Indian Journal of Agricultural Sciences **92** (10): 1253–1257, October 2022/Article
https://doi.org/10.56093/ijas.v92i10.120837

Molecular characterization of necromenic nematode *Pristionchus pacificus* and associated bacteria: A new record

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Received: 31 January 2022; Accepted: 6 September 2022

ABSTRACT

The vast diversity of nematodes has adapted to endure in different trophic groups as free living and parasites of plants and animals. In search of potential entomopathogenic nematodes (EPNs), soil samples were collected from, the rhizosphere of Apple orchard in Darima, Nainital, Uttarakhand, India north-western Himalaya in August 2019. The whole experimental activity carried out at ICAR-Vivekanada Parvatiya Krishi Anusandhan Sansthan, Almora during 2019–2021. In present study, *Pristionchus pacificus* was isolated using *Corcyra* bait trap, identified based on morphological and molecular approaches, studied its host range, evaluated the phylogenetic tree of *P. pacificus* and isolated and characterized the nematode associated bacteria. The reproduction potential and frequency of emergence of *P. pacificus* differs significantly among the tested hosts on *Corcyra*, *Spodoptera* and *Holotrichia* larvae. The molecular diagnostics of associated bacteria based on 16S gene displayed the highest homology with *Myroides marinus* strain. The discovery of *P. pacificus VLPP01* represents the first record from India and Himalayan region.

Keywords: *Myroides marinus*, Necromenic nematode, North-western Himalaya, Phylogenetic analysis, *Pristionchus pacificus*

The nematodes are highly diverse organisms (Jones *et al*. 2013). The role differs from ecological indicators, model organisms, biological control of insect pests and biotic stress in various crops (Singh *et al*. 2021). The value of entomopathogenic nematodes as an eco-friendly option to combat insect pests has inspired us to conduct a survey to isolate native EPNs of North western Himalayan region. During the search for potential EPNs, soil samples were collected from different hilly regions. *Pristionchus pacificus* was recovered from the rhizospheric soil sample of an Apple orchard from the locality of Darima, Nainital district, Uttarakhand, India. *P. pacificus* is reported as a model nematode for developmental biology and evolutionary studies (Hong and Sommer 2006). The available biotechnological advances have utilized *P. pacificus* in numerous ways from decoding genome to investigating functional genomics using CRISPR technology. *P. pacificus* is found globally and more than 150 isolates have been reported from soil and scaraebid beetles (Kanzaki *et al*. 2011). The genus Pristionchus belongs to the family of Neodiplogasteridae shows necromancy with beetles of the Scarabaeidae family. The larvae resume normal life by feeding on microbiome that grows on carcass of host insect. The newly emerged juveniles search for a new host and enters the larval body through the natural openings. The invaded IJs do not kill the host but patiently wait for the host to die and then they feed on the microbes grown on dead carcass (Rae *et al*. 2008). The genus Pristionchus contains many species including *P. pacificus* which reflects the behaviour of specific necromancy with scaraebid beetles. The identification of *P. pacificus* is difficult due to its morphological similarity with other species of Pristionchus genera (Herrmann *et al*. 2006). Therefore, ITS DNA sequence-based identification and phylogenetic analysis is more, accurate and robust way to identify Pristionchus at the species level (De Ley *et al*. 2005). In this study, we have (1) isolated and identified *P. pacificus* using molecular approach; (2) studied the host range of *P. pacificus*; (3) evaluated the phylogenetic tree of *P. pacificus* and (4) isolated and characterized the associated bacteria.

MATERIALS AND METHODS

Rearing insect host: The study was carried out at ICAR-Vivekanada Parvatiya Krishi Anusandhan Sansthan, Almora during 2019–2021. The larvae of *Corcyra cephalonica* reared broken corn seeds. The larvae of *Holotrichia* spp. collected from the infested soybean fields from Experimental Farm, ICAR-Vivekanada Parvatiya Krishi Anusandhan Sansthan,

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Hawalbagh, Almora (29.63º N and 79.63º E, 1250 amsl). The grubs were surface sterilized twice with 70% ethyl alcohol, autoclaved double distilled water, reared on potato slices at temperature of 25±2ºC, RH 65±5% for 48 h in a large sterile cylindrical plastic container containing autoclaved FYM and sandy loam soil in the ratio 1:1. The larvae of *Spodoptera litura* were collected from field and reared on tomato plants. The insect *S. litura* were identified based on morphological traits following PM, E. (2015) standards.

Soil sample collection and baiting: A total of 40 soil samples (each 200 g) were collected from different locations around Almora and Nainital districts of Uttarakhand located in North-western Himalaya, in August 2019. Soil samples placed in labelled plastic bags and carried to laboratory. The GPS coordinates of samples were recorded with GPS data exa mobile application 2.2.05.249. The average monthly rainfall was 351.07 mm (August month), mean maximum temperature 29.71°C and mean minimum temperature 22.39°C. The soil was baited for entomopathogenic nematode using *C. cephalonica* larvae with 30 g of soil in plastic container. Four larvae of *C. cephalonica* were placed in soil and container was inverted and placed at room temperature under dark conditions. The mortality of dead larvae was observed 12 days post-baiting and dead were individually rinsed with water and 70% ethanol to remove surface contamination and transferred to white trap (White 1927) for nematode extraction.

Morphological and molecular characterization: The harvested nematode samples were collected, concentrated and processed for nematode fixation following methodology of Seinhorst I and II (Seinhorst J W 1959). For molecular characterization, the genomic DNA were extracted using lysis buffer from nematodes in eppendorf tube. The isolated lysates were stored at -20ºC. The extracted DNA were amplified using universal r-DNA ITS and LSU primer set (ITS 5'-TTGATTACGTCCCTGCCCTTT-3' 5'-TTTCACTCGCCGTTACTAAGG-3') (LSU 5'-ACAAGTACCGTGAGGGAAAGTTG-3' 5'-TCGGAAGGAACCAGCTACTA-3') using PCR. The amplified products were separated and visualized using 5 µl of PCR products under 1.5% agarose gel electrophoresis in TAE buffer stained with EtBr. The obtained result compared with gene ruler marker of (100 bp). The Positive amplicons were purified by using FavorPrep™ GEL/PCR Purification Kit. The Purified Product was sequenced with Sanger's method of DNA sequencing for both directions. The input FASTA sequence of as query (length 874) were entered to NCBI nucleotide blast web server for standard database search set, to find highly similar sequences following megablast.

For Phylogenetic analysis, sequences showing highest similarity were selected and retrieved to FASTA file for phylogenetic analysis. *Koerneria lucani* (AB597244.1) were chosen as outgroup accession. The evolutionary history was inferred by using the maximum likelihood method, neighbour joining and maximum parsimony statistical method following Tamura-Nei model. Evolutionary analyses were conducted in MEGA X.

Test of reproduction potential of Pristionchus pacificus *on different insect host*: The isolated nematodes were reinoculated on *Corcyra*, *Spodoptera* and *Holotrichia* larvae for multiplication and purification of isolated nematodes. Insect larvae were placed in 9 cm petri dish lined with double layered moist filter paper containing 0, 10, 50, 100, 500 and 1000, 2500, 5000, 10000 nematodes/ml suspension in three replications. The Petri dishes were placed in a plastic tray and incubated at 25±1°C in dark. The dead larvae were placed on white trap at room temperature for 10 days and the nematodes emerging from cadaver were collected and washed with double distilled water. The data on emergence period from different insect host post mortality (days) and frequency of *P. pacificus* emerged from different insect hosts were recorded.

Isolation, identification and phylogenetic analysis of associated bacteria: Fifty surface sterilized nematodes were collected and crushed with micropestle and streaked on NBTA medium. Single colony were picked and incubated in LB media for 3 days at 27°C with 250 RPM. Bacterial DNA was extracted following modified CTAB method and PCR amplified with 16S universal primer (F 5'-GAGTTTGATYHTGGCTCAG-3' R 5"-GAGTTTGATYHTGGCTCAG-3"). DNA sequencing was performed on Sanger platform. The obtained reads were imported to NCBI BLASTn algorithm for homology search. Multiple sequence alignment (using Muscle) and phylogeny reconstructed using MEGA X with maximum likelihood statistical method at 2000 bootstrap replication following Tamura-Nei model. *Sphingobacterium spiritivorum* GenBank: AJ459411.1 were chosen as an outgroup.

Statistical analysis: The recorded data were analysed using one-way analysis of variance (ANOVA) following the standard procedure (Gomez and Gomez 1984). The post hoc test of means compared using Duncan's multiple range test (DMRT) at 5% level of significance (P< 0.05) using the statistical tool SPSS for windows version 16.

RESULTS AND DISCUSSION

Pristionchus specimen was located in a 20-year Apple orchard at Darima, Uttarakhand province of north-western Himalayan region (Table 1). The soil was characterized to have sandy loam texture, with 0.105 dS/m EC, 6.59 *p*H and organic matter content 10.6 g/kg. The grub of *Holotrichia* spp. was found frequently in the soil of sample collection sites. The nematode was found in in one out of 40 baiting samples. The nematode harvested post baiting on white trap used for species confirmation. Morphological characterization of isolated nematode showed the typical features of Diplogastridae family and conspecific to *Pristionchus pacificus*. The body pasture remained straight post fixation, cylindrical oesophagus, long and wide stoma, presence of dorsal tooth, tail elongated conical. Measurements of nematodes could not be conducted due to lack of expertise in drawing and measurement. To ensure the morphological identification of *P. pacificus*, the

Table 1 Physico-chemical properties of collected soil sample

Parameter	Unit	Control	Method	Reference
*p*H		6.59	1:2 soil-water suspension method	Jackson (1967)
EC (1:2 soil-water suspension)	dS/m	0.105	1:2 soil-water suspension method	Bower and Wilcox (1965)
Organic carbon	g/kg	10.6	Modified Walkley and Black method	Jackson (1967)
Mineralizable-N	kg/ha	467.6	Subbiah and Asija method	Subbiah and Asija (1956)
Available P	kg/ha	49.32	Olsen method	Olsen *et al*. (1954)
Available K	kg/ha	128.2	Ammonium Acetate method	Schollenberger and Simon (1945)
Available Zn	mg/kg	2.77	DTPA-$CaCl_2$ –TEA extraction method	Lindsay and Norvell (1978)
Available Mn	mg/kg	5.4		
Available Cu	mg/kg	1.78		
Available Fe	mg/kg	24.7		
Soil texture	-	Sandy loam	Jar test method	

molecular characterization was conducted. The genomic DNA of nematode were successfully extracted using lysis buffer and purity of DNA spectral quality assessed at 260/280 ratio through Thermo Scientific NanoDrop 2000 Spectrophotometer was found to be 1.7 with concentration of 875 ng/µl. The r-DNA cistron 18S SSU and 28S LSU (D2/D3) gene were successfully amplified for the nematode DNA. BLAST search of DNA sequence displayed significant similarity of 99.32% identity with *P. pacificus* (KY914570.1) with 100% query cover and 0.0 E-value. The Blast of D2-D3 sequence displayed 92.79% identity with 98% query cover and 0.0 E-value to *P pacificus* (KY914570.1) (Hassani-Kakhki *et al*. 2013). The identified isolate was designated as *P. pacificus* VLPP01 (Vivekanada Lab *Pristionchus pacificus* 01). The phylogenetic tree based on maximum likelihood of 40 aligned sequences of 18S SSU reflected 5 different clades, in which newly isolated *P. pacificus* are grouped under clade 1 with other isolates of *P. pacificus* (Fig 1). Maximum likelihood analysis placed the isolate of *P. pacificus* independently with single branch at the bottom of clade 1. 28 LSU sequence search in BLASTn showed highest similarity to organism *P. pacificus* (KY914570.1) with 96.86% (Hassani-Kakhki *et al*. 2013). The 28 LSU gene-based phylogeny supports the identity and indicates that the isolated nematode belongs to *Pristionchus* and has independent origin but shares comman ancestor with other known isolates under study. Evolutionary descent of different *Pristionchus* spp. indicated that the isolated nematode is related to other isolates of *P. pacificus.* The sequences of forward and reverse of rDNA cistrons were deposited at NCBI GenBank database under accession codes MZ506757 (874 bp), MZ506758 (874 bp), MZ853186 (667 bp) and MZ853185 (667 bp). *P. pacificus* were inoculated on *Corcyra*, *Holotrichia* and *Spodoptera* which demonstrated the reproduction potential on these three hosts. The reproduction potential and frequency of emergence of *P. pacificus* differed among the tested hosts. The emergence of juveniles was observed post inoculation of nematode concentration of 50, 100, 500, 1000, 2500, 5000 and 10000 nematodes/ml suspensions. However, no multiplication and emergence of nematode was observed at inoculation dosage of 0, 10, 50 nematodes per ml. The data analysis on emergence period of nematodes from insect body indicates significant difference between the tested insect hosts. *P. pacificus* took shortest duration in completion of life cycle and emergence on *Corcyra* larvae and longest duration from mortality to emergence of nematodes in *Holotrichia* (Fig 2). The emergence of nematodes from larval body of *Corcyra*, *Spodoptera* and *Holotrichia* started from 6th, 7th and 9th days and continued to 15th, 16th, 29th day, respectively. The frequency of *P. pacificus* emerged from different insect hosts were found significantly higher for *Holotrichia* followed by *Spodoptera* and *Corcyra* larvae (Fig 2). Our study also suggests that with an increase in concentration of nematodes/ml inoculated on the insect host, the time period taken in emergence reduced and the frequency of emergence of *P. pacificus* increased on different insect hosts. The molecular diagnostics of associated bacteria based on 16S gene displayed the highest homology with *Myroides marinus* strain GN-5 MN220571.1 (98.28%) belonging to family Flavobacteriaceae. Forward and reverse sequences *M. marinus* strain VLBM01 submitted to GenBank with accession ID OK402026 and OK405021.

P. pacificus have been established as a satellite system to investigate evolutionary developmental biology (Hong and Sommer 2006, Sommer and McGaughran 2013). *Pristionchus* spp. is known to be associated with beetles and frequently have been isolated from different scarab beetle host (Herrmann *et al*. 2010). Its isolation from rhizospheric soil and baiting through *Corcyra* indicates their possibility of occurrence freely in soil, infection and reproduction. Its preference to *Corcyra* host is the first description for *P. pacificus*. Similar observation of association with lepidopteran host *Galleria melonella* has been reported previously (Li *et al.* 2015). PCR based molecular diagnostics of nematode has been a commonplace due to simple handling, reliability and rapid species identification (Powers 2004). Tracing the phylogenetic relation using rDNA cistron of 18S and 28S has exhibited interesting results in unravelling nematode evolution (Holterman *et al*. 2006,

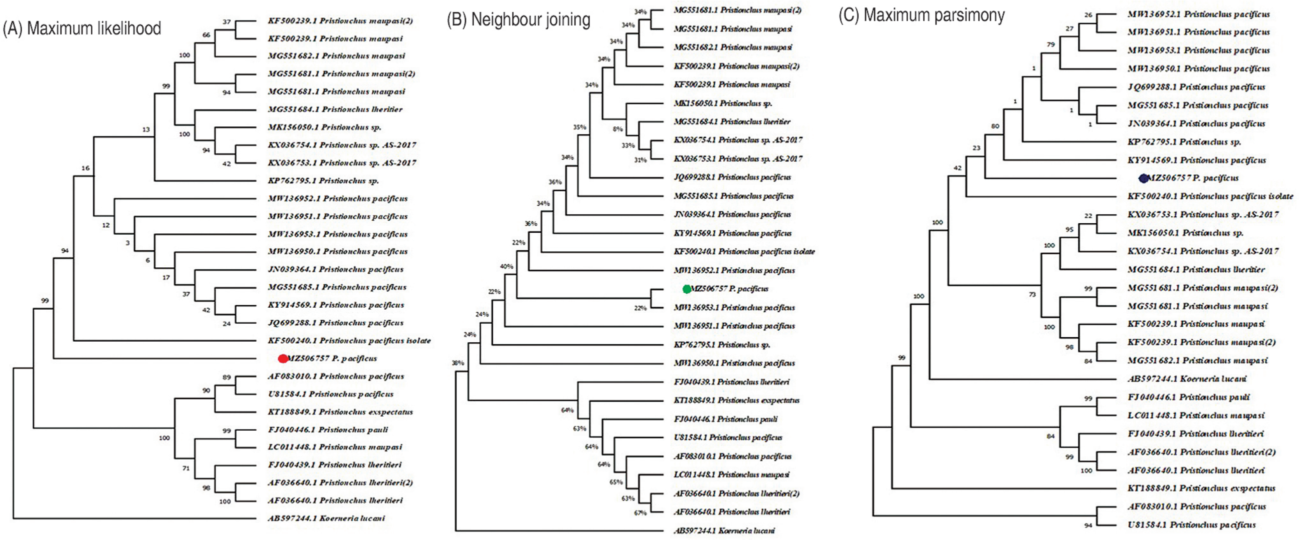

Fig 1 Phylogenetic tree based on 18S SSU gene sequence indicating the position of *P. pacificus* isolates in comparison to similar isolates based phylogenetic relationship. AB597244.1 (*Koerneria lucani*) were chosen as outgroup and bootstrap value 2000 (A) Maximum likelihood; (B) Neighbour joining; (C) Maximum parsimony.

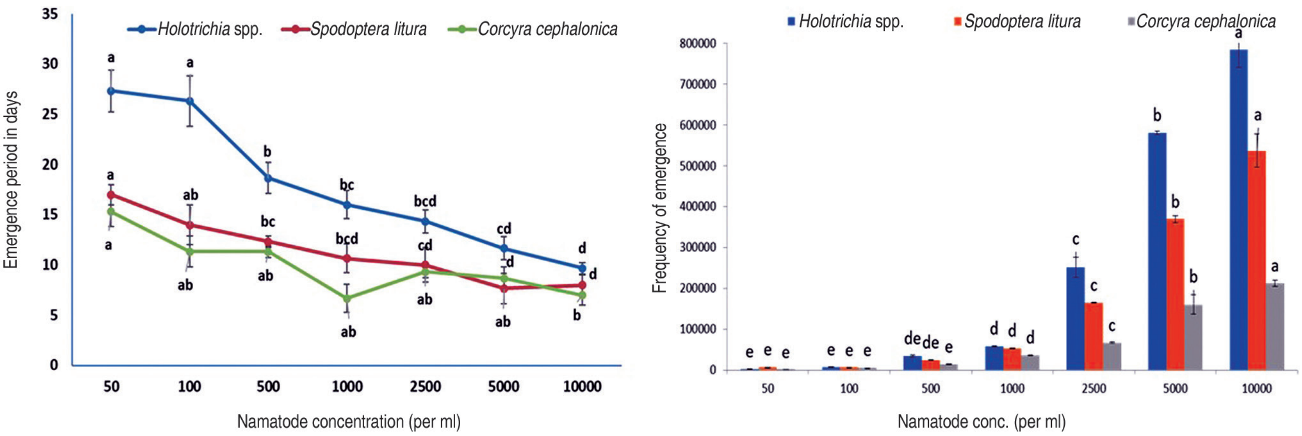

Fig 2 Emergence period from different insect hosts post mortality (days) and frequency of *P. pacificus* emerged from different insect hosts.

Pereira and Baldwin 2016). We identified and report for the first-time presence of *P. pacificus* isolate from India using 18S and 28S rDNA based diagnostic markers and the collection site have been marked to recover nematode for future use. The reconstructed phylogenetic tree reveals and confirms the identity and relativeness as the sister of another *P. pacificus*. This is the first time we evaluated and describe the comparative effect on larval host preferential by *P. pacificus*. Similarly, the shortest time duration required by *P. pacificus* for completion of life cycle and emergence from *Corcyra* followed by *Spodoptera* and *Holotrichia*. The obtained result has significance in choosing suitable host for cultivation of *P. pacificus* for research purpose. The isolated nematode was observed to be associated with bacterial strain of *Myroides marinus.* The NCBI BLASTn algorithm search displayed greatest similarity to *M. marinus* strain GN-5 MN220571.1 (98.28%) belonging to family Flavobacteriaceae. This is the first report of association of *M. marinus* bacteria to a nematode *P. pacificus* despite its frequent occurrence in aquatic, terrestrial habitats and clinical specimens (Cho *et al*. 2011). Up to our knowledge and literature search, the discovery of *P. pacificus VLPP01* is the first report from India and Himalayan region. Given the fact, the current sampling was limited to the two districts of NWH region and the real distribution and diversity of *Pristionchus* spp. might be greater and depends upon the research need based sampling and efforts in other part of the country. Nevertheless, the morphological and molecular based diagnostics conducted in this study infers following conclusions: first, the discovery of *P. pacificus VLPP01* is first evidence from India which expands our knowledge about the biogeography of *P. pacificus* globally. Our efforts on morphology and molecular taxonomy indicates the use of integrative approach to confirm the species despite the cosmopolitan distribution and reports from many countries (Herrmann *et al*. 2006, Kanzaki *et al*. 2012,) *P. pacificus* has not been reported earlier from India. Further sampling in other part of country may change our perspective on availability and biogeography and diversity of *P. pacificus* in India. Second, *P. pacificus* can be cultivated successfully on three larval insect hosts, viz. *Corcyra*, *Spodoptera* and *Holotrichia*. Earlier *P. pacificus* was known to multiply on scarab beetle host. Hence it expands our understanding on its insect hosts for multiplication (Herrmann *et al*. 2010, Kanzaki *et al*. 2012, Li P *et al*. 2015). Here, we suggest that *P. pacificus* can be cultivated on three insect hosts other than NGM media (Pires-daSilva 2013). Test of reproduction potential of *P. pacificus* on different larval insect host expands our understanding about culture maintenance and mass multiplication of *P. pacificus*. Third, the isolation of bacterial strain *M. marinus from P. pacificus* is the first report of association of *M. marinus* from nematode body. The relationship between *P. pacificus* and bacterial strain *Myroides marinus* needs further investigation.

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