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Rep-PCR based genomic fingerprinting of isolates of *Leptosphaeria maculans* from Poland

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Abstract

Leptosphaeria maculans, the ascomycete fungus which causes blackleg disease of oilseed rape, has been considered for a long time as a single species divided into 'aggressive' and 'non-aggressive' pathogenicity groups which differ in their economic importance. However, the development of accurate biochemical and molecular characterisation methods has demonstrated that the world-wide *L. maculans* population actually comprises at least two species. The aim of this research was to assess the ability of rep (repetitive element based)-PCR genomic fingerprinting methods, initially developed for bacterial identification, to characterise a collection of 90 isolates of *L. maculans* from Poland, in comparison with reference isolates from the IBCN (International Blackleg of Crucifers Network) collection. REP (repetitive extragenic palindromic)-, ERIC (enterobacterial repetitive intergenic consensus)-, and BOX primers for rep-PCR genomic fingerprinting, and primers derived from LMR1, a *L. maculans* specific repeated element, were tested. Rep-PCR and LMR1-based analyses were able to discriminate the different components of the species complex and to evaluate the genetic diversity within each member of the complex. These analyses suggested that Polish populations of *L. maculans* mainly belong to the 'non-aggressive' species, rather than the 'aggressive' species which is prevalent in Western Europe, Canada and Australia.

Introduction

Blackleg (stem canker) of oilseed rape, caused by *Leptosphaeria maculans* (Desm.) Ces. et de Not. (anamorph: *Phoma lingam* [Tode ex Fr.] Desm.), is a damaging disease of crucifers world-wide. Severe epidemics on oilseed rape have been reported since oilseed rape became one of the main oilseed crops (Gugel and Petrie, 1992). For instance, the disease can cause losses of up to 60 million ECU per annum in the UK (Fitt et al., 1997). The disease is spread by contaminated crop residues and by infested seeds (Hall, 1992). Ascospore release from *L. maculans* pseudothecia on residues leads to infection of new crops in neighbouring areas, whereas seed infection disseminates the pathogen over longer distances (Gladders and Musa, 1980).

L. maculans isolates originating from oilseed rape have been ascribed to one of two pathogenicity groups, referred to as 'highly virulent' and 'weakly virulent', or 'aggressive' and 'non-aggressive' (for reviews, see Rouxel et al., 1994; Williams and Fitt, 1999). Koch et al. (1989) demonstrated that these groups consistently differ in their *in vitro* production of sirodesmin toxins. Therefore, Balesdent et al. (1992) proposed the terms Tox^+ and Tox^0 for these two groups. Other morphological and biochemical differences support the hypothesis that considerable biological differences exist between the two groups (Williams and Fitt, 1999). Moreover, whereas matings have been successfully

obtained *in vitro* between isolates belonging to either the Tox^+ (Mengistu et al., 1993; Gall et al., 1994) or the Tox^0 (Somda et al., 1997; B. Volke et al., pers. com.) group, attempted matings between these two groups have always failed (Venn, 1979; Petrie and Lewis, 1985; Somda et al., 1997), suggesting there is a genetic barrier between the two groups. Furthermore, molecular data, including DNA sequence analyses of the rDNA genes and intergenic spacers regions, have confirmed that the Tox^+ and Tox^0 isolates belong to two different ascomycete species (Koch et al., 1991; Morales et al., 1993; Balesdent et al., 1998). Phylogenetic analyses of Tox^0 isolates demonstrated additional diversity within the Tox^0 group. On the basis of RFLP patterns, three different Tox^0 groups, referred to as NA1, NA2 and NA3, were identified (Koch et al., 1991; Gall et al., 1995). Fungal strains identified as *L. maculans* can be also isolated from cruciferous weeds belonging to *Erysimum, Lepidium, Thlaspi, Sysimbrium* and *Descurainia* species (Petrie, 1969).

Poland is one of the major oilseed rape producing countries in central Europe. The incidence of stem canker in winter oilseed rape has increased since the late 1970s (Frencel, 1983), and the disease completely devastated some crops in north-western Poland in the mid 1980s (Frencel et al., 1991). In the 1990s, stem canker symptoms were commonly observed on oilseed rape in all regions (Jedryczka et al., 1994), so that breeders have been encouraged to include disease resistance in their breeding strategies. There is therefore an urgent need for *L. maculans* population studies in Poland. Previously, *L. maculans* isolates from many geographically distant localities were characterised on the basis of their pathogenicity and sirodesmin production (Jedryczka et al., 1994; Kachlicki and Jedryczka, 1994). Preliminary results indicate the predominance of Tox^0 isolates in Poland (Jedryczka et al., 1994; Kachlicki and Jedryczka, 1994; Gall et al., 1995). Both this observation, and the continued increase of stem canker incidence in Poland, appear to contradict the suggestion that infection caused by Tox^0 isolates only has a little effect on crop yield (Gugel and Petrie, 1992).

To characterise *L. maculans* isolates collected from infected oilseed rape plants in Poland, PCR-based genomic DNA fingerprinting tools were developed to identify components of the *L. maculans* species complex. The strategy chosen was to generate genomic fingerprints via PCR, using primers derived from the DNA sequence of repetitive-elements (i.e. rep (repetitive element based)-PCR) (Rademaker et al., 1997). The primers were derived from the 'repetitive extragenic palindromic' (REP) sequence (Gilson et al., 1984), the 'enterobacterial repetitive intergenic consensus' (ERIC) sequence (Hulton et al., 1991), the conserved repeated bacterial DNA element 'BOX' (Martin et al., 1992), and LMR1, a 5.3 kb repeated element specific to the Tox^+ group of *L. maculans* (Taylor and Borgmann, 1994). The discrimination efficiency and reliability of rep-PCR were assessed using worldwide reference isolates from the International Blackleg of Crucifers Network (IBCN) (Rouxel and Séguin-Swartz, 1995) and previously characterised isolates from Europe. Subsequently, rep-PCR was used to characterise a collection of 90 Polish isolates.

Materials and methods

Fungal isolates and culture maintenance. Oilseed rape (*Brassica napus* L. var. *oleifera*) plants displaying stem lesions with pycnidia were collected at the end of the growing season (i.e. early July), from different regions of Poland (Table 1). Eighty-three single-conidium isolates were obtained from these stem lesions. Seven single-conidium isolates (PhL2 to PhL8) were obtained from lesions on leaves in the autumn. Isolates representative of the seven groups of *L. maculans* identified in the IBCN collection (i.e. Tox^+, Tox^0 NA1, Tox^0 NA2, Tox^0 NA3, 'Thlaspi', 'Lepidium' and 'Erysimum'), were used as reference isolates (Table 2). One *Phoma nigrificans* isolate, obtained from *Thlaspi arvense* in Poland, one *Phoma* sp. isolated from carnation, and 14 previously characterised *L. maculans* isolates from Denmark, France, Poland and the UK were also used (Table 2).

Polish isolates were stored at 4 °C on oat kernels. Isolates in the IBCN collection, and other reference isolates were stored on agar at 4 °C under paraffin oil. Isolates were cultured at 20 °C on V8-juice agar medium with a 12 h photoperiod.

DNA purification. *L. maculans* conidia were collected from 12-day-old sporulating cultures. To release conidia from pycnidia, the cultures were flooded with 5 ml of sterile distilled water and rubbed with a rod. Conidia were separated from mycelium by filtration through sterile glass wool. Aliquots of conidia suspension (ca. 10^8) were added to 100 ml of modified Fries liquid medium in Roux bottles (Balesdent et al., 1992).

Table 1. The origin and classification of Polish Tox^+ or Tox^0 *Leptosphaeria maculans* isolates

Symbol	Region	Area of Poland	Year	Group
Ph 0, Ph 2	Poznan	Western	1984	Tox^0 NA1
Ph 3	Szczecin	North-western	1984	Tox^0 NA1
Ph 4	Szczecin	North-western	1987	Tox^0 NA1
Ph L2 to Ph L4	Poznan	Western	1990	Tox^0 NA1
Ph L5	Poznan	Western	1991	**Tox**$^+$
Ph L6 to Ph L8	Elblag	Northern	1991	Tox^0 NA1
Ph 24, Ph 35	Poznan	Western	1992	Tox^0 NA1
Raw 1, Raw 5	Szczecin	North-western	1992	Tox^0 NA1
Raw 4	Szczecin	North-western	1992	**Tox**$^+$
Rym 7	Koszalin	North-western	1992	Tox^0 NA1
PL 34	Warszawa	Central	1992	Tox^0 NA1
Ph L9, Ph S1	Walbrzych	South-western	1993	Tox^0 NA1
Ph Bial	Koszalin	North-western	1993	**Tox**$^+$
Ph Gosc	Jelenia Gora	South-western	1993	Tox^0 NA1
Ph Trzy	Szczecin	North-western	1993	Tox^0 NA1
Ph EP, Cer A, PL 1, PL 2	Poznan	Western	1994	Tox^0 NA1
PL 3 to PL 8, PL 10, PL 28	Kalisz	Central	1994	Tox^0 NA1
PL 11, PL 12, PL 27	Sieradz	Central	1994	Tox^0 NA1
PL 14 to PL 17, PL 30, PL 31	Lublin	Eastern	1994	Tox^0 NA1
PL 18 to PL 25, PL 32, PL 33	Zamosc	Eastern	1994	Tox^0 NA1
PL 26	Tarnobrzeg	Eastern	1994	Tox^0 NA1
PL 29	Chelm	Eastern	1994	Tox^0 NA1
PL 35 to PL 43	Poznan	Western	1995	Tox^0 NA1
PL 44 to PL 46, PL 48, PL 49	Leszno	Western	1995	Tox^0 NA1
PL 47	Leszno	Western	1995	**Tox**$^+$
PL 50, PL 51	Legnica	South-western	1995	Tox^0 NA1
PL 52 to PL 55, PL 57 to PL 61, PL 63 to PL 67	Wroclaw	South-western	1995	Tox^0 NA1
PL 56	Wroclaw	South-western	1995	**Tox**$^+$
PL 68, PL 70	Opole	South-western	1995	Tox^0 NA1
PL 69	Opole	South-western	1995	**Tox**$^+$

Four-day-old mycelium was harvested by vacuum filtration onto sterile muslin, then frozen at $-20\,^{\circ}C$ and freeze-dried. DNA was extracted as previously described (Balesdent et al., 1998). DNA from two *Botrytis cinerea* isolates (Table 2) were supplied by C. Lévis (INRA Versailles, France).

Primers. REP (REP1RI: 5′-IIIICGICGICATCIGGC-3′ and REP2I: 5′-ICGICTTATCIGGCCTAC-3′) and ERIC (ERIC1R: 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and ERIC2: 5′-AAGTAAGTGACTGGGGTGAG-CG-3′) primers were described by Versalovic et al. (1991); the sequence of the BOX primer (BOX A1R: 5′-CTACGGCAAGGCGACGCTGACG-3′) was described by Versalovic et al. (1994). Primers LMR1F2: 5′-GAGGACTAAACCTAGCGCAG-3′, LMR1F3: 5′-AAGAGCACAGGAGAAGCCAC-3′ (forward primers), and LMR1R2: 5′-TGGTGATAG AGCGATAGG-3′ and LMR1R3: 5′-ACTTACTCTG-CCTACGCTACCC-3′ (reverse primers) were designed from the published sequence of the repetitive *L. maculans* 5.3 kb element LMR1 (Genebank accession number m77515), using the Prime program of the Wisconsin software Package, version 9.0 (Genetics Computer Group, Madison, WI). 5′ bases of the primers LMR1F2, LMR1F3, LMR1R2 and LMR1R3 are located at base numbers 1883, 3422, 3175 and 5072 of the published sequence, respectively. All primers were synthesised by Life Technologies, Gaithersburg, USA.

PCR conditions. The 25-μl reaction mix in a 0.5-ml microtube contained 25 ng DNA, 200 μM of dATP, dGTP, dCTP and dTTP, 1.30 mM of each primer, 1 unit Taq DNA polymerase (Appligene) in Appligene

Table 2. Characteristics of reference isolates used

Original isolate number	IBCN number	Geographical origin	Isolated from	Originator	Strain type[a]	Reference[b]
Reference Leptosphaeria maculans *isolates from the IBCN*						
92-01-2	65	Canada	*Thlaspi arvense*	G. Séguin-Swartz	Thlaspi	3
Leroy	80	Canada	*B. napus*	G.A. Petrie	Tox^+	6
Unity	81	Canada	*B. napus*	G.A. Petrie	Tox^0 NA2	6
Ery-2	83	Canada	*Erysimum* sp.	G. Séguin-Swartz	Erysimum	3
Lep-2	84	Canada	*Lepidium* sp.	G. Séguin-Swartz	Lepidium	3
PHW1268[c]	91	USA	*B. oleracea*	P.H. Williams	Tox^0 NA3	4, 6
PHW1270[d]	93	France	*B. oleracea*	P.H. Williams	Tox^0 NA1	4, 6
Other Leptosphaeria maculans *isolates*						
H5		France	*B. napus*	C. Gall	Tox^+	2
Q5, Q118		France	*B. napus*	D. Ansan-Melayah	Tox^+	1
ISH10, ISH15		France	*B. napus*	D. Ansan-Melayah	Tox^+	1
WHA		UK	*B. napus*	B. Fitt	Tox^0 (NA1)	3
Ph3		Poland	*B. napus*	M. Jedryczka	Tox^0 (NA1)	3
PL 53		Poland	*B. napus*	M. Jedryczka	Tox^0 (NA1)	3
Raw 5		Poland	*B. napus*	M. Jedryczka	Tox^0 (NA1)	3
Fp2		Denmark	*B. napus*	L. Butterworth	Tox^+	3
DL3		UK	*B. napus*	B. Fitt	Tox^+	3
DL32		UK	*B. napus*	B. Fitt	Tox^+	3
E(Box)126b		UK	*B. napus*	B. Fitt	Tox^+	3
Ak(1)10		UK	*B. napus*	B. Fitt	Tox^+	3
Other fungal species						
Phoma nigrificans:						
Phb		Poland	*T. arvense*	E. Lewartowska		3, 5
Phoma sp.		Poland	Carnation	E. Lewartowska		
Botrytis cinerea:						
MUCL 34651			Rose	MUCL culture collection		
MUCL 652			Grapevine			

[a] Strain type, according to the originator or to the reference.
[b] 1, Ansan-Melayah et al., 1997; 2, Balesdent et al., 1992; 3, Balesdent et al., 1998; 4, Gall et al., 1995; 5, Jedryczka et al., 1995; 6, Koch et al., 1991.
[c] formerly named PHW129.
[d] formerly named PHW126.

dilution buffer. PCR amplifications were done in a Perkin-Elmer DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Norwalk, CT). PCR conditions were (i) 30 cycles of 1 min at 94 °C, 1 min at 52 °C or 60 °C and 1 min at 72 °C for LMR1-based PCR, (ii) cycles for REP and ERIC described by Versalovic et al. (1991), (iii) cycles for BOX described by Versalovic et al. (1994). Annealing temperatures were 40 °C for REP-PCR, 52 °C for ERIC-PCR and 53 °C for BOX-PCR. Amplified DNA fragments were separated by electrophoresis in 1.4% agarose gel in 1× TBE. DNA bands were stained with ethidium bromide, visualised under 280 nm UV light and photographed with a Polaroid system.

Results

Rep-PCR genomic fingerprinting. ERIC-, REP- and BOX-PCR all generated complex banding patterns which were specific for each of the seven components of the '*L. maculans* species complex', and for the *P. nigrificans* isolate (Figures 1–4). As compared to reference isolates, each rep-PCR fingerprint allowed an unequivocal assignment of unknown isolates to one of the seven groups of the species complex, i. e., Tox^+, Tox^0 NA1, Tox^0 NA2, Tox^0 NA3, 'Lepidium', 'Thlaspi' and 'Erysimum'.

Depending on the taxonomic sub-unit, 15–21 DNA fragments, ranging from 0.3 to 5 kb, were obtained with

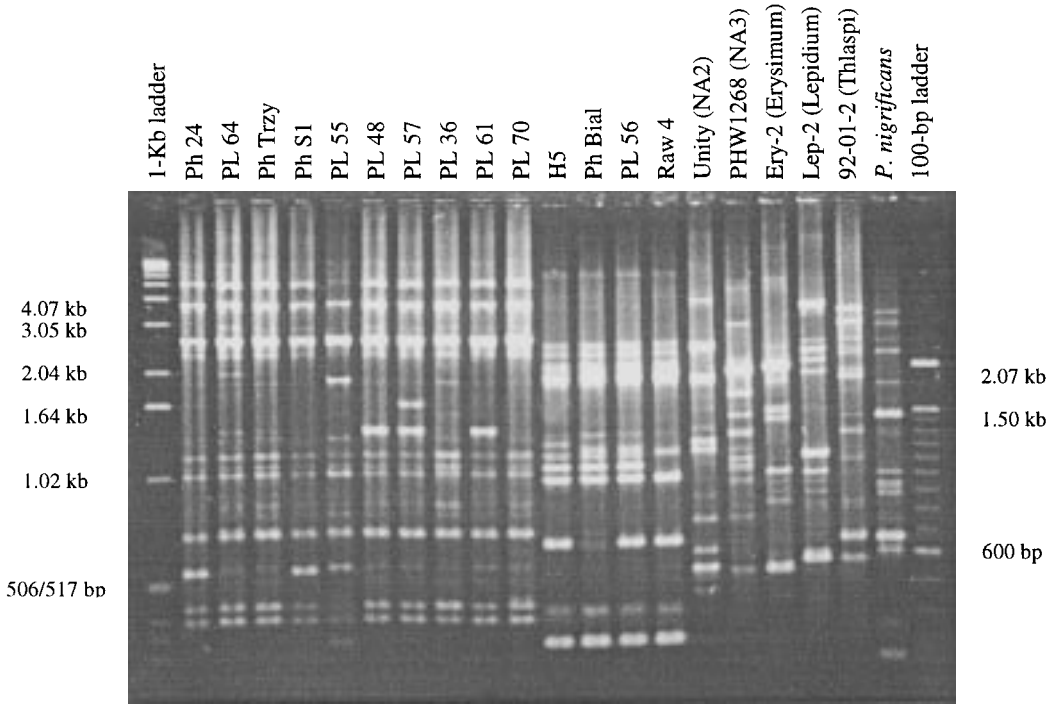

Figure 1. Banding patterns of members of the '*L. maculans* species complex' following ERIC-PCR. Tox^0 NA1 isolates: lanes 2–11; Tox^+ isolates: lanes 12–15; reference isolates from other subgroups: lanes 16–21.

ERIC-PCR (Figure 1). The profiles were reproducible for all isolates within the Tox^0 NA1 and Tox^+ subgroups. Fifteen bands were conserved for all NA1 isolates, whereas seven major bands (595, 600, 620, 1420, 1640, 1880 and 1950 bp) were found to be polymorphic between Tox^0 NA1 isolates (Figure 1). Six bands (740, 1150, 1230, 1250, 1280 and 1300 bp) out of 18 bands were polymorphic between the four Tox^+ isolates (Figure 1).

The number of amplicons generated by the REP primers ranged from 13 for the 'Lepidium' isolate to 18 for the 'Erysimum' isolate (Figure 2). The observed size range of the major bands was 0.2–5 kb (Figure 2). DNA polymorphisms were few among the 11 Tox^+ isolates examined (Figure 2). Polymorphisms were also few when Tox^0 NA1 isolates from the UK or Poland were compared; one major band (3.86 kb) was missing for one UK and two Polish isolates (Figure 2A, B). Four minor bands (2.53, 2.64, 2.73 and 2.76 kb) were highly polymorphic (Figure 2A, B).

Using the BOX primer, up to 26 DNA fragments were amplified per isolate (Figure 3). Unlike ERIC- and REP-PCR, the bands were of uniform intensity within a lane. Few polymorphisms were observed within the Tox^+ and the Tox^0 NA1 groups (Figure 3).

LMR1-based PCR generated very different profiles for the primer pairs LMR1F2/LMR1R2 or LMR1F3/LMR1R3. The primer pair LMR1F2/LMR1R2 generated the 1.3 kb fragment expected for all Tox^+ isolates, together with a few additional faint bands, whether the annealing was at 52 °C or 60 °C (data not shown). No major bands were observed for other isolates (data not shown). Using the LMR1F3/LMR1R3 primer pairs, the 1.6 kb band expected was amplified for Tox^+ isolates only, when hybridisation was at 60 °C (data not shown). Unexpectedly, when annealing was at 52 °C, the LMR1F3/LMR1R3 combination of primers also generated complex and consistent fingerprints for the other members of the *L. maculans* complex, and for unrelated fungal species (e.g. *Phoma nigrificans* or *Botrytis cinerea* (teleomorph *Botryotina fuckeliana*)) (Figure 4). Depending on the isolates, the fingerprints comprised 1–6 major bands and up to 22 minor bands. No polymorphism in the major bands was observed between the NA1 isolates from Poland, France or the

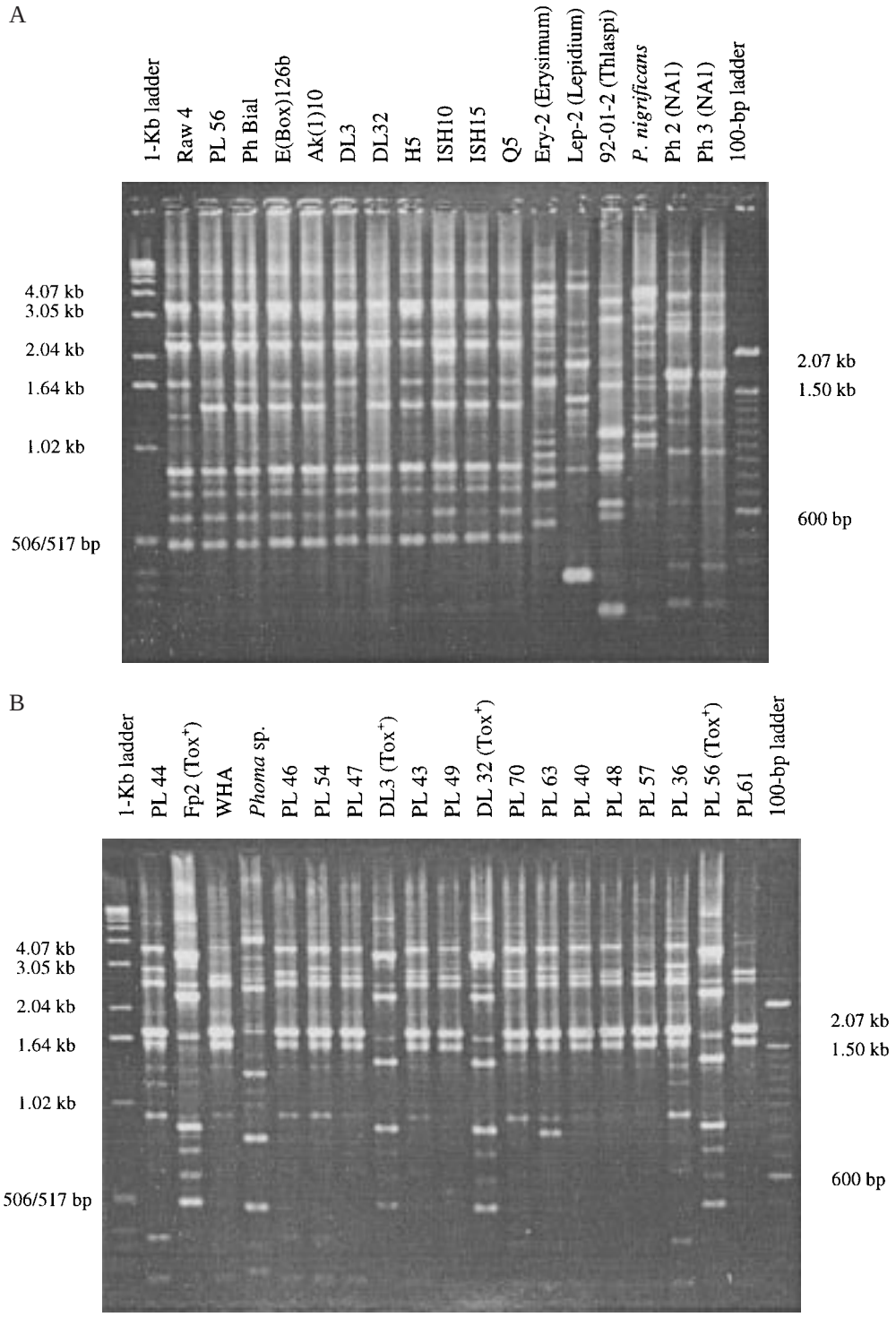

Figure 2. Banding patterns of members of the '*L. maculans* species complex' following REP-PCR. A. Polymorphism between Tox^+ isolates. Tox^+ isolates: lanes 2–12; other members of the species complex: lanes 13–18. B. Polymorphism between Tox^0 NA1 isolates. Control NA1 UK isolate (WHA): lane 4; *Phoma* sp. isolated from carnation: lane 5; Tox^+ isolates: lanes 3, 9, 12, 19; Polish Tox^0 isolates: lanes 2, 6–8, 10–11, 13–18, 20.

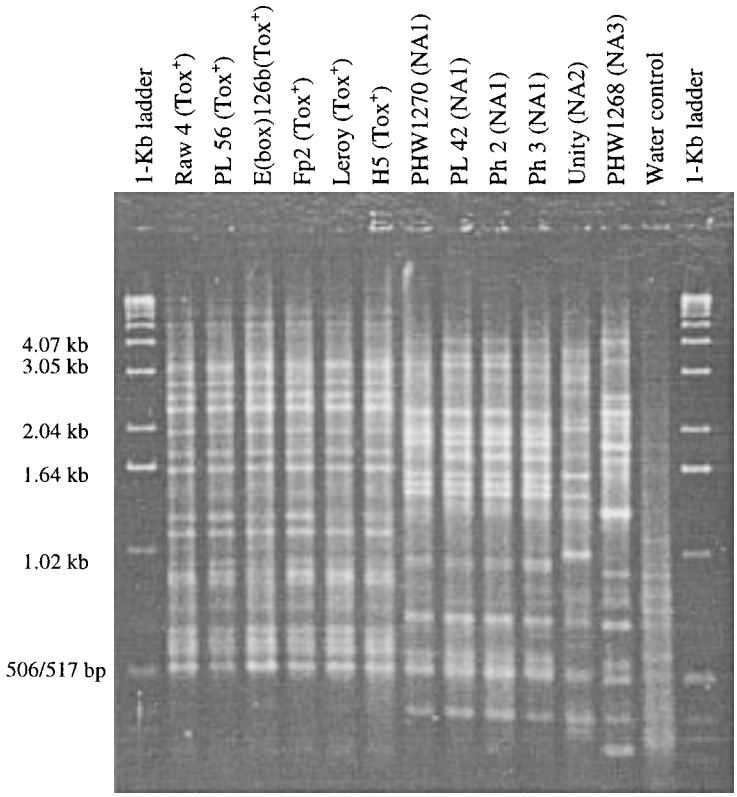

Figure 3. Banding patterns of members of the '*L. maculans* species complex' following BOX-PCR. Tox^+ isolates: lanes 2–7; Tox^0 NA1 isolates: lanes 8–11; reference isolates from other subgroups: lanes 12, 13; water control: lane 14.

UK. Minor bands were polymorphic within the NA1 group in the 0.74–1.02 kb and the 1.49–1.93 kb size ranges (Figure 4).

Rep-PCR analysis of Polish populations of L. maculans. The collection of Polish isolates was characterised using both ERIC- and REP-PCR (Figures 1, 2 and data not shown). The identification of selected isolates was confirmed with BoxA and LMR1-PCR (Figures 3 and 4). From the collection of 90 isolates, 84 isolates (i. e. 93.3%) were classified as Tox^0 NA1 and only six isolates (6.7%) were Tox^+ (Table 1). All isolates identified as Tox^+ were isolated from widely separated crops in the western part of Poland, which has warmer winter weather than the eastern part of Poland. The Tox^+ isolates Raw 4 and Ph Bial were isolated in 1992 and 1993, respectively, in north-western Poland. Due to the influence of the Baltic Sea, this region is characterised by the mildest winters and the smallest range of temperature extremes in Poland. The Tox^+ isolates Ph L5 (isolated in 1991) and PL 47 (isolated in 1995) came from the central-western Poland, and PL 56 and PL 69 (isolated in 1995), from south-western Poland. All the other isolates, classified as Tox^0 NA1, originated from regions of intensive oilseed rape production in Poland, from the western, central and eastern locations.

Discussion

Rep-PCR generates DNA fingerprints by amplifying sequences between randomly dispersed repetitive sequences in a genome (Rademaker et al., 1997; George et al., 1998). The rep-PCR method was designed for species or strain differentiation within prokaryotes (Rademaker et al., 1997). Recently, rep-PCR fingerprinting was used to characterise *Aspergillus* (van Belkum et al., 1993), *Fusarium* (Edel et al., 1995) and *Verticillium* (Arora et al., 1996). In the present study, REP, ERIC and BOXA primers generated reproducible

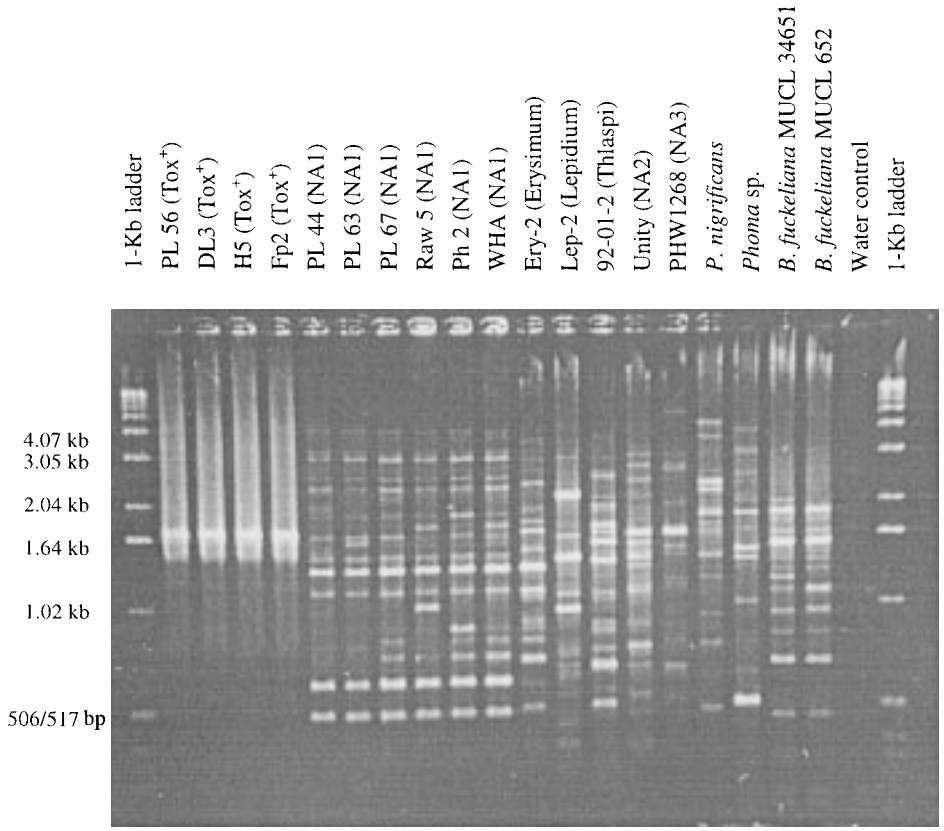

Figure 4. Banding patterns of members of the '*L. maculans* species complex' or other fungi, following LMR1-PCR, using the LMR1F3-LMR1R3 primer combination at low stringency (52 °C). Tox^+ isolates: lanes 2–5; Tox^0 NA1 isolates: lanes 6–11; other members of the species complex: lanes 12–17; *Phoma* sp. isolated from carnation: lane 18; *B. cinerea* isolates: lanes 19, 20; water control: lane 21.

and specific fingerprints for all members of the *L. maculans* species complex, confirming that ERIC-, REP- and BOX-like sequences are present in fungal genomes and represent useful targets for isolate characterisation.

The use of endogenous repetitive DNA sequences to generate fingerprints following PCR has been reported for a fungal pathogen of rice, *Magnaporthe grisea*, and was also termed rep-PCR (George et al., 1998). In *L. maculans*, a 5.3 kb repetitive DNA element, termed LMR1, was found in a Tox^+ isolate. LMR1 was absent in most of the Canadian Tox^0 isolates (Taylor and Borgmann, 1994), which probably belong to the NA2 subgroup (Gall et al., 1995). LMR1 is an AT-rich element, containing numerous very short direct and inverted repeats. This paper reports isolate characterisation using inwardly faced primers chosen either to amplify part of the element containing a few short direct /inverted repeats (LMR1F2/LMR1R2) or to amplify part of the element containing most or all of the short direct /inverted repeats (LMR1F3/LMR1R3). LMR1R3 itself is located in a region of the element rich in short direct repeats. In agreement with the data of Taylor and Borgmann (1994), one major band of the expected size (1.3 kb) was amplified for all Tox^+ isolates using the LMR1F2/LMR1R2 combination, whereas no amplification was observed for all other members of the species complex. Similar results were obtained with the LMR1F3/LMR1R3 primer pair, using a hybridisation temperature of 60 °C. These results confirm that LMR1 is specific for the Tox^+ group and suggest that LMR1-based PCR is a valuable tool for discriminating Tox^+ isolates from all other components of the species complex, as proposed by Taylor and Borgmann (1994). When used at a lower hybridisation temperature (52 °C), the LMR1F3/LMR1R3 primers generated fingerprints

specific for each member of the species complex. Moreover, complex profiles were also obtained for unrelated fungal species, like *B. cinerea*. The fact that these profiles were generated using PCR conditions which were less stringent than those of Taylor and Borgmann (1994) suggests imperfect matching of the primers with the target DNA. However, the consistency and specificity of the fingerprints generated suggest that the LMR1F3/LMR1R3 primers correspond to sequences conserved at some extent in all fungal species analysed, and constitute a new primer pair for rep-PCR fingerprinting of fungi.

All the rep-PCR based methods used were able to discriminate each member of the *L. maculans* species complex. Restriction profiling of the ITS region, following PCR amplification using spores of *L. maculans*, was recently described as a convenient tool for routine analyses of *L. maculans* populations (Balesdent et al., 1998). In contrast to this method, the rep-PCR fingerprints also demonstrated polymorphisms within each of the Tox^+ and Tox^0 NA1 groups. It is therefore suggested that the combined use of the different rep-PCR methods could be a convenient tool for discriminating individual isolates, within each of the $Tox^+$and Tox^0 NA1 groups, for epidemiology or population genetic studies.

Knowing the profiles generated by ERIC-, REP-, BOX-, and LMR1-PCR for the seven taxonomic subunits of the *L. maculans* species complex, the majority of the 90 Polish *L. maculans* isolates were characterised as Tox^0 NA1 isolates, since Tox^+ isolates represented only 6% of the isolates obtained from profound stem lesion and 14% of the isolates obtained from leaf lesions. For comparison, the Tox^0 group represented 65% of the isolates in a German collection analysed by Koch et al. (1989) (comprising more than 350 isolates), but a later assessment suggested a change in the German population of *L. maculans*, since only 11% of 341 isolates analysed in the 1990s were Tox^0 (Kuswinanti et al., 1995). Tox^+ isolates currently represent 66% of the French *L. maculans* population, with large geographic disparities (Balesdent et al., 1997). In the UK, the majority of strains belong to the Tox^+ group (M. Jedryczka et al., unpublished data). Tox^+ isolates are predominant in Australia (Plummer et al., 1994) and in Canada (Petrie et al., 1985). All Tox^+isolates from Poland were gathered in the western part of the country, although they were from widely separated fields. It is now difficult to determine whether Tox^+ isolates have been present in Poland at a low incidence for many years, since this is the first report on the characterisation of such a large collection of Polish isolates, or whether Tox^+ isolates have been introduced to Poland recently on the seeds from other countries. The presence of Tox^+ isolates in several separated regions seems to support the suggestion that they are of foreign origin. It is now important to continue our survey of the Polish *L. maculans* populations, to analyse whether Tox^+ population is currently increasing in Poland.

The high incidence of Tox^0 isolates in the Polish population analysed does not seem compatible with the severe damage attributed to *L. maculans* on *B. napus* in Poland (Frencel et al., 1991), therefore questioning whether Tox^0 are truly 'weakly pathogenic', as usually described in the literature (Williams and Fitt, 1999). However, this result is consistent with previous data: (i) both Tox^+ and Tox^0 isolates are known to be able to produce leaf lesions in field conditions, although the lesions produced by the two groups of isolates may be slightly different (Ansan-Melayah et al., 1997). This result was recently confirmed in experiments with artificial inoculations with ascospores of each group of isolates (Biddulph et al., 1999). (ii) Four Polish Tox^0 NA1 isolates were more aggressive than two French Tox^0 NA1 isolates or reference isolates from the NA1 and NA3 subgroups, in cotyledon inoculation experiments (Gall et al., 1995); (iii) 24 world-wide 'weakly virulent' isolates were all able to cause stem lesions following stem inoculation (Sippel and Hall, 1995). Although the mean of the lesion lengths differed significantly between Tox^+ and Tox^0 isolates, lesion lengths of the least aggressive Tox^+ isolate and of the most aggressive Tox^0 isolate did not differ significantly (Sippel and Hall, 1995); (iv) The pathogenicity of nine Tox^0 and one Tox^+ Polish isolates (Ph2, Ph3, Ph24, Ph35, PhL2-Ph L7, Table 1) was compared with that of two Tox^+ French isolates, using three different inoculation protocols, i.e. cotyledon inoculation, stem inoculation and soil inoculation, on *B. napus* cultivars Lirajet and Primor. Differences in aggressiveness were observed between the different Polish Tox^0 isolates (Jedryczka et al., 1994). At least one of the isolates, Ph L7, was always more aggressive than French and Polish Tox^+ isolates included in the study. All these data suggest that the role of Tox^0 isolates in blackleg disease of oilseed rape has to be further investigated. The predominance of Tox^0 isolates in Poland, contrasting with the predominance of Tox^+ isolates in Western Europe provides a unique opportunity to compare the effects of Tox^0 and Tox^+ isolates on oilseed rape.

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