BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF INDIAN ISOLATES OF *NEOVOSSIA INDICA*

A THESIS SUBMITTED TO THE
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IN BIOTECHNOLOGY.

BY

RENU DATTA

PLANT MOLECULAR BIOLOGY UNIT,
DIVISION OF BIOCHEMICAL SCIENCES,
NATIONAL CHEMICAL LABORATORY,
PUNE 411 008 (MAHARASHTRA) INDIA.

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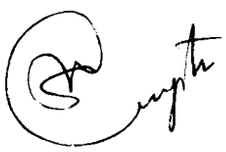
Renu Datta

Renu Datta

DECLARATION

Certified that the work incorporated in the thesis entitled **"BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF INDIAN ISOLATES OF *NEOVOSSIA INDICA*"**, submitted by Miss **RENU DATTA** was carried out under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.



(Dr. Mrs. Vidya S. Gupta)

Research Guide

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Punjab Agricultural University

Ludhiana, Punjab, India

Scientist involved from each institute

Dr. V.S.Gupta

N.C.L

Dr. P.K.Ranjekar

N.C.L

Dr. H.S.Dhaliwal

P.A.U

Dr. Harjit Singh

P.A.U.

LIST OF ABBREVIATIONS

bp : base pairs

kbp :kilo base pairs

mbp : mega base pairs

gm : grams

μg : micrograms

ng : nanograms

ml : millilitre

μl : microlitre

U : units of enzyme

rpm : revolutions per minute

dCTP : deoxycytidine 5' triphosphate

dGTP : deoxyguanosine 5' triphosphate

dATP : deoxyadenosine 5' triphosphate

dTTP : deoxythymidine 5' triphosphate

Tris : tris- hydroxymethyl amino methane

SDS : sodium dodecyl sulfate

EDTA : ethylene diamine tetra acetic acid

TAE : Tris- acetate EDTA buffer

CTAB : Hexadecyl tri-methyl ammonium bromide

Tm : melting temperature

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Introduction : Karnal bunt of wheat

Wheat ranks high amongst the major cereal crops as it provides food for 100 million people in 43 countries around the world. Agronomic practices have played major role in maximizing the yield potential of dwarf and semi dwarf wheat varieties. Application of fertilizers (N, P and K) and micro-nutrients on the basis of soil testing and weed management has created tremendous impact on wheat yield. However, wheat is under constant attack of many fungal and bacterial pathogens who bring down the quantity as well as quality of wheat grain.

Karnal bunt (KB) of wheat caused by basidiomycetous fungus *Neovossia indica* (= *Tilletia indica*) is one of the major diseases of wheat in view of global concern. It was first reported at Botanical Research Station, Karnal, Haryana State in Northwest India by Mitra (1931). It appeared as a serious disease in late 1960s due to its sporadic occurrence in all the regions of north India and now is of common occurrence in India. Factors such as large scale cultivation of high yielding, uniformly flowering, dwarf *aestivum* wheat varieties such as WL711, HD2009, HD2329, PBW154 etc., excessive use of nitrogenous fertilizers and free movement of commercial wheat grain across the country have been responsible for spread of the pathogen (Gill *et al.*, 1993).

Neovossia indica (*N. indica*) causes more quality losses than quantitative losses in contrast to other pathogens who mainly affect the yield of plant. In context of infected wheat grain quality, Mehdi *et al.* (1973) have reported that the wheat flour becomes unacceptable for human consumption with just more than 1% KB infection. Apart from India, Karnal bunt has also been reported from other parts of world such as Pakistan (Munjal, 1975), Iraq (CMI, 1989), Nepal (Singh *et al.*, 1989), Mexico (Duran, 1972) and

recently from USA (Ykema *et al.*, 1996). This disease is of global importance, due to the quarantine restrictions imposed on export of commercial wheat grain across the countries having KB infection, leading to substantial economic losses and reduction in foreign exchange.

1) Taxonomy and life cycle

There has been a lot of controversy regarding taxonomic position of Karnal bunt pathogen due to similarity in morphological characters with genera *Tilletia* and *Neovossia* (Gill *et al.*, 1993). Although Commonwealth Mycological Institute has approved its placement under genus *Tilletia,* yet it is known by both the names. *Neovossia indica* is classified as below :

Kingdom : Fungi

Division : Eumycota

Sub-division : Basidiomycotina

Class : Teliomycetes

Order : Ustilaginales

Family : Teliaceae

Genus : *Tilletia / Neovossia*

Species : *indica*

N. indica survives through dormant teliospores which remain viable up to 5 years, although their germination efficiency decreases due to long storage. Cytological teliospores are diploid (2N), thick walled, ellipsoidal in shape with an average of 35μm in diameter and olive brown to dark brown in color (Gill *et al.*, 1993). Teliospores obtained just after harvesting are in dormant phase, however, this dormancy can be broken down

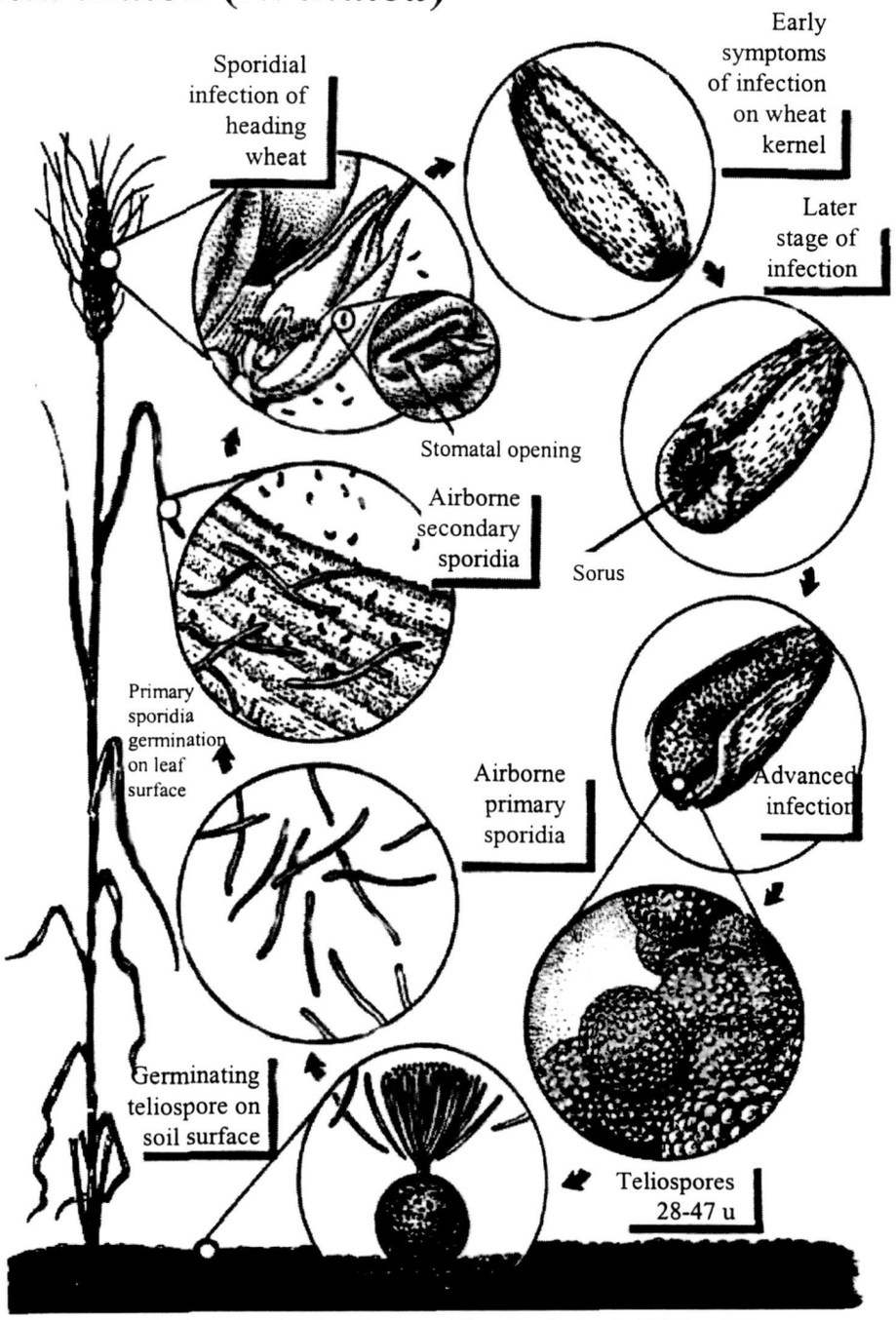
by exposing teliospores to 80^0C for 15 minutes. They show maximum activity after one year and germination capacity reduces with increase in the period. Under natural environmental conditions, teliospores survive on soil surface, wheat straw and in air. They germinate on the onset of favorable conditions and produce primary sporidia which are haploid (N), large in size and filliform in shape. These filliform sporidia do not infect the host plant, but multiply to produce either mycelium or secondary sporidia which are sickle shaped. They are known as allantoid sporidia and act as actual source of infection to the host. They travel up to flag leaf of plant by air currents and splashes of water where they multiply and pass through boot leaf to infect the ear head (Dhaliwal *et al.*, 1983). The pathogen is heterothallic in nature i.e. it requires two opposite mating types for producing disease symptoms. Although it grows on many plants but it produces disease only on wheat after dikaryotization. Sporidia multiply to form mycelium which spreads to wheat kernel. Teliospores are produced in huge number during maturation of the kernels. They drop down to soil surface during harvesting and threshing of grains and carry forward the disease to the next season. Figure 1 describes the life cycle of *N. indica.*

2) Symptoms, survival and dissemination of the pathogen

Neovossia indica is not detectable under field conditions until grain hardening stage. It appears in the form of sori filled with approximately 10,000 black, dormant spores called teliospores on germinal end of the grain. It is also known as partial bunt on the basis of its characteristic symptoms (Cashion and Luttrell, 1988). A fishy odour is emitted from KB infected grains due to presence of trimethylamine. The whole ear head is never infected as in case of smuts where the ear head converts into spores (Fig. 2).

Karnal Bunt Life Cycle

Tilletia indica (*N. indica*)



Courtesy : Bonde & Smilanick In : Bunts and Smuts of wheat, 1997, pp141

Figure 1 : Life cycle of *Neovossia indica*

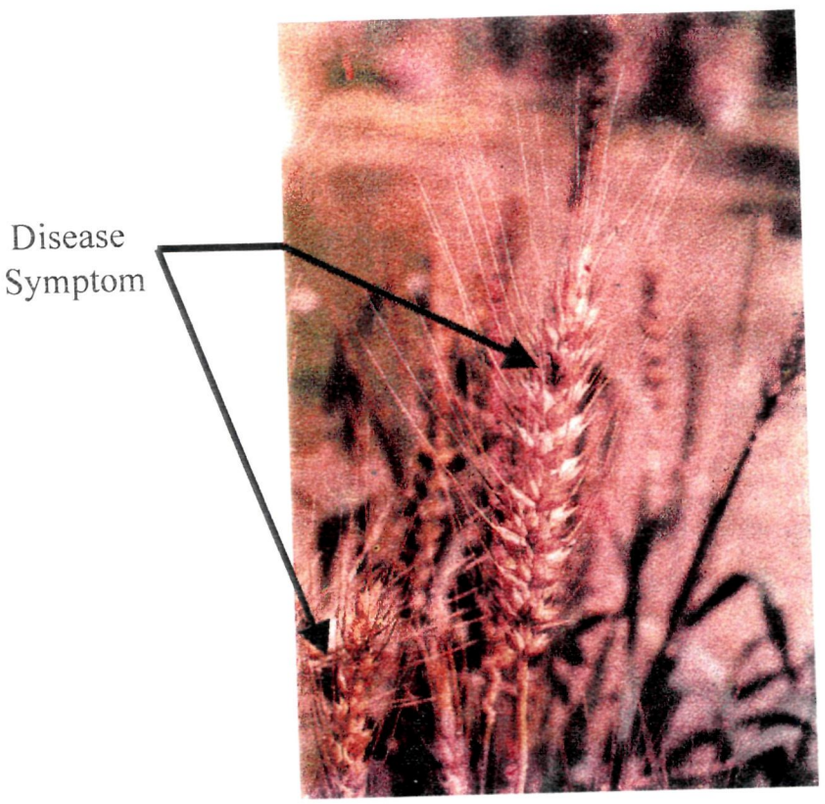


Figure 2 : Field photograph showing symptoms of Karnal bunt.

Teliospores survive on and under the soil surface for 3-7 years, however, aging affects viability of the spores. In general, maximum germination is recorded in one year old teliospores. Teliospores are microscopic and very light and are carried away easily with air currents, farm machinery used during agricultural operations and during transport of wheat seed (Warham, 1986). Life cycle of *N. indica* (Fig. 1) is almost similar to other smut fungi although symptoms vary in appearance. *Tilletia controversa* causing dwarf bunt of wheat infects young ovaries and converts kernel into sorus of teliospores and sterile cells (Goates, 1997) whereas in Karnal bunt only a few grains are affected.

3) Contribution of meteorological factors in disease outbreaks

Environmental parameters play a key role in deciding the disease epiphytotics, in addition to teliospore load in the soil and cultivation of susceptible wheat varieties (Gill *et al.*, 1993). Among the meteorological factors, temperature and humidity are the main parameters responsible for development and spread of the disease. Humidity plays a critical role for germination of teliospores where a minimum of 82% humidity is required for optimum germination of spores. Dhaliwal (1989) has observed a rapid decline in the germination of sporidia at 76% humidity. Factors including high relative humidity, low temperature, continuous rainfall and foggy and cloudy weather, for 13 days or more from the period of ear emergence to anthesis stage, have favoured the disease development in different years resulting in epiphytotics at various places (Munjal, 1971; Aujla *et al.*, 1977, 1986; Singh and Prasad, 1978; Singh *et al.*, 1983; Dhiman *et al.*, 1984; Nagarajan, 1991). Uniform distribution of rainfall from boot stage to ear emergence stage increases probability of the disease outbreaks. Spread of Karnal bunt is affected by changes in climatic conditions and hence there is a wide fluctuation in disease severity every year.

Data from Indian survey reports the occurrence of disease epiphytotic during the years 1976, 1978, 1979, 1981-83, 1986, 1987, 1990 and 1991 (Gill *et al.*, 1993; Singh *et al.*, 1997). However, Joshi *et al.* (1980) have reported that although significant losses are observed in a few wheat fields where pathogen is surviving continuously for the last few years, quantitative losses are only 0.2-0.5% of total production. Cultivation of *Triticum aestivum* cultivars WL711 and Arjun which increased the disease incidence have been replaced by tolerant varieties such as HD2329, WL1562, HD2285 etc (Singh *et al.*, 1997).

4) Disease control

Integrated management programmes to combat Karnal bunt include mulching, chemical treatment, biological control and breeding for disease resistant varieties.

a) Mulching

It involves proper irrigation of soil followed by covering with transparent polythene sheets, resulting in maintenance of higher temperature in the mulched soil. Teliospores loose their viability during summers because of soil mulching at different depths of soil surface (Gill *et al.*, 1993). Inter cropping of wheat with chickpea has been useful to reduce Karnal bunt incidence and a significant reduction (63-75%) in KB incidence has been reported using polythene sheet in the inter row space of wheat during tillering stage (Singh *et al.*, 1992). However, this approach is not very practical due to cost of polythene sheet as limiting factor over large areas of cultivation.

b) Chemical treatments

All the fungicides used against KB infected seeds have been found to be fungistatic in action. A few mercurials are known to show lethal activity against teliospores. Most

effective fungicide found so far is Propiconazole which shows no residue on wheat seeds and straw during harvesting (Aujla *et al.*, 1989). In general, fungicides can restrict the activity of teliospores but are unable to eliminate their activity totally. Further they have the limitation of not being cost effective in case of cultivation over large areas.

c) Bio-control agents

Certain fungal and bacterial species have been tested for their antagonistic potential against growth of *N. indica*. It has been found that a few species of *Trichoderma*, *Gliocladium* and fluorescent *pseudomonads* and bacterial cultures suppress the germination of *N. indica* teliospores. Amer (1995) has reported that when *Trichoderma lignorum* is used as soil inoculant under field and glass house conditions, there is a significant decline in the teliospore germination and an increase in growth of the host plants. Later, in a more detailed study by Singh *et al.* (1997), a few compounds were isolated from the antagonistic organisms and were purified by thin layer chromatography. Bioassay of these purified compounds against *N. indica* mycelium concluded that *T. lignorum* showed maximum antagonistic activity against growth of *N. indica* thus supporting the previous findings by Amer (1995). Karnal bunt incidence can be reduced using above mentioned approaches but a requirement of disease free seed in the commercial wheat grain market yet maintains the challenge (Singh, 1997). Ultimate remedy to this problem thus will be the development of disease resistant varieties.

d) Genetic resistance

Genetic studies involving resistance to Karnal bunt have indicated presence of at least 8 resistant genes, which are dominant over susceptible genes, and they show additive effect. A few resistant lines from India, China and Brazil along with a synthetic hexaploid

and a commercial *durum* cultivar Altar 84 show promising resistance in India and Mexico against Karnal bunt (Rajaram and Fuentes-Davila, 1997). In other studies done in India by Bag (1995) and Singh *et al.* (1996), Indian hexaploid lines HD29, HP1531 and W485 have been found to be effective resistant sources against Karnal bunt. Nelson *et al.* (1998) have reported the tagging of Karnal bunt partial-resistance genes on chromosome arms 3BS and 5AL in the recombinant inbred lines from a cross between a resistant synthetic wheat (*Triticum turgidum* 'Altar 84' x *T. tauschii*) and the susceptible common wheat cultivar 'Opata 85'. Knowledge about variability in the fungus regarding the existence of pathogenicity, however, is a pre-requisite to evaluate the host germplasm for resistance against the pathogen and for designing future wheat breeding strategies.

5) Genetic analysis of pathogen

Information about the amount and distribution of genetic variation in the pathogen is an essential step for designing management strategies. Mitra (1935) identified two races of *N. indica* using teliospore size as a morphological marker. However, later studies by Bansal *et al.* (1984) and Peterson *et al.* (1984) concluded that these markers were not stable as they were dependent on climatic conditions and hence were not suitable for variability analysis. Compared to morphological criteria, biochemical and virulence analyses of variability are more stable approaches to study genetic variation in the pathogen (Mathre, 1995).

a) Virulence typing

Pathogenic analysis of various plant pathogens has been performed using a set of differential cultivars to group isolates/strains in their defined races based on the presence and expression of different virulence genes in different isolates. Well-defined races have

been found in wheat bunt fungi *T. carries* and *T. controversa* on the basis of virulence genes present in the pathogen (Hoffmann and Mertzger, 1976). Ou (1980) has detected a high degree of instability within the pathogenic races of *Magnaporthe grisea* but fast evolution of the pathogen has been considered responsible for this pathogenic instability within a race (Leung *et al.*, 1988). Existence of the race concept in *N. indica* is controversial. Aujla *et al.* (1987) have reported presence of 4 races in India on the basis of differential pathogenic reactions of *N. indica* isolates. However, this possibility has been ruled out in recent studies by Singh *et al.* (1995) and Bonde *et al.* (1996) who consider that *N. indica* is a sexually reproducing dikaryotic fungus where sexual phase is responsible for causing infection. Virulence genes are assorted each year due to sexual recombinations resulting in a population with constant variation.

Attempts have been made to study the genetic basis of host-pathogen interaction in various systems as described by Flor (1942). Such studies have been reported from other fungi such as rice blast pathogen *M. grisea, Cladosporium fulvum* causing leaf curl in tomato and *Bremia lactucae* causing downy mildew disease in lettuce (De wit, 1992). A few attempts have been made in the past years to analyze variable patterns among *N. indica* isolates on the basis of their pathogenic reactions on host cultivars. A comparison of Indian and Mexican isolates of *N. indica* has been carried out using inoculation method but no significant differences have been found in their virulence reactions on five spring wheat cultivars (Royer and Rytter, 1985). In another comprehensive study, Singh *et al.* (1995) have analyzed the pathogenic behaviour of eight Indian isolates of *N. indica* on twenty two host differentials and have concluded that percent disease incidence needs to be taken as a criteria for selecting early generation material resistant against Karnal bunt. In another study, aggressiveness of *N. indica* isolates from India, Pakistan and

Mexico has been compared on eight wheat lines (Bonde *et al.*, 1996). Indian isolates have been found to be moderately virulent and Mexican isolates to be the most virulent isolates. In these studies, a significant correlation ($P< 0.5$) has been observed between percent disease incidence and extent of infection in the seeds.

b) Biochemical markers

Biochemical markers have been used in the past years to differentiate closely related species of *Tilletia*. Phenol soluble polypeptide extracts from teliospore walls of wheat bunt fungi such as *T. tritici*, *T. laevis* and *T. contoversa* have been compared to differentiate these fungi (Kawchuck *et al.*, 1988). It has been concluded that though races and collections of these bunt fungi have morphological and physiological features of their respective species and possess different combinations of virulence genes, they all give similar polypeptide patterns. Out of 415 polypeptides used for this analysis, 359 are present in all the races and collections and rest 56 polypeptides are present only in few races and collections. No species specific and race specific polypeptides have been detected leading to a conclusion that these fungi should be considered as varieties of one species.

Isozyme markers have been exploited to study genetic diversity in various fungal pathogen systems. High level of variation exists in sexual populations of *Puccinia graminis* f. sp. *tritici* (Burdon and Roelf, 1985) and asexual populations of *Rychosporium secalis* (Mc Donald *et al.*, 1989). Isozymes have also been used for studying genetic variation among different species of *Tilletia* (Bonde *et al.*, 1988, 1989). It has been shown that out of 36 isozyme loci, fifteen are polymorphic having allelic variation in *Tilletia* isolates. Average similarity coefficient is high (0.83) among *T. indica* isolates in comparison to that of *T. barclayana*. The similarity coefficient between *T. indica*

and *T. barclayana* is extremely low (0.04) suggesting large differences in their isozyme profiles leading to a large genetic variation in *T. indica* and *T. barclayana*.

6) DNA based markers : a useful tool for analysis of pathogen population

Apart from morphological, pathogenic and biochemical analysis, knowledge about the genetic make up of the pathogen is an essential requirement to prevent its epidemic spread. DNA markers which are easily accessible, abundant and highly polymorphic have brought out a revolution in the field of basic studies on fungi involving various aspects such as epidemiology, systematics and population biology and provide a refined and informative differentiation of the pathogen isolates. Further they are more stable and are unaffected by environmental changes and, therefore, are more effective to act as a diagnostic tool for identification of pathogen. They do not have deleterious or strong epistatic effects thus allowing multiplexing of markers. Co-dominant DNA markers particularly allow identification of all possible genotypes having numerous alleles for some markers (Michelmore and Hulbert, 1987). Virulence reactions of the fungal isolates obtained by biological typing are not consistent for subsequent seasons due to environmental constraints. These results can not be reproduced and compared in different geographic locations due to restrictions on transport of pathogenic fungi (Weising *et al.*, 1991). However, information gathered by using DNA markers is stable and can be exploited anywhere as DNA is unaffected by changes in climatic conditions (Weising *et al.*, 1991). Moreover, DNA markers scan many loci simultaneously in the genome in comparison to biological typing leading to a refined classification of pathogen isolates (Rajebhosale, 1998). A wide variety of DNA markers have been used for various studies

in fungi. A brief description of the important molecular markers and their usage in fungal analysis is given below.

a) Hybridization based markers

RFLP markers follow Mendelian inheritance and have their origin in DNA rearrangements occurring through evolutionary processes such as point mutations within restriction enzyme recognition site sequences, insertions or deletions within the fragments and unequal crossing over (Schlotterer and Tautz, 1992; Joshi *et al.*, 1999). The first genetic map of human genome has been constructed using RFLP markers (Botstein *et al.*, 1980). RFLP markers in nuclear and mitochondrial genomes are routinely used for studying genetic structure of most of plant-pathogenic fungi. They include repetitive DNA probes also which can analyze different loci in the genome simultaneously (Weising *et al.*, 1995). However, hybridization based markers are more suitable for fungi that can be grown on artificial medium since more amount of DNA is required for practical application of RFLP markers.

i) Mitochondrial DNA markers

Fungal mitochondrial (mt) DNA has been widely used as a source of molecular markers for studying genetic variability, taxonomy and evolutionary aspects (Michelmore and Hulbert, 1987). It is advantageous to use mtDNA because it is easy to purify, small in size and exists in relatively high copy number. It is inherited independent of the nuclear genome and exhibits more conserved sequences with little possibility of recombinations. Closely related individuals of a species have been differentiated using mtDNA as probe (Sock *et al.*, 1994). A mitochodrial DNA fragment of 2.3 kb has been found to distinguish *T. indica* from other *Tilletia* species such as *T. barclayana, T. foetida, T. carries, T. controversa* and *T. fusca* (Ferreira *et al.*, 1996). Primers designed from this

partially sequenced fragment have amplified the same sized fragment in 17 isolates of *T. indica* whereas this amplicon is absent in 25 isolates of other bunt fungi. In a study by Smith *et al.* (1996), another pair of primers is developed from mtDNA of *T. indica* to design a PCR-based method for identification of *T. indica*. Specificity of these primers is checked using 78 isolates of *T. indica* and 79 isolates of other species of *Tilletia* including 69 isolates of *T. barclayana*, 2 of *T. tritici*, 1 of *T. laevis*, 4 of *T. controversa* and 3 of *T. fusca*. Specificity of amplification in isolates of *T. indica* has been further confirmed by southern hybridization of mtDNA clones as probes. Thus the primers obtained from mtDNA have the potential for pathogen identification. Similar studies have been carried out in other fungi also to differentiate closely related individuals of a species as well as to classify different species of a genus. Eighty one isolates of *Trichoderma harzianum* (colonizing mushroom compost) have been clustered in three major groups by RFLP analysis of mtDNA (Muthumeenakshi *et al.*, 1994). Group 2 isolates, which are aggressive colonizers of mushroom compost, are clearly distinguished from isolates of other two groups. In a recent study by Torriani *et al.* (1999), restriction analysis of mtDNA has distinguished *Saccharomyces cerevisiae* and *S. bayanus* and revealing polymorphism among *S. cerevisiae* isolates.

ii) Repetitive elements as DNA markers

Repetitive elements from genomic library have been most widely used to study genetic variation in fungi. Moderately repetitive DNA sequences have been particularly used, as they are dispersed throughout the genome and many loci can be assessed using one probe. A family of dispersed repetitive elements has been shown to generate complex and stable fingerprint patterns in *M. grisea* isolates (Hammer *et al.*, 1989). This family called as MGR sequences is highly conserved among *M. grisea* isolates pathogenic to rice

(Hammer *et al.*, 1989). Specific genomic DNA probes have been isolated for pathotype identification in *Entomophaga grylli* which show host specific variance in grasshopper subfamilies (Bidochka *et al.*, 1995).

iii) Double stranded (ds) RNAs

Double stranded RNAs occur widely in fungi including plant pathogens. They usually appear as encapsidated viral genomes or rarely as unencapsidated molecules (Zhang *et al.*, 1994). Double-stranded RNA of approximate size of 3000-4500 bp has been reported to be present in *N. indica* genome, suggesting that this pathogen is a host for mycoviruses (Beck *et al.*, 1990). However, so far these dsRNAs have not been used as marker for genetic variability studies in *N. indica*. They have been extensively used to study intraspecific variation among various *Puccinia* spp. where the patterns obtained have been correlated to pathogenicity in *P. striiformis* (Newton *et al.*, 1985). However, in similar studies no such correlation has been found between polymorphisms of dsRNAs and pathogenicity in *M. grisea* where various races of *M. grisea* are used for analysis (Hunst *et al.*, 1986). Double-stranded RNAs are not considered to be suitable markers for diversity analysis due to the limitation that they may not be faithfully transferred during asexual subculture (Anagnostakis and Day, 1979; Hunst *et al.*, 1986).

b) PCR based markers

Due to its efficiency, sensitivity and simplicity, polymerase chain reaction has revolutionized the field of molecular biology (Saiki *et al.*, 1985). Moreover, it requires very small amount of DNA which is more suitable in case of obligate pathogens such as those for rusts and mildews. PCR is preferred over other techniques due to many such advantages. Introduction of PCR-based markers such as Random amplified polymorphic DNA (RAPD) and microsatellites has accelerated studies on genome analysis as well as

identification of plant pathogens (Weising *et al.*, 1995). Among the available PCR based markers, following markers have been widely used in fungi.

i) Random Amplified Polymorphic DNA markers (RAPD)

They are commonly called as RAPD markers (William *et al.*, 1990) and are most popularly used for diversity analysis in fungi. RAPDs involve use of GC-rich primers of arbitrary sequence (10 mers) to generate ampilfication products with genomic DNA. Distinct, variable, reproducible and polymorphic profiles have been obtained for all the fungal species tested so far (Bulat and Mironenko, 1996; Weising *et al.*, 1995). RAPD analysis has been done to differentiate Italian isolates of *Ascochyta rabiei* (Fischer *et al.*, 1995). RAPDs have been used as diagnostic tools to differentiate pathogenic isolates of *Fusarium solani* from nonpathogenic isolates (Achenbach *et al.*, 1996). In another study, RAPD analysis has classified different populations of an ascomycete *Hypoxylon truncatum* into two distinct groups corresponding to different stomatal types and suggesting that these two forms of stomata represent two distinct species (Yoon and Glawe, 1993). *Phytophthora cinnamomi* causing diseases in cultivated fruit trees and plantations of two Cinnamomum species have been differentiated into mating types using RAPDS (Chang *et al.*, 1996). It has been observed that RAPD primers OPS-13 and OPE-06 amplify fragments of sizes 2.7 and 1.3 kb, respectively, in all the isolates of A1 mating type which are absent in A2 mating type. Strains of *Agaricus bisporus* have been differentiated using RAPD markers to identify two haploid nuclear components of a heterokaryotic strain so as to verify artificial synthesis of heterokaryons and transmission of genetic loci to progeny (Khush *et al.*, 1992). RAPD markers have revealed DNA polymorphism among the races and among single-spore isolates within races of *P. striiformis* from diverse geographic areas of North America (Chen *et al.*, 1993).

Preliminary studies on bunt fungi are based on teliospore morphology which is not helpful in distinguishing closely related species of *Tilletia.* DNA markers are being exploited to classify species of bunt fungi. There are a few reports on the diversity analysis of *T. indica* and its allied species using RAPD markers. Gang and Weber (1996) have analyzed variation among races of wheat bunt fungi such as *T. caries, T. foetida* and *T. controversa* using RAPD markers. A high level of genetic variability has been observed between species, races and even individuals of a race thus distinguishing these bunt fungi, however, no race- or species specific markers have been identified. In another study, Boyd and Carris (1997) have successfully separated 16 isolates of *T. fusca* complex infecting variety *Vulpia* from *Bromi-tectorum* and *guytiana* infecting *Bromus* on the basis of RAPD analysis. Isolates of *T. indica, T. barclayana* and other allied species have been distinguished clearly using RAPD markers (Pimentel *et al.*, 1998).

Although RAPD markers are powerful tools for fingerprinting studies and variability analysis between and within fungal species, they have certain technical limitations. RAPDs are dominant markers and are not suitable for analyzing genetic variation in basidiomycetes and oomycetes fungi which are mainly heterokaryotic, diploid or polyploid. In such cases RAPDs can not differentiate homozygotes and heterozygotes without a progeny test (Lynch and Milligan, 1994).

ii) Simple sequence repeats (SSR)

Simple sequence repeats are tandem repeats with short motifs of 1-10 bp in length, dispersed throughout the genome. They are the most probable sites of mutations resulting in polymorphisms and have been used as probes for genetic variability studies (Weising *et al.*, 1991). Recently, these sequences are being used as primers in PCR to amplify polymorphic loci. Simple sequence repeats, also known as microsatellites, have been

used for rapid identification and differentiation of species of yeast (Lieckfeldt *et al.*, 1993) and strain variation in *Cryptococcus neoformans* (Meyer *et al.*, 1993; Meyer and Mitchell, 1995). In fungal systems, microsatellite sequences have been used for diversity studies among the isolates of fungus *Epichloe typhina* (Groppe *et al.*, 1995). A set of three microsatellite primers has been developed for population studies in filamentous fungi by Bridge *et al.* (1997). The microsatellite sequences have also been used in evolutionarily diverse genomes for variability analysis by Gupta *et al.* (1994) and it is concluded that tetra nucleotide sequences are the most effective primers in generating polymorphic profiles and are very commonly used to analyze various animal and plant systems (Joshi *et al.*, 1999). Reproducibility is one of the main criteria for DNA typing using microsatellites. Use of anchored simple sequence repeats as PCR primers make the primers specific to target sequences thus resulting in higher reproducibility in amplification profiles. These anchored SSRs, known as Inter-simple-sequence-repeat (ISSR), have been used in a variety of eukaryotic systems for genetic variability studies (Zeitkewicks *et al.*, 1994). 5' anchored microsatellites have been used as primers to study genetic variation among and within different fungal species of Phycomycetes, Oomycetes and Basidiomycetes also (Hantula *et al.*, 1996).

7) Applications of molecular markers in relation to pathogen analysis

Previous approaches to control the spread of pathogen include cultural practices, crop rotation, soil solarization, treatment with fungicides at different stages of plant growth and breeding for disease resistance. Use of molecular markers in combination with virulence typing has broadened the scope of the strategies to incorporate resistance genes in plants against the pathogen.

a) Phylogenetic and evolutionary studies

DNA markers provide a significant contribution for classification of pathogen populations at species level when criteria of morphological differences are variable. Internal transcribed spacer (ITS) region of rDNA has been used to study phylogenetic relationships among wheat bunt fungi such as *T. controversa, T. tritici* and *T. laevis* causing different types of bunt diseases of wheat (Shi *et al.*, 1996). RFLP analysis of ITS region of rDNA clustered *T. controversa* in haplotype A and *T. tritici* and *T. laevis* in haplotype B. This combined data suggested the origin of dwarf bunt and common bunt fungi from a common ancestral population that later on differentiated into two sub-lineages. This conclusion is in agreement with previous finding that these bunt species were earlier closely related but later evolved into distinct pathogens with different life cycles and adaptive characteristics (Kawchuk *et al.*, 1988). Similar findings have been reported from other systems also. One such example is of *Sclerospora graminicola* causing downy mildew of pearl millet. It is well known that host and pathogen co-evolve, as host puts directional selection on the pathogen isolates (Mc Donald, 1997). Downy mildew resistance of pearl millet was shown to be broken down in a specific genotype NHB 3 grown at Durgapura (Rajasthan, India) in 1977. However, same genotype regained the resistance against *Sclerospora graminicola* population when it was reintroduced after a gap of 3 years (Singh and Singh, 1987). From this observation, it can be concluded that a pathogen population specific to a genotype can disappear within 3 years. Later on, in a similar study using DNA analysis of the pathogen by Sastry (1998), it has been shown that the pathogen specific to a particular host genotype adapts slowly to new genotype and cannot rapidly revert back to its original host.

b) Molecular mapping and tagging of genes

Genetic maps are now available in various pathogenic and nonpathogenic fungi using DNA markers. For example, Sweigard *et al.* (1992) have developed the molecular linkage map of *M. grisea* using RFLP markers where two avirulence genes and mating type locus have been mapped to chromosomes 1, 2 and 6, respectively. An AFLP linkage map of *Phytophthora infestans*, causing late blight of potato, has been reported where two AFLP markers are linked with mating type locus in linkage group 3 (Lee *et al.*, 1997). In another attempt, microsatellite markers have been used to develop a linkage map of chickpea blight pathogen *Ascochyta rabiei* and to localize virulence and mating type loci (Geistlinger *et al.*, 1997). It is reported that microsatellite sequences $(CAA)_5$, $(GAA)_5$, $(GACA)_4$ and $(GTTTGG)_3$ are abundant in *Ascochyta* genome. Genetic maps have also been reported from nonpathogenic fungi such as *Neurospora crassa* (Mertzenberg and Grotelueschen, 1992) and *Sacchromyces cerevisiae* (yeast) (Mortimer *et al.*, 1992) where mating type loci of *Neurospora* and *Sacchromyces*were have been mapped in their respective linkage maps.

Thus some information is available on *N. indica*-wheat system and other host pathogen systems to design strategies to control the disease using different methods. However, knowledge about the genome and genetics of *Neovossia indica* virulence is very fragmentary which is a prerequisite for incorporation of durable resistance in wheat.

8) Genesis of thesis and its organization

As explained earlier in Introduction, *Neovossia indica,* the causal agent of Karnal bunt of wheat, has posed a major threat to Indian subcontinent due to restrictions imposed on movement of the commercial wheat grain thus bringing economic losses. The most

onomical, ecofriendly and effective approach to control the spread of the pathogen is rough cultivation of disease resistant varieties. However, knowledge of the pathogen)pulation is a vital criterion to assess resistance and design management strategies to corporate useful R genes in the desired background. Realising the importance of ıthogen analysis, a collaborative project between Punjab Agricultural University, ıdhiana and National Chemical Laboratory, Pune was planned to study the pathogen ing biological as well as molecular approaches with the following objectives :

- To assess pathogenic reactions of all the isolates on various host cultivars to study their virulence patterns and their interaction with the host system.
- To identify homologous markers from *N. indica* isolate for assessment of genetic variability.
- To assess the potential of PCR based markers (ISSR) in differentiation of *N. indica* isolates, which can be further useful in identification and differentiation of the isolates.
- To assess the genetic diversity among Indian isolates of *N indica* using virulence typing and molecular markers. It involves use of selected number of isolates from different geographic regions of India, so as to serve as a base for analysis of larger pathogen population.

he thesis is organized into four chapter as follows :

hapter 1 : Introduction

hapter 2 : Materials and Methods

Chapter 2

Materials and Methods

Materials and Methods

1) Fungal material
2) Host material
3) Virulence typing of *N. indica* isolates
4) DNA extraction
5) Construction of genomic library and identification of repeat clones
6) Characterization of repeat clones
7) RFLP analysis
8) PCR amplification and gel electrophoresis
9) Statistical data analysis

Materials and Methods

1) Fungal material

Seven isolates of *Neovossia indica*, in the form of dormant teliospores were collected from infected seed samples, from different geographic locations of North western India. It is a major wheat growing region in the Indian subcontinent with Punjab state as the largest producer of wheat, hence it was represented more in the collection. Figure 3 gives the distribution of *N. indica* isolates used in the present study. All the isolates were multiplied on *Triticum aestivum* cv. WL711, a susceptible cultivar. Teliospores were dusted on sterile water under aseptic conditions and were incubated at 20^0C for 2-3 weeks for germination. A bunch of sickle shaped primary sporidia was visible on the tip of promycelium emerging out of germinating teliospore. Figure 4 shows the germination of teliospore with emerging promycelium. Primary sporidia were transferred to PDA (Potato-Dextrose-Agar) slants and were subcultured subsequently to purify the isolates (Munjal, 1974). Purified isolates were multiplied and maintained on PDA slants at 20^0C and 80% humidity.

2) Host material

Thirteen cultivars of three genotypes of wheat germplasm namely hexaploid aestivum wheat (AABBDD genome), tetraploid durum wheat (AABB genome) and the wheat-rye hybrid triticale (AABBRR genome) with diverse pedigree were used as host cultivars for assessing the pathogenic reactions of the seven isolates under present investigation. Selection of these cultivars was based on their characters related to quality, yield and disease resistance. Table 1 gives the list of cultivars used in the present study.

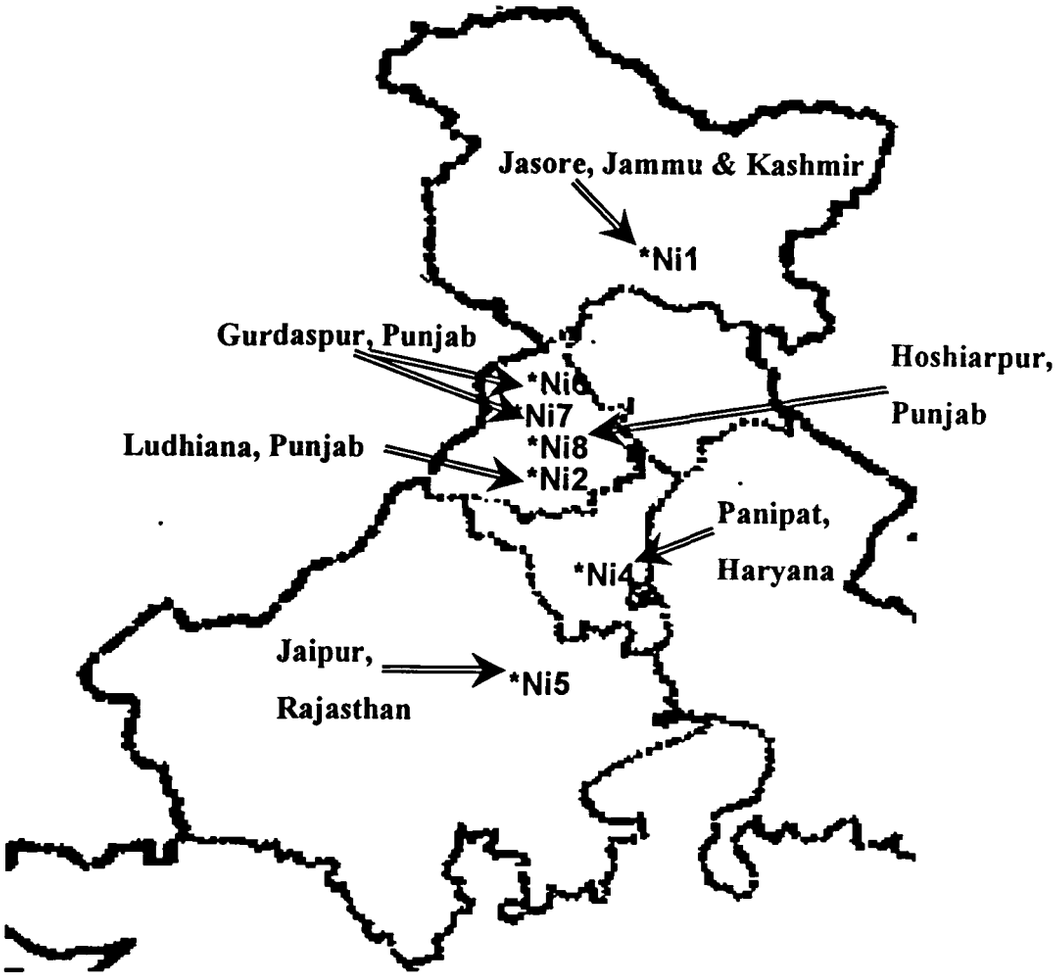


Figure 3 : Distribution of *N. indica* isolates in Northern India (*N. indica* isolates were received as gift from Dr. P.P.S. Pannu, Assistant Plant Pathologist, Department of Plant Pathology, Punjab Agricultural University, Ludhiana-141004, Punjab, India)

Table 1 : Parentage of different host lines of cultivated wheats and triticale used for virulence typing of Karnal bunt causing fungus.

No.	Line	Pedigree
	Triticum durum	
1.	PBW 34	ANHINGA/FLAMINGO
2.	PDW 215	DWL 5031/DWL 5002
	Triticosecale	
3.	TL 419	ARS/KAL
4.	TL 1210	CINNAMON/RAJ 821/3/INIA/2* TURKE/Y60/TARM
	Triticum aestivum	
5.	PBW 343	ND/VG 9144/KAL/BB/3/YACO$^{S'}$ /4/VEERY # 5
6.	WL 711	S 308/CHRIS//KALYANSONA
7.	WL 1562	KALYANSONA/JUSTIN/UP 301
8.	SONALIKA	II 54-388/AN/3/YT54/N1OB/LR 64
9.	PBW 248	HD 2258/HD 2160
10.	PBW 154	HD 2160/HD 2177
11.	HD 29	HD 2160/HD 1977//HD 1949/HD 1944/3/HD 2136
12.	HD2329	HD 1962/E4870//K65/3/HS 1553/UP 262
13.	PBW 138	RAVI 43/HD 2177

3) Virulence typing of *N. indica* isolates

The isolates were multiplied individually on the susceptible hexaploid wheat cultivar WL711 by injecting 1-2 ml of fungal inoculum (10,000 sporidia/ml of water) with hypodermal syringe at awn emergence stage of the plant under field conditions as described by Aujla *et al.* (1982) (Fig. 5). Inoculum of each isolate was revived using PDA medium slants in the same way as mentioned above. Each of the host lines, listed in Table 1, was grown in one meter long paired rows with 10 plants in each row at four different dates at an interval of one week in the month of November. Every host line was inoculated with each of the seven isolates (10,000 sporidia/ml), at each date of sowing, during awn emergence stage following the method of syringe inoculation. On an average 20 heads were inoculated for each host-isolate combination. The inoculated heads were harvested and threshed separately by hand approximately after 80-90 days of inoculation. Symptoms of Karnal bunt were evident during grain hardness as a small black dot at the germinal end of the grain. Infected ear heads were harvested and disease data were scored using grading scale 0-4, depending on the extent of infection on the grain. Figure 6 depicts the grades of infection on grains of WL 711. Disease scoring was analyzed by calculating parameters such as percent disease incidence, coefficient of infection and primary infection sites (PIS). The host lines were considered to be resistant when kernel infection percentage was below 5% (Fuentes-Davila, 1996). Percent disease incidence was calculated as described by Aujla *et al.* (1982) using following formula :

$$\frac{\text{Total no. of infected grains}}{\text{Total no. of grains}} \times 100$$

Figure 4 : Photograph showing teliospore germination

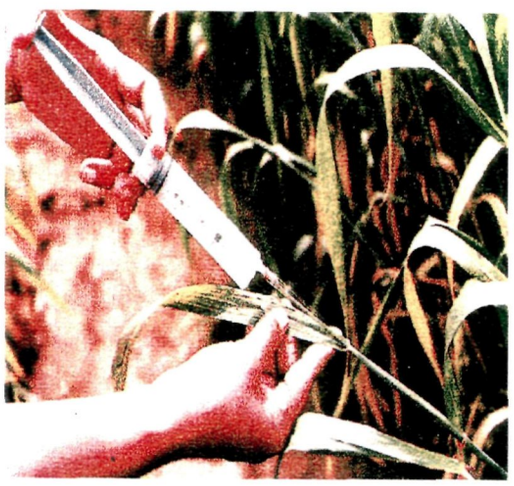


Figure 5 : Field photograph showing inoculation method.

Figure 6 : Photograph showing disease scoring scale.

Courtesy : Gill *et al.*, 1993

Coefficient of infection was calculated using following formula-

$$C.I. = \Sigma^{4}_{i1} \frac{X \times Y}{N} \times 100$$

Where, i - grade of infection (i = 0 to 4), N - total number of grains, X - numerical value of i-th grade of infection and Y - No. of grains of i-th grade of infection (Singh *et al.*, 1995).

Number of primary infection sites was counted by tracing the infection from rachis and rachilla separately in each of the infected ear heads. When the grade of infection decreased from rachis/rachilla in an orderly manner, the primary infection site was considered to be one whereas if the grade of infection scored was fragmented, then those many primary infection sites were considered in rachis/rachilla. All the experiments were repeated for two consecutive years at Punjab Agricultural University fields at Ludhiana.

4) DNA extraction

Mycelia of all the *N. indica* isolates were grown on 100 ml of MGYP (Malt extract 0.3%, glucose 1%, yeast extract 0.3%, peptone 0.5%) broth in 250 ml conical flask at 20^0C for 2-3 weeks by inoculating a bit of isolates maintained on PDA slants (Warham, 1986). Mycelium of each isolate was harvested by decantation of the broth. Mycelium obtained was dried in tissue paper to remove medium contents and ground with liquid nitrogen to powder form. The latter was homogenized by adding extraction buffer (25mM Tris-HCl, pH 8.0 and 10mM EDTA) 4 times the weight of mycelium and was kept without shaking till it reached room temperature (RT). Following procedure of DNA extraction is a modification of a protocol described by Rajebhosale *et al.* (1997). $1/10^{th}$ volume of 10% sodium dodecyl sulphate (SDS) was added to extraction buffer, suspension was mixed gently and kept for incubation at 60^0C for 10 min. Then 5M NaCl

was added to the mixture to make the effective concentration of NaCl as 1.4M and mixed thoroughly. 1/5th volume of 10% hexadecyltrimethyl ammonium bromide (CTAB) was added and the incubation was continued at 60^{0}C for 10 min. Each sample was extracted twice with equal volume of mixture of chloroform-isoamyl alcohol (24:1), centrifuged at 10,000 rpm for 15 min at room temperature. DNA was precipitated with 0.6 volumes of isopropanol from the aqueous layer and dissolved in 200µl of 10mM Tris-HCl, pH 8.0 and 1mM EDTA (TE buffer). RNAase A (2.5mg/ml in 10mM Tris-HCl, pH 7.0) was added to the dissolved DNA to make the final concentration of RNAase A as 25µg/ml and kept for incubation at 37^{0}C for 1 h. Concentration of DNA was estimated by comparing known concentrations of λ DNA on 0.8% agarose gel.

5) Construction of genomic library and identification of repeat clones

N. indica isolate (Ni7) DNA was digested with different concentrations of restriction endonuclease *EcoRI* ranging from 1unit/µg-25 units/µg of DNA by serial dilution method with conditions as per manufacturer's instructions (Promega, USA) and by incubating at 37^{0}C for 16 h (Sambrook *et al.*, 1989). The digestion patterns were checked on agarose gel by loading 10µl (≈ 500 ng) aliquot from reaction mixture. Digested samples were pooled and column purified using QIA-quick gel extraction kit (Quagen, USA) with following steps. Buffer QX1 was added 3 times the weight of gel slice and was incubated at 50^{0}C for 10 min. Isopropanol was added in 1:1 ratio followed by gentle mixing. Then it was loaded on to the column and centrifuged briefly. Column was washed with 0.75ml of PE buffer and was spun twice briefly. Finally, TE buffer was added to the column and DNA was obtained in collection tube by centrifuging at 12,000 rpm for 5 min. All the

further protocols used for library construction were as per the instruction manual from Stratagene for Lambda Zap II System (Stratagene, USA). *EcoRI* digested and purified Ni7 DNA sample was ligated with λ Zap II / *EcoRI* cut / CIAP treated arms. The ligated samples were then packaged using Gigapack II packaging extracts (Stratagene, USA) as per the instruction manual along with a positive control given in the kit for checking packaging efficiency. The packaged ligation product was plated on NZY top agar (NaCl 0.5%, $MgSO_4.7H_2O$ 0.2%, yeast extract 0.5%, NZ amine 1% and agarose 0.7%) containing X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (Isopropylthio-β-D-galactoside) to check number of background plaques (blue in colour) and recombinant plaques (white in colour). On an average, out of 300 plaques per plate 3 were nonrecombinant and 297 were recombinants. The titer of the library was calculated to be $2.2*10^5$ pfu/μg of λ arms. Library was amplified using instructions as given in instruction manual (Stratagene, USA) and titer was adjusted to get 5,000 pfu/plate for further screening.

Genomic library was screened to isolate putative repetitive clones. Plaque lifts in duplicate were done from 3 plates each of size 150 mm using hybond-N membrane of same size. Baked membrane lifts were prehybridized in solution containing 5x SSPE (3.6M NaCl, 0.2M sodium phosphate and 0.2M EDTA), 5x Denhardt's, 0.1% SDS and 0.1% defatted milk powder at 60^0C for 3-5 hours. They were probed using Ni7 genomic DNA radiolabeled with α-P^{32}-dATP (5.5×10^9 cpm/μg) using random primer labeling kit (BRIT, India) and incubated at 60^0C for 18-20 h to identify putative repetitive clones. Blots were washed with 1xSSC (3.0M NaCl and 0.3M Na-citrate) and 1% SDS at RT for 15 min followed by a hot wash at 60^0C for 10 min; a RT wash with 0.1xSSC and 1%

SDS for 15 min followed by a hot wash at 60^0C for 5 min. Probed blots were exposed to X-ray film using intensifying screen at -70^0C. The films were developed after 6 h and overnight exposure. After co-relating the signals on autoradiograms with membrane lifts and plaques on the plates, isolated positive putative clones were picked up and purified through three rounds of plaque formation, lifting, hybridization and autoradiography. Purified clones were subjected to *in vivo* excision of pBluescript SK(-) phagemid from λ ZAP II vector using Ex Assist/Solar system exactly as per instructions given in the manual from Stratagene (USA). Same clones were used as probes for further RFLP analysis.

6) Characterization of repeat clones

Restriction endonuclease mapping of these repetitive DNA clones was done by digesting the plasmid DNAs with various restriction enzymes such as *BamHI, EcoRI, EcoRV, HindIII, KpnI, PstI, SmaI, XbaI,* and *XhoI* and resolving them on 1% agarose gels in TAE (0.04M Tris-acetate, pH 8.0 and 0.001M EDTA) buffer system. Sizes of the fragments generated were calculated by using computer software SEQAID (Rhoads and Roufa, 1989). Assuming genome size of *N. indica* as $4.7*10^7$ bp (Anderson *et al.*, 1992), copy number estimation of each repeat clone was done by quantitative dot blot method and the number of copies were calculated using following formula -

$$M = n * D * P * 660 / K * 6.022 * 10^{23}$$

where, M = amount of insert DNA in picograms, n = number of copies, D = amount of genomic DNA in μg, P = number of bp of the DNA fragment whose copy number is to be determined and K = haploid DNA content in gram (Rajebhosale, 1998).

7) RFLP analysis

DNAs of all seven *N. indica* isolates were digested with various restriction enzymes such as *BamHI, EcoRI, EcoRV, HindIII, HinfI* and *PstI*. However, *BamHI, EcoRI* and *PstI* gave good digestion patterns and were used for RFLP analysis. Digestions were set up according to the manufacturer's instructions (Promega, USA). Ten units of enzyme per μg of fungal DNA were used to ensure complete digestion followed by electrophoresis on 0.9% agarose gel in 1X TAE buffer, pH 8.0 till loading buffer (0.25% Bromophenol blue and 30% glycerol in water) front reached 3/4 of the gel. Digested samples were checked on transilluminator with 254nm wavelength and then transferred to Hybond-N membrane (Amersham, UK) using vacuum blotting apparatus (Vacublot, Pharmacia-LKB, USA). Blotting of digested genomic DNAs involved following steps- (a). Depurination of the in-gel DNA samples with 0.25N HCl (pH 2.0) for 15 min. (b). Denaturation using 1.5M NaCl and 0.5M NaOH (pH 9.0-10.0) for approximately 20-25 min. (c). Neutralization using 0.5M Tris-HCl and 1.5M NaCl (pH 7.5) for 15-20 min. Transfer of DNA samples to Hybond-N was done using 20x SSC for 2 h. Blotted membrane was baked at 80^0C for two hours. Baked blots were prehybridized in solution containing 5x SSPE, 5x Denhardt's, 0.1% SDS and 0.1% defatted milk powder at 60^0C for 3-5 h. They were probed with putative repetitive clones radiolabeled with α-P^{32}-dATP (5.5 x 10^9 cpm/μg) using random priming labeling kit (BRIT, India) and incubated at 60^0C for 18-20 h. Blots were washed with 2x SSC and 1% SDS at RT for 20-30 min followed by a hot wash at 60^0C for 10 min; 1xSSC and 1% SDS at RT for 15 min followed by a hot wash at 60^0C for 5 min. Probed blots were exposed to X-ray film using intensifying screen at -70^0C for required time.

8) PCR amplification and gel electrophoresis

Microsatellite based primers of 16-18 nucleotides in length (UBC set # 9) were obtained from Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada and used for polymerase chain reaction (PCR) amplifications. Genomic DNAs were amplified in 10x PCR buffer containing 15mM $MgCl_2$, 200µM dNTPs, 15 picomoles of primer and 30ng of genomic DNA in each 25µl reaction volume with 1 unit of Taq polymerase (Perkin Elmer, USA). Reaction volumes were overlaid with mineral oil. Amplification reactions were performed on Perkin Elmer PCR machine model 480 programmed for 35 cycles with a cycle in the beginning at 94^0C for 5 min followed by denaturation at 94^0C for 1 min, annealing as per Tm for 1 min and extension at 72^0C for 2 min with a final extension at 72^0C for 5 min. Amplified products were resolved on 1.8% agarose gels and stained with ethidium bromide (stock 10 mg/ml) for 15-20 min. The gels were photographed using black and white 200 ASA film on Pentax SLR camera. These experiments were repeated at least 3 times in order to confirm the amplification profiles.

9) Statistical data analysis

Data on percent disease incidence, coefficient of infection and the number of primary infection sites were analyzed statistically using analysis of variance (Dayton and Stunkard, 1971) to estimate standard error associated with each mean and to work out cultivar x isolate interaction.

Autoradiograms and gel photographs available on RFLP and ISSR analysis, respectively, were scored by difference in band pattern of seven isolates on the basis of presence/absence of bands. Similarity indices were calculated using following formula :

$$X_D = 2N_{AB} / (N_A + N_B)$$

where N_{AB} is the number of bands present in both lanes under consideration; N_A is the total number of bands in lane A and N_B is the total number of bands in lane B (Nei, 1973). The values of similarity indices were averaged and plotted in the form of similarity matrix. Matrix generated on the basis of molecular marker data based on RFLP and ISSR markers individually as well as together was used for the construction of dendrograms using software TAXAN 4.0 (Swartz, 1989). In order to study the correlation between molecular data and virulence data, disease reactions of 13 host lines employed for assessing the virulence were used as characters whereas isolates were taken as operational taxonomic units (OTUs). Data were scored as 1 for susceptible cultivars and 0 for resistant cultivars.

Correlation coefficient between data on ISSR primers and virulence patterns as well as RFLP data and virulence patterns of *N. indica* isolates were calculated separately using Word Excel (Window 95). The significance of the correlation coefficient was calculated by using 't Test' formula (Panse and Sukhatme, 1985) as given below :

$$t = \frac{r}{\sqrt{n-2}} \sqrt{1-r^2}$$

where n = no. of pairs of samples, r = estimate of correlation coefficient obtained from n pairs.

Chapter 3

Results

Results

Section I

Pathogenicity analysis of *Neovossia indica* isolates

1) Percent disease incidence
2) Coefficient of infection
3) Primary infection sites from rachis
4) Primary infection sites from rachilla
5) Primary infection sites from rachis and rachilla
6) Coefficient of correlation
7) Cluster analysis based on virulence data

Section II

Identification and characterization of repeat elements from *Neovossia indica*

1) Construction of genomic library of *N. indica*
2) Repeat DNA content and identification of repetitive elements
3) Characterization of repeat elements
    a) Development of RE map for repeat elements
    b) Estimation of repeat element frequency in *N. indica* genome

Section III

Genetic diversity analysis of *Neovossia indica* isolates

1) **Genetic diversity analysis of *N. indica* isolates using homologous repeat elements**

   Cluster analysis based on hybridization data

2) **Genetic diversity analysis of *N. indica* isolates using ISSR-PCR markers**

   a) **Variation in amplification at dinucleotide repeat due to 3' and 5' anchor**

   b) **Generation of unique bands**

   c) **Cluster analysis based on ISSR data**

3) **Cluster analysis on the basis of combined molecular data**

4) **Correlation between virulence and molecular approaches**

Section I

Pathogenicity analysis of *N. indica* isolates

To study the aggressiveness and variation in virulence reactions shown by the isolates of *Neovossia indica* under present study, disease reactions of 13 wheat host lines including 3 cultivated species of wheat as listed in Table 1 were studied using different isolates of *N. indica*. The disease incidence was scored using various parameters such as percent disease incidence, coefficient of infection (C. I.), primary infection sites (PIS) from rachis, primary infection sites (PIS) from rachilla and primary infection sites (PIS) from both rachis and rachilla.

1) Percent disease incidence

Incidence of Karnal bunt (percent infection) on different host lines with individual isolates under artificial inoculation conditions is given in Table 2. Contrasting disease incidence was observed on different host lines inoculated with the same isolate. As is evident from Table 2, no disease was seen on *Triticum durum* cv. PBW 34 inoculated with isolate Ni8 whereas a high incidence of disease (36.2 %) was noticed on *Triticum aestivum* cv. PBW 138 with the same isolate. Similarly *T. durum* cv. PDW 215 gave a very low disease incidence (0.6 %) as compared to *T. aestivum* cv. PBW 138 (48.5 %) against isolate Ni1. Interestingly, isolate Ni2 did not show much variation (0.0 %-7.0 %) in its virulence reaction on all the host lines analyzed, indicating Ni2 to be the least virulent in nature. Among *Triticum aestivum* cultivars alone, difference in disease incidence was clear against specific isolates. For example, a very low disease incidence (2.9%) was evident on to *T. aestivum* cv. PBW 248 as compared to *T. aestivum* cv. PBW

Table 2.: Karnal bunt incidence (percent infection) and disease reaction on different host lines inoculated with individual isolates of *Neovossia indica*

No	Line	Isolate							Weighted cultivar mean
		Ni 1	Ni 2	Ni 4	Ni 5	Ni 6	Ni 7	Ni 8	
	Triticum durum								
1	PBW 34	6.4 (S)[a] ± 3.2[b]	0.3 (R) ±0.3	16.0 (S) ±1.6	5.1 (S) ±0.2	6.7 (S) ±2.5	13.1(S) ±1.3	0.0 (R) ±0.0	4.78 ±2.96
2	PDW 215	0.6 (R) ±0.3	0.6 (R) ±0.5	12.9 (S) ±5.6	3.1 (R) ±1.3	6.5 (S) ±2.4	1.1 (R) ±1.1	0.3 (R) ±0.3	2.64 ±1.45
	Triticosecale								
3	TL 419	18.8(S) ±4.6	2.6 (R) ±1.3	29.0 (S) ±4.9	13.1 (S) ±4.0	2.1 (R) ±1.3	9.9 (S) ±3.0	13.4 (S) ±3.1	11.68 ±3.73
4	TL 1210	3.7 (R) ±0.7	0.4 (R) ±0.3	8.3 (S) ±2.7	4.2 (R) ±2.0	1.3 (R) ±0.4	5.7 (S) ±1.8	6.0 (S) ±1.8	4.19 ±1.47
	Triticum aestivum								
5	PBW 248	2.9 (R) ±1.3	0.0 (R) ±0.0	38.9 (S) ±5.8	19.1 (S) ±4.3	12.3 (S) ±3.8	10.8 (S) ±3.0	1.1 (R) ±1.1	7.64 ± 3.02
6	PBW 343	17.2 (S) ±4.6	0.3 (R) ±0.3	5.3 (S) ±1.5	16.9 (S) ±3.7	3.1 (R) ±1.4	8.5 (S) ±3.4	4.2 (R) ±1.7	9.2 ±3.39
7	WL 1562	21.6 (S) ±7.0	2.0 (R) ±1.1	33.0 (S) ±12.9	13.9 (S) ±5.1	4.8 (R) ±2.0	15.4 (S) ±4.0	10.4 (S) ±2.7	12.81 ±1.12
8	SONALIKA	23.6 (S) ±2.7	0.9 (R) ±0.6	20.6 (S) ±7.2	19.2 (S) ±3.3	2.6 (R) ±0.6	13.5 (S) ±2.3	6.87 (S) ±1.65	13.48 ±2.81
9	PBW 154	8.5 (S) ±1.8	0.4 (R) ±0.3	8.2 (S) ±4.0	7.3 (S) ±2.5	3.6 (R) ±0.8	9.0 (S) ±2.4	4.3 (R) ±1.6	5.99 ±1.96
10	HD 29	5.9 (S) ±1.7	1.2 (R) ±0.8	0.4 (R) ±0.2	6.2 (S) ±2.2	1.5 (R) ±0.8	2.6 (R) ±1.0	2.3 (R) ±0.9	3.41 ±1.57
11	HD 2329	40.5 (S) ±5.3	7.0 (S) ±3.5	46.3 (S) ±8.8	31.8 (S) ±6.3	16.1 (S) ±2.6	37.6 (S) ±4.7	24.9 (S) ±3.7	30.11 ±5.11
12	PBW 138	48.5 (S) ±4.7	2.0 (R) ±1.5	47.0 (S) ±9.1	46.3 (S) ±4.1	10.8 (S) ±2.3	51.0 (S) ±6.2	36.2 (S) ±5.7	35.96 ±5.01
13	WL 711	49.3 (S) ±5.6	4.2 (R) ±1.3	39.5 (S) ±5.9	66.8 (S) ±4.7	15.1 (S) ±2.8	30.5 (S) ±4.8	32.9 (S) ±6.4	34.04 ±4.78
	Weighted isolate mean	20.23 ±3.77	1.80 ±0.04	24.61 ±8.02	21.77 ±4.01	6.61 ±1.95	16.26 ±3.7	11.95 ±3.24	

[a]Disease reaction, S = Susceptible (disease score > 5.0%), R = Resistant (disease score < 5.0%);

[b]Standard error of mean.

138 (48.5%) against isolate Ni1. Similarly isolate Ni4 showed a differential reaction on two different *T. aestivum* lines, HD 29 (0.4%) and HD 2329 (49.4%). However, no such contrasting variations were noticed in case of *durum* cultivars although only two cultivars were considered for analysis against all the isolates.

Disease incidence was also analyzed by comparing two *N. indica* isolates with respect to one cultivar. It was observed to be as high as 66.8 % with Ni5 as compared to 4.2 % with isolate Ni2 when inoculated on *T. aestivum* cv. WL 711. Similarly *T. aestivum* cv. Sonalika showed 0.9% of disease with isolate Ni2 as compared to a higher disease incidence of 23.6% with isolate Ni1. Similarly a low disease incidence (10.8 %) in case of Ni6 as compared to a high disease incidence (51.0%) in case of Ni7 was seen on *T. aestivum* cv. PBW 138. However, *T. aestivum* cv. HD 29 did not show significant susceptible reactions against any of the isolates under present study indicating HD29 probably to be the most resistant *aestivum* cultivar next to durum cultivars. Similarly, a wheat-rye hybrid *Triticosecale* cv. TL 1210 also showed immune responses against four isolates of *N. indica*. Thus a considerable differential interaction was evident between the host lines and the pathogen isolates. Further analysis of variance for disease incidence (Table 3) showed that cultivars and isolates both differed significantly for disease level and there was significant host/pathogen interaction as evidenced by mean sum of squares as 5868.25 at 0.01 probability when cultivar-isolate combinations were considered.

2) Coefficient of infection

A wide range of variation was observed among all the isolate-cultivar combinations using coefficient of infection (C. I.) as one of the parameters for pathogenicity analysis. Table 4 summarizes the C. I. of *N. indica* isolates against host lines. When *T. durum* cultivars

Table 3.: Analysis of variance of disease incidence.

No.	Source	Degree of freedom	Mean sum of squares	F value
1	Cultivars	12	22407.46	411.07**
2	Isolates	6	15338.97	281.40**
3	Cultivars * Isolates	72	6115.39	112.19**
4	Error	1680	54.51	
5	Total	1770		

**Significant at P = 0.01

Table 4.: Coefficient of infection on different host lines inoculated with individual isolates of *Neovossia indica*

No	Line	Isolate							Weighted cultivar mean
		Ni 1	Ni 2	Ni 4	Ni 5	Ni 6	Ni 7	Ni 8	
	Triticum durum								
1	PBW 34	2.58 ± 1.55	0.31 ± 0.31	---	2.46 ± 0.92	1.61 ± 1.30	---	0.0 ± 0.0	1.66 ± 0.73
2	PDW 215	0.14 ± 0.1	0.0 ± 0.0	---	1.77 ± 0.89	1.97 ± 0.88	0.57 ± 0.57	0.0 ± 0.0	0.82 ± 0.69
	Triticosecale								
3	TL 419	10.14 ± 4.11	1.48 ± 0.72	---	8.56 ± 2.79	3.07 ± 3.08	6.18 ± 2.41	7.51 ± 2.44	6.94 ± 3.02
4	TL 1210	3.55 ± 0.71	0.43 ± 0.43	4.99 ± 1.94	2.15 ± 0.70	0.99 ± 0.37	4.49 ± 1.60	4.47 ± 1.39	3.17 ± 1.17
	Triticum aestivum								
5	PBW 248	1.28 ± 0.65	0.0 ± 0.0	---	9.54 ± 2.82	3.14 ± 1.90	1.26 ± 1.26	0.0 ± 0.0	2.94 ± 1.88
6	PBW 343	2.90 ± 1.82	0.15 ± 0.15	1.19 ± 0.0	7.18 ± 1.74	1.31 ± 0.73	4.46 ± 2.21	2.48 ± 1.11	4.01 ± 1.90
7	WL 1562	8.53 ± 3.35	1.33 ± 0.88	54.61 ± 1.75	9.51 ± 5.45	2.48 ± 1.3	7.47 ± 2.23	5.19 ± 1.48	6.97 ± 3.04
8	SONALIKA	11.08 ± 1.67	0.55 ± 0.55	10.84 ± 8.94	10.63 ± 2.21	0.98 ± 0.30	6.76 ± 1.22	3.72 ± 1.02	6.92 ± 1.97
9	PBW 154	4.5 ± 1.06	0.30 ± 0.21	0.35 ± 0.16	4.18 ± 1.92	1.05 ± 0.22	4.36 ± 1.15	2.24 ± 0.95	2.86 ± 1.08
10	HD 29	2.46 ± 0.84	0.47 ± 0.47	---	3.32 ± 1.22	1.33 ± 0.70	1.06 ± 0.44	1.18 ± 0.47	1.74 ± 0.88
11	HD 2329	19.15 ± 3.11	5.0 ± 2.3	25.87 ± 5.78	16.42 ± 4.15	5.38 ± 1.10	17.58 ± 3.26	11.10 ± 1.95	14.41 ± 3.11
12	PBW 138	25.94 ± 3.07	1.48 ± 1.25	20.66 ± 7.6	23.18 ± 2.66	4.42 ± 0.79	25.82 ± 2.96	21.87 ± 3.61	19.80 ± 3.20
13	WL 711	24.70 ± 4.18	1.98 ± 0.69	16.49 ± 3.42	31.23 ± 3.17	0.0 ± 0.0	15.57 ± 3.05	16.79 ± 5.20	17.80 ± 3.71
	Weighted isolate mean	10.22 ± 2.37	1.07 ± 0.83	16.06 ± 7.12	11.38 ± 2.78	2.14 ± 0.87	9.46 ± 2.45	6.57 ± 2.17	

were compared with those of *T. aestivum*, in general *durum* cultivars showed a lower C. I. than *aestivum* cutivars. For example, *a* very low coefficient of infection (0.14) was found on *T. durum* cv. PDW 215 as compared to a high coefficient of infection (25.94) on *T. aestivum* cv. PBW 138 with respect to isolate Ni1. Similarly isolate Ni5 gave a lower C. I. of 1.77 on *T. durum* cv. PDW 215 as compared to a higher C. I. of 31.23 on *T. aestivum* cv. WL 711. A low C. I. was evident on *T. durum* cv. PBW 34 against all the isolates. On the other hand, a wide range of variation in C. I. was seen within *aestivum* cutivars. A low C. I. (0.35) was noticed on *T. aestivum* cv. PBW 154 as compared to high C. I. (54.61) on *T. aestivum* cv. WL 1562 in case of isolate Ni4. Similarly no infection was observed in case of *T. aestivum* cv. PBW 248 whereas 21.87 C. I. was clear on *T. aestivum* cv. PBW 138, with respect to isolate Ni8. In case of interactions of two isolates on one cultivar, considerable differences in C. I. values were seen. *T. aestivum* cv. WL 1562 gave a C. I. of 1.33 with isolate Ni2 as compared to C. I. of 54.61 with isolate Ni4. Similarly no infection was observed on *T. aestivum* cv. PBW 248 with isolate Ni8 as against 9.54 C. I. with isolate Ni5 on the same cultivar. However, *T. aestivum* cv. HD 29 showed low C. I. values for all the isolates under present study. Further analysis of variance for C. I. (Table 5) showed that the cultivars and isolates differed significantly with respect to this parameter. As observed for percent disease incidence, the cultivar-isolate interaction was significant at 0.01probability for this parameter as well.

3) Primary infection sites from rachis

Primary infection sites (PIS) were calculated from rachis for all the possible isolate-cultivar combinations. Table 6 and 7 describe the number of primary infection sites and their analysis of variance, respectively, for each isolate-host combination. There were

Table 5.: Analysis of variance of coefficient of infection.

No.	Source	Degree of freedom	Mean sum of squares	F value
1	Cultivars	12	4641.20	156.93**
2	Isolates	6	3494.07	118.14**
3	Cultivars * Isolates	72	1265.36	42.78**
4	Error	1227	29.57	---
5	Total	1317	---	---

**Significant at P = 0.01

Table 6.: Primary infection sites (PIS) from rachis on different host lines inoculated with individual isolates of *Neovossia indica*

No	Line	Isolate							Weighted cultivar mean
		Ni 1	Ni 2	Ni 4	Ni 5	Ni 6	Ni 7	Ni 8	
	Triticum durum								
1	PBW 34	0.85 ± 0.46	0.5 0 ± 0.50	---	1.2 ± 0.53	0.60 ± 0.60	---	0.0 ± 0.0	0.71 ± 0.61
2	PDW 215	0.20 ± 0.13	0.0 ± 0.0	---	0.22 ± 0.15	1.16 ± 0.60	0.40 ± 0.40	0.0 ± 0.0	0.33 ± 0.33
	Triticosecale								
3	TL 419	3.42 ± 1.10	0.67 ± 0.29	---	3.00 ± 0.82	1.50 ± 1.50	2.5 ± 1.00	2.85 ± 0.79	2.50 ± 0.98
4	TL 1210	0.89 ± 0.17	0.10 ± 0.32	1.67 ± 0.64	0.43 ± 0.17	0.26 ± 0.09	1.32 ± 0.44	1.24 ± 0.40	0.89 ± 0.32
	Triticum aestivum								
5	PBW 248	0.44 ± 0.24	0.0 ± 0.0	---	2.67 ± 0.64	1.29 ± 0.71	0.25 ± 0.25	0.0 ± 0.0	0.91 ± 0.50
6	PBW 343	2.33 ± 0.88	0.20 ± 0.20	1.0 ± 0.0	3.2 ± 0.76	1.43 ± 0.57	2.8 ± 1.39	1.2 ± 0.20	2.14 ± 0.93
7	WL 1562	2.23 ± 0.71	0.37 ± 0.26	6.0 ± 1.0	2.0 ± 0.81	0.89 ± 0.35	2.23 ± 0.55	1.78 ± 0.59	1.75 ± 0.62
8	SONALIKA	2.74 ± 0.34	0.0 ± 0.0	2.25 ± 1.60	2.1 ± 0.35	0.5 ± 1.17	1.32 ± 0.17	1.0 ± 0.25	6.99 ± 0.32
9	PBW 154	1.16 ± 0.24	0.2 ± 0.13	0.17 ± 0.17	1.31 ± 0.51	0.48 ± 0.13	1.23 ± 0.32	0.55 ± 0.71	0.82 ± 0.27
10	HD 29	0.61 ± 0.16	0.14 ± 0.14	---	1.10 ± 0.40	0.12 ± 0.09	0.65 ± 0.21	0.52 ± 0.23	0.51 ± 0.23
11	HD 2329	4.64 ± 1.48	2.0 ± 0.90	6.27 ± 1.14	3.47 ± 0.73	1.41 ± 0.32	3.61 ± 0.51	2.71 ± 0.47	3.37 ± 0.66
12	PBW 138	6.1 ± 0.62	0.58 ± 0.41	3.62 ± 1.05	4.65 ± 0.45	1.39 ± 0.30	5.65 ± 0.64	4.77 ± 0.65	4.36 ± 0.62
13	WL 711	4.56 ± 0.76	0.58 ± 0.19	3.44 ± 0.57	5.61 ± 0.94	0.0 ± 0.0	4.19 ± 0.66	3.92 ± 3.46	3.82 ± 0.69
	Weighted isolate mean	2.43 ± 1.5	0.38 ± 0.27	3.36 ± 1.28	2.64 ± 0.56	0.73 ± 0.08	2.35 ± 0.15	1.73 ± 0.15	

Table 7.: Analysis of variance of primary infection sites from rachis.

No.	Source	Degree of freedom	Mean sum of squares	F value
1	Cultivars	12	204.23	137.04**
2	Isolates	6	151.08	101.38**
3	Cultivars * Isolates	72	54.35	36.47**
4	Error	1229	1.4903	---
5	Total	1319	---	---

**Significant at P = 0.01

significant differences between cultivars and isolates. More number of PIS were observed to be present in case of Ni4 on *T. aestivum* cv. HD 2329 (6.27) as well as in case of Ni1 on *T. aestivum* cv. PBW 138 (6.1). Contrary to this, no PIS were observed in *durum* cultivars PBW 34 and PDW 215 with isolates Ni2 and Ni8, respectively.

4) Primary infection sites from rachilla

Primary infection sites (PIS) from rachilla were calculated as seen in Table 8. Number of PIS was found to be higher in case of rachilla (0-12.28) than in case of rachis (0.0-6.27). No PIS were evident on *T. durum* cv. PDW 215 whereas 7.91 PIS were present on *T. aestivum* cv. WL711 with respect to isolate Ni1. A lower number of PIS (0.5) was observed on *Triticosecale* cv. TL1210 as compared to the highest number of PIS (12.28) on *T. aestivum* cv. WL711 against Ni5. Isolates Ni2 and Ni6 gave least number of PIS on all the cultivars as compared to other isolates. Analysis of variance as described in Table 9 revealed that the cultivar-isolate interaction was highly significant with respect to number of primary infection sites from rachilla as well.

5) Primary infection sites from rachis and rachilla

Number of primary infection sites from rachis and rachilla were added (Table 10) to calculate total number of primary infection sites per spike. Ni5 gave the highest number of PIS (18.67) on *T. aestivum* cv. WL711 as compared to the lowest number of PIS (0.78) on *T. durum* cv. PDW 215 followed by Ni4 with maximum PIS (17.5) on *T. aestivum* cv. WL1562 and minimum PIS (0.67) on *T. aestivum* cv. PBW 154. Isolates Ni2 and Ni6 gave minimum PIS on all the cultivars as compared to other isolates. In general *T. durum* cultivars showed less number of PIS with all the isolates as compared to *T. aestivum* and *Triticale* cultivars. *T. aestivum* cv. HD 29 showed minimum PIS suggesting its resistance

Table 8.: Primary infection sites (PIS) from rachilla on different host lines inoculated with individual isolates of *Neovossia indica*

No	Line	Isolate							Weighted cultivar mean
		Ni 1	Ni 2	Ni 4	Ni 5	Ni 6	Ni 7	Ni 8	
	Triticum durum								
1	PBW 34	1.44 ± 0.66	0.0 ± 0.0	---	1.1 ± 0.32	0.8 ± 0.58	---	0.0 ± 0.0	0.74 ± 0.59
2	PDW 215	0.0 ± 0.0	0.0 ± 0.0	---	0.55 ± 0.29	0.33 ± 0.21	0.20 ± 0.20	0.0 ± 0.0	0.20 ± 0.21
	Triticosecale								
3	TL 419	3.71 ± 1.13	0.78 ± 0.43	---	1.87 ± 0.74	0.5 ± 0.5	2.5 ± 0.92	1.46 ± 0.57	1.89 ± 0.36
4	TL 1210	0.85 ± 0.21	0.0 ± 0.0	0.89 ± 0.35	0.50 ± 0.17	0.18 ± 0.08	1.63 ± 0.64	1.34 ± 0.4	0.87 ± 0.19
	Triticum aestivum								
5	PBW 248	0.55 ± 0.38	0.0 ± 0.0	---	4.0 ± 1.13	1.86 ± 1.24	0.50 ± 0.50	0.0 ± 0.0	1.32 ± 0.87
6	PBW 343	1.33 ± 1.33	0.20 ± 0.20	0.0 ± 0.0	2.4 ± 0.59	0.57 ± 0.57	2.0 ± 0.71	1.0 ± 0.31	1.45 ± 0.87
7	WL 1562	3.15 ± 1.10	0.37 ± 0.26	11.5 ± 5.52	2.1 ± 0.70	0.84 ± 0.34	3.18 ± 0.96	1.71 ± 0.50	2.18 ± 0.83
8	SONALIKA	2.26 ± 0.40	0.33 ± 0.33	1.5 ± 1.5	1.81 ± 0.33	0.23 ± 0.13	1.64 ± 0.34	1.0 ± 0.32	1.43 ± 0.39
9	PBW 154	1.25 ± 0.18	0.0 ± 0.0	0.5 ± 0.78	1.1 ± 0.56	0.35 ± 0.11	1.59 ± 0.50	0.24 ± 0.08	0.77 ± 0.46
10	HD 29	0.51 ± 0.21	0.14 ± 0.14	---	0.67 ± 0.29	0.17 ± 0.08	0.27 ± 0.16	0.28 ± 0.16	0.36 ± 0.21
11	HD 2329	5.82 ± 0.76	1.8 ± 0.97	6.27 ± 1.47	3.82 ± 0.94	1.62 ± 0.46	5.87 ± 0.78	3.16 ± 0.60	4.15 ± 0.80
12	PBW 138	7.23 ± 0.90	0.33 ± 0.33	3.75 ± 1.64	5.18 ± 0.64	1.72 ± 0.38	7.55 ± 0.84	7.08 ± 1.36	5.34 ± 0.89
13	WL 711	7.91 ± 1.20	0.63 ± 0.17	5.61 ± 0.94	12.28 ± 1.30	0.0 ± 0.0	7.76 ± 1.24	5.42 ± 1.23	6.64 ± 1.19
	Weighted isolate mean	2.79 ± 2.11	0.35 ± 0.27	4.07 ± 1.80	3.07 ± 0.70	0.68 ± 0.33	3.28 ± 0.81	2.03 ± 0.62	

Table 9.: Analysis of variance of primary infection sites from rachilla.

No.	Source	Degree of freedom	Mean sum of squares	F value
1	Cultivars	12	467.00	249.93**
2	Isolates	6	262.41	140.44**
3	Cultivars * Isolates	72	122.03	65.31**
4	Error	1229	1.8685	---
5	Total	1319	---	---

**Significant at P = 0.01

Table 10.: Total number of primary infection sites (PIS) on different host lines inoculated with individual isolates of *Neovossia indica*

No	Line	Isolate							Weighted cultivar mean
		Ni 1	Ni 2	Ni 4	Ni 5	Ni 6	Ni 7	Ni 8	
	Triticum durum								
1	PBW 34	2.0 ± 1.05	0.5 ± 0.5	---	2.3 ± 0.88	1.4 ± 1.17	---	0.0 ± 0.0	1.45 ± 1.12
2	PDW 215	0.2 ± 0.13	0.0 ± 0.0	---	0.78 ± 0.36	1.5 ± 0.76	0.6 ± 0.6	0.0 ± 0.0	0.53 ± 0.43
	Triticosecale								
3	TL 419	7.14 ± 1.9	1.44 ± 0.71	---	4.87 ± 1.51	2.0 ± 2.0	5.0 ± 1.88	4.31 ± 1.33	4.39 ± 1.80
4	TL 1210	1.74 ± 0.35	0.1 ± 0.1	2.55 ± 1.0	0.93 ± 0.32	0.44 ± 0.15	2.91 ± 1.1	2.58 ± 0.77	1.73 ± 0.69
	Triticum aestivum								
5	PBW 248	1.0 ± 0.53	0.0 ± 0.0	---	6.67 ± 1.66	3.14 ± 1.91	0.75 ± 0.75	0.0 ± 0.0	2.20 ± 1.30
6	PBW 343	3.67 ± 2.12	0.4 ± 0.4	1.0 ± 0.0	5.6 ± 1.26	2.0 ± 0.98	4.8 ± 2.0	2.2 ± 0.5	3.59 ± 1.6
7	WL 1562	5.38 ± 1.70	0.75 ± 0.49	17.5 ± 4.51	4.1 ± 1.36	1.74 ± 0.68	5.41 ± 1.47	3.5 ± 0.96	3.93 ± 1.33
8	SONALIKA	5.81 ± 1.03	0.33 ± 0.33	3.75 ± 3.09	3.92 ± 0.64	0.73 ± 0.23	2.97 ± 0.46	2.0 ± 0.5	3.22 ± 0.79
9	PBW 154	2.4 ± 0.52	0.12 ± 0.13	0.67 ± 0.33	2.37 ± 0.96	0.84 ± 0.16	2.82 ± 0.72	0.79 ± 0.22	1.59 ± 1.15
10	HD 29	1.10 ± 0.32	0.28 ± 0.28	---	1.73 ± 0.64	0.29 ± 0.15	0.92 ± 0.34	0.81 ± 0.37	0.90 ± 0.40
11	HD 2329	10.46 ± 1.31	3.8 ± 1.71	12.54 ± 2.55	7.29 ± 1.50	3.04 ± 0.60	9.48 ± 1.18	5.87 ± 1.05	7.53 ± 1.32
12	PBW 138	13.5 ± 1.48	0.92 ± 0.74	7.37 ± 2.6	9.82 ± 1.0	3.1 ± 0.5	13.21 ± 1.27	11.85 ± 1.73	9.74 ± 1.38
13	WL 711	12.48 ± 1.72	1.21 ± 0.31	9.06 ± 1.48	18.67 ± 1.92	0.0 ± 0.0	11.95 ± 1.86	9.33 ± 1.71	10.46 ± 1.78
	Weighted isolate mean	5.46 ± 3.71	0.73 ± 0.52	3.76 ± 2.94	5.70 ± 1.15	1.41 ± 0.52	5.63 ± 1.27	3.6 ± 1.04	

towards *N. indica* isolates. Analysis of variance based on total number of PIS from rachis and rachilla showed significant differences between isolates and cultivars (Table 11) at 0.01 probability.

6) Coefficient of correlation (r)

Statistical analysis of the virulence data as detailed earlier indicated an association between percent disease incidence and primary infection sites per ear head (Table 2 and 11). The cultivars having higher disease incidence with individual isolates had higher number of infection sites with those isolates. Correlation coefficients (r) of primary infection sites (PIS) from rachis, rachilla and both were studied with respect to percent disease incidence which were found to be 0.92, 0.91 and 0.93 along with degree of determination (r^2) as 0.84, 0.82 and 0.86, respectively. Primary infection sites from rachis, rachilla and both were correlated to coefficient of infection also and correlation coefficients were found to be 0.91, 0.94 and 0.95 along with degree of determination (r^2) as 0.83, 0.88 and 0.90, respectively. Values of correlation coefficient with percent disease incidence and coefficient of infection were highly significant at 0.01 probability.

There was, however, no association between grade of infection and percent disease incidence. Exceptionally, in certain cases, lines having lower incidence of disease with certain isolates also had lower grade of infection with those isolates. The examples were *T. aestivum* cv. HD 29/Ni2 and *T. durum* cv. PBW 34/Ni4 which had low percent disease incidence (0.4% and 0.2%, respectively) as well as the lowest grade of infection as 1.0 with both the isolates.

Table 11.: Analysis of variance of total number of primary infection sites.

No.	Source	Degree of freedom	Mean sum of squares	F value
1	Cultivars	12	1258.92	321.80**
2	Isolates	6	834.83	213.97**
3	Cultivars * Isolates	72	334.91	85.61**
4	Error	1229	3.9121	---
5	Total	1319	---	---

**Significant at P = 0.01

7) Cluster analysis based on virulence data

The average similarity indices were calculated for virulence and avirulence reactions of *N. indica* isolates and plotted in the form of a similarity matrix as shown in Table12. This Table indicates that the least similarity index value is 0.308 for the pair Ni2 and Ni4 indicating these isolates to be more diverse and hence they do not cluster in the dendrogram. Similarly the maximum similarity index value is 0.860 for the isolates Ni1 and Ni7 suggesting these isolates to be closer and thus leading to group in the dendrogram.

Table 12 : Similarity matrix of *N. indica* isolates based on their pathogenic reactions against host lines

Ni 1	1.000						
Ni 2	0.462	1.000					
Ni 4	0.692	0.308	1.000				
Ni 5	0.769	0.385	0.769	1.000			
Ni 7	0.860	0.615	0.692	0.615	1.000		
Ni 8	0.692	0.769	0.538	0.615	0.846	1.000	
Ni 6	0.538	0.769	0.538	0.615	0.692	0.846	1.000
OTU	Ni 1	Ni 2	Ni 4	Ni 5	Ni 7	Ni 8	Ni 6

Figure 7 depicts the dendrogram based on pathogenic reactions of *N. indica* isolates. Two major groups are formed where group I clusters isolates Ni1, Ni7, Ni5 and Ni4. Isolates Ni1 and Ni7 are closest among all the isolates and are moderately virulent in their pathogenic reactions. Isolate Ni5 joins the isolates Ni1 and Ni7 at a comparatively low similarity value (0.76). Position of isolate Ni5 in the dendrogram can be explained on the basis of its more virulent reaction on the resistant lines PBW 248 and PBW 154 as

compared to Ni1 and Ni7. Isolate Ni4 shows less similarity to Group I isolates due to its most virulent nature as is evident from Table 2. It is well differentiated from other isolates probably due to its pathogenic reaction on the resistant durum PDW 215. Group II clusters low virulent isolates where isolates Ni6 and Ni8 are found to be the closest as they show almost similar pathogenic reactions in our analysis. These isolates although show higher similarity in the dendrogram, are well distinguished from each other by giving contrasting reactions on the lines PBW 248 and WL 1562 thus explaining the presence of different avirulence genes with respect to their corresponding resistant genes on the host lines. Ni2 is the least virulent isolate and hence joins the group at a lower similarity value.

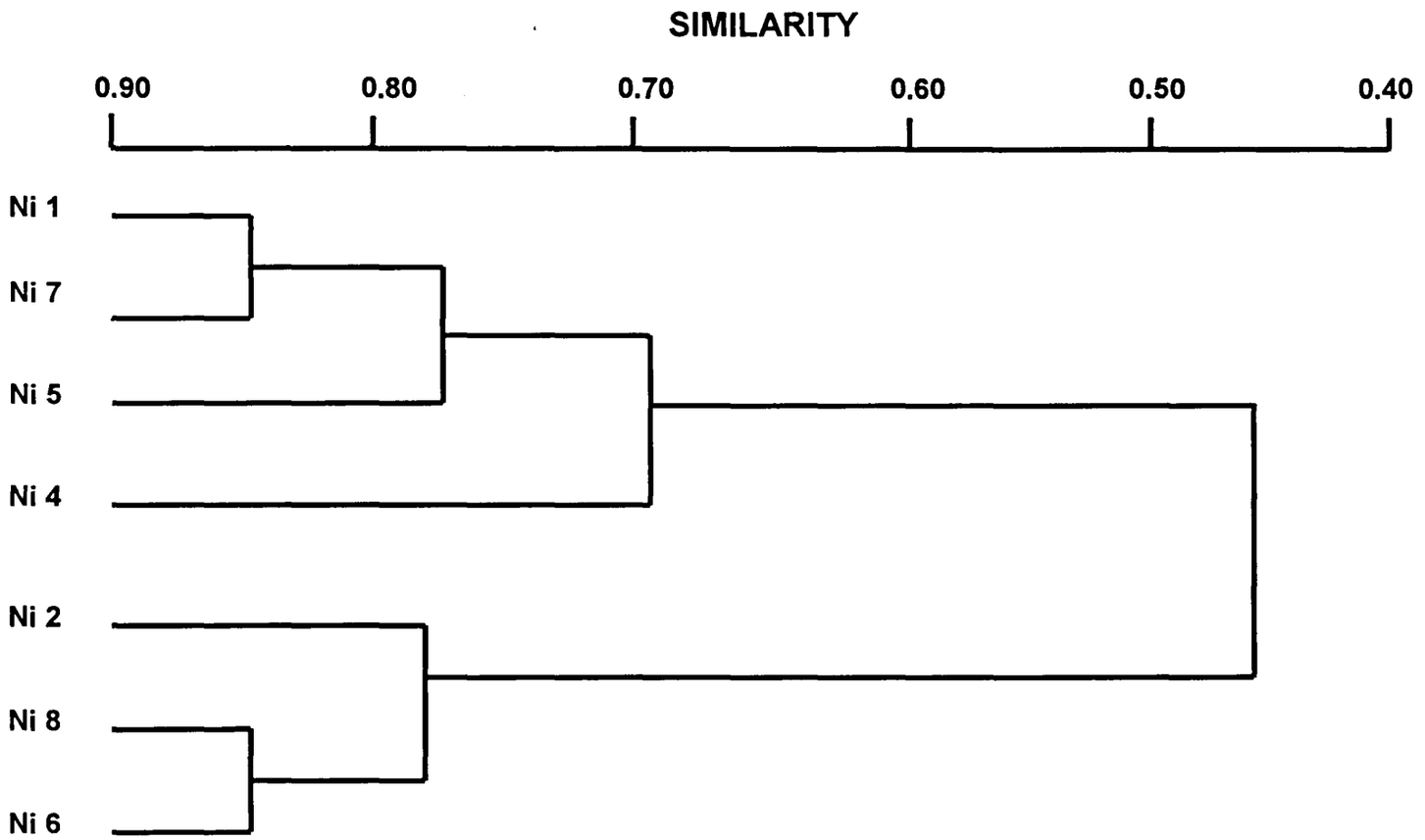


Figure 7 : Dendrogram indicating clustering of *N.indica* isolates based on pathogenicity analysis

Section II

Identification and characterization of repeat elements from *Neovossia indica*

The genetic diversity of *Neovossia indica* isolates collected from North western region in India was studied using two multilocus marker systems including repeat elements from *N. indica* genome and Inter-simple-sequence repeat markers. This section deals with isolation and characterization of repeat elements from *N. indica* which were further used for diversity analysis.

1) Construction of genomic library of *N. indica*

Based on pathogenicity analysis, isolates Ni4 and Ni5 were observed to be highly virulent, Ni1, Ni7 and Ni8 to be moderately virulent and Ni2 and Ni6 to be less virulent. To construct a genomic library, moderately virulent isolate Ni7 was selected and its DNA was digested using serial dilutions of restriction enzyme *EcoRI* in a concentration range of 12u/μg-0.0023u/μg DNA. DNA fragments in the range of 10.0 kb-0.5 kb as revealed in Fig. 8, were further purified from the agarose gel. Purified, digested genomic DNA of Ni7 was checked to confirm the presence of intact overhangs by analyzing efficiency of self ligation.

Figure 9A highlights gel electrophoresis of ligated genomic DNA along with the test insert given in the kit. The molecular sizes of ligated DNA of Ni7 as well as test insert are comparable to each other (lanes 1 and 2, respectively) as well as to uncut genomic DNA of Ni7 (lane 3) and λ DNA (lane L) indicating intact *EcoRI* compatible ends of the digested Ni7 DNA. The *EcoRI*/λZAPII arms were ligated with different concentrations of Ni7 insert DNA. The ligation profiles are depicted in lanes 1, 2 and 3

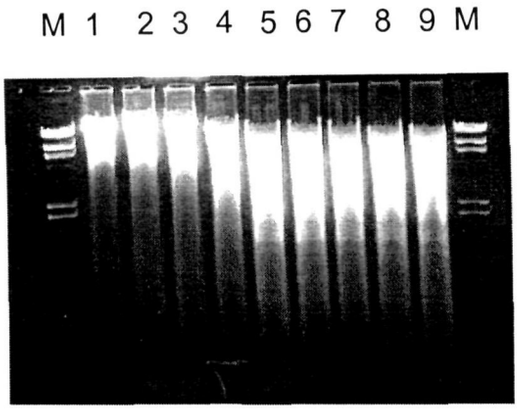


Figure 8 : Agarose gel electrophoresis of Ni7 genomic DNA digestions with various dilutions of *EcoRI*. Lane M represents Lambda DNA/*HindIII* marker. Lane 1 contains 0.0046 units of *EcoRI*/2μg of genomic DNA and lanes 2-9 contain 1μg of genomic DNA with 0.092 units (2), 0.185 units (3), 0.375 units (4), 0.75 units (5), 1.5 units (6), 3 units (7), 6 units (8) and 12 units (9) of *EcoRI*.

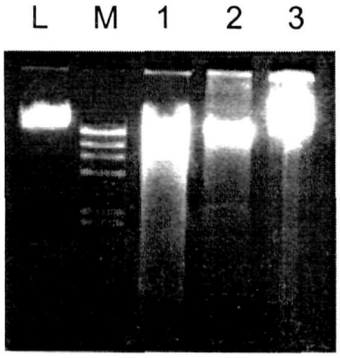


Figure 9 A : Agarose gel electrophoresis of self ligated genomic DNA of Ni7 cut with RE *EcoRI* along with test insert. Lanes L & M represent Lambda DNA uncut and Lambda DNA cut with *HindIII*, respectively. Lanes 1-3 contain test insert (1), test insert ligated (2) and self ligated genomic DNA cut with *EcoRI* (3).

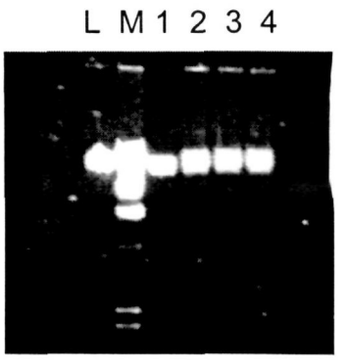


Figure 9 B : Agarose gel electrophoresis of different concentrations of *EcoRI* digested genomic DNA of Ni7 ligated with Lambda arms along with self ligated Lambda arms as positive control. Lane L & M represent Lambda DNA uncut and Lambda DNA cut with *HindIII*, respectively. Lanes 1-4 contain ligated 1μg of genomic DNA(1), 0.5 μg of genomic DNA (2), 0.25 μg of genomic DNA (3) and test insert control DNA (4) with Lambda arms

along with a test insert in lane 4 of Fig. 9B. A combination of 0.25 μg insert with 1.0 μg λ ZAP II DNA (Fig. 9B, lane 3) gave the best ligation. After confirming the ligation, packaging of the ligated DNA was carried out to establish a genomic library representing 22 genomes of Ni7. Use of λ ZAP II system offers two advantages, first it is highly efficient in cloning up to 10.0 kb insert fragments and secondly it provides convenient methodology to convert λ clones into plasmid clones without undergoing another round of restriction enzyme digestion, ligation and transformation.

2) Repeat DNA content and identification of repeat elements

Genomic library of *N. indica* was screened using Ni7 genomic DNA as probe for calculating repetitive DNA content initially and for identifying repetitive elements for further analysis. Figure 10 represents a picture of one such autoradiogram indicating positive signals in plate hybridization assay. Based on hybridization signals revealed after six hours of exposure, amount of total repetitive DNA was estimated to be 1% of the total genomic DNA of *N. indica.*

Twenty-four putative repetitive plaques were identified after three rounds of plaque purification and *in vivo* excision was performed to convert the phage clones into plasmid clones. These plasmids were digested with *EcoRI* to elute the inserts. Only those clones that gave intense hybridization signals, within 6 hours of exposure, were selected. Figure 11A shows the insert sizes of three repetitive clones which were finally selected. Lanes 2, 4 and 6 represent the undigested plasmids whereas lanes 3, 5 and 7 show respective sizes of the inserts to be 3700, 4800 and 500 bp, respectively. These clones were southern hybridized with genomic DNA after blotting on to hybond-N memebrane as shown in Fig. 11B. The intense signals in lanes 3, 5 and 7 represent the respective

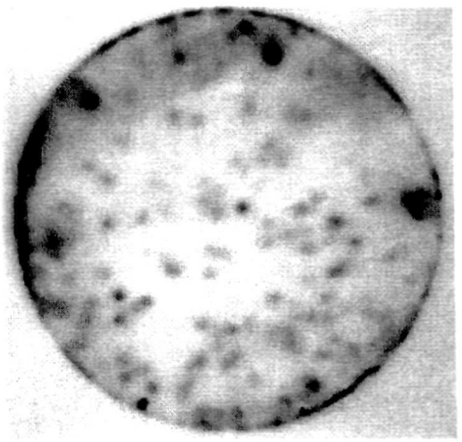


Figure 10 : Screening of genomic library of *N. indica* isolate Ni7 constructed in Lambda ZAP II system using genomic DNA of Ni7 as probe

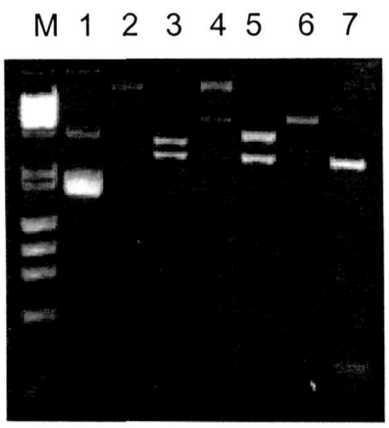


Figure 11 A : Agarose gel electrophoresis of genomic clones uncut/cut with *EcoRI*. Lane M represents lambda DNA/ *HindIII* marker. Lanes 1-7 contain PUC-18 DNA uncut (1), pNiR9 DNA uncut (2), pNiR9 DNA cut (3), pNiR12 DNA uncut (4), pNiR12 DNA cut (5), pNiR16 DNA uncut (6), pNiR16 DNA cut (7)

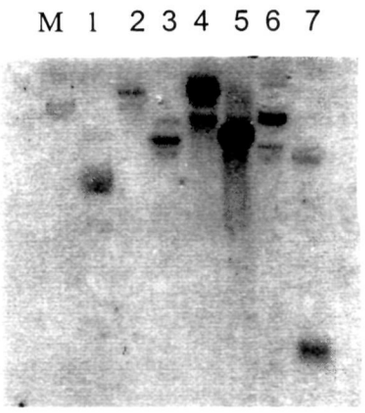


Figure 11 B : Autoradiogram of genomic clones uncut/cut with *EcoRI* hybridized with genomic DNA of Ni7. The lane sequence is as given in Figure 10 A

inserts hybridized to genomic DNA of Ni7. These repetitive clones termed as pNiR9, pNiR12 and pNiR16 carrying insert size of 3700, 4800 and 500bp, respectively, were further analyzed.

3) Characterization of repeat elements

In an initial attempt of characterizing repeat elements identified in the present investigation, restriction endonuclease (RE) map and copy number estimation were carried out for each of the three repeat clones.

a) Development of RE map for repeat elements

The repeat clones pNiR9, pNiR12 and pNiR16 were digested with various restriction enzymes to identify RE sites within insert DNA fragment and the same gel was blotted and hybridized to α-P^{32}-dATP labeled Ni7 genomic DNA. As is seen in Fig. 12A, single digestion of repetitive element pNiR9 shows one internal site each for restriction endonucleases *BamHI* (lane 2), *HindIII* (lane 5) and *XbaI* (lane 8). Fig. 12B highlights the digestion pattern of insert fragment pNiR16 showing no site for any enzyme except for *EcoRI* (lane 3). Single digestion profiles of insert fragment pNiR12 as shown in Fig. 12C, indicate two sites for *BamHI* (lane 2) and one each for *PstI* (lane 7), *XbaI* (lane 8) and *XhoI* (lane 9). These gel electrophoretic profiles of the insert fragments were blotted to nylon membrane and southern hybridized with genomic DNA of Ni7 to confirm the results. The results of hybridization are depicted in Fig. 13 A, B and C. Repeat element pNiR16 did not show internal site for any restriction enzyme used in the present analysis, hence double digestions of insert fragments pNiR9 and pNiR12 were set up to plot the restriction sites on the respective insert fragments as shown in Fig. 14A and 15A, respectively. The double digested patterns of the insert fragments of pNiR9 and pNiR12

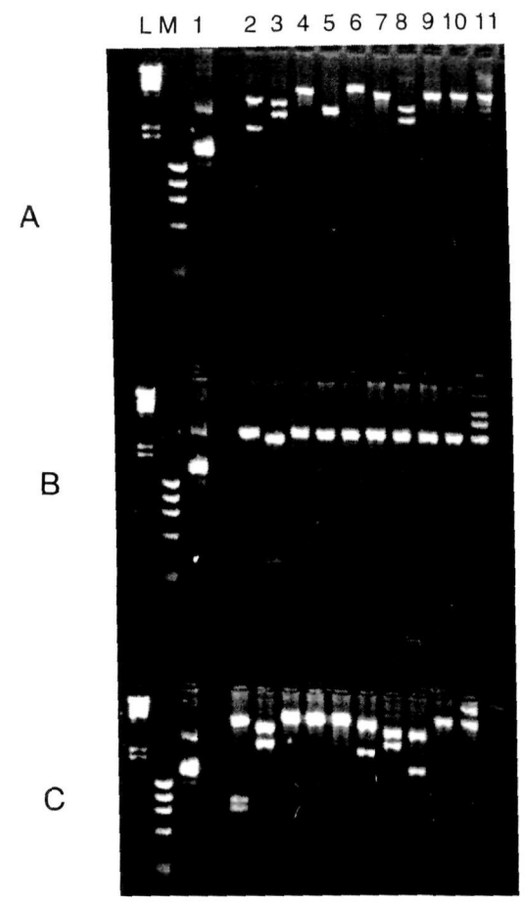


Figure 12 : Agarose gel electrophoresis of insert fragment of pNiR9 (A), pNiR16 (B) and pNiR12 (C), digested with restriction enzymes *BamHI* (2), *EcoRI* (3), *EcoRV* (4), *HindIII* (5), *KpnI* (6), *PstI* (7), *XbaI* (8), *XhoI* (9), *SmaI*(10) and uncut pNiR9 (11). Lanes L & M represent lambda DNA/ *HindIII* and X-174 DNA/*HaeIII*, respectively. Lane 1 represents PUC-18 DNA uncut.

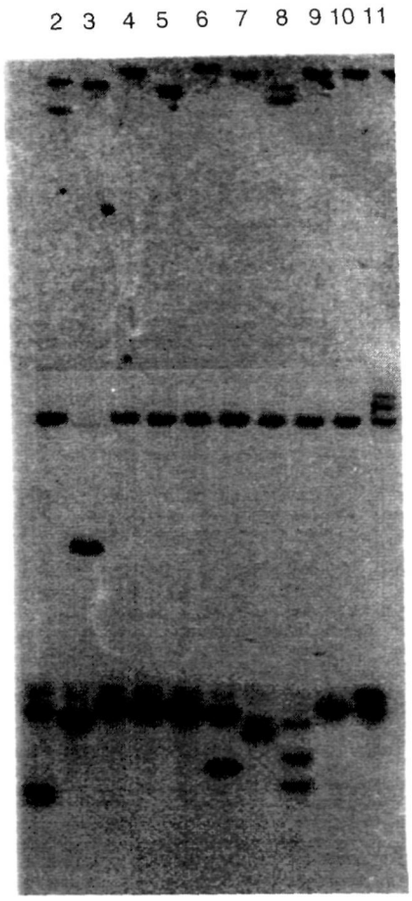


Figure 13 : Autoradiogram of insert fragment of pNiR9 (A), pNiR16 (B) and pNiR12 (C), digested with various restriction enzymes and hybridized with genomic DNA of Ni7. Lane sequence is as given in Fig. 12

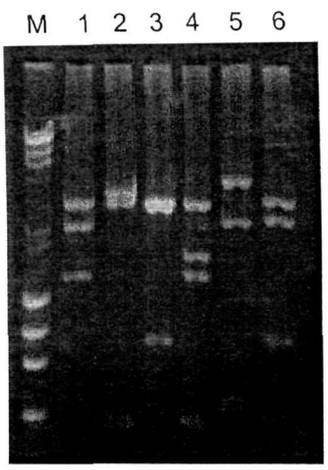


Fig. 14 A

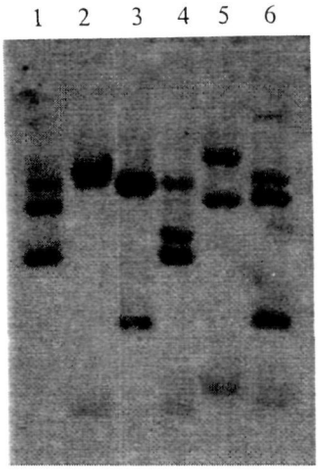


Fig. 14 B

Figure 14 A-B : Agarose gel electrophoresis (A) and Autoradiogram (B) of double digestion of insert fragment of pNiR9 digested with restriction enzymes *EcoRI/BamHI* (1), *EcoRI/HindIII* (2), *EcoRI/XbaI* (3), *BamHI/HindIII* (4), *BamHI/XbaI* (5), *XbaI/HindIII* (6) and hybridized with Ni7 genomic DNA. Lane M represents lambda DNA/*HindIII* and ϕX-174 DNA/*HaeIII*

Fig. 15 A

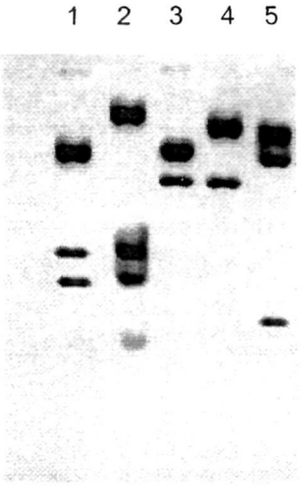


Fig. 15 B

Figure 15 A-B : Agarose gel electrophoresis (A) and Autoradiogram (B) of double digestion of insert fragment of pNiR12 digested with restriction enzymes *EcoRI/BamHI* (1), *PstI/BamHI* (2), *EcoRI/PstI* (3), *EcoRI/XbaI* (4), *EcoRI/XhoI* (5) and hybridized with Ni7 genomic DNA. Lane M represents lambda DNA/*HindIII* and ϕX-174 DNA/*HaeIII*

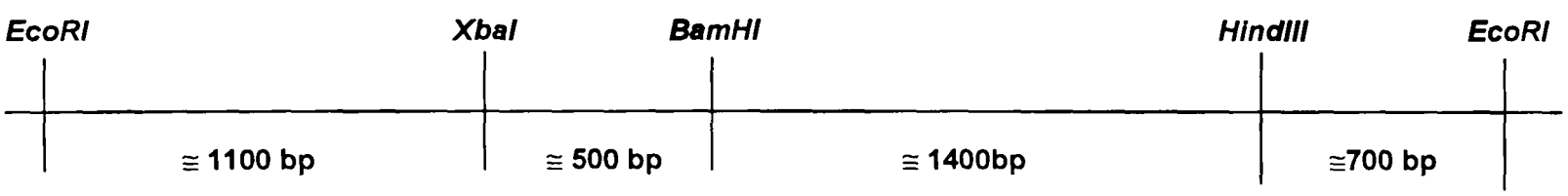


Figure 16 A : Restriction endonuclease map of insert fragment pNiR 9.

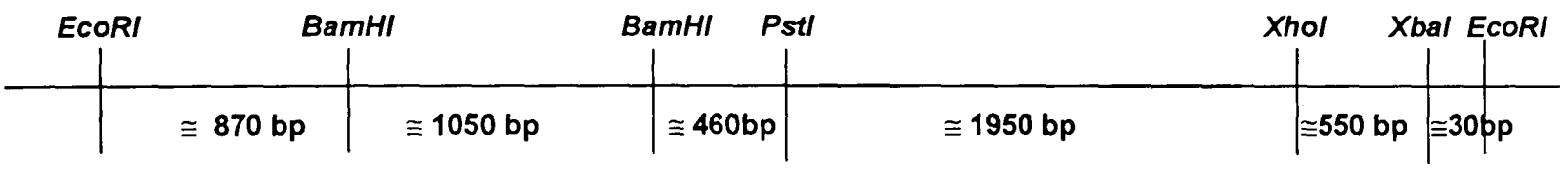


Figure 16 B : Restriction endonuclease map of insert fragment pNiR 12.

were confirmed by blotting and hybridization with genomic DNA of Ni7 as indicated in Fig. 14B and 15B. RE maps of inserts pNiR9 and pNiR12 were constructed on the basis of single and double digestions. RE map of pNiR9 as given in Fig. 16A shows one site each for *BamHI*, *HindIII* and *XbaI* whereas RE map of pNiR12 shows two internal sites for *BamHI* and one site each for *PstI*, *XhoI* and *XbaI* as seen in Fig. 16B.

b) Estimation of repeat element frequency in *N. indica* genome

The repeat sequences are known to be present in high copy number in the genome. Hence copy number of each of the repeat elements pNiR9, pNiR12 and pNiR16 was estimated by quantitative dot-blot analysis. On the basis of molecular weight of each of the repetitive elements, the amount of repeat DNA element required for a known copy number equivalent in the genomic DNA was calculated and loaded against the known concentration of genomic DNA of Ni7. Each blot was hybridized to α-P^{32}-dATP labeled respective insert fragment. The dot-blot autoradiogram of copy number estimation of insert of pNiR9 is shown in Fig. 17 where 25, 50, 100 and 200 copies of insert are loaded in lane A against the known concentrations of 1µg and 2µg of genomic DNA of Ni7 in lane B. Each autoradiogram so obtained was analyzed by densitometric scanning using gel documentation system (GDS 9000, UVP USA). The intensities of the dots in the genomic DNA were calculated and compared with the known copies of the repeat element pNiR9. Approximately 32 copies of pNiR9 were estimated to be present in the genome of *Neovossia indica*. Same method of calculations was followed for copy number estimation of insert fragments pNiR12 and pNiR16. Dot-blots of pNiR12 and pNiR16 are shown in Fig. 18 and 19, respectively. Concentration of 0.5 µg and 1.0 µg of genomic

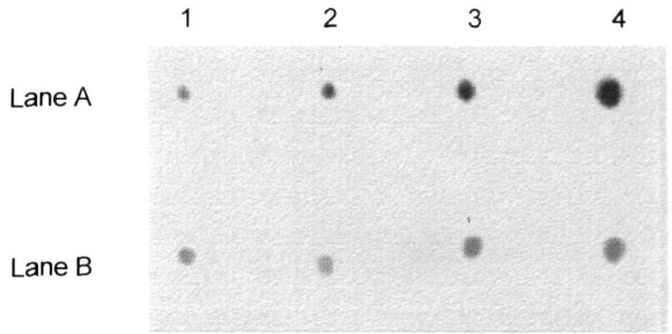


Figure 17 : Quantitative dot-blot of pNiR9. Lane A contains 25 (1), 50 (2), 100 (3) and 200 (4) copies of the insert DNA loaded against 1.0 µg (1, 2) and 2.0 µg (3, 4) genomic DNA of Ni7 in lane B.

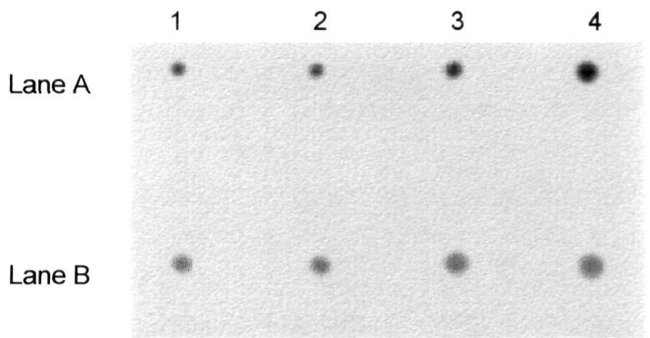


Figure 18 : Quantitative dot-blot of pNiR12. Lane A contains 25 (1), 50 (2), 100 (3) and 200 (4) copies of the insert DNA loaded against 0.5 µg (1, 2) and 1.0 µg (3, 4) genomic DNA of Ni7 in lane B.

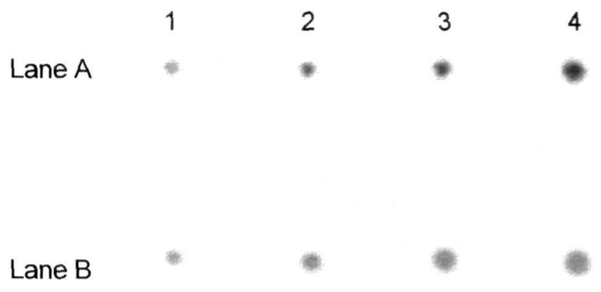


Figure 19 : Quantitative dot-blot of pNiR16. Lane A contains 25 (1), 50 (2), 100 (3) and 200 (4) copies of the insert DNA loaded against 0.5 µg (1, 2) and 1.0 µg (3, 4) genomic DNA of Ni7 in lane B.

DNA of Ni7 were used for these insert fragments. Sixty one copies of pNiR12 and 64 copies of NiR16 were estimated to be present in the genome of *Neovossia indica*.

Section III

Genetic diversity analysis of *N. indica* isolates

The genetic diversity analysis of *N. indica* isolates, from North western region of India, was carried out using two marker systems namely repeat elements and ISSR primers.

1) Genetic diversity analysis of *N. indica* isolates using homologous repeat elements

Many restriction enzymes such as *AluI*, *BamHI*, *EcoRI*, *EcoRV*, *HinfI*, and *PstI*, were initially attempted to get good digestion of *N. indica* DNAs. Only three restriction enzymes *BamHI*, *EcoRI* and *PstI* were finally used for variability analysis. The DNAs of 7 isolates namely Ni1, Ni2, Ni4, Ni5, Ni6, Ni7 and Ni8 representing North western region in India were digested with these three enzymes to get digestion products in the range of 20-0.1 kb. The digested products were separated on 0.9% agarose gels in TAE buffer and southern blotted on to nylon membranes. Repetitive clones pNiR9, pNiR12 and pNiR16 were used to hybridize with these blots to study their potential as probes for diversity analysis and the variability existing in *N. indica* isolates (Fig. 20-22).

As seen in Fig. 20, the repeat clone pNiR9 generates ≈ 8-17 different bands with *EcoRI* (lanes 1-7) and *BamHI* (lanes 15-21) whereas ≈ 10-20 bands in *PstI* (lanes 8-14) digested *N. indica* DNAs in the range of 22.0-2.0 kb. It is further apparent that the *N. indica* isolates Ni1 and Ni7 show similar hybridization profiles in case of *EcoRI* digestion (lanes 3 and 4), but they can be distinguished well by using *PstI* digests (lanes 10 and 11) where two bands of approximate size of 10.0 and 7.0 kb are present in Ni7/*PstI*/pNiR9 profile. *N. indica* isolate Ni8 DNA (lanes 5, 12 and 19) digested with *EcoRI*, *BamHI* and *PstI* gives less but different bands on hybridization. DNAs of Isolates Ni1 (lanes 3 and

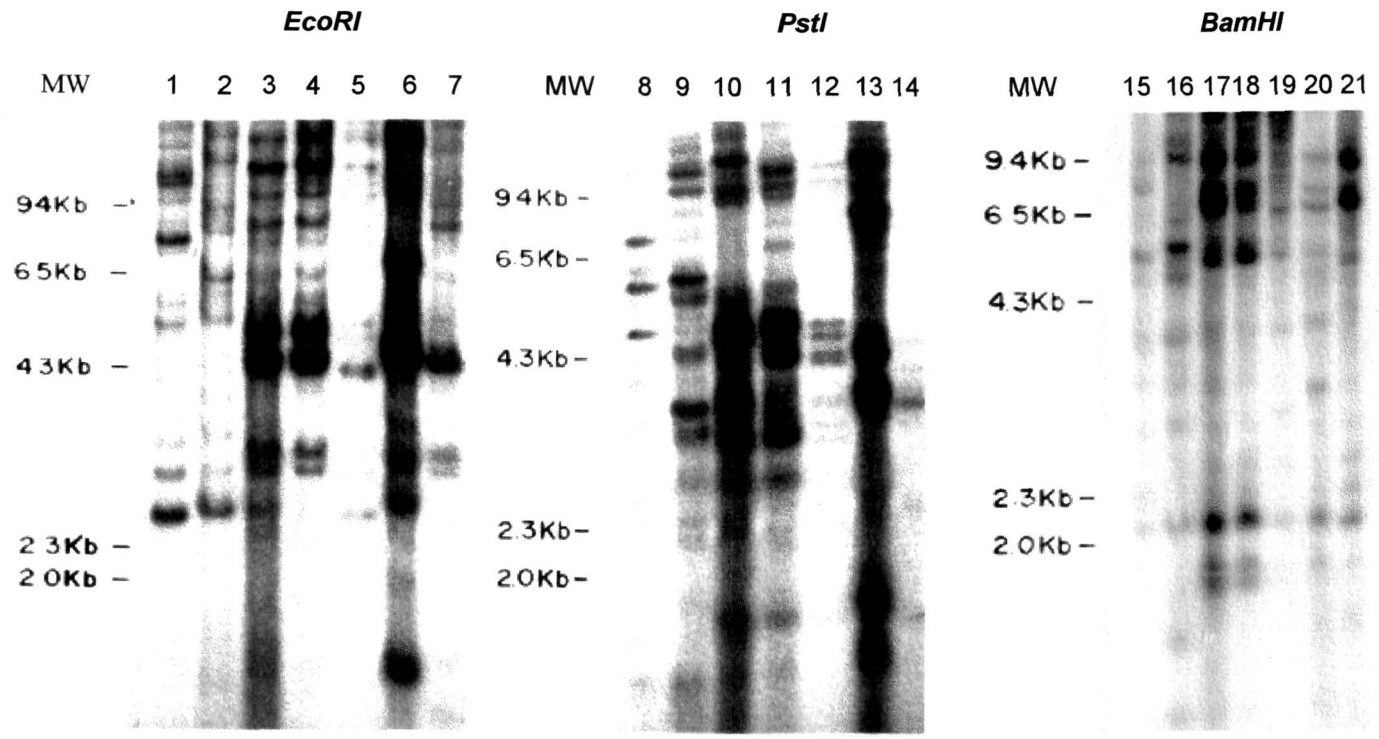


Figure 20 : Southern hybridization profiles of pNiR9 with genomic DNA digests of *N. indica* isolates. Lanes 1-7 contain isolates Ni4(1), Ni5(2), Ni1(3), Ni7(4), Ni8(5), Ni6(6) and Ni2(7) digested with *EcoRI*, lanes 8-14 with *PstI* digested *N. indica* DNAs and lanes 15-21 with *BamHI* digested *N. indica* DNAs, with same sequence of *N.indica* isolates as in lanes 1-7.

10), Ni7 (lanes 4 and 11) and Ni6 (lanes 6 and 13) digested with *EcoRI* and *PstI* show intense signals as well as more number of bands with repeat clone pNiR9 suggesting higher number of copies of a repeat in a specific environment.

Figure 21 depicts the hybridization patterns of pNiR12 with different *N. indica* isolates DNAs. The repeat clone pNiR12 gives ≈ 2-5 bands with *EcoRI* (lanes 1-7) and *PstI* (lanes 8-14) digested DNAs and ≈ 2-9 bands with *BamHI* (lanes 15-21) digested DNAs within 9.4-2.0 kb range. The repeat clone pNiR12 does not seem to be much dispersed in the genome since number of bands is less as compared to that generated by repeat clone pNiR9 as seen in Fig. 20. An intense band of approximately 4.8 kb in case of *EcoRI*, two of size 6.0 kb and 3.0 kb in *PstI* and three of size 4.0 kb, 3.0 kb and 1.3 kb in *BamHI* digested isolates suggest the presence of at least one internal site for *PstI* and two internal sites for *BamHI* may be present in the insert fragment of pNiR12. This observation is further supported by restriction enzyme map of pNiR12 (Fig. 16B).

The repeat clone pNiR16 gives ≈ 14-21 bands in case of *EcoRI* (lanes 1-7) whereas ≈ 8-18 bands in *PstI* (lanes 8-14) digested DNAs of *N. indica* isolates in the range of 9.4-1.0 kb as seen in Fig. 22. It did not give any scorable pattern with *BamHI* digested DNAs of *N. indica* isolates. As is evident from Fig. 22, *N. indica* isolates Ni6 and Ni2 (lanes 6 and 7) show many bands in common in case of *EcoRI* digestion whereas Ni1 and Ni7 show similar patterns in case of *EcoRI* (lanes 3 and 4) as well as *PstI* (lanes 10 and 11) digestion. It is very interesting that Ni1 and Ni7 can be distinguished in only two hybridization profiles such as pNiR9/*PstI* and pNiR12/*PstI*. These results suggest that both probe and the enzyme combination are important to obtain fingerprints of *N. indica* isolates.

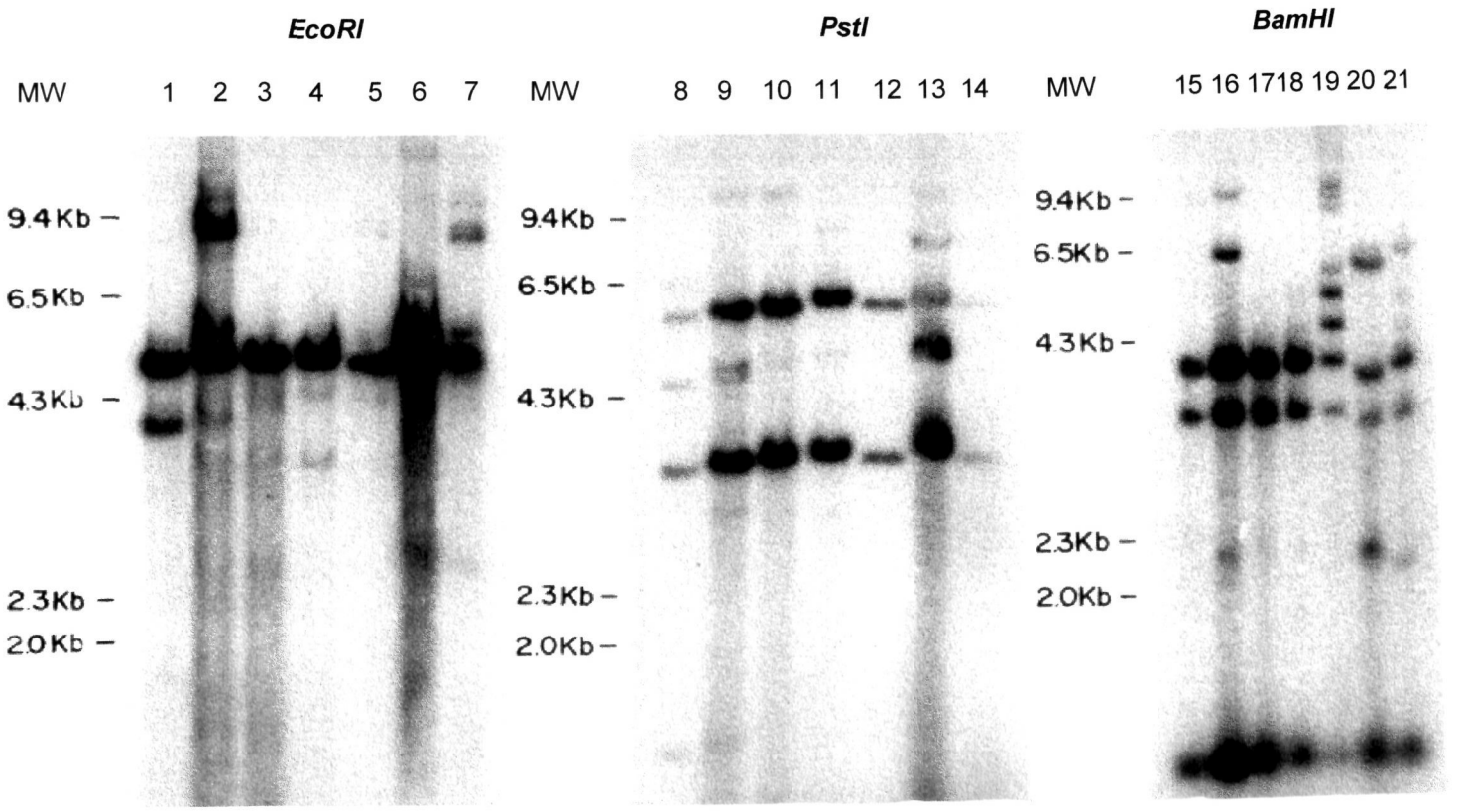


Figure 21 : Southern hybridization profiles of pNiR12 with genomic DNA digests of *N. indica* isolates. Lanes 1-7 contain *N. indica* isolates digested with *EcoRI,* lanes 8-14 *PstI* digested isolates and lanes 15-21 *BamHI* digested isolates with same sequence as given in Fig. 20.

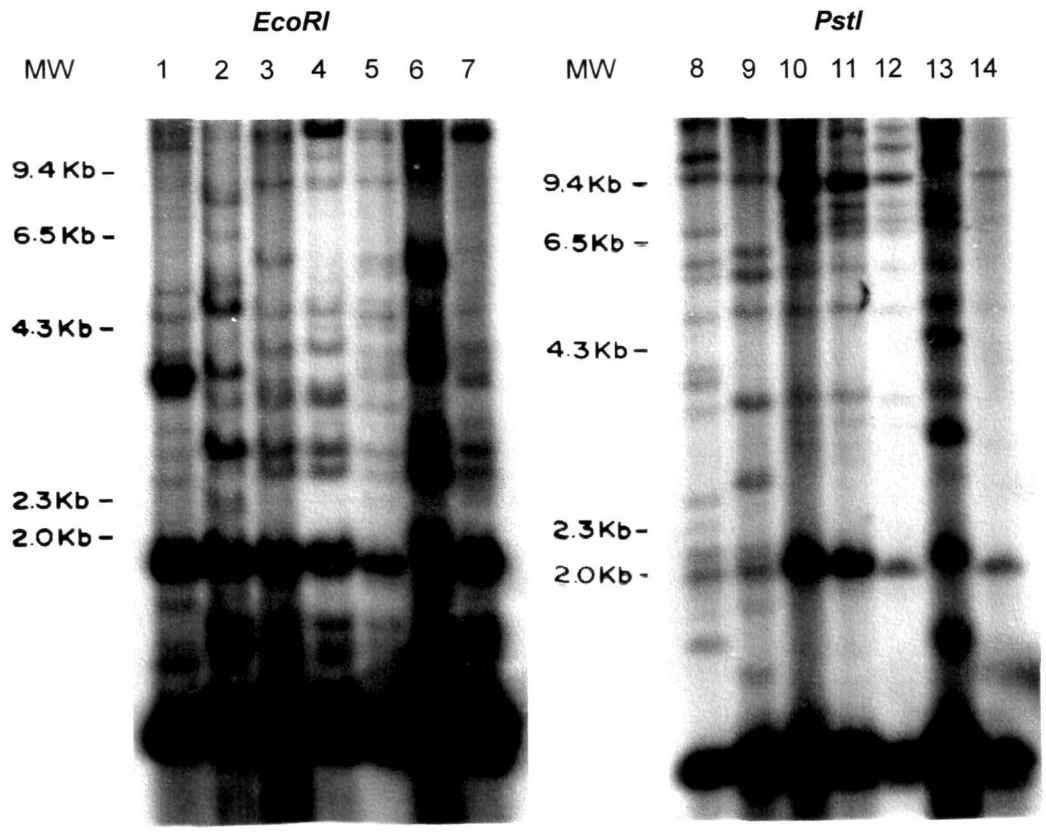


Figure 22 : Southern hybridization profiles of pNiR16 with genomic DNA digests of *N. indica* isolates. Lanes 1-7 contain *N. indica* isolates digested with *EcoRI,* lanes 8-14 *PstI* digested isolates with same sequence as given in Fig. 20.

Cluster analysis based on hybridization data

The hybridization data obtained by repeat elements was utilized to calculate similarity indices of *N. indica* isolates in pairwise manner for each probe enzyme combination. These values were averaged and plotted in a form of similarity matrix as given in Table 13. As is clear in Table 13, the least similarity index value is 0.51 for the pair Ni4 and Ni7 indicating these isolates to be more diverse and hence do not cluster in the dendrogram. Similarly the maximum similarity index value is 0.83 for the isolates Ni1 and Ni7 indicating these isolates to be genetically closer leading to a cluster in the dendrogram.

Table 13. : Similarity matrix obtained on the basis of repeat clones.

Ni4	1.000						
Ni5	0.516	1.000					
Ni1	0.535	0.596	1.000				
Ni7	0.512	0.526	0.836	1.000			
Ni8	0.516	0.549	0.746	0.723	1.000		
Ni6	0.526	0.559	0.653	0.638	0.596	1.000	
Ni2	0.573	0.549	0.615	0.62	0.671	0.596	1.000
OTU	Ni4	Ni5	Ni1	Ni7	Ni8	Ni6	Ni2

The dendrogram constructed on the basis of hybridization profiles obtained by repeat elements gives a view of genetic variation among *N. indica* isolates. As is evident in Fig. 23, at a cut off value of 0.73 similarity, out of seven *N. indica* isolates, three isolates namely Ni1, Ni7 and Ni8 group together. The boot strap values for this cluster are as high as 99.3 and 93.1, respectively thus suggesting that the grouping of these three isolates is robust based on the molecular markers used under present study. Isolates Ni6 and Ni2

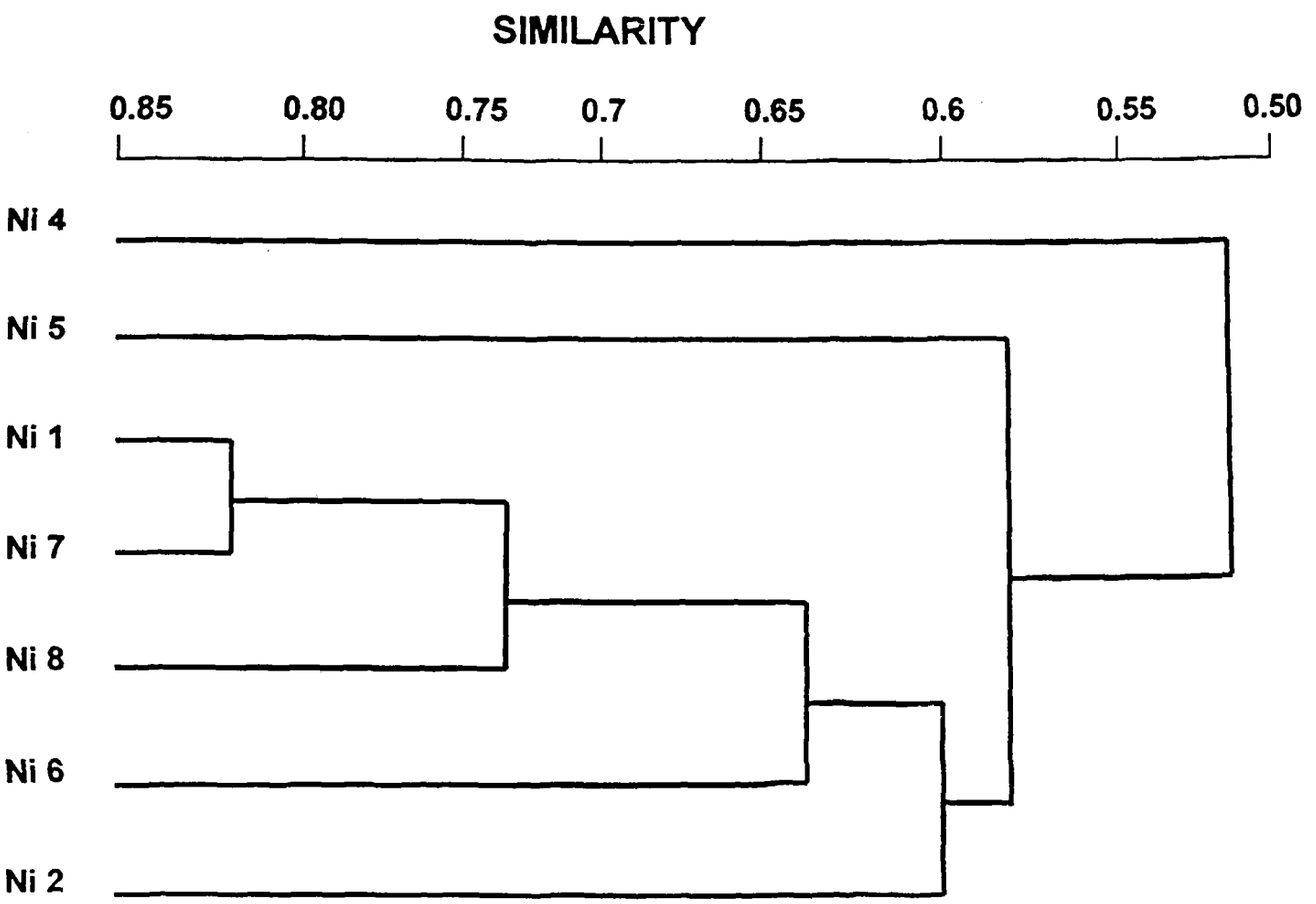


Fig. 23: Dendrogram indicating clustering of *N. indica* isolates based on repeat element data

further group with these three but at lower boot strap values indicating that these two might change their positions in the cluster. The remaining two isolates Ni4 and Ni5 do not show much similarity with rest of the isolates.

2) Genetic diversity analysis of *N. indica* isolates using ISSR-PCR markers

Seventy seven primers were screened with seven *N. indica* isolates out of which 27 primers (35%) gave amplification. Out of 27 primers, 16 primers were found to be monomorphic and 11 primers gave polymorphic profiles with 7 isolates of *N. indica*. Among the 77 primers used, 56 primers were of dinucleotide nature with a specific anchor at 3' or 5' end. Other primers used were either tri- or tetra- or pentanucleotide repeats with no anchor. Hence the effect of anchor could be studied in case of dinucleotide repeat only. Polymorphic amplification profiles were obtained with 3'/5' anchored dinucleotide repeat primers. Detailed description of these repeat sequences is given later in the section. Primers with $(AT)_n$ sequences did not give scorable patterns and hence were not used for further study.

Among trinucleotide repeats including 11 different primers, no amplification was observed with repeats $(AGC)_n$, $(AGT)_n$, $(GGC)_n$, $(GAA)_n$, $(GTT)_n$ and $(TGC)_n$. Only one primer $(ATG)_n$ generated a polymorphic pattern and 4 primers containing $(ACC)_n$, $(CCG)_n$, $(CTC)_n$ and $(TAT)_n$ repeats gave monomorphic profiles. Similarly out of seven tetranucleotide primers, one primer $(GGAT)_n$ gave amplification and polymorphic pattern whereas other six primers containing repeats $(GATA)_n$, $(GACA)_n$, $(CCCT)_n$, $(CTAG)_n$, $(TGCA)_n$ and $(GATA\text{-}GACA)_n$. did not give any amplification. Among 3 pentanucleotide repeats, primer $(GGGTG)_n$ was found to be polymorphic, $(GGAGA)_n$ was monomorphic and $(CTTCA)_n$ did not show amplification in *N. indica* genome.

Although monomorphic primers were not useful for variability analysis, they provided a valuable information regarding frequent presence of these repeats in all the isolates. Polymorphic primers identified in the present study mainly included AG, GA, GT, CA, TC, AC, ATG, GGAT and GGGTG repeat sequences. A total of 129 bands were scored with 11 primers out of which only 3 bands were found to be monomorphic in all the seven isolates suggesting high level of polymorphism obtained with these 11 primers.

Representative pictures of amplification using di, tri, tetra and penta nucleotide repeat sequences are given, where amplification products are obtained in the range of 3.0-0.3 kb. Figure 24 represents the amplification of dinucleotide repeat $(AG)_nC$ and $(AG)_nG$. As is evident from amplification profile in this figure, lanes 5 and 6 show similar pattern whereas lane 2 shows two bands and only one band is amplified in lane7. An intense band of 700 bp is seen in all the isolates in case of $(AG)_nG$ repeat (lanes 8-14). Lanes 12 and 13 show similar amplification patterns. Figure 25 represents gel electrophoretic profiles obtained with dinucleotide repeats $(CA)_nG$ and $(TC)_nC$. Highly polymorphic patterns are obtained with these repeats in all the isolates. However, the number of bands obtained with $(TC)_nC$ are less (lanes 8-14) as compared to those generated with $(CA)_nG$ (lanes 1-7). A unique ladder like pattern is observed in case of Ni8 (lane 6) with $(CA)_nG$ repeat. Gel electrophoretic profiles obtained with repeats $(AC)_nC$ and $(GA)_nYC$ are seen in Fig. 26 where polymorphic amplification profiles are present with both the repeats. However, lanes 1 and 2, and 8 and 9 show similar profile. Figure 27 describes the amplification profile of a dinucleotide repeat $(GT)_nYC$ and a trinucleotide primer $(ATG)_n$. It is very interesting to note that many bands are generated using these repeats.

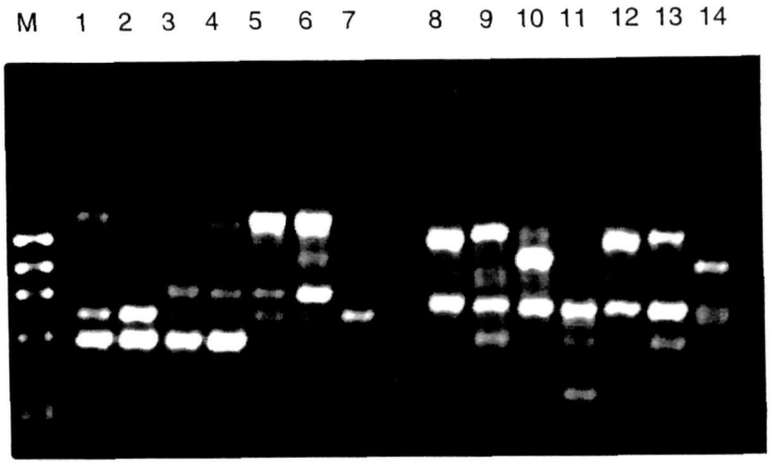


Figure 24 : Agarose gel electrophoretic profiles of genomic DNAs of *N. indica* isolates amplified using repeat (AG)nC (lanes 1-7) and repeat (AG)nG (lanes 8-14). Lane M indicates marker ϕX-174 DNA/*HaeIII* and lanes 1-7 contain isolates Ni1(1), Ni2(2), Ni4(3), Ni5(4), Ni7(5), Ni8(6) and Ni6(7). Lanes 8-14 contain same sequence of *N. indica* isolates as in lanes 1-7

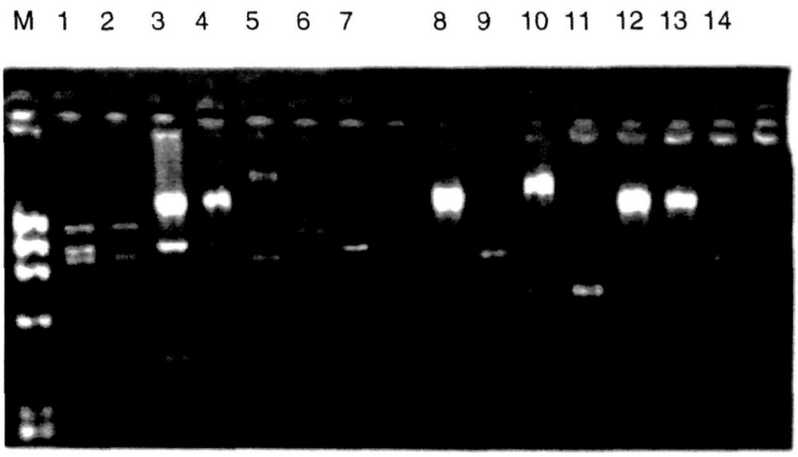


Figure 25 : Agarose gel electrophoretic profiles of genomic DNAs of *N. indica* isolates amplified using repeat (CA)nG (lanes 1-7) and repeat (TC)nC (lanes 8-14). Lane M indicates marker ϕX-174 DNA/*HaeIII* and sequence of isolates in lanes 1-7 and 8-14 is same as given in Fig. 24

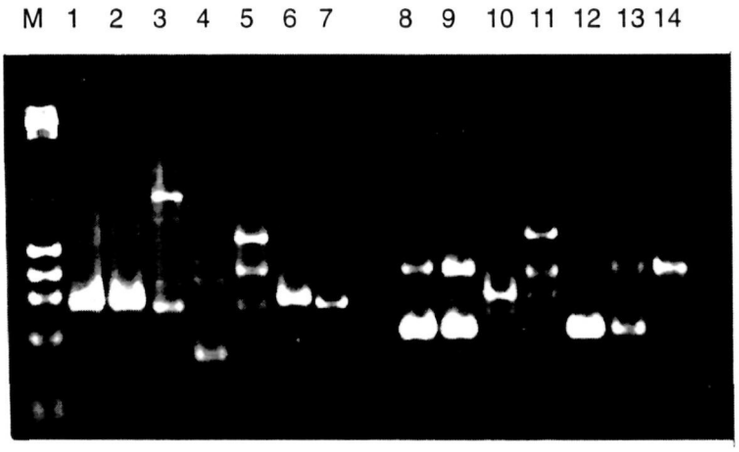


Figure 26 : Agarose gel electrophoretic profiles of genomic DNAs of *N. indica* isolates amplified using repeat (AC)nC (lanes 1-7) and repeat (GA)nYC (lanes 8-14). Lane M indicates marker ϕX-174 DNA/*HaeIII*. Lanes 1-7 and 8-14 contain isolates in the same sequence as given in Fig. 24

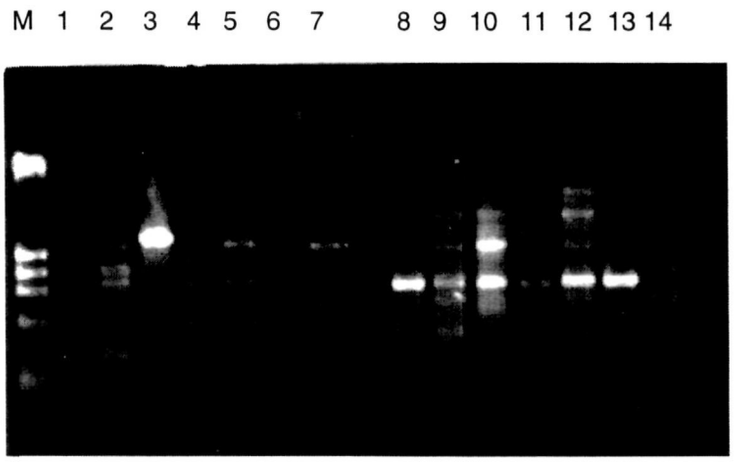


Figure 27 : Agarose gel electrophoretic profiles of genomic DNAs of *N. indica* isolates amplified using repeat (GT)nYC (lanes 1-7) and repeat (ATG)n (lanes 8-14). Lane M indicates marker ϕX-174 DNA/*HaeIII*. Lanes 1-7 and 8-14 contain isolates in the same sequence as given in Fig. 24

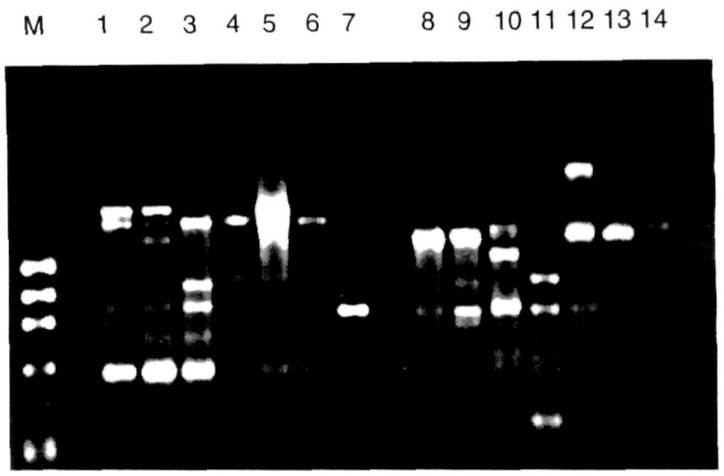


Figure 28 : Agarose gel electrophoretic profiles of genomic DNAs of *N. indica* isolates amplified using repeat (GGAT)n (lanes 1-7) and repeat (GGGTG)n (lanes 8-14). Lane M indicates marker ϕX-174 DNA/*HaeIII*. Lanes 1-7 and 8-14 contain isolates in the same sequence as given in Fig. 24

However, each lane shows a specific pattern thus resulting in clear differentiation of *N. indica* isolates. Figure 28 depicts a view of amplification profiles of tetra- and pentanucleotide repeats $(GGAT)_n$ and $(GGGTG)_n$. Lanes 1 and 2 show similar pattern with $(GGAT)_n$ while a common band of approximately 1.4 kb in size is observed in all the isolates with $(GGGTG)_n$. Isolate Ni6 (lanes 7 and 14) shows one or two bands in each case indicating less number of these repeats in its genome.

a) Variation in amplification at dinucleotide repeat due to 3' and 5' anchor

Table 14 gives the details about amplification profiles of 56 ISSR dinucleotide primers used in our analysis. Out of these, 19 primers give amplification profiles of which only 8 primers are polymorphic. The primers containing $(GT)_n$ repeat give amplification with 70% of the attempted anchors (5 out of 7 primers) suggesting abundance of this repeat in *N. indica* genome. $(GA)_n$, $(AG)_n$ and $(CA)_n$ repeats show amplification with three out of seven primers among which $(GA)_n$ and $(CA)_n$ repeats generate polymorphic profile with just one primer whereas $(AG)_n$ repeat generate polymorphic pattern with all the three amplified primers suggesting the presence of variation at inter $(AG)_n$ repeat loci. $(AC)_n$ repeat shows amplification with two out of seven primers, one of which is polymorphic. The repeats $(TG)_n$, $(CT)_n$ and $(TC)_n$ give amplification with just one primer out of seven, generating monomorphic patterns for $(TG)_n$ and $(CT)_n$ repeats whereas $(TC)_n$ give polymorphic profile. Thus among the dinucleotide repeats, $(GT)_n$ is found to be the most abundant while $(AG)_n$ to be the most polymorphic and thus informative in *N. indica* genome. Differences in amplification profiles are also observed with one repeat primer due to the presence of different anchors at 3'/5' end. As is indicated in Table 14, the dinucleotide repeat $(GA)_n$ shows no amplification product with 3' anchors T, A, YT and

Table 14.: Variation in amplification reaction due to change in anchor

Sr No	Primer No	Repeat	3'/5' Anchor	Effect on amplification profile
1.	UBC819	GT	A	+
2.	UBC820		C	+
3.	UBC821		T	+
4.	UBC849		[a]YA	-
5.	UBC850		YC	+++
6.	UBC851		YG	-
7.	UBC890		[b]VHV	+
8.	UBC810	GA	T	-
9.	UBC811		C	+
10.	UBC812		A	-
11.	UBC840		YT	-
12.	UBC841		YC	+++
13.	UBC842		YG	+
14.	UBC885		[c]BHB	-
15.	UBC807	AG	T	+++
16.	UBC808		C	+++
17.	UBC809		G	+++
18.	UBC834		YT	-
19.	UBC835		YC	-
20.	UBC836		YA	-
21.	UBC884		[d]HBH	-
22.	UBC816	CA	T	+
23.	UBC817		A	-
24.	UBC818		G	+++
25.	UBC846		[e]RT	-
26.	UBC847		RC	-
27.	UBC848		RG	+
28.	UBC888		BDB	-
29.	UBC825	AC	T	-
30.	UBC826		C	+++
31.	UBC827		G	-
32.	UBC855		YT	-
33.	UBC856		YA	-
34.	UBC857		YG	-
35.	UBC889		[f]DBD	+

Table 14. continued

36.	UBC828	TG	A	-
37.	UBC829		C	-
38.	UBC830		G	-
39.	UBC858		RT	-
40.	UBC859		RC	-
41.	UBC860		RA	-
42.	UBC891		HVH	-
43.	UBC813	CT	T	-
44.	UBC814		A	-
45.	UBC815		G	-
46.	UBC843		RA	-
47.	UBC844		RC	-
48.	UBC845		RG	-
49.	UBC886		VDV	+
50.	UBC822	TC	A	-
51.	UBC823		C	+++
52.	UBC824		G	-
53.	UBC852		RA	-
54.	UBC853		RT	-
55.	UBC854		RG	-
56.	UBC887		DVD	-

[a]Y = C,T; [b]V = A,C,G ; [c]B = C,G,T ; [d]H = A,C,T; [e]R = A,G; [f]D = A,G,T

- = No amplification, + = amplification/monomorphic, +++ = polymorphic

5' anchor BHB, monomorphic patterns with anchors C and YG and polymorphic profiles with anchor YC. Similarly repeat $(CA)_n$ shows no amplification product with 3' anchors A, RT, RC and with 5' anchor BDB, generate amplification patterns with anchors T and RG whereas polymorphic profiles are generated with anchor G. Such diverse and polymorphic profiles are evident in Fig. 24 using the repeat $(AG)_n$ with different anchors. The above results suggest that the anchor nucleotide plays an important role in revealing polymorphism in ISSR-PCR amplification reaction probably due to the abundance of these repeats in *N. indica* genome.

b) Generation of unique bands

Table 15 lists the average number of bands per isolate, polymorphic bands between pairs as well as the unique bands obtained with different primers. A pentanucleotide repeat $(GGGTG)_n$ gives maximum number of polymorphic bands (3.6) whereas a dinucleotide $(TC)_n$ repeat generates minimum number of polymorphic bands (0.95). Although pentanucleotide (GGGTG) primer is the most polymorphic primer, the percentage of polymorphism is not as high as dinucleotide primer $(AG)_nG$ which generates almost similar number of polymorphic bands. Some bands are unique to individual *N. indica* isolates as listed in Table 15. Out of 11 polymorphic repeat primers, 8 repeat sequences give amplification profiles which could differentiate all the isolates except isolate Ni1 due to the presence of one or more bands which are specific to a particular isolate. This suggests a need to design more primer sequences to differentiate larger set of *N. indica* isolates. Isolate Ni5 gives maximum number of unique bands followed by Ni4, Ni8 and Ni7 whereas Ni6 and Ni2 show only one unique band each. Isolate Ni1 does not give any

Table 15.: Average no. of polymorphic bands and unique bands generated in *N. indica* isolates

Sequence of Primer	Average no. of bands per isolate n±SD*	Average no. of polymorphic bands between pairs	Unique bands generated	
			Isolate No.	**Mol. wt in bp±SD*
AGAGAGAGAGAGAGAGT	2.71±2.1	1.46	Ni8	306±10.8
AGAGAGAGAGAGAGAGC	3.0±1.3	1.56	---	---
AGAGAGAGAGAGAGAGG	4.85±1.0	3.46	Ni4	1142±10.8
			Ni5	695±10.8
			Ni5	290±10.8
CACACACACACACACAG	4.14±2.3	2.46	Ni8	602 ± 5.97
			Ni8	547 ± 5.97
			Ni8	700 ± 5.97
			Ni4	454 + 5.97
TCTCTCTCTCTCTCTCC	1.71±0.7	0.95	Ni5	1316 + 5.97
			Ni6	927 + 5.97
ACACACACACACACACG	2.42±1.6	1.21	Ni4	2604 + 6.99
			Ni7	1524 + 6.99
			Ni5	1221 + 6.99
			Ni7	1101 + 6.99
			Ni5	996 + 6.99
			Ni5	537 + 6.99
GAGAGAGAGAGAGAYC	1.71±0.7	1.12	Ni5	1524 + 6.99
GTGTGTGTGTGTGTGTYC	3.28±1.6	2.17	---	---
ATGATGATGATGATGATG	4.0±1.5	2.96	---	---
GGATGGATGGATGGAT	4.14±1.9	2.37	Ni2	1682 + 8.6
GGGTGGGGTGGGGTG	6.0±2.44	3.6	Ni4	1505 + 8.6
			Ni4	424 + 8.6
			Ni5	398 + 8.6

*SD = Standard Deviation

**Mol. wt = Molecular weight in bp was estimated by using computer software Sequaid (Rhoads and Roufa 1989)

unique band with any of the primers used in the present study. The overall data thus indicates that a key can be suggested for identification of *N. indica* isolates.

Among the amplification profiles generated with polymorphic repeat primers, a few bands are common to all the isolates. As seen in Fig. 24, a common band of ≈ 700 bp is present in all the isolates (lanes 8-14). Similarly a common band of ≈900 bp is seen in Fig. 27 (lane 8-14) and a band of ≈ 1600 bp is amplified in all the isolates in Fig. 28 (lane 8-14). These common bands may represent the conserved sequences in *N. indica.* and can be further exploited to confirm their specificity for *N. indica* isolates.

c) Cluster analysis based on ISSR data

Based on ISSR gel electrophoretic profiles, similarity indices of *N. indica* isolates were calculated in pair wise combinations for all the primers. These similarity values were averaged and plotted in the form of a similarity matrix as given in Table 16.

Table 16. : Similarity matrix obtained on the basis of ISSR repeat sequences.

Ni1	1.000						
Ni2	0.790	1.000					
Ni4	0.552	0.552	1.000				
Ni5	0.543	0.524	0.571	1.000			
Ni7	0.695	0.695	0.571	0.600	1.000		
Ni8	0.648	0.629	0.543	0.552	0.705	1.000	
Ni6	0.657	0.638	0.552	0.505	0.619	0.610	1.000
OTU:	Ni1	Ni2	Ni4	Ni5	Ni7	Ni8	Ni6

As indicated in the table, that the least similarity index value is 0.52 for the pair Ni2 and Ni5 indicating these isolates to be more diverse and hence do not cluster in the dendrogram. Similarly the maximum similarity index value is 0.79 for the isolates Ni1

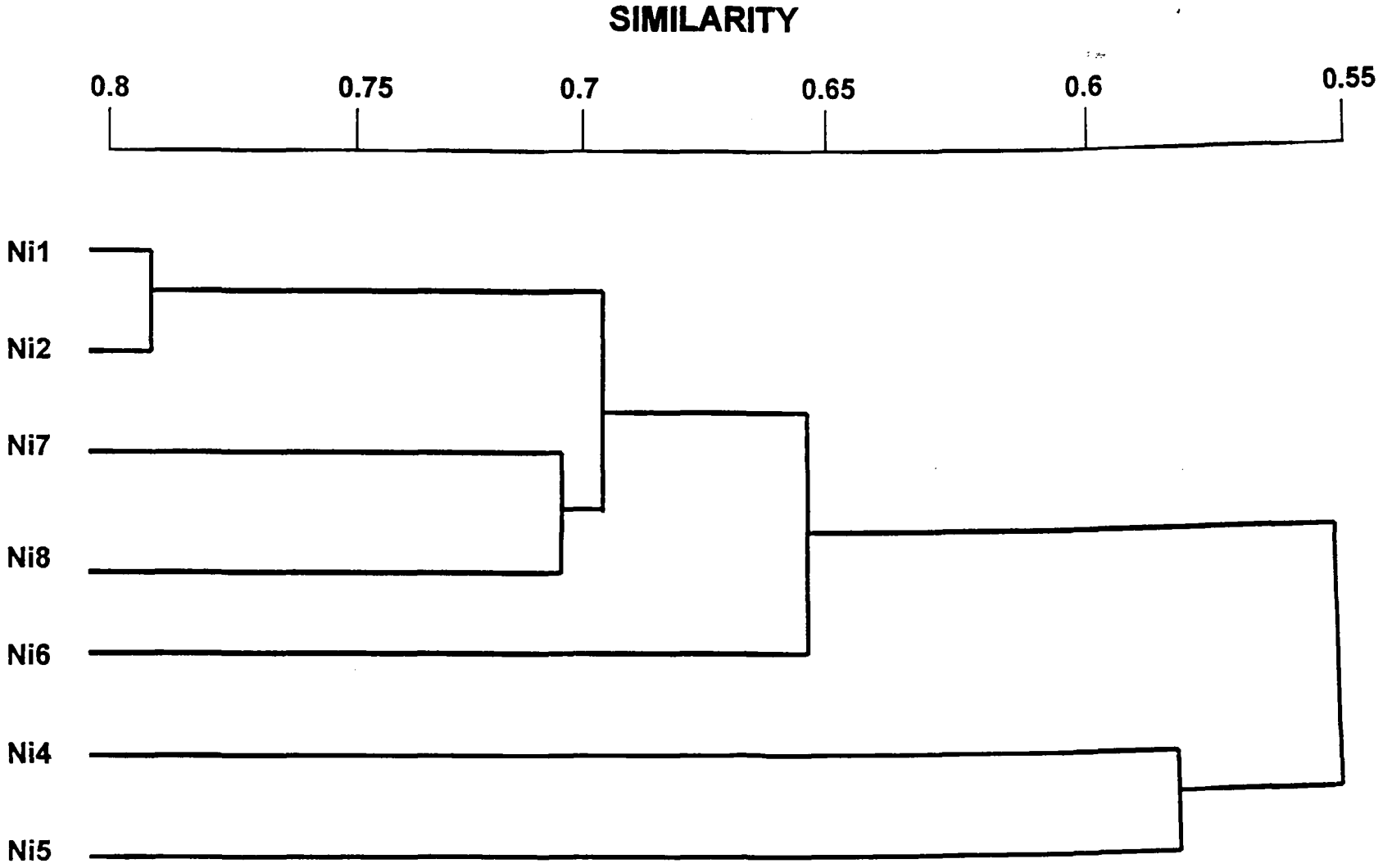


Fig. 29: Dendrogram indicating clustering of *N. indica* isolates based on ISSR marker data

and Ni2 indicating these isolates to be closer genetically and leading to a cluster in the dendrogram.

Molecular data of ISSRs were used to construct a dendrogram as shown in Fig. 29. It can be seen from this figure that at a similarity of 0.65, isolates Ni1, Ni2, Ni7, Ni8 and Ni6 group under one major cluster. In this cluster, isolates Ni1 and Ni2 are the closest and group at 0.8 similarity. Similarly isolates Ni7 and Ni8 group at 0.71 similarity whereas isolate Ni6 groups with these isolates at a comparatively low similarity value of 0.65. Isolates Ni4 and Ni5 group together at a 0.57 similarity making another cluster but join the major cluster at 0.55 similarity thus explaining the least similarity with this cluster. This is supported by maximum number of unique bands obtained with isolates Ni4 and Ni5 in ISSR analysis suggesting that these isolates are genetically more diverse.

3) Cluster analysis on the basis of combined molecular data

The similarity indices of all the pair wise combinations of both the molecular approaches used in the present work were averaged to create an average similarity matrix as indicated in Table 17.

Table 17. : Average similarity matrix obtained on the basis of combined molecular data.

Ni1	1.000						
Ni2	0.673	1.000					
Ni4	0.541	0.566	1.000				
Ni5	0.579	0.541	0.535	1.000			
Ni7	0.789	0.645	0.531	0.550	1.000		
Ni8	0.714	0.657	0.525	0.550	0.717	1.000	
Ni6	0.629	0.881	0.566	0.535	0.619	0.651	1.000
OTU:	Ni1	Ni2	Ni4	Ni5	Ni7	Ni8	Ni6

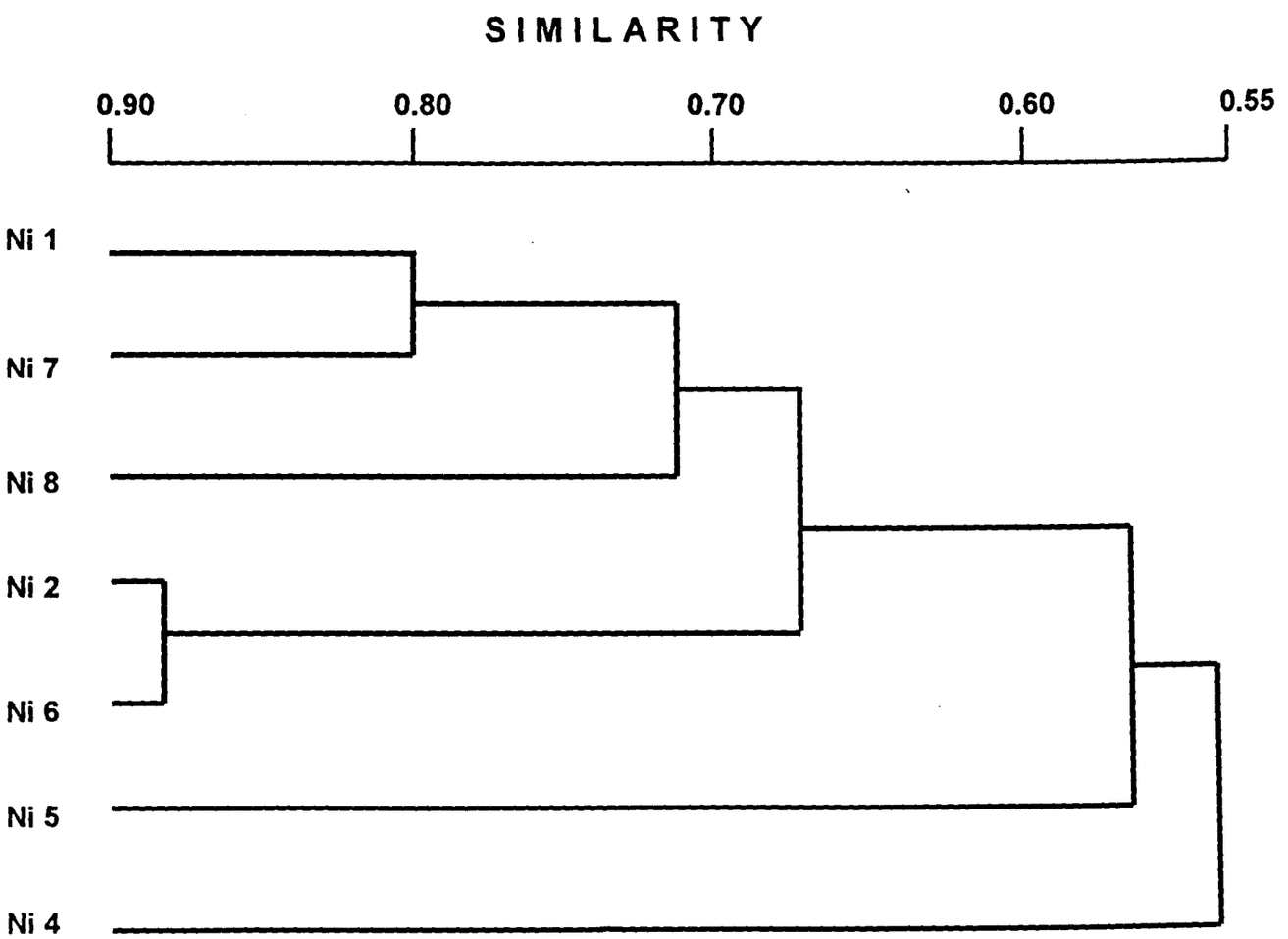


Figure 30 : Average dendrogram indicating clustering of *N.indica* isolates based on molecular data.

The table shows the highest similarity index value of 0.88 between isolates Ni2 and Ni6 indicating these isolates to be genetically closer. Similarly the lowest similarity index value is 0.52 between Ni4 and Ni8 indicating these isolates to be more diverse.

The data obtained using repetitive elements and ISSR markers were combined to generate an average dendrogram as shown in Fig. 30. Isolates Ni1 and Ni7 are found to be the closest in this cluster analysis and the grouping is also supported by the cluster analysis based on biological and repeat element data (Fig. 7 and 23). However, these isolates group away in the dendrogram based on ISSR markers (Fig. 29). Isolates Ni4 and Ni5 show least similarity to rest of the isolates in the dendrograms based on individual markers as well as average dendrogram. Ni2 groups with Ni6 at the highest similarity of 0.88 in average dendrogram but deviates from its position in the individual dendrograms based on molecular data.

4) Correlation between virulence and molecular approaches

Statistical analysis of virulence and molecular data was carried out using similarity matrices to calculate correlation coefficient and to compare it with statistical table at 0.01 probability level. Correlation coefficient of repeat elements and ISSR primers with that of biological data is 0.73 and 0.77, respectively. Such significant values of correlation coefficient indicate a positive correlation between molecular and biological typing. Avirulence data of *N. indica* isolates based on resistant cultivars such as PDW215, TL1210 and HD29, shows low correlation to the molecular data thus suggesting less involvement of avirulence loci as compared to virulence loci in our molecular analysis. Among the repeat elements, pNiR9 and pNiR16 with restriction enzymes *BamHI* and *PstI*, respectively show higher correlation as 0.73 and 0.76, respectively, compared to

other enzymes. Similarly, ISSR repeats $(TC)_nC$ and $(GGGTG)_n$ show high correlation coefficients as 0.78 and 0.76, respectively, suggesting the linkage of these repeats to virulence loci in the fungal genome.

Chapter 4

Discussion

Discussion

1) Importance of Karnal bunt in international scenario
2) Analysis of host-pathogen interactions
   a) Biological typing demonstrates the existence of gene-for-gene relation in *N. indica-wheat* system
   b) More than one resistance genes exist for *N. indica*
   c) Gene-for-gene interaction for minor resistance and avirulence genes
3) DNA markers provide an alternative approach for pathogenicity analysis of *N. indica* isolates
   a) Importance of ISSR markers in *N. indica*
   b) Cluster analysis based on biological and molecular data
4) Conclusions
5) Future perspectives

Discussion

1) Importance of Karnal bunt in international scenario

Neovossia indica causing Karnal bunt of wheat is one of the most important pathogens of wheat and has become significantly important due to following reasons :

i) KB infection alters the chemical composition of grains and reduces the palatability of the flour. Although bunted grains do not produce any harmful toxins, yet the grains with more than 3% infection are rejected by milling industry due to the fishy odour released from spores.

ii) It affects quality and quantity of the grains as Karnal bunt (KB) affected ear heads show less number of grains as compared to healthy ear heads. Moreover, the infected grains are reduced in size in comparison to healthy grains.

iii) Yield losses caused by *N. indica* are not significant however, indirect cost of chemical treatment of seed to restrict spread of KB and avoiding growing of high yielding wheat varieties susceptible to KB leads to substantial financial losses. The cost of quarantine, inspection and value of loss of exports due to KB further add to the financial losses indirectly.

Karnal bunt has become an international issue due to the strong implications of phytosanitary and quarantine regulations on the movement of wheat across the infected countries. In India, major investment is incurred to maintain the domestic as well as international quarantine for exporting disease free wheat. In Mexico, quarantine regulations contribute 50% of the economic cost resulting in substantial economic losses and wheat export as well. Wheat importing countries desire for zero tolerance levels in

order to restrict the entry of pathogen as once introduced *N. indica* cannot be eradicated. Karnal bunt infected countries are supposed to face a challenge posed by the pathogen.

India is the second largest producer of wheat in the world where wheat is consumed next to rice. Since the main product of wheat in Indian sub-continent is chapati, roti and parantha, more than 80% area is under cultivation of *aestivum* bread wheat whereas only 12% of the area is occupied by durum wheat. I, therefore, included more lines from hexaploid wheat than *durum* and *triticale* varieties in the present study. Secondly, the North western region of India is the main wheat growing region. About 90% production of wheat in India is from this region which is affected by Karnal bunt. Identification and differentiation of pathogen populations from affected regions is a prerequisite to design management strategies and for exploiting host germplasm for identification of resistance sources. Realizing the extreme importance of the pathogen, biological and molecular approaches were used in the present study for variability analysis of isolates of *N. indica* from North western region of India.

2) Analysis of host-pathogen interactions

a) Biological typing demonstrates the existence of gene-for-gene relation in *N. indica-wheat* system

In the present study, differential reactions of 13 host lines of wheat to 7 isolates of *N. indica* were carried out. These studies are important in order to understand the host-pathogen interactions and the basis of virulence mechanism. In this section, I have attempted to discuss these interactions with the help of some important results. The differential reactions of host lines to different isolates (Table 2) observed in the present study suggestd the existence of gene-for-gene relationship in this host-pathogen system.

Only certain reaction patterns required to satisfy the existence of gene-for-gene relationship are discussed in more details.

Table 18 : Disease reactions of a pair of host lines against a pair of isolates. : R_1 and r_1 are the alleles for resistance and susceptibility in the host ; and v_1 and V_1 are alleles for virulence/avirulence at corresponding loci for pathogenicity in the pathogen.

Host line	Isolate	
	Ni4 (v_1v_1)	**Ni8 (V_1V_1)**
PBW 248 (R_1R_1)	Susceptible (S)	Resistant (R)
	39.2% disease incidence	1.2% disease incidence
PBW 138 (r_1r_1)	Susceptible (S)	Susceptible (S)
	40.6% disease incidence	34.7% disease incidence

Gene designations in the tables are provisional as no genetic studies have been done to confirm these designations. Table 18 describes the interaction of two isolates Ni4 and Ni8 with that of two wheat cultivars PBW 248 and PBW 138. As it is seen in this Table, isolate Ni4 is virulent on both the host lines whereas the incidence of disease with isolate Ni8 is very low on PBW 248 as compared to that on PBW 138. This behaviour may be explained by assuming that Ni4 possesses a gene for virulence corresponding to the gene for resistance in PBW 248 while isolate Ni8 carries an avirulence allele at this locus for pathogenicity. Little disease incidence of 1.2% observed on PBW 248 with the isolate Ni8 possessing the corresponding gene for avirulence can be explained by the fact that

the pathogen isolates are not distinct pathotypes but are heterogeneous populations. The frequency of occurrence of the avirulence allele in Ni8 isolate population may not be 100%. Thus the low incidence of the disease on PBW 248 may be due to the presence of the allele for virulence (at this locus) in this pathogen population at a low frequency. Assuming that the dikaryotization of compatible mating types takes place inside the host followed by gene-for-gene interaction, the susceptible reaction should occur only if both the mating partners possess the alleles for virulence. If one or both of the mating haploid hyphae have the allele for avirulence, the establishment of disease shall not take place. This argument is in agreement with our present observation that the incidence of disease is highly correlated with the number of infection sites as is evident from Tables 2 and 11 in results. The incidence of disease may, therefore, correspond to the frequency of virulence allele in the pathogen isolate i.e. population. If the frequency of corresponding virulence allele is high, the probability of presence of the alleles for virulence in both haploid mating types will also be high. These arguments are based on the assumption that gene-for-gene interaction takes place after dikaryotization and major gene (s) for resistance interact (s) with the corresponding gene (s) for avirulence. It is of course a possibility that a hypersensitive type of reaction may occur and inhibit the pathogen beyond primary site of infection. This indicates that gene-for-gene hypothesis where for each resistance gene in the host there exists an avirulence gene in the pathogen, holds true for *N. indica*-wheat system.

b) More than one resistant genes exist for *N. indica*

According to gene-for-gene relationship, a pair of host lines needs to give exactly opposite reaction when tested with a pair of isolates if more than one resistance genes are

present in host-pathogen system. Such reactions have been observed among various host/isolate combinations in our study (Table 2) which support gene-for-gene relationship. Only a few examples are listed in the form of Tables 19 A and B.

Table 19 A and B : Disease reactions of a pair of host lines against a pair of isolates. V_1/v_1, V_2/v_2 and V_3/v_3 are alleles for avirulence/virulence in the pathogen corresponding to three resistance loci in the host, R_1/r_1 , R_2/r_2 and R_3/r_3, respectively.

Table 19 A

Host line	**Isolate**	
	Ni4 (V_1 - v_2v_2)	**Ni5 (v_1v_1 V_2 -)**
PBW 34 (r_1 r_1 R_2 R_2)	Susceptible (S)	Resistant (R)
PBW 343 (R_1 R_1 r_2 r_2)	Resistant (R)	Susceptible (S)

Table 19 B

Host line	**Isolate**	
	Ni1 (v_1v_1 V_3 -)	**Ni4 (V_1 - v_3v_3)**
PBW 343 (R_1 R_1 r_3 r_3)	Susceptible (S)	Resistant (R)
PBW 248 (r_1 r_1 R_3 R_3)	Resistant (R)	Susceptible (S)

As indicated in Table 19A, cultivar PBW 34 is susceptible to isolate Ni4 while resistant to Ni5 and cultivar PBW 343 shows exactly opposite reaction. Same holds good for cultivars PBW 343 and PBW 248 with isolates Ni1 and Ni4 as seen in Table 19B. In above cases, the incidence of disease on the resistant line is the function of frequency of allele for virulence at the locus corresponding to the gene for resistance. The lower incidence of disease on the resistant line is mainly due to the less frequency of virulence allele at that locus.

Data in Tables 19A and 19B further suggest presence of more than one resistant genes in the host cultivars and the corresponding more than one avirulence genes in the isolates. Singh *et al.*(1995) have previously observed that the genotypes with two genes for resistance have expressed a higher level of resistance than the genotypes with a single gene for resistance. The probability of presence of virulence alleles at both the loci in dikaryotizing mating types would be the product of square of frequency of virulence alleles at those loci. This probability would be lower in mating partners having the virulence alleles at either of the loci (to the same isolate). Therefore, a line having two resistance genes at two different loci will show low incidence of disease to an isolate than a host line having a resistance gene at either of the loci to the same isolate. Thus the incidence of disease on the host lines possessing no major gene for resistance would be expected as 100.0% or close to 100.0%. However, in our studies an average incidence of 100.0% was not observed on any susceptible line (Table 2) which can be explained as follows : i) Environmental conditions affect the expression of disease (as the establishment of pathogen on host requires specific favorable humidity and temperature conditions during particular stage of heading) and the failure of fusion of opposite mating types for dikaryotization reduces incidence of disease and ii) Minor genes for resistance

may also inhibit the secondary spread from the site of infection through systemic infection.

c) Gene-for-gene interaction for minor resistance and virulence genes

It has been reported that the minor genes can also operate on gene-for-gene basis (Parlevliet & Zadoks, 1977). Figure 31 gives the graphic presentation of differential pathogenic reactions of *N. indica* isolates on three host lines. As shown in this Figure, there is contrasting difference in the incidence of disease on two susceptible lines HD 2329 and WL 711 tested with isolate Ni5. This might be due to the presence of minor genes for resistance at more number of loci in HD2329 than WL 711. However, a lower disease incidence is observed on WL 711 than HD 2329 when tested with isolate Ni7 indicating the occurrence of gene-for-gene interaction for the minor genes also. Although there is no association of low incidence of disease with lower grade of infection, the low grade of infection observed in certain host line/isolate combinations can be attributed to minor genes. However, the wide range of grade of infection observed in many host line/isolate combinations suggests that the expression of grade of infection is highly variable and is probably influenced by environment.

No incidence of disease on PBW 248 with Ni2 and on PBW 34 with Ni8 may be attributed to some sort of general resistance due to hardening of the cell walls of rachis stomata inhibiting primary infection through rachis. Alternatively, the frequency of allele(s) for virulence in these isolates corresponding to the gene(s) for resistance in the host lines may be equivalent to zero.

In the present discussion,although certain assumptions including gene-for-gene interaction after dikaryotization in the host and presence of virulence/avirulence genes in

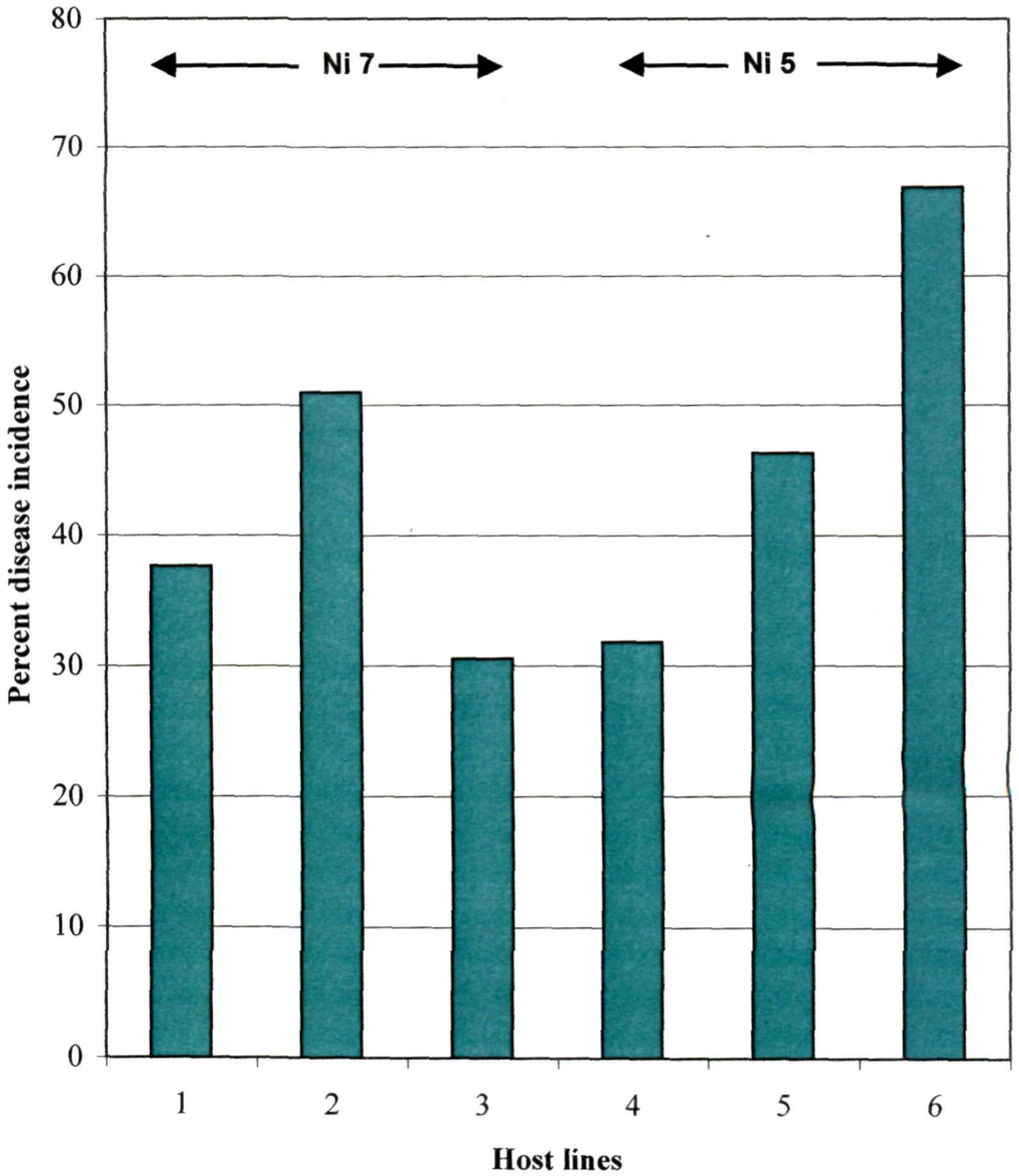


Figure 31 : Differential pathogenic reactions of *N.indica* isolates Ni7 and Ni5 on three host lines HD2329 (1,4), PBW138 (2,5) and WL711(3,6).

the pathogen corresponding to their respective genes in the host, have been made, the contrasting levels of disease in different host line/pathogen isolate interactions do not rule out the existence of gene-for-gene relationship. Therefore, it can be stated safely that the variability among different isolates is due to the variability in the frequency of virulence/avirulence alleles at different loci for pathogenicity. Although the fixation of resistance level at 5% is arbitrary, it makes classification of disease reactions workable so that comparatively more resistant genotypes could be selected for practical use. This indicates that the frequency of corresponding avirulence allele is considerably high in the pathogen isolate (more than 95%) to make the host line practically useful for resistance against that isolate. On the basis of virulence reactions of *N. indica* isolates, it can be suggested that the frequency of virulence alleles is considerably high at maximum number of loci in isolate Ni4 followed by Ni5 and Ni1 (Table 2) whereas it is the lowest in case of isolate Ni2. Previously, similar kind of host-pathogen interaction has been reported for gene-for-gene relationship in three host/pathogen systems namely *Magnaporthe grisea* (Valent *et al.*, 1991), *Melampsora lini* (Flor, 1942) and *Cladosporium fulvum*. (Lindhout *et al.*, 1989). Avirulence (avr) genes in these pathogen isolates have been identified using differential set of cultivars and have been further used for cloning and characterization in order to identify the nature of avirulence genes (De Wit, 1992).

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

Although biological typing provides a valuable information about the pathogenicity of the isolates, it has a few limitations. As it is clear from detailed field analysis of *N. indica* isolates in the present study, the infection and multiplication of fungal spores are highly

influenced by environmental conditions with special reference to temperature and humidity. A change in any of these factors can alter the pathogenic reactions of these isolates using same cultivars in successive seasons. Moreover, biological typing becomes a tedious, laborious and time consuming method while adding more host genotypes to differentiate two fungal isolates showing same pathogenic reactions. Finally, there is a quarantine regulation on transport of pathogenic fungi where the results obtained with a particular set of fungal isolates/host cultivars can not be exploited by the researchers in other parts of the world (Weising *et al.*, 1991). Thus there is a need to attempt more suitable and efficient approaches to overcome the limitations of pathogenicity analysis using biological methods. In recent years several types of DNA markers have been generated and used as most reliable tools for pathogen analysis. I, therefore, attempted to use molecular markers for analyzing agronomically important pathogen, *N. indica*, of wheat.

a) Importance of ISSR markers in *N. indica*

PCR-based methods have been used to assess genetic variability in yeast, fungi and plants (Lieckfeldt *et al.*, 1993; Bebeli *et al.*, 1997). In general pathogenic fungi which can be multiplied and maintained on artificial medium have wide choice of DNA markers such as hybridization based and PCR based markers. However, it is not the same in case of *N. indica* as it is a slow growing fungus and it can not be maintained on artificial medium for longer period. Hence it is difficult to extract large amounts of DNA required for hybridization based markers. PCR based markers were, therefore, used in the present investigation to study the polymorphism among *N. indica* isolates. They are more appropriate for use in *N. indica* as they are simple, fast in operation, and no background

information is required to use such markers. ISSR markers are considered to be more reliable than any other random PCR based markers because the primers used are long enough (16-18 bp) to withstand high annealing temperature thus maintaining stringent amplification conditions as compared to RAPD-PCR in which low annealing temperature is essential due to small (10-mers) size. Secondly, ISSR primers amplify fragments specific to the target sequence due to the presence of anchors (presence of 1 to 3 different bases apart from the core repeat) at 5'/3' position. Such 5'anchored microsatellite primers of random sequences have been recently used for analysis of genetic variation among fungal species of Phycomycetes, Ascomycetes and Basidiomycetes (Hantula *et al.*, 1996) where species specific amplification profiles have been obtained for identification of different fungal species as well as races within a fungal population. Because of such additional advantages of ISSR primers apart from being multilocus in nature, I used them in analysis of Indian isolates of *N. indica*.

In the analysis based on ISSR markers, use of 3' and 5' anchors with dinucleotide repeats has played an important role in generating a number of amplification products. Among dinucleotide repeat primers, $(GT)_n$ has been found to be the most abundant whereas maximum polymorphism is generated with $(AG)_n$ repeat which suggests the occurrence of variation at these loci. Among trinucleotide repeats, $(ATG)_n$ has generated polymorphic profiles whereas monomorphic patterns have been obtained with other repeats suggesting that the loci flanked by such repeats might be conserved in *N. indica* genome. However, a critical analysis of the sequence homology between the amplified products still needs to be explored to confirm the above statement. Monomorphic primers are not useful for variability analysis but provide valuable information regarding the frequency of these repeats in the isolates. The results in the present investigation,

describing a high level of genetic variability among *N. indica* isolates, are supported by the previous findings of Pimentel *et al.* (1998) where they have detected a higher degree of genetic variation among isolates of *N. indica* as compared to its allied species using RAPD markers and ITS region of rDNA as RFLP marker.

b) Cluster analysis of *N. indica* isolates based on biological and molecular data

All the markers including virulence data, repetitive elements and ISSR markers used in the present study have generated specific profiles for *N. indica* isolates. It was observed that the estimated genetic diversity and relative discriminatory power are marker dependent in our study. For example, dendrogram based on virulence typing data has formed two major clusters with outgrouped isolate Ni4 (Fig. 7). Similarly dendrogram based on the repetitive element data has also grouped the isolates according to their virulence patterns showing maximum homology between Ni1 and Ni7 and minimum similarity with Ni4 and Ni5 (Fig. 23). However, a different grouping of *N. indica* isolates has been obtained with ISSR markers. As shown in Fig. 29, isolates Ni4 and Ni5 although show minimum similarity to other isolates, they form a separate cluster at a very low similarity value. Remaining five isolates namely Ni1, Ni2, Ni7, Ni8 and Ni6 show deviation in their positions in this dendrogram compared to other dendrograms. Finally, an average dendrogram (Fig. 30) based on repeat elements and ISSR markers both, has grouped the isolates in a manner similar to that with repeat elements (Fig. 23) except for deviation in the position of Ni2. This dendrogram was similar to that based on biological and molecular approaches together (Figure not shown) except for a minor variation in the scale of similarity. As shown in these studies, *N. indica* isolates Ni4 and Ni5 show highly virulent reaction and are diverse from other isolates on the basis of their virulence pattern

(Datta *et al.*, 1999). These two isolates show least homology on the basis of DNA markers suggesting that they are genetically diverse. Isolates Ni2, Ni6 and Ni8 show low virulent reaction and show less similarity with other isolates and group together in the dendrogram based on combined data. However, their position changes in each cluster analysis suggesting that their placement is not robust. On the contrary, *N. indica* isolates Ni1and Ni7 show similar pathogenic reaction and on the basis of our combined data they cluster together at a high similarity value suggesting that they are genetically similar. Thus the dendrogram clusters the isolates in such a manner that it also reflects on their pathogenic relationships. As it is assumed that isolates showing similar phenotype are genetically related (Canten, 1987), it is very interesting to note that present molecular data support this assumption. It may be possible that the DNA polymorphisms identified in the present investigation somehow reflect on virulent/avirulent loci in the genome arranging them in the virulence order in the dendrogram.

Efforts have been made previously to correlate pathogenicity data with the data generated by molecular markers in a few plant pathogens such as *M. grisea* (Levy *et al.*, 1993) and *Puccinia recondita* f. sp. *tritici* (Kolmer *et al.*, 1995) and a positive correlation has been reported. DNA profiles have also found to be associated with avirulent/virulent population of *Leptosphaeria maculence* by Goodwin and Annis (1991). An association of molecular markers with virulence has been analyzed as a way to supplement the pathogenicity variation analysis on differential cultivars (Leung *et al.*, 1993). The present work shows a positive correlation of molecular markers in general and more specifically with repeat elements pNiR9/*BamHI* and pNiR16/*PstI*, and ISSR repeats $(TC)_nC$ and $(GGAT)_n$, with virulence patterns of *N. indica* isolates on susceptible lines. This needs to be further studied in detail to identify markers for virulence.

4) Conclusion

The present data on biological typing of Indian isolates of *N. indica* has provided some guidelines for exploiting wheat germplasm to identify *N. indica* resistant genotypes. The possible existence of gene-for-gene hypothesis has given an insight about the presence of different resistant genes in the host cultivars. Molecular approaches have been in frequent use for characterization and classification of fungal pathogens. The present investigation is the first report of genetic variability analysis of *N. indica* isolates using repeat elements and 3'/5' anchored SSRs. Identification of repetitive elements has provided a new insight in analyzing population structure of *N. indica* isolates. The preliminary characterization of repeat sequences will greatly help in designing STS markers for molecular analysis as it is well known that maintaining *N. indica* isolates is very difficult and only PCR approach with requirement of low quantity of DNA from teliospores will be a practical strategy for field application. More primers, including additional microsatellite sequences, need to be used to confirm the correlation between genetic diversity and virulence. In conclusion, these molecular markers will be useful to study the geographic distribution of various *N. indica* isolates as well as will monitor the introduction and emergence of more virulent pathogen populations in various geographical areas.

5) Future perspectives

Biological typing is a primary requirement to look for resistant sources in the host germplasm. In the present study, *T. aestivum* cv. HD 29 and *T. durum* cv. PDW 215 have shown maximum immunity to *N. indica* isolates and hence it would be worthwhile to use these lines in the future breeding programmes. The virulence typing has led to an

important finding of the presence of gene-for-gene relationship in this host pathogen system. It will be interesting and worthwhile to use this information as baseline to explore the existence of avirulence genes in the pathogen and in-turn identify resistant (R) genes in the host. The repeat elements identified in the present investigation have been shown to be potential DNA markers for genetic diversity analysis. The information thus obtained can be extended on a larger pathogen population. It would also be worthwhile to subclone and sequence the repeat elements, so as to identify unique sequences conserved in *N. indica*. The ISSR-PCR has also yielded common bands in all the 7 isolates of Karnal bunt and can be further assessed for their use in identification of *N. indica* from its allied species. These approaches thus will be useful in molecular diagnosis of this disease. Thus the preliminary analysis carried out in the present study can lead to many in depth prospective studies in *Neovossia indica*.

Summary

Summary of the thesis entitled 'Biological and molecular characterization of Indian isolates of *Neovossia indica*'

Background information

Neovossia indica, a basidiomycetous fungus is the causal agent of Karnal bunt of wheat in major wheat growing regions all over the world. Wheat grains infected with Karnal bunt emit a fishy odour due to the presence of trimethylamine which makes the products unacceptable for human consumption with just more than 1% infection (Mehdi *et al.,* 1973). Thus the fungal pathogen *N. indica* is important because it causes more quality losses than quantity losses. Though the quantitative losses caused are not much, the investment of funds by Indian agricultural industry for maintaining the national and international quarantine regulations results in decline in foreign exchange and Indian economy. Karnal bunt was considered as minor disease in the begining but it has turned out to be a disease of serious concern after the introduction of high yielding, dwarf wheat varieties in India. It is a major threat to Indian subcontinent due to restrictions imposed on the movement of commercial wheat grain thus bringing economic losses.

The knowledge of pathogen population is vital criteria to assess resistance and guide breeders to incorporate useful R genes in the desired background. Efforts involving virulence typing of *N. indica* have been initiated in the recent years. Bonde *et al.,*(1996) found a significant relation between percent disease incidence and number of infection sites in the infected ear heads by analyzing pathogenic reactions of *N. indica* isolates from India, Pakistan and Mexico. In a more comprehensive study based on virulent reactions of Indian isolates of *N. indica*, Singh *et al.,*(1995) described percentage of

infected grains as an important criterion for selection of early generation material for resistance to Karnal bunt. Although virulence typing gives a valuable information about the pathogenic behaviour of the fungal isolates, this approach is not very efficient regarding reproducibility of the pathogenic reactions of the isolates due to environmental constraints. Among the biochemical markers, isozymes have been used to differentiate Indian and Mexican isolates of *N. indica* (Bonde *et.al.*,1989). However, this approach is more appropriate to study interspecific genetic variations as compared to genetic variability analysis within the species. Use of DNA based markers provide an alternative approach to study reproducible and reliable differentiation of the isolates under present study. Molecular analysis of different species of *Tilletia* was done using RAPD markers (Bonde *et.al.*, 1996; Shi *et.al.*, 1996; Gang & Weber 1996). A species specific marker of *T. indica* was identified using mitochondrial DNA as a probe (Ferreira *et.al.*,1996).

Objectives

Present work was initiated with the following objectives:

- Assessment of pathogenic reactions of all the isolates on various host cultivars to study their virulence patterns and their interaction with the host system.
- To identify homologous markers from λ ZAP II library of *N. indica* isolate Ni7 for assessment of genetic variability.
- To assess the potential of PCR based markers (ISSR) in differentiation of *N. indica* isolates, which can be further useful in isolate identification, and differentiation.
- To assess the genetic diversity among Indian isolates of *N indica* using virulence typing and molecular markers. It involves the use of selected number of isolates from

different geographic regions of India, so as to serve as a base for analysis of larger pathogen population.

Results

Important research findings are summarized under following headings.

1) Pathogenicity analysis of *N. indica* isolates

N. indica isolates Ni1-Ni8 were collected, isolated, revived, purified, multiplied and maintained on PDA (Potato-Dextrose-Agar) slants. All the isolates were tested for their field reactions on a set of host genotypes of durum wheat, triticale and aestivum wheat. Inclusion of different wheat genotypes will help to facilitate breeding programs involving identification and transfer of resistance genes from resistant durum and triticale genotypes to popular hexaploid wheat cultivars.

Percent disease incidence and number of infection sites were the two main parameters used to analyze the pathogenic reactions of *N indica* isolates with respect to their host cultivars. Disease data were scored and results were concluded by devising a scale : Resistant (R) = 5.0% disease incidence, Moderately resistant (MR) = 6-10% and Susceptible > 10%. Based on this scale, contrasting percent disease incidence was observed on the cultivars against same isolate. It was found that isolate Ni4 gave high disease incidence on *Triticum aestivum* cv. PBW248 (39.2%) whereas no disease was found to be present on *T. aestivum* cv. HD29 (0.4%). Similarly data was analyzed using same cultivar against two isolates and it was found that host line *T. aestivum* cv Sonalika was highly susceptible (23.9%) against isolate Ni1 but resistant (2.8%) against isolate Ni6. Out of 13 wheat lines used for pathogenic reactions of *N .indica* isolates even the resistant host lines such as *T. durum* cv PDW215, *Triticosecale* cv. TL1210 and *T.*

aestivum cv. HD29 were distinguished on the basis of their reaction patterns to individual isolates. Thus it was suggested that different KB resistant genes probably existed in these cultivars and there was significant genetic diversity for pathogenicity in *N. indica* isolates. A positive correlation was found between between percent disease incidence and number of infection sites per ear head. Coefficient of correlation between these two parameters was as high as 0.92 and high degree of determination (r^2=0.84) indicated that the percent incidence of disease was highly influenced by infection sites per earhead.

2) Identification and characterization of repeat elements from *N. indica*

Repetitive elements were used in the present diversity analysis due to a few advantages. Repetitive sequences are dispersed throughout the genome, thus a single repeat element can assess many loci simultaneously thus saving considerable time and effort in grouping the isolates. Sufficient polymorphism can be detected by using multicopy clones as they are the prime sites of mutations in comparison to single copy or low copy clones.

A genomic library of *N. indica* isolate Ni7 was constructed in λ ZAP II system and was screened to isolate putative repetitive clones. Amount of total repetitive DNA was calculated to be 1% of total genomic DNA of *N. indica* which was confirmed by the calculations of insert size and copy number of the three identified repeat clones. Repetitive elements were isolated and were tested for their potential as repetitive probes on the basis of the radioactive signal obtained with plaque lifts. All the repetitive clones were used for molecular analysis. These repetitive elements generated complex hybridization profiles producing fingerprint patterns of all the seven *N. indica* isolates under present study. Molecular weights of the repeat elements pNiR9, pNiR12 and pNiR16 were calculated to be 3700 bp, 4800 bp and 500 bp, respectively. Further

characterization of these repeat clones was done by restriction endonuclease mapping and copy number estimation. Restriction endonuclease map of pNiR9 showed one internal site each for restriction enzymes *BamHI, HindIII and XbaI.* Approximately 32 copies of pNiR9 were estimated to be present in the genome. Restriction endonuclease map of pNIR12 showed two internal sites for *BamHI* and one internal site each for *PstI, XhoI* and *XbaI.* Approximately 61 copies of pNiR12 were estimated to be present in the genome. Restriction endonuclease map of repetitive element pNiR16 did not show any internal site for restriction endonucleases used in the present study. Approximately 64 copies of pNiR16 were found to be present in the genome. Copy no. of repetitive elements estimated in the present investigation is supported by the previous findings (Hammer *et al.*, 1989).

Identification of repetitive elements will provide a new insight in analyzing population structure of *N. indica* isolates, useful in future wheat-breeding programs.

3) Genetic diversity analysis of *N. indica* isolates

a) Genetic diversity analysis of *N. indica* isolates using repeat elements

Only three restriction enzymes *BamHI, EcoRI* and *PstI* were finally used for variability analysis. Repetitive clones pNiR9, pNiR12 and pNiR16 were used to study their potential as probes for diversity analysis and the variability existing in *N. indica* isolates. Repeat clones pNiR9 and pNiR16 generated more number of bands with *EcoRI and PstI* digested genomic DNAs of *N. indica* isolates suggesting higher number of copies of these repeats in specific environment. However pNiR16 did not give any scorable pattern with *BamHI* genomic DNA digests. The repeat element pNiR12 was not much dispersed in the genome but the hybridization profiles obtained with all the three restriction enzymes

suggested the presence of at least one internal site for *PstI* and two internal sites for *BamHI*.

b) Genetic diversity analysis of *N. indica* isolates using ISSR-PCR markers

Inter-simple-sequence-repeat (ISSR) markers are the markers in which a single anchored microsatellite primer is used to generate complex fingerprint patterns in plants and animals due to multilocus nature of microsatellites (Zeitkiewicz *et.al*, 1994 ; Gupta *et.al* , 1994). ISSR-PCR was performed using primers of 16-18 bp in length with 3'/5' anchors, obtained from University of British Columbia (USA). Seventy-seven primers were used to analyze their potential to study genetic variation among *N. indica*. Eleven primers were polymorphic generating reproducible and highly specific electrophoretic profiles. Dinucleotide repeats $(GA)_n$ and $(GT)_n$ were found to be abundant in the genome. Most of the tri, tetra and penta-oligonucleotides did not show amplification with *N. indica* DNAs. Change in the anchor at 3'/5' position of ISSR primers generated variable patterns in all the isolates except Ni1, using different primers. Isolate Ni5 was found to have maximum number of unique bands followed by Ni4, Ni8, Ni7, Ni6 and Ni2. Cluster analysis based on molecular data showed that at a similarity value of 0.65 most of *N. indica* isolates were grouped under one major cluster. The isolate Ni4 was found to be the most virulent and outgrouping in the dendrogram as well.

Conclusion

Potential of biological and molecular approaches including virulence typing, genomic repetitive elements (RFLP) and Inter-simple-sequence-repeats (PCR) was examined to study the genetic diversity among Indian isolates of *N. indica*. Our studies with *N. indica* isolates showed the presence of different KB resistant genes in the host lines resulting in more genetic variation. Three repetitive elements identified from genomic library were

found to be good, polymorphic probes which can be further subcloned and sequenced so as to identify unique sequences in *N. indica*. ISSR markers generated reproducible polymorphic patterns along with common bands in all the isolates. Thes approaches will be useful in molecular diagnosis of *Neovossia indica*.

List Of Publications

1) **R Datta,** Harjit- Singh, V.S. Gupta, P.K. Ranjekar and H.S. Dhaliwal (1999) Differential host-pathogen interaction of cultivated wheats with karnal bunt (*Neovossia indica*) : Existance of gene - for - gene relation. **Accepted for publication in Plant Breeding.**

2) 2) **R Datta,** M.D. Rajebhosale, H.S. Dhaliwal, Harjit- Singh, P.K. Ranjekar and V.S. Gupta. (1999) Intraspecific genetic variability analysis of *Neovossia indica* causing Karnal bunt of wheat using repetitive elements. **Accepted for publication in Theoratical and Applied Genetics.**

3) 3) **R Datta,** M.D. Rajebhosale, H.S. Dhaliwal, Harjit- Singh, P.K. Ranjekar and V.S. Gupta. (1999) ISSR-PCR : a useful tool for genome analysis of *Neovossia indica.* **To be communicated shortly.**

Conferences attended

1) International Symposium on Bacterial genetics and Pathway engineering , from June 4,1997 - June 6,1997 held at NEERI, Nagpur.

2) International conference on Disease and pest management for sustainable agriculture from Nov. 10,1997 - Nov.15, 1997 held at IARI, New Delhi.

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