PCR-RFLP PATTERN ANALYSIS OF ENTOMOPATHOGENIC NEMATODES ISOLATED FROM AGRO-ECOSYSTEM FOR IMPLICATING THEIR GENETIC DIVERSITY

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ABSTRACT: Entomopathogenic nematodes (EPNs) (Rhabditida: Steinernematidae and Heterorhabditidae) were isolated from cultivated lands in Karur region of Tamil Nadu, India. The ITS (internal transcribed spacer) rDNA sequence similarity of isolated EPNs revealed that the nematode strains *Steinernema siamkayai*, *S. pakistanense* and *Heterorhabditis indica* were more prevalent in cultivated eco-systems of that region. PCR-RFLP patterns obtained from 10 different restriction enzymes have shown that these strains were phylogenetically are more or less closely related within species, but they are genetically diverse from location to location in that region. Among 25 nematode strains, 19 strains were phylogenetically related to *S. siamkayai* (strain CM2) isolated from Belgium. Three strains of *Steinernema* sp. were related to *S. pakistanense* strain isolated from USA and remaining three *Heterorhabdus* sp. similar to *H. indica* strain cohen 21 from USA. Thus, intra-specific relationships among strains of these nematodes would emphasis that geographic distribution and environmental conditions may be associated to bring such genetic variations and divergence in the genomes of the nematodes.

KEY WORDS: *Steinernema*, *Heterorhabdus*, Cultivated lands, ITS region, PCR-RFLP, Genetic variation.

Entomopathogenic nematodes (EPNs) belonging to the genera *Heterorhabditis* carry symbiotic of *Photorhabdus luminescens* with different subspecies and *Steinernema* carry symbiotic of *Xenorhabdus* spp. possess bioinsecticides, antimicrobial and antiviral activities are the curious potential characters. However, genetic variations in these nematodes are one of the determinants for survival of endosymbiotic and non-symbiotic bacteria in the respective hosts. Different nematode species and strains exhibit differences in survival, infectivity and efficacy against particular insect pest. A phylogenetic sketch is a necessary component of the comparative method in evolutionary biology and provides to study gene flow, population structure, biogeography, co-evolution, co-adaptation, co-speciation, and historical ecology (Hominick et al., 1997). Strengthening biosystematics research is extremely essential for the accurate identification of the species and knowing their genetic diversity, which forms the basis for exploiting the use of vast biodiversity resources, as well as their management.

A number of molecular techniques have been used for EPNs identification, including isoenzyme patterns (Akhurst, 1987), total protein patterns (Poinar & Kozodoi, 1988) and immunological techniques (Jackson, 1965). Restriction fragment length polymorphism (RFLP) detection within total genomic DNA is a reliable taxonomic tool that can be used for the identification of single nematode

(Curran & Webster, 1989; Smits et al., 1991; Reid & Hominick, 1993). The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (Hillis et al., 1991; Powers et al., 1997) has already reported to be a consistent marker for identifying species within each genus of EPNs. It could provide the requisite heritable characteristic for a thorough phylogenetic analysis (Vrain et al., 1992; Joyce et al., 1994; Cherry et al., 1997). The advantages and taxonomic suitability of this marker include the potential for PCR amplification and sequencing by universal primers, forced uniformity of paralogues via rapid determined evolution, variation due principally to point mutations, apparent independence of variable sites, and phylogenetic information appropriate for species level investigations (Adams, 1998). Moreover, molecular approaches can be enhancement by morphological, morphometric and cross breeding techniques to confirm the identification of reputed new species (Hominick et al., 1997).

The genetic differentiation among species is the result of the interaction of random chance, migration, and natural selection. Various field isolates are often different from one another at different degrees. Therefore, the present work was aimed to identify the genetic differences of EPN strains isolated from different locations of cultivated lands in Karur region of Tamil Nadu, India using PCR-RFLP profiling.

MATERIALS AND METHODS

EPN populations

A total of 25 populations of the *Heterorhabditis* and *Steinernema* species were used for this study (Table 1). The samples were obtained from cultivated lands of Karur region of Tamil Nadu, India. EPNs were isolated from *Galleria mellonella* trapping method according to Bedding and Akhurst (1975). Each isolate was given a code as follows KAR (for Karur) followed by serial number.

DNA extraction and PCR amplification of the ITS-rDNA

Genomic DNA was extracted by grinding the IJs in a mortar and pester with liquid nitrogen until a fine white powder was produced. The powder was collected and 1 ml of extraction buffer (10 mM Tris-Cl pH = 8, 1% SDS, 0.4 M NaCl, 5 mM EDTA) was added. Then 50 µl proteinase K solution (0.01 g/ml) was added and the mixture was incubated for 30 min at 37 ºC and subsequently for 60 min at 65 ºC. The solution was twice extracted with equal volumes phenol-chloroform- isoamyl alcohol (25:24:1) and finally with chloroform- isoamyl alcohol (24:l). DNA was precipitated from the final aqueous phase by adding 2 volumes of 96 % ethanol and 0.1 volume of 3M ammonium acetate and placing the mixture at - 70ºC for 30 min. The precipitated DNA was centrifuged in a micro centrifuge and dried under vacuum at room temperature. The pellet was resuspended in 50 µl of TE-buffer (0.01 M Tris-Cl pH - 7.5, 0.001 M EDTA) and stored at 4ºC. The nucleic acid concentration was measured in a spectrophotometer at 260 nm. The DNA sample was run by agarose gel electrophoresis using 1 kb markers of lambda DNA as a standard.

The ITS region of rDNA sequence in each strain was amplified by PCR (polymerase chain reaction) and the primers used this study were designed according to Joyce et al. (1994b). PCR reaction were carried out, 100ng Genomic DNA, 1µl of dNTP mix (2.5mM each), Forward primer and Reverse Primer 10 pmole, 5 µl of *Taq* buffer (10X), 3U of *Taq* DNA polymerase enzyme and nuclease

free water was added make up the volume 5µl. PCR running condition was set as 35 cycles of 94°C for 1 min, 55°C for 1min, and 72°C for 2 min.

PCR-RFLP pattern analysis

PCR products (5µl) of every isolates were digested with 0.5µl of restriction endonucleases *Pst*I, *Pvu*II, *Sau*3AI, *Sal*I, *Alu*I, *Hpa*II, *Hha*I, *Hind*III, *Hinf*I and *Xha*I according to the manufacturer's instructions with the total volume of 20µl. The digested products were run on a 2% agarose gel, at 100V for 2 hours and photographed using GeneTech gel documentation. A genetic relationship between each pair of strains was valuated as presence or absence of DNA restriction fragment.

RESULTS

The samples collected from different cultivated lands showed positive for the presence of the EPNs (25 strains) of the genus *Steinernema* and *Heterorhabditis*. The PCR products of each strain has composed of a part of the ITS region and a part of 18S, rDNA gene and they were yielded a single fragment with a length of approximately 776-784 bp. Blast similarity search results showed that ribosomal DNA (ITS region of nematodes) sequences of each isolate have a maximum sequence similarity with closely related with the sequences of EPNs belonged to the same genera. It reflected to confirm these isolates were belonged to *Steinernema* and *Heterorhabditis* sp. Phylogenetic bootstrapping values of scaled in the phylogenetic tree was supported the correspondence of every isolates with their species and promising their distinctiveness as separate species (data not shown). As the results of ITS rDNA sequence similarities and phylogenetic inference, 19 isolates were grouped under the members of *S. siamkayai*, the 3 isolates were the members of *S. pakistanense* and the remaining 3 *Heterorhabditis* isolates were belonged to *H. indica*.

PCR-RFLP pattern analysis

The PCR-RFLP profiles of the isolated EPNs strains were performed with 10 restriction enzymes. The restriction patterns obtained with *Pst*I; *Pvu*II; *Sau*3AI; *Sal*I; *Alu*I; *Hpa*II; *Hha*I; *Hind*III; *Hinf*I and XhaI are represented in Table 2 and Figure 1. The counting numbers of bands generated from each restriction enzyme digestion (PCR-RFLP) was chosen for genetic variation analysis in which more distinct band and numbers of RFLP fragments indicated strain specific variations in the ITS sequences of strains isolated from this region. The genetic variants of these strains isolated from different location of Karur region so that this criterion would be more appropriate to distinguish the strains. PCR-RFLP variant analysis of each strain revealed a little or no discernible intra- specific variability was observed within the species of *Steinernema*. The isolate *S. siamkayai* isolated from Krishnarayapuram, Kulithalai and Aravakurichi locations was same strains, while isolate *S. pakistanense* was existed in Kulithalai and Karur similarly. The strain *H. indica* was found in Krishnarayapuram and Karur are closely resembles. Although these strains have been identified by morphology and genetic variants in ITS regions of rDNA, they have some genetic polymorphic differences in these genomes. Thus, a polymorphically distinct character of these strains was analyzed by RFLP fragments.

*Sau*3AI and *Hha*I digested fragments showed that the strains KAR01, KAR04 and KAR05 have the same genetic polymorphism in ITS sequences. More number of fragments has been generated by restriction enzymes *Pst*I, *Alu*I and *Xha*I from

the strains KAR01 and KAR05 as shown in Table 2. *Sal*I digested fragments of ITS sequences were highly varied from strains to strains indicated its genetic variability. Strain KAR04 has a low number of RFLP fragments and same genetic polymorphisms were found in the fragments generated by *Sau3A*I and *Hha*I. However, *Xha*I digested of KAR36 and KAR518 fragments showed a high variability. Other RFLP fragments of ITS sequences of *H. indica* were highly varied even those strains isolated from same locality of Karur region.

Apart from the genetic variations of these strains, the highly homologous genetic polymorphism in *S. siamkayai* have been detected in the RFLP fragments resulted from the digestion of *Sau*3A (KAR12, KAR140, KAR723, KAR240 and KAR571), *Hha*I (KAR12, KAR140, KAR723, KAR240, KAR571 and KAR06), *Alu*I (KAR12, KAR140, KAR723 and KAR240), *Hind*III (KAR723, KAR240, KAR571 and KAR06), *Hinf* I (KAR723, KAR240 and KAR571). The fragments of *Sal*I and *Hha*I showed the strains specific similarity between KAR 723 and KAR 240, respectively. All the strains significantly varied in the fragments of *Pst*I and *Hha*I whereas *S. pakistanense* and *H. indica* have also genetically differed in the fragments of *Pst*I, *Hha*I, *Hpa*II and *Xha*I. A high possibility of polymorphic variation detected in *S. siamkayai* was in the strain KAR06. Results of RFLP pattern analysis revealed that the nematode *H. indica*, *S. siamkayai* and *S. pakistanense* were highly variable in their genetic diversity that was dependent on the location of isolation.

DISCUSSION

In the present investigation the geographical origin and habitat can influence the morphometric characteristics of the isolates can change respective to those provided in the bibliography based on abiotic factors and host availability (Stock et al., 2000; Poinar, 1992). These morphometric differences are resulted due to intra-specific variability (Stock et al., 2000; Stock & Reid, 2004). Soil nematodes in general are morphologically very conservative so that the higher level taxonomy in this phylum is hampered by a lack of suitable characters and morphology is unreliable as an indicator of genetic differentiation of species. Systematic of EPNs is still in a state of instability and transition. DNA sequencing has become one of the most utilized molecular approaches for inferring phylogenetic history. In our studies, primers have been used to amplify ITS region, including the partial 18S rDNA gene which provides the insights necessary to develop a strong, morphologically based taxonomy according to Hominick et al. (1996). Thus, phylogenetic analysis revealed that 19 isolates were grouped under *S. siamkayai,* 3 isolates were *S. pakistanense* and the remaining 3 isolates were belonged to *H. indica*.

RFLP analysis of PCR amplified products from specific regions of the genome is a powerful taxonomic tool that can be used for identification of single nematodes. Amplified products of the ITS region have, upon restriction enzyme digestion, yielded many RFLP fragments for several rare strains that can be used for species identification of *Steinernema* isolates (Reid, 1994). This PCR-RFLP profiling reported the genetic divergence of 19 *S. siamkayai*, 3 *S. pakistanense*, and 3 *H. indica* populations. It resulted that the strains *H. indica*, *S. siamkayai* and *S. pakistanense* were highly variable in the RFLP patterns analyzed from the ITS sequences, which were dependent on the location of isolation. This may be resulted due to intrinsic and extrinsic factors, host, genome dynamics and horizontal gene transfer and evolutionary process. These factors could be influenced to bring such genetic variations among EPNs strains isolated from the

same location. The present attempt is useful for identification of EPNs at species level, and to know the species-specific genetic variation to that region. This will provide a new vision to select a species or strain for bio-control testing for controlling the pest in a selected zone.

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Table 1. EPN isolates recovered from Karur Region of Tamil Nadu, South India and their identity based on PCR-RFLP patterns of the amplified ITS rDNA region.

Isolate	Species/Strain	Provinces	Crops
KAR511	*S. siamkayai*	Krishnarayapuram	Sugarcane
KAR704	*S. siamkayai.*	Krishnarayapuram	Sugarcane
KAR36	*H. indica*	Krishnarayapuram	Banana
KAR211	*S. siamkayai*	Krishnarayapuram	Cotton
KAR240	*S. siamkayai*	Krishnarayapuram	Paddy
KAR01	*S. pakistanense*	Kulithalai	Sugarcane
KAR05	*S. pakistanense*	Kulithalai	Banana
KAR09	*S. pakistanense*	Kulithalai	Banana
KAR12	*S. siamkayai*	Kulithalai	Sunflower
KAR140	*S. siamkayai.*	Kulithalai	Paddy
KAR120	*S. siamkayai*	Aravakurichi	Sugarcane
KAR210	*S. siamkayai*	Aravakurichi	Sugarcane
KAR742	*S. siamkayai*	Aravakurichi	Banana
KAR571	*S. siamkayai*	Aravakurichi	Sunflower
KAR104	*S. siamkayai*	Aravakurichi	Paddy
KAR06	*S. siamkayai*	Aravakurichi	Cotton
KAR25	*S. siamkayai*	Aravakurichi	Cotton
KAR23	*S. siamkayai*	Aravakurichi	Groundnut
KAR220	*S. siamkayai*	Karur	Sugarcane
KAR707	*H. indica*	Karur	Sugarcane
KAR 881	*Steinernema* sp.	Karur	Sugarcane
KAR723	*Steinernema* sp.	Karur	Banana
KAR518	*Heterorhabditis* sp.	Karur	Sunflower
KAR04	*Steinernema* sp.	Karur	Cotton
KAR21	*Steinernema* sp.	Karur	Cotton

Table 2. PCR-RFLP patterns generated by digestion of the ITS region from *Steinernema* and *Heterorhabditis* isolates with 10 restriction enzymes. Numbers stand for different RFLP fragments yielded by a single enzyme digestion; species with the same number specify identical patterns; species with different numbers specify different patterns.

Species	RFLP patterns of individual restriction enzymes									
	***Pst*I**	***Pvu*II**	***Sau*3AI**	***Sal* I**	***Alu*I**	***Hpa*II**	***Hha*I**	***Hind*III**	***Hinf* I**	***Xha*I**
H.indica										
Kar 36	5	2	3	3	2	3	1	1	1	1
Kar 518	3	3	3	1	4	1	1	1	4	4
Kar 707	4	1	3	1	4	1	3	1	3	3
S.pakistanense										
Kar 01	4	1	4	6	4	1	1	2	2	2
Kar 04	4	3	1	4	1	2	1	1	1	1
Kar 05	4	2	4	1	4	1	1	1	2	3
S.siamkayai										
Kar 06	5	5	5	1	3	1	6	1	4	4
Kar 12	5	1	3	3	3	1	4	1	4	4
Kar 140	4	1	2	3	3	1	4	1	4	4
Kar 240	4	4	3	1	3	1	5	1	3	3
Kar 571	5	1	3	5	4	1	3	1	3	5
Kar 723	4	1	3	3	3	1	4	1	4	4

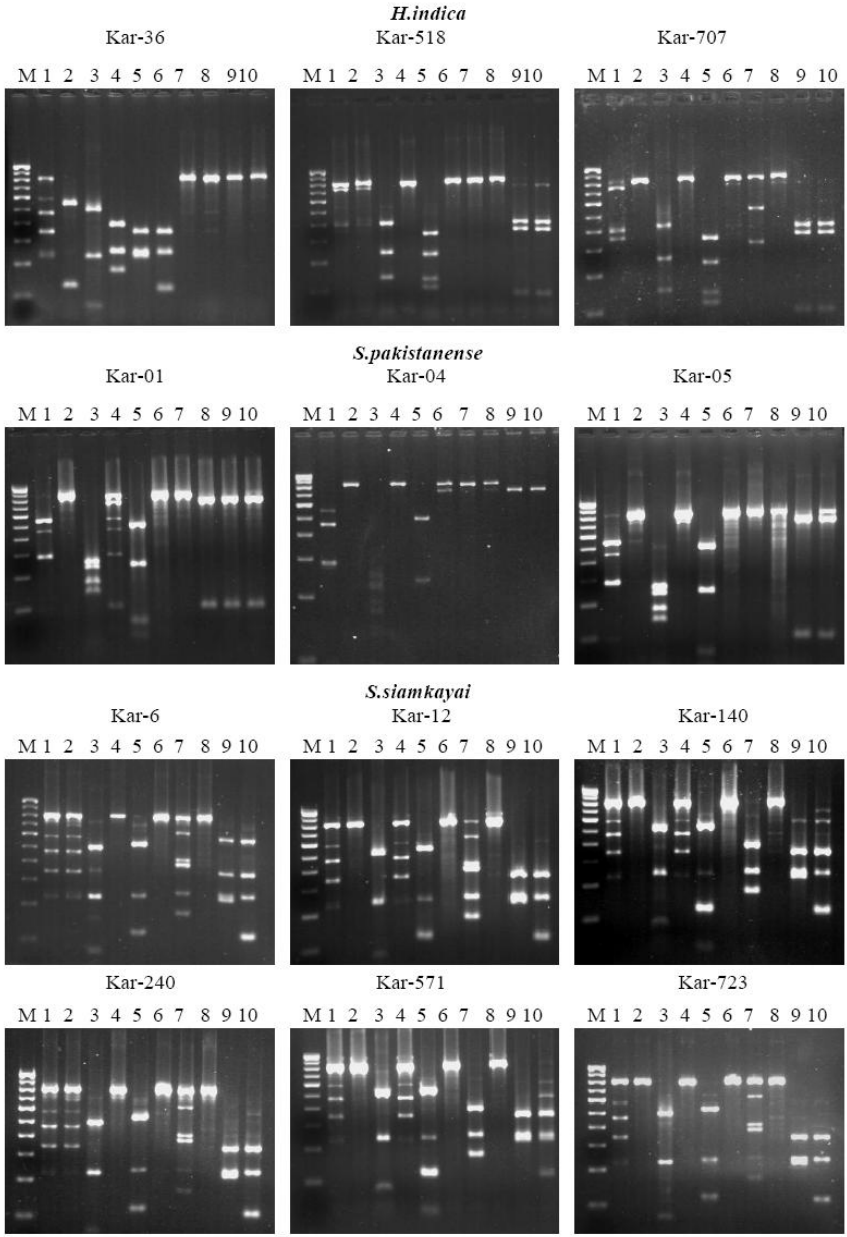

Figure 1. Agarose Gel electrophoresis banding patterns obtained from restriction digestion of the PCR-amplified ITS region of *H. indica*, *S. pakistanense* and *S. siamkayai* isolates. The PCR-amplified ITS regions were digested with the 10 restriction enzymes. Lanes 1-10 indicate the following enzymes: M- Molecular markers (100Kbp) 1.*Pst* I; 2.*Pvu* II; 3.*Sau* 3 AI; 4.*Sal* I; 5.*Alu* I; 6.*Hpa* II; 7.*Hha* I; 8.*Hind* III; 9.*Hinf* I; 10.*Xha* I.