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Host reaction and molecular genotyping to identify pathotype variability in rice root-knot nematode (*Meloidogyne graminicola)* populations

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ABSTRACT

Six rice cultivars, Annapurna, Ramakrishna, TKM 6, Zenith, Tetep and Tadukan were evaluated for their resistance/ susceptibility reaction against four rice root-knot nematode (Meloidogyne graminicola) populations from CRRI farm, Amana and Kamakhyanagar in Odisha and from Trichy in Tamil Nadu. Each rice cultivars showed differential reaction pattern to nematode populations, indicating the existence of variation in virulence pattern of root-knot nematode populations. Genomic polymorphisms between four rice root-knot populations were assessed by using 17 random primers. A total of 142 bands were amplified, out of which 95(66.9%) were polymorphic. The number of bands per primer ranged from two to fourteen with an average of 8.35 bands per primer. Forty four unique bands were identified, which could be developed into diagnostic markers to identify rice root-knot nematode population. UPGMA cluster analysis classified four populations into two major groups at 48% of genetic similarity. All the Odisha populations were included in first major cluster while Trichy population was included in second major cluster. The host reaction and molecular genotyping of four populations of the rice root-knot nematode indicates the existence of pathotype variability.

Key words: Meloidogyne graminicola, host plant, rice, root-knot nematode, virulence

Root-knot nematodes (RKN) belonging to the genus *Meloidogyne* are major agricultural pests of a wide variety of economically important crops (Williamson and Gleason, 2003). The rice root-knot nematode, *Meloidogyne graminicola* Golden and Birchfield is one of the potent nematode pests of rice in nurseries, uplands, well drained medium land paddies, lowland rain-fed and deep water ecosystem. In India, root-knot nematode is mainly prevalent in the state of Assam, Odisha, Tamil Nadu, Kerala, Tripura, West Bengal, Bihar, Uttar Pradesh, Panjab, Haryana, Himachal Pradesh, Jammu and Kashmir and Madhya Pradesh (Prasad *et al*, 1987). Epidemic proportion of root-knot nematode has been noticed in recent past in Karnataka (Prasad *et al*, 2001). Thus, the most feasible alternative is to use resistant cultivars to manage the pest. Studies on pathotype variability are an essential prerequisite for successful deployment of resistance genes.

RKN are known to be highly variable. They exhibit extreme cytogenetic diversity and their mode of reproduction ranges from obligatory amphimixis to obligatory parthenogenesis (Triantaphyllou, 1985). Detailed information on identity and variability of root-knot nematodes is still lacking. Rapid and accurate identification is important for understanding the host-parasite relationships and implementing appropriate management options. Traditional methods of identification of root-knot nematode are based on morphology (Van der Beek *et al*., 1998), differential host range test (Sasser and Triantaphyllou, 1977) and isozyme (Eshenshade and Trintaphyllou, 1990). However, these methods are time consuming, developmental specific and influenced by environment (Eshenshade and Trintaphyllou, 1990). The PCR based randomly amplified polymorphic DNA (RAPD) marker technique is simple, quicker, and cost effective and does not depend on developmental stage (Welsh and McClelland., 1990). The technique has been extensively used for accurate estimation of genetic, taxonomic and phylogenetic relationships of root-knot nematodes (Blok *et al*. 1997; Randing *et al*., 2002).

In the present study, therefore, RKN populations of Odisha (Amana, Kamakhyanangar and Cuttack) and Tamil Nadu (Trichy) were used to study

host reaction and assess the genetic diversity and relationships with pathotype variability of root-knot nematode populations.

MATERIALS AND METHODS

Soil samples from rice field, infested with *Meloidogyne graminicola* were collected from Central Rice Research Institute farm, Cuttack, Aman, Kamakhyanagar (all in Odisha) and Trichy of Tamil Nadu. The soil samples were mixed with autoclaved soil and filled in four different plastic trays. Two grams of Annapurna seeds were sown and allowed to germinate. After 50 days of germination, the roots were carefully uprooted, washed and presence of egg masses were checked under microscope. Subsequently, the roots with egg masses were blended for three seconds, placed on tissue paper supported over a wire mesh for 24-48 hrs incubation. The hatched out larvae were collected in water, counted and re-inoculated on single freshly raised 10-15 days old healthy seedling of Annapurna in pots. The infested Annapurna plants were harvested for gall collection after 45-50 days of inoculation. The roots were uprooted, washed and galls were collected by using forceps. Then galls were dissected and mature female nematode handpicked under microscope using needle and forceps. Matured female nematodes were stored in 1.5 eppendorf tube containing 0.9% KCl solution till genomic DNA isolation. The nematodes were maintained on Annapurna plants under greenhouse conditions.

Five donors, Ramakrishna, TKM6, Zenith, Tetep and Tadukan and one susceptible rice cultivar, Annapurna were evaluated for their resistance reaction against four nematode populations of Odisha (CRRI farm, Amana and Kamakhyanagar) and from Trichy in Tamil Nadu. Single plant of each cultivar was grown in plastic pots containing 1:1 mixture of sterile sand and soil. Complete randomized design was followed with six replications. Annapurna and Ramakrishna served as standard checks for susceptibility and resistance, respectively. When seedlings were 10 days old, they were infected with 2000 freshly hatched second stage juveniles of rice RKN by exposing roots. Plants were uprooted 50 days after inoculation and root system was gently cleaned and the average number of eggs and fresh weight of roots per plant were recorded.

Genomic DNA was isolated from female nematodes following the modified method (Anonymous, 1993). Two hundred females were soaked in 50 μl of extraction buffer {50 mM Tris-HCl (pH 8.0), 25 mM EDTA, 50 mM NaCl, 1% SDS} for 10 minutes, and then homogenized in 1.5 ml of eppendorf tube with sterilized polypropylene pestle. Then, it was ringed with additional 350 μl of extraction buffer. Eight microliter of Proteinase-K (10 mg ml^{-1}) was added and incubated at 37^0C for 1 hr followed by addition of 400 μl of equilibrated phenol. The samples were emulsified and centrifuged at 12,000 rpm for 10 min at 4^0C. The upper layer was carefully transferred to another tube and an equal volume containing 200 μl of phenol and 200 μl of chloroform: isoamyl alcohol (24:1) was added, mixed well and the solution was centrifuged at 12,000 rpm for 10 min at 4^0C. Then, the supernatant was transferred to a new tube and ten microliter of RNase (10 mg/ml) was added and incubated for 1 hr at 37^0C. 400 microlitre of chloroform: isoamyl alcohol (24:1) was added, mixed well and centrifuged at 12,000 rpm at 4^0C for 10 min. The upper aqueous layer was carefully transferred to another eppendorf tube and the eight mirolitre of NaCl (5M) and twice the volume of ice cold absolute alcohol was added to it. An overnight incubation was carried out at -20^0C followed by centrifugation at 12,000 rpm for 10 minutes. The DNA pellet was washed with 100μl of 70% ethanol, dried at room temperature and re-suspended 50 μl of TE buffer. The quality and quantity of genomic DNA was estimated by using spectrophotometer and agarose gel electrophoresis. The samples were diluted in $T_{10}E_1$ buffer to get final concentration of 12 ng μl^{-1} and stored at -20^0C for further analysis.

Seventeen random decamer primers were used for DNA amplification. These primers were from commercially available RAPD primer kits (Operon Technologies, Almeda California, USA). The PCR amplification was performed in a 20 Ml reaction mixture volume containing 24 ng of DNA, 1X PCR buffer {75 mM Tris-HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$}, 200 µM dNTP mix (MBI Fermantas, Lithuania, USA), 5 pmol of primer, 2 mM of magnesium chloride and 1 U of *Taq* (*Thermus aquaticus*) DNA polymerase enzyme(Biotools, Spain). A single primer was included in each PCR reaction. The PCR amplification was carried out in Thermo Hybaid Thermal Cycler for 45 cycles under following PCR conditions: initial denaturation at 94^0C for 2 min, denaturation at 94^0C for 1 min, annealing at 36^0C for 1

min and extension at 72^{0}C for 2 min for 45 cycles and final extension at 72^{0}C for 5 min. Four microliter of loading buffer was added amplified PCR products, mixed well, centrifuged briefly to collect drops from wall of tube. Twelve microliter of amplified products of each sample was loaded on 1.5% agarose gel in 1X TBE buffer to separate the amplified fragments. The electrophoresis was done for about 3 hours at 60 volts. The molecular weight marker (100 bp ladder plus) was used to compare the molecular weights of amplified products. After electrophoresis the gel was stained with ethidium bromide for 20 min. The gel was visualized under UV trans-illumination and was photographed using Gel-Doc system (Fluor Chem^{TM} 5500, Alpha Innotech, USA). Individual bands within lanes were assigned to a particular molecular weight comparing with the DNA molecular weight marker (100bp plus DNA ladder, MBI Fermentas, Lithuania).

The amplified bands were scored as present (1) or absent (0) for each primer population combination. The data was entered into a binary matrix (0/1) and subsequently analysed using the computer package NTSYS-pc Version 2.02 (Rolf, 1998). The total number of bands per primer, percentage of polymorphic bands, common bands and unique bands were calculated to assess diversity of bands amplified by a RAPD primer.

Jaccard's similarity coefficients were calculated and used to assess the genetic relationship among nine rice brown planthopper. A dendrogram was constructed using unweighted pair group method using arithmetic averages (UPGMA). The Cophenetic correlation coefficient (Lapointe and Legendre, 1992) was calculated to measure the goodness of fit of clusters.

RESULTS AND DISCUSSION

Based on the number of eggs produced after completion of second life cycle by the RKN, Annapurna showed susceptible reaction to all the four rice RKN populations while five donors, Ramakrisna, Tetep, Zenith, TKM6 and Tadukan showed different intensities of resistance to RKN populations (Table 1). TKM6 showed moderate resistance to all the RKN populations. Ramakrishna showed moderately resistance to Cuttack population while resistant reaction to Amana, Kamakhyanagar and Trichy populations. Tadukan showed highly resistant reaction to Cuttack population, resistant reaction to Amana, Kamakhyanagar and moderately resistant reaction to Trichy populations. Tetep showed resistant reaction to Cuttack and Trichy populations while moderately resistance to Amana and Kamakhyanagar populations. Zenith showed highly resistant reaction to Cuttack population, resistant reaction to Kamakhyanagar and Trichy populations while moderately resistance to Amana populations. Host reaction pattern indicated that all the four populations showed different virulence pattern. Amana and Kamakhyanagar populations showed closer virulence reaction pattern as compared to Amana and Cuttack populations.

Table 1. **Reaction of rice cultivars to different populations of rice root-knot nematode**

Variety/Population	Ramakrishna	Annapurna	Tetep	Zenith	Tadukan	TKM6
Amana	R(1891)	S(7029)	MR(2269)	MR(2487)	R(1248)	MR(2608)
CRRI farm	MR (2099)	S(4359)	R(1620)	HR(975)	HR(944)	MR(2285)
Kamakhyanagar	R(1428)	S(5059)	MR(2584)	R(1307)	R(1759)	MR(2536)
Trichy	R(1528)	S(4065)	R(1965)	R(1328)	MR(2417)	MR(2263)

< 1000-highly resistant (HR), 1001-2000-resistant(R), 2001-3000-moderately resistant (MR),
> 3001-susceptible (S). Number in parenthesis denotes mean number of eggs (non transformed values)

In order to find the efficiency of RAPD primers for differentiation of populations, the discriminating power (D) of each primer was calculated following formula, $Dj = 1-Cj = 1-\sum Pi\ (NPi-1)/(N-1)$, where Dj is discriminating power of j^{th} primer, Pi is frequency of i^{th} band, Cj confusion probability of j^{th} primer (Tessier *et al*., 1999). Further, in order to know minimum number of primer required to identify and differentiate genotypes from each other primer using formula, $Xj = \{N(N-1)/2\}Cj$.

A total of 142 bands were amplified by seventeen random primers, of which 95 (66.9%) are

Table 2. Amplification pattern of bands produced by random primers

RAPD Primer	Total no. of bands	No of poly-morphic	No of common bands	Size of bands (bp) bands	D*
OPA2	10	2	9	600-2200	0.15
OPA20	8	0	8	600-1875	0
OPD2	4	2	4	420-2250	0.333
OPH8	10	3	8	500-1900	0.283
OPH9	4	3	3	600-970	0.583
OPH17	7	6	3	350-2200	0.81
OPH18	11	10	7	500-2800	0.697
OPH19	5	3	4	650-1200	0.4
OPH20	14	13	6	400-2750	0.821
OPN2	9	6	5	340-2650	0.630
OPN3	2	0	2	640-900	0
OPN9	4	2	2	550-1100	0.5
OPN11	10	5	7	440-3100	0.4
OPO16	8	6	6	630-2700	0.542
OPR1	11	11	7	300-2600	0.803
OPR2	14	13	10	220-1725	0.726

*D - discriminating power of a primer

polymorphic (Table 2). The number of bands per primer ranged from two (OPN3) to fourteen (OPH20 and OPR2) with an average of 8.35 bands per primer. The size of the amplified bands varied from 220bp (OPR2) to 3800bp (OPR3). Fifteen primers were polymorphic between populations while two primers, OPA20 and OPN3 were monomorphic. One primer, OPR1 amplified 11 bands, all being polymorphic. The amplification pattern with some random primers is shown in the figure 1.

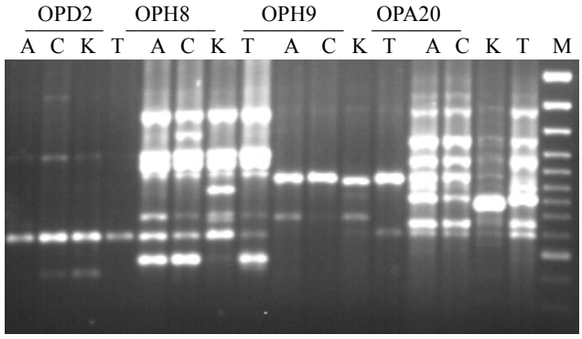

Fig. 1. Amplification pattern of genomes of rice root-knot nematode populations obtained with random primers, OPD2, OPH8, OPH9 and OPA20

M- 100bp plus DNA ladder, random primers are indicated on the top of the gel. Lanes: A- Amana, C-Cuttack, K-Kamakshyanagar, T-Trichy

Ninety seven (68.3%) bands were found to be common. Number of common bands varied from 2 (OPN3 and OPN9) to 10 (OPR2). Forty six bands were amplified in all the RKN populations. Such common bands amplified with different primers could represent species-specific markers and their additional characterization would be useful to determine their potential in distinguishing *Meloidogyne graminicola* from other *Meloidogyne* spp.

A total of forty four (30.99%) unique bands were amplified by fourteen primers (Table 3). Three primers, OPA20, OPD2 and OPN3 could not amplify any unique band. Number of unique bands amplified by the rest of 14 primers varied from one (OPA2, OPH9 and OPH19) to 8 (OPH20)(Table 2). Six primers, OPH17, OPH18, OPN2, OPR1, OPR2 and OPR3 amplified four unique bands each followed by three bands by primer, OPN11. Sixteen unique bands were amplified RKN population from Kamakhyanagar followed by 12 bands each in populations from CRRI and Trichy. Three unique bands were amplified in population from Amana. Seventeen bands were amplified only in RKN population of Odisha while 12 bands were amplified only in Trichy population. These unique bands could be developed into diagnostic markers to identify particular population of RKN.

The discriminating power of random primers varied from 0 (OPA20 and OPN3) to 0.821(OPH20), with an average of 0.49 (Table 2). Ten primers, OPH9, OPH17, OPH18, OPH20, OPN2, OPN9, OPO16, OPR1, OPR2 and OPR3 showed more than 0.5 discriminating power. These primers are very useful in discriminating rice root-knot nematode populations.

Similarity index of pair-wise comparisons estimated on the basis of all the 17 primers ranged from 0.456 to 0.743 with an average of 0.558. Amana population showed highest similarity (i.e. 0.743) with Kamakhyanagar population while Trichy population showed least similarity (i.e. 0.456) with Kamakshyanagar population. Amana population showed 0.627 similarities with Cuttack population while Kamakhyanagar population showed 0.541 similarities with Cuttack population. High genetic similarity (i.e. average: 0.637) between these three Odisha populations indicated that all the three populations could have common origin.

Cluster analysis based on UPGMA classified four populations into two major groups at 48% of genetic

similarity (Fig. 2). The Cophenetic correlation coefficients (r = 0.949) revealed the reliability and stability of clustering. First major group was further sub-grouped into two sub-clusters, IIA and IIB at 58.5% genetic similarity. Cuttack population was included in sub-cluster IA while Amana and Kamakhyanagar populations were included in IIB. Trichy population was included in major group II (Table 3). Trichy population was differentiated from others at 52% genetic diversity, indicating that Trichy is a different pathotype than other nematode populations.

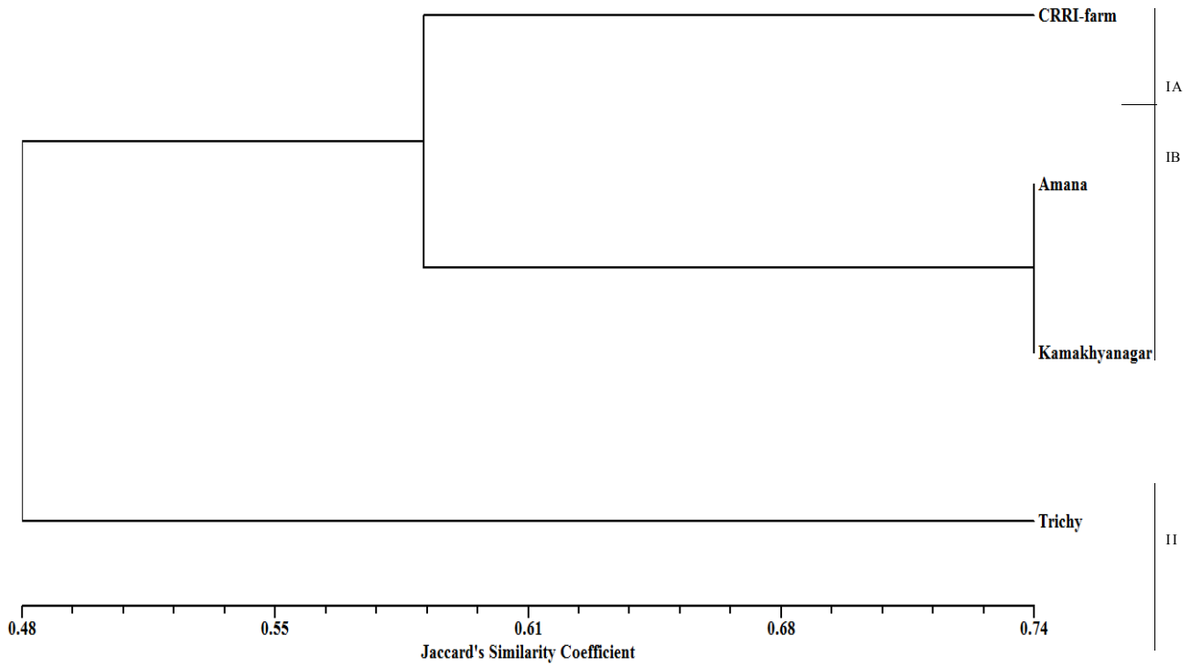

Fig. 2. Dendrogram showing genetic relationships between rice root-knot nematode populations.

All the individual populations of rice root-knot nematode included in the study could be distinguished precisely from each other. The highly informative primer, OPH20 could differentiate all the RKN populations. Each population could be identified and differentiated by the unique bands. Virulence pattern indicated that each rice root-knot nematode population is different from other (Table 1). Molecular genotyping also indicated that Trichy is a different pathotype than other nematode populations although genetic diversity does exist among Odisha populations.

Semblat *et al*. (2000) investigated relationships between molecular fingerprints and virulence reaction pattern of 17 populations belonging to three major parthenogenetic species of *Meloidogyne* against the tomato *Mi* resistant gene. The result demonstrated that virulent populations do not share a common origin and DNA polymorphism is independent of virulence, which is presumably under host selection. Randing *et al* (2002) used RAPD markers to characterize the genetic diversity and relationships between root-knot nematodes (*Meloidogyne* spp.) of coffee in Brazil. A high level infraspecific polymorphism was detected in *Meloidogyne arenaria*, *Meloidogyne exigua*, and *Meloidogyne hapla* compared with the other species tested. Pokharel *et al.* (2007) recovered thirty-three root-knot nematode isolates from soil samples of rice-wheat fields in Nepal. The isolates were characterized using morphology, host range and DNA polymorphism analyses. Results indicated the phenotypic similarity of the Nepalese isolates with *Meloidogyne graminicola*, with minor variations. Phylogenetic analyses indicated that all Nepalese isolates formed a distinct clade with known isolates of *M. graminicola.*

The present study revealed that the RAPD marker technique is extremely useful for rapid identification of genetic polymorphisms in rice root-knot nematode populations. The molecular genotyping with

Table 3. Unique bands amplified in different rice root-knot nematode populations

Name of RAPD primer	No. of unique bands	Population along with size of unique bands (bp)
OPA2	1	Cuttack (1600)
OPH8	2	Kamakhyanagar(675, 925)
OPH9	1	Kamakhyanagar(850)
OPH17	4	Cuttack (500),Kamakhya-nagar (700), Trichy (600, 950)
OPH18	4	Cuttack (650), Kamakhya-nagar(655, 1250), Trichy(445)
OPH19	1	Kamakhyanagar(700)
OPH20	8	Amana(510, 900, 1340), Cuttack (600), Kamakhyanagar(420, 1100, 1200), Trichy(400)
OPN2	4	Kamakhyanagar(750, 2650), Trichy(510, 1400)
OPN9	2	Amana(925), Trichy(1100)
OPN11	3	Kamakhyanagar(400, 510), Trichy(420)
OPO16	2	Amana(1100, 1325)
OPR1	4	Amana(370, 470, 1400), Trichy (590)
OPR2	4	Amana(390, 1300), Kamakhya-nagar(750, 895)
OPR3	4	Amana(480), Trichy(300, 620, 1100)
Total	44	

RAPD markers showed that Trichy population is different from Odisha populations although genetic diversity does also exist among Odisha populations. Virulence studies also indicated that Trichy population is a different pathotype than other nematode populations studied.

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