RAPD analysis of Indian isolates of *Xenorhabdus* spp. associated with entomopathogenic nematodes, *Steinernema* spp.

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Abstract. The bacterial symbionts of entomopathogenic nematodes *Steinernema* and *Heterorhabditis*, *Xenorhabdus* and *Photorhabdits* spp., respectively, have been identified to offer potential alternative to chemical pest control because of their insecticidal toxins associated with the bacteria. Isolation and identification of these bacteria from indigenous nematodes are useful, as they possess insecticidal activities. Four Steinernematid species, *Steinernema carpocapsae*, *S. feltiae* and *S. tami* recovered from Indian soils (PDBC Collection) were used for isolation of their respective symbiotic bacteria, *Xenorhabdus* spp. Establishing the identity of *Xenorhabdus* bacterial isolates or species based on phenotypic and cultural characteristics is difficult because most strains are phenotypically very similar and fail to give positive results in many classical tests of identification and lack of sufficient members per taxon. In view of this, these bacterial isolates were subjected to RAPD analysis to evolve an easy method for their identification by using about 20 random primers and if they could be used as RAPD markers. It was observed that the overall percentage of similarity among these four isolates ranged from 22.61% to 61.73% with a mean of 41.5%. Dendrogram analysis among the four populations showed *X. nematophilus* (SCX3) and *Xenorhabdus* sp. (SCX6) were more closely related among the isolates and *X. nematophilus* (SCX3) and *X. bovenii* (SCX7) were the most distantly related. The present findings showed that four isolates of *Xenorhabdus* obtained from different geographical parts of India were genetically different from one another indicating that RAPD analysis with eleven primers can be used for the assessment of genetic variability of *Xenorhabdus* bacteria associated with *Steinernema* spp.

Keywords. Entomopathogenic nematodes, indigenous isolates, RAPD, *Steinernema* spp., variation, *Xenorhabdus* spp.

INTRODUCTION

Bacteria belonging to the genera *Xenorhabdus* and *Photorhabdus* are mutualistically associated with insect-pathogenic nematodes of the families of *Steinernematidae* and *Heterorhabditidae*, respectively (Akhurst, 1983). The nematode-bacterium complex exhibits great potential as a biological agent for insect pest control in cryptic environments (Kaya et al., 1985). The free-living 3rd instar infective juvenile embed cells of the symbiotic bacterium in the intestinal vesicles (Bird et al., 1983) and releases them after penetrating into the host and feeds on multiplied bacteria and disintegrate insect tissues (Kondo et al., 1988, Poinar et al., 1967). *Xenorhabdus* belongs to family Enterobacteriaceae (Thomas and Poinar, 1979) and it is composed of four variedly described species. *Xenorhabdus nematophilus* is the symbiont of *Steinernema carpocapsae* (Akhurst and Boemare, 1988) and *X. bovenii* is a symbiont of *S. feltiae* and *S. poinarii*. *X. poinarii* is a symbiont of *S. glaseri* and unnamed *Steinernema* spp. (Akhurst, 1986, Curran, 1989) and *X. beddingii* is a symbiont of two unnamed *Steinernema* spp. (Akhurst and Boemare, 1990). The previous studies involving molecular techniques, such as, DNA-DNA hybridization studies (Boemare et al., 1993), RFLP of 16SrRNA gene (Brunel et al., 1997) and preliminary sequenced based investigations of *Xenorhabdus* phylogeny (Raine et al., 1995) have been confined only to genus level and were successful in segregating one genus from the other. *Xenorhabdus* sp. isolated from the nematode, *Steinernema thermophilum* was reported from Indian soils (Ganguly et al., 2000). 16SrRNA gene sequencing indicated identity and phylogenetic position towards an individual taxon within the
phylogenetic dendrogram of Xenorhabdus type strains (Somvanshi et al., 2006). Very little work is reported on the genetic analysis/identification of these bacteria even at species level from India.

**MATERIALS AND METHODS**

**Source of nematodes and their bacterial symbionts**

Monoxenic nematode cultures of Steinernema carpocapsae, S. riobrave, S. feltiae and S. tami (Anonymous, 1999) were obtained from Project Directorate of Biological Control, Bangalore and were used for isolation of bacteria and for further experiments. A Xenorhabdus bacterium isolated and morphologically identified from Steinernema carpocapsae weiser was (Nagesh et al., 2002) used in the study along with three other Xenorhabdus isolates from Steinernema riobrave, S. feltiae and S. tami as described by Woodring and kaya (1988).

The isolation of bacteria from the above mentioned nematodes were done by infecting newly molted last instars larvae of the greater wax moth, Galleria mellonella, and exposed to infective juveniles at the rate of 25/larva. After 24-48 h the dead larvae were surface-sterilized by dipping them in 95% ethanol and igniting them. The cadavers were aseptically opened with sterile forceps and the bacteria from the haemolymph was streaked onto NBTA medium (Nutrient Agar with Bromothymol Blue) and incubated for 2 days at 25°C. The plates were examined for colony characteristics on nutrient agar as described by (Akhurst, 1980). Single isolated colonies were subcultured and maintained as slants for further studies. The isolated bacteria from each nematode species were designated an identification as given in Table 1.

**DNA preparation**

A single isolated colonies from Xenorhabdus isolates viz., SCX3, SCX6, SCX7, SCX8 was freshly inoculated in nutrient broth and incubated overnight on a orbital shaker (Scigenics) at 150 RPM 25°C. The cells were harvested by centrifugation at 4000g for 10 mins at 4°C. The harvested cells were taken for total genomic DNA isolation as per the protocol of Nasiri et al., with some modification. The cells were resuspended in TE-1 buffer (10mM Tris HCl, pH 7.6, 10mM MgCl₂, 1mMEDTA) and lysed with the addition Lysozyme (5mg/ml, Genei India) 37°C and with a incubation of 15-min. Additionally, 10% SDS (AMRESCO) was added and mixed thoroughly by pipetting. The lysed solution was added with 5M sodium chloride (AMRESCO) and centrifuged to separate the debris. The DNA present in the clear supernatant was precipitated with the addition of two volumes (V/V) of absolute ethanol (MERCCK) at room temperature and mixed by inverting and separated by centrifuged for 5 min. The DNA pellet was dehydrated and suspended in TE-2 buffer (10mM Tris HCl, pH 8.0, 1 mM EDTA pH 8.0) and incubated at 56°C for 15 min in a water bath. DNA concentration was determined by spectrophotometer (Biomate-5) at A²600.

**Primers**

The twenty random primers were obtained from Operon Technologies Inc.) containing 10-base oligonucleotide primers. The primers studied are OPA-13, OPA-18, OPS-13, OPC-06, OPC-09, OPC-011, OPC-012, OPX-04, OPH-15, OPH-16, OPH-18, OPH-19, OPA-15, OPA-16, OPA-17, OPC-07, OPC-08, OPS-16, OPC-13 and OPC-17.

**PCR amplification**

RAPD- PCR amplification was carried out in 200 µl PCR tubes using programmable thermal cycler (Bio-Rad 1-cycler, U.S.A). Each 25 µl reaction mix comprising of 100ng of template DNA, 1 µl of each primer (20 p mol/µl), 200mM of each dNTP (ppendorf), 5U of Taq DNA polymerase and 1×Taq buffer (Genei, India). The Taq buffer contained of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, and 0.08% Nonidet P40 and 1.5 mM MmCl₂. The reaction were overlayed with 50 µl of light mineral oil (Genei India). The PCR cycling program was 1 cycle of 94°C -1 min; 39 cycles of 1 min-94°C, 1.5 min-50°C, 1 min-72°C and 1 cycle of 72°C for 10 min. PCR products were stored at 4°C until required.

**Electrophoresis of the PCR products**

The PCR amplified products were then resolved on 1.5% agarose gel (AMESCO) with Tris borate acetate buffer. 20 µl of the PCR products were mixed with 5 µl of the loading dye and loaded in the wells of the gel. 5 µl of lambda ECOR1+Hind III double digest (Genei, India) DNA marker was loaded along with the samples in a separate lane. Electrophoresis was carried out at 50 V for three hours and the gel was captured in Transilluminator Gel doc System (Bio-Rad).
**RESULTS AND DISCUSSION**

In the present investigation, we have used RAPD analysis as a first screen to identify genetic variation among the four Indian isolates *Xenorhabdus* species viz., *X. nematophilus* (SCX3), *X. bovenii* (SCX6), *Xenorhabdus* sp. (SCX7) and *Xenorhabdus* sp. (SCX8) associated with *Steinernema carpocapsae*, *S. riobrave*, *S. feltiae* and *S. tami* respectively (Table 1). Out of twenty decamer primers used only eleven primers exhibited polymorphism and discriminating banding patterns (Table 2). Banding patterns with OPA-13, OPA-18, OPS-13, OPC-06, OPC-09, OPC-011, OPC-012, OPH-04, OPH-18, OPH-19, OPS-13 revealed the presence of amplicons of a variety of sizes (Fig 1, 2 and 3) and generated a set of bands ranging from 500-5000bp. The primer OPC-09 was the most differentiating primer for the ability to type *Xenorhabdus* isolates and produced 12 polymorphic bands whereas primer OPC-12 generated least number of 2 bands among the primers studied. Eleven primers revealed at least one scorable band with all the strains or isolates; Only 1 primer OPC-06 did not produce bands with SCX3 and SCX6. These decamer primers generated a total of 62 consistent polymorphic bands and data was analyzed for polymorphism. The percentage of primer producing scorable bands with different isolates is shown in Table 3.

The average percentage similarity between different isolates based on shared DNA fragments is shown in Table 4. Similarity matrix indicated that isolates of *X. nematophilus* (SCX3) and *Xenorhabdus* sp. (SCX6) were the closest group/isolates and shared percent similarity of 61.73. A minimum similarity (22.72%) was observed between *X. nematophilus* (SCX3) and *X. bovenii* (SCX7). These observations are presented in the form of a dendrogram (Fig. 4).

Based on the cluster analysis, *X. nematophilus* (*S. carpocapsae*) was more closely related to *Xenorhabdus* sp. (*S. riobrave*) compared to *X. bovenii* (*S. feltiae*) and *Xenorhabdus* sp. (*S. tami*). *Xenorhabdus nematophilus* and *X. bovenii* from Bangalore were found to be dissimilar and are distantly related. The polymorphic data reveals *X. nematophilus* (SCX3) and *Xenorhabdus* sp. (SCX6) from Bangalore and Gujarat was closely related. Fisher-le-saux *et al.* (1998) based on Restriction Fragment Length polymorphisms reported that among the *Xenorhabdus* isolates of Caribbean region *X. nematophilus* (*S. carpocapsae*) was more closely related to *X. bovenii* (*S. feltiae*) compared to *Xenorhabdus* sp. (*S. riobrave*). The observed differences could be due to the restricted diversity and co-evolution of *Xenorhabdus* species in a given geographical location like India. A high degree of *Xenorhabdus* diversity was observed with the wide diversity of associated steinernema nematode except in the case of *X. Poinarii* where the isolates were associated with two nematode species, *S. glaseri* and *S. cubanum*.

### Table 2. Number of polymorphic bands provided with the isolates used for percentage similarly analysis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Number of polymorphic bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPC - 06</td>
<td>GAACGGACTC</td>
<td>5</td>
</tr>
<tr>
<td>OPA - 18</td>
<td>AGGTGACCGT</td>
<td>7</td>
</tr>
<tr>
<td>OPA - 13</td>
<td>CGACACCCAC</td>
<td>6</td>
</tr>
<tr>
<td>OPH - 18</td>
<td>GAATCGGCA</td>
<td>3</td>
</tr>
<tr>
<td>OPH - 04</td>
<td>GGAAGTGGCC</td>
<td>4</td>
</tr>
<tr>
<td>OPC - 09</td>
<td>CTCACCCGTC</td>
<td>12</td>
</tr>
<tr>
<td>OPC - 11</td>
<td>AAAGCTGGG</td>
<td>11</td>
</tr>
<tr>
<td>OPC - 12</td>
<td>TGTCATCCCC</td>
<td>2</td>
</tr>
<tr>
<td>OPC - 13</td>
<td>GTGCTTCCG</td>
<td>5</td>
</tr>
<tr>
<td>OPH - 19</td>
<td>CTCAGGCGCC</td>
<td>6</td>
</tr>
<tr>
<td>OPS - 13</td>
<td>TTCGTTCCTG</td>
<td>5</td>
</tr>
<tr>
<td>Total number of polymorphic bands</td>
<td>62</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Percentage of scorable bands produced by primers with four isolates.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>% of scorable bands</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Xenorhabdus nematophilus</em> (SCX3)</td>
<td>83.33</td>
</tr>
<tr>
<td><em>Xenorhabdus</em> sp. (SCX6)</td>
<td>83.33</td>
</tr>
<tr>
<td><em>Xenorhabdus bovenii</em> (SCX7)</td>
<td>100</td>
</tr>
<tr>
<td><em>Xenorhabdus</em> sp. (SCX8)</td>
<td>91.6</td>
</tr>
</tbody>
</table>
Table 4. Average percent similarly between isolates using different primers.

<table>
<thead>
<tr>
<th></th>
<th>Xenorhabdus nematophilus (SCX3)</th>
<th>Xenorhabdus sp. (SCX6)</th>
<th>Xenorhabdus bovenii (SCX7)</th>
<th>Xenorhabdus sp. (SCX8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xenorhabdus nematophilu (SCX3)</td>
<td>100</td>
<td>61.73</td>
<td>22.72</td>
<td>30.15</td>
</tr>
<tr>
<td>Xenorhabdus sp. (SCX6)</td>
<td>-</td>
<td>100</td>
<td>34.28</td>
<td>32.95</td>
</tr>
<tr>
<td>Xenorhabdus bovenii (SCX7)</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>41.09</td>
</tr>
<tr>
<td>Xenorhabdus sp. (SCX8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

(Hominick et al., 1997). The genetic studies of local isolates from geographically related regions are very important because of the fact that single bacterial isolate can be associated with two or more species of Steinernema.

The preliminary findings of our study using RAPD-profiles with selected primers suggest that Indian isolates viz., *X. nematophilus*(SCX3), *Xenorhabdus* sp. (SCX6) *X. bovenii* (SCX7) and *Xenorhabdus* sp. (SCX8) were genetically different from each other. Although this is the first report on genetic analysis of Indian isolates of
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Xenorhabdus species through RAPD, a complimentary study with RFLP and 16SrRNA gene sequencing with more number of geographical isolates is in progress to confirm the findings.

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LITERATURE CITED


(Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and proposal to transfer Xenorhabdus luminescens to a new genus, Photorhabdus gen. nov. International Journal of systematic Bacteriology 43, 249-255.


Dear Dr. Vidya,

Your above-mentioned article has been accepted for the publication in Journal of Biological Control Volume 21 no. 2 year 2007.

As all the authors must become members of the society, please arrange to remit membership fee in respect of the following authors:

1. Dr. H.S. Vidya Rs. 175/- towards annual membership for the year 2007
2. 
3. 
4. 

The amount should be sent in the form of demand draft drawn in favour of Society for Biocontrol Advancement payable at Bangalore.

With personal regards,

Yours sincerely,

Dr. H.S. Vidya

Chief Editor