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Biochemical diagnostic markers for differentiating *Steinernema abbasi, S. siamkayai* and three new strains from four different agro-climatic zones of India*

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Entomopathogenic nematodes belonging to the genus *Steinernema* are obligate and lethal parasites of insects. They are currently being used as biological control agents for the management of several insect pests of crops. They can also be referred to as bio-pesticidal nematodes. Their species and strains are known to exhibit different efficacies against particular pests in different conditions (Gaugler 2002). Therefore, new species are always being sought. India's geographical area has been demarcated into 15 agro-climatic regions. The biopesticidal nematodes isolated from a particular condition are generally more efficacious for the pests prevailing at the same condition. Whenever new isolates are discovered, their identification is not always straightforward. Therefore, there has been much confusion over the taxonomy and nomenclature of these nematodes, with many isolates being mis-identified by standard morphological criteria. *Steinernema thermophilum* Ganguly & Singh 2000 is the first new species of this genus described from India. Thereafter, two more species *S. seemae* and *S. masoodi* were described by Ali *et al*. (2005). Eight other species of *Steinernema*, viz *S. abbasi* Elawad *et al*. 1997, *S. bicornutum* Tallosi *et al*. 1995, *S. carpocapsae* Weiser 1955, Wouts *et al*. 1982, *S. feltiae* (Filipjev 1934) Wouts *et al*. 1982, *S. glaseri* Steiner 1929, Wouts *et al*. 1982, *S. riobrave* Cabanillas *et al*. 1994, *S. siamkayai* Stock *et al*. 1998, *S. tami* Luc *et al*. 2000, have been recorded from this country (Ganguly 2003; Ganguly *et al*. 2005). Species-specific isozyme patterns, revealed through polyacrylamide slab gel electrophoresis, have been found useful for differentiating the species of *Steinernem*a as well as *Meloidogyne* (Esbenshade and Triantaphyllou 1985, 1990, Molinari *et al.* 2005). The isozyme techniques were soon replaced by RAPD (random amplified polymorphic DNA), RFLP (restriction fragment length polymorphism) of ribosomal DNA and base sequencing of the ITS region of rDNA (Dubey *et al*. 2009, Eddington *et al*. 2009, Malan *et al*. 2008, Stock *et al.* 2001), which are frequently used as supplementary tools in species descriptions. These techniques, though robust, remain of limited use for large surveys because they are costly and time-consuming procedures. Instead, isozyme patterns may prove to be an excellent tool for the identification of newly isolated strains of *Steinernema* spp obtained during routine surveys, as indicated by Ganguly and Pandey (2006).

For further strengthening the robustness of this technique for species differentiation, and revalidating our earlier observations, the present investigation was undertaken in 2009 to characterize the isozyme profiles of esterase (EST, E.C. 3.1.1.1) and superoxide dismutase (SOD, E.C.1.15.1.1) of five newly isolated native species *(S. abbasi*, *S. siamkayai* and three unidentified species) of *Steinernema*, and to explore their utility for differentiating the species/strains that are being isolated from different parts of the world.

Live cultures of infective juveniles of entomopathogenic nematodes of the genus *Steinernema (S. abbasi* strain IARI-EPN-*as*1, *S. siamkayai* strain IARI-EPN-*or*1; and three unidentified strains of *Steinernema* IARI-EPN-*wb*5 and IARI-EPN-*wb*2 and IARI-EPN-*jk*1) were maintained on the last instar larvae of greater wax moth, *Galleria mellonella*. Based on the ITS region of rDNA, the three unidentified strains exhibited respectively 91, 92 and 93% similarity with *S. carpocapsae*; while the two strains IARI-EPN *as*1 and IARI-EPN-*or*1 strains exhibited 99% similarity with *S. abbasi* and *S. siamkayai*, respectively (Ganguly Sushilkumar, unpublished). The gene sequences of rDNA were submitted in NCBI GenBank, Maryland, USA. The details of the strains used and their respective NCBI GenBank Accession Numbers are given in Table 1. Live populations of infective juveniles in sterile distilled water were stored at 15 °C. About 5 000 Ijs (infective juveniles) in 1 ml suspension were taken in 1.5 ml Eppendorf tubes and centrifuged at 8,000 rpm (2,147 g) for 5 min. The supernatant was discarded and pellet was homogenized with 40 µl of extraction buffer containing 20%

*Short note

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glycerol and 2% Triton-X-100. Additional extraction medium was added to increase the total volume of crude samples to about 100 µl and centrifuged at 10 000 rpm (3,354 g) for 10 min. at 4 °C. Clear supernatant was introduced immediately into the electrophoretic cell or stored at –80 °C until use.

The esterase and superoxide dismutase isozymes of IJs were determined by negative polyacrylamide mini slab gel electrophoresis in a mini slab gel apparatus (Genei Bangalore, India). A 5% acrylamide stacking gel and 7% acrylamide separation gel were used. About 25 µl of samples were loaded into the wells of the gel. The electric potential was set at 35 volts for the first 40–45 min. This allows stratification of proteins in the sample according to their relative charge. Afterwards, voltage was increased to 150 volts until the marker dye moved to the lower end of the gel.

Esterase activity was determined by incubating the gel at 37°C for 1 hr in a freshly prepared solution of Fast blue RR salt (15 mg), EDTA (7.5 g) dissolved in 25 ml of 0.1 M sodium phosphate buffer *p*H 7.1 and naphthyl acetate (20 mg dissolved in 0.5 ml acetone). Stain solution was poured off when the enzyme bands were sufficiently visible. Distilled water was added to wash the gel from any remaining stain solution (Harris Hopkinson 1976).

SOD isozymes were determined by incubating the gel at 37 °C for 20 min. in a stain solution containing 7.5 mg Sodium EDTA, 4 mg Riboflavin and 10 mg NBT in 100 ml Tris buffer *p*H 8.2 (0.61 g Tris dissolved in 100 ml distilled water). The gel was removed from the incubator and placed under fluorescent light. The bands of SOD activity were resolved as clear areas in the gel.

The stained gels were dried, scanned by means of a scan jet (Hewlett Packard), analyzed as digital images and then printed on photo quality paper. The species-specific enzyme phenotypes were named with capital letter of the respective species, followed by the number of isozymes, while non-specific enzyme phenotypes were labeled as 'N' followed by the number of isozymes. For each phenotype, the presence of a band (1) or its absence (0) was scored. Cluster analysis was conducted using NTSYS software and UPGMA method based on Jaccard's similarity matrix.

Isozymic patterns of esterase (EST) and superoxide dismutase (SOD) showed polymorphism, which could differentiate *S. abbasi*, *S. siamkayai* and three unidentified strains of *Steinernema* from four different agro-climatic zones of India (Table 1, Figs 1–3).

EST patterns (Fig 1) showed good polymorphism among *Steinernema* species. Strain IARI-EPN-*wb*5 exhibited one distinct band at Rf 0.07. Strain IARI-EPN-*wb*2 possessed five species specific bands at Rf 0.37, 0.42, 0.49, 0.77 and 0.84. Strain IARI-EPN-*as*1 of *S. abbasi* showed two species specific bands of Rf 0.30 and 0.44. *S. siamkayai* strain IARI-EPN-*or*1 and *S. carpocapsae* strain IARI-EPN-*jk*1 exhibited three bands of similar Rf values (0.30, 0.61, 0.88) but IARI-EPN-*jk*1 had one unique band at Rf 0.35 (Fig 1).

Isozyme profiles of superoxide dismutase revealed non-specific phenotypes with one band at Rf 0.43 for the strains IARI-EPN-*wb*5 and *S. abbasi* strain IARI-EPN-*as*1. Rest three strains showed species-specific phenotypes with one band at Rf 0.55 for IARI- EPN- *wb*2, three bands at Rf 0.18, 0.29 and 0.46 for *S. siamkayai* strain IARI- EPN- *or*1 and one band at Rf 0.47 for strain IARI- EPN- *jk*1 (Fig 2).

The dendrogram obtained from hierarchical cluster

Table 1 Enzyme phenotypes of esterase (EST) and superoxide dismutase (SOD), for five species / strains of *Steinernema* from four agro-climatic zones of India

Nematode species / strains[a] (NCBI GenBank acc. no.)	Origin (locality, state)	Geographic location	Agro-climatic zones	Enzyme phenotypes*	
				EST	SOD
Steinernema sp. strain IARI-EPN-*wb*5	Bankura, West Bengal	85°50'E – 89°50'E longitude and 21°10'N – 27°38'N latitude	Lower Gangetic Plains Region (3)	W1	N1
Steinernema sp. strain IARI-EPN-*wb*2 (GenBank Acc. No.FJ418045)	Bardhaman, West Bengal	85°50'E – 89°50'E longitude and 21°10'N – 27°38'N latitude	Lower Gangetic Plains Region (3)	W'5	W'1
S. abbasi strain IARI-EPN-*as*1 (GenBank Acc. No.FJ715946)	Jorhat, Asom	89°42'E – 96°E longitude and 24°8'N – 28°2'N latitude	Eastern Himalayan Region (2)	A2	N1
S. siamkayai strain IARI-EPN-*or*1 (GenBank Acc. No.GQ353373)	Bhubaneswar, Odisha	81°27'E – 87°29'E longitudes and 17°49'N – 22°34'N latitude	East Coast Plains and Hills Region (11)	O3	O3
Steinernema sp. strain IARI-EPN-*jk*1 (GenBank Acc. No.FJ418046)	Jammu and Kashmir	37°26'E – 80°30'E longitude and 32°17' – 36°58'N latitude	Western Himalayan Region (1)	J4	J1

[a]:Sequence of ITS region of rDNA deposited in NCBI GenBank, Maryland, USA.

*Species-specific enzyme phenotypes denoted by W (IARI-EPN-*wb*5), W' (IARI-EPN-*wb*2), A (IARI-EPN-*as*1), O (IARI-EPN-*or*1), J (IARI-EPN-*jk*1) ; and non-specific by N; Number following these letters indicate number of bands. Figures in parentheses indicate the number allotted to the agro-climatic zone

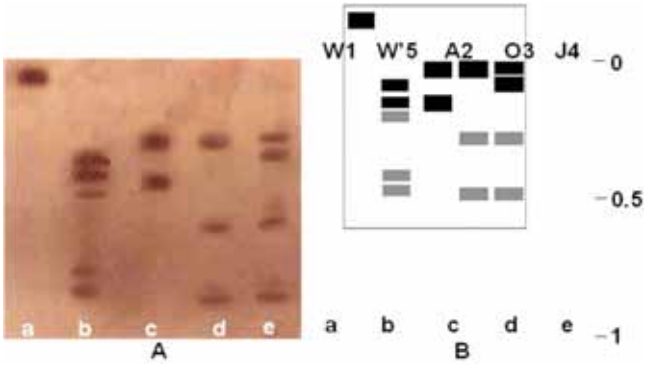

Fig 1 Esterase isozyme profiles (*A*) and their enzyme phenotypes (*B*), of infective juveniles of six Indian species of *Steinernema*. a: *Steinernema* sp. strain IARI-EPN-*wb*5; b: *Steinernema* sp. strain IARI-EPN-*wb*2; c: *S. abbasi* strain IARI-EPN-*as*1; d: *S. siamkayai* strain IARI-EPN-*or*1; e: *Steinernema* sp. strain IARI-EPN-*jk*1.

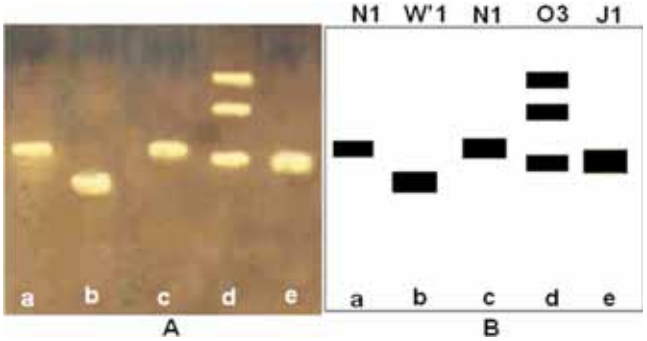

Fig 2 Superoxide dismutase isozyme profiles (*A*) and their enzyme phenotypes (*B*), of infective juveniles of six Indian species/ strains of *Steinernema*. a: *Steinernema* sp. strain IARI-EPN-*wb*5; b: *Steinernema* sp. strain IARI-EPN-*wb*2; c: *S. abbasi* strain IARI-EPN-*as*1; d: *S. siamkayai* strain IARI-EPN-*or*1; e: *Steinernema* sp. strain IARI-EPN-*jk*1.

analysis based on esterase and SOD isozymes (Fig 3) placed strains IARI-EPN-*wb*5, *S. abbasi* strain IARI-EPN-*as*1 and IARI-EPN-*wb*2 in one cluster (Cluster I) showing 82% similarity between the latter two, while 73.3% similarity between these two strains and IARI-EPN-*wb*2. Two strains IARI-EPN-*jk*1 and *S. siamkayai* strain IARI-EPN-*or1* form Cluster II with 70.5% similarity with each other. Both the clusters remained distinct with only 58% closeness.

Isozymic profiles of b-esterase, superoxide dismutase, isocitrate dehydrogenase and malate dehydrogenase have already been found to differentiate the species as well as their intra-specific variants in the genus *Meloidogyne* (Esbenshade Triantaphyllou 1985, 1990; Molinari *et al.* 2005) and for elucidating their phylogenetic relationships.

The present study has demonstrated the utility of esterase and superoxide dismutase isozymes as diagnostic markers for differentiating five strains/species of *Steinernema*, as is evident from the species-specific enzyme phenotypes obtained for almost all the strains studied using both the enzymes. These findings are in accordance with the earlier

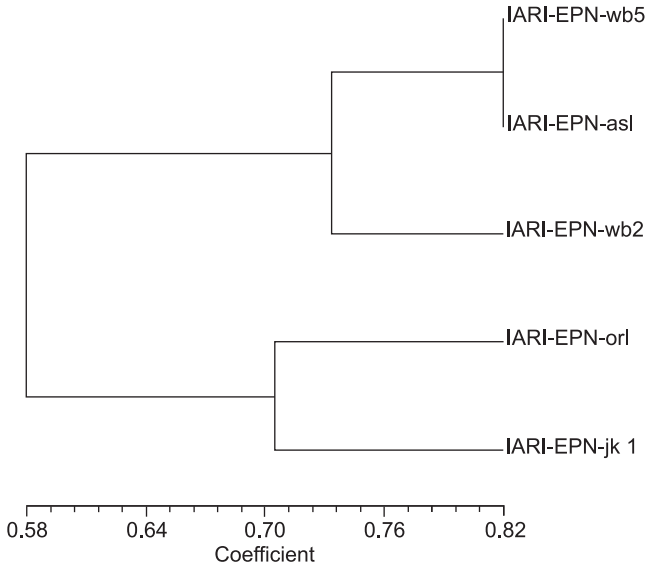

Fig 3 Dendrogram showing inter-relationships among the *Steinernema* species based upon esterase and superoxide dismutase isozymic profiles (only species names are indicated)

work done by Ganguly *et al.* (2006, 2009) who also reported the utility of esterase, catalase and superoxide dismutase isozymic profiles for differentiating six strains/species of *Steinernema*. Earlier, Ganguly Pandey (2006) could differentiate five species of *Steinernema (S. thermophilum, S. carpocapsae, S. siamkayai, S. glaseri, S. riobrave)* and an unidentified strain IARI-EPN-*mg*1 based on esterase isozymic profiles. Later, Ganguly *et al*. 2009 found esterase profiles to be more useful than superoxide dismutase for differentiating the *Steinernema* species. In this study, all the five species could be differentiated based on esterase as well as superoxide dismutase. Further studies on isozymic profiles of several populations of *Steinernema* comprising different species and strains, will yield useful information for the revalidation of these diagnostic markers. Distinctive isozymic profiles of esterase and superoxide dismutase obtained in the present study did indicate the possibility of all the test strains to be distinct species.

Keeping in view the high biocontrol potential of *Steinernema* species, their strains are continuously being isolated from different parts of the world and several strains are already in queue waiting for identification. Combination of esterase and superoxide dismutase isozymic profiles from infective juveniles, supplemented with morphological details, can be useful for preliminary screening and differentiation of *Steinernema* species.

SUMMARY

It has been demonstrated that the five newly isolated strains representing *S. abbasi*, *S. siamkayai* and three unidentified ones, are distinctive from each other based on esterase and superoxide dismutase isozymic profiles, which

clearly showed the unidentified strains to be different from each other. Furthermore, the three newly isolated unidentified strains are also indicated to be distinct species. However, it needs to be supplemented with the morphological characteristics of their different life stages. Two unidentified species of *Steinernema* strains IARI-EPN-*wb*5 and IARI-EPN-*wb*2, even though representing same agro-climatic zone, have been found to be placed in different clades, thus showing heterogeneity, perhaps, for harbouring varied insect pests. Further studies on host range of these strains will be of immense value for exploiting their biocontrol potential against specific insect pests prevailing in their respective niche agro-climatic zones.

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