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Occurrence of *Polymyxa graminis* Ribotypes in Germany and Their Association with Different Host Plants and Viruses

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The plasmodiophorid *Polymyxa graminis* transmits plant viruses to cereal crops such as wheat, rye, barley and triticale. Soil samples from different locations and cereal host plants were analyzed for the presence of *P. graminis* ribotypes I and II, and tested for the occurrence of soil-borne viruses. *P. graminis* sequences mainly from fields in Germany used for virus resistance trials, but also from a site each in Poland and Denmark were obtained and deposited in the European Nucleotide Archive. The interactions between the components of the pathogen complex – vector ribotype and virus – and the host are discussed.

Keywords: *P. graminis* f. sp. *temperata*, *P. graminis* f. sp. *tepida*, ribotypes I and II, soilborne viruses, SBCMV, SBWMV, WSSMV

Introduction

Polymyxa graminis (Ledingham 1939) is an endoparasitic protist classified in the order Plasmodiophorida (Braselton 1995; Bulman et al. 2001; Bulman and Braselton 2014) that parasitizes roots of grasses and cereal species such as wheat, rye, triticale and barley, but also sorghum, pearl millet, rice, maize, groundnut and sugarcane. *P. graminis* is economically important because of its ability to transmit a number of different virus species, including soil borne cereal mosaic virus (SBCMV), soil borne wheat mosaic virus (SBWMV), wheat spindle streak mosaic virus (WSSMV), barley mild mosaic virus (BaMMV), barley yellow mosaic virus (BaYMV) (Kanyuka et al. 2003; Kühne 2009; Tamada and Kondo 2013), peanut clump virus (PCV) (Thouvenel and Fauquet 1981), sorghum chlorotic spot virus (Kendall et al. 1988) and rice stripe necrosis virus (Lozano and Morales 2009).

Ward et al. (1994, 1998 and 2005) have described different *P. graminis* ribotypes based on sequence differences in the ribosomal rDNA region. Differences in ecological requirements and rDNA sequences among *P. graminis* isolates have led Legreve et al. (2002) to propose five special forms (*formae speciales*): *P. graminis* f. sp. *temperata* (ribotype I),

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f. sp. *tepida* (ribotype II), f. sp. *tropicalis* (ribotype III), f. sp. *subtropicalis* (ribotype IV) and f. sp. *colombiana* (ribotype V). Recently, Cox et al. (2014) proposed the new ribotype VI for a sequence obtained from northeast Australia. Two of the ribotypes (f. sp. *temperata* = ribotype I, and f. sp. *tepida* = ribotype II) are found in temperate areas and are responsible for the transmission of economically important viruses such as SBCMV, SBWMV and WSSMV, as well as BaMMV and BaYMV to cereals. Some host specificity of the *P. graminis* special forms was proposed: f. sp. *temperata* was found to be more frequent on barley, and f. sp. *tepida* – on wheat and other cereals (Ward et al. 2005; Vaianopoulos et al. 2007).

Dieryck et al. (2011) have shown that the PCV transmission efficiency depends on the *P. graminis* special forms. *P. graminis* f. sp. *subtropicalis* transmitted PCV to barley and wheat, whereas f. sp. *tropicalis* transmitted PCV to barley, wheat, pearl millet and sorghum. *P. graminis* f. sp. *temperata* did not transmit PCV, even though *in vivo* acquisition of PCV had occurred. Recently Smith et al. (2013) have reported greater sequence variation in *P. graminis* than described so far; at least two new ribotypes have been recognized. More effort is needed to elucidate the specifics of the host-vector ribotype-virus associations. According to Smith et al. (2013) the host range for *Polymyxa* spp. may be wider than thought: *P. betae* and *P. graminis* type I sequences were detected in Arabidopsis (Smith et al. 2011), and *P. graminis* type II sequences have been found in DNA from the roots of forest trees (Kwaśna et al. 2008).

Kastirr and Widera (1988) had reported earlier that *P. graminis* was common throughout Eastern Germany. In an attempt to extend this knowledge we have analyzed by PCR and qPCR samples of soils and plant roots from experimental field sites in different regions of Germany used for virus resistance trials, as well as some samples from abroad for the presence of different *P. graminis* ribotypes.

Materials and Methods

Different host plants and soil samples were obtained from fields in different regions of Germany, from Stubbum (Denmark), from an experimental field in Poland and from Saudi-Arabia (Derab, Al-Riyadh).

DNA extraction from root material was done according to the following protocol:

https://www.diversityarrays.com/files/DArT_DNA_isolation.pdf; DNA from soil was extracted as in Ward et al. (2004) with modifications. One ml of extraction buffer (120 mM Na_2HPO_4, 2% CTAB, 1.5 M NaCl) was added to approx. 0.5 g soil in a screw-cap tube containing 6 steel ball bearings (3 mm diameter). After homogenizing for 30 sec (Minilys, Peqlab) the material was centrifuged for 5 min at 5000 rpm. The supernatant was mixed in a fresh tube with an equal volume of chloroform, mixed for 30 sec and centrifuged for 5 min at 14,000 rpm. The supernatant was mixed with an equal volume of isopropanol and kept for 1 h at room temperature. After centrifugation for 10 min at 14,000 rpm, the pellet was washed with 75% ethanol twice, dried and finally dissolved in 50 µl TE pH 8.0.

Primers

The primers for the *P. graminis* detection were designed by lining up known *P. graminis* ribotype I and ribotype II sequences using CLUSTAL Omega (McWilliam et al. 2013).

The chosen primers AZtempF and AZtepF (forward primers for ribotypes I and II, respectively) hybridize to the 18S gene of the *P. graminis* sequence, the AZtempR and AZtepR primers (reverse primers for ribotypes I and II, respectively) – to a part of ITS1 of the *P. graminis* genome that shows sequence differences between ribotypes I and II (primer nucleotide sequences shown in Table 1). For the wheat samples from Saudi-Arabia, generic fungal ITS primers ITS4 (5'TCCTCCGCTTATTGATATGC3') and ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') were used (White et al. 1990) for amplification, since it was not known what *Polymyxa* ribotypes to expect from these samples.

Table 1. Primers used for the amplification of *P. graminis* f. sp. *temperata* (ribotype I) and *tepida* (ribotype II)

Ribotype	Primer name	Sequence region	Primer sequence
temperata ribotype I	AZtempF	nts 263–284 in Y12824	5'CAGCAATGGCCGGAGCGCGGTT3'
	AZtempR	nts 398–419 in Y12824	5'CACATAGCCCACAATCCACATG3'
tepida ribotype II	AZtepF	nts 282–303 in Y12826	5'CAGCAATGGCCGGCGCGCGTCC3'
	AZtepR	nts 430–450 in Y12826	5'TCCATCCCGGTATCCAGCAAC3'

nts = nucleotides.

The nucleotide sequences of the primers used for the detection of the Furoviruses SBCMV and SBWMV, and the Bymovirus WSSMV (Fomitcheva et al. 2008) are shown in Table 2.

Table 2. Primers used for the detection of soil-borne viruses (Fomitcheva et al. 2008)

Virus	Primer name	Sequence region	Primer sequence
SBWMV	PGRV4a	nts 385–404 RNA2	5'CTGCGACTCACGCTTACATA3'
	PGRV4b	nts 1047–1066 RNA2	5'TAACCGCTTTGGGATGATAG3'
WSSMV	PGRV7a	nts 1–20 RNA1	5'CAACCGTTTTCTCAGGACTT3'
	PGRV7b	nts 797–817 RNA1	5'GCTTTCTCATTCCAACTATCG3'
SBCMV	SBCMVfor_RNA2	nts 456–475 RNA2	5'ACTTACCCATTTAGGTGTAA3'
	SBCMVrev_RNA2	nts 1414–1433 RNA2	5'TTATAATCACGCAAGTACCT3'

nts = nucleotides.

RNA extraction and standard RT-PCR for virus detection

RNA was prepared from leaf material using RNA tri-liquid (Bio&Sell, Feucht, Germany). The primers used for detection of SBCMV, SBWMV and WSSMV are detailed in Fomitcheva et al. (2008). Reverse transcription was performed with M-MLV Reverse transcriptase (Promega) for 60 min at 37 °C, the PCR was carried out using Taq DNA polymerase (MP).

Cycling conditions for the PCR reaction were as follows: 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s; 72 °C for 10 min. The PCR products were analyzed on a 1.2% agarose gel.

PCR and qPCR for detection of P. graminis

PCR was performed as follows: initial 3 min denaturation step at 96 °C; 30 cycles of 30 s 96 °C, 1 min 54 °C and 1 min 72 °C; finally, 10 min at 72 °C.

The qPCR was carried out in a qTower 2.2 (Analytik Jena) using a SYBR Green No-ROX Mix (Bioline). The primer pairs AZtempF/AZtempR and AZtepF/AZtepR were used for the detection of ribotype I and ribotype II, respectively. The initial denaturation at 95 °C for 2 min was followed by 40 cycles of 15 s 95 °C, 15 s 58 °C and 30 s 72 °C. The resulting product underwent a melting curve analysis (from 60 °C to 95 °C) to verify the specificity of amplification products.

Cloning of P. graminis *sequences*

PCR products were cloned in the pGEM-T vector, and insert-containing clones were analyzed by sequencing (GATC, Cologne). The sequences were compared to the database using BLAST (Altschul et al. 1990). Cloned *P. graminis* sequences were used as positive controls in PCR and qPCR.

A *P. graminis* sequence with the GenBank accession number Y12824 (Ward and Adams 1998) served as reference sequence for ribotype I, and Y12826 (Ward and Adams 1998) was used as reference sequence for ribotype II.

Results

In 2010, cereal plants from three different experimental field sites in Germany were examined for the presence of *P. graminis* ribotypes. In Heddesheim, all cereal species tested (winter wheat, winter triticale, winter rye and winter barley) contained *P. graminis* ribotype II (f. sp. tepida). All of these plants were also infected with SBWMV. SBWMV is the only soil borne virus known to occur at the Heddesheim site. In Schleesen only ribotype I (f. sp. temperata) was detected and three out of the four plants tested were infected by WSSMV, the only soil borne virus confirmed for the Schleesen site.

Ribotype I was found in Gödnitz in a winter wheat plant; this plant was infected by both the furovirus SBCMV and the bymovirus WSSMV. Triticale and rye were infected with SBCMV, and the barley (cv. Maris Otter) was free of virus. Triticale, rye and barley contained *P. graminis* ribotype II.

Between 2010 and 2015, in order to obtain a more detailed picture, the fields in Schleesen, Heddesheim and Gödnitz were revisited and a number of additional locations were investigated for the occurrence of *P. graminis* ribotypes. The results shown in Table 3 indicate that both ribotypes I and II are ubiquitous throughout Germany, and that they can occur together at the same location. Furthermore, we have shown the presence

Table 3. Presence of *P. graminis* ribotypes in soil samples from different locations

Location	Viruses present in field	*Polymyxa* ribotype present	Ribotype sequence determined
Altenhagen, Im Moor (Lower Saxony)		II	II
Altenhagen, Im trockenen Kamp (Lower Saxony)		II (2012)	II
Bornum (Lower Saxony)	BaYMV, BaMMV	I	
Eickeloh (Lower Saxony)	SBCMV, WSSMV	II (2010) I , II (Smith et al. 2013)	II
Schladen (Lower Saxony)	BaYMV, BaMMV	I, II (Smith et al. 2013)	
Wietze (Lower Saxony)	SBCMV, WSSMV	I (2007)	I
Bormkoppel (Schleswig-Holstein)	SBWMV (New York strain)	I, II (2013)	I
Westerrade (Schleswig-Holstein)	SBWMV (New York strain)	I, II (2013)	I, II
Gödnitz (Saxony-Anhalt)	SBCMV, WSSMV	I, II (2010, 2014)	I, II
Lindau (Saxony-Anhalt)	SBCMV, WSSMV	II	II
Schleesen (Saxony-Anhalt)	WSSMV	I	I
Walternienburg (Saxony-Anhalt)	SBCMV, WSSMV	II (2013, 2015)	II
Heddesheim (Baden-Württemberg)	SBWMV (Nebraska strain)	II (2010), I (2015)	I, II
Stubbum (Denmark)	SBCMV	II (2011)	II
Pulawy (Poland)		I, II (2013)	I, II

f. sp. *temperata* = ribotype I, f. sp. *tepida* = ribotype II. The results of Smith et al. (2013) for Eickeloh and Schladen were included.

of ribotype II in Stubbum (Denmark), and, for the first time, both ribotypes I and II in soil from an experimental field in Pulawy (Poland).

Wheat plants obtained from Saudi-Arabia were tested with universal primers ITS4 and ITS5 for the presence of *P. graminis*. No *P. graminis* sequences were detected, and the only sequences derived from the material showed homologies to common soil bacteria.

Table 4 summarizes pathogen combinations detected in individual triticale field plants from experimental fields in Germany: all possible pathogen combinations occur. *Poly-*

Table 4. Pathogen combinations detected in individual triticale field plants

Location	Cultivar	*P. graminis* ribotypes	Virus
Gödnitz	undefined	I, II	SBCMV
Gödnitz	PZO10401 (T)	II	WSSMV, SBCMV
Westerrade	Valentino (T)	I	SBWMV
Eickeloh	Valentino (T)	II	SBCMV
Schleesen	Trinidad (T)	I	WSSMV
Heddesheim	Trinidad (T)	II	SBWMV
Lindau	undefined	II	WSSMV, SBCMV

DNA for *Polymyxa* detection was from roots, RNA for virus detection from leaf material of the same plant.

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Y12824          CAGCAATGGCCGGAGCGCGGTTGAACTTTTGTAAATTTGGACGACTAGA 50
FN393968        --------------------------------------------------
LN651079        CAGCAATGGCCGGAGCGCGGTTGAACTTTTGTAAATTTGGACGACTAGA 49
LN651065        CAGCAATGGCCGGAGCGCGGTTGAACTTTTGTAAATTTGGACGACTAGA 49
LN651072        CAGCAATGGCCGGAGCGCGGTTGAACTTTTGTAAATTTGGACGACTAGA 50
LN651066        CAGCAATGGCCGGAGCGCGGTTGAACTTTTGTAAATTTGGACGACTAGA 50
LN651069        CAGCAATGGCCGGAGCGCGGTTGAACTTTTGTAAATTTGGACGACTAGA 50
LN651070        CAGCAATGGCCGGAGCGCGGTTGAACTTTTGTAAATTTGGACGACTAGA 50
Schl-31BI15     CAGCAATGGCCGGAGCGCAGTTGAACTTTTGTAAATTTGGACGACTAGA 49
Y12826          CAGCAATGGCCGGCGCGCGTCCGAACTTTTGTAAATTTGGTCGACTAGA 50
KF535917        --GCAATGGCCGGCGCGCGTCCGAACTTTTGTAAATTTGGACGACTAGA 50
AM075823        --------------------------------------------------
LN651073        --GCAATGGCCGGCGCGCGTCCGAACTTTTGTAAATTTGATCGACTAGA 50
LN651068        CAGCAATGGCCGGCGCGCGTCCGAACTTTTGTAAATTTGGTCGACTAGA 49
LN651075        CAGCAATGGCCGGCGCGCGTCCGAACTTTTGTAAATTTGGTCGACTAGA 49
LN651076        CAGCAATGGCCGGCGCGCGTCCGAACTTTTGTAAATTTGGTCGACTAGA 49
LN651077        CAGCAATGGCCGGCGCGCGTCCGAACTTTTGTAAATTTGGTCGACTAGA 49
LN651078        CAGCAATGGCCGGCGCGCGTCCGAACTTTTGTAAATTTGGTCGACTAGA 49
Y12826          CAGCAATGGCCGGCGCGCGTCCGAACTTTTGTAAATTTGGTCGACTAGA 50
LN651074        CAGCAATGGCCGGCGCGCGTCCGAACTTTTGTAAATTTGGTCGACTAGA 50
LN651071        CAGCAATGGCCGGCGCGCGTCCGAACTTTTGTAAATTTGGTCGACTAGA 50
LN651067        CAGCAATGGCCGGCGCGCGTCCGAACTTTTGTAAATTTGGTCGACTAGA 50

Y12824          GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC 100
FN393968        ----------------------------------CTGCGGAAGGATC  13
LN651079        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC  99
LN651065        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC  99
LN651072        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC 100
LN651066        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC 100
LN651069        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC 100
LN651070        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC 100
Schl-31BI15     GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC  99
Y12826          GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC 100
KF535917        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC 100
AM075823        ------------------------------------------GATC   4
LN651073        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC 100
LN651068        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC  99
LN651075        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC  99
LN651076        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC  99
LN651077        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC  99
LN651078        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC  99
LN651074        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC 100
LN651071        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC 100
LN651067        GGAAGAAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC 100
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Figure 1. Lineup of *P. graminis* sequences (partial 18S rRNA gene and ITS1 sequence)
The sequences shown cover part of the 18S rRNA gene and part of the ITS1 sequence (shown in italics). Y12824: reference sequence for ribotype I; Y12826: reference sequence for ribotype II. The primer sequences AZtempF and AZtempR (for amplification of ribotype I) and AZtepF and AZtepR (for amplification of ribotype II) are underlined

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Y12824          ATTAGCGTTGAATTGGTCTTGGTGCCATGCGAAAAACATGTGGATTGTGG 150
FN393968        ATTAGCGTTGAATTGGTCTTGGTGCCATGCGAAAAACATGTGGATTGTGG  63
LN651079        ATTAGCGTTGAATTGGTCTTGGTGCCATGCGAAAAACATGTGGATTGTGG 149
LN651065        ATTAGCGTT-AATTGGTCTTGGTGCCATGCGAAAAACATGTGGATTGTGG 148
LN651072        ATTAGCGTT-AATTGGTCTTGGTGCCATGCGAAAAACATGTGGATTGTGG 149
LN651066        ATTAGCGTT-AATTGGTCTTGGTGCCATGCGAAAAACATGTGGATTGTGG 149
LN651069        ATTAGCGTT-AATTGGTCTTGGTGCCATGCGAAAAACATGTGGATTGTGG 149

LN651070        ATTAGCGTT-AATTGGTCTTGGTGCCATGCGAAAAACATGTGGATTGTGG 149
Schl-31BI15     ATTAGCGTT-AATTGGTCTTTGTGCCATGCGAAAAACATGTGGATTGTGG 148
Y12826          ATTAGCGTTGAATGG---TTGTTGCCATTTCGTAAAAATGTGGATCGTCT 147
KF535917        ATTAGCGTTGAATGG---TTGTTGCCATTTCGTAAAAATGTGGATCGTGT 147
AM075823        ATTAGCGTTGAATGG---TTGTTGCCATTTCGTAAAAATGTGGATCGTGT  51
LN651073        ATTAGCGTTGAATGG---TTGTTGCCATTTCGTAAAAATGTGGATCGTCT 147
LN651068        ATTAGCGTTGAATGG---TTGTTGCCATTTCGTAAAAATGTGGATCGTCT 146
LN651075        ATTAGCGTTGAATGG---TTGTTGCCATTTCGTAAAAATGTGGATCGTCT 146
LN651076        ATTAGCGTTGAATGG---TTGTTGCCATTTCGTAAAAATGTGGATCGTCT 146
LN651077        ATTAGCGTTGAATGG---TTGTTGCCATTTCGTAAAAATGTGGATCGTCT 146
LN651078        ATTAGCGTTGAATGG---TTGTTGCCATTTCGTAAAAATGTGGATCGTCT 146
LN651074        ATTAGCGTTGAATGG---TTGTTGCCATTTCGTAAAAATGTGGATCGTCT 147
LN651071        ATTAGCGTTGAATGG---TTGTTGCCATTTCGTAAAAATGTGGATCGTCT 147
LN651067        ATTAGCGTTGAATGG---TTGTTGCCATTTCGTAAAAATGTGGATCGTCT 147

Y12824          GCTATGTGACCCCTCTGTG----- 169
FN393968        GCTATGTGACCCCTCTGTG-----  82
LN651079        GCTATGTGA--------------- 158
LN651065        GCTATGTGA--------------- 157
LN651072        GCTATGTGA--------------- 158
LN651066        GCTATGTG---------------- 157
LN651069        GCTATGTG---------------- 157
LN651070        GCTATGTG---------------- 157
Schl-31BI15     GCTATGTGA--------------- 157
Y12826          CTGTTGCTGGATACCGGGATGGAA 175
KF535917        CTGTTGTCGGATATCGGGATGGAA 175
AM075823        CTGTTGTCGGATATCGGGA-----  70
LN651073        CTGTTGCTGGATACCGGGATGGAA 171
LN651068        CTGTTGCTGGATACCGGGATGGAA 170
LN651075        CTGTTGCTGGATACCGGGATGGAA 170
LN651076        CTGTTGCTGGATACCGGGATGGAA 170
LN651077        CTGTTGCTGGATACCGGGATGGAA 170
LN651078        CTGTTGCTGGATACCGGGATGGAA 170
LN651074        CTGTTGCTGGATACCGGGATGGAA 171
LN651071        CTGTTGCTGGATACCGGGATGGAA 171
LN651067        CTGTTGCTG--------------- 156
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Figure 1 (cont.)

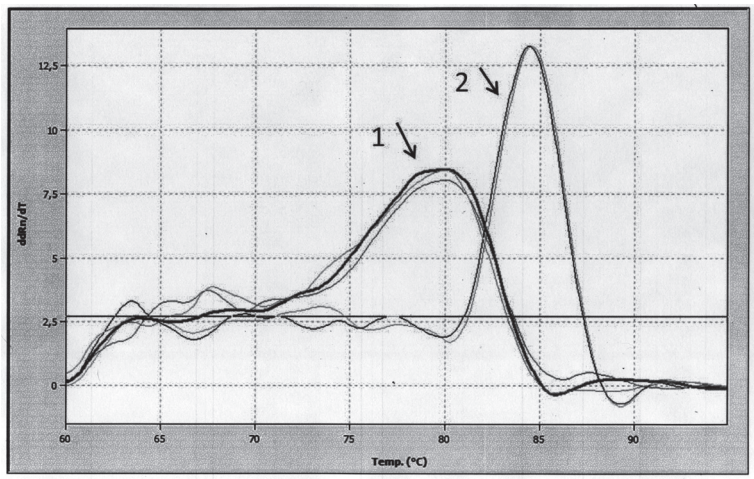

Figure 2. Melting temperature analysis after qPCR using primers that detect f. sp. *temperata* (ribotype I) Peak 1: non-template control and sample Wietze (ribotype I) with ribotype II-specific primers; Peak 2: sample Wietze and positive control (ribotype I plasmid DNA) with ribotype I-specific primers

myxa was detected in DNA prepared from roots, and RNA for virus detection was prepared from leaf material of the same plant.

A number of *P. graminis* cDNA clones were generated from samples analyzed in this study, and their sequences were obtained (GATC). The DNA sequences were compared to *P. graminis* database entries, especially accession numbers Y12824 (F1, ribotype I) and Y12826 (F51, ribotype II) (Ward and Adams 1998). A lineup of these nucleotide sequences is shown in Fig. 1; there is a clear difference between ribotype I and ribotype II sequences.

The sequences have been submitted to the European Nucleotide Archive as an environmental set (http://www.ebi.ac.uk/ena/data/view/LN651065-LN651079).

qPCR was used to specifically detect the *P. graminis* ribotypes I and II in DNA extracted from root and soil samples. The cloned sequences were utilized as positive controls. Melting curve analysis was used to verify the specificity of the amplified products (Fig. 2).

Discussion

The results of the analysis of a small number of plants from three different locations in 2010 provided a snapshot only of the *P. graminis* ribotypes situation in Germany. Therefore, in the following years samples from different locations in Germany and abroad were analyzed regarding the presence of *P. graminis* ribotypes. Both ribotypes I and II were found to be ubiquitous in Germany, and they can occur together at the same location. This was also the situation reported previously by Smith et al. (2013); Vaianopoulos et al. (2007) and Ward et al. (2005) in Europe, and recently for southwest Australia (Cox et al. 2014).

Also, we have shown the presence of ribotype II in Stubbum (Denmark) and of both ribotypes I and II in soil from Pulawy (Poland). In the Czech Republic, the presence of *P. graminis* ribotype I was reported earlier by Ketta et al. (2011). We failed to detect *P. graminis* in wheat plants from Saudi-Arabia using universal primers ITS4 and ITS5. It cannot be excluded that using different primer sets would have led to the dection of *P. graminis,* or that wheat samples from other parts of Saudi-Arabia would have contained *P. graminis*. Yilmaz et al. (2011) did not detect *P. graminis* in samples from the Anatolian part of Turkey; however, in the Samsun province of Turkey, in Lebanon, Iran and Syria the presence of *P. graminis* was reported (Sahandpour and Izadpanah 1999; Anonymous 2007; Mouhanna et al. 2008; Erkan and Yilmaz 2009). Details on ribotype or sequence information were not available.

The partial 18S/ITS1 *P. graminis* sequences obtained by us from the different locations were compared to database entries. Generally, we found 99% homology with the corresponding reference sequence (Y12824 for ribotype I, and Y12826 for ribotype II; Ward and Adams 1998). The cloned *P. graminis* sequences were used as positive controls in qPCR experiments to specifically detect the two ribotypes.

We have obtained separate sources of *P. graminis* ribotype I (Wietze) and of *P. graminis* ribotype II (Lindau). Collected root meal was tested to verify the presence of the expected ribotype by qPCR. Figure 2 illustrates a melting curve analysis of qPCR products to verify the presence of ribotype I only in the Wietze sample. These sources of *P. graminis* ribotypes will be useful for further studies on transmission specificity.

There are still questions over the host specificity and preferences of ribotypes I and II (Smith et al. 2013), and the significance of their co-occurrence in temperate regions is not clear. *P. graminis* ribotypes I and II each can infect several species of small grains, including wheat, barley, triticale and rye; ribotype I can infect even Arabidopsis (Smith et al. 2011, 2013). It is still not clear if there is any preference of ribotypes I or II to transmit SBCMV, SBWMV or WSSMV. In addition, these ribotypes are also able to transmit BaYMV and BaMMV to barley. In our field virus resistance trials involving different grain species (wheat, rye, and triticale) infection took place with the viruses reported for the field in question independent of the *P. graminis* ribotype present as long as there was viruliferous vector present in the soil. Both ribotypes I and II can transmit SBCMV, SBWMV and WSSMV. Plants co-infected with SBCMV and WSSMV are found regularly during our field trials (also see Table 4).

There are differences in aggressiveness of the pathogen complexes at different locations that could be due, for example, to *P. graminis* inoculum distribution, *P. graminis* ribotype efficiency in virus transmission, the virus strain present or differences in climate and soil characteristics. Dieryck et al (2011) have suggested the existence of a co-evolution mechanism between PCV, vector and host plant. This could lead to local adaptation, which could be a reason for differences in aggressiveness. Therefore, it is important to elucidate the host plant-*P. graminis* ribotype-virus interactions because they could influence the outcome of virus resistance trials.

Some progress has been made in dissecting other pathogen complexes involving plasmodiophorids. For example, the difference in aggressiveness between BNYVV isolates

was found to be independent of the *Polymyxa betae* vector population (Bornemann and Varrelmann 2011). Tuitert et al. (1994) found that the total number of *P. betae* resting spore clusters formed in roots of partially BNYVV-resistant cultivars and a susceptible cultivar did not differ at 6 and 12 weeks after inoculation. This agrees with Ratti et al. (2004); they reported that *P. graminis* levels did not differ significantly between susceptible and SBCMV-resistant wheat varieties.

Gau et al. (2013) have recently presented a detailed population genetic characterization of *Spongospora subterranea*, another plasmodiophorid that infects – and transmits potato mop-top virus to – potato.

Plasmodiophorids are ubiquitously distributed in agricultural and natural habitats, with a high cryptic diversity. Neuhauser et al. (2014), investigating cross-kingdom host shifts of phytomyxid parasites, found new plasmodiophorid lineages in soil, suggesting a higher diversity than previously known. They showed a high 18S-type diversity of plasmodiophorids in soil ecosystems, but did not identify an association pattern between 18S type and host plant species. Taking into account research on other phytomyxids will inform our efforts to study the economically important plasmodiophorid species regarding lifestyle, risk of spread, climate influence, and diversity and speciation, and will ultimately lead to better disease management.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.

Anonymous 2007. The first report of *Polymyxa graminis* Led. in Northern Syria. Arab and Near East Plant Protection Newsletter **45**:39.

Bornemann, K., Varrelmann, M. 2011. Analysis of the resistance-breaking ability of different Beet necrotic yellow vein virus isolates loaded into a single *Polymyxa betae* population in soil. Phytopathol. **101**:718–724.

Braselton, J.P. 1995. Current status of the plasmodiophorids. Crit. Rev. Microbiol. **21**:263–265.

Bulman, S.R., Kühn, S.F., Marshall, J.W., Schnepf, E. 2001. A phylogenetic analysis of the SSU rRNA from members of the plasmodiophorida and phagomyxida. Protist. **152**:43–51.

Bulman, S.R., Braselton, J.P. 2014. Rhizaria: Phytomyxea. In: Mc Laughlin, D.J., Spatafora, J.W. (eds), The Mycota VII, 2nd ed. Systematics and Evolution, Part A. Springer-Verlag. Berlin-Heidelberg, Germany. pp. 99–112.

Cox, B.A., Luo, H., Jones, R.A.C. 2014. *Polymyxa graminis* isolates from Australia: Identification in wheat roots and soil, molecular characterization, and wide genetic diversity. Plant Dis. **98**:1567–1575.

Dieryck, B., Weyns, J., Doucet, D., Bragard, C., Legreve, A. 2011. Acquisition and transmission of peanut clump virus by *Polymyxa graminis* on cereal species. Phytopathol. **101**:1149–1158.

Erkan, E., Yilmaz, N.D.K. 2009. Determination of virus diseases in wheat growing areas of Samsun province. Anadolu J. of Agric. Sci. **24**:67–75.

Fomitcheva, V., Kastirr, U., Habekuss, A., Kühne, T. 2008. Diagnostic multiplex RT-PCR analysis for the detection of soil-borne mosaic viruses and their natural vector *Polymyxa graminis*. In: Rush, C.M., Proc. 7th symp. of the international working group on plant viruses with fungal vectors, pp. 43–48.

Gau, R.D., Merz, U., Falloon, R.E., Brunner, P.C. 2013. Global genetics and invasion history of the potato powdery scab pathogen, *Spongospora subterranea* f. sp. *subterranea*. PLOS ONE **8**:e67944.

Kanyuka, K., Ward, E., Adams, M.J. 2003. *Polymyxa graminis* and the cereal viruses it transmits: a research challenge. Mol. Plant Pathol. **4**:393–406.

Kastirr, U., Widera, A. 1988. Erste Ergebnisse zum Vorkommen der Vektoren des Gerstengelbmosaik-Virus und Rübenwurzelbärtigkeits-Virus in der DDR und deren Vermehrung an ihren Wirtspflanzen (Occurrence of the vectors of barley yellow mosaic virus and beet necrotic yellow vein virus in the German Democratic Republic and multiplication on their host plants-1st results). Arch. Phytopathol. Plant Prot. **24**:93–101. (in German)

Kendall, T.L., Langenberg, W.G., Lommel, S.A. 1988. Molecular characterization of Sorghum chlorotic spot virus, a proposed furovirus. J. Gen. Virol. **69**:2335–2345.

Ketta, H., Zouhar, M., Rysanek, P. 2011. First report of *Polymyxa graminis* f. sp. temperata, a vector of soil-borne cereal viruses in the Czech Republic. Plant Dis. **95**:353.

Kühne, T. 2009. Soil-borne viruses affecting cereals – known for long but still a threat. Virus Res. **141**:174–183.

Kwaśna, H., Bateman, G.L., Ward, E. 2008. Determining species diversity of microfungal communities in forest tree roots by pure-culture isolation and DNA sequencing. Appl. Soil Ecol. **40**:44–56.

Ledingham, G.A. 1939. Studies on *Polymyxa graminis*, n. gen. n. sp., a plasmodiophoraceous root parasite of wheat. Can. J. Res. C. **17**:38–51.

Legreve, A., Delfosse, P., Maraite, H. 2002. Phylogenetic analysis of *Polymyxa* species based on nuclear 5.8S and internal transcribed spacers ribosomal DNA sequences. Mycol. Res. **106**:138–147.

Lozano, I., Morales, F. 2009. Molecular characterization of rice stripe necrosis virus as a new species of the genus Benyvirus. Eur. J. Plant Path. **124**:673–680.

McWilliam, H., Li, W., Uludag, M., Squizzato, S., Park, Y.M., Buso, N., Cowley, A.P., Lopez, R. 2013. Analysis tool web services from the EMBL-EBI. Nucleic Acids Res. **41** (Web server issue); W597–600.

Mouhanna, A.M., Choueiri, E., Langen, G. 2008. First report of *Polymyxa betae* and *Polymyxa graminis* in Lebanon. J. Plant Pathol. **90**:585–589.

Neuhauser, S., Kirchmair, M., Bulman, S., Bass, D. 2014. Cross-kingdom host shifts of phytomyxid parasites. BMC Evol. Biol. **14**:33.

Ratti, C., Budge, G., Ward, L., Clover, G. 2004. Detection and relative quantitation of soil-borne cereal mosaic virus (SBCMV) and *Polymyxa graminis* in winter wheat using real-time PCR (TaqMan®). J. Virol. Methods **122**:95–103.

Sahandpour, A., Izadpanah, K. 1999. Report of *P. graminis* and a virus resembling wheat soilborne mosaic virus from Fars. Iranian J. Plant Pathol. **35**:61.

Smith, M.J., Adams, M.J., Ward, E. 2011. Evidence that Polymyxa species may infect *Arabidopsis thaliana*. FEMS Microbiol. Letters **318**:35–40.

Smith, M.J., Adams, M.J., Ward, E. 2013. Ribosomal DNA analyses reveal greater sequence variation in *Polymyxa* species than previously thought and indicate the possibility of new ribotype-host-virus associations. Env. Microbiol. Rep. **5**:143–150.

Tamada, T., Kondo, H. 2013. Biological and genetic diversity of plasmodiophorid-transmitted viruses and their vectors. J. Gen. Plant Pathol. **79**:307–320.

Thouvenel, J.C., Fauquet, C. 1981. Further properties of peanut clump virus and studies on its natural transmission. Ann. Appl. Biol. **97**:99–107.

Tuitert, G., Vanoorschot, P.M.S.M., Heijbroek, W. 1994. Effect of sugar-beet cultivars with different levels of resistance to beet necrotic yellow vein virus on transmission of virus by *Polymyxa betae*. Eur. J. Plant Pathol. **100**:201–220.

Vaianopoulos, C., Bragard, C., Moreau, V., Maraite, H., Legreve, A. 2007. Identification and quantification of *Polymyxa graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* on barley and wheat. Plant Dis. **91**:857–864.

Ward, E., Adams, M.J., Mutasa, E.S., Collier, C.R., Asher, M.J.C. 1994. Characterization of *Polymyxa* species by restriction analysis of PCR-amplified ribosomal DNA. Plant Pathol. **43**:872–877.

Ward, E., Adams, M. 1998. Analysis of ribosomal DNA sequences of *Polymyxa* species and related fungi and the development of genus- and species-specific PCR primers. Mycol. Res. **102**:965–974.

Ward, L.I., Fenn, M.G.E., Henry, C.M. 2004. A rapid method for direct detection of *Polymyxa* DNA in soil. Plant Pathol. **53**:485–490.

Ward, E., Kanyuka, K., Motteram, J., Kornyukhin, D., Adams, M.J. 2005. The use of conventional and quantitative real-time PCR assays for *Polymyxa graminis* to examine host plant resistance, inoculums levels and intraspecific variation. New Phytologist **165**:875–885.

White, T.J., Bruns, T., Lee, S., Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (eds), PCR Protocols: a Guide to Methods and Applications. Academic Press. New York, USA. pp. 315–322.

Yilmaz, N.D.K., Lyons, R.L., Smith, M.J., Kanyuka, K. 2011. Investigation of soilborne mosaic virus diseases transmitted by *P. graminis* in cereal production areas of the Anatolian part of Turkey. Eur. J. Plant Pathol. **130**:59–72.