

Revision of *Pseudocercospora*-like species causing eyespot disease of wheat

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Three species and two varieties of *Pseudocercospora* Deighton are known to be associated with the eyespot disease complex of graminicolous hosts. Based on the production of lateral conidial branches, and conidiogenous cells with flat to protuberant, unthickened scars, three species had been disposed to *Ramulispora* Miura. The description of the teleomorph in *Tapesia* (Pers.) Fuckel supported this view. Although these species are regarded as varieties of *Ramulispora herpotrichoides* (Fron) Arx by some workers, a clear distinction could be made between them in the present study based upon morphology and DNA banding patterns. *Tapesia yallundae* Wallwork & Spooner (anam. *Ramulispora herpotrichoides*) is distinguished from *R. herpotrichoides* var. *acuformis* Nirenberg. Furthermore, the latter variety is erected to species level as *Tapesia acuformis* (Boerema, Pieters & Hamers) Crous stat. nov. [anam. *R. acuformis* (Nirenberg) Crous stat. nov.]. *Pseudocercospora anguioides* Nirenberg is reallocated to *Ramulispora* as *R. anguioides* (Nirenberg) Crous comb. nov. Furthermore, *P. aestiva* does not represent a species of either *Pseudocercospora* or *Ramulispora*, and its generic status remains uncertain.

Drie spesies en twee variëteite van *Pseudocercospora* Deighton is in die oogvleksierte-kompleks van graan-gewasse geïdentifiseer. Weens die voorkoms van laterale konidiale vertakkings en konidiogene selle met plat of uitstaande, onverdikte aanhegtingslokusse, is drie spesies in die genus *Ramulispora* Miura geplaas. Die daaropvolgende beskrywing van die teleomorf in *Tapesia* (Pers.) Fuckel het hierdie besluit ondersteun. Alhoewel sommige navorsers die drie spesies as variëteite van *Ramulispora herpotrichoides* (Fron) Arx beskou, kon die huidige studie die spesies onderskei op grond van morfologie en DNA bandpatrone. *Tapesia yallundae* Wallwork & Spooner (anam. *Ramulispora herpotrichoides*) word onderskei van *R. herpotrichoides* var. *acuformis* Nirenberg. Laasgenoemde variëteit word ook tot spesievlak verhef as *Tapesia acuformis* (Boerema, Pieters & Hamers) Crous stat. nov. [anam. *R. acuformis* (Nirenberg) Crous stat. nov.]. *Pseudocercospora anguioides* Nirenberg word geplaas in *Ramulispora* as *R. anguioides* (Nirenberg) Crous comb. nov. *P. aestiva* verteenwoordig ook nie 'n spesie van of *Pseudocercospora* of *Ramulispora* nie, en sy generiese status bly onseker.

Keywords: *Pseudocercospora aestiva*, *Ramulispora acuformis*, *R. anguioides*, *R. herpotrichoides*, *Tapesia acuformis*, *T. yallundae*, *Triticum aestivum*.

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Introduction

The fungus commonly associated with eyespot disease symptoms of wheat was first described as *Cercospora herpotrichoides* Fron (Sprague 1936). Based on the unthickened and inconspicuous conidial scars, several *Cercospora* species were reallocated to *Pseudocercospora* Deighton (Deighton 1973). Two varieties of *P. herpotrichoides* (Fron) Deighton were distinguished, namely *P. herpotrichoides* var. *herpotrichoides* and *P. herpotrichoides* var. *acuformis* Nirenberg (Nirenberg 1981). In addition, *P. anguioides* Nirenberg and *P. aestiva* Nirenberg were also described from eyespot lesions on cereals in Germany (Nirenberg 1981). *P. herpotrichoides* var. *herpotrichoides* and var. *acuformis* were regarded comparable with the respective wheat and rye pathotypes of this fungus (King & Griffen 1985; Sanders *et al.* 1986; Julian & Lucas 1990).

In a study of the various *Mycosphaerella* Johanson species and their respective anamorphs (von Arx 1983), *P. herpotrichoides* was reallocated to the genus *Ramulispora* Miura which was restricted to graminicolous hosts. This decision was based on the fact that scars on conidiogenous cells are usually protuberant, conidia are acicular, multi-septate, with truncate, unthickened bases, and frequent lateral branches. Von Arx (1983) stated that too many genera were available for taxa with hyaline or subhyaline conidiogenous structures and unthickened scars, justifying a redefinition of the genus *Ramulispora*. Conidiogenous cells of *Ramulispora* were determined as elongate sympodial with often

protuberant conidial scars. Furthermore, the acicular, multi-septate conidia of *R. sorghi* (Ellis & Everh.) Olive & Lefebvre were shown to form lateral branches or secondary conidia. Olive *et al.* (1946) also observed that conidia disarticulate into separate cells, usually forming mucoid masses.

Corlett (1991) listed several species of *Pseudocercospora* which have teleomorphs in *Mycosphaerella*. The description of the teleomorph of *P. herpotrichoides* as *Tapesia yallundae* Wallwork & Spooner (1988) further supports its separation from *Pseudocercospora*.

New combinations for the varieties of *P. herpotrichoides* were subsequently introduced as *R. herpotrichoides* var. *herpotrichoides* and var. *acuformis* (Nirenberg) Boerema, Pieters & Hamers (Boerema *et al.* 1992). Braun (1993) furthermore reduced *P. anguioides* to an additional variety of *R. herpotrichoides*. Because of the uncertainty surrounding the generic placement of *P. aestiva*, the latter species was not included in *Ramulispora*.

Using isozyme and mitochondrial DNA (mtDNA) banding patterns, *Pseudocercospora herpotrichoides* var. *herpotrichoides*, *P. herpotrichoides* var. *acuformis* and *P. anguioides* were clearly distinguished from *P. aestiva* (Julian & Lucas 1990; Priestly *et al.* 1992; Nicholson *et al.* 1993). The aim of the present study, therefore, was to investigate the generic and species status of the four taxa discussed above by means of scanning electron microscopy (SEM) and random amplified polymorphic DNA (RAPDs).

Materials and Methods

Scanning electron microscopy

Verified isolates of *R. herpotrichoides* var. *herpotrichoides* (South Africa, Western Cape, Klipheuwel, *Triticum aestivum*, G.F. Campbell, Sept. 1992, GC-21), var. *acuformis* (UK, *T. aestivum*, J. Lucas, isolate 22-8), var. *anguioides* (England, Rothamstead, *T. aestivum*, G.L. Bateman, 1986, isolate 86.45.64C), *P. aestiva* (Germany, *T. aestivum*, H. Nirenberg, BBA 64002, CBS 497.80) and *R. sorghi* (India, *Sorghum bicolor*, Rawla, 1971, IMI 153077; Nigeria, *S. bicolor*, E. Harris, 1960, IMI 81785) were used to compare their conidial morphology using SEM and light microscopy. Sporulation of *R. herpotrichoides* and *P. aestiva* isolates was obtained after 2 weeks on synthetic nutrient agar (SNA) (Nirenberg 1981) at 15°C under near-ultraviolet light. *R. sorghi* sporulated after 3 weeks on distilled water agar at 20°C in the dark.

Specimens were cut from colonized agar and prepared for cryo-SEM. Colonized agar plugs were flash-frozen (-212°C) in liquid nitrogen under vacuum, transferred to the preparation chamber, and then to the SEM chamber where the frozen samples were sublimated (-80°C) to remove ice particles. Samples were sputter-coated with gold in the preparation chamber for 75 s under 1.2 kV at -170°C. Specimens were viewed under 5 kV at -188°C with a Jeol JSM 6100 scanning electron microscope.

DNA isolation

DNA isolation was made from representative verified strains (cited above), selected from a larger study where 100 isolates of *R. herpotrichoides* were characterized (Campbell, Janse & Crous, in prep.). Colonized agar plugs from 14-day-old colonies grown on potato-dextrose agar (PDA) were inoculated in 200 ml liquid cultures (8 g/l yeast extract and 5 g/l glucose). Cultures were incubated at 25°C for 10 days on an orbital shaker and the mycelia harvested by filtration (Whatman No. 1 filter paper). Freeze-dried mycelia mixed with river sand was ground to a fine powder with a mortar and pestle. 20 ml Lysis buffer [3 % (v/v) SDS; 1 % (v/v) 2-mercaptoethanol; 50 mM Tris (pH 7.2) and 50 mM EDTA] was added and the mixture was incubated at 65°C overnight. Cetyltrimethylammonium bromide (CTAB) and 5 M NaCl were then added to a final concentration of 1% (v/v) and 0.7 M, respectively, and incubated for 4 h at 65°C. The mixture was extracted with phenol/chloroform/isoamylalcohol (25:24:1) (PCI) (Sambrook *et al.* 1989), and followed by two extractions with chloroform/isoamylalcohol. The resulting aqueous fraction was mixed with 7.5 M NH₄OAc and 0.54 volumes isopropanol and incubated at -20°C overnight to precipitate the nucleic acids. After centrifugation at 5000 r/min for 5 min, the pellet was washed with 70% (v/v) ethanol, vacuum-dried and dissolved in 300 µl TE buffer [10 mM Tris (pH 8.0) and 1 mM EDTA]. The sample was treated with RNase A (10 mg/ml) and incubated at 37°C for 90 min after which the DNA was PCI-extracted, precipitated with isopropanol in the presence of NH₄OAc, washed, vacuum-dried and redissolved in 300 µl TE buffer.

RAPD analysis

The 10-mers used as random primers were purchased from Operon Technologies (Kit E, Operon Technologies Inc. Alameda CA94501, USA). *Taq* polymerase together with its 10× concentrated buffer [100 mM Tris-HCl (pH 8.3, 20°C); 15 mM MgCl₂; 500 mM KCl] was obtained from Boehringer Mannheim (Johannesburg, South Africa). PCR reaction mixtures (50 µl final volumes) contained 25 ng genomic DNA, dATP, dCTP, dGTP and dTTP each at 200 µM final concentration, 100 nM oligonucleotide primer, 1X *Taq* polymerase buffer and 1 unit of *Taq* polymerase. 1 mM MgCl₂ was also added. Each reaction was overlaid with 100 µl of mineral oil to prevent evaporation. The random sequence primers used were OPE-14 5'TGCGGCTGAG and OPE-15 5'ACGCACAACC. Amplifications were conducted in a Hybaid Omnigene (Hybaid Ltd., Waldegrave Rd., Middlesex, UK) thermal cycler. Samples were subjected to 60 repeats of the following cycle: 1 min at 94°C, 1 min at 36°C and 2

min at 72°C. After the last cycle, a final extension step of 72°C for 5 min was included, followed by cooling to 4°C until recovery of the samples. Electrophoresis of aliquots (15 µl) of amplification products was through 1.4% agarose gels using 1X TAE buffer (Sambrook *et al.* 1989) at a constant voltage of 150 V for 1 h. Gels were subsequently stained with ethidium bromide and visualized by illumination with ultraviolet light.

Results and Discussion

Generic placement

The description of the teleomorph of *R. herpotrichoides* var. *herpotrichoides* as a species of *Tapesia* in contrast to *Mycosphaerella* (Wallwork & Spooner 1988), clearly separated the anamorph from *Pseudocercospora*. Furthermore, based on the research of King (1990), *T. yallundae* var. *acuformis* Boerema, Pieters & Hamers (1992) has also recently been described as the teleomorph of *R. herpotrichoides* var. *acuformis*.

Von Arx (1983) placed several *Pseudocercospora* species in *Ramulispora* based on their hosts being members of the Poaceae, and conidia having the ability to form lateral branches (Figures 1 & 2). The latter structures were clearly shown for *R. sorghi* and *R. herpotrichoides* with and without septa dividing the lateral branches from the conidia. Although outgrowths from conidia appeared to be lateral branches in the present study, some had a septum dividing them from the primary conidium. Short conidia were also observed among primary conidia of *R. herpotrichoides*, suggesting that a lateral branch could also develop into a secondary conidium (Figures 3 & 4).

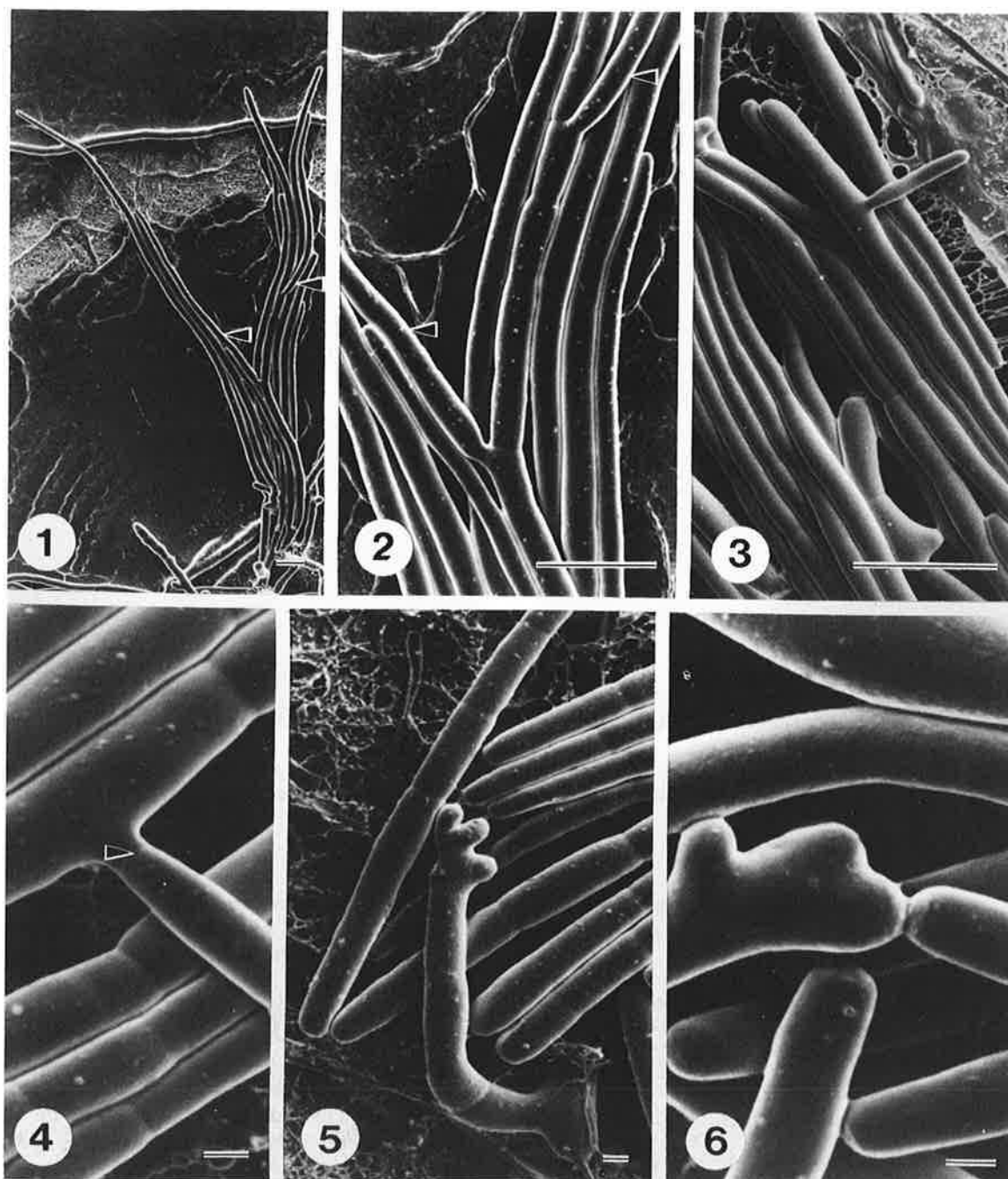
The first report of sporulation by spores in *R. herpotrichoides* was made by Foex (1914). In a detailed study, Chang and Tyler (1964) observed secondary conidia to form directly from primary conidia, as well as conidia forming on short secondary conidiophores present on the primary conidia. Microcyclic conidiation, also known as iterative germination or precocious sporulation, was defined by Smith *et al.* (1977) as the recapitulation of conidiation following conidial germination without an intervening phase of mycelial growth. However, in the present study conidiation following conidial germination was not observed, but secondary conidia were found to develop directly on the surface of primary conidia, which we consider to be distinct from microcyclic conidiation where a short conidiophore is present. Secondary conidia were observed for *R. herpotrichoides* var. *herpotrichoides*, var. *acuformis* and var. *anguioides*, and were distinct from those formed via microcyclic conidiation (*sensu* Fernandez *et al.* 1991).

From these observations it is clear that the three varieties discussed above are presently best retained in *Ramulispora*, as they cannot be accommodated in *Pseudocercospora*. However, the generic placement of *P. aestiva* remains uncertain. The present data suggest that the discovery of its teleomorph and further molecular studies will be needed to resolve the generic affinities of *P. aestiva*.

Species delimitation

Ramulispora herpotrichoides var. *herpotrichoides* and var. *acuformis*

Several morphological criteria are used to distinguish var. *herpotrichoides* and var. *acuformis*. Conidia of var. *herpotrichoides* are curved, whereas those of var. *acuformis* are straight (Nirenberg 1981). Our SEM study showed that var. *herpotrichoides* as well as var. *acuformis* have polyblastic conidiogenous cells (Figures 5 & 6). When cultured on PDA, var. *herpotrichoides* has fast-growing colonies with smooth margins, whereas *R. herpotrichoides* var. *acuformis* has slow-growing colonies with feathery margins (Lange-de la Camp 1966; Scott *et al.* 1975). The two



Figures 1–6 Scanning electron micrographs of *Ramulispora sorghi*, *R. acuformis* and *R. herpotrichoides*. **1 & 2.** Conidia of *R. sorghi* with lateral branches (arrows; scale bar 10 μ m). **3.** A primary conidium of *R. acuformis* giving rise to what initially appeared to be a lateral branch but subsequently developed into a secondary conidium (scale bar 10 μ m). **4.** A septum (arrow) dividing the secondary and the primary conidium of *R. acuformis* (scale bar 1 μ m). **5.** Polyblastic conidiogenous cell giving rise to conidia of *R. acuformis* (scale bar 1 μ m). **6.** Conidium of *R. herpotrichoides* attached to a polyblastic conidiogenous cell (scale bar 1 μ m).

varieties also differ in their virulence to wheat and rye, with var. *herpotrichoides* being more virulent to wheat than to rye, and var. *acuformis* being equally virulent to wheat and rye (Lange-de la Camp 1966; Hollins *et al.* 1985). Furthermore, an ultrastructural study showed that the infection process on a wheat coleoptile was also different for var. *herpotrichoides* and var. *acuformis* (Daniels *et al.* 1991). Distinct mating populations have recently also been isolated for var. *acuformis* and var. *herpotrichoides* in England (P. Dyer, pers. commun.), confirming the observations by King (1990). Molecular markers, including isozymes, restriction fragment length polymorphisms (RFLPs) and RAPDs, have successfully been used to distinguish var. *herpotrichoides* and

var. *acuformis* (Julian & Lucas 1990; Nicholson *et al.* 1991, 1993; Priestley *et al.* 1992; Thomas *et al.* 1992; Nicholson & Rezanoor 1994).

Using RAPDs, the present study supported the distinction made between var. *herpotrichoides* and var. *acuformis* based on general morphology, culture characteristics, pathology, and molecular studies of other workers discussed above. The *F*-values obtained with two primers in the present study indicated var. *herpotrichoides* to have 50% similarity to var. *acuformis* (Figure 7, Table 1). This value was observed to be slightly lower (25–40%) when additional primers and a wider range of isolates were used (Campbell, Janse & Crous, in prep.). Freeman *et al.*

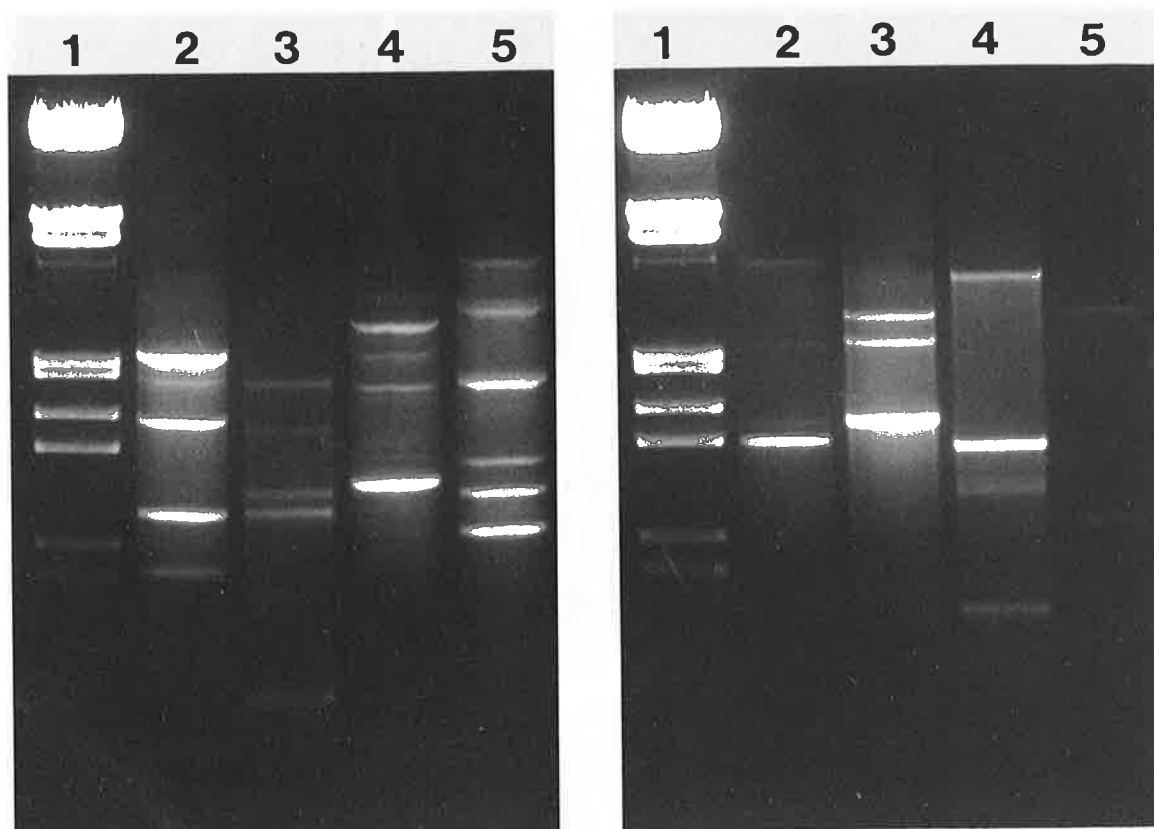


Figure 7 Amplified, polymorphic DNA bands after RAPD analysis, using primer OPE-14 (left) and OPE-15 (right). Lane 1: Phage lambda DNA digested with *EcoRI* and *HindIII* was used as a standard. Lane 2: *R. herpotrichoides*; Lane 3: *R. acuformis*; Lane 4: *R. anguioides*; Lane 5: *P. aestiva*.

Table 1 Combined *F*-values (% similarity) following RAPD analysis (2 primers) of *R. herpotrichoides*, *R. acuformis*, *R. anguioides* and *P. aestiva*

	<i>R. herpotrichoides</i>	<i>R. acuformis</i>	<i>R. anguioides</i>	<i>P. aestiva</i>
<i>R. herpotrichoides</i>				
<i>R. acuformis</i>	50			
<i>R. anguioides</i>	17	9		
<i>P. aestiva</i>	9	29	8	

(1993) regarded isolates of *Colletotrichum* Corda with 80% or more similarity as belonging to the same species and those with 35% or less similarity as belonging to different species. Isolates with intermediate levels of similarity were regarded as being in a stage of evolutionary separation (Freeman *et al.* 1993). The low percentage similarity in their DNA profiles, together with the other differences discussed above, show that var. *herpotrichoides* and var. *acuformis* should be seen as two separate species, as indicated below.

Holomorph: *Tapesia yallundae* Wallwork & Spooner, Trans. Br. mycol. Soc. 71: 703 (1988).

Anamorph: *Ramulispora herpotrichoides* (Fron) Arx, Proc. K. ned. Akad. Wet. C86 (1): 36 (1983). *Cercospora herpotrichoides* Fron, Annls Sci. agron. fr. 4, 1: 11 (1912). *Pseudocercospora herpotrichoides* (Fron) Deighton, Mycol. Pap. 133: 46 (1973).

Holomorph: *Tapesia acuformis* (Boerema, Pieters & Hamers) Crous stat. nov. *T. yallundae* Wallwork & Spooner var. *acuformis* Boerema, Pieters & Hamers, Neth. J. Pl. Path. 98: 1 (1992).

Anamorph: *Ramulispora acuformis* (Nirenberg) Crous stat. nov. *Pseudocercospora herpotrichoides* (Fron) Arx var. *acuformis* Nirenberg, Z. PflKrankh. PflSchutz 88: 244 (1981). *Ramulispora herpotrichoides* (Fron) Arx var. *acuformis* (Nirenberg) Boerema, Pieters & Hamers, Neth. J. Pl. Path. 98: 1 (1992). *R. herpotrichoides* (Fron) Arx var. *acuformis* (Nirenberg) U. Braun, Nova Hedwigia 56: 423 (1993).

Ramulispora herpotrichoides var. *anguioides*

This variety was originally described as a distinct species of *Pseudocercospora* by Nirenberg (1981), who noted its colonies on PDA to be velvety with even margins, and grey-pink. Conidia were described as forming on simple, seldomly branched conidiophores as shown in Figure 8. Successful protoplast fusion between *R. herpotrichoides*, *R. acuformis* and *R. herpotrichoides* var. *anguioides* has recently been done by Hocart and McNaughton (1994), reflecting on the relatedness of species within a genus. The successful recovery of interspecific hybrids indicated that *R. herpotrichoides*, *R. acuformis* and *R. herpotrichoides* var. *anguioides* are relatively closely related.

Isozyme electromorphs have been successfully used to distinguish *formae speciales* of *Puccinia* Pers. (Newton *et al.* 1985), aggressive and non-aggressive strains of *Ophiostoma ulmi* (Buisman) Nannf., Svenska & Skogsfor. (Bernier *et al.* 1983), as well as between species of *Phytophthora* de Bary (Hall *et al.* 1969) and *Peronosclerospora* Ito (Micales *et al.* 1988). This suggests that isozymes are useful to distinguish inter- as well as intra-

specific variation. This technique has also been used to show var. *anguioides* as distinct from *R. herpotrichoides*, *R. aciformis* and *Pseudocercospora aestiva* (Julian & Lucas 1990; Priestley *et al.* 1992).

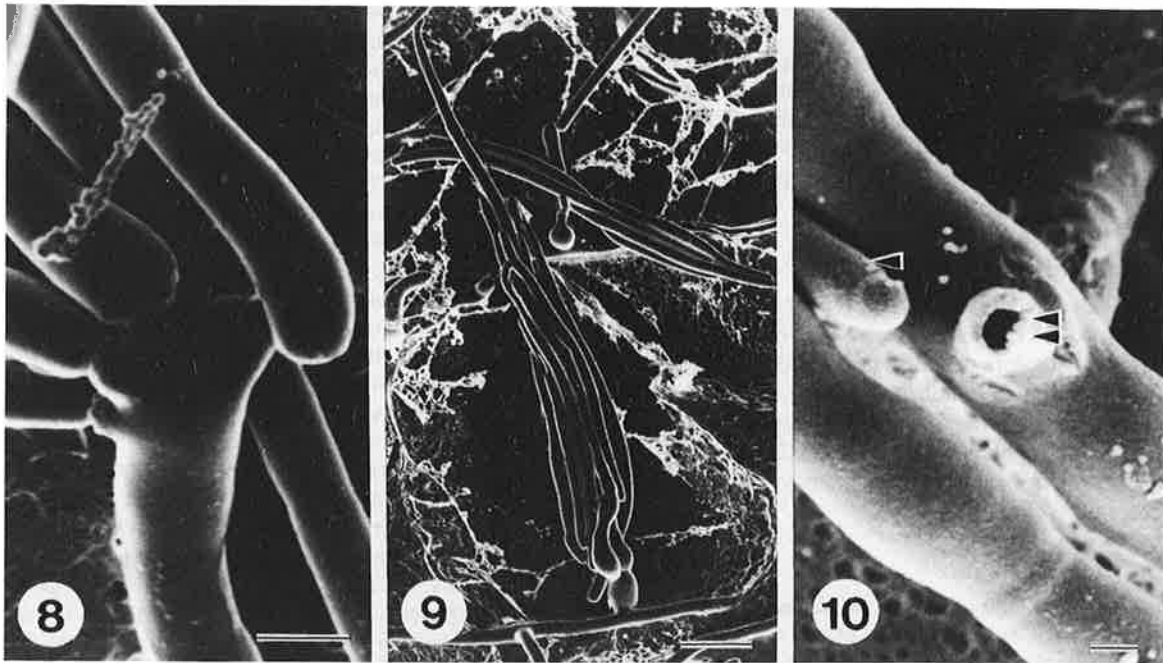
R. herpotrichoides and *R. aciformis* could also be clearly differentiated from each other using mitochondrial DNA profiles (Nicholson *et al.* 1993). Using a mitochondrial DNA probe from *R. herpotrichoides*, Nicholson *et al.* (1993) found that strong hybridization occurred to DNA of var. *anguioides*. However, strong hybridization did not occur to DNA of *P. aestiva*, indicating that var. *anguioides* may be more closely related to *R. herpotrichoides* than *P. aestiva*. Results obtained using RAPDs in the present study support the findings of Nicholson *et al.* (1993).

F-values showed var. *anguioides* to have 17, 9 and 8% similarity to *R. herpotrichoides*, *R. aciformis* and *P. aestiva*, respectively (Figure 7, Table 1). We therefore conclude that var. *anguioides* should be retained as a separate species.

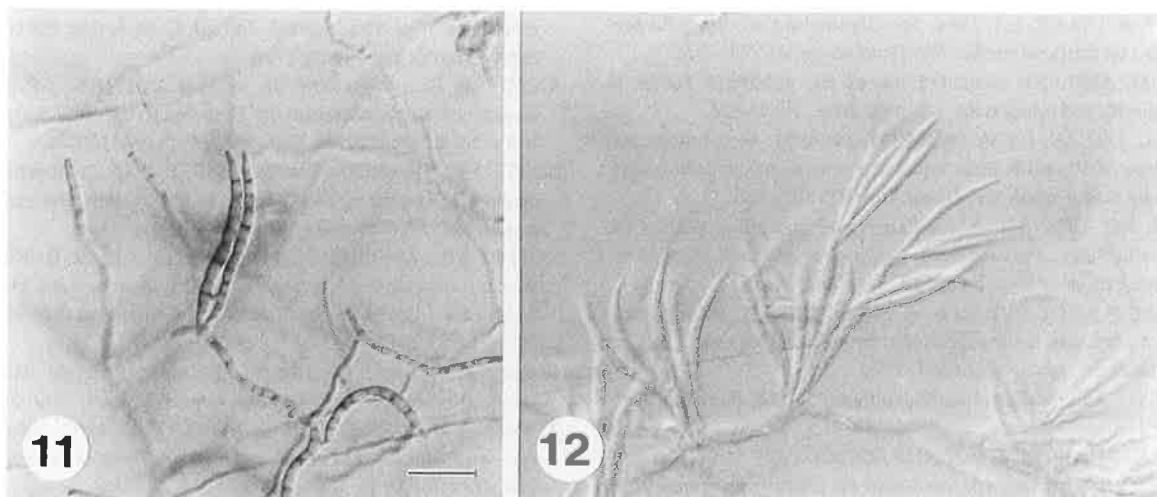
Ramulispora anguioides (Nirenberg) Crous comb. nov. *Pseudocercospora anguioides* Nirenberg, Z. PflKrankh. PflSchutz 88: 244 (1981). *Ramulispora herpotrichoides* (Fron) Arx var. *anguioides* (Nirenberg) U. Braun, Nova Hedwigia 56: 423 (1993).

Pseudocercospora aestiva

Nirenberg (1981) made no morphological distinction between



Figures 8–10 Conidiophores, conidiogenous cells and conidia of *Ramulispora anguioides*, *R. herpotrichoides* and *Pseudocercospora aestiva*. **8.** Conidia of *R. anguioides* developing from an elongated polyblastic conidiogenous cell (scale bar 1 μ m). **9.** Scanning electron micrograph of the conidiophore of *R. herpotrichoides* with ampulliform conidiogenous cells giving rise to an aggregated, slimy mass of conidia (scale bar 10 μ m). **10.** Mature, swollen conidium of *P. aestiva* giving rise to a secondary conidium (arrow) via an inconspicuous conidiogenous locus (double arrow, scale bar 1 μ m).



Figures 11 & 12 Conidia and conidiogenous cells of *Pseudocercospora aestiva* (scale bar 10 μ m). **11.** Conidia developing from an inconspicuous polyblastic conidiogenous cell. **12.** Mature, swollen primary conidia giving rise to a fascicle of secondary conidia.

conidiophores and conidiogenous cells of the three *Ramulispora* species and *P. aestiva*. In the present study it was found that all four taxa have polyblastic conidiogenous cells. However, the conidiogenous cells of *R. herpotrichoides* were frequently swollen and ampulliform, whereas those of *P. aestiva* were mostly inconspicuous. *P. aestiva* also produced fascicles of secondary conidia from older, swollen conidia. These secondary conidia were morphologically similar to the primary conidia (Figures 8–12).

Isozyme and RFLPs have indicated a dubious relationship between *P. aestiva* and the three *Ramulispora* species (Julian & Lucas 1990; Priestley *et al.* 1992; Nicholson *et al.* 1993). Using RAPDs, the present study supports these results, and also shows *P. aestiva* to be distinct from the three species of *Ramulispora*. *F*-values between *P. aestiva* and *R. herpotrichoides*, *R. acufiformis* and *R. anguoides* were 9, 29 and 8%, respectively, indicating practically no relationship (Figure 7, Table 1).

In conclusion, the use of molecular techniques in *Ramulispora* is shown to enhance the taxonomic importance of seemingly insignificant morphological differences. Results obtained with SEM and RAPDs indicate that the four taxa studied are distinct species, and that *P. aestiva* is probably not a species of *Ramulispora*.

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