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Random amplified polymorphic DNA analysis of a *Globodera pallida* population selected for virulence

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Summary – Selection of a *Globodera pallida* population on the partially resistant potato cultivar Darwina (Marijke × VTN[2] 62.33.3) for seven generations resulted in an increase of reproduction rate indicating a higher ratio of virulent genotypes in the selected population. Random amplified polymorphic DNA (RAPD) patterns of the selected and the unselected population were generally very similar with 40 primers tested. With two of these primers, however, two additional non-homologous DNA fragments were amplified in the selected population. RAPD analyses of single cysts revealed that the DNA properties leading to these amplified fragments were highly enriched in the selected population. One of the two DNA fragments was cloned and used to probe amplified DNA from sixteen *G. pallida* populations. Hybridization was only observed with populations showing higher reproduction rates on c.v. Darwina. This may indicate a correlation of the DNA fragment with a particular type of virulence.

Résumé – *Analyse d'ADN polymorphe par amplification au hasard chez une population de* Globodera pallida *sélectionnée pour sa virulence* – La sélection, pendant sept générations, d'une population de *Globodera pallida* pour sa virulence envers le cultivar de pomme de terre partiellement résistant Darwina (Marijke × VTN[2] 62.33.3) provoque une augmentation du taux de reproduction indiquant un rapport élevé de génotypes virulents au sein de la sélection ainsi réalisée. Les modèles d'ADN polymorphes amplifiés au hasard (RAPD) appartenant aux populations sélectionnée et non sélectionnée se sont révélés généralement très similaires pour les 40 amorces testées. Pour deux de ces amorces cependant, deux fragments additionnels non homologués d'ADN ont été amplifiés dans la population sélectionnée. L'analyse par RAPD de kystes isolés démontre que les propriétés de l'ADN conduisant à ces fragments amplifiés sont très enrichies en ce qui concerne la population sélectionnée. L'un de ces fragments d'ADN a été cloné et utilisé pour tester l'ADN amplifié de seize populations de *G. pallida*. Une hybridation n'est observée qu'avec les populations montrant un taux de reproduction élevé sur le cv. Darwina, ce qui peut indiquer une corrélation entre le fragment d'ADN et un type particulier de virulence.

Key-words : RAPD, DNA marker, virulence, selection, *Globodera pallida*.

Populations of the potato cyst nematode, *Globodera pallida,* exhibit clear differences in virulence, i.e. the rate of reproduction on plants carrying a resistance property. By means of biotesting on plant clones with different types of resistance, Kort *et al.* (1977) classified *G. pallida* populations in three pathotypes, Pa1, Pa2 and Pa3.

As postulated by Andersen and Andersen (1982), all individuals of a true pathotype should be homogeneous with respect to the particular virulence gene(s) distinguishing them from other pathotypes. This proposal apparently does not hold for the pathotypes Pa2 and Pa3 as was already pointed out by Trudgill (1985) and Stone (1985). In the scheme of Kort *et al.* (1977) these pathotypes are distinguished on the basis of their multiplication rate *(Pf/Pi)* on the *Solanum vernei* hybrid VTN[2] 62.33.3 (Pa2 : $Pf/Pi < 1$; Pa3 : $Pf/Pi > 1$). As shown by Turner *et al.* (1983) and Turner (1990), strains with high levels of virulence against hybrid 62.33.3 were selected from populations of both pathotypes during propagation on this host for up to eleven generations. Therefore, the difference between these Pa2 and Pa3 populations before selection may not be due to different types of virulence genes in the two pathotypes but rather to different proportions of individuals carrying the same types of virulence genes.

The strong increase in reproduction rate upon selection observed by Turner *et al.* (1983) and Turner (1990) suggests a substantial shift of the initial mixture of avirulent and virulent genotypes towards the virulent genotypes. Molecular markers for virulent genotypes may therefore be obtained by comparing a selected population with its unselected counterpart. In the present work we have tried this approach using the RAPD-PCR technique (Williams *et al.,* 1990; Caetano-Anollés *et al.,* 1991) for comparing amplified DNA fragments of a *G. pallida* population unselected and selected for virulence.

Material and methods

Propagation and virulence testing of *G. pallida* populations

The selected population " Kalle " was obtained by maintaining the *G. pallida* population " Kalle " (virulence group Pa2/3) for 7 years in a microplot on the resistant potato cv. Darwina (pedigree: Marijke × VTN[2] 62.33.3). Other *G. pallida* populations used in this study were continuously propagated on the susceptible cv. Clivia. Virulence against the Darwina genotype was estimated from the number of newly formed cysts on " Darwina " as percentage of those on " Clivia ". Tests were performed under glass at ten times replication in 500 ml plastic pots containing steam-sterilized soil. Inoculum was eighteen cysts per pot giving an initial population density of seven eggs/g soil. After plant senescence cysts were extracted and the number of newly formed cysts counted.

Extraction of nematode DNA

DNA was extracted from 50 mature brown cysts applying the procedure of Dellaporta *et al.* (1983) with modifications. For investigation of intra-population heterogeneity single cysts were extracted likewise. In the following protocol, quantities referring to single cyst extraction are given in parentheses. Fifty cysts (one cyst) were ground on ice in a small manually operated glass homogenizer (W. O. Schmidt Laboratoriumsbedarf, Braunschweig) in 150 µl (10 µl) of extraction buffer (100 mM Tris-HCl pH 8.0; 500 mM NaCl; 50 mM Na-EDTA pH 8.0; 10 mM mercaptoethanol) and transferred to a 1.5 ml Eppendorf tube. The homogenizer was washed with 100 µl (8 µl) of extraction buffer which was then added to the homogenate. After addition of 15 µl (1.2 µl) of 20 % SDS and mixing, the homogenate was incubated for 10 min at 65 °C. Then 50 µl (6 µl) of 5 M potassium acetate was mixed in, the sample was incubated for 20 min on ice and centrifuged at 25 000 *g* for 20 min. The pellet was discarded and the supernatant transferred to a fresh tube. Nucleic acids were precipitated twice, once with 0.6 vol of isopropanol and once with 0.1 vol of 3 M sodium acetate/2.5 vol of ethanol. After the final centrifugation the pellet was washed with 70 % ethanol and dissolved in 400 µl (10 µl) of sterile water. Nucleic acid concentration was estimated from the intensity of ethidium bromide fluorescence (Sambrook *et al.*, 1989 *a*).

RAPD analysis

Ten-base oligonucleotide primers of random sequence were purchased from Operon Technologies (Alameda, U.S.A.). PCR was carried out in 25 µl containing 2 ng of template DNA, 5 U of Taq DNA polymerase Stoffel fragment (Perkin Elmer Cetus), 100 µM of each dNTP (Boehringer Mannheim), 0.2 µM of a single primer and 4 mM $MgCl_2$ in 1 × reaction buffer (10 mM KCl, 10 mM Tris-HCl pH 8.3). Using a Perkin Elmer Cetus Thermal Cycler (TC-480), the reaction mixture was heated to 94 °C for 5 min, submitted to 45 cycles of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min, and finally heated to 72 °C for 10 min. After the PCR, 10 µl aliquots of the reaction mixture were resolved by 2 % agarose gel electrophoresis and visualized by staining in 0.5 µg/ml ethidium bromide.

Cloning and labelling of an amplified DNA fragment

A 0.43 kb DNA fragment amplified from the selected population " Kalle " (cf. Results) was eluted from the electrophoresis gel, purified with the QUIAEX procedure (QUIAGEN, Hilden, FRG), ligated into the pGEM-T vector (Promega) and cloned in *E. coli* NM 522. The recombinant plasmid was isolated by the alkaline lysis method (Sambrook *et al.*, 1989 *b*) and cut with restriction enzymes to release the DNA insert which was separated by electrophoresis in 2 % agarose and purified by the QUIAEX procedure. The DNA fragment was labelled with digoxigenin-11-dUTP (Boehringer, Mannheim) by random priming according to the instructions of the manufacturer.

DNA hybridization

DNA fragments amplified by RAPD-PCR from various *G. pallida* populations were separated by electrophoresis in 2 % agarose, diffusion-blotted with 0.4 N NaOH to a positively charged nylon membrane (N + membrane, Boehringer, Mannheim) and fixed by heating the membrane to 80 °C for 2 h. Hybridization of the blot with the digoxigenin-labelled 0.43 kb DNA fragment and chemiluminescence detection were carried out according to the instructions provided with the DIG nucleic acid detection kit (Boehringer, Mannheim).

Results

Virulence of selected and unselected *G. pallida* " Kalle "

Thirteen *G. pallida* populations representing a wide range of virulence within the Pa2/3 group exhibited relative reproduction rates on cv. Darwina ranging from 0.4 % to 18 % of reproduction on the susceptible cv. Clivia (Fig. 1). Selection of *G. pallida* " Kalle " on Darwina for seven generations resulted in an increase of relative reproduction rate from 2 % to 13 % (Fig. 1).

Differences in RAPD patterns between selected and unselected *G. pallida* " Kalle "

DNA extracted from 50 pooled cysts of *G. pallida* " Kalle " was used to establish suitable reaction conditions for RAPD analysis as described above. The reproducibility of the RAPD patterns was examined using different template DNA concentrations in the PCR. Control PCR reactions with omission of the template DNA did not yield detectable amplification products

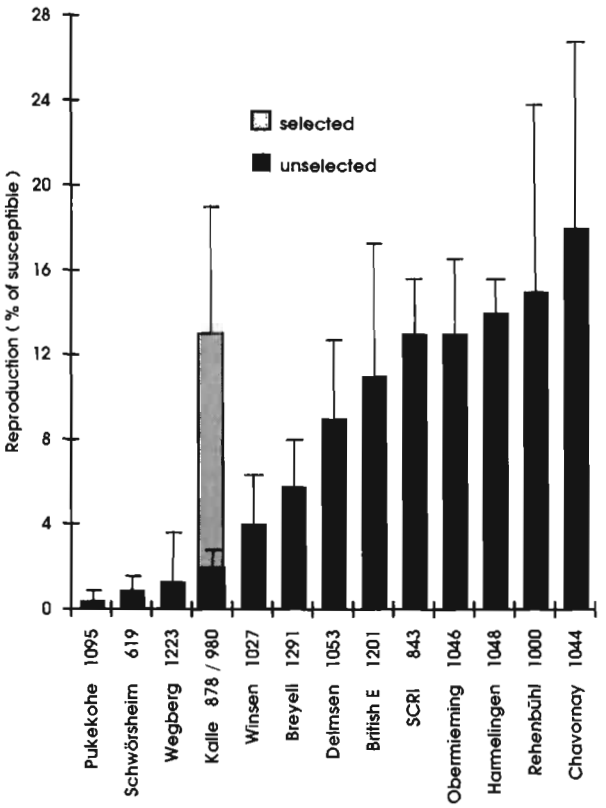

Fig. 1. *Relative reproduction rates of* Globodera pallida *populations (virulence group Pa2/3) on cv. Darwina, expressed by the number of newly formed cysts as percentage of those on the susceptible cv. Clivia. The number of newly formed cysts on cv. Clivia is given for each population. Experimental error limits are indicated on the columns.*

thereby excluding the possibly that any of the fragments seen with genomic DNA were actually primer artefacts.

Fourty different ten base primers from the Operon kits B and E were tested to produce RAPD patterns to see if they would differentiate between the selected and unselected " Kalle " populations. Different RAPD patterns were obtained with these primers (experiments not shown). However, striking differences between the patterns of the selected and the unselected population were only observed with primers B-07 (5'-GGTGACG-CAG-3') and E-06 (5'-AAGACCCCTC-3') (Fig. 2). With each of these primers, RAPD patterns differed by an additional DNA fragment in the selected population. The size of the additional fragments was 1.0 kb with primer B-07 and 0.43 kb with primer E-06, respectively. The 0.43 kb DNA fragment was isolated and cloned. When blots containing the 1.0 kb DNA fragment were probed with the labelled 0.43 kb fragment, no hybridization signal was obtained (not shown). Homologies between the two DNA fragments could therefore be excluded.

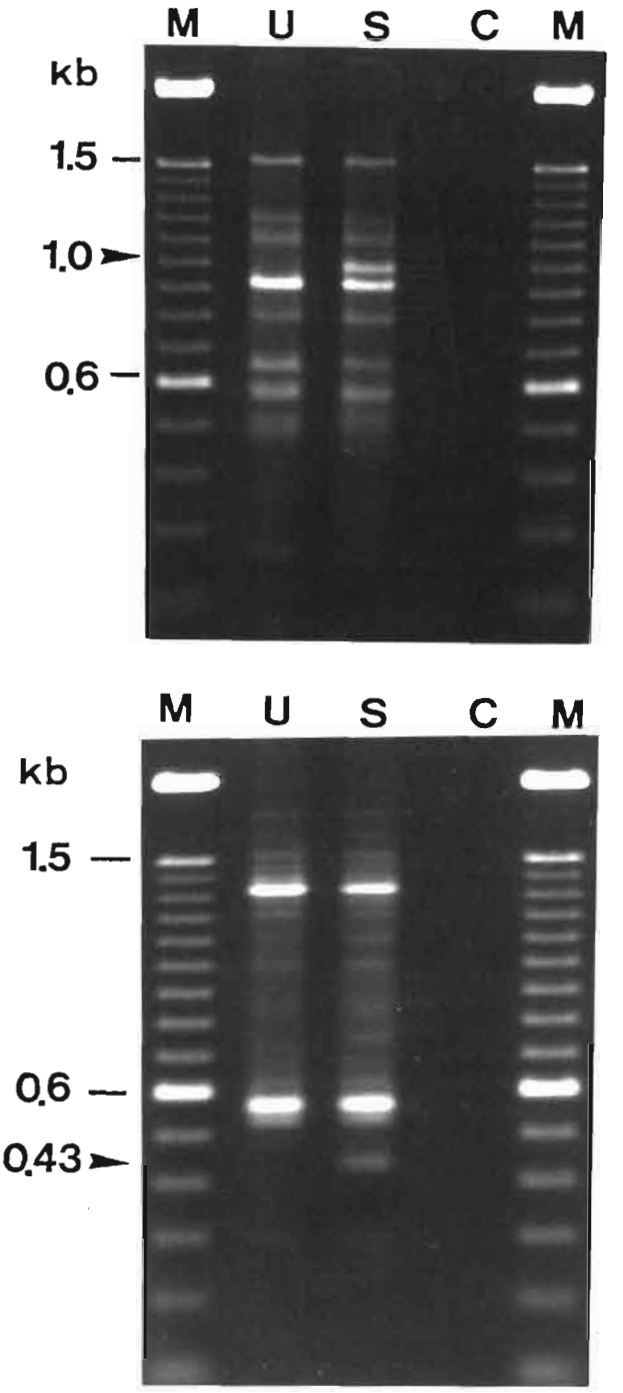

Fig. 2. *RAPD analyses with extracts of 50 pooled cysts from the unselected (U) and the selected (S) population " Kalle ". Random primers B-07 (top) and E-06 (bottom) were used for amplification of 2 ng template DNA. Control PCR was carried out with omission of the template DNA (lanes C). DNA size markers (100 bp ladder, BRL) were applied in lanes M. The arrows indicate DNA fragments of approximately 1.0 kb and 0.43 kb observed only in the selected population.*

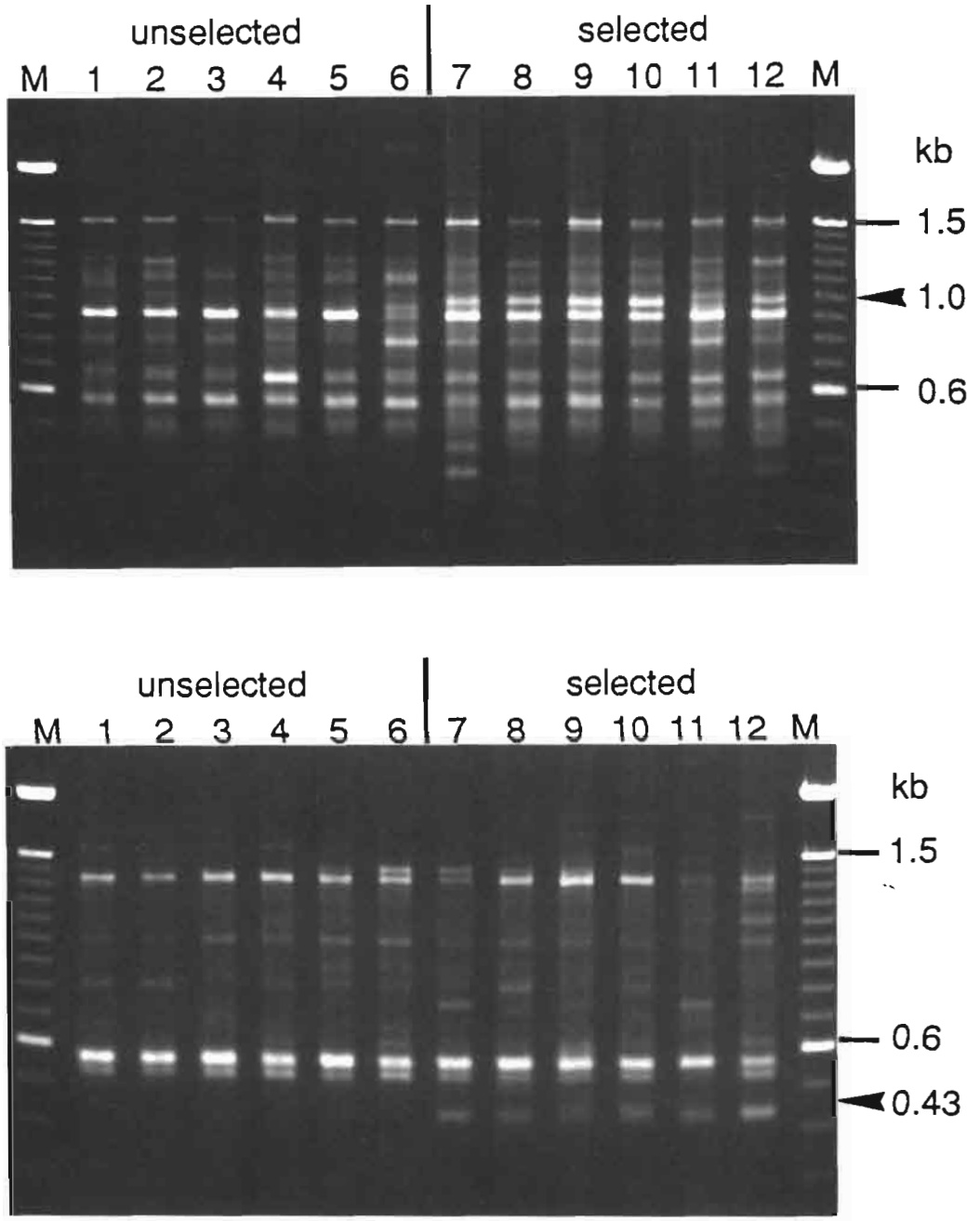

Fig. 3. *Representative RAPD analyses of six single cysts, each from the unselected and the selected population "Kalle". Random primers B-07 (top) and E-06 (bottom) were used for amplifying 2 ng of template DNA. DNA size markers are as in Fig. 2 (lanes M). The arrows indicate DNA fragments of approximately 1.0 kb and 0.43 kb observed only in the selected population.*

DNA extracted individually from 50 single cysts was also examined to check if the DNA properties leading to the additional amplified fragments was uniformly distributed in the selected population or present in only a fraction of the cysts. Representative RAPD analyses obtained with single cyst extracts are shown in Fig. 3. In general, patterns of the same type were obtained as with DNA from bulk extraction of 50 cysts. Occasionally, however, unexpected patterns were seen, as shown in Fig. 3 top, lane 6 and bottom, lane 12.

The additional DNA fragments amplified in the virulence-selected population "Kalle" were clearly visible

in RAPD analyses of single cysts (Fig. 3). The 1.0 kb fragment amplified with primer B-07 was observed with 47 out of 50 cysts from the selected population and in one out of 50 cysts from the unselected population. The 0.43 kb fragment produced with primer E-06 was found with 48 out of 50 cysts fom selected and in none out of 50 cysts from unselected " Kalle ".

Amplification of the 0.43 kb DNA fragment in other *G. pallida* populations

To check for a possible correlation of the 0.43 kb and 1.0 kb DNA fragments with virulence, the occurrence of the respective sequences in other *G. pallida* populations should be investigated. So far, only the cloned 0.43 kb fragment was used as a hybridization probe. DNA from thirteen Pa2/3 populations representing a wide range of virulence (Fig. 1) and from three Pa1 populations was amplified using primer E-06. The PCR products were separated by electrophoresis, blotted and hybridized with the labelled 0.43 kb DNA fragment (Fig. 4). Amplified DNA from the selected population " Kalle " was applied as a control yielding a strong hybridization signal of the 0.43 kb fragment. Corresponding hybridization signals were also obtained with seven out of eight Pa2/3 populations showing higher degrees of virulence on cv. Darwina (*cf.* Fig. 1). No hybridization was observed with five less virulent Pa2/3 populations including the unselected population " Kalle " and with the three Pa1 populations tested.

Discussion

The virulence ranking of *G. pallida* populations on the basis of relative reproduction rates on cv. Darwina (Fig. 1) is in agreement with the results of more elaborate biological testing (Mugniéry *et al.*, 1989). As expected, the unselected population " Kalle " ranks among the least virulent Pa2/3 populations. Selection on cv. Darwina for seven generations resulted in a 6.5-fold increase in relative reproduction rate on this host, ranking the selected population " Kalle " near population " SCRI " which is recognized as a relatively highly virulent *G. pallida* population (Mugniéry *et al.*, 1989). We may conclude that the selected population " Kalle " contains a substantially higher ratio of virulent genotypes than the original population " Kalle ".

RAPD analysis has been successfully used to assess genetic variability between populations of *Heteroda schachtii* and *H. cruciferae* (Caswell-Chen *et al.*, 1992), *Meloidogyne* spp. (Cenis, 1993) as well as *G. pallida* and *G. rostochiensis* (our group, unpubl.). Due to the diversity of available primer sequences and the sensitivity of the PCR, RAPD analysis may well have the potential of identifying DNA markers for virulence. The problem of identifying these DNA markers may be approached in different ways including RAPD analysis of selected virulent lines of the nematodes (Bakker *et al.*, 1993). Our approach of comparing a selected population with its

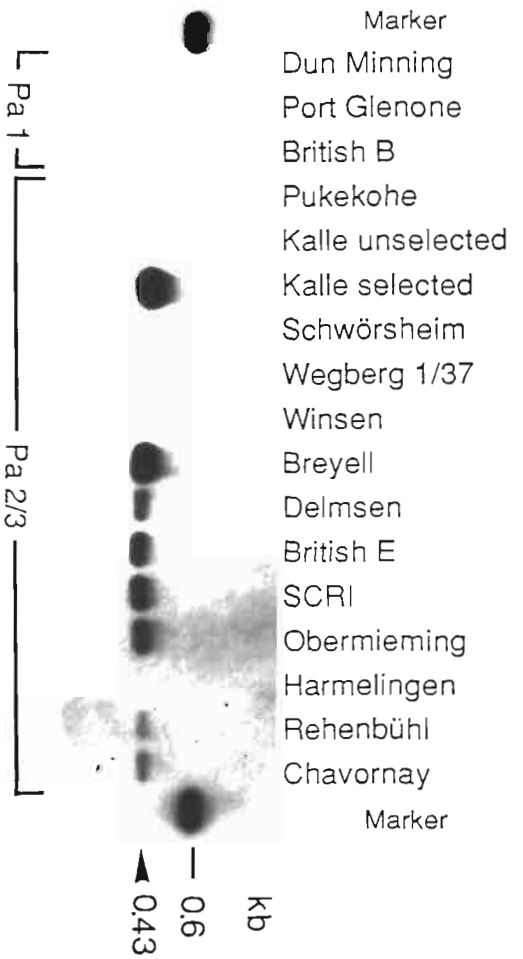

Fig. 4. *Hybridization of the 0.43 kb DNA fragment from the selected population " Kalle " with amplified DNA from other* G. pallida *populations. DNA was amplified using primer E-06, separated electrophoretically, blotted and hybridized with the labelled 0.43 kb fragment.*

unselected counterpart requires that RAPD patterns are not grossly changed upon selection for virulence because this would complicate identification of virulence-specific DNA fragments. Our results show that this condition was met since RAPD patterns of the selected and unselected population " Kalle " were almost indistinguishable from most of the ten base primers tested. A virulent and virulent genotypes making up the population " Kalle " may therefore not differ very much in most of their genetic features.

The two additional DNA fragments amplified in the selected population " Kalle " may be tentatively attributed to DNA characteristics specific for a virulent genotype. RAPD analyses of single cysts revealed amplification of these fragments with less than 2 % of the

unselected and more than 94 % of the selected cysts. The increase in frequency of the additional DNA fragments upon selection appears to be much higher than expected from the observed increase in virulence from 2 to 13 % relative reproduction rate. However, since one cyst contains several hundred J2, the additional DNA fragments amplified from a single cyst may in fact originate from only a fraction of larvae belonging to a virulent genotype. RAPD analyses of single animals (e.g. J2, young or unmated females) would therefore be useful to check for a direct connection of the additional DNA fragments with a virulent genotype (experiments in progress).

The exclusive hybridization of the 0.43 kb DNA fragment with amplified DNA from Pa2/3 populations showing higher degrees of virulence on cv. Darwina provides further evidence for a relationship of this sequence with a virulent genotype. The Pa1 populations and the less virulent Pa2/3 populations tested may either lack the respective DNA sequence or contain a small amount of it below the detection limit of our hybridization procedure, as we find with the unselected population " Kalle ".

The absence of a hybridization signal with the highly virulent population " Harmelingen " may indicate that the ability of this population to multiply on cv. Darwina is perhaps mediated by other type(s) of virulence not related to the sequence of the 0.43 kb DNA fragment. Further information may be obtained from hybridization experiments with the nonhomologous 1.0 kb DNA fragment which still has to be cloned (experiments in progress). Selection experiments using plants with other types of resistance are also necessary to obtain a sufficient number of DNA markers for covering the different types of virulence existing in *G. pallida* populations.

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