***Polymyxa graminis* Isolates from Australia: Identification in Wheat Roots and Soil, Molecular Characterization, and Wide Genetic Diversity**

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Abstract

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Polymyxa graminis is an obligate parasite of roots and an important vector of viruses that damage cereal crops in different parts of the world. In 2011 and 2012, *P. graminis* was identified infecting 11 wheat root samples from three widely dispersed locations in southwest Australia. Its presence was detected by polymerase chain reaction (PCR) and confirmed by DNA sequencing of the transcribed regions of its ribosomal RNA genes (rDNA) and observing sporosori of characteristic morphology and size in stained wheat roots. Also, when soil samples were collected from two locations where *P. graminis* was found and wheat bait plants grown in them, *P. graminis* was detected in their roots by PCR. Ribosomal DNA sequences of six southwest Australian isolates were obtained from wheat roots, and one northeast Australian isolate from barley roots. When these seven *P. graminis* sequences were compared with others from GenBank by phylogenetic analysis, three southwest Australian isolates were classified as *P. graminis* f. sp. *temperata* (ribotypes Ia and Ib), and three as f. sp. *tepida* (ribotypes IIa and IIb). *P. graminis* f. sp. *temperata* and *tepida* both occur in

temperate growing regions of other continents and are associated with transmission of soil-borne viruses to cereal crops. The *P. graminis* isolate from northeast Australia was sufficiently distinct from the five existing sequence groups for it to be placed into a newly proposed grouping, ribotype VI, which also included an isolate from tropical West Africa. However, when randomly collected wheat leaf samples from 39 field crops from 27 widely dispersed locations, 21 individual wheat plant samples collected from low lying areas within 21 fields at 11 locations, and wheat bait plants growing in five soil samples from two locations were tested by reverse transcription (RT)-PCR for the presence of *Soil-borne wheat mosaic virus*, *Soil-borne cereal mosaic virus*, *Wheat spindle streak mosaic virus*, and furoviruses in general, no virus infection was detected. These findings suggest at least three *P. graminis* introductions into Australia, and the occurrence of f. sp. *temperata* (ribotype I) and f. sp. *tepida* (ribotype II) suggests that, if not already present, soil-borne cereal viruses are likely to become established should they become introduced to the continent in the future.

Plasmodiophorids were traditionally considered to be fungi until phylogenetic analysis of their small subunit ribosomal DNA sequences classified them within the protists (6,10). The plasmodiophorid virus vector genus *Polymyxa* is most closely related to *Ligniera* and *Sorosphaera*, and more distantly to *Plasmodiophora* and the virus vector genus *Spongospora* (36). The two species of *Polymyxa* that act as virus vectors, *P. graminis* and *P. betae*, were formerly distinguished largely on the basis of host range, as they are morphologically very similar. *P. graminis* mainly infects cereals and grasses, while *P. betae* mainly infects *Chenopodiaceae* (4). However, their ribosomal DNA sequences are now known to distinguish them (30,36,37).

P. graminis is an obligate biotrophic parasite of plant roots (13,17). It has a biphasic life cycle, with (i) a sporogonic phase resulting in production of sporosori containing resting spores which produce primary zoospores, and (ii) a sporangial phase, resulting in production of zoosporangia which produce secondary zoospores (5,13). Each phase is initiated by the attachment of zoospores and penetration of epidermal or root hair cells. Secondary

zoospores have the ability to initiate either phase of the cycle. Molecular diversity and phylogeny of *P. graminis* isolates were studied using restriction fragment length polymorphisms (RFLPs) and sequencing of transcribed regions of ribosomal RNA genes (18,24,36,37). A high level of sequence diversity occurs between *P. graminis* isolates, with >20% differences across the 18S, 5.8S, and internal transcribed spacer rDNA regions. Several rDNA subgroups (ribotypes) of *P. graminis* occur (18,21,22,36,38). Biological and molecular studies revealed differences in host range, temperature requirements, and genome characteristics, leading to the proposal to divide *P. graminis* into five *forma speciales* (ribotype designations in parentheses): f. sp. *temperata* (I), *tepida* (II), *tropicalis* (III), *subtropicalis* (IV), and *colombiana* (V) (18). *P. graminis* f. sp. *temperata* and *tepida* occur in cereal crops in temperate regions multiplying best at temperatures of 15 to 20°C, whereas isolates belonging to the other three *forma speciales* preferred higher growth temperatures (21). An additional temperate ribotype (VI) was proposed subsequently (39), but later placed within f. sp. *tepida* (ribotype IIb) (30). Whether temperate, tropical, and subtropical ribotypes can be distinguished based on host specificity or host preference remains unclear (22,23,30,34). For example, in one study, *temperata* was found more frequently on barley, and *tepida* on wheat, but different ribotypes often occurred together in the same soil or plant (34).

P. graminis is not considered a plant pathogen in its own right, but poses a significant threat to agriculture because of its ability to transmit agronomically important viruses that cause severe diseases in several major crop species. These crop hosts include wheat, barley, oats, pearl millet, rice, rye, and sorghum (1,22). *P. graminis* is a vector of at least 14 different soil-borne plant viruses belonging to the genera *Furovirus*, *Bymovirus*, *Pecluvirus*, and *Benyvirus*. Soil-borne viruses it transmits that infect wheat include:

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*The e-Xtra logo stands for “electronic extra” and indicates that a supplementary table is available online, and that Figure 5 appears in color online.

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the furoviruses *Soil-borne wheat mosaic virus* (SBWMV), *Soil-borne cereal mosaic virus* (SBCMV), and *Chinese wheat mosaic virus* (CWMV), and the bymoviruses *Wheat spindle streak mosaic virus* (WSSMV) and *Wheat yellow mosaic virus* (WYMV), and *Aubian wheat mosaic virus* (genus unassigned). These viruses occur in parts of Europe, Asia, and North America, and SBWMV in New Zealand (13,15,16). In addition, two pecluviruses it transmits, *Indian peanut clump virus* (IPCV) and *Peanut clump virus* (PCV), occur in the Indian subcontinent and Africa, respectively. IPCV and PCV sometimes cause damaging diseases of wheat growing under subtropical conditions (11,31). Different soil-borne viruses have been associated with distinct types of *P. graminis*. Temperate *f. sp. temperata* (ribotype I) and *f. sp. tepida* (ribotype II) are associated with WSSMV, SBCMV, SBWMV, *Barley yellow mosaic virus* (BaYMV), and *Barley mild mosaic virus* (BaMMV) infection in cereal crops (16,30,37). *P. graminis f. sp. tropicalis* (ribotype III) and *f. sp. subtropicalis* (ribotype IV) are associated with pecluviruses (18,22), and *f. sp. colombiana* (ribotype V) with *Rice stripe necrosis virus* (RSNV) (24). Viruses can be present in *P. graminis* during all stages of its life cycle and can persist inside its thick-walled resting spores, remaining viable in the soil for decades. Virus is acquired and transmitted either when zoospores penetrate host cells and transfer their contents into the cytoplasm, or at the young plasmodium stage when only a thin membrane boundary separates cytoplasm of *P. graminis* from that of its host (5,31).

In Australia, *P. graminis* was detected in 2009 in the roots of a barley crop in Wondai, Queensland, in subtropical northeast Australia, and was identified as *P. graminis f. sp. tepida* according to polymerase chain reaction (PCR) results (33). The only other record of *P. graminis* in Australia was an unconfirmed observation of its sporosori in *Poa annua* roots in 1959 in Murrumburra, which has a warm temperate climate and is in southeast New South Wales, central eastern Australia (3). No soil-borne viruses of cereal crops have been recorded in Australia (12). Southwest Australia, which has a Mediterranean type climate, hosts an extensive grainbelt which is the biggest wheat producing area in Australia. Cereals are sown in the grainbelt in late autumn or early winter and harvested in late spring, and are entirely rainfed, with the majority of the annual rainfall occurring between late fall (autumn) and early spring (May to September). The amount of rainfall is greatest near to the coast (high rainfall zone) and least further inland (low rainfall zone). Fields are large (up to 250 ha in size), and the cultural practices used are low input (minimum tillage and retained stubble) with wheat being grown continuously or in rotation with canola, grain legumes (field pea or lupin) or annual legume pastures.

This paper reports the detection in 2011 and 2012 of *P. graminis* in the roots of wheat plants sampled at three locations in southwest

Australia. It was also detected in the roots of wheat bait plants grown in soil samples from two of these locations. Identification of *P. graminis* was confirmed by PCR, microscopic observation of sporosori in stained roots, and characterization of isolates by sequencing of transcribed regions of ribosomal DNA. Phylogenetic analysis of *P. graminis* of six isolates from southwest and one isolate from northeast Australia revealed presence of *f. sp. temperata* (ribotype I), *f. sp. tepida* (ribotype II), and a new sequence group proposed in this study, ribotype VI.

Materials and Methods

Plant and soil collection. During September and October in 2011 and September in 2012, 100 leaf samples were collected for virus testing from each of 39 wheat crops growing in the southwest Australian grainbelt (Fig. 1). This leaf sampling involved 20 crops at 13 different locations in 2011 and 19 crops at 14 locations in 2012 (Supplementary Table 1). These locations were at widely dispersed sites, ranging from Geraldton in the northern grainbelt to Esperance in the south coastal grainbelt (a distance of 896 km) in 2011, and from Perth to Esperance (a distance of 740 km) in 2012. The 100 samples/crop were all taken at random by walking through the crop in a W-shaped pattern and sampling a leaf every 5 m. No potentially symptomatic plants were sampled or present. In addition, whole wheat plant samples with roots were collected from low lying areas within 21 fields. These fields were at Esperance (4 locations, 12 samples) and Pingelly (1 location, 1 sample) in 2011, and Northam (2 locations, 2 samples), Kulin (3 locations, 3 samples), and Department of Agriculture and Food (DAFWA) irrigated field plots in South Perth (2 plots, 3 samples) in 2012 (Fig. 1). Five soil samples were also collected in the immediate vicinity of plant samples at Esperance (3 samples) in 2011 and South Perth (2 samples) in 2012. Dried barley root samples obtained from Wondai, Queensland and known to be infected with *P. graminis* (33) were kindly provided by J. M. Thompson and K. J. Owen. These were used as positive controls for PCR tests.

DNA extraction and PCR. Roots were washed in tap water to remove soil and dried on paper towels. A subsample of approximately 1 g of root material was ground to a fine powder under liquid nitrogen using a mortar and pestle. DNA was extracted from 80 mg of ground root powder using a DNeasy Plant Mini kit (Qiagen) as per manufacturer's instructions. DNA was diluted 1:10 with RNase-free water prior to PCR. PCRs were done using *Polymyxa* spp. primer pair Pxfwd1/Pxrev7 and *P. graminis* primer pair Pgfwd2/Pxrev7 (36). Each 20- μ l reaction mixture contained 1 μ l of diluted DNA, 1 $\times$ PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 μ M each primer, and 0.5 U *Taq* polymerase (Qiagen). PCR cycling conditions were 94°C for 3 min, followed by 35 cycles of 94°C for

Fig. 1. Map of Australia showing locations where 21 whole wheat plant samples were collected from low lying areas within fields to test their roots for presence of *Polymyxa graminis*. Numbered dots represent locations sampled: 1. South Perth; 2. Northam; 3. Pingelly; 4. Kulin; 5. Esperance. *P. graminis* was detected at three of these locations (numbers of positive samples in parentheses): South Perth (3), Northam (2), Esperance (6).

30 s, 56°C for 1 min, 72°C for 1 min, with a final extension at 72°C for 10 min. Products were analyzed on a 2% agarose gel in TAE buffer and stained with ethidium bromide.

RNA extraction and reverse transcription (RT)-PCR. Root samples (80 mg/sample) ground under liquid nitrogen as described above for DNA extraction were also used for RNA extraction. For 100 leaf samples, 1-cm-long leaf pieces were subsampled from each leaf and pooled for one extraction. With individual plant samples, five leaves from each plant were pooled. Pooled leaf samples were ground to a fine powder under liquid nitrogen in a mortar and pestle, and RNA extractions carried out on 100 mg of ground leaf material using a Spectrum Plant Total RNA kit (Sigma) as per manufacturer's instructions. Leaves and roots were tested for the presence of soil-borne viruses by RT-PCR, using a OneStep RT-PCR kit (Qiagen), as per manufacturers' instructions, with specific primer pairs WSSMVc2F/WSSMVc2R for WSSMV (35), SBWMV-EF/SBWMV-UNIR for SBCMV (8), SBMV L2/SBMV R2 for SBWMV (9), and universal primers Furo1-F/Furo2-F for furoviruses (28). Virus-infected dried leaf samples of each virus obtained from DSMZ (Germany) were used as positive controls.

Sequencing and phylogenetic analysis. To obtain ITS region sequences from root samples containing *Polymyxa* spp., PCR was done on root DNA extractions using primer pairs Psp1/Psp2rev (20), Psp1/ITS 4 (40), and Pgf2/ITS4 (37). Amplicons were purified using QIAquick PCR purification kit (Qiagen) where one band was present. When multiple bands were present, they were separated by agarose gel electrophoresis and purified using QIAquick Gel extraction kit (Qiagen). Both strands of PCR products were sequenced directly with the primers used for amplification in an AB 3730 DNA Analyzer using Big Dye v 3.1 Terminator chemistry (Applied Biosystems, Victoria, Australia). Overlapping sequences were aligned and edited using Geneious v 6, and submitted to the GenBank database. Nucleotide (nt) sequence data for 46 *P. graminis* rDNA-ITS region sequences from GenBank that included the complete ITS 1, 5.8S rRNA gene, and ITS 2 were aligned in Mega v 5 using ClustalW, and manually edited. Lengths of sequences ranged from 411 to 485 bases. A *P. betae* sequence was included in the alignment as an outgroup. Phylogenetic analysis was carried out in Mega v 5 (32) using the neighbor-joining method. The alignment and analysis was then repeated, with sequences trimmed to the end of the 5.8S gene, so that an extra 16 sequences with partial coverage of the ITS region could be included. Sequence lengths ranged from 257 to 310 bases.

Microscopy. A 0.5-g subsample of washed dried roots (sample Northam P5) was cleared and stained with trypan blue (26). Root pieces were mounted in lactic acid on microscope slides and observed under a compound microscope at magnifications up to ×400. Photomicrographs of *P. graminis* sporosori were taken with an Olympus UC30 microscope/camera system.

Soil bait tests. Soil collected from South Perth (samples SP22 and SP28) and Esperance (samples GG, E2, and W12), was spread out on newspaper to air dry. The dried soil samples were diluted with sterile sand at a 1:3 vol/vol ratio and added to clean 18-cm-diameter pots, one pot per soil sample. Sterile potting mix containing sand was used as a control. Wheat seeds (cv. Calingiri) were surface sterilized with 2.5% NaClO and planted into pots, four seeds per pot. Pots were placed on a free-draining metal grill in a growth cabinet, 16°C night and 20°C day with a 12-h photoperiod, and watered daily. After 2 months, pot contents were removed and roots of individual plants were washed free of all soil and sampled for DNA and RNA extraction. Leaves were sampled for RNA extraction. PCR assays (for root extracts) and RT-PCR assays (for leaf extracts) were used to test the samples as described above.

Results

PCR testing of field samples. *Polymyxa* spp. were detected by PCR using the *Polymyxa* spp. primer pair Pxfwd1/Pxrev7 in DNA extracted from 14 out of 21 wheat root samples from Esperance (9 samples), South Perth (3 samples), Northam (1 sample), and Kulin (1 sample) (Table 1). Eleven samples gave a band with *P. graminis* type I and II specific primer pair Pgfwd2/Pxrev7, including one which gave no band with primer pair Pxfwd1/Pxrev7. The approximate size of the PCR products for the three samples from South Perth (290 bp) corresponded to the published size for *P. graminis* type I (36), and one of these samples produced two bands (290/230 bp) with primer pair Pgfwd2/Pxrev7, indicating ribotypes I and II were both present (Fig. 2). Five samples from Esperance gave a smaller band (approx. 290 bp) with *Polymyxa* spp. primer pair Pgfwd1/Pxrev7 corresponding to ribotype I, and a larger band (320 bp), corresponding to ribotype II with primer pair Pgfwd2/Pxrev7. One sample each from Esperance and Northam also produced a second PCR band with primer pair Pgfwd1/Pxrev7, indicating the presence of ribotypes I and II. One sample from Kulin and three from Esperance gave a 290-bp band with primer pair Pgfwd1/Pxrev7, but no band with the ribotype I and II primer pair Pgfwd2/Pxrev7. This corresponds most closely to a ribotype III

Table 1. *Polymyxa* spp. polymerase chain reaction (PCR) product sizes obtained from wheat root field samples

Sample ID	Location	Cultivar	PCR product size (bp) with <i>Polymyxa</i> spp. primer sets ^a	
			Pxfwd1/Pxrev7	Pgfwd2/Pxrev7
E2	Esperance	Sapphire	290	320
E2-1	Esperance	Sapphire	...	...
E2-2	Esperance	Sapphire	290	320
E2-3	Esperance	Sapphire	290	...
W12	Esperance	Sapphire	290	320
W12-1	Esperance	Sapphire	290/320	320
W12-2	Esperance	Sapphire	290	...
W12-3	Esperance	Sapphire	290	320
GG	Esperance	Unknown	290	320
GG-1	Esperance	Unknown	...	...
GG-2	Esperance	Unknown	290	...
TOS	Esperance	Sapphire	...	...
Pingelly	Pingelly	Unknown	...	...
SP 22a	South Perth	Unknown	290	290
SP 22b	South Perth	Unknown	290	290/320
SP 28	South Perth	Unknown	290	290
KE	Kulin	Mace	...	...
KW	Kulin	Mace	...	...
KWH	Kulin	Mace	290	...
P38	Northam	Magenta	...	290
P5	Northam	Benu	320/290	320
Qld control	Wondai	Dictator (barley)	330	330

^a From Ward and Adams (36).

result. One sample from Northam produced a 290-bp band with primer pair Pgfwd2/Pxrev7, but no band with primer pair Pgfwd1/Pxrev7, indicating ribotype I presence. RT-PCR tests on the roots or leaves of the 21 individual field plant samples, or 100 leaf samples taken at random from 39 wheat crops, did not detect WSSMV, SBCMV, SBWMV, or with the generic furovirus primer pair, any other soil-borne virus.

DNA sequencing. Wheat field sample extracts in which *P. graminis* was detected by PCR were amplified for sequencing using three sets of primers in order to obtain complete ITS region sequences. Seven new *P. graminis* sequences were obtained, three from the Northam, two from the South Perth, and one each from Esperance and Queensland samples (Table 2). Considerable sequence variation was found within the Australian isolates sequenced, their nt sequence identities ranging from 73.9 to 100%. Two distinct isolates were obtained from South Perth samples, one with 99.6% nt identity with *P. graminis* ribotype II isolate 'Kent' from wheat in the UK, the other whose sequence was identical to a ribotype I isolate 'Ag' from Switzerland (see Table 3 for full list of isolates compared). The sequence obtained from Esperance most closely resembled that of isolate 'garden' from the UK (99.7% identity). Three sequences were obtained from the Northam samples, one of which was identical to the Esperance sequence. The other two shared closest nt identities (97.9 to 99.8%) with ribotype I isolates 'C13SH' and 'Oto4' from Belgium. Five barley root samples from Queensland were sequenced, and all had an identical *P. graminis* sequence, which had 99.9% identity with sorghum isolate 'BF-209' from Burkina Faso. A second PCR band was also produced from Queensland barley samples with primers Psp1/Psp2rev and sequenced, but it was more closely related to *P. betae* sequences. In addition, partial sequences were obtained from a sample from Kulin, and one from Esperance. These two partial sequences shared some homology with other *P. graminis* sequences, but not enough sequence information was obtained to identify them.

Phylogenetic analysis. The tree generated using *P. graminis* sequences that included all of the ITS 1 and 2 regions revealed great sequence diversity. The complete set of isolates compared (Table 3) grouped into six distinct clades with strong bootstrap support, corresponding to the five previously recognized ribotypes, and one

new clade (Fig. 3). There was sufficient diversity within clades I and II for separation of both into two smaller subclades, a and b. Northeast Australian isolate QldB was placed into a new clade which contained only isolate BF-209 from Burkina Faso in West Africa. This clade was closest to clade V, which contained one isolate each from South America and Africa, but was distant enough and with reasonable bootstrap support to be considered a separate clade, clade VI. One South Perth isolate was located with three European isolates in subclade Ib, and the other South Perth isolate grouped with six European isolates in subclade IIa. Clade III contained eight isolates from the United States, Africa, and India, while clade IV consisted of two isolates from India and one from Pakistan.

When the phylogenetic analysis was repeated with sequences trimmed to ITS 1 and 5.8S gene only such that isolates without ITS 2 could be included, another tree with six major clades was produced (Fig. 4). Isolates with complete ITS region sequences were all within the same clades as previously. However, clades III, V, and VI changed positions in this phylogenetic tree due to use of shorter lengths and greater numbers of sequences. Northam isolate P38 was in subclade Ia with isolates from North America and Europe, and isolate NorP5S was in subclade Ib with SP1. The remaining Northam isolate NorP5B, and Esperance isolate EspW12B were located in subclade IIb with an isolate from the UK.

Observation of *P. graminis* in wheat roots. Photomicrographs of *P. graminis* observed in stained sections of wheat roots from Northam (sample P5) are shown in Figure 5A and B. Multiple sporosori were observed, their appearance corresponding to descriptions of *P. graminis* sporosori in wheat given by Ledingham (17) and Barr (4). Individual resting spores were approximately 4 µm in diameter.

Soil bait tests. *Polymyxa* spp. and *P. graminis* ribotypes I and II were detected by PCR in the roots of wheat bait plants grown in soil collected from one of two South Perth samples and all three Esperance samples (Table 4). No *Polymyxa* spp. were detected in the roots of control plants grown in potting mix. RT-PCR tests detected neither the three soil-borne viruses tested for WSSMV, SBCMV, SBWMV, or furoviruses in general in leaf samples from these wheat bait plants.

Fig. 2. Agarose gel showing polymerase chain reaction products with a, *Polymyxa* spp. primers and b, *Polymyxa graminis* type I and II specific primers. Lanes 1 to 3: South Perth wheat root samples, lane 4: uninfected wheat roots, lane 5: *P. graminis* +ve barley roots Qld, lane 6: no template control, M: 100-bp DNA ladder.

Table 2. *Polymyxa graminis* sequences obtained from Australian cereal root samples

Isolate	Location	Accession	Primers used	Sequence length (b)	Region included	Closest sequence match	% Nucleotide identity
EspW12	Esperance	KF657737	Psp1/Psp2rev	518	ITS1	AMO75823 UK garden soil	99.7
NorP5B	Northam	KF535917	Psp1/Psp2rev	520	ITS1	As above	99.7
NorP5S	Northam	KF535918	Psp1/Psp2rev	470	ITS1	AM110920 Belgium, rye	97.1
NorP38	Northam	KF535919	Psp1/Psp2rev	471	ITS1	AM259279 Belgium, barley	99.8
SP2	South Perth	KF535920	Pgf2/ITS4	493	ITS 1 and 2	AJ841287 UK wheat	99.6
SP1	South Perth	KF535921	Psp1/ITS4	672	ITS 1 and 2	EU244488 Switzerland	100
QldB	Queensland	KF535922	Psp1/ITS4	695	ITS 1 and 2	HE565477 Burkina Faso, Sorghum	99.8

Discussion

In this study, presence of *P. graminis* was confirmed for the first time in southwest Australia, and for the second time in Australia as a whole. It was found by PCR in roots of 11 wheat plants from low-lying areas within wheat fields or field plots at three widely dispersed locations in southwest Australia, and characteristic *P. graminis* sporosori were observed microscopically in stained root

samples. *P. graminis* was also detected by PCR in roots of wheat bait-plants grown in soil samples from two of these locations. Ribosomal DNA sequencing of root samples from the field in southwest Australia revealed the presence of genetically distinct *P. graminis* isolates with wide nt diversity across their internal transcribed spacer regions. Phylogenetic analysis of *P. graminis* ITS rDNA sequences produced trees similar to those previously reported (30,39), but with the inclusion of seven new Australian iso-

Table 3. Details of *Polymyxa graminis* isolates with rDNA internal transcribed spacer region sequences used to establish sequence identities and for phylogenetic analysis

Accession number	Isolate name	Geographical origin	Isolation source	Reference
AJ010424	Meta	Colombia	Rice	24
AJ311572	B1	Belgium	Barley	18
AJ311573	C1	Canada	Barley	18
AJ311574	F11	France	Barley	18
AJ311575	I1-1	India	Sorghum	18
AJ311576	I1-20	India	Sorghum	18
AJ311577	I9	India	Sorghum	18
AJ311578	I10	India	Pearl millet	18
AJ311579	P1	Pakistan	Sorghum	18
AJ311580	S6	Senegal	Pearl millet	18
AJ841287	Kent	UK	Wheat	39
AM075820	Afr2	Cote d'Ivoire	Rice	39
AM075823	Garden	UK	Wheat	39
AM110920	C13Sh	Belgium	Rye	None
AM259275	C14Oto	Belgium	Barley	34
AM259276	C160c	Belgium	Barley	34
AM259277	C11Fc	Belgium	Wheat	34
AM259278	C12Fs	Belgium	Wheat	34
AM259279	Oto4	Belgium	Barley	34
AM259280	Oc6	Belgium	Barley	34
AM259281	Oc12	Belgium	Barley	34
AM259282	Fc17	Belgium	Wheat	34
AM259283	Fc18	Belgium	Wheat	34
AM259284	Ff24	Belgium	Wheat	34
EU244487	Sg	Switzerland	Unknown	None
EU244488	Ag	Switzerland	Unknown	None
FN393966	CoWob11	UK	Arabidopsis	29
FN393967	LeWil7	UK	Arabidopsis	29
FN393968	LeWob8	UK	Arabidopsis	29
FN393969	CoWil1	UK	Arabidopsis	29
FN393970	CoWil7	UK	Arabidopsis	29
FN393971	CoWob3	UK	Arabidopsis	29
FN393972	Cowob10	UK	Arabidopsis	29
FN393973	LeWob34	UK	Arabidopsis	29
FN393974	LeWi13	UK	Arabidopsis	29
FN393975	CoWob29	UK	Arabidopsis	29
GQ892044	Austria	Austria	Poa	25
HE565475	Se-141	Senegal	Unknown	19
HE565476	BF-207	Burkina Faso	Sorghum	19
HE565477	BF-209	Burkina Faso	Sorghum	19
HE860044	Wob25	UK	Soil	30
HE860045	Sch1	Germany	Barley	30
HE860046	Sch2	Germany	Barley	30
HE860047	87Sum6	USA	Wheat	30
HE860048	87Sum8	USA	Wheat	30
HE860049	108Sum5	USA	Wheat	30
HE860050	108Sum18	USA	Wheat	30
HE860051	3Ger1	Germany	Rye	30
HE860052	3Ger2	Germany	Rye	30
HE860053	7Ger12	Germany	Rye	30
HE860054	Ken21	UK	Soil	30
HE860055	Ken5	UK	Soil	30
HQ378505	Czech	Czech	Barley	14
KF657737	EspW12B	Australia	Wheat	This study
KF535917	NorP5B	Australia	Wheat	This study
KF535918	NorP5S	Australia	Wheat	This study
KF535919	NorP38	Australia	Wheat	This study
KF535920	SP2	Australia	Wheat	This study
KF535921	SP1	Australia	Wheat	This study
KF535922	QldB	Australia	Barley	This study
Y12824	F1	UK	Barley	36
Y12825	I1-229	India	Sorghum	36
Y12826	F51	UK	Oat	36
Y12827	F67 <i>P. betae</i>	Netherlands	Sugar beet	36

lates revealing even greater sequence diversity. Six clades with strong bootstrap support could be distinguished on both trees, clades I to V corresponding to the five recognized ribotypes. All temperate forms of *P. graminis* f. sp. *temperata* (ribotype Ia and Ib) and f. sp. *tepida* (ribotype IIa and IIb) were identified among the southwest Australian isolates, sometimes within the same sample. In addition, inclusion of an isolate from Queensland, northeast Australia revealed presence of a new sequence group proposed in this study, ribotype VI.

No virus particles were observed by electron microscopy in leaf samples from the Queensland barley crop where *P. graminis* was first detected in Australia (33). In this study, RT-PCR testing for

WSSMV, SBCMV, and SBWMV, and for furoviruses in general, was undertaken on (i) leaf survey samples collected at random from 39 wheat crops growing at 27 widely dispersed locations in the southwest Australian grainbelt; (ii) 21 individual wheat plant samples, which included 14 with *Polymyxa* spp. in their roots, collected from low-lying areas within 21 fields at 10 locations in the grainbelt and one irrigated Metropolitan field plot location; and (iii) samples from bait plants grown in five soil samples from *P. graminis* infested locations, three from the grainbelt and two from Metropolitan field plots. However, no viruses were detected in any of these samples. More extensive sampling of wheat crops, especially targeting those growing in high rainfall grainbelt zones prone

Fig. 3. Neighbor-joining relationship tree for the rDNA internal transcribed spacer nucleotide sequences of 46 *Polymyxa graminis* isolates from GenBank, and three from this study (shown in bold). A *Polymyxa betae* sequence is included as an outgroup. Details of sequences are given in Table 3. Tree branches were bootstrapped with 10,000 replicates. Numbers at nodes indicate bootstrap scores >60%. Evolutionary distances were computed using the Maximum Composite Likelihood Method. Scale bar represents a genetic distance of 0.02 for horizontal branch length. The phylogenetic tree was generated using MEGA version 5 (32).

to water-logging, is warranted before any conclusions can be drawn about the presence or absence of soil-borne wheat viruses in the region.

The six sequenced southwest Australian *P. graminis* isolates shared sequence identities with temperate isolates belonging to ribotypes I and II, found in wheat, barley, rye, and garden soil in Europe. These ribotypes are associated with transmission of SBCMV, SBWMV, and WSSMV. Moreover, isolate SP1 closely resembled the short 258-bp *P. graminis* isolate cv. 1393-06

sequence (accession no. EU826169) found associated with SBWMV in wheat in New Zealand (16), as both sequences matched ribotype Ib Swiss isolate Ag (accession no. EU244488), and UK isolate CoWob3 (accession no. FN393971). The sequence of the Queensland isolate from barley was very different, being almost identical to a tropical *P. graminis* isolate from sorghum in Burkina Faso (19).

Based on PCR data, Thompson et al. (33) had suggested previously that the Queensland barley samples, which were all from the

Fig. 4. Neighbor-joining relationship tree for the partial rDNA internal transcribed spacer nucleotide sequences of 62 *Polymyxa graminis* isolates from GenBank, and seven from this study (shown in bold). A *Polymyxa betae* sequence is included as an outgroup. Details of sequences are given in Table 3. Tree branches were bootstrapped with 10,000 replicates. Numbers at nodes indicate bootstrap scores >60%. Evolutionary distances were computed using the Maximum Composite Likelihood Method. Scale bar represents a genetic distance of 0.02 for horizontal branch length. The phylogenetic tree was generated using MEGA 5 (32).

Fig. 5. Photomicrographs of sporsori of *Polymyxa graminis* in roots of wheat cv. Benu from Northam, WA. Roots cleared and stained with trypan blue and observed at **A**, $\times 200$ and **B**, $\times 400$ magnifications.

same location, contained *P. graminis* ribotype II, but noted that there might have also been a second ribotype present, possibly tropical ribotype III. According to PCR results from southwest Australia, three samples from Esperance and one from Kulin contained *P. graminis* ribotype III, but this was not confirmed by sequencing, in which only a partial sequence from the Kulin sample was obtained. Moreover, the sequence obtained for isolate SP1, which placed it in ribotype Ib, had some sequence variation in the region where primer Pgfwd2 binds, so this might have prevented its detection by the ribotype I and II specific PCR primers. In an assessment based on PCR alone, such a result would have incorrectly indicated it was ribotype III instead of ribotype Ib. The samples from Esperance and Kulin might therefore have contained similar ribotype Ib isolates rather than ribotype III. Further studies are required on new samples from northeast Australia to establish whether *P. graminis* ribotypes I, II, III, and other ribotypes are present there, and in southwest Australia to establish whether ribotype III is present. Smith et al. (30) recently reported the presence of *P. graminis* ribotype III in California which has a Mediterranean-type climate like that of southwest Australia, so its possible presence in southwest Australia or subtropical northeast Australia would seem consistent with the U.S. experience.

P. graminis isolates found around the world show a high degree of genetic diversity (30). In this study, with some of the wheat root samples, PCR tests with the two primer pairs gave double bands or two different-sized bands, which might indicate the presence of more than one *P. graminis* ribotype or, possibly, of another *Polymyxa* species. One partial sequence from Kulin and another from

Table 4. Detection of *Polymyxa graminis* by polymerase chain reaction (PCR) in the roots of wheat bait plants grown in soil collected from locations where *P. graminis* was found^a

Soil sample	Collection location	Number of plants positive with primer sets	
		Pxfwd1/Pxrev7	Pgfwd2/Pxrev7
SP 22	South Perth	0/4	0/4
SP 28	South Perth	2/4	2/4
E2	Esperance	4/4	2/4
GG	Esperance	3/4	0/4
W12	Esperance	3/4	3/4
Potting mix control	...	0/4	0/4

^a Four wheat plants were planted per pot, then tested individually by PCR.

Esperance shared some homology with other *P. graminis* sequences, but were too short to identify them. When a second PCR band produced from Queensland samples with primers Psp1/Psp2rev was sequenced, it was more closely related to *P. betae*. Because *Polymyxa* spp. are obligate biotrophs and can only be maintained in the roots of plants, it is often difficult to obtain high quality DNA extracts for sequencing that are sufficiently free from host DNA and other root organisms, especially from field samples. In the future, to help overcome problems with sequencing samples containing different *Polymyxa* ribotypes or species, propagation of single spore cultures on host plants in semisterile cultures (2) or cloning of PCR products would be preferable.

Agriculture only commenced within the Australian continent 226 years ago following European colonization, and many crop pathogens found elsewhere in the world are absent. However, the finding of (i) *P. graminis* isolates belonging to two different ribotypes, and two different subclades within each of them, in a small-scale survey in southwest Australia, and (ii) a distinct *P. graminis* ribotype in northeast Australia, would suggest that there have already been multiple introductions to the Australian continent. A widespread survey involving many more samples, locations, and regions would be required to establish whether *P. graminis* is widespread in the soils of Australia's different regional grainbelts. IPVC is seedborne in millet and peanut (27). Also, there is evidence that SBCMV may be seedborne in wheat (7), and also spread through surface contamination of seeds with viruliferous *P. graminis* resting spores so soil-borne viruses are likely to be introduced in this way to new regions. Assuming that this has not occurred already or remains undetected as yet, if soil-borne wheat viruses were to arrive in Australia via viruliferous resting spore contaminated imported wheat seed or infested soil material, they could become a widespread threat to cereal production, especially in high rainfall zones, as the vector is already established. Moreover, once soil-borne viruses are established in an agricultural area, they cannot be eradicated from the soil due to the resilience and persistence of viruliferous *P. graminis* resting spores. Chemical control is not effective or environmentally acceptable, and the principal way to control such virus diseases effectively once they become established is through development of virus-resistant cultivars (15). There are cereal breeding programs breeding for resistance to soil-borne viruses transmitted by *P. graminis* in many other countries, but the molecular diversity of virus strains is providing an ongoing challenge to their effectiveness (15). No such cereal breeding for resistance to soil-borne viruses has been undertaken in Australia, but pre-emptive breeding for this purpose should be considered as a precautionary measure, especially for wheat because of its significance as the country's most important and largest agricultural export commodity.

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