DNA homology between *Pyrenophora japonica* and *P. teres*

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Pyrenophora isolates associated with net- and spot-type net blotch of barley were compared with verified isolates of *P. teres* f. *teres*, *P. teres* f. *maculata* and *P. japonica* using total DNA banding patterns, morphology, symptomatology and mating studies. DNA of South African and overseas spot- and net-type isolates digested with *Hae* III and *Msp* I showed 74–100% similarity. Furthermore, spot- and net-type isolates could be mated to produce viable progeny, indicating *P. teres* to have two-allele heterothallism. Single-ascospores inoculated on differential barley cultivars produced spot, net or mixed symptoms, suggesting that recombination has occurred. Based on the high degree of homology in DNA banding patterns between *P. japonica* and *P. teres* f. *maculata*, as well as similarities in symptom expression on differential cultivars and general morphology, *P. japonica* is proposed as synonym of *P. teres*.

Net blotch disease of barley (*Hordeum vulgare* L. *emend.* Bowden), caused by *Pyrenophora teres* Drechsler (anamorph *Drechslera teres* [Sacc.] Shoemaker), is an important disease of this crop in South Africa, as well as elsewhere in the world (Khan, 1987; Steffenson, Webster & Jackson, 1991; Louw *et al.*, 1994*b*). Two types of leaf symptoms are associated with net blotch disease, namely a spot- and a net-type lesion. Originally *P. japonica* S. Ito & Kurib. (anamorph *Drechslera tuberosa* [G. F. Atk.] Shoemaker) was described as the pathogen causing spot symptoms, whereas *P. teres* was associated with net type lesions (Ito & Kuribayashi, 1931; Shoemaker, 1962). McDonald (1967) reported that he could mate isolates of the spot and net type, and referred to the spot isolates as mutant strains of *P. teres*. Using strains from Denmark, Smedegård-Petersen (1971) repeated these matings, and concluded that they were two *formae* of the same biological species, for which he proposed the names *P. teres* f. *teres* (net-type symptoms) and *P. teres* f. *maculata* Smedegård-Petersen (spot-type symptoms).

In a comparison of *Pyrenophora* spp. occurring on graminicolous hosts, Sivanesan (1987) retained *P. japonica* and *P. teres* as two separate species. Scott (1991) subsequently reported the anamorph of *P. japonica* from spot symptoms of barley in South Africa, while Louw, Crous & Holz (1994*a*) reported the teleomorph from barley stubble, and from matings of spot-type isolates in culture. Using A + T-rich DNA (AT-DNA) polymorphisms (Freeman, Pham & Rodriguez, 1993), Louw *et al.* (1994*b*) found banding patterns of conidial and ascospore strains (spot-type), and conidial strains (net-type) to be similar, and concluded that they were *formae* of the same species as proposed by Smedegård-Petersen (1971). The aim of the present study, therefore, was to determine if *P. japonica* is a separate species, or if it should be treated as synonym of *P. teres*, using the similarity of DNA banding patterns generated by *Hae* III and *Msp* I digestion, mating studies, general morphology and symptomatology.

MATERIALS AND METHODS

Sampling of isolates

Leaves showing characteristic spot- and net-type symptoms were collected in barley fields in the Western Cape, the major barley producing area in South Africa. Overseas reference isolates were obtained from Denmark, Japan, Canada and Australia (Table 1). These included the type strain of *P. teres* f. *maculata*, and a verified isolate of *P. japonica* collected by Ito, who described the species. Other than the verified isolate of *P. japonica* and several South African spot-type isolates reported as *P. japonica* (Scott, 1991), no additional isolates of *P. japonica* could be obtained. Furthermore, no original type culture of *P. teres* was ever lodged for study.

Mating studies

All isolates listed in Table 1 were single-spored and mated in all possible combinations on barley agar (BA) (water agar plates containing pieces of autoclaved barley stubble). Agar plugs (3 mm diam.) of the respective isolates (two isolates per plate) were mated by placing them on either side of the stubble. Three replicate plates were used for each combination. Plates were sealed with Parafilm and incubated at 15 °C under nuv light to induce pseudothecial formation, which was rated 6 months after inoculation.

Morphology

Dried leaves were placed in moist chambers and incubated at 15° under nuv light to induce sporulation. Conidiophores and

Table 1. *Pyrenophora* isolates used in this study

Species/ accession no.	Location	Source
P. teres f. *maculata*		
Cal 5	South Africa	J. P. Louw
Cal 8	South Africa	J. P. Louw
Mor 2	South Africa	J. P. Louw
Hop 1	South Africa	J. P. Louw
MP 1	South Africa	J. P. Louw
MP 4	South Africa	J. P. Louw
MP 6	South Africa	J. P. Louw
MP 7	South Africa	J. P. Louw
Nap 4	South Africa	J. P. Louw
Pt90/10	Denmark	J. C. Reeves
Pt90/13a	Denmark	J. C. Reeves
Pt90/13b	Denmark	J. C. Reeves
Pt90/14	Denmark	J. C. Reeves
CBS 228.76	Denmark	CBS
1550 WRS T1	Canada	A. Tekauz
1510 WRS V	Canada	A. Tekauz
P. teres f. *teres*		
Mor 5	South Africa	J. P. Louw
Riv 8	South Africa	J. P. Louw
Nap 7	South Africa	J. P. Louw
Nap 5	South Africa	J. P. Louw
KH 334	Australia	T. N. Khan
Pt90/8a	Denmark	J. C. Reeves
1572 WRS F1	Canada	A. Tekauz
1697 WRS D2	Canada	A. Tekauz
P. japonica		
CBS 281.31	Japan	CBS

conidia were mounted in lactophenol cotton blue and examined under the × 100 (oil) objective. The formation of secondary conidiophores and microcyclic conidiation were also noted.

Symptom verification

Symptom expression of South African and overseas isolates were verified on barley cultivars Stirling and B87/14 (susceptible to net- and spot-type isolates, respectively). Plants were inoculated in the two leaf stage in a glasshouse using the technique described by Louw *et al.* (1994*b*). Plants were examined for symptom expression 2 wk after inoculation. Leaves were harvested from plants showing the most characteristic symptoms of either spot-type or net-type net blotch, and dried. Harvested leaves were subsequently used for further characterization of the isolates.

DNA isolation

Louw *et al.* (1994*b*) showed South African spot- and net-type isolates to have similar banding patterns. Therefore, only one representative isolate of each South African lesion type was used in the present study. Single-conidial isolates of selected spot- and net-type collections were grown on purity agar (Louw *et al.*, 1994*a*) (Table 1). Plugs of 7 d old cultures were transferred into 200 ml glucose-yeast extract broth (GYEB) (Zumpetta, 1976), and incubated for 14 d at 26° in the dark. Mycelia were harvested and DNA extracted following the method described by Louw *et al.* (1994*b*). Total DNA was subjected to digestion with *Hae* III and *Msp* I for 4 h according to the recommendations of the suppliers (Boehringer Mannheim, Johannesburg, South Africa).

The DNA was separated on a Hoefer Scientific Instruments horizontal electrophoresis unit (HE 99) using a 1% (w/v) agarose gel. The gels were run at 6 V cm^{-1} in a 0·5 × TBE buffer (pH 8·0) (Sambrook, Fritsch & Maniatias, 1989). After separation, the DNA was stained with 20 µl ethidium bromide (10 mg ml^{-1}) in 600 ml 0·5 × TBE (pH 8·0) for 60 min and destained in 0·5 × TBE (pH 8·0) for at least 120 min. The DNA banding patterns on the agarose gels were photographed using type 55 Polaroid film. Phage lambda DNA digested with the restriction enzymes *Eco*R I and *Hin*d III was used as a molecular weight standard. The similarity between two isolates was determined using the *F* value of Nei & Li (1979).

RESULTS

Mating studies

Of the 25 single-spored isolates that were mated in all possible combinations on BA (Table 1), several combinations were observed to form protopseudothecia. However, pseudothecia with viable ascospores were only formed in three combinations, namely Pt90/14 (Danish spot-type isolate) × MP4 (South African spot-type isolate); Pt90/8a (Danish net-type isolate) × Nap 5 (South African net-type isolate), and Pt90/10 (Danish spot-type isolate) × KH334 (Australian net-type isolate). Eleven single-ascospores (A1–A11) were obtained from the progeny of Pt90/10 × KH334. The latter isolates were further characterized in pathogenicity studies on the barley cultivars Stirling and B87/14 as described previously.

Morphology

Conidiophores were observed to be variable in shape, with those of spot-type isolates being in the range 25–160 × 10–18 µm, (0–)4(–7)-septate, and those of net-type isolates being 18–120 × 10–18 µm, (0–)3(–5)-septate (Louw *et al.*, 1994*b*). Conidia of spot-type isolates were in the range 25–146 × 10–18 µm, olivaceous brown, cylindrical, tapering to acutely rounded bases. Conidia of net-type isolates were 25–100 × 10–18 µm, olivaceous brown, cylindrical, tapering to subtruncate bases. Although both types formed secondary conidiophores, microcyclic conidiation was more common among net-type isolates. Conidia of the eleven ascospore isolates were in the range 30–130 × 12–18 µm in culture, and could not be classed as either spot- or net-type isolates based on their morphology.

Symptom verification

The verified isolates caused typical net- or spot-type lesions. Of the 11 isolates derived from the progeny of Pt90/10 × KH334, A1 and A2 induced net-type symptoms characterized by longitudinal and transverse dark brown striations. Isolates A5–A11 induced spot-type symptoms

characterized by elliptical to oval dark brown spots, surrounded by a chlorotic zone. Isolates A3 and A4 produced net-type, spot-type and atypical symptoms on the same leaves. On cv. B87/14 they predominantly produced spot-type symptoms, while atypical net-type symptoms were visible as transverse striations. Although net-type symptoms were the most commonly observed on cv. Clipper, the atypical transverse striations and spot-type lesions were also present.

AT-DNA polymorphisms

Differences between isolates appeared as the presence/absence of individual bands. The bands that were used for determining the % similarity varied in size between 4 and 21 kb. Although *F*-values (% similarity) were calculated for each pair of isolates with each endonuclease, only the combined *F*-values (Hausner, Reid & Klassen, 1993) for each pair with both endonucleases are given, as the two data sets were consistent with each other. The *F*-values varied between 74 and 100% similarity (Table 2).

DISCUSSION

The DNA banding patterns of South African and overseas spot- and net-type isolates showed extensive homology in molecular size (74–100% similarity; Figs 1, 2). Furthermore, *P. teres* f. *maculata* (CBS 228.76, type strain) and *P. japonica* (CBS 281.31, verified strain) showed high homology with one another (93% similarity; Table 2). Three net-type isolates (Pt90/8a, KH334 and 16997WRSD2) showed lower similarity when compared with spot-type isolates. However, KH334 (net) could mate with Pt90/10 (spot), indicating this to be acceptable variation within the net-type population. Although little variation could be detected in the banding patterns of isolates from different geographic locations (Figs 1, 2), it is interesting to note that both the South African spot- and net-type isolates have a higher % similarity to the overseas net-type than spot-type isolates (Table 2). From these data it appears that in interpreting AT-rich DNA banding patterns generated by *Hae* III and *Msp* I digestion, a species of *Pyrenophora* can be defined as having a minimum of 70% similarity between isolates.

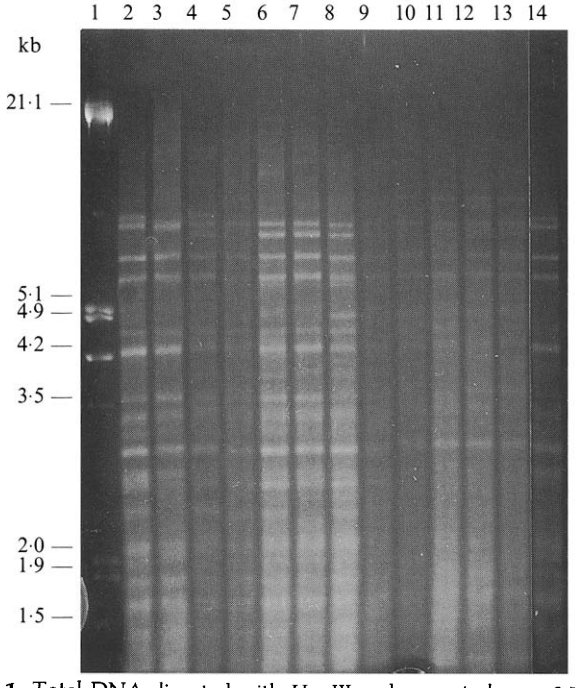

Fig. 1. Total DNA digested with *Hae* III and separated on a 0·7% agarose gel. Lane 1: Molecular weight standard. Lane 2: *P. teres* f. *maculata*, type, CBS 228.76 (Denmark). Lane 3: *Drechslera japonica fide* Ito, CBS 281.31 (Japan). Lanes 4–10: Spot type isolates Pt90/10, Pt90/13a, Pt90/13b, Pt90/14 (Denmark), 1510WRSV, 1550WRST1 (Canada), Cal 5 (South Africa). Lanes 11–14: Net type isolates Pt90/81 (Denmark), KH334 (Australia), 1697WRSD2 (Canada), Nap 4 (South Africa).

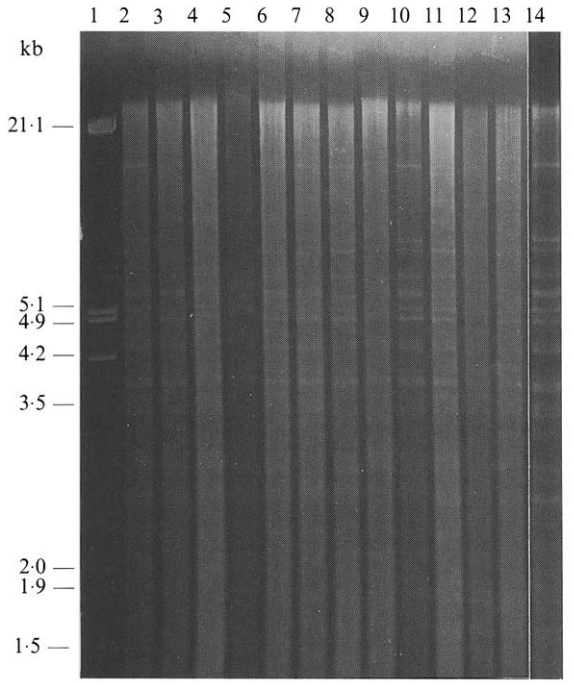

Fig. 2. Total DNA digested with *Msp* I and separated on a 0·7% agarose gel. Lane 1: Molecular weight standard. Lane 2: *P. teres* f. *maculata*, type, CBS 228.76 (Denmark). Lane 3: *Drechslera japonica fide* Ito, CBS 281.31 (Japan). Lanes 4–10: Spot type isolates Pt90/10, Pt90/13a, Pt90/13b, Pt90/14 (Denmark), 1510WRSV, 1550WRST1 (Canada), Cal 5 (South Africa). Lanes 11–14: Net type isolates Pt90/8a (Denmark), KH334 (Australia), 1697WRSD2 (Canada), Nap 4 (South Africa).

In contrast to a previous mating study with South African spot- and net-type isolates (Louw *et al.*, 1994*b*), the present study obtained three matings with viable progeny, the teleomorph of which is described below.

Ascomata black, globose, superficial, setose, becoming ostiolate with age, 300–600 × 200–400 µm. *Setae* dark brown, septate, thick-walled, cylindrical, smooth, becoming lighter in colour and warted towards the acute apices, up to 500 µm long, 8–11 µm wide at the first basal septum. *Asci* clavate to cylindric-clavate, straight or curved, bitunicate, thick-walled, stalked, 2–8-spored, 235–300 × 30–45 µm. *Ascospores* hyaline, becoming yellow-brown, ellipsoidal, rounded at apices, muriform with 3–4 transverse septa, and 0–3 vertical septa

Table 2. Combined *F*-values (% similarity) following restriction analysis of DNA of 13 isolates of *Pyrenophora teres*

	CBS 228.76	CBS 281.31	Pt90/10	Pt90/13a	Pt90/13b	Pt90/14	1510 WRS V	1550 WRS T1	Cal 5	Pt90/8a	KH 334	1697 WRS D2	Nap 4
CBS 228.76	—												
CBS 281.31	92·9	—											
Pt90/10	88·1	83·6	—										
Pt90/13a	87·3	86·3	88·9	—									
Pt90/13b	86·2	88·9	87·7	86·8	—								
Pt90/14	87·7	90·6	89·3	84·6	98·2	—							
1510 WRS V	88·1	85·7	88·1	83·6	96·6	94·7	—						
1550 WRS T1	84·2	88·9	75·9	75·5	86·2	85·2	93·1						
Cal 5	89·3	80·8	80·0	74·5	88·9	86·8	85·7	88·8	—				
Pt90/8a	76·4	78·1	77·8	76·0	90·6	84·6	87·3	86·8	94·1	—			
KH 334	75·5	81·6	76·9	79·2	86·3	84·0	83·0	86·3	93·9	95·8	—		
1697 WRS D2	75·5	81·6	76·9	79·2	86·3	84·0	83·0	86·3	93·9	95·8	100	—	
Nap 4	89·3	84·6	87·3	82·4	85·2	85·2	85·7	84·6	92·3	82·4	81·6	81·6	—

which occur primarily in the two central cells, constricted at septa, 45–65 × 14–30 µm.

In his monograph of *Pyrenophora*, Sivanesan (1987) described *Pyrenophora japonica* to have asci 225–400 × 35–45 µm, and ascospores with three transverse and 1–2 vertical septa, 40–65 × 17·5–30 µm. *P. teres* was described with asci being 175–275 × 32–60 µm, and ascospores with 3–4 transverse and 1–2 vertical septa, 36–65 × 14–28 µm. The teleomorph obtained in the present study has large asci and ascospores, comparable with those of *P. japonica*, whereas ascospore septation shows a better correlation with that ascribed to *P. teres*.

By mating a Danish spot-type isolate (Pt90/10) with an Australian net-type isolate (KH334) in the present study, we managed to repeat the findings of McDonald (1967) and Smedegård-Petersen (1971), who also reported mating spot- and net-type isolates. Smedegård-Petersen (1972) found that when inoculating isolates derived from several asci per pseudothecium, spot, net, as well as a mixture of the two symptoms could be obtained. The matings with single-ascospore cultures and mixed symptom expression on hosts inoculated with the progeny in the present study, suggest that *P. teres* may have a two-allele heterothallic mating system resulting in recombination. More successful matings, and other more sensitive molecular techniques would be required, however, to prove this.

McDonald (1967) regarded *P. japonica* to be a mutant strain of *P. teres*, and stated that the latter is made up of numerous clones adapted to particular environments. Although Smedegård-Petersen (1971) stated that the Danish spot-type of *P. teres*, namely *P. teres* f. *maculata*, showed great similarity to *P. japonica*, he did not reduce the latter to synonymy, and neither did Sivanesan (1987). In light of this Scott (1991) suggested that based on certain morphological differences the name *P. japonica* should rather be used for the spot-type net blotch of barley. The present study has proven, however, that based on the high % similarity in DNA banding patterns, as well as sexual compatibility, the spot-type and net-type net blotch are caused by two *formae* of the same species. Furthermore, although the verified strain of *P. japonica* (CBS 281.31) could not be induced to mate with *P. teres*, it produced similar leaf spots on the various differential cvs, had a similar general morphology, and shared 93% similarity with the DNA profiles of *P. teres* f. *maculata* (CBS 228.76). Based on these results the following synonymy is therefore proposed:

Pyrenophora teres Drechsler, *J. Agric. Res.* **24**, 656 (1923)
P. japonica S. Ito & Kurib., in Ito, *Proc. imp. Acad. Japan* **6**, 353 (1930)
Anamorph: *Drechslera teres* (Sacc.) Shoemaker, *Can. J. Bot.* **37**, 881 (1959)
Helminthosporium teres Sacc., *Michelia* **2**, 558 (1882)
H. hordei Eidam, *Landwirt, Breslau* **27**, 509 (1891)
H. tuberosum G. F. Atk., *Bull. Corn. Univ.* **3**, 47 (1897)
Drechslera tuberosa (G. F. Atk.) Shoemaker, *Can. J. Bot.* **37**, 881 (1959)
H. fragosoi Bubák, *Hedwigia* **57**, 13 (1915)
H. japonicum S. Ito & Kurib., *J. Fac. Agric. Hokkaido Univ., Japan* **29**, 108 (1931)
Drechslera japonica (S. Ito & Kurib.) Shoemaker, *Can. J. Bot.* **37**, 881 (1959)
H. secalis Whitehead & Dickson, *Mycologia* **44**, 757 (1952)

Other than the conspecificity between *P. japonica* and *P. teres*, the latter is also morphologically similar to the anamorphs of *P. graminea* S. Ito & Kurib. apud Ito and *P. hordei* Wallwork, Lichon & Sivan. Smedegård-Petersen (1972) stated that *P. graminea* is morphologically similar to *P. teres*, but causes a leaf stripe disease of barley, and cannot infect leaves directly *via* conidia. Although he was able to mate the spot- and net-types of *P. teres*, and induce pseudothecia in *P. graminea*, no matings were reported between the two species. Bulat & Mironenko (1990) used randomly amplified polymorphic DNA (RAPDs) to demonstrate that *P. teres* and *P. graminea* are intraspecific forms of the same species. Using the same technique, however, Reeves & Ball (1991) could distinguish between isolates of *P. teres* as well as *P. graminea*, suggesting that further research is necessary to characterize the relationship between these two species.

Pyrenophora hordei was recently described as a new pathogen from barley in Australia (Wallwork, Lichon & Sivanesan, 1992), and also reported from this host in South Africa (Scott, 1994). Although the *Drechslera* anamorph is morphologically similar to that of *P. teres*, the teleomorphs are distinct, as well as their disease symptoms. In preliminary inoculations done

with a South African isolate, both differential cultivars Stirling and B87/14 were highly susceptible to *P. hordei*, suggesting that the occurrence of this pathogen may seriously influence resistance breeding strategies in future.

Further research will therefore be conducted to characterize the relationship between *P. teres*, *P. graminea* and *P. hordei*, and to determine the influence of recombination on the inheritance of pathogenicity and fungicide resistance.

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