Theor Appl Genet (2000)100:569–575

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Intraspecific genetic variability analysis of *Neovossia indica* causing Karnal bunt of wheat using repetitive elements

Received: 20 April 1999 / Accepted: 29 July 1999

Abstract *Neovossia indica* (*Tilletia indica*), causing Karnal bunt of wheat, affects major wheat growing regions all over the world. Karnal bunt ranks as one of the major diseases of wheat causing quality losses and monetary losses due to international quarantine regulations. The present work is the first report of a genetic diversity analysis of Indian isolates of *N. indica.* A library of *N. indica* isolate Ni7 was constructed in a λZAPII system, and three repetitive elements were identified for molecular analysis. These repetitive elements generated complex hybridization profiles producing fingerprint patterns of all seven isolates. Copy-number estimation of these three elements, pNiR9, pNiR12 and pNiR16, indicated the presence of 32, 61 and 64 copies, respectively. Cluster analysis based on hybridization patterns grouped together moderately virulent isolates Ni1, Ni7 and Ni8, thus suggesting a positive correlation between virulence typing and cluster analysis based on molecular data. Variability analysis of *N. indica* isolates will aid in checking new resistant sources in host germplasm.

Key words Karnal bunt · Repetitive elements · Genome diversity · RFLP

Introduction

Neovossia indica (*Tilletia indica*), a basidiomycetous fungus causing Karnal bunt (KB) of wheat, was first reported in Northern India by Mitra (1931), subsequently in Pakistan (Munjal 1975), Nepal (Singh et al. 1989), Iraq (CMI 1989), and Mexico (Duran 1972) and recently in the USA (Ykema et al. 1996). Susceptible host cultivars are infected by air-borne sporidia at the flowering stage, and disease symptoms are evident in the form of teliospores during hardening of the grains (Gill et al. 1993). A fishy odour is emitted from infected seeds due to the presence of trimethylamine which makes the products unacceptable for human consumption when little more than 1% of the grains are infected (Mehdi et al. 1973). Strict quarantine regulations have been imposed on the movement of commercial wheat grain and wheat germplasm in view of the invasion of this pathogen into new areas (Royer and Rytter 1988). Conventional approaches for controlling this disease consist of the adoption of various cultural practices such as crop rotation for longer periods, the sowing of disease-free seeds, adjustment of the nitrogen balance in the soil and adjustment of the time of irrigation to minimize disease incidence (Mitra 1937, Bedi et al. 1949; Munjal 1974; Goel et al. 1977; Singh and Prasad 1978; Gill 1979; Aujla et al. 1981, Singh and Singh 1985). Control through fungicides is not completely effective as the disease is seed-and-soil-borne (Singh et al. 1985). Most of the fungicides, except for a few mercurials, are ineffective as they are fungistatic in action. Hence, the most economical, ecofriendly and effective approach to control the disease is the cultivation of resistant varieties. The main sources of resistance against Karnal bunt have been the Indian, Chinese and Brazilian wheats (Fuentes-Davila and Rajaram 1994). A new range of genetic variability for resistance to Karnal bunt has been observed in synthetic hexaploid wheats derived from *Triticum turgidum*×*Triticum tauschii* crosses (Villareal et al. 1996). Resistant sources have also been identified among other wild *Triticum* species particularly those carrying the A genome (Pannu et al. 1994). However, extensive knowledge of the pathogen population is a vital criterion in assessing resistance and guiding breeders to incorporate useful R genes into the desired background.

Efforts involving virulence typing and molecular analysis of *N. indica* have been initiated in recent years.

Communicated by P.M.A. Tigerstedt

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Bonde et al. (1996) found a significant relation between percentage disease incidence and number of infection sites in the seed by analysing the virulence patterns of fungal isolates from India, Pakistan and Mexico. In a more comprehensive analysis of pathogenic reactions of Indian isolates of *N. indica*, Singh et al. (1995) described the percentage of infected grains as a criterion for selecting early generation material for resistance to Karnal bunt. Although virulence typing provides valuable information about the pathogenic behavior of fungal isolates, this approach is not yet very efficient with respect to the reproducibility of pathogenic reactions of fungal isolates due to environmental constraints. Moreover, it is a laborious, tedious, time-consuming technique that can not differentiate isolates showing the same pathogenic reaction.

Due to limitations in virulence typing, there have been attempts to carry out molecular analyses of different species of *Tilletia*. A species-specific marker for *T. indica* was identified using mitochondrial DNA as a probe as well as PCR based method (Ferreira et al. 1996; Smith et al. 1996). Molecular analyses of different species of *Tilletia* were also carried out using random amplified polymorphic DNA (RAPD) markers (Bonde et al. 1996; Shi et al. 1996; Gang and Weber 1996; David & Darrell 1996). To date there has been no report of a genetic analysis of *N. indica* using DNA markers. Considering the importance of this approach in pathogen identification, the work was initiated to identify a few repeat elements from the genomic library of *N. indica* and to assess their potential in characterizing different isolates.

Materials and methods

Fungal material

Seven isolates of *Neovossia indica* were collected from different geographic locations of northern India. All the isolates were revived, multiplied and maintained on PDA (potato dextrose agar) slants at 20°C and 80% humidity. Table 1 gives a list of the *N. indica* isolates used in the present study.

DNA extraction

Mycelia of all the *N. indica* isolates were grown in MGYP (0.3% malt extract, 1% glucose, 0.3% yeast extract, 0.5% peptone) broth at 20°C for 14 days. Mycelium of each isolate was harvested and ground with liquid nitrogen to a powder form. The latter was homogenized by adding extraction buffer (25 m*M* TRIS-HCl pH 8.0, 10 m*M* EDTA) four times the weight of the mycellium and kept stable until it reached room temperature. The remainder of the protocol, with minor modifications, was that described by Rajebhosale et al. (1997).

Table 1 List of *Neovossia indica* isolates used in present study[a]

Sr. no.	Isolate	Geographic origin
1	Ni1	Jasore, Jammu and Kashmir
2	Ni2	Ludhiana, Punjab
3	Ni4	Panipat, Haryana
4	Ni5	Jaipur, Rajasthan
5	Ni6	Gurdaspur, Punjab
6	Ni7	Gurdaspur, Punjab
7	Ni8	Hoshiarpur, Punjab

[a] Cultures were received as gift from Dr. P.P.S. Pannu, Assistant Pathologist, Department of Plant Pathology, Punjab Agricultural University, Ludhiana 141004, India

Construction of genomic library

A genomic library of *N. indica* isolate Ni7 DNA was constructed in λZAPII/*Eco*RI cut/CIAP-treated system (Stratagene USA). *N. indica* isolate Ni7 DNA was digested with serial dilutions of *Eco*RI and then column-purified using a QIA-quick gel extraction kit (Qiagen USA) to remove all the impurities. Purified DNA was ligated with predigested λ arms with *Eco*RI along with the positive control provided in the kit. Ligated DNA was packaged using a Gigapack III Gold Packaging Extract (Stratagene USA). Phage infection was done with *E. coli* strain XL1-Blue at an O.D. (optical density)$_{600}$ equivalent to 0.5; infected cells were incubated at 37°C for 20 min and then plated on NZY-top agarose with IPTG (Isothiopropyl-thio-β-D-galactose) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Plates were incubated overnight at 37°C for plaque formation. Titer of the library was calculated to be $2.2 \cdot 10^5$ pfu/ug DNA.

Identification of repeat elements

The genomic library was screened for isolating putative repetitive clones. Plaque lifts in duplicate were done on Hybond-N membranes (Amersham, UK) as per the manufacturer's instructions. Baked blots of plaque lifts were prehybridized in a solution of 5× SSPE, 5× Denhardt's, 0.1% SDS and 0.1% milk powder at 60°C for 3–5 h. They were probed with genomic DNA of *N. indica* isolate Ni7 radiolabeled with α-[P^{32}]-dCTP by the random priming method (BRIT, India) and incubated at 60°C for 18–20 h. Blots were washed with 2×SSC and 1% SDS; 1×SSC and 1% SDS and 0.1×SSC and 1% SDS at 60°C. Probed blots were exposed to X-ray film using an intensifying screen at –70°C. After co-relating the signals on the audioradiograms with plaques on the plates, we picked out and purified positive putative clones. *In vivo* excision was done as per the instructions in the Stratagene manual, and the selected clones were used as probes for further RFLP analysis. Restriction endonuclease mapping of the repetitive clones was done by digesting the plasmid DNAs with various restriction enzymes such as *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Sma*I, *Xba*I, and *Xho*I, and the sizes of the fragments generated were calculated by using computer software SEQUAID (Rhoads and Roufa 1989). Assuming the genome size of *N. indica* to be 4.7×10^7 bp (Anderson et al. 1992), we estimated the copy-number of each repeat clone by the quantitative dot blot method.

Restriction fragment length polymorphism (RFLP) analysis

DNAs of all seven *N. indica* isolates were digested with restriction enzymes, *Eco*RI, *Bam*HI and *Pst*I according to the manufacturer's instructions (Promega, USA). Ten units of enzyme per microgram of fungal DNA were used to ensure complete digestion followed by electrophoresis on a 0.9% agarose gel in 1× TAE buffer (0.04 *M* TRIS acetate, 0.001 *M* EDTA, pH 8.0). Blotting was using a vacuum blotting apparatus (Vacublot, Pharmacia LKB) with Hybond-N membranes (Amersham UK), and the blotted membranes were baked at 80°C for 2 h. Prehybridization, hybridization and washing of blots were performed as described above. Radiolabeling of the repeat clones and hybridizations were performed as described by Sambrook et al. (1989).

Statistical data analysis

Autoradiograms were scored by differences in the banding patterns of the seven isolates on the basis of presence/absence of bands. Similarity indices (Nei 1973) were used to create a similarity matrix which was utilized to construct the dendrogram using software *TAXAN 4.0* (Swartz 1989).

Results

Content of repetitive DNA and selection of repeat clones

A moderately virulent *N. indica* isolate Ni7 was used for construction of the genomic library in λZAPII/*Eco*RI-cut/CIAP treated system (Sratagene, USA). The lambda ZAPII system has two advantages: first, it is highly efficient in the construction of a λ library and, secondly, it provides convenient methodology to convert λ clones into plasmid clones without undergoing another round of restriction enzyme digestion, ligation and transformation. The genomic library was screened using Ni7 genomic DNA as the probe for calculating repetitive DNA content and for identifying repetitive elements. The amount of total repetitive DNA was calculated to be 1% of the total genomic DNA of *N. indica.* Twenty-four putative repetitive plaques were identified, and in vivo excision was performed. The resulting clones were further screened and only those clones that gave intense hybridization signals within 6 h of exposure were selected. Three such repetitive clones, pNiR9, pNiR12 and pNiR16, with an insert size of 3700 bp, 4800 bp and 500 bp, respectively, were used for RFLP analysis of *N. indica* isolates.

Hybridization of repetitive DNA sequences with *N. indica* genomic DNAs

Many restriction enzymes were tested in attempts to obtain a good digestion of *N. indica* DNAs of which only three restriction enzymes, namely *Eco*RI, *Pst*I and *Bam*HI, were finally used for variability analysis. Repetitive clones pNiR9, pNiR12 and pNiR16 were used as probes to analyse the *N. indica* isolates showing different virulence patterns.

As seen in Fig. 1, the repeat clone pNiR9 generated approximately 8–17 different bands with *Eco*RI, whereas approximately 10–20 bands were observed in *Pst*I-digested *N. indica* DNAs, all in a range of 2.0–22 kb. Figure 1 also reveals that *N. indica* isolates Ni1 and Ni7 show similar hybridization profiles (lanes 3, 4), but they can be distinguished well using *Pst*I digests (lanes 10, 11). *N. indica* isolate Ni8 (lanes 5, 12) digested with both *Eco*RI and *Pst*I gave fewer but different bands upon hybridization. Isolates Ni1 (lanes 3, 10), Ni7 (lanes 4, 11) and Ni6 (lanes 6, 13) showed intense signals with repeat clone pNiR9 suggesting a higher number of copies of a repeat in a specific environment.

The repeat clone pNiR12 produced approximately 2–5 bands with *Eco*RI- and *Pst*I-digested DNAs of *N. indica* isolates and approximately 2–9 bands with *Bam*HI-digested DNAs of *N. indica* isolates within a 2.0 to 9.4 kb range (Fig. 2). The repeat clone pNiR12 was not widely dispersed throughout the genome since number of bands is fewer than that shown by the repeat clone pNiR9. An intense band of approximately 4.8 kb in the case of *Eco*RI, 2 intense bands of sizes 6.0 kb and 3.0 kb in *Pst*I- and 3 intense bands of sizes 4.0 kb, 3.0 kb and 0.5 kb in *Bam*HI-digested isolates suggest that at least one internal site for *Pst*I and two internal sites of *Bam*HI may be present in the insert fragment pNiR12. This observation was supported by the restriction enzyme map of pNiR12.

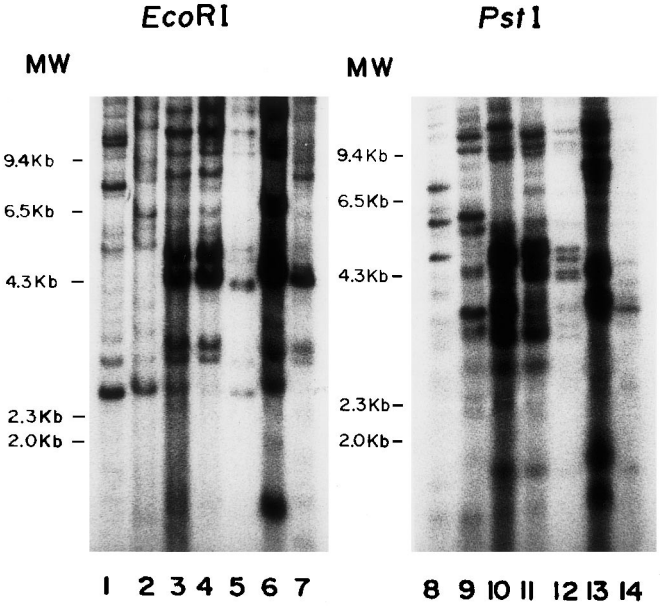

Fig. 1 Southern hybridization profiles of pNiR9 with genomic digests of *N. indica* isolates. *Lanes 1–7* contain isolates Ni4 (*1*), Ni5 (*2*), Ni1 (*3*), Ni7 (*4*), Ni8 (*5*), Ni6 (*6*) and Ni2 (*7*) digested with *Eco*RI, *lanes 8-14* contain the same sequence of *N. indica* isolates as in *lanes 1-7* only digested with *Pst*I

The repeat clone pNiR16 produced approximately 14–21 bands in *Eco*RI-digested DNA of *N. indica* isolates, whereas there were approximately 8–18 bands in the *Pst*I-digested, all in the range of 2.0–9.4 kb (Fig. 3). It did not give any scorable pattern with *Bam*HI-digested DNAs of *N. indica* isolates. As is evident from Fig. 3, *N. indica* isolates Ni6 and Ni2 (lanes 6, 7, respectively) had many bands in common in the case of *Eco*RI, whereas Ni1 and Ni7 showed a similar pattern with *Eco*RI (lanes 3, 4) and *Pst*I (lanes 10, 11). It is very interesting that Ni1 and Ni7 could be distinguished in only two hybridization profiles such as pNiR9/*Pst*I and pNiR12/*Pst*I. These results suggest that both probe and the enzyme combination are important to obtain polymorphism.

Cluster analysis of hybridization data

The dendrogram constructed on the basis of the hybridization profiles obtained by repeat elements provided an indication of the genetic variation present among *N. in-*

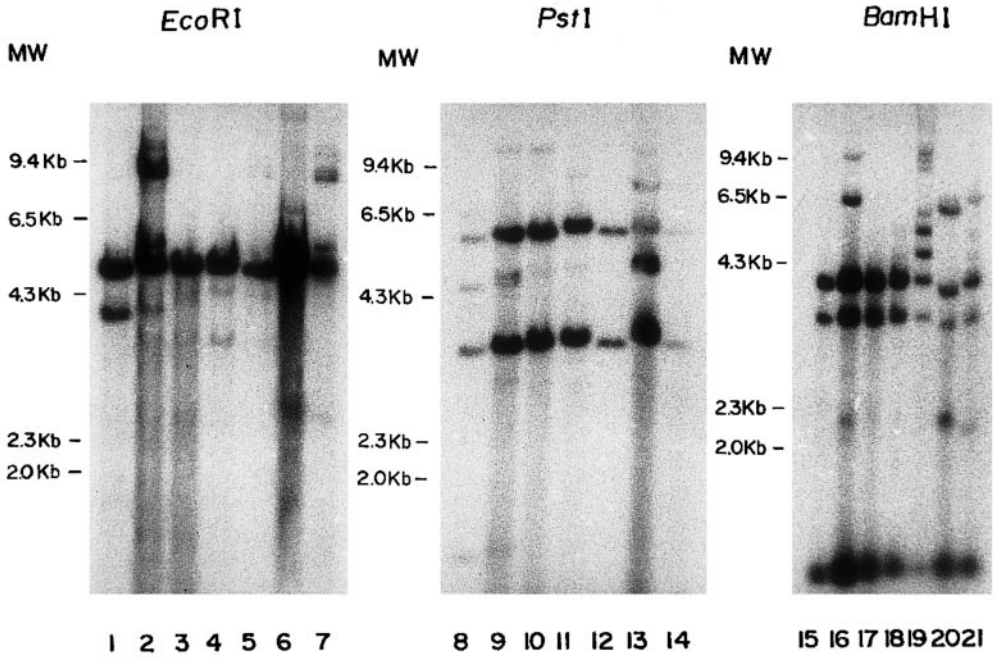

Fig. 2 Southern hybridization profiles of pNiR12 with genomic digests of *N. indica* isolates. *Lanes 1-7* contain isolates (Ni4 (*1*), Ni5 (*2*), Ni1 (*3*), Ni7 (*4*), Ni8 (*5*), Ni6 (*6*) and Ni2 (*7*) digested with *Eco*RI; *lanes 8-14* contain the same sequence of *N. indica* isolates as in *lanes 1-7* only digested with *Pst*I; and *lanes 15-21* contain the same sequence of *N. indica* isolates as in *lanes 1-7*, only digested with *Bam*HI

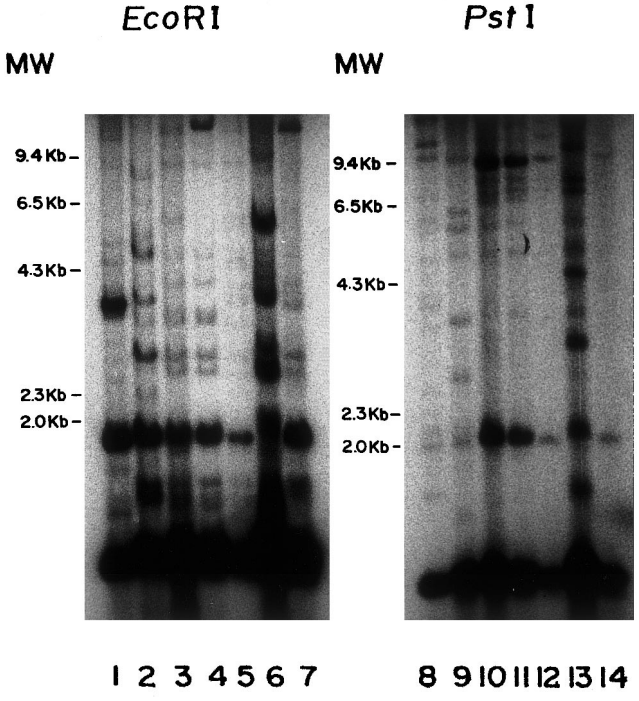

Fig. 3 Southern hybridization profiles of pNiR16 with genomic digests of *N. indica* isolates. *Lanes 1-7* contain isolates Ni4 (*1*), Ni5 (*2*), Ni1 (*3*), Ni7 (*4*), Ni8 (*5*), Ni6 (*6*) and Ni2 (*7*) digested with *Eco*RI and *lanes 8-14* contain the same sequence of *N. indica* isolates as in *lanes 1-7* only digested with *Pst*I

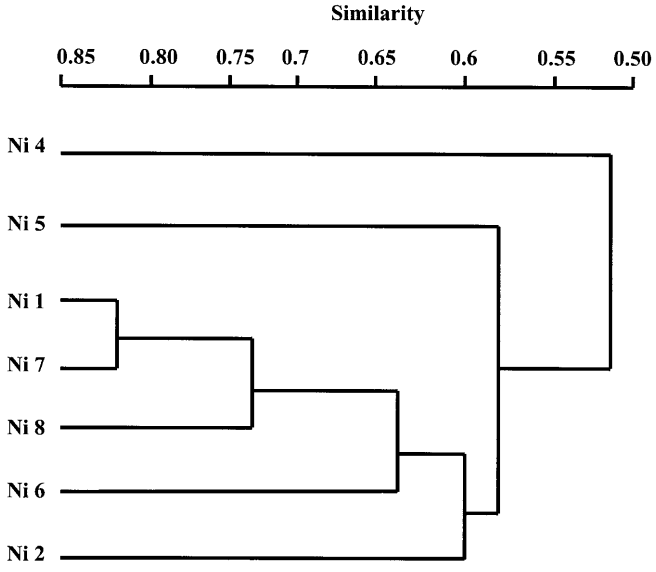

Fig. 4 Dendrogram indicating clustering of *N. indica* isolates based on hybridization patterns generated using repeat elements pNiR9, pNiR12 and pNiR16

dica isolates. As is evident from Fig. 4, at a cut off value of 0.73 similarity, out of seven *N. indica* isolates, three isolates Ni1, Ni7 and Ni8 are grouped together. The boot strap values for this cluster are as high as 99.3 and 93.1, respectively, thus suggesting that the clustering of these three isolates obtained using the molecular markers of present study, is robust. Isolates Ni6 and Ni2 further group with these three but at lower boot strap values, indicating that these two can change their positions in the cluster. The remaining two isolates, Ni4 and Ni5, are outgrouped.

Characterization of repeat elements

As an initial attempt to characterize the repeat elements identified in the present investigation, we made a restriction endonuclease map and estimated the copy number for each clone. Repetitive element pNiR9 showed one

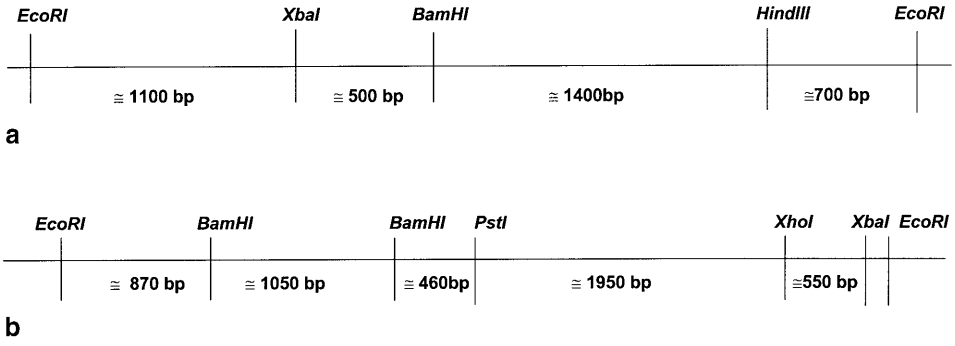

Fig. 5a, b Restriction endonuclease map of pNiR9 (**a**) and pNiR12 (**b**)

internal site each for restriction endonucleases *Bam*HI, *Hin*dIII and *Xba*I (Fig. 5a), while the restriction endonuclease map of pNiR12 showed two internal sites for *Bam*HI and one site each for *Pst*I, *Xho*I and *Xba*I (Fig. 5b). The restriction endonuclease map of repetitive element pNiR16 did not show any internal sites for the endonucleases used in the present study. Approximately 32, 61 and 64 copies of pNiR9, pNiR12 and pNiR16, respectively, were estimated to be present in the genome.

Discussion

N. indica survives through sexual reproduction as it is a heterothallic fungus. There is no well-developed race concept based on the virulence patterns of different *N. indica* isolates on different host genotypes and genetics of the pathogen. However, Aujla et al. (1987) reported the existence of races among 21 *N. indica* field collections from different geographic locations of Punjab and Himachal Pradesh. Recent studies conducted by Singh et al. (1995) and Bonde et al. (1996) once again did not indicate any physiological specialization in this pathogen. Isozyme analysis has been useful in differentiating at least Indian and Mexican isolates of *N. indica* (Bonde et al. 1985). While this approach has also been useful in analysing interspecific and intraspecific genetic variations to some extent, it can detect only significant differences in enzyme structure compared to DNA-based markers that can detect even a minute change in the genome.

Pathogenicity analysis of *N. indica* isolates

Biological analysis of the *N. indica* isolates in present study was done by field inoculations to check the level of aggressiveness of the isolates (Datta et al. 1997). A considerable differential reaction was observed between host lines and fungal isolates indicating genetic variation among *N. indica* isolates. The order of the virulence pattern of *N. indica* isolates obtained on the basis of number of susceptible reactions on the host lines for each isolate was as follows – Ni4>Ni5>Ni1>Ni7>Ni8>Ni6>Ni2. An individual isolate showed a differential reaction against two host cultivars and, similarly, an individual host cultivar gave different reactions against two *N. indica* isolates. Thus, Datta et al. (1997) suggested that different KB resistance genes probably exist in the these cultivars and that there is genetic variability for pathogenicity in the isolates. However, they also suggested that though the virulence typing explains the pathogenic behaviour of fungal isolates, it is a rough estimate. Further, it is influenced by environmental conditions which make the reproducibility of the results difficult in successive seasons even under controlled conditions. Secondly, it is very tedious, laborious and time-consuming and is unable to differentiate isolates showing same pathogenic reaction. Moreover, it is not convenient and practical for analyzing a larger set of fungal isolates and host lines. As there is a quarantine regulation on the transport of pathogenic fungi, the results obtained with a particular set of fungal isolates/host cultivars can not be exploited by researchers in other parts of the world (Weising et al. 1991). Thus, there is a need to exploit phenotypic markers, biochemical markers and molecular markers to overcome the limitations of pathogenicity analysis.

DNA markers provide an alternative approach for pathogenicity analysis of *N. indica* isolates

Our analysis has indicated that there is a low percentage of repetitive DNA (1%) in the *N. indica* genome. This is in agreement with findings in some other fungal genomes where repetitive sequences are present in a low to moderate number of copies (Hammer et al. 1989; Valent and Chumley 1991). In the present study, repeat elements were isolated from *N. indica* that gave polymorphic hybridization patterns among *N. indica* isolates. These repeat elements can be further used to detect more diverse *N. indica* isolates and to tap new sources of resistance in host germplasm.

As shown in our previous studies, *N. indica* isolates Ni4 and Ni5 show a highly virulent reaction and are diverse from other isolates on the basis of their virulence pattern (Datta et al. 1997). These two isolates are outgrouped on the basis of our molecular markers, also suggesting that they are genetically diverse. Isolates Ni2 and Ni6 show a low virulent reaction and are very loosely placed in the cluster in the present study. On the contrary, *N. indica* isolates Ni1, Ni7 and Ni8 show a similar pathogenic reaction, and on the basis of our molecular data

they are grouped together, suggesting that they are genetically similar. Thus, the dendrogram clusters the isolates in such a manner that it also reflects on the pathogenic relationships. As it is assumed that isolates showing a similar phenotype are genetically related (Canten 1987), it is very interesting to note that our molecular data support this assumption. It may be possible that the DNA polymorphisms identified in the present investigation are limited to virulent/avirulent loci in the genome, thereby classifying them into respective clusters in the dendrogram. At present, collection of *N. indica* isolates is mainly confined to the northern region of Indian subcontinent, which is the main wheat growing zone of the country, however studies can be extended to other areas also.

The preliminary characterization of repeat sequences will greatly help in designing sequence-tagged-site markers for molecular analysis as it is well-known that maintaining *N. indica* isolates is very difficult and that DNA extractions from teliospores will be sufficient only for PCR reactions (Gang and Weber 1995). In conclusion, the identification of repetitive elements will provide a new insight in analysing the population structure of *N. indica* isolates that will be useful in future wheat breeding programmes.

Acknowledgements We are thankful to Dr. P.P.S. Pannu for providing *N. indica* isolates. R. Datta is grateful to Council of Scientific and Industrial Research, New Delhi, for the award of a Senior Research Fellowship.

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