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Differentiation of Indian isolates of *Neovossia indica* by RAPD-PCR and clustering based on teliospore morphology

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Teliospore morophology of Indian isolates of *Neovossia indica* were studied by light and scanning electron microscopy. The data subjected to ANOVA showed significant difference at 1% level among the isolates. UPGMA analysis divided the isolates into two major clusters. The ultrastructure of the isolates were found to be substantially similar. The isolates analysed by random amplified polymorphic DNA analysis (RAPD), yielded polymorphic bands and UPGMA clustering of these divided the isolates into major clusters. The cluster analysis is indicative of the fact that variability exists among various isolates belonging to different geographical parts of Northern India. The clustering based on RAPD analysis has no correlation with the geographical distribution as well as their grouping based on teliospore morphology. Teliospore morphology is a variable character, therefore it is imperative to use approaches involving DNA polymorphism for searching variability among fungal isolates.

Key words : Variability, Karnal bunt, teliospore morphology, RAPD

INTRODUCTION

Karnal bunt or partial bunt of wheat, caused by *Neovossia indica* [Mitra] Mundkur (= *Tilletia indica* Mitra), was first reported from India on *Triticum aestivum* L., (Mitra, 1931). It has since been reported from Mexico, Afghanistan, Pakistan, Nepal, Iraq and Iran Infection of wheat grains has also been reported from Arizona and California, USA.

Moderate temperature, high relative humidity or free moisture, cloudiness and rainfall during anthesis has been reported to favour disease development (Agarwal *et al.*, 1976 ; Bedi *et al.*, 1949).

Since Karnal bunt is seed borne disease in addition to being air and soil borne, strict quarantine regulations exist regarding movement of wheat into USA and other countries where Karnal bunt has not yet established (Loke and Watson 1975). Due to several interceptions of teliospores in wheat shipments entering the USA from Mexico, it has become a significant concern to the USA wheat industry and USDA.

Variability in pathogenicity, morphological, cultural, physiological, biochemical and izozyme analysis characteristics of *N. indica* have been studied (Bonde *et al.*, 1984).

Historically, smut fungi have been described, classified and identified based on the host characteristics of the sori and teliospores. It is generally assumed that smut fungi are host - specific, but recent studies have revealed that the determination of relationships between smut fungi is much more complicated. many apparently distinct species are able to hybridize, but to what extent is unclear. Moreover, often there is very little variation in teliospore morphology useful for distinguishing species. In the recent studies additional morphological as well as molecular characteristics have been undertaken to elucidate the existence of variability among diferent isolates of *N. indica* prevalent in India.

MATERIALS AND METHODS

Collection of isolates

Twenty samples of *N. indica* on wheat were collected from various parts of Northern India viz., Punjab, Haryana, Himachal Pradesh, Uttar Pradesh, Delhi and it's vicinity during April, 1997 and April, 1999 (Table 1). The teliospores of these samples were taken from infected heads and subjected to following investigations.

Morphological Studies

I) Light microscopy : One hundred dried teliospores of each sample were first bleached in concentrated H_2O_2 for 30-40 min to enable their counting of the number of meshes per teliospore diameter and characteristics of reticulum. Samples were rehydrated in Shear's mounting fluid and observed at 2000X magnification with an Olympus BX50 microscope. Measurements for length, width of the teliospores and sterile cells. gelatinoid sheath, width and height of reticulum and length of apiculus were taken with the help of ocular micrometer.

II) Scanning Electron Microscopy : Air dried teliospores were dusted on small pieces of double sided adhesive tape, mounted on specimen stub, sputter-coated with goldpalladium under vacuum, ca. 20 nm for 4.5 min, 7.5 mA. The specimens were then observed and photographed in a LEO 435 VP SEM, operated at 15kv following the procedure of Vanky (1997).

Production of monosporidial lines

Teliospores of *N. indica* are dikaryotic in nature and hence it is essential to obtain monosporidial haploid cells for molecular analysis. Teliospores from single sorus of each isolates were surface sterilized and dusted over petri plates containing 15-20 ml sterile distilled water and incubated at 17°C for 15 days. The floating teliospores were steaked on the surface of Potato - Dextrose Yeast extract agar (PDA + 0.1% Yeast extract) with the help of a sterile pasture pipette and incubated at 17°C. The whitish fungal colonies developed after 2-3 days of incubation and started producing sporidia after another 3-4 days. The monosporidial lines of these isolates were obtained by using chamber micromanipulator and kept at 17°C in Potato Dextrose Yeast extract agar for about 3 weeks to produce pure colonies. After the formation of colony, the cultures were transferred to Potato Sucrose Broth (PSB) at 17°C on an orbital shaker incubator at 120 rpm for 20 days and subsequently the mixtures of sporidia and mycelial growth in PSB from single sporidial cultures were harvested by filtration through Whatman filter paper No 41. These cultures were then rinsed with sterile distilled water and frozen at -20°C. The frozen samples were used for DNA extraction.

DNA Extraction

Because of the slow growth of *N. indica*, a modified procedure from the cetyltrimethylammonium bromide (CTAB) method of Shi *et al.* (1996) was followed. 0.2-0.3g of compressed frozen mycelium of each isolate was grounded in pre - cooled sterile mortar and pestle using liquid nitrogen. The fine frozen powder was transfered to a sterile centrifuge tube. Pre-warmed at 65°C for 1 h. After incubation an equal volume of chloroform / isoamyl alcohol (24:1) was added and mixed gently for centrifugation at 10000 rpm, 25°C for 10 min. The aqueous phase was transferred to a new sterile tube and the DNA was precipitated with 0.6 volume of cold isopropanol and 0.1 volume of Sodium acetate (3M, pH 5.2) for 25 min at room temperature. DNA was pelleted by centrifugation at 10000 rpm for 10 min at 25°C. The pellet was washed with cold 70% ethanol and resuspended in 100 µl dH_2O and frozen at -20° until needed for PCR amplificatioin.

Purification of DNA

A stock solution of RNase-A was prepared @10 mg/ml in 10mM Tris-HCl pH 8.0 and 15mM Sodium Chloride. The solution was boiled for 10 min to destroy DNase From the stock, 2µl of RNase-A was added to the crude DNA sample and incubated at 37°C for 1 h. The DNA concentration of the samples and its purity was determined by taking ultraviolet obsorbavce at 260 nm and 280 nm in a spectrophotometer.

Random Amplification of Polymorphic DNA (RAPD) conditions

Twenty five 10 mer oligonucleotides from sets P, S, M and N (Operon Technologies, Inc, USA) were used as single primers for the amplification of sequences. The PCR reaction was performed in a Gene-Cycler (Bio-RAD, USA), in a 25 µl volume containing 4 µl of 1X reaction buffer (10 mM Tris-HCl, pH 8.3 and 50 mM KCl), 5 mM $MgCl_2$, 0.5 unit of Taq DNA polymerase, 100 µM each of dATP, dCTP, dGTP and dTTP (GENEI, Bangalore, India), 1 µM of 10 mer primer and 25 ng of template DNA. The amplification conditions were : initial step of denaturation at 94°C for 2 min followed by 40 cucles of denaturation at 92°C for 1 min, primer annealing at 37°C for 5 min. Samples of 25 µl PCR products were mixed with 3 µl of 10X loading dve (0.25% bromophenol blue 0.25% evanel and 40% sucrose W/V) and spun briefly in a microfuge before loading. The amplification products were analysed on 1.2% agarose gel contaning 0.2 µg/ml of ethidium bromide in 1X TAE buffer running at 60 volts for three hours and visualized under UV light and photographed on Polaroid 667 film under UV light. The DNA size marker used was λDNA double digested with *Eco*RI and *Hin*dIII.

Data analysis

1) *Morphological data analysis*

The data obtained from morphological studies was analysed by two methods viz.,

I) Analysis of Variance :The Analysis of Variance (ANOVA) was performed by using GLM procedure of PC SAS (SAS Institute, 1989). The mean comparison of the studied characters of the isolates was separately done using Duncan's Multiple Range Test (DMRT).

II) *Hierarchical Cluster Analysis*

The dissimilarity matrix was calculated from standardised morpjological characters data using Euclidean measure of distance. Unweighted pair group method using arithmetic average (UPGMA) was selected to generate grouping.

2) *RAPD data analysis*

Each amplification product was considered as RAPD marker. Gels were scored on the basis of the presence (1) or absence (0) of each band for all isolates. All amplifications were repeated at least twice and only reproducible bands were considered for analyses. Jaccard's similarity coefficient values for each pairwise comparison between isolates were calculated and a similarity coefficient matrix was constructed. This matrix was subjected to unweighted pair-group method for arithmetic average analysis (UPGMA) to generate a dendrogram using average linkage procedure. All the numerical taxonomic analysis were conducted using software NTSYS-pc, version 1.80 (Exeter software,NY).

RESULTS

The teliospores of twenty isolates of *N. indica* were subjected to morphological characterisation for the parameters like, teliospore length and width, sterile cells length and width, gelatinoid sheath thickness and length of apiculus (Table 2). One hundred teliospores were taken up from infected wheat from 26.76 µm for Ni6 to 32.76 µm respectively. The teliospores did not show much variation as to the number of meshes per spore diameter as well as length and width of reticulation. The average thickness of gelationoid sheath of all the isolates of *N. indica* was 4-7 µm. The data for apiculus length

Table 1: Different isolates of *Neovossia Indica* collected from various parts of Northern India.

Isolate No.	Location	Date of collection	Isolate No.	Location	Date of Collection
Ni1	Delhi	Apr. 97	Ni11	Yamuna Nagar, Haryana	May. 98
Ni2	HAU, Hissar, Haryana	Apr. 98	Ni12	Kashipur, U.P.	May. 98
Ni3	HAU, Hissar, Haryana	Apr. 98	Ni13	Muzaffarnagar, U.P.	May. 98
Ni4	HAU, Hissar, Haryana	Apr. 98	Ni14	Yamuna Nagar Haryana	May. 98
Ni5	HAU, Hissar, Haryana	Apr. 98	Ni15	Roper, Punbjab	May. 98
Ni6	PAU, Ludhiana, Punjab	May, 98	Ni16	Pant Nagar, U.P.	May. 98
Ni7	PAU, Ludhiana, Punjab	May, 98	Ni17	Batala, Punjab	May. 98
Ni8	Gurdaspur, Punjab	May, 98	Ni18	Bilaspur, U.P.	May. 98
Ni9	Muzaffarnagar, U.P.	May, 98	Ni19	Muzaffarnagar, U.P.	May. 98
Ni10	Jalandhar, Punjab	May, 98	Ni20	HUA, Hissar, Haryana	Apr. 98

Table 2 : Comparison of the mean of studied characters of *Neovossia indica* using Duncan's Multiple Range Test.

Isolate no.	Spore length		Spore width		S*. Cell Length		S*. Cell Width		Meshes per spore diam.		Reticulim width		Reticulim Height		Giatinoid Sheath		Apiculu
	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean
Ni1	30.00	D	27.78	BC	21.96	CD	17.75	GHI	8.93	F	3.83	AB	4.11	BC	5.11	CD	0.58
Ni2	28.47	E	26.16	E	19.45	E	16.19	K	8.56	KI	3.87	A	4.31	A	5.45	A	0.61
Ni3	30.89	CD	24.92	F	21.38	DE	17.52	GHI	8.47	FGH	3.80	AB	4.22	AB	5.19	BC	2.26
Ni4	32.07	AB	26.60	DE	24.42	A	18.96	CDH	8.61	GHI	3.79	ABC	4.3	A	5.36	AB	2.45
Ni5	28.28	EF	25.32	F	20.63	EFG	17.08	IJ	8.67	GH	3.74	BC	4.12	BC	5.06	CDE	0.96
Ni6	26.79	G	24.09	G	21024	DE	18.01	FGH	8.44	I	3.78	ABC	4.02	CDE	5.10	CDE	1.26
Ni7	26.86	G	23.34	G	18.21	I	15.65	K	8.53	HI	3.67	CD	4.08	BCD	5.02	CDEF	1.42
Ni8	27.36	FG	23.89	G	19.80	GH	16.41	JK	8.78	FG	3.61	D	4.00	CDE	4.99	CDEFG	1.03
Ni9	30.83	CD	26.31	DE	21.37	DE	17.14	IJ	9.20	F	3.37	E	3.79	F	4.77	HI	1.18
Ni10	31.76	ABC	26.20	DG	23.25	B	19.69	BC	9.22	DE	3.31	EF	3.62	G	4.62	I	1.17
Ni11	32.37	AB	29.39	A	24.34	A	20.81	A	0.41	CD	3.19	FG	4.11	BC	5.11	CD	1.33
Ni12	31.94	ABC	27.07	CD	23.18	B	20.00	B	9.56	BC	3.21	FG	3.82	F	4.81	GH	1.01
Ni13	31.82	ABC	28.41	B	22.82	BC	18.61	EF	9.72	AB	3.17	G	4.07	BCD	5.09	CDE	0.75
Ni14	32.76	A	30.07	A	21.51	DE	18.74	EF	9.77	AB	3.15	G	4.11	BC	5.07	CDE	1.13

Table 3 : Correlation of characters of different isolates of *Neovosia indica.*

	Spore length	Spore width	S*Cell length	S*Cell width	Meshes per Spore dim.	Reticulum width	Reticulum height	Gelatinoid Sheath	Apiculus length
Spore Length	1.00								
Spore Width	0.842	1.00							
S*Cell Length	0.679	0.513	1.00						
S* Cell Width	0.731	0.620	0.895	1.00					
Meshes per spore dim.	0.761	0.780	0.263	0.513	1.00				
Reticulum Width	–0.675	–0.660	–0.250	–0.531	–0.951	1.00			
Reticulum Height	–0.196	–0.049	0.023	–0.179	–0.488	0.588	1.00		
Gelatinoid Sheath	–0.220	–0.057	0.031	–0.173	–0.509	0.607	0.980	1.00	
Apiculus length	0.084	–0.331	0.313	0.106	–0.349	0.300	0.238	0.203	1.00

* : Sterile cells

Table 4 : ANOVA of 20 isolates of *Neovossia indica for the studied characters*

sv	df	MS								
		Length length	Width width	Length length	Width width	Meshes/Sp. Spore dim.	Width width	Height height	Gelatin Sheath	Apicul. length
Isolate	19	374.641	345.152	263.541	177.022	24.120	8.303	3.776	4.541	21.258
Error	1980	12.537	8.078	11.263	7.049	0.475	0.170	0.308	0.388	5.036
CV (%)	—	11.60	10.61	15.59	14.67	7.49	12.02	13.84	12.45	194.00

varied as the samples examined consisted of both young and mature teliospores. In young teliospores the length of the apiculus went up to 10 µm whereas in mature teliospores it was very small or even absent.

Under SEM teliospores showed prominent thick projections of outer layer which were irregular with blunt margins due to which the surface looked rough.

The morphological characteristics were also evaluated in terms of correlation between various parameters (Table 3). It was observed that width and height of reticulum are negatively correlated with teliospore and sterille cells length and width. The thickness of gelatinoid sheath was found to be negatively correlated with teliospores length and

width. The other parameters showed a positive correlation with each other.

The ANOVA showed significant differences at 1% level for and between the isolates of *N. indica* (Table 4). The high coefficient of variance obtained for apiculus length is due to the fact that the observation varied from 0-10 µm owing to the presence of both young and mature teliospores.

The denderogram obtained for morphological characters divided the *N. indica* isolates into two major clusters, "A" cluster consists of Ni14, Ni20, Ni13, Ni12, Ni16, Ni11, Ni15, Ni18, Ni9, Ni19, Ni17, and Ni10 and "B" cluster consists of Ni1, Ni3, Ni4, Ni7, Ni8, Ni5, Ni6 and Ni2. All the isolates from U.P. belonged to cluster "A" whereas isolates from Punjab and Haryana were distributed in both "A" and "B" cluster. The major clusters "A" and "B" were further divided into sub groups. These did not show any correlation as to the geographical distribution (Fig. 1).

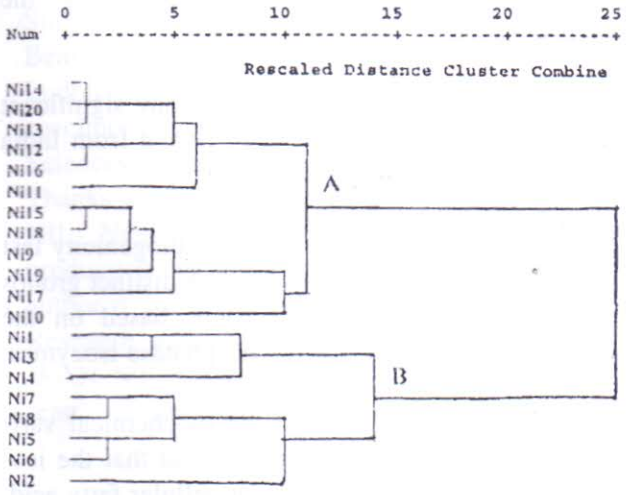

Fig. 1 : Dengrogram of *Neovossia indica* isolates resulting from UPGMSA cluster analysis based different characters of teliospores and sterile cells.

The isolates of *N.indica* were subjected to purification by obtaining monosporidial lines. The hyphae were then multiplied on potato sucrose broth to raise mycelia mass for DNA extraction. However, only 11 isolates of *N. indica* (Ni1, Ni4, Ni5, Ni8, Ni9, Ni10, Ni11, Ni12, Ni13, Ni18 and Ni20) yielded sufficient mycelium for DNA extraction and were further taken up for RAPD analysis.

Optimisation of RAPD markers

Good quality DNA of concentration 100 ng/µl was obtained from approximately 200-300 mg of frozen mycelial mass. Standardization of the PCR protocol was done by changing the variables like template DNA, Taq DNA polymerase and magnesium chloride. The optimum amplification was obtained by using the reaction mix having 25ng template DNA, 0.5 unit Taq polymerase, 5mM $MgCl_2$, 1 µm primer and 100 µM dNTP in reaction volume of 25 µl.

Twenty five random 10 mer Operon primer from OPP, OPS, OPM and OPN series were screened. Seventeen primers were selected for doing RAPD analysis on the basis of reproducible and scorable polymorphic bands obtained (Table 5).

Table 5 : Primers used and the number of total and polymorphic bands produced in *Neovossia indica* isolates.

Primer	Sequence (3'-5')	Total bands	Polymorphic bands	Polymorphic percentage	Monomorphic bands
OPM06	CTGGGCAACT	9	9	100	0
OPM07	CCGTGACTCA	8	8	100	0
OPM13	GGTGGTCAAG	5	5	100	0
OPN02	ACCAGGGGGA	NA	NA	NA	NA
OPN05	ACTGAACGCC	NA	NA	NA	NA
OPN17	CATTGGGGAG	NA	NA	NA	NA
OPN01	GTAGCACTCC	NA	NA	NA	NA
OPP02	**TCGGCACGCA**	**18**	**17**	**94.40**	**1**
OPP03	**CTGATAGGCC**	**18**	**18**	**100**	**0**
OPP04	GTGTCTCAGG	12	12	100	0
OPP06	**GTGGGCTGAC**	**17**	**14**	**82.35**	**3**
OPP08	ACATCGCCCA	13	13	100	0
OPP09	**GTGGTCCGCA**	**18**	**18**	**100**	**0**
OPP10	TCCCGCCTAC	NA	NA	NA	NA
OPP16	CCAAGCTGCC	7	7	100	0
OPP19	GGGAAGGACA	11	11	100	0
OPP20	GACCCTAGTC	12	12	100	0
OPS01	CTACTGCGCT	4	4	100	0
OPS02	CCTCTGACTG	NA	NA	NA	NA
OPS03	CAGAGGTCCC	NA	NA	NA	NA
OPS05	TTTGGGGCCT	12	12	100	0
OPS06	GATACCTCGG	NA	NA	NA	NA
OPSO8	TTCAGGGTGG	6	6	100	0
OPS09	TCCTGGTCCC	4	4	100	0
		174	170		4

* Not Amplified

RAPD analysis

A total of 174 bands were obtained for the 17 primers investigated out of which 170 were polymorphic. Primers OPP2, OPP3 and OPP9 amplified maximum number of polymorphic bands. The size of the fragments obtained varied from 0.5 to 4 kb.

UPGMA cluster analysis

The data obtained from RAPD analysis of 11 isolates of *N.indica* with 17 primers was subjected to UPGMA analysis. A dendrogram for these 11 isolates was prepared using the similarity coefficient of RAPD marker. The UPGMA clustering also divided the isolates of *N. indica* into two major groups. Out of this 4 isolates from U.P. subjected to cluster analysis, 3 of them Ni12, Ni13 and Ni18 belong to cluster "II". In cluster "I" there were two subgroups cousisting of Ni1, Ni9, Ni4 and Ni5 belonged to "Ia" and Ni10 and Ni11 belonged to "Ib". Cluster "II" also showed two major divisions "Ic" consisting only of Ni12 and "Id" having Ni13, Ni18 and Ni20. All the isolates in cluster "II" of RAPD analysis belong to cluster "A" of morphologically similar characters.

DISCUSSION

The variablility within species and generation of smut fungi is a product of genetic recombination during sexual reproduction. The progeny from a single generation may differ from each other and from the parent in virulence, host specificity and other characteristics (Fischer and Holton,1957).

Physiological specialization in *N. indica* was first reported by Mitra (1931). He observed differences in the size of teliospores collected from Karnal, India and Peshawar, Pakistan and speculated two races.

Mitra (1935) regarded two different collections of *N. indica* as two physiological forms differing in teliospore size.

Munjal (1970) differentiated seven physiologic races on the basis of serology and their host reaction. The pathotypes could not be differentiated on the basis of the morphological studies as the shape and colour of teliospores in the samples resembled a lot. Four pathotypes in 21 collections of *N. indica* from Punjab and Himachal Pradesh, India have been reported (Aujla *et al.*, 1987).

Singh and Singh (1988) reported that since teliospore size is influenced by environmental variation, there are no large differences in size of the teliospores from different locations and varieties, hence it can not be used as a differentiating character for the collections of *N. indica.*

Observations of Bansal *et al.*, (1984) indicated that average size of teliospores from various samples of wheat varieties collected from Punjab, Haryana and Uttar Pradesh differed significantly but the teliospore size from Himachal Pradesh and Jammu and Kashmir did not differ significantly within themselves in comparison to the type specimen. Apparently there is no difference in teliospore size between the type specimen and the samples studied. He concluded that the teliospore size is a slightly variable character and is influenced by the environmental factors.

Peterson *et al.*, (1984) did not find any significant differences among isolates of *N. indica* from India and Mexico for teliospore diameter.

Sharma *et al.*, (1998) carried out pathogenicity test and isozyme analysis and grouped 5 distinct groups using pathogenicity and 2 groups based on the analysis of esterase and acid phosphatase isozymes.

Singh *et al.*, (1998) studied the biochemical variability of different isolates and found that the isolates varied considerably in their cellular fatty acid. Lipids analysis could not distinguish between the strains, but autoradiography allowed the strains to be distinguished clearly.

SEM studies of teliospores of *N. indica* by Aggarwal *et al.*, (1998) showed that episporium surface looked rough and the stretchability of perisporium did not keep pace with the advancing maturity. Khanna and Payak (1968) also reported that teliospore projections (exospore ornamentation) under light microscopy were truncate with

flattened to occasionally curved tips that sometimes develop tears or become forked. From the results obtained, it is concluded that the ultrastructure of the different isolates of *N. indica* were found to be substantially similar.

The cluster analysis is indicative of the fact that variability exists among different isolates of *N. indica* between and within different geographical regions of Northern India. It points towards the fact that further investigation are needed to closely elucidate the variability spectrum of this important pathogen in relation to it's pathogenicity.

The results of this preliminary study based on RAPD analysis indicate that DNA fingerprints generated can be used as a tool in distinguishing isolates of *N. indica*.

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