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Morphological and molecular characterization of potato cyst nematode populations from The Nilgiris

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ABSTRACT

Quarantine species of potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, were reported from Nilgiris during 1961. The studies were carried out at ICAR-CPRS, Muthorai, Ooty and ICAR-CPRI, Shimla during 2015–17. To investigate the distribution of PCN, soil samples were collected from potato growing areas of Nilgiris and were identified based on morphological criteria and ITS-1 region. Molecular characterization using ITS-1 region specific primers revealed the presence of pure population of *G. rostochiensis* in 50% of the samples, *G. pallida* in 10.7% of the samples, mixed population in 28.6% of the samples and absence of both the species in 10.7% of the samples. The phylogenetic analysis inferred by the sequence of the ITS-1 region confirmed 92–100% genetic similarities in *Globodera* spp. Seventeen isolates of *G. rostochiensis* showed 92–99% genetic similarity and rest four 92–100% similarities. Whereas, genetic similarity among the ten isolates of *G. pallida* was 96.1–99.4%. In the morphometric characters J_2s of *G. rostochiensis* exhibited shorter body length (459.8 µm) than *G. pallida* (493.7 µm). *G. rostochiensis* and *G. pallida* had difference in mean stylet length (21.1 µm and 23.4 µm respectively), hyaline tail terminal length (28.3 µm and 24.2 µm respectively) and shape of stylet knob. Highest mean value of vulval basin-anus distance (65.3 µm), number of cuticular ridges between vulval basin-anus (18.4) and Granek's ratio (4.0 µm) was recorded in *G. rostochiensis* than *G. pallida.* Therefore, the present study will help to take appropriate and region specific PCN management decisions according to species dominance in that area.

Key words: *G. rostochiensis*, *G. pallida*, Molecular characterization, Morphology, ITS-1 region

Potato cyst nematode (PCN), *Globodera pallida* (Stone) and *G. rostochiensis* (Woll.) are the important endoparasitic nematodes causing considerable damage worldwide in potato. Potato protection against PCN is complicated because its eggs can remain dormant and viable within the cyst for 30 years (Evans and Trudgill 1992). In India, PCN was first detected from a field in Vijayanagaram farm in Udhagamandalam, Nilgiri hills, Tamil Nadu at an elevation of 2125 m above mean sea level (Jones 1961) and later on, their occurrence was also reported from Kodaikanal hills (Prasad 2008). Under Indian conditions, an initial level of even two larvae of PCN per gram of soil results in overall yield reduction of 65%. Although, PCN has been reported from the Nilgiri and Kodaikanal hills of Tamil Nadu and adjoining hills of Kerala and Karnataka, but has become a serious endemic pest of potato in Nilgiris region due to intensive cultivation of potato throughout the year (Aarti *et al.* 2016). Traditionally, species of PCN are distinguished based on different morphological characters (Yu *et al*. 2010). Besides this, some new molecular approaches have also been used to recognize PCN. RAPD analysis used to differentiate *G. pallida* populations, including some P_1A isolates, which were distinct from the typical UK gene pool (Folkertsma *et al*. 1996). Knoetze *et al.* (2006) evaluated the potential of ITS size and sequence variation as a means of identifying a wide range of plant-parasitic nematode groups. In this study we used ITS-1 region specific primers for characterization of PCN species in soil samples collected from Nilgiri hills and compared these results with morphological and morphometric characters of both the species of PCN**.**

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MATERIALS AND METHODS

Survey and collection of soil samples: Random survey was conducted in three talukas (Udhagamandalam, Kundha and Coonoor) of the Nilgiris district during 2015–16 where potato is being grown in all three seasons (summer, autumn and spring) at an elevation of 1500–2600 m amsl. A total of 159 soil samples from 28 locations were collected with a shovel from root zone area of potato. Cysts were extracted

using 60 mesh sieve from 100 ml of soil samples using Fenwick's apparatus. The collected cysts were used for further molecular and morphological studies. Molecular works were carried out at ICAR-CPRI, Shimla during 2016–17 and the results were statistically analyzed (Panse and Sukhatme 1985).

Molecular analysis

DNA extraction: Total 150 cysts of *Globodera* species were used for DNA extraction using DNeasy Blood & Tissue Kits (Qiagen) (Cat. No. 69504) following manufacturer's procedure and quantified using a nano-drop spectrophotometer (Thermo scientific Nano-drop 2000C [Waltham, Massachusetts], USA) and stored at 4°C for further study.

PCR amplification: ITS-1 region specific primer sets Fro1 (5-'ACACATGCCCGCTGTGTATG-3'); Rro1 (5'-GATGGGAAAAAGCTGGCC-3') (*G. rostochiensis*, amplicon size 411 bp) and Fpa2 (5'-TCAACAATGTATGGACAGCG -3') Rpa1 (5'-GGCACGTACGACATGGAATA-3') (*G. pallida*, amplicon size 239 bp) (Vejl *et al.* 2002) were used to distinguish PCN species. PCR amplification was carried out using 20 µl reaction mix comprising: Emerald Amp GT PCR master mix (2x Premix) 10 µl; primer (10 pmol) 1 µl each, DNA extracted from cysts 6 µl and sterile water 3 µl in a 0.2 ml PCR tube. Amplification condition were an initial denaturation step 5 cycle (94 sec at 94°C, 45 sec at 64°C and 90 sec at72°C), 15 cycle (60 sec at 94°C, 45 sec at 61°C and 90 sec at 72°C) and 15 cycle (60 sec at 94°C, 45 sec at 60°C and 90 sec at 72°C) and last 8 min for elongation at 72°C to terminate the reaction. The PCR reaction was performed using Applied Biosystem GenAmp PCR system 9700 (Foster City, California, United States). The PCR products were separated by electrophoresis in Tris-Borate-EDTA (TBE) buffered 1.5% agarose gels with ethidium bromide and visualized in Gel Doc system (ZENITH, Model Gel ProCCD9 2010).

Sequencing and phylogenetic analysis: PCR products were purified using Qiagen PCR purification kit following the manufacturer's protocol and the sequencing reactions were run on an automatic sequencer (ABI 377; Applied Biosystems). Sequence was determined by using the 0.8pmol reverse primers of *G. rostochiensis* (5'-GATGGGAAAAAGCTGGCC-3') and *G. pallida* (5'-GGCACGTACGACATGGAATA-3'). Sequence information was assembled using DNASIS software and a BLAST search at NCBI was performed in order to confirm PCN species (Altschul *et al.* 1990). The sequence data containing ITS-1 region were aligned using the Clustal Womega software with the published gene sequence of *Globodera* species. Sequence alignments were manually edited using BioEdit in order to improve the multi-alignment (Hall 1999). Out-group taxa for each dataset of ITS-1 region were selected according to the results of previously published data. A dendrogram was produced from these similarities using UPGMA model.

Morphological identification: Based on the molecular characterization results 10 cysts and 15 second stage juveniles (J_2s) from two different groups of samples, viz. Pure and mixed population of *Globodera* species were taken for morphological and morphometric characterization. The J_2s were obtained by soaking cysts in potato root exudates. Measurements were made using an inverted microscope Nikon EFD-3 connected to a digital camera Nikon DS-Ri 1. Photomicrograph of the specimens were analysed with Nikon Application system on the images and dimensions were expressed using formula proposed by De Man (1880). Morphological identification of PCN was based on the measurements of main diagnostic characters of second stage juveniles (J_2), viz. body length, stylet length, stylet knob shape, hyaline tail terminal length and cyst vulval basin diameter, distance between vulva and anus, Granek´s ratio (anus-fenestra distance: fenestra diameter) and number of cuticular ridges between vulval basin to anus. Stylet shape and other parameters were taken from the heat fixed juveniles and cysts vulval cone mounted on glycerine. Results of morphological characterization of the cysts were compared with those of the J_{2S} contained in the same cysts. The cysts were air dried and gold plated for scanning electron microscopy studies (SEM).

RESULTS AND DISCUSSION

In Udhagamandalam taluka, the population ranged from 12–61 to 116–466 cysts/100 ml soil. The minimum cyst population mean was recorded in Mullikorai village 45.00 (12-61 cysts/100 ml soil) and maximum was recorded in Muthorai 283.83 (116-466 cysts/100 ml soil). In Kundha taluka, the population ranged from 5–11 to 116–300 cysts/100 ml soil, Peduhatti area recorded less cysts population mean 8.25 (5–11 cysts/100 ml soil) while in Ithlar recorded the maximum cysts population mean 230.80 (114–300 cysts/100 ml soil). Kettipalada area of Connoor taluka recorded less cyst population (9–22 cysts/100 ml) while maximum population (3-404 cysts/100 ml soil) was recorded from porthyhada (Table 1). The high proportions of infested fields in Nilgiris are explained by the temperate climate and continuous availability of host throughout the year.

Molecular analysis: PCR analysis was performed using species specific primers for ITS-1 region to understand the distribution of *Globodera* spp. in twenty eight samples. Amplification of the ITS-1 region yielded excepted size of bands for *G. rostochiensis* (411 bp) and *G. pallida* (239 bp). In Udhagamandalam, pure population of *G. rostochiensis* was noticed in nine locations, viz. Killkavati, Balcola, Mekeri, Manalada, Glenmorgan, Mullikorai, Hoshatti, Muthorai Palada and Nanajnadu, whereas mixed population in two locations, viz. Fernhill and Muthorai. In Kunda, mixed populations of PCN were recorded in five locations, viz. Appokodu, Redhill, Porthyhada, Kalakorai and Peduhatti. Pure population of *G. pallida* was recorded in three locations, viz. Gandhi kandi, Ithlar and Saparhada while *G.rostochiensis* in one location at Lorane. In Coonor,

Table 1 Details of soil samples collected during survey

Taluka	Altitude (msl)	Location	No. of samples	Cysts/100 cc soil
Udhaga-mandalam	2240-2268	Fernhill	6	68.00±29.64 (33-106)
		Palada	8	67.00±45.72 (24-140)
		Balcola	5	46.40±27.25 (6-74)
		Mekeri	8	91.88±31.95 (56-156)
		Manalada	8	151.50±64.08 (64-238)
		Glenmorgan	5	51.80±29.22 (16-83)
		Muthorai	6	283.83±144.77 (116-466)
		Mullikorai	4	45.00±22.58 (12-61)
		Hoshatti	4	69.75±45.62 (15-121)
		Killcohatty	6	233.83±88.80 (82-318)
		Nanajnadu	8	191.75±76.50 (56-305)
		Mean	68	118.25
Kundah	2876-2882	Gandhi kandi	3	98.67±78.00 (21-177)
		Ithlar	5	230.80±77.13 (114-300)
		Appokodu	4	106.00±28.20 (72-137)
		Redhill	6	178.33±74.71 (52-245)
		Lorane	5	63.00±38.43 (16-106)
		Portyhada	8	178.13±84.60 (44-270)
		Kalakorai	5	139.60±99.62 (13-238)
		Saprhahada	4	13.00±7.07 (4-20)
		Peduhatti	4	8.25±2.5 (5-11)
		Mean	44	114.26
Coonor	1850-1858	Thumanatty	8	69.25±37.93 (14-126)
		Ketti	6	75.33±39.78 (26-126)
		Wellington	5	32.40±20.35 (4-52)
		Lovedale	4	37.00±24.73 (2-59)
		Kettipalada	5	17.40±5.23 (9-22)
		Kenduari	4	157.50±90.67 (45-259)
		Kollimali	10	208.20±158.63 (3-404)
		Santur	5	52.40±25.17 (18-80)
		Mean	47	81.19

*Cyst count/100 cc soil, presented as mean ± standard deviation and within range in parentheses.

pure population of *G. rostochiensis* was recorded in four locations, viz. Ketti, Wellington, Lovedale and Santur while mixed population in one location at Thumanatty and there was no PCN in three locations, viz. Kettipalada, Kenduari and Kollimali. The molecular characterization revealed the presence of pure population of *G. rostochiensis* in 50% of the samples, *G. pallida* in 10.7% and mixed population in 28.6% of samples. It may be due to variation in temperature and altitude as reported (Hlaoua *et al.* 2008, Prasad 2008) which favors multiplication of both the species of *Globodera* during the cropping season. Furthermore, for the first time our data showed the mixed occurrence of *G. rostochiensis* and *G. pallida* in main potato growing talukas of The Nilgiris and *G. rostochiensis* is dominant in many regions of Udhagamandalam, Kundah and Coonor taluka. Present results are in confirmation with earlier report of Umarao *et al.* (2002), where they indicated the dominance of *G. rostochiensis* in field samples of Nilgiris. It was also reported that the *G. rostochiensis* could develop into females only when the maximum temperature is below 24°C (Kaczmarek *et al.* 2014).

Sequencing and phylogenetic analysis: Intra-specific variations among *G. pallida* and *G. rostochiensis* population were 4–7 and 4–14 nucleotides respectively. Most variable population was noticed from Wellington (Well23) and Porthyhada (Por17) (Fig 1). In dendrogram, *G. rostochiensis* clusters were well separated from *G. pallida* group. There were several groups in the two species clusters corresponding to different subgroups. However, later two sub groups were formed in *G. rostochiensis*. The majority of *G. rostochiensis* populations appeared in a single large group consisting 17 out of 28 isolates. Sequences of these 17 isolates were different when compared with the reference sequences. Similarly, Subbotin *et al*. (2000) have also found several haplotypes within the genome of *G. rostochiensis*. This could be either due to the nature of genome in different isolates or random effect of genetic drift (Picard *et al*. 2006). However, variation in sequence did not

change the species identity of the nematode. While, four isolates (Gr-Hos9, Gr-Pedu20, Gr-Por17, Gr-San 28) were grouped into separate sub-clade which showed similarity with reference sequences of *G. elligtoneae* from Oregon (JFT39896, JF739885); *G. rostochiensis* from Poland (GQ294517), Canada (GQ294517, GQ294513); *G. tabaucm* from USA (DQ847114, DQ847115). The present study showed genetic similarities that ranged from 92.0-100% among all the populations of *Globodera.* While it ranged from 92–99% amongst the seventeen isolates of *G. rostochiensis* and 92–100% was recorded in the four isolates (Gr-Hos9, Gr-Pedu20, Gr-Por17 and Gr-San28) of *G. rostochiensis,* which grouped as sister species. The genetic similarity among the 10 isolates of *G. pallida* was 96.1 to 99.4%. Sequences of ten isolates were similar with the group of reference sequences, viz. European population (KJ409622, KJ409623), Ukraine (AJ606687), Gourie clone G2 (HQ670248), Clone 3-Aa (DQ847111), Solvenia population (HF583248) and P4A population (HQ670269). Genetic diversity exists among the PCN populations in Nilgiris as it was an introduced organism in Nilgiri hills (India) along with the potato from Britain (Jones 1961) and having two species, *G. rostochiensis* (3 pathotypes) and *G. pallida* (2 pathotypes) (Prasad 2008). Genetic structure of PCN might have changed due to number of generation after introduction in Nilgiris and favorable climatic condition across the potato growing seasons. Our data confirm that *G. rostochiensis* may represent a species complex as already reported (Madani *et al*. 2010, Subbotin *et al*. 2011). Our results are in agreement with the hypothesis given by Den Nijs (1992) that cross hybridization between both the species that may generate new genotypes. So this technique could be used for nematode populations which were directly sampled from potato field and in both new and old cysts (Vejl *et al.* 2002). The same approach to distinguish species of *Globodera* spp. was also followed by Knoetze *et al*. (2006) and Skantar *et al*. (2007).

Morphological analysis: Morphometric analysis of 10 cysts and 15 J_2s per population showed the presence of pure population of *G. rostochiensis, G. pallida* and mixed population of *Globodera* spp. The characteristics that vary most are larval length and cyst size. Our measurements indicate that J_2s of *G. rostochiensis* exhibited shorter body 459.8 µm than *G. pallida* (493.7 µm) **(**Fig 2). The clear differences were recorded between mean stylet length (21.1 µm and 23.4 µm), hyaline tail terminal length (28.3 µm and 24.2 µm) and knobs shape (Table 2). Our observations were in agreement with data obtained by different researchers (Skantar *et al*. 2007, Yu *et al*. 2010).

Morphometric characters of cyst perineal pattern revealed clear differences between the populations of *G.*

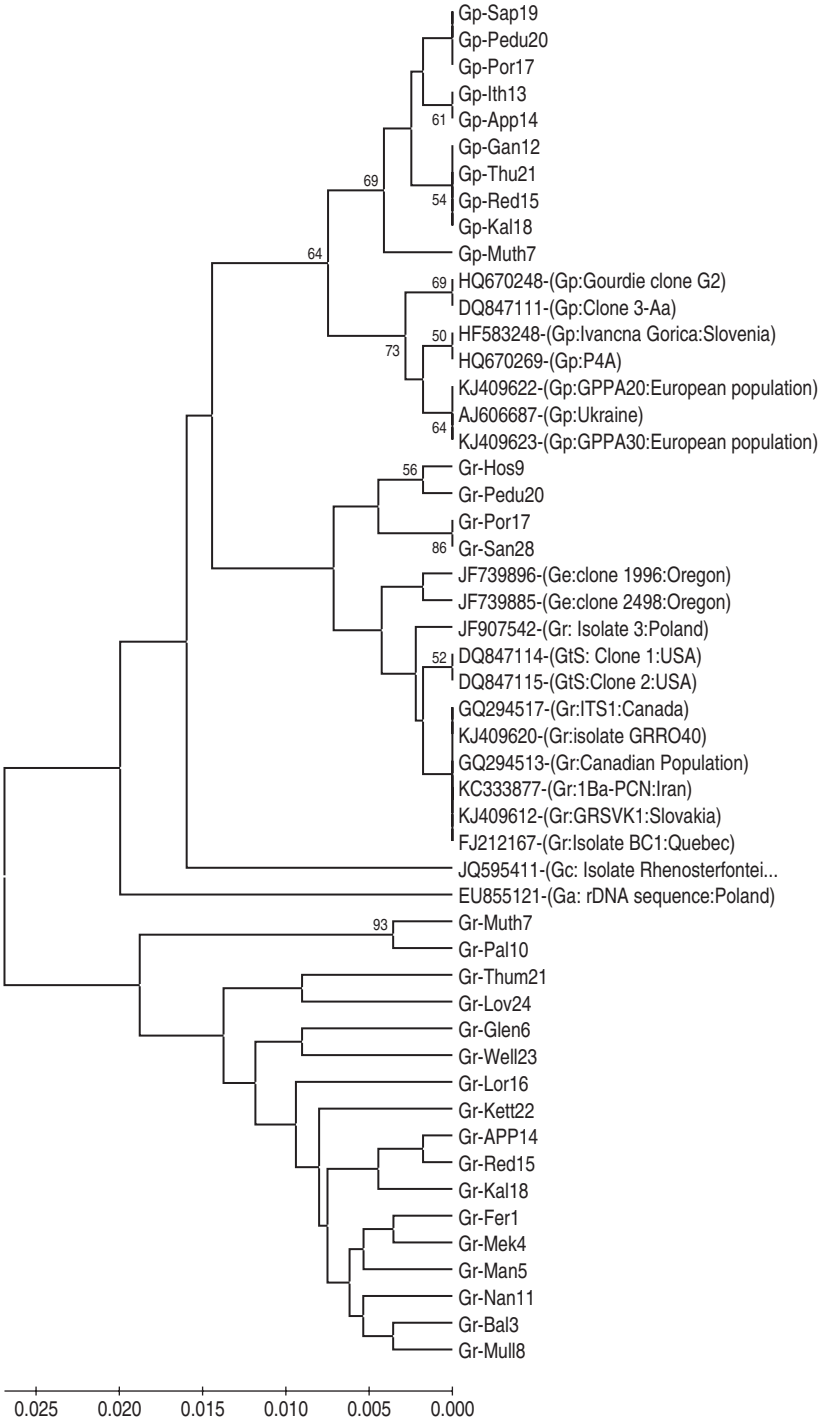

Fig 1 Phylogenetic tree of ITS-1 containing region describing the evolutionary relationships between the PCN species, *G. rostochiensis* and *G. pallida* collected from The Nilgiris using UPGMA model. Branch lengths are proportional to the distances. Number at Nodes indicates bootstrap values.

Table 2 Morphometric characters of J_2s and cysts of *Globodera* spp.

Character	Juvenile (n=15)	
	G. rostochiensis	*G. pallida*
Body length	459.8 ± 37.6 (390.7-497.9)	493.7 ± 43.3 (428.5-554.9)
Stylet length	21.1 ± 0.9 (18.4-22.0)	23.4 ± 0.8 (21.9-24.2)
Stylet knob	Round	Pointed
Hyaline tail terminal length	28.3 ± 3.9 (22.1-32.1)	24.2 ± 5.7 (19.1-31.8)
Character	*Cyst (n=10)*	
	G. rostochiensis	*G. pallida*
Body length excluding neck	615.5 ± 58.8 (514.8-684.2)	456.5 ± 42.5 (405.1-539.3)
Body width	557.7 ± 30.13 (511.9-611.8)	416.8 ± 52.5 (370.3-487.4)
Neck length	143.5 ± 35.1 (112.8-224.3)	143.5 ± 83.9 (78.1-148.9)
Distance from vulval basin to anus	65.3 ± 24.6 (35.5-92.1)	56.8 ± 15.8 (35.0-69.7)
Number of cuticular ridges between vulval basin to anus	18.4 ± 2.7 (14-21)	11.8 ± 2.2 (10-15)
Granek's ratio	4.0 ± 0.7 (2.5-5.4)	2.7 ± 0.6 (1.9-3.4)

Note: All the measurements are in µm, presented as mean ± standard deviation and within range in parentheses.

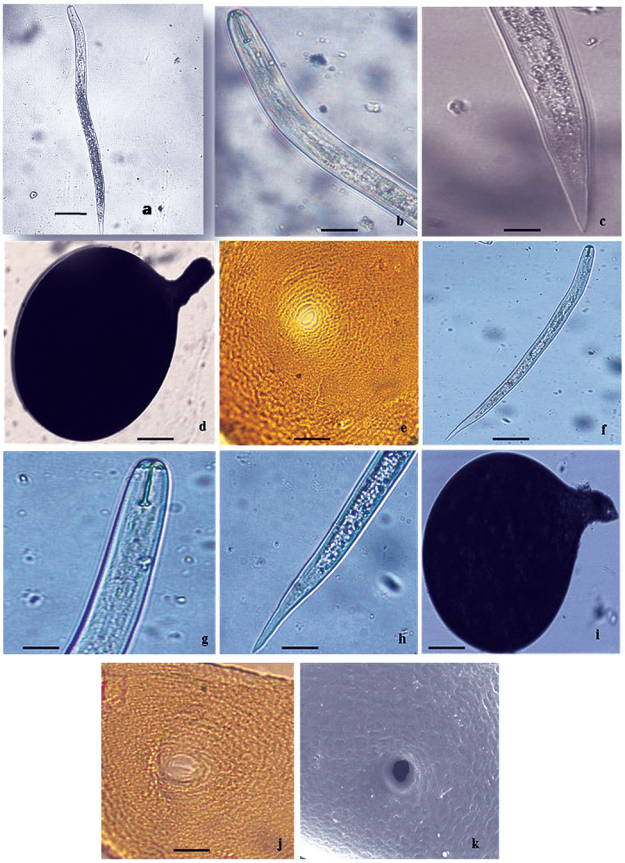

Fig 2 Photomicrographs: (a-e) second stage juvenile and cyst of *G. rostochiensis* associated with potato in The Nilgiris; a. entire body; b. head and stylet; c. tail; d. spherical shaped cysts; e. perineal region of cysts; (f-h): second stage juvenile and cyst of *G. pallida* associated with potato in The Nilgiris; f. entire body; g. head and stylet; h. tail; i. spherical shaped cysts; j. perineal region of cysts (arrows indicate vulval basin and anus); k. SEM of ridges in perineal area (Scale Bar=10µm).

rostochiensis and *G. pallida*. The Granek's ratio is the most reliable morphological characteristic for species morphological designation. Samples of Udhagamandalam, Coonor and Kundah have shown the highest mean, standard deviation, and range values of vulval basin-anus distance 65.3±24.6 (35.5–92.1), number of cuticular ridges between vulval basin and anus 18.4±2.7 (14–21) and Granek's ratio 4.0±0.7 (2.5–5.4) (Fig 2) (Table 2) which was within the ranges of *G. rostochiensis* (Gitty and Maafi 2010, Violeta *et al.* 2010).

Perineal pattern of cysts and J_2 stylet characteristics distinguished PCN species, but sometimes these diagnostic characters may overlap among various populations of different species (Baldwin and Mundo-Ocampo 1991).

The study indicated that now a day's mixed population of *Globodera* is occurring in lower altitude where the day temperature is more than 24ºC during cropping season. This may be due to development of new pathotype or generation due to cross hybridization between both the species and variation in cooler sub-tropical climatic conditions in Nilgiris which differ than European climate. Knowledge of the distribution and identification of *Globodera* species in Southern hills of Tamil Nadu are valuable for the design of effective management strategies as well as regulatory measures in order to prevent the spread of potato nematodes into new areas, and to keep nematode density below damage levels in infested areas. Use of resistant varieties may help to manage the nematode's population to levels that allow successful and profitable potato production.

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