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RESEARCH ARTICLE

Mermithid parasitism of shoot borer (*Conogethes punctiferalis*) infesting ginger and turmeric and its biocontrol potential

Senthil Kumar C. M.　|　**Jacob Thuruthumkara Kurian**　|　**Devasahayam Selvakaran**　|　**Hariharan Vasudevan**　|　**Sharon D'Silva**

Division of Crop Protection, ICAR, Indian Institute of Spices Research, Kozhikode, India

Correspondence

Dr C. M. Senthil Kumar, Division of Crop Protection, ICAR, Indian Institute of Spices Research, Marikunnu P.O., Kozhikode 673 012, Kerala, India.
Email: senthilkumarcm@spices.res.in; cmskm@yahoo.com

A mermithid nematode parasitic to larvae of *Conogethes punctiferalis*, a serious pest of ginger and turmeric was recorded under field conditions during 2015 at Peruvannamuzhi (Kerala, India). The infection reached epizootic levels during July to September 2015 exceeding 50% mortality in host insect populations and the parasitism ranged from 18.2 to 80.6% and 17.9 to 66.7% in *C. punctiferalis* collected from ginger and turmeric, respectively. The level of host parasitism by the mermithid was positively correlated with rainfall and negatively influenced by maximum temperature. Molecular analysis of the partial 18S small ribosomal subunit gene region and phylogenetic analysis with other mermithid sequences available in the GenBank indicated that the present nematode clustered with other nematodes belonging to different genera and hence could not be attributed to a specific genus. The pairwise Kimura 2-parameter (K2P) distance revealed that the closest taxon to the study nematode was an undescribed mermithid species reported to infect slugs with a K2P distance of 0.009. The epizootics caused by this mermithid nematode in the natural populations of *C. punctiferalis* suggest its potential as a biocontrol agent against a polyphagous pest of international importance. The results of our studies provide a basis for using this nematode as a biocontrol agent for developing integrated pest management strategies against *C. punctiferalis*.

KEYWORDS

18S, biocontrol, *Conogethes punctiferalis*, ginger, mermithidae, turmeric

1　|　INTRODUCTION

Mermithid nematodes were considered as potential biocontrol agents against insects as early as in the 1960s (Welch, 1964). The infective juveniles or pre-parasites of the nematodes encounter a host insect while the latter spends a part of its life cycle in the soil or the nematodes can crawl up low growing plants to infect an insect feeding on the leaves or stems (Nickle, 1981). Once inside the host insect, the nematodes grow through several larval stages and leave the host as fully grown non-feeding post-parasites. The egress of the nematodes from the insect leads to host mortality. The post-parasites move into the soil where they moult to adults, mate and lay eggs (Q. Li, Sun, Wang, & Liu, 2009). Mermithid nematodes have been reported to be infective to all major orders of insects, including Orthoptera, Coleoptera, Lepidoptera and Diptera (Nickle, 1981). Although mermithids

have several advantageous attributes as biocontrol agents including environmental safety, high lethality, host specificity, ease of application and potential for long-term recycling in the environment (Kerry & Hominick, 2002; Petersen, 1985), the difficulties in traditional taxonomy of juvenile stages (Poinar, 1979) and lack of protocols for mass culturing many of the species for field release limit their successful deployment as biocontrol agents against insect pests (Kerry & Hominick, 2002).

Ginger (*Zingiber officinale* Rosc.) and turmeric (*Curcuma longa* L.) are herbaceous plants native to South Asia and their dried rhizomes yield the spice of commerce. India is a leading producer of ginger and turmeric in the world and the shoot borer, *Conogethes punctiferalis* Guenée is considered as a serious pest of these crops in India. The pest is widely distributed in Asia and Australia and highly polyphagous attacking more than 120 host plants including many agriculturally

important crops (Shashank, Chakravarthy, Raju, & Bhanu, 2014). In ginger and turmeric, the larvae of the insect bore into pseudostems and feed on the growing shoot resulting in yellowing and drying of infested pseudostems. The presence of bore holes on the pseudostems through which frass is extruded and the withered central shoot are the characteristic symptoms of pest infestation (Devasahayam & Koya, 2004, 2007). Significant yield losses were reported in ginger (Koya, Balakrishnan, Devasahayam, & Banerjee, 1986) and turmeric (Devasahayam, Jacob, & Koya, 2010), when more than 50% of the pseudostems in a clump were damaged by the pest. Although several larval and pupal parasitoids have been recorded on the pest (Devasahayam, 1996), none of them could be developed into a successful biological control agent for the pest and the present control measures rely only on synthetic chemical pesticides. In this study, we report parasitism of *C. punctiferalis* by a mermithid nematode (Mermithidae: Nematoda) and discuss its biocontrol potential in integrated pest management (IPM) programmes.

The objectives of this study were to identify the nematode parasitizing *C. punctiferalis* by molecular tools; to establish its phylogenetic relationship with other reported mermithid species and to record the levels of parasitism in *C. punctiferalis* populations in relation to weather parameters such as rainfall and temperature. The biological control potential of the nematode by causing epizootics in *C. punctiferalis* populations is discussed.

2 | MATERIALS AND METHODS

2.1 | Study area

The study was conducted during July to December 2015 in the research farm of ICAR—Indian Institute of Spices Research (IISR), located at Peruvannamuzhi (11°35′0″N, 75°49′0″E), Kozhikode District, Kerala, India. The annual total rainfall, number of rainy days, maximum and minimum temperature of the location during the study period was 4,243 mm, 164 days, 32.9 and 23.1°C, respectively.

2.2 | Raising of crops

Fifty beds each of ginger (variety: *IISR-Rejatha*) and turmeric (variety: *IISR-Prathibha*) (40 plants/bed) were planted in rows on raised beds of 1 m × 3 m as a rain-fed crop at a spacing of 25 cm × 25 cm. The seed rhizomes were sown during the second week of June 2015 and the crops were raised following all standard agronomic practices (ICAR-Indian Institute of Spices Research, 2015a, 2015b), except for plant protection measures. Fresh pseudostems ceased to emerge after November and the crop was harvested ~230 days after planting in January 2016.

2.3 | Seasonal incidence of mermithid

To study the seasonal incidence of the mermithid in *C. punctiferalis* populations, pseudostems harbouring live larvae as indicated by extrusion of fresh frass through the bore holes (Figure 1a,b) were collected at fortnightly intervals from the beds crop-wise and the larvae were carefully extracted from the pseudostems in the laboratory. The larvae
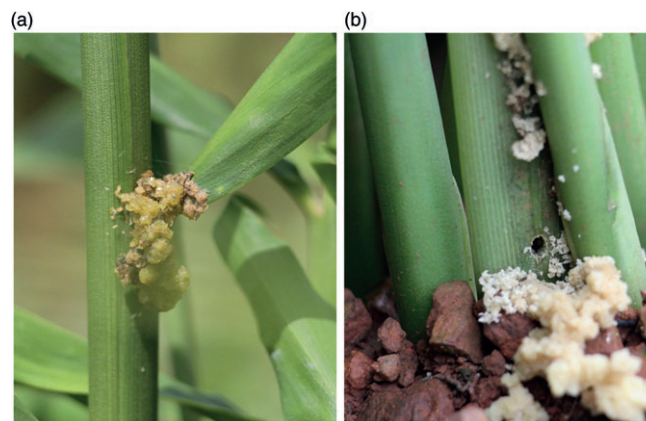

FIGURE 1 *Conogethes punctiferalis* infested shoots with extrusion of frass in (a) ginger and (b) turmeric

were transferred crop-wise to plastic trays (25 cm diameter) covered with a nylon mesh at the rate of 15 larvae per tray and were maintained on fresh ginger or turmeric pseudostems according to their host plants under ambient natural conditions (28–32 ± 2°C, 60–70% relative humidity and a 12:12 hour day:night photoperiod). The number of larvae examined on each crop varied from 13 to 182 per month depending on field incidence of the pest. The emergence of mermithid nematodes was recorded daily until the larvae died because of exit of nematodes or until the larvae pupated.

2.4 | Influence of rainfall and temperature on mermithid parasitism

The influence of abiotic factors on the extent of mermithid parasitism in *C. punctiferalis* was studied by correlating the number of mermithid parasitized larvae with the prevailing rainfall and temperature at fortnightly intervals. The meteorological data during the study period was collected from the Meteorology Unit of ICAR-IISR at Peruvannamuzhi.

2.5 | Molecular identification of the mermithid

The mermithid nematode was identified by sequencing the 18S small ribosomal subunit (SSU) nuclear gene. Genomic DNA was extracted by grinding two post-parasitic nematodes individually, each collected from *C. punctiferalis* infesting ginger and turmeric using QIAmp DNA Mini Kit (Qiagen, Germany) according to manufacturer's instructions. PCR amplification was performed in a 25 μL reaction volume containing 2.5 μL of 10× PCR buffer, 2.0 μL of dNTPs (2.5 mM each), 0.75 μL each of (20 μM) forward and reverse primer pairs: 18S-5F and 18S-9R (Vandergast & Roderick, 2003), 1 μL of genomic DNA (~10–20 ng), 0.2 μL of 5 units μL^{-1} *Taq* DNA polymerase (Sigma, USA) after making the remaining volume with PCR grade water (Himedia, India). The PCR conditions were as described by Vandergast and Roderick (2003) without modifications. Five microlitre of the PCR product was loaded onto a 0.8% wt/vol agarose gel, stained with ethidium bromide, visualised and bi-directional sequencing was carried out in ABI 3730 XL DNA Analyser using the same set of PCR primers at SciGenom Labs Private Ltd., Kochi, India. Trimmed sequences were manually edited using Bioedit software (Hall, 1999), checked for homologous sequences using BLAST

(Basic Local Alignment Search Tool) search and a sequence was deposited in GenBank with accession no. MF893191. Specimens of nematodes preserved in 70% alcohol were deposited in the Biocontrol Repository of ICAR-IISR with accession number IISR-MN-01.

2.6 | Phylogenetic analysis

The 18S SSU sequence of the isolated mermithid nematode was multiple aligned with gene sequences of published taxa (Mazza et al., 2017; Tobias, Jorge, & Poulin, 2017) retrieved from the GenBank using ClustalW (Larkin et al., 2007) and the alignment was refined using Gblocks (Dereeper et al., 2008). The pairwise genetic distance between taxa was calculated using Kimura 2-parameter (K2P) model (Kimura, 1980) in MEGA 7 (Kumar, Stecher, & Tamura, 2016). An optimal evolutionary model for Maximum Likelihood (ML) analysis was selected based on the lowest score value of Bayesian Information Criterion available in MEGA 7. Gaps in alignment were treated as missing data and the ML tree was constructed using K2 + G substitution model with five gamma categories (Kimura, 1980). Bootstrapping was performed with 500 replicates. *Xiphinema americanum* (Tobias et al., 2017) was used as outgroup. Similarly, Bayesian inference (BI) analysis was performed with the MrBayes online tool available in www.phylogeny.fr following the default parameters (Dereeper et al., 2008).

2.7 | Statistical analysis

The relationship between abiotic factors and extent of mermithid parasitism of *C. punctiferalis* was analysed using Pearson's correlation coefficient (*R*) and linear regression analyses. Significance among factors was considered at the level of $p \leq 0.05$.

3 | RESULTS

3.1 | Mermithid parasitism in *C. punctiferalis*

The occurrence of the pest in both ginger and turmeric was first observed 45 days after sowing of the crop. Parasitism of the pest by mermithid nematodes was first recorded in July and August in ginger and turmeric populations respectively and continued till the persistence of the pest until December. Parasitized larvae of *C. punctiferalis* were pale, sluggish and appeared similar to non-parasitized larvae in their shape and size. The number of post-parasitic nematodes that emerged from a single larva ranged from 1 to 5 in number. The nematodes exit the host larvae either through the anal opening or through the epicranial suture of the larval head (Figure 2a,b). However, in cases of super infections, the mermithids always emerged through the anal opening of the larva and egress of nematodes through multiple exits was not observed. Freshly emerged post-parasitic nematodes were creamy or pale white. The length of the post-parasitic nematodes ranged from 2.5 to 21.4 cm (average length: 9.0 ± 0.5 cm) and was indirectly proportional to the extent of host parasitism by the nematodes. The post-parasitic nematodes had a tail-like appendage present at the posterior end and with a blunt end anterior head tip (Figure 2d,e). Immediately after emergence of the parasites, the host larva turned dark, became flat, deprived of its contents and died (Figure 2c).

Mortality of *C. punctiferalis* because of mermithid parasitism during the crop periods ranged from 18.2 to 80.6% and 17.9 to 66.7% in ginger and turmeric, respectively. The infection reached epizootic levels during July to September, leading to more than 50% mortality in *C. punctiferalis* (Figures 3 and 4).
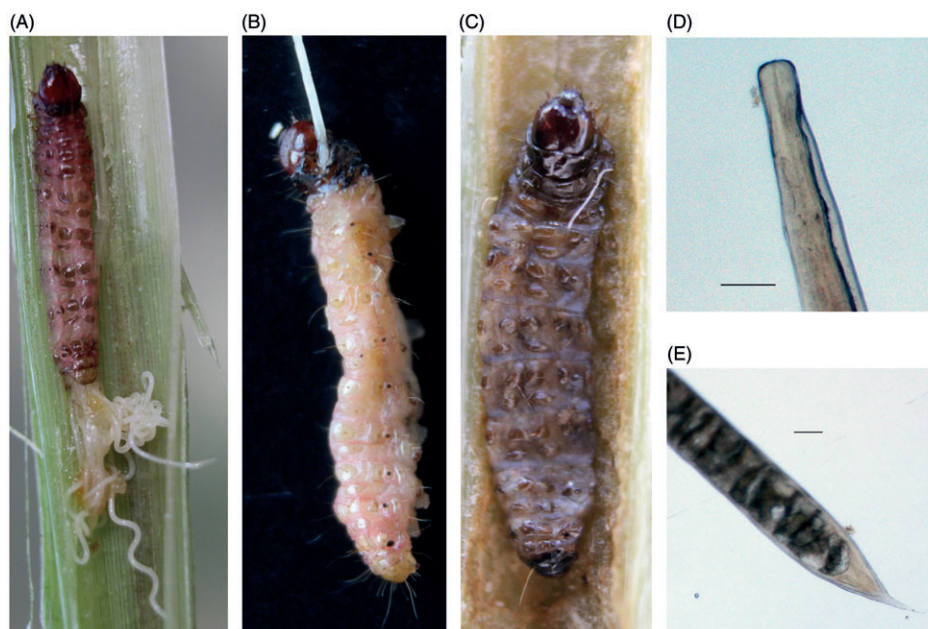

FIGURE 2 (a) Egression of post-parasitic mermithid nematodes through the anal opening; (b) exit of a mermithid nematode through the epicranial suture; (c) dead *Conogethes punctiferalis* larva upon exit of mermithid nematodes; (d) head of post-parasitic juvenile (scale bar: 99.5 μm) and (e) tail region of post-parasitic juvenile with papillae (scale bar: 102 μm)
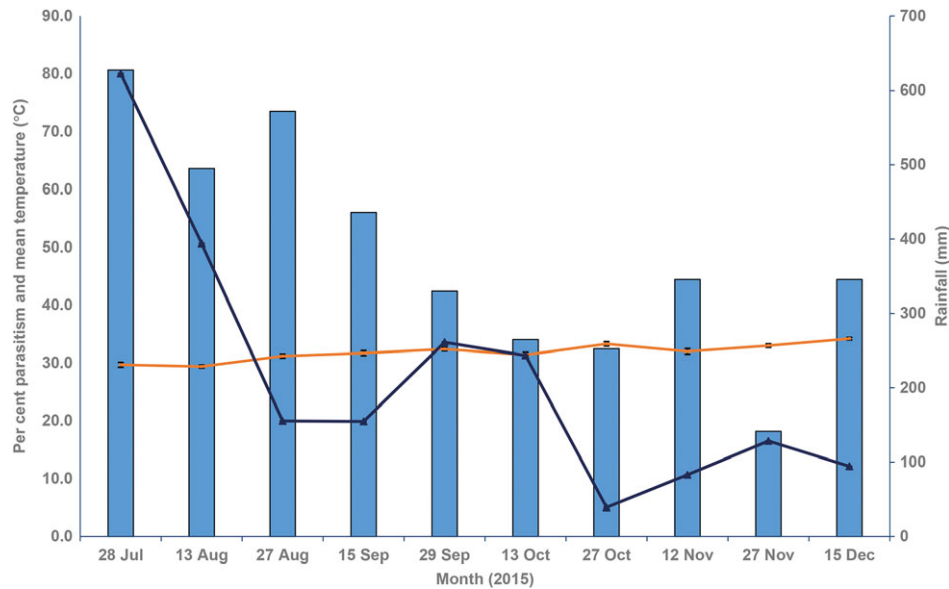
FIGURE 3 Seasonal prevalence of mermithid parasitism in *Conogethes punctiferalis* infesting ginger in relation to mean maximum temperature (±SE) and rainfall. (▉) % Mortality; (——) Max. temp; (—▲—) total rainfall (mm)

3.2 | Influence of weather parameters on mermithid parasitism

A significant positive correlation (p = 0.047, 0.031, respectively) was observed between the level of parasitism and rainfall in both ginger and turmeric (r = 0.638, 0.678, respectively). Mermithid induced mortalities in *C. punctiferalis* infesting ginger and turmeric were high (81 and 67%, respectively) during July to August, coinciding with the period of maximum rainfall during the year and the parasitism declined with decrease in rainfall (Figures 3 and 4). Maximum temperature had a significant negative correlation (p = 0.020, 0.013, respectively) with the level of mermithid incidence in *C. punctiferalis* populations infesting ginger and turmeric (r = −0.714 and −0.746, respectively). The months that recorded higher temperatures during the cropping season also had lower incidence of mermithid parasitism in *C. punctiferalis* infesting ginger and turmeric (Figures 3 and 4).

3.3 | Molecular analysis

Molecular sequencing of the 18S SSU gene region of the mermithid nematodes yielded a sequence length of about 811 bp. BLAST homology search for the submitted sequence showed that it was 98% identical to mermithid nematodes (MF192960, AY374415, FJ982324 and KU177046) reported to attack slugs, spiders and a lepidopteran host with query coverage varying from 95 to 100%. Phylogenetic analysis of the 18S SSU sequences by ML and BI methods did not differ in tree topologies and hence only the BI tree is presented here (Figure 5). The tree grouped the nematodes into five clades with bootstrap values higher than 50%. Clade 1 included the genera *Ovomermis*, *Agamermis*, *Octomyomermis* and *Hexamermis*; Clade 2 included the genera *Thaumamermis*; Clade 3 included the genera *Amphimermis*, *Romanomermis*, *Gastromermis*, *Isomermis*, *Limnomermis* and *Strelkovimermis*; Clade 4 included the genera *Mermis* and *Allomermis* and Clade 5 included the genera *Heleidomermis*. The present nematode is

included in Clade 1, which contains five genera of mermithids and thus both the methods of analysis could not resolve the inner relationships among various genera. The pairwise K2P distance between the study nematode and those belonging to the same clade ranged from 0.009 to 0.030, the closest being two undescribed species of mermithids (MF192960 and FJ982324) attacking slugs (Table S1).

4 | DISCUSSION

The incidence of *C. punctiferalis* on both ginger and turmeric was observed 45 days after sowing and continued till December. Mermithid parasitism of *C. punctiferalis* populations in ginger and turmeric initiated in July and August, respectively, and continued throughout the period of occurrence of the pest in these crops. The length of a nematode was dependent on the number of parasites developed in a host with longer nematodes emerging from simple parasitism and shorter nematodes in the case of super parasitized larva. A similar observation was made on *Hexamermis* sp. parasitizing *Cydia hemidoxa* infesting black pepper, which was attributed to the limited host-bound food resources available to the nematodes for development (Devasahayam & Koya, 1994). Superparasitism and nutrient availability also play a significant role in sex selection and population regulation of mermithid nematodes (Sanad, Sun, Shamseldean, Wang, & Gaugler, 2017).

Three modes of mermithid entry into the host tissue have been reported. Firstly, the infective juveniles directly penetrate through the host cuticle; secondly, the insect may accidentally feed on the mermithid eggs laid on the host plants and thirdly, through an intermediate or paratenic insect host as reported from ants, wasps and spiders (Kaiser, 1991; Poinar, 1985; Yoshida, 2014). However, it remains unclear how the mermithid infection takes place in the case of *C. punctiferalis*. Usually, the insect lays eggs singly on leaf margins and
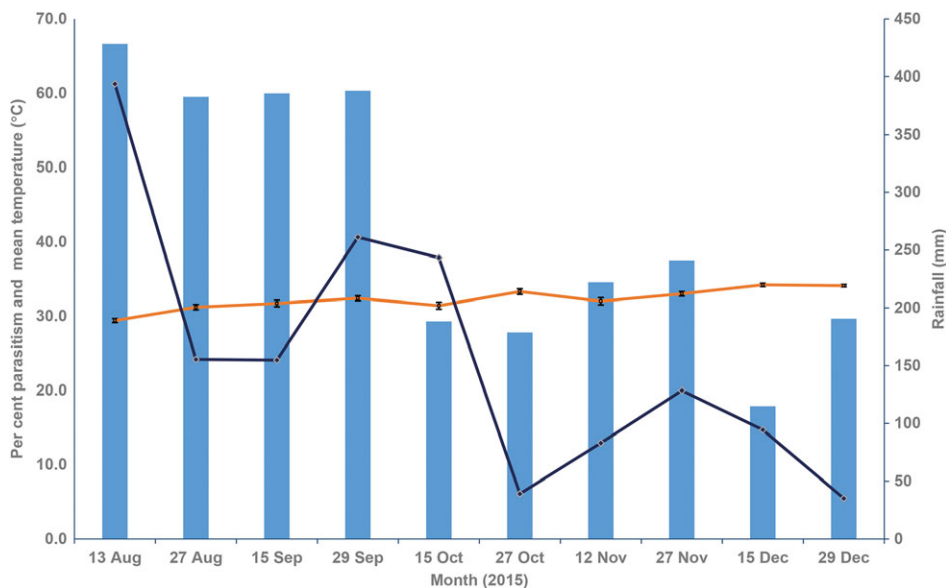
FIGURE 4 Seasonal prevalence of mermithid parasitism in *Conogethes punctiferalis* infesting turmeric in relation to mean maximum temperature (±SE) and rainfall. (■) % Mortality; (—□—) Max. temp; (—◆—) total rainfall (mm)

the neonates feed along the leaf margins and bore into the pseudostem to feed and pupate. The possibility of the mermithid adults laying eggs on the leaf surfaces and the neonates accidentally feeding on them could not be ruled out. Alternatively, the infective juveniles in the soil can crawl up a short distance on the pseudostem to reach the bore holes of the insects as in the case of *Ovomermis sinensis* infecting tobacco budworm, *Helicoverpa armigera* (Q. Li et al., 2009). The question of *C. punctiferalis* as a paratenic host of the mermithid is ruled out because the insect is phytophagous and the nematodes emerged out

upon completing their parasitic life cycle leading to host mortality (Kubo, Ugajin, & Ono, 2016). Terrestrial mermithids can infect host insects directly from the soil or after short migration up a plant stem (Nickle, 1981). Further experimental studies are required to clearly understand the infection pattern of these mermithids.

Severe epizootics by mermithid species can result in mortality rates exceeding 50% and often higher than 90% (Bhatnagar, Pawar, Jadhav, & Davies, 1985; Devasahayam & Koya, 1994; Micieli, Risso, Achinelly, de los Milagros Villar, & Muttis, 2012). In our studies, the
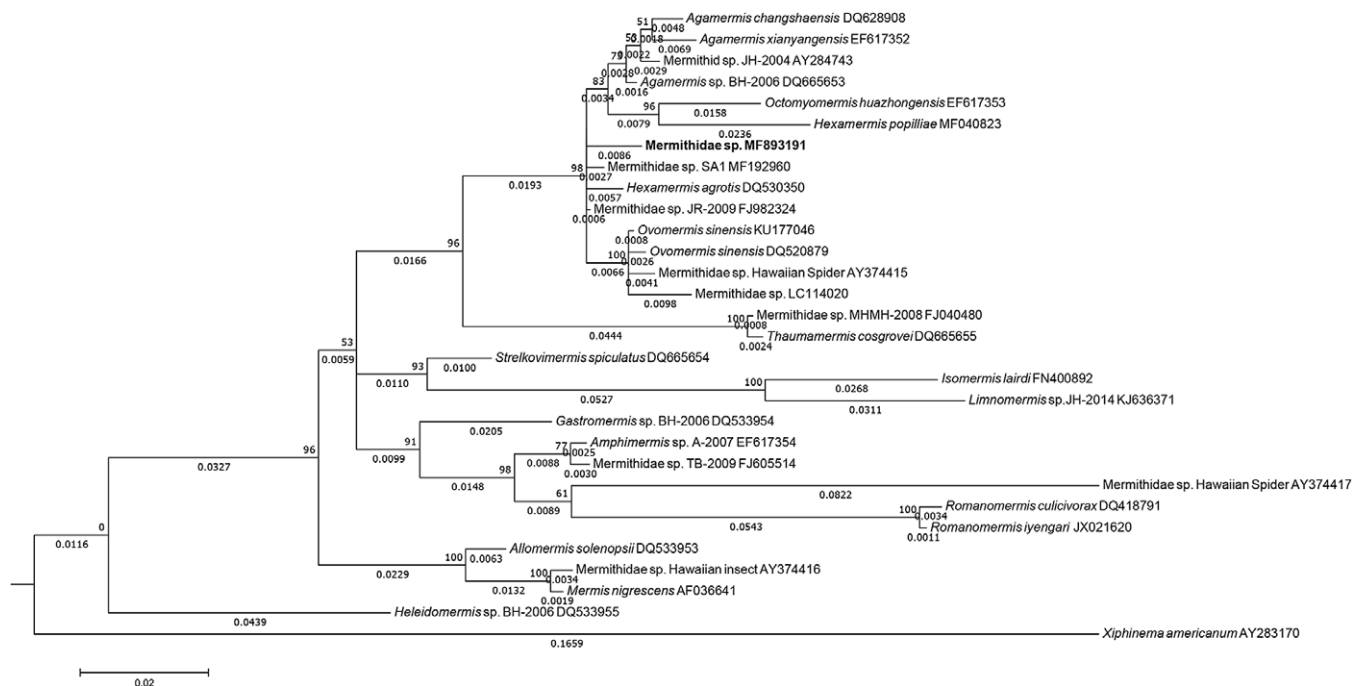

FIGURE 5 Bayesian tree based on nucleotide sequences of partial 18S small ribosomal subunit gene sequences of different nematode taxa. The evolutionary distances are in the units of the number of base substitutions per site. Values below the branch indicate branch length. *Xiphinema americanum* was used as outgroup

highest mortality because of mermithid epizootics reached 80.6 and 66.7% in ginger and turmeric *C. punctiferalis* populations, respectively. This finding is in conformity with the results of earlier studies involving nematodes of the genus *Ovomermis* and *Hexamermis* that attack many important lepidopteran pests. For example, the average parasitism of *Mythimna seperata* by *O. sinensis* ranged from 40 to 90% and that of *Agrotis ipsilon* by *Hexamermis agrotis* ranged from 44.0 to 67.6% (Chen, Jian, & Ren, 1991; F. C. Li & Xiong, 2005). The studies indicated that there was a decline in the per cent mermithid parasitism of *C. punctiferalis* after September. *C. punctiferalis* larvae attack only tender shoots and new shoot production in both ginger and turmeric reduced from October onwards, bringing down the insect larval populations and thereby lowering the per cent larval parasitism by the mermithids. In December, the crops started drying showing signs of maturity.

Our studies showed that rainfall and temperature play crucial roles in the level of mermithid parasitism of *C. punctiferalis*. It has been reported that mermithid prevalence is strongly influenced by temperature and rainfall (Baker & Capinera, 1997; Chen et al., 1991; Devasahayam & Koya, 1994; Prabhakar, Prasad, & Venkateswarlu, 2010), and levels of infection most likely change from year to year depending upon summer rainfall levels (Presswell, Evans, Poulin, & Jorge, 2015). In general, mermithid nematodes require a moist environment to infect their host (Helden, 2008; Poinar, 1975) and were found to occur in regions receiving 800 to 1,200 mm annual rainfall (Kaiser, 1991). Both ginger and turmeric were grown as rain-fed crops and the study area received copious amount of rainfall (3,539 mm) during the cropping season from June to December. During this period, the soil as well as the crop remained consistently moist and were conducive for the infective juveniles to crawl up the pseudostem and infect the *C. punctiferalis* to cause high levels of parasitism. The relationship between temperature and nematode parasitism has been well documented in many nematode parasites (Brown & Platzer, 1977; Galloway & Brust, 1977) and in general, high humidity and cool temperatures positively influence the survivorship of nematodes (Arthurs, Heinz, & Prasifka, 2004) because the eggs and adults, which both occur outside the host, are susceptible to desiccation (Poinar, 1975). The high level of moisture and moderate temperature that prevailed in the study location (Figures 3 and 4) might have provided ideal conditions for high levels of mermithid parasitism in *C. punctiferalis*.

Morphological identification of mermithid nematodes relies on adult samples, which are free living in soil (Vandergast & Roderick, 2003) and it is often difficult to identify them because of lack of diagnostic taxonomic characters (Mazza et al., 2017; Presswell et al., 2015) and hence molecular tools offer a powerful alternative for the characterisation of nematodes. Phylogenetic analysis based on 18S SSU sequences of our mermithid along with sequences of mermithids belonging to different genera showed that this nematode belongs to Cluster 1, which contains several important genera like *Ovomermis*, *Agamermis*, *Octomyomermis* and *Hexamermis*. However, we could not place the present mermithid under any specific genus or species. The tree in our study almost resembled to earlier reports (Kubo et al., 2016; J. Y. Wang, Xu, Liu, & Wang, 2007) and the recently reported phylogenetic trees for mermithids (Mazza et al., 2017; Tobias et al., 2017). The subtle differences noticed in the trees could be because of

exclusion or inclusion of certain sequences for the phylogenetic analysis by different workers. For example, Tobias et al. (2017) have not included *Hexamermis popilliae* in their analyses, whereas Mazza et al. (2017) excluded many unidentified mermithid species in their analysis. We observed three main clusters in the tree similar to Tobias et al. (2017) and in contrast to the four clusters reported by Mazza et al. (2017). However, if we apply the same yard stick of Mazza et al. (2017), we will have a total of five clusters with Cluster 1 being further subdivided into three sub-clusters containing the genera, *Romanomermis*, *Amphimermis*, *Gastromermis* under Sub-cluster 1, *Allomermis* and *Mermis* under Sub-cluster 2 and *Limnomermis*, *Isomermis* and *Strelkovimermis* under Sub-cluster 3. The variation in trees could be because of high divergence between molecular clock rates inside the tree and also because of the paucity of genetic data for many of the described taxa (Mazza et al., 2017; Tobias et al., 2017). This could be overcome by including more taxa in future analyses. Availability of molecular sequences for all the described mermithid taxa is very important to clearly understand the evolutionary relationships among various taxa.

Mermithids belonging to genera *Ovomermis*, *Agamermis*, *Octomyomermis* and *Hexamermis* which are related to our mermithid have significant biocontrol potential against several economically important pests. For example, *O. sinensis* infect a wide range of insect pests including, *Mythimna separata*, *H. armigera*, *Spodoptera litura*, *S. exigua*, *Plutella xylostella*, *Pieris rapae*, *A. ipsilon*, *A. segetum*, etc. and attempts have been made to mass culture this nematode for biological control of pests in China (Chen et al., 1991; G. X. Wang, Chen, & Chen, 2001). Similar reports are also available on *Agamermis* sp. against whitebacked and brown plant hoppers (Choo & Kaya, 1990), *Hexamermis* sp. and *Mermis* sp. against orthopterans, dermapterans, coleopterans, dipterans, hymenopterans and lepidopterans (Achinelly & Camino, 2008; Platzer, Mullens, & Shamseldean, 2005), and *Octomyomermis* sp., *Romanomermis* sp. and *Heleidomermis* sp. against mosquitoes (Platzer et al., 2005; Poinar, 2001).

The high mortality of *C. punctiferalis* induced by the mermithid in the present study indicates that they have good biocontrol potential and can play a significant role in the natural control of the pest. In Kerala, apart from mermithid nematodes, parasitisation by hymenopterans is also observed. However, hymenopterous parasitoids are observed during post-monsoon period (October to December) and peak parasitisation levels can reach up to 28% during November (Devasahayam, Senthil Kumar, & Jacob, 2018). The heavy rainfall period (June to August) may not be suitable for hymenopterous parasitoids but is ideal for parasitisation by mermithid nematodes as observed in the present study. This high level of parasitisation by mermithid nematodes during the early stages of the crop is helpful in bringing down the initial field populations of the pest, which would be more amenable for other methods of management including the use of parasitoids, thereby considerably reducing the use of conventional insecticides. The difficulty in mass rearing of mermithids can be addressed by rearing them in vivo as in the case of vector insects (Santamarina & Perez, 1997). Thus, mermithid nematodes can form an important component of biocontrol while developing IPM strategies for the eco-friendly management of *C. punctiferalis* on ginger and turmeric.

In conclusion, mermithid parasitism was recorded in *C. punctiferalis*, a serious insect pest of spice crops like ginger and turmeric and many other economically important crops. The epizootic efficiency of the nematode also indicated that they can play a significant role in the natural control of this pest. The positive correlation between rainfall and mermithid infection will also be helpful in scheduling the timing of application of insecticides to control this pest in IPM packages. Our future studies aim at morphological identification of the nematode up to species level and to develop mass rearing techniques for the nematode for field releases and successful management of the pest by augmenting this natural enemy as an effective biological control agent against *C. punctiferalis*.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.