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Genetic Variability among Different Populations of Root Knot Nematodes Based on Their Encumbrance Response to *Pasteuria* Isolates Using PCR-RFLP

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A great variable response was observed when PP-3 and PP-J encumbered with 116 populations of root knot nematode (RKN) at two different temperatures (25 ± 2°C and 30 ± 2°C) and concentrations (10^4 and 10^5 spores/ml). The PCR reaction amplified intergenic region between cytochrome oxidase subunit II gene (COII) and large subunit of rRNA gene (lrRNA) of the mitochondrial genome of different RKN species. The primer C2F3 and 1108 identified *M. incognita* with the highest frequency (52.6%) followed by *M. javanica* (36.8%) and *M. arenaria* (10.5%). The sizes of PCR products were 1.7 kb for *M. incognita* and *M. javanica* populations while populations of *M. arenaria* produced 1.1 kb fragment. The digestion with *Hinf* I yielded three different fragment length patterns on 1.5 % agarose gel. From current research it is concluded that intra-*Meloidogyne* genetic variability exist in RKN populations which have better encumbrance with *P. penetrans*.

Keywords **:** attachment, bacterial parasite, genetic diversity, root knot nematode

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Pasteuria penetrans has potential for its development as a biological control agent of economically important root knot nematodes (Sayre and Starr, 1985). These hyperparasites start their infection by adhering to the cuticle of infective juveniles (J2s), then penetrate the body wall to enter the host root, usually after the nematode has set up a feeding site (Chen et al., 1997). They either act as restraining the nematode migration toward the roots or by reducing their reproduction (Brown and Smart, 1985; Davies et al., 1988).

Adhesion of spores to the cuticle plays a vital role in the activity of the pathogen when it is used as a biological nematicide (Stirling et al., 1986). Attachment studies of isolates of *Pasteuria* to different populations and strains of RKN showed a high degree of variation and it is host specific (Davies et al., 1988; Espanol et al., 1997).

This obligate parasitic bacterium exhibits host specificity at two stages in the nematode life cycle; firstly, endospore isolates differ in their ability to encumber the J2s of particular species and strains of root-knot nematodes (Davies, 2005; Davies et al., 2001) and second, endospore populations differ in adhesion to different life stages of the same strain as exemplified by differential attachment to males (Davies and Williamson, 2006).

Identification of RKN is a prerequisite to develop an effective cropping system, resistant cultivars and biocontrol agents (Cenis, 1993). Molecular approaches have made significant contribution to the detection and identification of RKNs (Randig et al., 2002; Ward et al., 2004). Polymerase chain reaction (PCR) based techniques are a

strong diagnostic tool for accurate detection of pathogens and have also been widely used for the identification of nematodes (Han et al., 2004; Henson and French, 1993; Martin et al., 2000; Schaad and Frederick, 2002). However, no comprehensive study has been conducted for genetic variability assessment in RKN in Pakistan using molecular tools. Hence, the present study was aimed to *in-vitro* encumbrance of *P. penetrans* with different RKN populations at different temperatures and concentrations, and to identify intra-*Meloidogyne* genetic variability in RKN populations which have better encumbrance with *P. penetrans,* collected from different ecological regions of Pakistan through PCR-RFLP of an intergenic region between cytochrome oxidase subunit II (COII) and 16S ribosomal mitochondrial genes.

Materials and Methods

Survey for the occurrence of different root knot nematodes in vegetable production areas of Punjab-Pakistan. A systematic survey was conducted for a reliable estimation of infestation of root knot nematode major vegetable production area of five Districts of Punjab Province [Faisalabad, Jhang, Khanewal, Multan and Rawalpindi (Fig. 1)]. From each sampling site ten samples were collected at random. Root samples were carefully lifted with trowel up to 15-20 cm depth from the rhizosphere of tomato and cucumber plants together with approximately 1 kg of adhering soil. Samples were put in polythene bags and data on host, locality and soil type etc. was recorded.

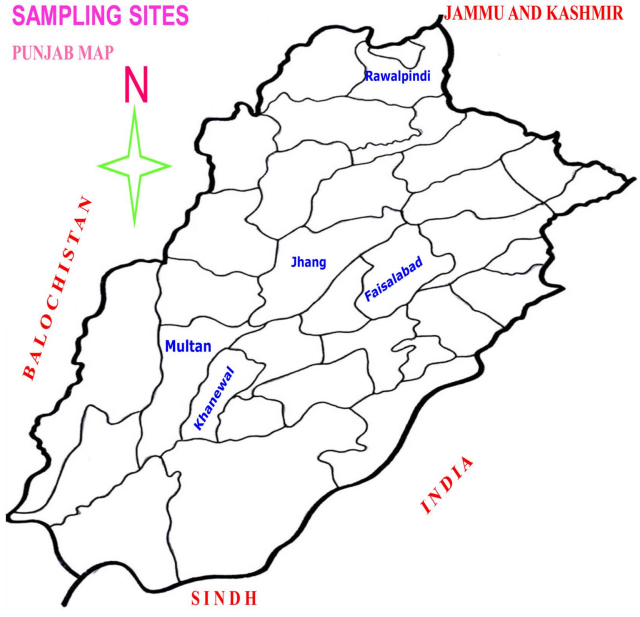

Fig. 1. Map of Punjab-Pakistan showing the five sampling Districts.

A total of 700 samples were collected from two vegetable hosts i.e. tomato and cucumber (350 samples from each host). These samples were transported in Nematology Laboratory of Plant Pathology Department, University of Agriculture, Faisalabad-Pakistan and were stored in refrigerator at 5°C (40°F) until processing.

In vitro encumbrance of *P. penetrans* with different root knot nematode populations at different temperatures and concentrations

KN populations used for encumbrance test. A total of 116 populations of RKN (two population/sampling site) were selected for their encumbrance to two isolates of *Pasteuria* (PP-3 and PP-J). The selection of these populations was made on the basis of RKN incidence in different sampling sites of five District of Punjab, Pakistan on two hosts (tomato and cucumber). During survey 27 out of 35 sampling sites were infested with RKN in case of tomato while in case of cucumber 31 out of 35 sampling sites were infested with RKN, hence 54 RKN populations (27 × 2 = 54, two RKN populations/sampling site) were selected from tomato and 62 RKN populations (31 × 2 = 62, two RKN populations/sampling site) were selected from cucumber.

Nematode inoculums. All RKN populations were purified by single egg mass culture and maintained on susceptible tomato cv. Money maker throughout the experiment. Root system bearing egg masses of each *Meloidogyne* population was washed, cut into pieces and treated with 1% Sodium hypochlorite (to facilitate the release of eggs from egg masses) two days before setting experiment (Hussey and Barker, 1973). The resulting egg suspension was placed in extraction dishes and incubated at 28°C for hatching. The newly hatched juveniles were collected after 2 days of incubation period and used in attachment studies.

Pasteuria endospore concentrations. Dry root powder (100 mg) containing mature endospores of two isolates of *Pasteuria* (PP-3 and PP-J) were grinded with a pestle and mortar in two ml of water. More water was added and sieved through 38 µm sieve (Stirling and Wachtel, 1980) and two suspensions of *Pasteuria* endospores were prepared in water (10^4 and 10^5 spores/ml) using Haemocytometer. Dilutions of the suspension were made using the following formula;

$$\frac{\text{Actual concentration}}{\text{Concentration to be made}} = \text{Dilution factor}$$

One ml of the spore suspension was pipetted to each Petri dish (5 cm in diameter) containing one ml of nematode suspension (50 J2s/ml). To facilitate the counting suspension was diluted by adding one ml of water. Each treatment was replicated ten times, completely randomized and incubated at 25 ± 2°C. Similarly, another set was prepared and incubated at 30 ± 2°C in an incubator.

Estimation of spore attachment. The Petri dishes were checked after one hour of exposure time and the number of spores attached/J2s on first 10 nematodes was counted under the microscope at 400× magnification. The mean of ten counts was calculated as one observation for each replication. Same procedure was repeated for each nematode population isolated from different hosts.

Assessment of genetic variability among different populations of root knot nematodes using PCR-RFLP of COII and large subunit of rRNA gene (lrRNA) of the mitochondrial genome

Nematode populations used in PCR. A total of 19 RKN populations (from tomato and cucumber) were subjected to PCR based analysis of intra-*Meloidogyne* genetic variability. The populations were selected on the basis of their encumbrance to two isolates of *P. penetrans* in the *in-vitro* encumbrance experiments (Table 1). Seventeen RKN populations were selected on the basis of their maximum encumbrance. Two populations which produced minimum encumbrance as compared to all the *Meloidogyne* populations were also included in the study.

DNA extraction. Total genomic DNA was extracted from single J2 using worm lysis buffer (WLB) [50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% Tween 20 (Sigma-Aldrich, UK), 0.01% gelatine, 60 µg/ml proteinase K (Fermentas, USA)]. An individual J2 was placed in 10

Table 1. List of 19 root-knot nematode populations selected on the basis of their encumbrance to two *Pasteuria* isolates (PP-3 and PP-J) and used in species differentiation using PCR-RFLP marker

Encumbrance	District	Tomato*		Cucumber**	
		PP-3	PP-J	PP-3	PP-J
Maximum	Faisalabad	FSD/T8	-	FSD/C4	FSD/C12
	Jhang	JNG/T11	JNG/T3	JNG/C2	-
	Khanewal	KHW/T6	KHW/T2	KHW/C9	KHW/C3
	Multan	MUL/T3	MUL/T10	MUL/C2	-
	Rawalpindi	RWP/T3	RWP/T7	RWP/C10	RWP/C2
Minimum	Rawalpindi. RWP/C5, RWP/C6 (with same response to PP-3 and PP-J)				

*FSD/T8, JNG/T11, KHW/T6, MUL/T3, RWP/T3, JNG/T3, KHW/T2, MUL/T10, RWP/T7 = RKN population code from tomato.
**FSD/C4, JNG/C2, KHW/C9, MUL/C2, RWP/C10, FSD/C12, KHW/C3, RWP/C2 = RKN population code from cucumber.

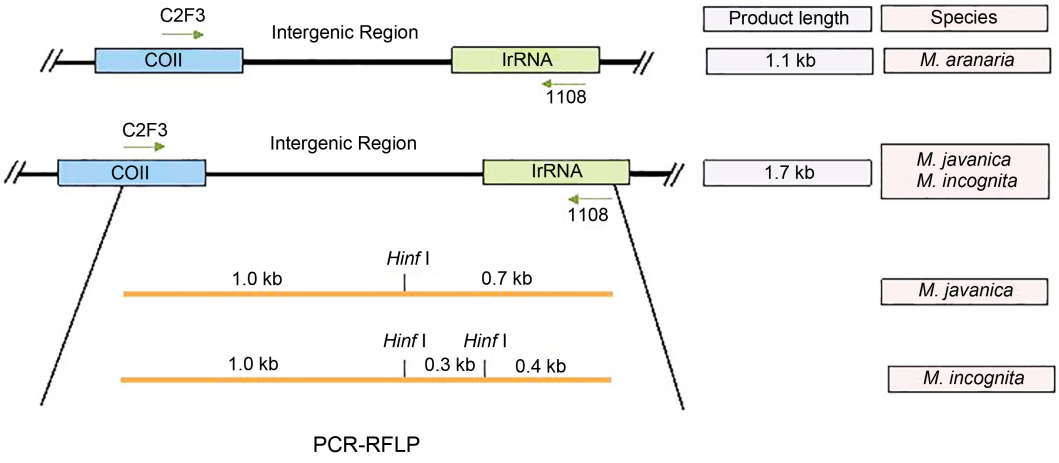

Fig. 2. Diagrammatic representation of primer binding sites on the *Meloidogyne* mitochondrial genome. Primer C2F3 anneals to the coding strand of the cytochrome oxidase subunit II (COII) gene and primer 1108 anneals approximately 450 bp downstream from the start of the lrRNA gene. The intergenic region varies in size among the different *Meloidogyne*.

Table 2. Composition of PCR reaction mixtures assembled for characterization of 19 root-knot nematode populations

Reagent	Concentration	Volume (µl)
PCR buffer	10×	2.0
$MgCl_2$	25.0 mM	1.6
dNTPs	2.5 mM	1.0
Primer forward (C2F3)	30.0 ng/µl	1.5
Primer reverse (1108)	30.0 ng/µl	1.5
Taq DNA polymerase	5.0 units/µl	0.2
Template DNA	-	5.0
Double distilled de-ionized water	-	7.2
Total volume		20.0 µl

Table 3. Temperature cycles used in PCR performed for characterization of 19 root-knot nematode populations

Steps	Temperature	Time	Number of cycles
Initial denaturation	94°C	5 min	1 (first)
Denaturation	94°C	60 s	
Annealing	55°C	60 s	35
Extension	72°C	1 min	
Final extension	72°C	7 min	1
Hold	4°C	Until turned off	

µl of WLB on a sterilized glass microscopic slide, cut into half with sterile micropipette tip, pressed, and lysate was transferred into 0.5 ml PCR tube. The tubes were frozen at –20°C for 10 min, incubated at 65°C for one hour followed by 95°C for 10 min and then immediately placed on ice. An aliquot of 5 µl of the suspension was used in PCR.

Polymerase chain reaction (PCR). The molecular characterization was conducted following protocol reported by Powers and Harris (1993). A fragment of intergenic region between COII and large subunit of rRNA gene (lrRNA) of the mitochondrial genome was amplified using forward primer C2F3 (5′-GGTCAATGTTCAGAAATTTGTGG-3′) and reverse primer 1108 (5′-TACCTTTGACCAATCACGCT-3′). Detailed description of primer design is given in Fig. 2. Polymerase chain reaction was performed in 20 µl reaction volume in 0.2 ml PCR tubes in a thermal cycler (Mastercycler gradient, Eppendorf Germany). Composition of the reaction mixture and temperature cycles is given in Table 2 and 3, respectively.

Gel Electrophoresis and Analysis. The PCR products were resolved on 1.5% agarose gel. In brief, agarose powder was dissolved completely in by sprinkling slowly over the stirring 1× Tris-Borate-EDTA (TBE) buffer and melted completely by heating in microwave oven in short intervals avoiding excessive foam formation and spitting out of the flask. Ethidium bromide was added at 0.5 µg/ml after cooling the solution to ~60°C and the solution was poured into the gel casting tray. After polymerization, the gel was placed in electrophoresis tank. The 6× DNA loading dye was added to the products and 10 µl of the mixture was pipetted into wells. First and last well of the gels were loaded with 1 kb DNA ladder (Fermentas, USA) for determination of exact size of amplicons. Electrophoresis was performed in 1× TBE buffer at 80 V for 1 h. Gels were visualized and documented using gel documentation system (NYXTECHNIK, UK).

Restriction analysis of PCR products. The PCR products of 1.7 kb in size were further subjected to restriction analysis to discriminate between *M. incognita* and *M. javanica* species through digestion with *Hinf* I enzyme. A 20 µl restriction reaction was assembled using 10 µl of amplified product, 7 µl of nucleases free water, 2 µl of 10× buffer R [10 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/ml BSA] and 1 µl (10 units) of *Hinf* I enzyme (Fermentas, USA). Contents were gently mixed, briefly centrifuged and incubated at 60°C for two hours. An aliquot of 10 µl of the restriction mixture was resolved on 1.5% agarose gel, stained with ethidium bromide and visualized through gel documentation system as described above.

Data analyses. Data were subjected to ANOVA and differences among the means were partitioned at $P = 0.05$ according to least significant difference (LSD) test (MSTAT version 3.1).

Results

In vitro encumbrance of *P. penetrans* with different RKN populations at different temperatures and concentrations

Encumbrance of PP-3 with different RKN populations at different temperatures and concentrations isolated from tomato. A great variable response was observed when PP-3 encumbered with different populations of RKN at two different temperatures (ambient and 30 ± 2°C) and concentrations (10^4 and 10^5 spores/ml). The encumbrance level of 54 populations of RKN isolated from tomato varied significantly (Table 4). Interspecific and intraspecfic variability in encumbrance of PP-3 with different populations of RKN was demonstrated. The encumbrance of PP-3 at concentration of 10^4 spores per ml at ambient temperature was as low as below ten spores attached to different populations of RKN collected from five District of Punjab. Rate

Table 4. Encumbrance of two *Pasteuria* isolates (PP-3 and PP-J) with different populations of *Meloidogyne* spp. isolated from tomato

Districts	Locality	Population code	*Meloidogyne* spp.	PP-3				PP-J			
				25 ± 2°C		30 ± 2°C		25 ± 2°C		30 ± 2°C	
				10^4	10^5	10^4	10^5	10^4	10^5	10^4	10^5
Faisalabad	JK farm	FSD/T1	*M. incognita*	3.93 cd[1]	13.70 bcd	12.63 de	19.87 e	15.57 ef	24.73 c	35.77 d	37.73 f
		FSD/T2	*M. incognita*	4.23 cd	14.30 bcd	14.87 bc	21.67 bcd	18.33 b	26.27 b	37.07 c	44.37 bc
	Jahangir Mor	FSD/T3	*M. incognita*	5.50 bc	14.80 bc	15.07 b	22.57 bc	16.63 de	23.73 cd	32.73 g	43.00 cd
		FSD/T4	*M. incognita*	4.66 cd	14.30 bcd	14.53 bc	21.83 bcd	18.03 bc	24.47 c	34.27 ef	43.33 cd
	Dhasian	FSD/T5	*M. incognita*	3.60 d	13.73 bcd	13.93 bcd	21.70 bcd	14.50 fg	19.70 fg	28.00 i	36.47 f
		FSD/T6	*M. incognita*	6.53 ab	15.37 ab	15.30 b	23.10 b	19.90 a	27.83 a	38.30 b	45.57 ab
	204RB	FSD/T7	*M. incognita*	3.93 cd	12.80 d	12.47 e	20.73 de	17.03 cd	21.67 e	33.13 fg	42.30 de
		FSD/T8	*M. incognita*	7.66 a	16.73 a	18.17 a	25.17 a	20.80 a	28.70 a	40.27 a	46.50 a
	53GB	FSD/T9	*M. incognita*	3.90 cd	13.33 cd	13.90 bcd	20.90 de	16.47 de	20.37 f	32.27 g	41.33 e
		FSD/T10	*M. incognita*	4.13 cd	14.40 bcd	14.33 bc	22.67 bc	14.80 fg	19.97 fg	31.93 g	42.03 de
	Nathu Chak	FSD/T11	*M. javanica*	6.96 ab	14.67 bcd	14.50 bc	22.57 bc	16.77 de	23.20 d	34.60 de	44.33 bc
		FSD/T12	*M. javanica*	3.73 d	13.33 cd	13.57 cde	21.40 cd	13.97 g	19.07 g	29.53 h	36.73 f
		LSD		1.425	1.63	1.286	1.267	1.108	0.9727	1.222	1.464
Jhang	Al Hafiz Farm	JNG/T1	*M. incognita* + *M. javanica*	6.20 ab	15.03 bc	14.87 abc	22.93 bcd	18.53 b	27.63 a	39.07 a	45.30 abc
		JNG/T2	*M. incognita* + *M. javanica*	2.96 d	13.13 f	13.43 cd	21.67 def	15.83 cd	21.03 f	33.20 f	42.43 e
	Chak No.204 JB	JNG/T3	*M. incognita*	4.93 bc	14.70 cde	15.00 abc	22.17 cde	20.53 a	27.90 a	40.07 a	45.87 a
		JNG/T4	*M. incognita*	3.33 d	13.10 f	13.10 d	19.80 g	18.37 b	26.87 ab	37.70 b	44.50 abcd
	Kosar Abad	JNG/T5	*M. incognita*	3.73 cd	14.77 bcd	14.20 bcd	22.50 bcde	17.73 b	24.10 e	35.03 de	44.17 cd
		JNG/T6	*M. incognita*	6.50 a	15.10 bc	15.63 ab	23.60 abc	18.53 b	27.87 a	39.60 a	43.90 cd
	Basti Usmana	JNG/T7	*M. javanica*	3.93 cd	13.70 cdef	13.63 cd	20.47 fg	18.13 b	25.53 cd	37.00 bc	45.73 ab
		JNG/T8	*M. javanica*	2.90 d	13.47 def	13.17 d	21.03 efg	15.00 d	19.93 g	34.33 ef	39.90 f
	Kot M.Yar (Shorkot)	JNG/T9	*M. incognita*	5.86 ab	14.73 bcd	15.00 abc	21.97 def	18.33 b	26.27 bc	37.07 bc	44.37 bcd
		JNG/T10	*M. incognita*	3.93 cd	13.30 ef	13.60 cd	21.70 def	16.60 c	24.73 de	36.27 bcd	43.67 de
	Gulgusht Farm	JNG/T11	*M. incognita*	6.80 a	16.57 a	16.27 a	24.90 a	15.37 d	21.03 f	36.13 cd	42.47 e
		JNG/T12	*M. incognita*	5.96 ab	15.73 ab	15.90 a	23.93 ab	17.63 b	25.77 cd	37.40 bc	44.27 bcd
		LSD		1.285	1.296	1.480	1.379	1.015	1.037	1.347	1.357
Khanewal	Khaliqa Abad	KHW/T1	*M. javanica*	3.53 bc	14.4	15.03 ab	22.57 ab	15.70 c	21.20 d	33.40 c	37.63 d
		KHW/T2	*M. javanica*	4.76 abc	14.9	15.43 a	22.57 ab	19.00 a	26.63 a	38.30 a	45.60 a
	Chak No. 125/15L	KHW/T3	*M. incognita* + *M. javanica*	3.60 bc	13.7	13.67 bc	21.43 bcd	16.30 c	23.73 b	36.00 b	41.00 c
		KHW/T4	*M. incognita* + *M. javanica*	5.30 ab	14.7	15.00 ab	21.97 bc	17.57 b	26.00 a	36.67 b	43.63 b
	10 Kassi	KHW/T5	*M. incognita*	3.03 c	13.9	13.97 bc	21.03 cd	13.63 d	17.77 e	28.27 d	35.73 e
		KHW/T6	*M. incognita*	5.46 a	15.0	15.53 a	23.37 a	16.50 bc	22.80 bc	34.23 c	40.20 c
	Tulamba	KHW/T7	*M. incognita*	3.63 bc	13.6	13.50 c	20.57 d	16.63 bc	22.03 cd	34.97 bc	39.90 c
		KHW/T8	*M. incognita*	4.03 abc	14.0	14.27 abc	22.67 ab	16.33 c	23.87 b	36.33 b	43.90 b
		LSD		1.63	-	1.333	1.237	1.131	1.166	1.620	1.299

of spore attachment at 10^5 spores per ml was approximately double that at 10^4 spores/ml (Table 4). At 30 ± 2°C, the encumbrance level also increased in all the RKN populations. The results revealed that with the increase in temperature and spore concentration the mean encumbrance level of *Pasteuria* with J2 cuticle increased. At least one population of RKN was selected from each District having maximum encumbrance with PP-3. These populations were FSD/T8 (Faisalabad), JNG/T11 (Jhang), KHW/T6 (Khanewal), MUL/T3 (Multan) and RWP/T3 (Rawalpindi).

Table 4. Continued

Districts	Locality	Population code	*Meloidogyne* spp.	PP-3				PP-J			
				25 ± 2°C		30 ± 2°C		25 ± 2°C		30 ± 2°C	
				10^4	10^5	10^4	10^5	10^4	10^5	10^4	10^5
Multan	Kotla Sadat	MUL/T1	*M. incognita*	4.9	15.03 ab	14.93 abcd	23.13 ab	17.73 bc	24.80 cde	35.80 de	41.40 c
		MUL/T2	*M. incognita*	5.8	14.97 ab	15.03 abc	22.47 abc	18.53 b	26.07 abc	37.37 bc	45.23 a
	Qadarpur	MUL/T3	*M. incognita*	5.9	15.73 a	15.47 a	23.90 a	18.07 b	25.57 bcd	36.57 cd	43.13 b
	Raan	MUL/T4	*M. incognita*	4.5	14.60 abc	15.17 ab	23.20 ab	16.07 de	20.73 g	33.63 fg	36.50 e
	Kambir Pur	MUL/T5	*M. incognita* + *M. javanica*	4.2	14.63 abc	13.93 bcd	22.77 ab	17.97 b	23.33 ef	34.80 ef	37.67 de
		MUL/T6	*M. incognita* + *M. javanica*	3.1	13.63 c	13.57 d	20.83 c	15.07 ef	20.90 g	33.13 g	37.37 de
	Kian Pur	MUL/T7	*M. incognita*	4.9	15.20 ab	15.03 abc	23.27 ab	17.80 bc	27.00 ab	38.40 b	46.00 a
		MUL/T8	*M. incognita*	4.4	14.57 bc	14.33 abcd	21.83 bc	15.87 def	22.00 fg	34.50 ef	38.20 d
	Abu-Al-Fatah	MUL/T9	*M. incognita*	4.0	13.70 c	14.03 abcd	22.03 bc	14.70 f	17.90 h	29.37 h	37.00 de
	Kotla	MUL/T10	*M. incognita*	4.3	14.97 ab	14.93 abcd	22.63 ab	19.70 a	27.57 a	39.77 a	46.23 a
	Saiqque	MUL/T11	*M. incognita*	4.1	13.73 c	13.67 cd	21.77 bc	16.60 cd	25.00 cde	35.60 de	40.90 c
	Abbad	MUL/T12	*M. incognita*	4.9	14.60 abc	14.70 abcd	22.37 abc	17.57 bc	24.00 de	35.33 de	40.50 c
		LSD		-	1.005	1.244	1.556	1.160	1.610	1.261	1.447
Rawal-pindi	Kango Juma	RWP/T1	*M. incognita*	5.80 a	15.70 ab	15.43 ab	23.60 ab	18.17 ab	25.30 ab	36.30 bc	42.30 b
		RWP/T2	*M. incognita*	5.03 abc	15.00 bc	15.17 abc	22.77 bc	15.03 de	19.97 d	32.67 e	36.70 c
	Kango	RWP/T3	*M. javanica*	6.16 a	16.20 a	16.50 a	24.53 a	17.73 ab	25.57 ab	37.37 b	42.47 b
	Bahadur	RWP/T4	*M. javanica*	3.03 d	13.63 d	12.77 e	20.20 f	14.63 e	18.03 e	30.00 f	36.20 c
	Qazi Abad	RWP/T5	*M. incognita*	4.10 bcd	13.90 d	13.60 cde	21.77 cde	18.07 ab	24.40 b	35.83 c	41.07 b
		RWP/T6	*M. incognita*	4.03 cd	14.00 cd	14.43 bcde	22.20 cd	17.10 bc	22.57 c	34.53 d	37.33 c
	Odiala	RWP/T7	*M. javanica*	5.93 a	15.73 ab	15.03 abc	22.93 bc	18.60 a	26.40 a	38.60 a	44.83 a
		RWP/T8	*M. javanica*	3.76 cd	13.80 d	13.20 de	20.67 ef	15.20 de	18.40 e	31.03 f	36.60 c
	Sawan	RWP/T9	*M. incognita*	4.73 abc	14.70 bcd	14.43 bcde	21.90 cd	16.33 cd	20.77 d	32.50 e	37.20 c
		RWP/T10	*M. incognita*	5.53 ab	15.63 ab	14.63 bcd	21.40 de	18.33 ab	25.77 ab	36.47 bc	41.60 b
		LSD		1.32	0.9869	1.619	1.074	1.318	1.478	1.221	1.405

[1] Means with in a column sharing the same letter are not significantly different from each other at $P = 0.05$ according to least significant difference test.

Encumbrance of PP-J with different RKN populations at different temperatures and concentrations isolated from tomato. The same 54 populations of RKN responded differently when encumbered with PP-J isolate of *Pasteuria*. Its attachment with different populations of RKN at two different temperatures and concentration was almost double than PP-3. The RKN populations which gave maximum encumbrance with PP-J were different from those RKN populations which showed maximum encumbrance with PP-3 (Table 4). Only FSD/T8 (Faisalabad) population showed maximum encumbrance with PP-3 as well as PP-J. The other four populations which gave maximum encumbrance were JNG/T3 (Jhang), KHW/T2 (Khanewal), MUL/T10 (Multan) and RWP/T7 (Rawalpindi).

Encumbrance of PP-3 with different RKN populations at different temperatures and concentrations isolated from cucumber. The encumbrance of 62 populations of RKN also varied with PP-3 at two different temperatures (ambient and 30 ± 2°C) and concentrations (10^4 and 10^5 spores per ml). The encumbrance level of PP-3 was maximum at 30 ± 2°C than ambient temperature and spore concentration was also directly related to attachment with nematode cuticle (Table 5). The populations selected on the basis of maximum attachment with PP-3 were FSD/C4 (Faisalabad), JNG/C2 (Jhang), KHW/C9 (Khanewal), MUL/C2 (Multan) and RWP/C10 (Rawalpindi).

Encumbrance of PP-J with different RKN populations at different temperatures and concentrations isolated from cucumber. A great variability of the number of spores attached to the cuticles of J2 was observed

Table 5. Encumbrance of two *Pasteuria* isolates (PP-3 and PP-J) with different populations of *Meloidogyne* spp. isolated from cucumber.

District	Locality	Population code	*Meloidogyne* spp.	PP-3				PP-J			
				25 ± 2°C		30 ± 2°C		25 ± 2°C		30 ± 2°C	
				10^4	10^5	10^4	10^5	10^4	10^5	10^4	10^5
Faisalabad	JK Farm	FSD/C1	*M. incognita*	4.63 cde[1]	11.00 e	13.70 de	18.00 g	14.87 de	22.20 c	34.00 cd	37.27 d
		FSD/C2	*M. incognita*	5.26 abcde	13.80 cd	15.70 bc	21.73 de	16.47 bc	22.43 c	35.23 bc	38.60 c
	Jahangir Mor	FSD/C3	*M. incognita*	5.93 abc	14.70 bc	16.27 abc	23.10 bc	17.03 abc	25.07 b	36.93 ab	41.00 b
		FSD/C4	*M. incognita*	6.86 a	16.23 a	17.73 a	24.80 a	17.77 ab	26.30 ab	36.90 ab	41.17 ab
	Chak. No 496	FSD/C5	*M. incognita*	5.63 abc	13.43 d	14.87 cd	19.70 f	14.67 de	20.00 def	25.57 f	36.57 de
		FSD/C6	*M. incognita*	6.50 ab	15.57 ab	15.60 bc	24.27 ab	17.30 ab	25.93 ab	36.70 ab	40.17 b
	Chak No.54	FSD/C7	*M. incognita*	3.70 e	8.967 f	12.70 e	16.37 h	13.87 ef	18.80 f	24.93 f	35.53 ef
		FSD/C8	*M. incognita*	4.90 bcde	13.93 cd	15.87 bc	20.80 ef	15.73 cd	20.27 de	30.30 e	35.90 ef
	Dhasian	FSD/C9	*M. incognita*	5.83 abc	13.90 cd	17.00 ab	19.90 f	16.37 bc	23.23 c	36.27 ab	40.50 b
		FSD/C10	*M. incognita*	5.53 abcd	13.40 d	16.70 ab	23.83 ab	15.63 cd	20.77 d	32.90 d	36.60 de
	204RB	FSD/C10	*M. incognita*	4.00 de	10.63 e	13.20 e	18.13 g	12.83 f	18.93 ef	25.97 f	35.23 f
		FSD/C12	*M. incognita*	6.40 ab	14.83 bc	17.03 ab	22.33 cd	18.33 a	26.67 a	37.57 a	42.27 a
		LSD		1.42	1.10	1.32	1.26	1.32	1.27	1.55	1.17
Jhang	Al Hafiz Farm	JNG/C1	*M. javanica*	4.7	12.90 ef	14.23 e	17.33 g	14.30 fg	20.53 fgh	32.27 efg	37.50 de
		JNG/C2	*M. javanica*	7.2	16.57 a	18.03 a	23.47 b	19.37 a	27.53 a	38.23 a	43.63 a
	Chak No.204	JNG/C3	*M. incognita*	5.8	13.93 cde	15.73 d	23.23 b	17.80 b	25.37 b	36.93 ab	41.20 b
		JNG/C4	*M. incognita*	5.0	13.87 cde	17.20 abc	22.00 cd	16.23 de	22.40 de	33.20 def	37.83 d
	Kosar Abad	JNG/C5	*M. javanica*	5.5	14.97 bc	17.87 a	22.60 bcd	14.90 efg	20.03 gh	27.67 i	36.37 de
		JNG/C6	*M. javanica*	5.6	14.17 cd	17.73 ab	24.63 a	17.67 bc	24.60 bc	35.30 bc	40.23 bc
	Bahsti Usmana	JNG/C7	*M. incognita*	4.6	13.30 def	16.63 bcd	20.63 e	13.67 g	19.43 h	29.37 h	37.50 de
		JNG/C8	*M. incognita*	5.1	12.63 f	15.97 d	21.73 d	16.03 de	21.27 efg	33.60 cde	39.93 bc
	Kot M. Yar	JNG/C9	*M. incognita* + *M. javanica*	5.3	14.60 bc	17.73 ab	22.63 bcd	15.63 def	20.67 efgh	30.93 gh	36.17 e
		JNG/C10	*M. incognita* + *M. javanica*	5.6	15.43 b	17.50 abc	22.87 bc	17.87 b	23.40 cd	34.37 cd	39.53 c
	Gulghasht Farm	JNG/C11	*M. incognita*	4.5	12.80 ef	16.37 cd	19.70 f	16.43 cd	21.83 def	32.00 efg	37.90 d
		JNG/C12	*M. incognita*	4.9	13.90 cde	16.63 bcd	20.73 e	15.30 def	21.23 efg	31.60 fg	36.97 de
		LSD		-	1.07	1.06	0.84	1.28	1.58	1.68	1.44
Khanewal	Raees Abad	KHW/C1	*M. incognita*	3.83 cd	7.30 f	11.57 f	16.00 fg	14.70 c	19.37 e	30.40 e	35.63 f
		KHW/C2	*M. incognita*	5.33 ab	14.33 abcd	16.83 ab	24.00 a	17.00 ab	21.53 d	32.33 d	38.57 bc
	Khaliqa Abad	KHW/C3	*M. javanica*	5.56 ab	14.93 ab	16.87 ab	23.63 ab	18.23 a	26.83 a	37.63 a	42.20 a
		KHW/C4	*M. javanica*	4.60 bcd	13.47 bcd	16.47 bc	22.37 c	15.10 c	20.10 e	30.50 e	36.33 ef
	Chak 125/15L	KHW/C5	*M. incognita*	5.53 ab	14.73 abc	16.97 ab	23.67 ab	17.57 a	24.90 b	35.30 bc	40.90 a
		KHW/C6	*M. incognita*	4.93 ab	13.53 bcd	16.70 abc	22.97 bc	15.80 bc	21.70 d	34.47 c	38.20 c
	Dass Kassi	KHW/C7	*M. incognita*	3.63 d	10.03 e	12.10 ef	15.63 g	13.03 d	19.20 e	30.87 de	36.57 def
		KHW/C8	*M. incognita*	4.73 abc	12.97 d	12.57 e	16.60 f	15.43 c	19.83 e	31.97 de	37.20 cde
	Kacha Koh	KHW/C9	*M. javanica*	5.83 a	15.77 a	17.43 a	24.30 a	17.83 a	25.97 ab	37.37 a	41.27 a
		KHW/C10	*M. javanica*	5.60 ab	14.00 bcd	15.90 c	20.73 d	17.17 a	23.37 c	36.70 ab	39.57 b
	Tulamba	KHW/C11	*M. incognita*	5.00 ab	13.27 cd	14.60 d	19.57 e	15.00 c	20.20 e	32.40 d	37.83 cd
		KHW/C12	*M. incognita*	5.30 ab	14.33 abcd	17.03 ab	23.97 a	15.80 bc	21.77 d	31.27 de	36.20 ef
		LSD		0.99	1.34	0.81	0.87	1.22	1.27	1.47	1.27

Table 5. Continued

District	Locality	Population code	*Meloidogyne* spp.	PP-3				PP-J			
				25 ± 2°C		30 ± 2°C		25 ± 2°C		30 ± 2°C	
				10^4	10^5	10^4	10^5	10^4	10^5	10^4	10^5
Multan	Kotla Saadat	MUL/C1	*M. javanica*	4.83 bc	13.77 bc	16.67 ab	21.63 ab	16.27 c	22.00 ef	32.77 e	37.57 de
		MUL/C2	*M. javanica*	6.56 a	15.20 a	17.23 a	22.80 a	18.50 a	26.80 a	38.03 a	41.63 a
	Qadir pur Raan	MUL/C3	*M. incognita*	5.60 ab	13.90 bc	16.30 ab	21.40 ab	14.87 d	20.70 fg	31.80 ef	38.17 de
		MUL/C4	*M. incognita*	5.86 ab	14.20 abc	16.80 ab	22.13 ab	17.97 ab	25.13 bc	36.33 b	39.67 bc
	Kambir pur	MUL/C5	*M. incognita*	3.90 c	12.20 d	11.43 e	16.73 e	13.10 e	20.17 gh	30.80 f	36.90 e
		MUL/C6	*M. incognita*	4.70 bc	13.13 cd	13.73 cd	19.00 c	16.67 bc	22.00 ef	33.17 de	38.90 cd
	Kian pur	MUL/C7	*M. incognita*	5.76 ab	13.93 bc	15.30 bc	20.93 b	14.53 d	20.67 fg	31.87 ef	37.97 de
		MUL/C8	*M. incognita*	5.06 bc	13.47 bc	14.27 cd	18.53 cd	17.23 abc	23.97 cd	34.90 c	38.57 cd
	Abu Al-Fatah Kotla	MUL/C9	*M. incognita* + *M. javanica*	3.93 c	11.00 e	11.77 e	16.03 e	13.90 de	19.00 h	30.97 f	37.47 de
		MUL/C10	*M. incognita* + *M. javanica*	5.03 bc	13.53 bc	13.40 d	17.13 de	16.40 c	22.80 de	34.50 cd	38.17 de
	Saddique Abad	MUL/C11	*M. javanica*	4.56 bc	13.87 bc	14.07 cd	18.63 cd	14.67 d	20.97 fg	34.17 cd	37.97 de
		MUL/C12	*M. javanica*	5.66 ab	14.47 ab	16.90 ab	20.77 b	18.03 ab	25.73 ab	36.33 b	40.53 ab
		LSD		1.16	1.11	1.61	1.44	1.26	1.29	1.32	1.32
Rawalpindi	Kango Juma	RWP/C1	*M. incognita*	4.16 bcde	12.73 de	13.03 def	18.03 e	16.17 cd	21.63 d	33.63 c	38.57 cd
		RWP/C2	*M. incognita*	5.70 ab	14.87 a	17.30 ab	23.60 a	18.90 a	26.70 a	37.73 a	42.00 a
	Kango Bhadur	RWP/C3	*M. incognita*	4.76 abcd	13.73 bcd	16.13 bc	21.33 b	16.90 bc	23.83 b	35.90 b	39.17 c
		RWP/C4	*M. incognita*	5.06 abc	13.33 cde	15.97 c	20.43 bcd	15.47 de	21.13 d	32.27 cde	38.23 cd
	Qazi Abad	RWP/C5	*M. arenaria*	3.23 de	5.06 h	7.30 h	9.16 h	5.66 g	8.800 g	9.83 g	12.93 h
		RWP/C6	*M. arenaria*	2.90 e	5.50 gh	6.46 h	8.26 h	6.00 g	8.23 g	9.40 g	12.60 h
	Mathian	RWP/C7	*M. incognita*	4.86 abc	12.97 cde	13.80 de	19.83 cd	14.50 e	18.73 ef	32.03 de	36.90 ef
		RWP/C8	*M. incognita*	4.56 bcd	12.50 e	12.57 ef	21.33 b	12.90 f	18.13 ef	30.97 e	36.17 fg
	Bunny	RWP/C9	*M. incognita*	5.20 abc	14.53 ab	16.67 abc	22.83 a	16.80 bc	23.43 bc	35.67 b	39.57 bc
		RWP/C10	*M. incognita*	6.20 a	15.17 a	17.70 a	23.77 a	17.67 b	24.77 b	36.20 b	40.60 b
	Sawan	RWP/C11	*M. incognita*	4.73 abcd	12.50 e	12.73 def	19.70 d	15.90 cd	19.43 e	32.30 cde	37.50 de
		RWP/C12	*M. incognita*	3.26 de	6.43 g	10.37 g	15.40 g	12.03 f	17.57 f	27.30 f	34.93 g
	Odiala	RWP/C13	*M. incognita*	3.76 cde	11.30 f	12.00 f	16.63 f	16.90 bc	22.27 cd	33.33 cd	38.90 c
		RWP/C14	*M. incognita*	5.00 abc	13.83 bc	13.83 d	21.03 bc	15.13 de	18.73 ef	31.40 e	35.87 fg
	LSD			1.39	0.96	1.13	1.15	1.16	1.31	1.38	1.25

[1] Means with in a column sharing the same letter are not significantly different from each other at $P = 0.05$ according to least significant difference test.

when same 62 populations of RKN were evaluated for encumbrance with PP-J at different temperatures and concentration (Table 5). Two populations showed maximum encumbrance with PP-3 and PP-J were JNG/C2 (Jhang) and MUL/C2 (Multan) while other three populations having maximum encumbrance were FSD/C12 (Faisalabad), KHW/C3 (Khanewal) and RWP/C2 (Rawalpindi). The two RKN populations from cucumber which produced least encumbrance throughout the *in-vitro* were RWP/C5 (Rawalpindi) and RWP/C6 (Rawalpindi).

Assessment of genetic variability among different populations of root knot nematodes using PCR-RFLP of CO II and large subunit of rRNA gene (lrRNA) of the mitochondrial genome. The primer set used to amplify intergenic region between CO II and large subunit of rRNA gene (lrRNA) of the mitochondrial genome. It produced single amplicon from each of the 19 samples (Fig. 3). A PCR product of 1.1 kb, expected from *M. arenaria* species, was obtained from the samples RWP/C5 and RWP/C6 which were chosen on the basis of minimum encumbrance, hence these samples were confirmed as *M. arenaria*. The seventeen samples, selected on the basis of maximum encumbrance, amplified a product of 1.7 kb related in size expected from *M. incognita* and *M. javanica* species (Fig. 3).

The *Hinf* I restriction analysis of 1.7 kb fragment to dif-

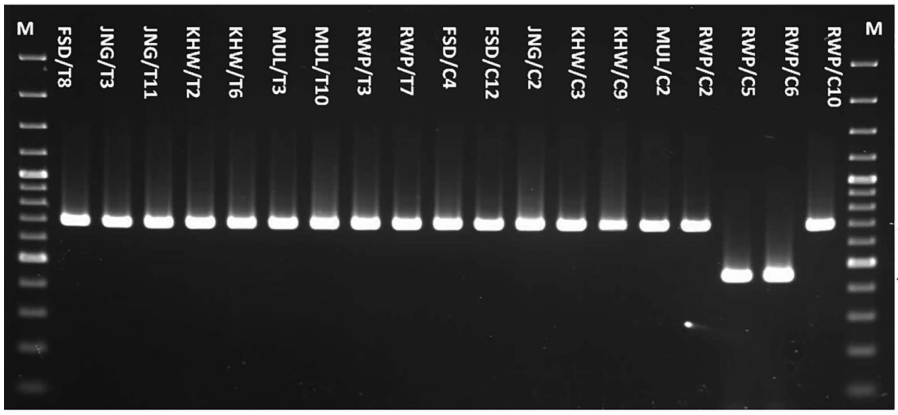

Fig. 3. Gel image of C2F3/1108 amplified PCR product of COII/LrRNA of mitochondrial genome. The 1.7 kb sizes of PCR products are characteristics of *M. incognita* and *M. javanica* while *M. arenaria* produce 1.1 kb fragment size. M lanes were loaded with 1 kb ladder.

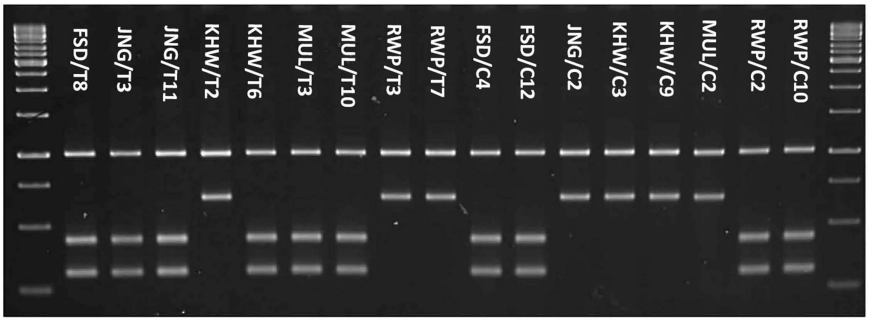

Fig. 4. Gel image of PCR products from 17 nematode populations restricted with *Hinf* I enzyme. Lanes showing two fragments of 1.0 and 0.7 kb were related *M. javanica* whereas lanes with three fragments of 1.0, 0.4 and 0.3 kb were related to *M. incognita*. The products were resolved on 1.5% agarose gel and stained with ethidium bromide. M lanes were loaded with 1 kb ladder.

ferentiate among *Meloidogyne* species determined single digestion site in samples KHW/T2, RWP/T3, RWP/T7, JNG/C2, KHW/C3, KHW/C9 and MUL/C2, generating two fragments of 1.0 and 0.7 kb (Fig. 4).

These restriction fingerprints related the samples to *M. javanica.* In rest of 10 samples including FSD/T8, JNG/T3, JNG/T11, KHW/T6, MUL/T3, MUL/T10, FSD/C4, FSD/C12, RWP/C2 and RWP/C10, an additional enzyme digestion site cleaved the 0.7 kb fragment to generate two more fragments of about 0.4 and 0.3 kb (Fig. 4). This restriction pattern related the samples to *M. incognita* species. In conclusion, *M. incognita* was found most prevalent in the 19 samples with the highest frequency (52.6%) followed by *M. javanica* (36.8%) and *M. arenaria* (10.5%).

Discussion

Encumbrance of *Pasteuria* endopsores to the cuticle plays an important role in biological management of root knot nematodes (Channer and Gowen, 1992; Espanol et al., 1997; Stirling, 1984; Stirling et al., 1986; Vagelas et al., 2012; Wishart et al., 2004). Different populations of *Pasteuria* endospores exhibit different levels of encumbrance to nematode cuticle. These variations have been attributed to differences in the surface composition of different species, races and RKN populations (Davies and Danks, 1992; Davies et al., 2008) and to the heterogenity of the *P. penetrans* endospore surfaces themselves (Davies et al., 1992). The specificity of endospore encumbrance to J2 cuticle has been studied biochemical and immunological methods. The results showed a high degree of heterogeneity both within and among different populations of *P. penetrans* (Davies and Redden, 1997; Preston et al., 2003). A carbohydrate-protein mechanism is involved in endospore encumbrance to *M. incognita* (Davies and Danks, 1993; Persidis et al., 1991) while according to Spiegel et al. (1996) carbohydrate residues, carbohydrate- recognition domains and a 250-kDa antigen on the J2 cuticle of *M. javanica* were shown to be involved in *Pasteuria* endospore attachment. According to Davies and Opperman (2006) collagen like proteins on *Pasteuria* spore core are involved for the adhesion of the endospore to J2 cuticle. These differences in attachment indicated complex interactions between the cuticle of the nematode and the surface of the *Pasteuria* endospore (Tian et al., 2007).

In current work interspecific and intraspecfic variability in encumbrance was observed with two *Pasteuria* isolates at different temperatures and concentrations. Temperature is one of several environmental factors that influence the ability of endospore to adhere and to infect *Meloidogyne* J2

(Hatz and Dickson, 1992). The rate of spore encumbrance to J2 increased with increasing temperature and current results are similar to previous works (Giannakou and Gowen, 2004; Singh and Dhawan, 1990; Stirling et al., 1990; Zareen et al., 2002). *Pasteuria penetrans* spores attached more readily at 22.5-30°C than 15°C (Stirling, 1981). However, Giannakou et al. (1997) observed greater attachment at 25 and 30°C but the maximum number of endospores was attached at 30°C (Ahmed, 1990; Hatz and Dickson, 1992; Orui, 1997). At higher temperature (above 30°C) the rate of encumbrance with J2 declined (Hatz and Dickson, 1992). Exposing nematodes to 30°C also increased rate of encumbrance. Freitas et al. (1997) pre-exposed *M. arenaria* J2 in 30°C water before exposure to endospores increased J2 receptivity to endospores when compared to treatments at 25°C and 35°C. The variable encumbrance at different temperatures is due to the fact that it is dependent on nematodes' mobility. At 30°C the mobility of nematodes is high while above 35°C or below 5°C nematodes have little activity (Taylor and Sasser, 1978). That's why at these temperatures attachment is decreased. Other reason is that, the bacterium developed more quickly within its host at 30°C and 35°C than at 25°C or below (Hatz and Dickson, 1992).

Encumbrance of *Pasteuria* endopsores to the J2 cuticle is also dependent on *Pasteuria* spore densities/concentrations. As the *Pasteuria* endospores are non-motile and successful encumbrance with J2 cuticle is only achieved when a suitable nematode comes under its vicinity (Chen and Dickson, 1998). The rate of encumbrance to J2 increased approximately five to six times for each 10-fold increase in numbers of *Pasteuria* endospores in the suspension Hewlett and Dickson (1993). Zareen et al. (2002) reported that encumbrance of *Pasteuria* endospore to J2 cuticle reduced with increase in dilution factor of spore suspension while Alves et al. (2004) provoked that in order to improve the endospore attachment, endospore concentration of the suspension is more important than to increase the agitation period of the nematodes in the bacterial suspension. *Pasteuria* endospore concentration in soil is also key factor for its infectivity (Stirling et al., 1990). Endospore densities in soil was positively correlated with percentage of J2 attached and number of spores/J2 (Talavera and Mizukubo, 2003).

The PCR reaction amplified intergenic region between cytochrome oxidase subunit II gene (COII) and large subunit of rRNA gene (lrRNA) of the mitochondrial genome. The primer C2F3 and 1108 identified *M. incognita* with the highest frequency (52.6%) followed by *M. javanica* (36.8%) and *M. arenaria* (10.5%). Heterogeneity in band size was detected in mitochondrial genome of different *Meloidogyne* species. The sizes of PCR products were 1.7 kb for *M. incognita* and *M. javanica* populations while populations of *M. arenaria* produced 1.1 kb fragment. Therefore, *M. arenaria* was easily distinguished from *M. incognita* and *M. javanica* based on band size of amplified PCR products. No band size variability was detected within species of *Meloidogyne*. The digestion with *Hinf* I yielded three different fragment length patterns on 1.5% agarose gel. First, restriction digestion of 1.7 kb *M. javanica* amplification product determined one enzyme digestion site and resulted in two fragments of 1.0 and 0.7 kb. Second, an additional enzyme digestion site on *M. incognita* product cleavage the 0.7 kb fragment to generate two more fragments of about 0.4 and 0.3 kb. Third, *M. arenaria* had no enzyme digestion site by *Hinf* I digestion.

For precise identification of *Meloidogyne* species, mitochondrial genome provides good source of genetic markers for identification (Blok et al., 2002; Hu and Glasser, 2006; Hugall et al., 1994; Jeyaprakash et al., 2006). Intraspecfic mitochondrial variation has been observed in a region of multiple nucleotide repeating units in *Meloidogyne*. Mitochondrial DNA has been applied not only for the identification of *Meloidogyne* species but also for the molecular differentiation or population genetic study of nematodes (Liu et al., 1999; Szalanski et al., 2000). In the present study intergenic region between cytochrome oxidase subunit II and 16S ribosomal mitochondrial genes have been amplified to determine intra-*Meloidogyne* genetic variability. The assay successfully differentiated isolates in to three types i.e. *M. incognita*, *M. javanica* and *M. arenaria* based on variation in fragment sizes of the PCR products/their restriction fingerprints. The similar studies have been conducted on characterization of USA (Powers and Harris, 1993) and Korean isolates (Han et al., 2004; Oh et al., 2009) of *Meloidogyne* species exploiting the same genetic markers. For *M. arenaria* C2F3/1108 amplified PCR product was of 1.1 kb in USA isolate (Powers and Harris, 1993; Powers et al., 2005) which is of the same size as found in current studies on Pakistani *M. arenaria* isolates. However, Oh et al. (2009) reported 1.7 kb fragment amplified from Korean *M. arenaria* isolates. PCR amplification product of Pakistani *M. javanica* isolates exhibited 1.7 kb product size and on digestion resulted into two band sizes of 1.0 and 0.7 kb which was similar to previous work of Powers and Harris, 1993. However, *Hinf* I digestion revealed results differing from those of previous study (Oh et al., 2009). Therefore *M. incognita*, *M. javanica* and *M. arenaria* has significant nucleotide variations depending upon different ecological origins.

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