MOLECULAR CHARACTERISATION OF *SAWADAEA* ANAMORPHS IN AUSTRALIA

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Abstract

In order to identify the species of *Sawadaea* in Australia from anamorphic material, the ribosomal DNA internal transcribed spacer region was amplified from a range of specimens and subjected to RFLP analysis. Specimens of known species were included for comparison. RFLP analysis revealed *S. bicornis* on *Acer negundo*, *A. rubrum* and *A. pseudoplatanus*, while *S. polyfida* var. *japonica* was found on *A. japonicum* and *A. palmatum*. This is the first record of S. *polyfida* var. *japonica* on *A. japonicum*. These results agree with reported host ranges for both species in Europe and Asia.

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Introduction

Sawadaea Miyabe is a genus of Erysiphales (Ascomycota) almost entirely restricted to the genus *Acer* (Aceraceae). The combination of conidial fibrosin bodies and ascomata (chasmothecia, according to Braun *et al.* 2002) forming multiple asci, as well as the formation of a microconidial state, distinguishes it from other genera. Braun (1987) accepted five species, the two most widely distributed being *S. bicornis* (Wallr. : Fr.) Homma and *S. tulasnei* (Fuckel) Homma. *Sawadaea bicornis* is the most commonly encountered species and occurs on a wide range of *Acer* spp. throughout Europe, the Middle East, Asia and New Zealand. *Sawadaea tulasnei* has been found in the same areas as *S. bicornis*, with the exception of New Zealand, and also occurs on a wide range of *Acer* spp.

The species are difficult to differentiate when only the anamorph is encountered. In Europe where only *S. bicornis* and *S. tulasnei* are known, these two species can be differentiated by the size of the conidia (Braun 1995). However, other taxa from Asia, *e.g. S. polyfida* (C.T. Wei) R.Y. Zheng & G.Q. Chen var. *polyfida*, *S. polyfida* var. *japonica* U. Braun & Tanda and *S. bomiensis* R.Y. Zheng & G.Q. Chen are poorly known and it is doubtful that these species can be differentiated by their anamorphs.

In Australia only the anamorphic state of *Sawadaea* species have been collected. There are 43 Australian specimens on *Acer* currently held in herbaria DAR, BRIP and VPRI. Most of these are held in VPRI. Cunnington *et al.* (2003) used sequences of the rDNA internal transcribed spacer region to match four anamorphic Australian specimens with sequences from either GenBank or from teleomorphic material obtained from Europe. Two were found to be *S. bicornis* and two were *S. polyfida* var. *japonica*. This was the first time that *S. polyfida* var. *japonica* had been found outside Japan.

The aim of this study was to further determine the identity of Australian specimens of *Sawadaea*, by analysing the ITS region of a range of specimens by RFLP analysis. Zeller & Levy (1995) successfully used RFLP analysis of the ITS region to resolve taxa within the *Golovinomyces cichoracearum* complex. The specimens identified previously by Cunnington *et al.* (2003) were included to act as representatives of known species.

Materials and Methods

Sixteen Australian specimens of *Sawadaea* on *Acer* were obtained from VPRI (Table 1). This included the four specimens sequenced by Cunnington *et al.* (2003). The host plants of two of these four were at that time unknown, but have since been determined as *A. rubrum* (VPRI 19042) and *A. japonicum* (VPRI 18722). For all specimens, a small amount of the fungus (approx 1 mm^3) was scraped from the infected leaf using a chisel shaped probe. This was put in a 1.5 mL Eppendorf tube containing 50 µL of 5% Chelex-100 (Biorad) containing 0.01% Triton X-100, and placed in a 94°C water bath for 1–2 hours. The tube was vortexed vigorously for 2–3 seconds and centrifuged for 10 minutes at 14000 rpm. The supernatant was used directly for the PCR.

The initial PCR was performed in 25 µL containing 1 µL DNA extract, 200 µM of each dNTP (Pharmacia Biotech), 1.5 mM $MgCl_2$, 2.5 µL 10× buffer, 4 ng each of primers PMITS1 and PMITS2 (Cunnington *et al.* 2003), 6% Tween 20 (Labchem) and 0.5 units of Amplitaq Gold (Perkin-Elmer). Primers PMITS1 and PMITS2 have previously been shown to have enhanced specificity for the ITS region of the Erysiphales (Cunnington *et al.* 2003). Reaction cycles were: 10 min. at 94°C, followed 35 cycles of: 1 min. at 94°C, 1 min. at 65°C, 2 min. at 72°C, and a final extension of 10 min. at 72°C. PCR products were detected by running 4 µL on a 1.4% agarose gel in TBE buffer. A nested PCR was performed in 50 µL as outlined above, but without Tween 20, using primers PMITS1 and ITS4 (White *et al.* 1990) at twice the previous concentration, and containing 1 µL of a 1:100 dilution of the first round product, or if no product was visible then an undiluted 1 µL. Cycling times were the same, but with an annealing temperature of 60°C.

Five microlitres of nested PCR product were digested in 10 µL reactions with six restriction endonucleases (Bresatec) *Msp*I, *Hinf*I, *Hin*6I, *Alu*I, *Csp*I and *Bsu*RI. Each 10 µL reaction contained 5 µL of nested PCR product, 10 U of enzyme and 1 µL of 10x buffer. Digestions were performed at 37°C overnight, after which 1 µL of loading dye was added. Products were run on a 3% agarose gel in TBE buffer. *Puc* digested with *Hpa*II (Progen) was used as a molecular weight marker.

Results

All specimens yielded a nested PCR product of approximately 650 bp. RFLP analysis separated the Australian specimens into three groups (Table 1). Group 1 contained the most specimens, including all those collected on *A. negundo*, *A. rubrum* and *A. pseudoplatanus*, and included the two specimens previously determined to be *S. bicornis* by Cunnington *et al.* (2003). Group 2 contained a single specimen on *A. japonicum*, known to be *S. polyfida* var. *japonica*. Groups 3 contained four specimens on *A. palmatum* and *A. japonicum*, including a specimen of *S. polyfida* var. *japonica*.

Discussion

Each of the three groups revealed by RFLP analysis included, at least one specimen that had previously been identified by Cunnington *et al.* (2003). The members of RFLP Group 1 are most likely to be *S. bicornis*, given the inclusion of both *S. bicornis* specimens and the relationship with the host species. The only species of *Sawadaea* listed by Braun (1987) on *A. negundo*, *A. rubrum* or *A. pseudoplatanus* is *S. bicornis*. Thus both host range and ITS sequence data support the widespread occurrence of *S. bicornis* in Australia.

Groups 2 and 3 were both determined to be *S. polyfida* var. *japonica*. Braun (1987) listed only *S. polyfida* var. *japonica* on *A. palmatum*, again supporting the ITS sequence based identification. However he did not record any powdery mildew on *A. japonicum*. Nomura (1997) lists *S. tulasnei* on *A. japonicum*, while Hirata (1966) listed *Sawadaea* sp. on *A. japonicum* from Japan. This is the first time that *S. polyfida* var. *japonica* has been recorded on *A. japonicum*. However, this is not surprising, as *A. palmatum* and *A. japonicum* are closely related species, both native to Japan (Suh *et al.* 2000). Although some ITS sequence variation was found between the two specimens sequenced, Cunnington *et al.* (2003) concluded that *S. polyfida* var. *japonica* is the best name for these specimens. This sequence variation may indicate that this species has been introduced into Australia on at least two occasions, as the two specimens were collected at disparate locations, *i.e.* Guilford, N.S.W. and Burnley, Vic.

Table 1. *Sawadaea* specimens grouped by ITS-RFLP analysis into their respective species. Fragment sizes for each restriction endonuclease are given in base pairs.

Specimens	Host	Location	*Msp*I	*Hinf*I
Sawadaea bicornis				
VPRI 18454	***A. negundo***	**Duffy, ACT**	240, 190, 180, 40	400, 260
VPRI 18496	***A. negundo***	**Duffy, ACT**	240, 190, 180, 40	400, 260
VPRI 18520	***A. negundo***	**South Yarra, Vic.**	240, 190, 180, 40	400, 260
VPRI 18825	***A. negundo***	**Farrer, ACT**	240, 190, 180, 40	400, 260
VPRI 19042*	***A. rubrum***	**Sassafras, Vic.**	240, 190, 180, 40	400, 260
VPRI 19048	***A. rubrum***	**Sassafras, Vic.**	240, 190, 180, 40	400, 260
VPRI 19596	***A. negundo***	**Kew, Vic.**	240, 190, 180, 40	400, 260
VPRI 19655	***A. negundo***	**Maldon, Vic.**	240, 190, 180, 40	400, 260
VPRI 19684*	***A. negundo***	**Duffy, ACT**	240, 190, 180, 40	400, 260
VPRI 19966	***A. pseudoplatanus***	**Perth, Tas.**	240, 190, 180, 40	400, 260
VPRI 21252	***A. negundo***	**Camberwell, Vic.**	240, 190, 180, 40	400, 260
Sawadaea polyfida* var. *japonica				
VPRI 18722*	***A. japonicum***	**Guildford, NSW**	190, 180(2), 70, 40	650
Sawadaea polyfida* var. *japonica				
VPRI 19183	***A. palmatum***	**Burnley, Vic.**	190, 180(2), 70, 40	400, 260
VPRI 19184*	***A. palmatum***	**Burnley, Vic.**	190, 180(2), 70, 40	400, 260
VPRI 19185	***A. palmatum***	**Burnley, Vic.**	190, 180(2), 70, 40	400, 260
VPRI 19190	***A. japonicum***	**Burnley, Vic.**	190, 180(2), 70, 40	400, 260

Specimens (cont'd)	*Csp*I	*Alu*I	*Hin6*I	*Bsu*RI
Sawadaea bicornis				
VPRI 18454	650	500, 120, 40	170, 160, 130, 90(2)	250, 150, 90
VPRI 18496	650	500, 120, 40	170, 160, 130, 90(2)	250, 150, 90
VPRI 18520	650	500, 120, 40	170, 160, 130, 90(2)	250, 150, 90
VPRI 18825	650	500, 120, 40	170, 160, 130, 90(2)	250, 150, 90
VPRI 19042*	650	500, 120, 40	170, 160, 130, 90(2)	250, 150, 90
VPRI 19048	650	500, 120, 40	170, 160, 130, 90(2)	250, 150, 90
VPRI 19596	650	500, 120, 40	170, 160, 130, 90(2)	250, 150, 90
VPRI 19655	650	500, 120, 40	170, 160, 130, 90(2)	250, 150, 90
VPRI 19684*	650	500, 120, 40	170, 160, 130, 90(2)	250, 150, 90
VPRI 19966	650	500, 120, 40	170, 160, 130, 90(2)	250, 150, 90
VPRI 21252	650	500, 120, 40	170, 160, 130, 90(2)	250, 150, 90
Sawadaea polyfida* var. *japonica				
VPRI 18722*	650	500, 120, 40	170, 130, 110, 90, 80, 50	235, 90, 80
Sawadaea polyfida* var. *japonica				
VPRI 19183	650	500, 120, 40	170, 130, 110, 90, 80, 50	235, 90, 80
VPRI 19184*	650	500, 120, 40	170, 130, 110, 90, 80, 50	235, 90, 80
VPRI 19185	650	500, 120, 40	170, 130, 110, 90, 80, 50	235, 90, 80
VPRI 19190	650	500, 120, 40	170, 130, 110, 90, 80, 50	235, 90, 80

* Specimens previously identified and sequenced by Cunnington *et al.* (2003).

There are 43 collections of powdery mildews fungi on *Acer* (presumably *Sawadaea* species) in herbaria BRIP, DAR and VPRI. Attempts were made to amplify the ITS region from most of these specimens; however, amplification was only successful for sixteen specimens. This was most likely owing to the poor growth of the specimens, as the methods used here have been shown to amplify the ITS region of most genera of Erysiphales, including *Sawadaea* (Cunnington *et al.* 2003). Of the 43 specimens present in Australian herbaria, sixteen were on *A. negundo*, three on *A. rubrum* and two on *A. pseudoplatanus*. Of these 21, eleven were shown here to be *S. bicornis*. There are 15 specimens on *A. palmatum* and two on *A. japonicum*. Of these 17, five were shown here to be *S. polyfida* var. *japonica*. Of the remaining specimens, one host is *A. circinatum*, while the others are labelled simply as *Acer* sp. Thus, this study has covered all (except for *A. circinatum*) species of *Acer* for which powdery mildew specimens have been collected in Australia. As the identifications based on ITS sequence variation have correlated exactly with the predicted identification based on host species, this demonstrates the usefulness of this approach for anamorphic stages, especially when only these are present, as in Australia. This new knowledge of the species on *Acer* will allow more confidence in identifying *Sawadaea* species in Australia.

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