–C). There were also differences in the temperature optima, ranging from 20° to 25°C for *M. shimabarensis*, while it was a narrower range (22.5°–25°C) for *M. polygoni-cuspidati* (Fig. 9C).

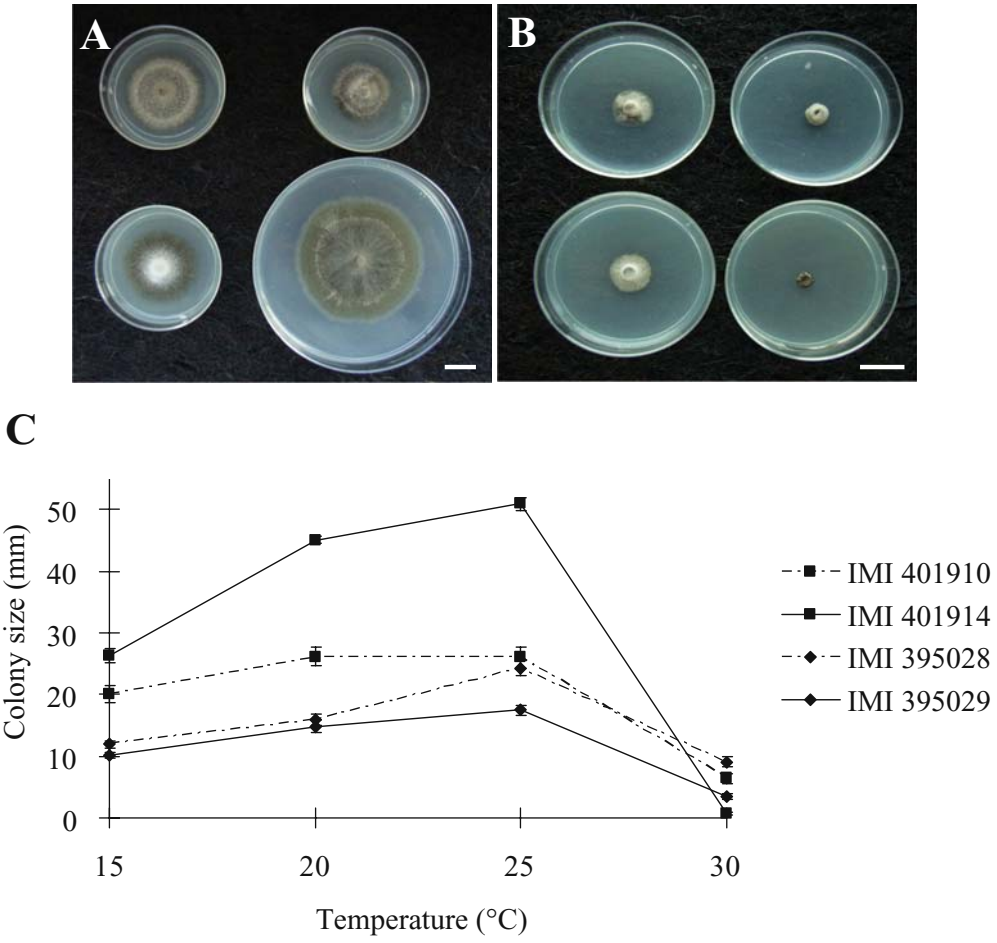


Fig. 9. The influence of temperature on the colony growth of four *Mycosphaerella* strains isolated from *Fallopia japonica* in Japan after 4 weeks under a 12-h light/12-h dark regimen. **A** 25°C. **B** 30°C. **A, B** *Top left*: *M. polygoni-cuspidati* (IMI 395028); *top right*: *M. polygoni-cuspidati* (IMI 395029); *bottom left*: *M. polygoni-cuspidati* (IMI 401910); *bottom right*: *M. shimabarensis* (IMI 401914). *Bars* **A**, **B** 1.5 cm. **C** Graphic representation of colony growths for IMI 401910, IMI 395028, IMI 395029 (*M. polygoni-cuspidati*), and IMI 401914 (*M. shimabarensis*)

Table 3. Morphological data of herbarium collections of *Mycosphaerella* species on *Fallopia japonica* from different regions of Japan

Species	Herbarium collection	Ascus		Ascospore		Spermatium
		Range	Mean	Range	Mean	
M. polygoni-cuspidati	Original[a]	40–50 × 6–9	ND	11.0–15.0 × 3.0–3.2	ND	ND
M. polygoni-cuspidati	IMI 401968	35–48 × 8–10	41.5 × 9.0	12.8–16.4 × 2.4–4.0	14.6 × 3.2	3.0–3.7 × 1.0–1.3
M. polygoni-cuspidati	IMI 393527	(40–)45–58 × 8–11	48.8 × 9.4	13.6–18.4 × 3.2–4.8	16.0 × 3.8	NM
M. polygoni-cuspidati	IMI 395027	NM	NM	14.8–18.4 × 3.2–4.0	16.3 × 3.6	NM
M. polygoni-cuspidati	IMI 395028	NM	NM	(13.6–)16.0–22.4 × 2.4–4.0	18.1 × 3.2	NM
M. shimabarensis	IMI 401914	30.5–40 × 7–9	34.4 × 7.9	7.2–12.0 × 2.4–4.0	9.7 × 3.1	NM

NM, not measured
ND, not described
[a] Data cited from Hara (1918), collected from Shizuoka Pref., on litter sample

Taxonomy and phylogeny

Morphological characteristics place the leaf spot pathogen *M. polygoni-cuspidati* firmly in the genus *Mycosphaerella*: features typical of that group include small, spherical, ostiolate ascomata; lack of interascal tissue; 8-spored, fissitunicate asci; and 2-celled, hyaline ascospores (Arx 1983, see Fig. 10). There were significant differences in both cultural and morphological characteristics between *M. polygoni-cuspidati* and *M. shimabarensis* (Table 3, Fig. 9A). Ascomata typical of *M. polygoni-cuspidati* occurred only within leaf spot lesions on both attached as well as fallen leaves, whereas *M. shimabarensis* was confined to freshly fallen leaves with no evidence of disease symptoms. It could be distinguished morphologically by aggregated and erumpent ascomata containing considerably smaller asci and ascospores.

Of the six strains of *M. polygoni-cuspidati* sequenced, five had identical ITS sequences and the sixth (AB434910) differed only by two separate C/T substitutions within the 400 bp analyzed (Fig. 11). No morphological or cultural differences were observed between this strain and the

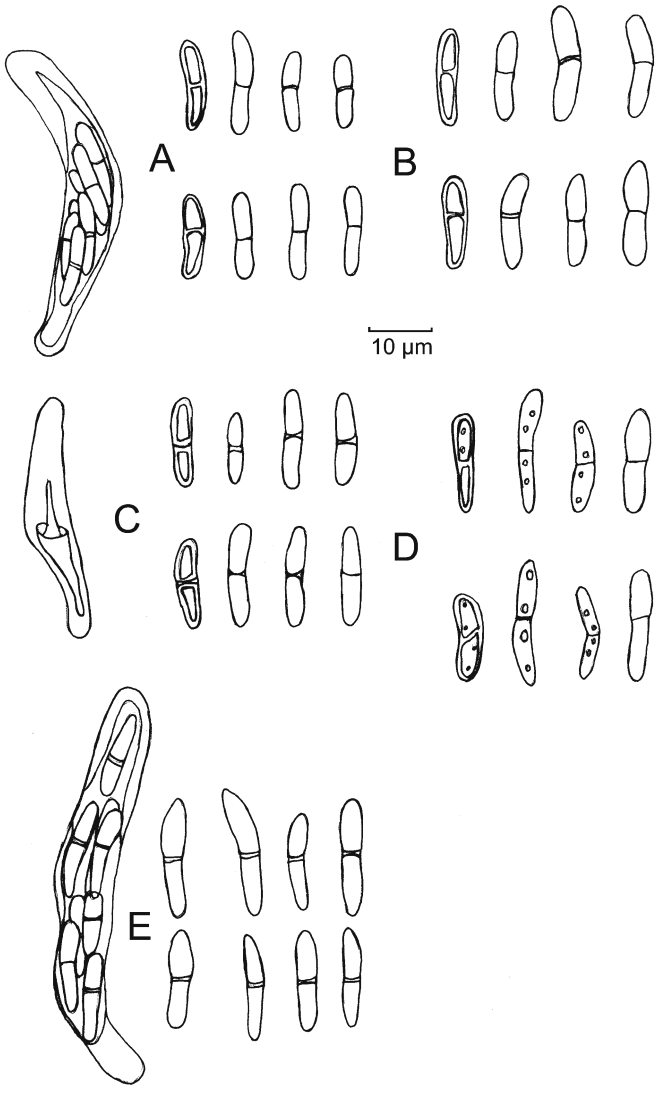


Fig. 10. Ascus and ascospore morphology of *Mycosphaerella polygoni-cuspidati* from diverse localities in Japan. **A** Ascus and ascospores ex Mt. Hiko, Fukuoka Pref., IMI 393529. **B** Ascospores ex Nishinagono, Hiroshima Pref., IMI 395027. **C** Young ascus and ascospores ex Kami-Naka, Kochi Pref., IMI 393527. **D** Ascospores ex Omura, Nagasaki Pref., IMI 395028. **E** Ascus and ascospores ex Yamaguchi, Yamaguchi Pref., IMI 401910

others. Blast searches of the *M. polygoni-cuspidati* sequences showed a closest match to ITS sequences of the Indonesian *Eucalyptus*-inhabiting species *M. sumatrensis* Crous & M.J. Wingf. (Crous et al. 2006) with approximately 97% similarity, and further analysis revealed 7 separate base pair differences between sequences of the two taxa. *M. shimabarensis* was found to be only distantly related to *M. polygoni-cuspidati*, and its ITS sequence was found to be most similar to *M. lateralis* Crous & M.J. Wingf. (Crous et al. 2006), another species from *Eucalyptus* with a *Dissoconium* anamorph (Crous et al. 1999). ITS sequences are not ideal for elucidating relationships within *Mycosphaerella* and its relatives (Crous et al. 2007) but are appropriate tools for identification of close relatives.

Mycosphaerella polygoni-cuspidati Hara, Byotyugai Zasshi 5: 617, 1918. Fig. 10

Neotypus (designated here): On living leaves of *Fallopia japonica* var. *japonica*, Mt. Hiko, Fukuoka Pref., Japan, 5 July 2005, collected by H.C. Evans, D. Kurose & R.H. Shaw, IMI 393529 (dried herbarium specimen).

Lesions amphigenous, circular, to 25 mm in diameter, often coalescing, chestnut- to chocolate-brown, grayish with age; with a darker, water-soaked halo, often surrounded by a chlorotic zone; veins on lower leaf surface bleached or whitened. Mycelium internal: intercellular around lesion, septate, branched, hyaline, 4–6 µm diameter; intracellular in older lesions, brown, 1.5–3 µm diameter, becoming swollen, to 6–8(–10) µm diameter. Spermogonia amphigenous, immersed to semi-erumpent, subepidermal, scattered, numerous, sometimes in concentric rings, both within and around lesions; black, globose, 60–80(–120) tall and (35)–60–80(–90) µm diameter. Spermatia rod-like, aseptate, hyaline, 3.0–3.7 × 1.0–1.3 µm, not germinating on agar. Ascomata pseudothecial, amphigenous, semi-erumpent, black, globose to subglobose, 100–120(–140) tall and 80–110 µm diameter, numerous, separate, rarely aggregated; ostiole apical, papillate, 10–20 µm diameter. Asci hyaline, fasciculate, aparaphysate, bitunicate, short-stalked or subsessile, 8-spored, ellipsoidal to cylindrical, straight to curved, 35–48(–55) × 8–10 µm. Ascospores bi- to multiseriate, hyaline, 1-septate, rather varied in form but typically slightly constricted at the median septum, widest in the middle of the apical cell and tapering toward the base, (11.0–)13.0–16.5(–17.5) × (2.0–)2.5–4.0 µm, without a sheath.

No species of *Mycosphaerella* has previously been cited in the specialist literature on the mycobiota of Japanese knotweed and its close relatives (Fowler and Holden 1994; Diaz and Hurle 1995), and this species was initially presumed to be undescribed. A further search of the Japanese mycological literature (Shirai and Hara 1927) revealed that a *Mycosphaerella* species had been described previously on *P. cuspidatum* (syn. *F. japonica*) by Hara (1918). Based on morphological data, specifically ascus and ascospore dimensions (Table 3), the leaf spot pathogen described here falls within the species concept. This taxon was originally misnamed as "*Mycosphaerella* (*Sphaerella*) *Polygoni cuspidatii*" (Hara 1918), but its name has been amended since as *Mycosphaerella polygoni-cuspidati* (Aptroot 2006). According to Hara (1918), Shimada collected the leaf litter sample in Shizuoka Pref. on October 1915, but apparently no material was conserved. Searches for authentic specimens in the national herbaria of Japan were unsuccessful. In accordance with Art. 9 of the International Code of Botanical Nomenclature (McNeill et al. 2006), a neotype has therefore been designated to fix application of the name.

In addition to the neotype designated above, numerous other herbarium collections of this species were made from natural stands of *F. japonica* (see Table 2). There are variations within these specimens, especially in dimensions of asci and ascospores (see Table 3); those of IMI 393527, IMI 395027, and IMI 395028 being consistently greater than the neotype. However, these differences do not appear to

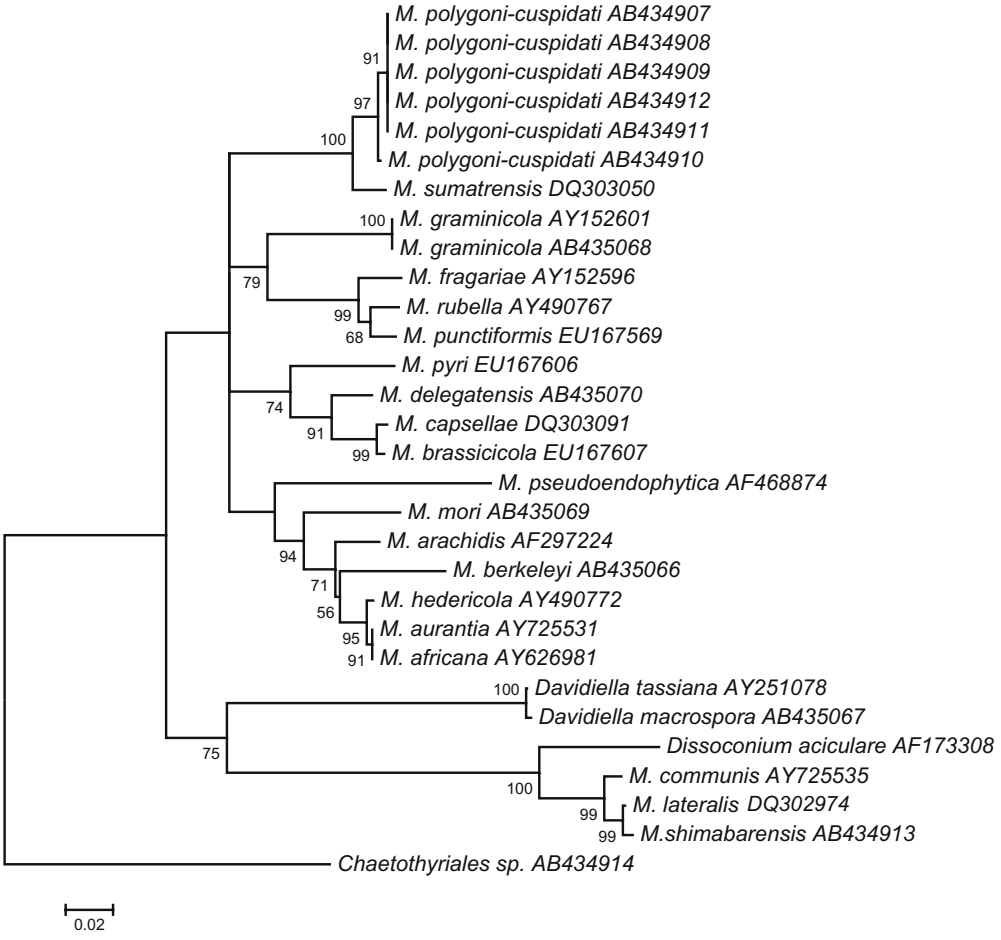


Fig. 11. Neighbor-joining phylogenetic tree of *Mycosphaerella* (*M.*) spp. and related taxa obtained by the Kimura two-parameter method, based on 400 bp of the internal transcribed spacer (ITS) region including the 5.8S rDNA sequences; 1000 bootstrap replicates were used, with values >50% displayed

correlate with cultural characteristics or ITS sequence (Figs. 9, 11).

Based on both field and experimental data, it is concluded that *M. polygoni-cuspidati* has a microcyclic or reduced life cycle in which the only functional spores are spermatia and ascospores. There is no evidence of a functional macroconidial stage or a true anamorph in the life cycle. The spermogonial stage of *Mycosphaerella* spp. has in the past often been assigned to the genus *Asteromella* Pass. & Thum. or designated as a microconidial synanamorph (Sutton 1980; van der Aa and Vanev 2002; Andrianova and Minter 2005). For example, that of *M. brassicicola* (Duby) Lindau, which has a similar reduced life cycle to the present species, is listed as *Asteromella brassicae* (Chevall.) Boerema & Kesteren (Boerema and van Kesteren 1964). In contrast to *M. brassicicola*, however, ascomata of *M. polygoni-cuspidati* have never been observed on inoculated plants (in vivo) or in culture (in vitro), despite testing various recommended humidity regimes and agar media (Snyder 1946; Nelson and Pound 1959), so it cannot be determined if the fungus is homo- or heterothallic. Indeed, in the field, ascomata are relatively uncommon, and most of the material collected over the 4-year period was dominated by the spermogonial stage. It could be argued that ascomata develop mainly after leaf fall, but there was no evidence of this from a detailed examination of leaf litter collected before and during the winter months.

Surveys showed that the first symptoms appeared mainly during June, occasionally towards the end of May, on established and developing plants, never before and never on the youngest growth stages. Thereafter, leaf spots increased in intensity, reaching a peak in September–October (Table 2). The presumed life cycle is thus: (1) maturation of ascospores during spring on fallen leaves shed during November–December as the plants die back from frost damage; (2) release of ascospores from leaf litter in May–June, well after emergence of Japanese knotweed plants in spring; infection of young leaves by airborne spores; (3) 2- to 4-week asymptomatic biotrophic or endophytic phase within leaves before necrosis and first appearance of leaf spots, typically in June–July; (4) synchronous development of spermogonia, both in and around lesion; (5) cross-fertilization or mating by exchange of spermatia through rain-splash or insect vectors: development of ascomata and maturation of ascospores; (6) wind-dispersed ascospores in July–August: infection of young leaves on maturing plants; (7) development of spermogonia and ascomata during August–September; (8) secondary infection cycle and spermogonial formation during October–November; and (9) plant death

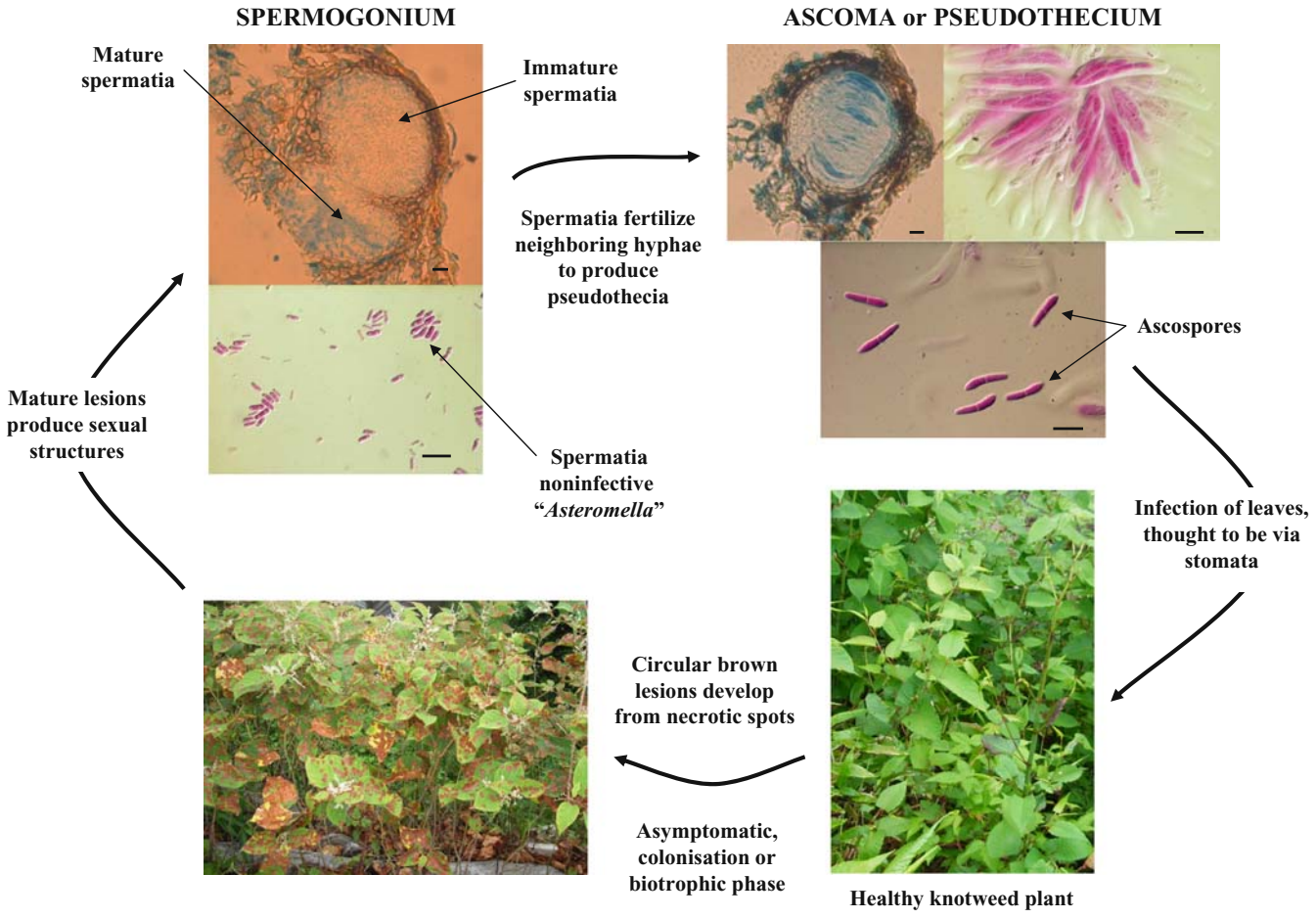


Fig. 12. Schematic representation of the proposed microcyclic life cycle of *Mycosphaerella polygoni-cuspidati*. *Bars* 10 µm

during November–December following first frosts, dependent on latitude and altitude.

Thus, *M. polygoni-cuspidati* has all the characteristics of a hemibiotroph: a prolonged or well-developed, latent or colonization phase, followed by a necrotic phase, and even the production of spermogonia within living tissues around the lesions. This putative life cycle is summarized in schematic form in Fig. 12.

Mycosphaerella polygoni-cuspidati is an extremely common and damaging pathogen of *F. japonica*, occurring throughout the natural range of its host plant. The ubiquitous nature of the leaf spot fungus is well illustrated by its incidental appearance in a botanical plate of "*Polygonum cuspidatum*," depicting the herbaceous plants of Japan (Kitamura and Murata 1961). In fact, the majority of leaf spots contain spermogonia rather than ascomata and in this respect, as well as in its life cycle, *M. polygoni-cuspidati* has much in common with the causal agent ring-spot disease of cabbage, *Mycosphaerella brassicicola* (Punithalingam and Holliday 1975). The crucial difference between these two species is that the ascomata of *M. brassicicola* are formed readily in the greenhouse as well as in vitro (Snyder 1946; Goetz et al. 1993). Unfortunately, this has never been replicated with *M. polygoni-cuspidati*, and it is supposed that the uniform conditions of the CT rooms and the lack of vectors are contributory factors. A further possibility is that the knotweed pathogen is heterothallic, in contrast to the proven homothallic condition of *M. brassicicola* (Snyder 1946).

Mycosphaerella shimabarensis H.C. Evans & P.F. Cannon, sp. nov. Fig. 13

Ascomata pseudotheciales, epiphylla, aggregata, subepidermalia, erumpentia, brunnea vel nigra, ostiolata, globosa (40–60 µm diametro) vel subglobosa (50–70 × 45–60 µm). Asci fasciculati, aparaphysati, bitunicati, subsessiles, octospori, obclavati vel subcylindrici, (21–)25–36(–40) × 6–8(–10) µm. Ascosporae biseriatae, imbricatae, hyalinae, guttulatae, mediano 1-septatae, ellipsoideae vel cuneiformes, cellulis basilibus et apicalibus obtusis, latissimae ad medium cellulae apicalis, attenuatae ad cellulum basalem, (6–)7.5–11 × 2–3(–4) µm. Spermogonia et anamorphosis ignotae.

Ascomata pseudothecial, epiphyllous, aggregated in groups typically along or near veins and midrib, subepidermal, erumpent, brown to black, ostiolate, globose (40–60 µm diameter) to subglobose (50–70 × 45–60 µm). Asci fasciculate, aparaphysate, bitunicate, with a thickened obtuse apex, subsessile, 8-spored, obclavate to subcylindrical,

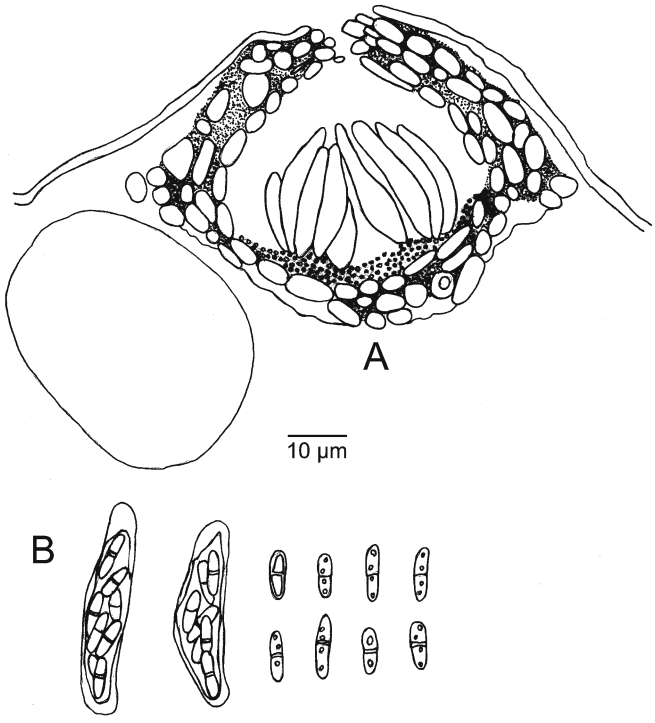


Fig. 13. Morphology of *Mycosphaerella shimabarensis* ex Shimabara, Nagasaki Pref., IMI 401914. **A** Mature ascoma showing indistinct ostiolar region lacking periphyses, with a developing ascoma to the *left*. **B** Asci and ascospores

(21–)25–36(–40) × 6–8(–10) µm. Ascospores unequally biseriate, overlapping, hyaline, guttulate, medianly 1-septate, ellipsoid to cuneiform, obtusely rounded at both ends, widest in the middle of the apical cell, tapering slightly toward the base, (6–)7.5–11 × 2–3(–4) µm. Spermogonia and anamorph unknown.

Colonies on PCA relatively fast growing at 23°C, reaching 38–40 mm after 4 weeks, grayish green, compact, with low mycelium, slightly raised centrally. No evidence of sporulation or spermogonial formation.

Etymology: Named after its type locality.

Holotype: On freshly fallen leaves of *Fallopia japonica* var. *japonica*, Shimabara, Nagasaki Pref., Japan, 8 July 2005, collected by H.C. Evans & R.H. Shaw, IMI 401914 (dried herbarium specimen).

The erumpent, aggregated ascomata of *M. shimabarensis* were found only on freshly fallen leaves without any evidence of the leaf spot infection. The cultures derived from ascospores could also readily be distinguished from the slower-growing, strongly stromatic colonies of *M. polygoni-cuspidati*, which invariably develop spermogonial initials. We conclude that *M. shimabarensis* is a cryptic endophytic fungus in living leaves of Japanese knotweed that sporulates only after natural senescence. There is no association with an anamorph, either on the host or in culture, and, although it is assumed that spermogonia are represented in the life cycle, no evidence of this stage was forthcoming. Phylogenetic analysis confirmed that this represents an undescribed taxon closely related to a complex of species from *Eucalyptus* hosts with *Dissoconium* anamorphs. Further revision of the genus *Mycosphaerella* may well lead to their separation at generic level, but this work is outside of our remit at present.

The two species of *Mycosphaerella* analyzed during this research project show clear differences in morphology, cultural characteristics, ITS sequence, and biology. *Mycosphaerella shimabarensis* appears to be a cryptic endophyte that does not cause noticeable damage to its host, and could possibly even be a mutualist. On the other hand, the leaf spot species *M. polygoni-cuspidati* has a considerable impact on plant vigor, and field observations and laboratory screening indicate high host specificity (unpublished data). These are strong indicators of its potential as a classical biological control agent for the sustainable management of Japanese knotweed. Pest risk assessments are still being completed, but it is hoped that, in the near future, *M. polygoni-cuspidati* will be the first exotic plant pathogen to be released in Europe for the control of an invasive alien weed.

Acknowledgments This work was undertaken within a project (VM03021) funded by a consortium made up of the Environment Agency, Network Rail, British Waterways, Welsh Assembly Government, South West of England Regional Development Agency and Defra (Department of Environment Food and Rural Affairs) all coordinated through Cornwall County Council. The quarantine studies were undertaken under Defra license no. PHL 182/4869. This study was also supported by a Grant-in-Aid for JSPS Fellows (No. 19-9902) and Scientific Research (No. 19580049) from the Japan Society for the Promotion of Science. The first author wishes to express his personal thanks to Dr. Masaru Matsumoto for his valuable suggestions during this study, and finally, the authors also thank Professor Masami Takagi and Ms. Yuko Inoue for their help in collecting samples.

References

Andrianova TV, Minter DM (2005) *Mycosphaerella jaczewskii.* IMI descriptions of fungi and bacteria 1625. CABI, Egham, Surrey

Aptroot A (2006) *Mycosphaerella* and its anamorphs: 2. Conspectus of *Mycosphaerella*. Centraalbureau voor Schimmelcultures, Utrecht

Arx JA von (1983) *Mycosphaerell*a and its anamorphs. Proc K Ned Akad Wet Ser C 86:15–54

Bailey JP, Conolly AP (2000) Prize-winners to pariahs: a history of Japanese knotweed s.l. (Polygonaceae) in the British Isles. Watsonia 23:93–110

Beerling DJ (1991) The testing of concrete revetment blocks resistant to growths of *Reynoutria japonica* (Japanese knotweed). Water Res 25:495–498

Boerema GH, van Kesteren HA (1964) The nomenclature of two fungi parasitizing Brassica. Persoonia 3:17–28

Child LE, Wade PM (2000) The Japanese knotweed manual. Packard, Chichester

Crous PW, Hong L, Wingfield MJ, Wingfield BD, Kang JC (1999) *Uwebraunia* and *Dissoconium*, two morphologically similar anamorph genera with different teleomorph affinity. Sydowia 51:155–166

Crous PW, Wingfield MJ, Mansilla JP, Alfenas AC, Groenewald JZ (2006) Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. II. Stud Mycol 55:99–131

Crous PW, Braun U, Groenewald JZ (2007) *Mycosphaerella* is polyphyletic. Stud Mycol 58:1–32

Diaz M, Hurle K (1995) Am Japanknoterich vorkommende Pathogene: Ansatz zu einer biologischen Regulierung. In: Bocker R, Gebhardt H, Konold W, Schmidt-Fischer S (eds) Gebietsfremde Pflanzenarten. Ecomed-verlag, Landesberg, pp 173–178

Fowler SV, Holden ANG (1994) Classical biological control for exotic invasive weeds in riparian and aquatic habitats-practice and prospects. In: Waal DC de, Child LE, Wade PM, Brock PM (eds) Ecology

and management of invasive riverside plants. Wiley, London, pp 173–182
Goetz M, Zombach W, Boyle C (1993) Life cycle of *Mycosphaerella brassicicola* (Duby) Lindau and ascospore production in vitro. J Phytopathol 139:298–308
Hara K (1918) Rakuyou oyobi kareha ni kiseisuru kinrui (in Japanese). Byotyugai Zasshi 5:617
Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120
Kitamura S, Murata G (1961) Colored illustrations of herbaceous plants of Japan (Choripetalae), vol II (in Japanese). Hoikusha, Osaka
Kurose D, Renals T, Shaw R, Furuya N, Takagi M, Evans H (2006) *Fallopia japonica*, an increasingly intractable weed problem in the UK: can fungi help cut through this Gordian knot? Mycologist 20:126–129
Mabey R (1998) Flora Britannica. Chatt and Windus, London
Makino T (1961) Makino's new illustrated flora of Japan (in Japanese). Hokuryukan, Tokyo
Makino T (2000) Newly revised Makino's illustrated flora of Japan (in Japanese). Hokuryukan, Tokyo
McNeill J, Barrie FR, Burdet HM, Demoulin V, Hawksworth DL, Marhold K, Nicolson DH, Prado J, Silva PC, Skog JE, Wiersema J, Turland NJ (2006) International code of botanical nomenclature (Vienna code). ARG Gantner Verlag, Koenigstein
Nelson MR, Pound GS (1959) The relation of environment to the ringspot (*Mycosphaerella brassicicola*) disease of crucifers. Phytopathology 49:633–640
Punithalingam E, Holliday P (1975) *Mycosphaerella brassicicola*. CMI descriptions of pathogenic fungi and bacteria 468. Commonwealth Mycological Institute (CMI), Kew, Surrey
Reinhardt F, Herle M, Bastiansen F, Streit B (2003) Economic impact of the spread of alien species in Germany. Umweltbundesamt, Berlin
Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
Shirai M, Hara K (1927) A list of Japanese fungi hitherto known, 3rd edn (in Japanese). Yokendo, Tokyo
Snyder WC (1946) Spermogonia versus pycnidia in *Mycosphaerella brassicicola*. Phytopathology 36:481–484
Sutton BC (1980) The Coelomycetes. Commonwealth Mycological Institute (CMI), Kew, Surrey
Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
van der Aa HA, Vanev S (2002) A revision of the species described in *Phyllosticta.* Centraalbureau voor Schimmelcultures, Utrecht
Waller JM, Lenné JM, Waller SJ (2001) Plant pathologist's pocket book, 3rd edn. CABI Publishing, Wallingford
White TJ, Bruns T, Lee SB, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Gelfand M, Sninsky D, White T (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322
Zhou Z, Miwa M, Nara K, Wu B, Nakaya H, Lian C, Miyashita N, Oishi R, Maruta E, Hogetsu T (2003) Patch establishment and development of a clonal plant, *Polygonum cuspidatum*, on Mount Fuji. Mol Ecol 12:1361–1373