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# *Heterorhabditidoides chongmingensis* gen. nov., sp. nov. (Rhabditida: Rhabditidae), a novel member of the entomopathogenic nematodes

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**Abstract**

During a recent soil sample survey in Eastern China, a new entomopathogenic nematode species, collected from the Chongming Islands in the southern–eastern area of Shanghai, was discovered. Morphological characteristics of different developmental stages of the nematode combined with molecular data showed that this nematode is a new genus of Rhabditidae, and described as *Heterorhabditidoides chongmingensis* gen. nov., sp. nov., for that it shares more morphological characteristics with heterorhabditids than with steinernematids. For males, the papillae formula of bursa is 1, 2, 3, 3, with constant papillae number in the terminal group, stoma tubular-shaped and about 1.5 head width; cheilorhabdions cuticularized, esophageal collar present and long, median bulb present. For infective juveniles, EP = 90 (80–105) μm, ES = 104 (92–120) μm, tail length = 111 (89–159) μm, and $a = 19.1$ (15–21). The percentages of the nucleotides A, T, C and G in the ITS1 regions of the new species are significantly different from those of heterorhabditids and other rhabditids. Molecular phylogenetic trees based on 18S rDNA and the internal transcribed spacer (ITS) sequences data revealed that the new entomopathogenic nematode species forms a monophyletic group, which is a sister group of the clade comprised of some genera of Rhabditidae.
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*Keywords:* Description; *Heterorhabditidoides chongmingensis* gen. nov., sp. nov.; Molecular; New species; Bacterial symbiont; Phylogenetic systematics; *Serratia*; Taxonomy

## 1. Introduction

Entomopathogenic nematodes (EPN) are obligate and lethal parasites of insects, and have been proved to be effective biological agents of soil-inhabiting insects and several other pest insects (Gaugler and Kaya, 1990; Berry et al., 1997; Ehlers, 1996; Gerritsen et al., 1998; Shields et al., 1999; Long et al., 2000; Mannion et al., 2000; Shapiro-Ilan et al., 2002). All the described EPN belong to the families Steinernematidae and Heterorhabditidae. Steinernematids and heterorhabditids are characterized by carrying specific symbiotic bacteria of the genus *Xenorhabdus* and *Photorhabdus* in their intestine, respectively (Kaya and Gaugler, 1993). Although entomopathogenic nematodes are (collectively) pathogenic to a wide variety of insect pests (Poinar, 1979), successful commercialization has been limited to relatively few insect species (Grewal and Georgis, 1999; Shapiro-Ilan et al., 2002). Due to the great need for more effective EPN for controlling insect pests and more new EPN symbiotic bacteria for developing bioactive by-products for various purposes, such as obtaining novel insecti-

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cidal toxin genes for developing transgenic insect-resistance crops or antimicrobial substances as agro- or medical-pharmaceuticals (Webster, 2002), interest in studying these nematodes has increased dramatically. The biological control potential of EPN has stimulated numerous surveys in an effort to find new, indigenous new species (Hominick et al., 1996; Mráček et al., 2006; Nguyen et al., 2006), and also to facilitate further research areas such as ecology, biodiversity, evolution, biochemistry, symbiosis and molecular genetics (Burnell and Stock, 2000; Li et al., 2007).

Numerous surveys have been conducted worldwide, and many new species of EPN have been recovered. Currently, 11 species have been reported in the family Heterorhabditidae and 45 valid species in the family Steinernematidae (Nguyen, 2005; Nguyen et al., 2006). The family Heterorhabditidae comprised of one genus *Heterorhabditis*. There are two genus, *Steinernema* Travassos 1927 and *Neosteinernema* (Nguyen and Smart, 1994), in the family Steinernematidae. All of the described species of EPN belong to Rhabditida.

We carried out several soil surveys searching for EPN in six provinces and in Shanghai city of Eastern China during the last two years. Seven EPN isolates have been found, six of which are heterorhabditids. Morphological characteristics combined with molecular data suggested that one isolate (DZ0503CMFT) is a new genus belonging to the family Rhabditidae. The new species shares the same bursa formula with Heterorhabditidae, and described herein as *Heterorhabditidoides chongmingensis* gen. nov., sp. nov.

## 2. Materials and methods

### 2.1. Origin of the nematode

The new species, *H. chongmingensis* gen. nov., sp. nov. (DZ0503CMFT), was obtained from a soil sample collected from Chongming Islands, in the southern–eastern area of Shanghai city, China, in 2005 using the insect bating method (Bedding and Akhurst, 1975). Five, last-instar *Galleria mellonella* larvae were placed in 250 ml plastic containers (five containers/samples) with moistened soil obtained from the collected sample. Containers were covered with a lid, turned up side down and kept at room temperature (20 ± 3 °C) (Stock et al., 1999). Water was added to samples over time to keep the soil moistened during bating. *G. mellonella* larvaes were checked every day and each dead larva was replaced by a fresh one. After 7 days dead insects were thoroughly rinsed in distilled water and placed in modified White traps (Kaya and Stock, 1997) until the emergence of third-stage, infective juveniles. To maximize the recovery of nematodes from the soil samples, a "second baiting round" was done after removing the dead *G. mellonella* (presumably infested with nematodes), as described by Uribe-Lorío et al. (2005).

All nematodes research were produced in *G. mellonella* larvae. Fifteen *G. mellonella* larvae were exposed to 2000 infective juveniles (IJs) in a Petri dish (60 × 15 mm) lined with two moistened filter papers at 20 ± 3 °C. The first-generation hermaphrodites and second-generation adult nematodes were obtained by dissecting infected, last-instar *G. mellonella* larvae 3–4 and 5–7 days, respectively. The third-stage, infective juveniles were collected during the first 3 days after initial emergence (approximately 14–17 days) from the cadavers (Nguyen and Smart, 1995a).

### 2.2. Morphological characterization

#### 2.2.1. Light microscopy

For morphological characterization of the isolate, 20 first and second-stage adults and 25 IJs were randomly selected from different *G. mellonella* cadavers. One male has selected as holotype of this new species. All the nematode samples were examined live and then heat-killed on glass slides in 60 °C in Ringer's solution. The heat-killed nematodes were placed in hot, triethanolamine–formalin (TAF) fixative (Kaya and Stock, 1997) and transferred to anhydrous glycerin for mounting (Seinhorst, 1959). Examination and measurements were performed with an Axio Imager A1 microscope (Carl Zeiss).

#### 2.2.2. Fluorescence microscope

For observing distribution and location of the symbiotic bacteria in the intestine and the whole nematodes, 100 adults and 100 IJs of *H. chongmingensis* gen. nov., sp. nov. were randomly selected from different *G. mellonella* cadavers and collected in a 1.5 ml Eppendorf tube, rinsed with Ringer's solution three times, individual nematodes was mounted and covered with a cover glass, and then examined live under fluorescence microscope (Axio Imager A1 microscope, Carl Zeiss).

#### 2.2.3. Scanning electron microscopy

IJs and first-generation adults were fixed in 3% glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.2 for at least 24 h at 4–8 °C (Nguyen and Smart, 1995b). Post-fixed with 2% osmium tetroxide solution for 12 h at 25 °C, dehydrated in a graded ethanol series, critical point dried with liquid $CO_2$, mounted on SEM stubs, coated with gold (Nguyen and Smart, 1995a,b), and examined using a Hitachi S-3000N scanning electron microscope (Hitachi).

### 2.3. Molecular characterization

#### 2.3.1. Polymerase chain reaction (PCR) amplification and sequencing

The method used for processing fresh samples for PCR amplification was that described by Joyce et al. (1994). A single female (or juveniles) was placed on a 70% ethanol swabbed microscope slide in 10 µl of worm lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP40, 0.45 Tween 20, 0.01% gelatin and 60 µg/ml proteinase K). NP40 and Tween 20 (Amresco) are the detergents available to aid in the degradation of the nematode pro-

teins. The nematode was cut in half and the pieces in lysis buffer transferred to a sterile 0.5 ml micro centrifuge tube on ice. The tubes containing nematodes and worm lysate were frozen at −80 °C for 10 min and then incubated at 65 °C for 1 h, followed by 10 min at 95 °C to inactivate the proteinase K in the worm lysis buffer. The lysates were cooled on ice and centrifuged at 12,000*g* for 2 min and 2.5 μl of the supernatant was used in the PCR reaction. It was important not to add too much of the debris at the bottom of the tube after centrifugation as this could inhibit the Taq polymerase (Hominick et al., 1997).

The primers for the amplification and sequencing of the internal transcribed spacer (ITS) and 18S rDNA of this new species were reported by Vrain et al. (1992) and Liu et al. (1997), respectively. The primers for amplification of ITS were 5′-TTG ATT ACG TCC CTG CCC TTT-3′ (forward), and 5′-TTT CAC TCG CCG TTA CTA AGG-3′ (reverse). For 18S rDNA amplification of this new species, the primers used were 5′-GGT GAA ACT GCG AAC GGC TCA-3′ (forward) and 5′-CCG GTT CAA GCC ATT GCG ATT-3′ (reverse).

PCR products were purified and sequenced by Invitrogen Co. (Shanghai, China).

*2.3.2. Sequences analysis and phylogenetic relationships*

Sequences of the ITS region and 18S rDNA of EPN and related nematodes were used in the taxonomic and phylogenetic analyses in this study. Sequences of the new nematode were determined in this study, other sequences obtained from Genbank (Table 1).

Sequences were assembled with Sequencing Analysis 3.0 and aligned with CLUSTAL X (Thompson et al., 1997) initially under the default alignment parameters. Molecular phylogenetic relationships between populations were reconstructed by Bayesin interference methods. Bayesin phylogenetic reconstruction was performed by using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). The model that best fitted the data was identified by the GTR + G model test using the program MRMODELTEST 2.0 (Nylander, 2004). Four cold Metropolis-coupled Markov chains Monte Carlo (MCMCMC) for 10,000,000 generations were run and one tree was retained every 100 generations, and a burn-in of 2,000,000 generations (Huelsenbeck and Ronquist, 2001).

*2.4. Symbiotic relationship test*

*2.4.1. Symbiotic bacteria strain isolation and identification of H. chongmingensis gen. nov., sp. nov.*

Symbiotic bacteria strains were obtained from the infective stages of *H. chongmingensis* gen. nov., sp. nov. by two methods. The first method was to crush *ca.* 100 surface-disinfected, IJs followed by streaking the product on nutrient-bromothymol blue agar plates (NBTA, nutrient agar, 0.0025% bromothymol blue, and 0.004% triphenyltetrazolium chloride medium) and incubated at 28 °C for 48 h (Akhurst, 1980). The second method was to streak onto NBTA plates a drop of hemolymph harvested from insects parasitized for by nematodes 24–48 h.

Table 1
Taxa used in this study

| Taxon | GenBank | Accession No. |
|---|---|---|
| | ITS | 18S rDNA |
| *Heterorhabditidoides chongmingensis* | EF503690 | EF503692 |
| *Caenorhabditis elegans* | X03680 | X03680 |
| *Heterorhabditis bacterophora* | AY321477 | — |
| *Heterorhabditis baujardi* | AF548768 | — |
| *Heterorhabditis downesi* | AY321482 | — |
| *Heterorhabditis floridensis* | DQ372922 | — |
| *Heterorhabditis indica* | AY321483 | — |
| *Heterorhabditis marelatus* | AY321479 | — |
| *Heterorhabditis megidis* | AY321480 | — |
| *Heterorhabditis mexicana* | AY321478 | — |
| *Heterorhabditis zealandica* | AY321480 | — |
| *Steinernema glaseri* | AF122015 | — |
| *Steinernema carpocapsae* | AF121049 | — |
| *Steinernema abbasi* | AY230158 | — |
| *Pellioditis typica* | AF036946 | — |
| *Longidorus elongatus* | AF511417 | |
| *Heterorhabditis bacterophora* | — | AF036593 |
| *Heterorhabditis zealandica* | — | AJ920368 |
| *Steinernema carpocapsae* | — | AF036604 |
| *Steinernema glaseri* | — | AY284682 |
| *Caenorhabditis briggsae* | — | U13929 |
| *Pellioditis mediterranea* | — | AF083020 |
| *Pellioditis marina* | — | AF083021 |
| *Rhabditella axei* | — | U13934 |
| *Rhabditis myriophila* | — | U13936 |
| *Rhabditis colombiana* | — | AY751546 |
| *Rhabditis blumi* DF5010 | — | U13935 |
| *Oscheius insectivora* | — | AF083019 |
| *Pellioditis typica* | — | U13933 |
| *Phasmarhabditis* sp. EM434 | — | EU196008 |
| *Pelodera teres* EM437 | — | AF083002 |
| *Chordodes morgani* | — | AF036639 |

Genomic DNA of the symbiotic bacteria was prepared following the method of Marmur (1961) and PCR amplification of the 16S rRNA gene was performed as described by Xu et al. (2003). Phylogenetic analysis was performed using the software MEGA version 3.1 (Kumar et al., 2001) after multiple alignment of data by CLUSTAL X (Thompson et al., 1997). Distances (distance options according to the Kimura two-parameter model; Kimura, 1980, 1983) and clustering were based on the neighbor joining (Saitou and Nei, 1987). Bootstrap analysis (1000 resamplings) was used to evaluate the topology of the neighbor joining tree (Felsenstein, 1985).

*2.4.2. Phenotypic characterization of the symbiotic bacteria*

All the tests were conducted at 28 °C. After 16–24 h, cellular morphology was examined under a light microscope, and motility on 0.25% (w/v) Luria–Bertani (LB) agar was monitored after 16 h. LB cultures were spotted on plates as described by Vivas and Goodrich-Blair (2001). Dye adsorption of bromothymol blue was assessed on NBTA agar (Akhurst, 1980). Pigmentation was test as described by Boemare et al. (1997). Bioluminescence was tested as

described by Peel et al. (1999). The emission of light was initially investigated by observing cultures on agar plates in a darkroom (under conditions of total darkness) for up to 20 min, then under fluorescence microscope. The light produced by aqueous suspensions of the strain was then measured by placing suspensions of these strains in the well of a tray and positioning the well directly above an opening to a photomultiplier tube connected to a microphotometer for reading of light output. A distilled water blank and a suspension of *E. coli* were used as negative controls. Extraneous light was excluded by the use of reflective foil to cover the tray and by enclosing the tray and photomultiplier tube in a light-tight box (White et al., 1987).

#### 2.4.3. Production of aposymbiotic nematodes (symbiont-free) IJs

*Heterorhabditidoides chongmingensis* gen. nov., sp. nov. was maintained by passaging through larval *G. mellonella* and harvesting on White traps (Woodring and Kaya, 1988). Nematodes also were cultivated on symbiotic bacteria lawns seeded with either 500 to 800 IJs or 1500 first-instar-juvenile-stage nematodes (isolation described below) and incubated at room temperature. Infective-juvenile-stage nematodes were harvested from bacterial lawns by placing the agar slab in a Petri dish lid floating in sterile deionized $H_2O$.

To produce aposymbiotic IJs of the nematodes in vivo conditions, the surface of the eggs of the nematodes were disinfected by crushing 40 mature females in sterile Ringer solution (NaCl 0.9%, w/v) with sodium hypochloride (10%, w/v) during 18 min (Sicard et al., 2004). The disinfected eggs were then rinsed twice with sterile Ringer and transferred to 'liver-agar' plates (Sicard et al., 2003) for incubation at 24 °C. Three weeks later, axenic (i.e. grown without any germs) IJs were obtained from these plates, which were then available for further experiment.

#### 2.4.4. Retention of bacteria by nematodes

As described by Ciche et al. (2001) with a small modification, the numbers of symbiont bacteria cells in the intestine of IJs nematodes were determined. For some experiments, 50–100 surface-sterilized nematodes were disrupted using a 0.1 ml microtissue grinder. The homogenate was then serially diluted and plated on LB agar. Alternatively, a 10 ml sample of a water suspension containing 10–50 IJs nematodes was placed in the depression of a sterile hanging drop slide and dried in a laminar flow hood for 5–10 min, and then each nematode was disrupted with a sterile scalpel while being examined under a 40× dissecting microscope. The disrupted nematodes were suspended in 0.1 ml of LB agar, and the scalpel blade was rinsed in this suspension. The material was then transferred to a tube containing 0.9 ml of LB agar. The slide depression and scalpel were rinsed three times before plating serial dilutions of the tube onto LB agar. Colonies were counted following incubation at 28 °C for 3 days.

#### 2.4.5. Pathogenetic relationship test between the nematode and the insect host

The methods used were that described by Bonifassi et al. (1999) with a small modification. Pathogenicity of IJs treated in two ways was tested by exposure to last-instar larva *G. mellonella* or by injecting the nematodes into last-instar larva *G. mellonella*. The exposed or injected larvae were stored at 25 °C. More than 50,000 native IJs (with symbiont in their intestines) in 0.8 ml water were placed onto filter paper in Petri dishes (10 cm diameter) targets with 50 last-instar *G. mellonella* larvae. Five thousand axenic IJs in 0.8 ml water were placed onto filter paper in Petri dishes (7 cm diameter) targets with 14 last-instar *G. mellonella* larvae. Twenty native IJs or 20 axenic IJs in 200 μl of sterile Ringer were injected into last-instar *G. mellonella* larvae with 25 replication of each, 25 controls were injected with sterile Ringer solution (20 larvae/each). For all these experiments, mortality was determined.

#### 2.4.6. Pathogenetic relationship test between the bacteria, supernatants of the bacteria and the insect host

Bacteria for injection experiments were grown in nutrient broth Bonifassi et al. (1999). After 24 h growth, they were centrifuged, the supernatants were collected and their pathogenicity was determined (Bowen and Ensign, 1998). The culture was then filter sterilized, and a 5 ml sample was injected into each of 20 fourth-instar *G. mellonella* larvae. The bacterial pellet was rinsed twice in sterile PBS buffer without Mg and Ca salts (8 g NaCl, 0.2 g KCl, 1.15 g $Na_2HPO_4$, 0.2 g $KH_2PO_4$, 1 L distilled $H_2O$). Different bacteria symbiont cell concentrations were obtained by dilution with sterile PBS. Colony forming units (CFUs) of dilutions were assessed by inoculation of 100 μl on nutrient agar in three Petri dishes, which allowed the calculation of the average CFUs/ml of each dilution. To test the pathogenicity of the bacteria, 20 last-instar larvae of *G. mellonella* were inoculated with 20 μl of each dilution, respectively, and incubated at 23 °C with food. The lethal dose to kill 50% of the injected insects ($LD_{50}$) was then estimated.

#### 2.4.7. The role of the symbiotic bacterial strain in the production of the new nematode test

To determine if nematodes can reproduce without their symbiotic bacteria, *G. mellonella* larvae was exposed separately (Mitani et al., 2004) to *H. chongmingensis* gen. nov., sp. nov. IJs with and without their symbiotic bacteria, and nematode reproduction was checked at every day after inoculation. The center eight wells of a 24-well tissue culture plate were each filled with 0.5 g of air-dried sand (<0.25 mm). IJs were concentrated to 500 IJs/ml. Twenty-five IJs in 50 μl of water were added to each well, followed by a single *G. mellonella* larva with an average weight of 300 mg. Two sets of plates were inoculated at the same time, each with three replicate plates and kept at room temperature. After 4 days, the cadavers from one set of plates were rinsed with tap water, dissected, and the nematodes in

each larva were counted with the aid of a dissecting microscope. These were repeated with the second set of plates after 10 days. Three replicates of the experiment were conducted on separate days.

## 3. Results

### *3.1. Heterorhabditidoides chongmingensis gen. nov., sp. nov.*

#### *3.1.1. Description (Table 2, Figs. 1–5)*

Morphometric measurements of the holotype and all developmental stages of the new EPN species are presented in Table 2.

*3.1.1.1. Males.* Body curved ventrally, like J-shaped, when heat-killed. Head is not truncate, swollen, six conical lips well developed, separate, each with a terminal papilla, with two pore-like amphids on the lateral side of lips on two conical lips and four labial papillae on the other four conical lips (Fig. 1A). Stoma tubular-shaped, about 1.5 head width; cheilorhabdions cuticularized (Fig. 2A and B). Metastom isoglottoid, with hemispherical swellings each bearing three or five warts. Pharynx with cylindrical corpus; Metacorpus swollen; Esophageal collar present and long. Isthmus distinct; basal bulb globose, valve distinct. Nerve ring surrounding isthmus located anterior to isthmus, cardia present, protruding into intestine, excretory

Table 2
Morphometrics (μm) of *Heterorhabditidoides chongmingensis* gen. nov., sp. nov., Presented as the means ± SD and the range

| Character | Holotype | Male | Herma | Female | IJ |
|---|---|---|---|---|---|
| *n* | 1 | 20 | 20 | 20 | 25 |
| Body length (*L*) | 933 | 1115 ± 151<br>(822–1400) | 1921 ± 251<br>(1640–2220) | 1143 ± 141<br>(809–1351) | 428 ± 25<br>(395–474) |
| Greatest body diam (*D*) | 40 | 46 ± 5.9<br>(37.7–62) | 104 ± 19.6<br>(76.5–135) | 55 ± 6.6<br>(44–67) | 22.6 ± 3.1<br>(19–29) |
| Stoma length | | | 10.8 ± 0.7<br>(9.8–12) | 9.7 ± 0.4<br>(8.9–10.3) | |
| Stoma width | | | 10 ± 0.6<br>(9.5–11.5) | 9.3 ± 0.5<br>(8.6–10.2) | |
| EP | 149 | 169 ± 18<br>(124–193) | 207 ± 27<br>(176–276) | 158 ± 14<br>(127–180) | 90 ± 7.5<br>(80–105) |
| NR | 111 | 115 ± 14<br>(88–133) | 143 ± 22<br>(105–176) | 123.4 ± 15<br>(102–156) | 74 ± 10.5<br>(63–100) |
| ES | 137 | 157 ± 18<br>(113–186) | 179 ± 22.6<br>(152–235) | 180 ± 14<br>(154–202) | 104 ± 8.2<br>(92–120) |
| Testis reflexion | 137 | 146 ± 25.9<br>(93–204) | | | |
| Tail length with sheath (*T*) | 25.5 | 29 ± 4.4<br>(22–38.8) | 90 ± 11.8<br>(75.3–117) | 81 ± 10.9<br>(67–102) | 111 ± 18.9<br>(89–159) |
| Tail length without sheath | | | | | 60 ± 11.7<br>(44–79) |
| Anal body diam (ABD) | 26.6 | 26 ± 3.1<br>(21–33) | 28 ± 5.5<br>(23–42.2) | 22.6 ± 1.7<br>(20–27) | 12 ± 1.6<br>(10–15) |
| Spicule length (SP) | 50 | 51 ± 8.2<br>(37–68) | | | |
| Spicule width | 4.4 | 4.2 ± 0.7<br>(3.3–5.5) | | | |
| Gubernaculum length (GU) | 24 | 24.6 ± 3.8<br>(20–33) | | | |
| *V* | | | 52 ± 1.5<br>(50.2–54.4) | 51 ± 1.5<br>(50–54.8) | |
| a | | | | | 19.1 ± 1.8<br>(15–21) |
| b | | | | | 4.1 ± 0.3<br>(3.6–4.4) |
| c | | | | | 3.9 ± 0.5<br>(2.9–4.9) |
| $D\% = EP/ES^{*} 100$ | 108 | 107 ± 1.4<br>(103–110) | | | 86 ± 1.4<br>(84–88) |
| $E\% = EP/T^{*} 100$ | | | | | 83 ± 8.7<br>(67–97) |
| $SW\% = SP/ABD^{*} 100$ | 185 | 195 ± 33.6<br>(112–269) | | | |
| $GS\% = GU/SP^{*} 100$ | 48 | 48 ± 3.2<br>(43.2–54.5) | | | |

*n*, number of specimens measured; EP, distance from anterior end to excretory pore; NR, distance from anterior end to nerve ring; ES, distance from anterior end to end of pharynx; *V*, distance from anterior end to vulva/body length × 100.

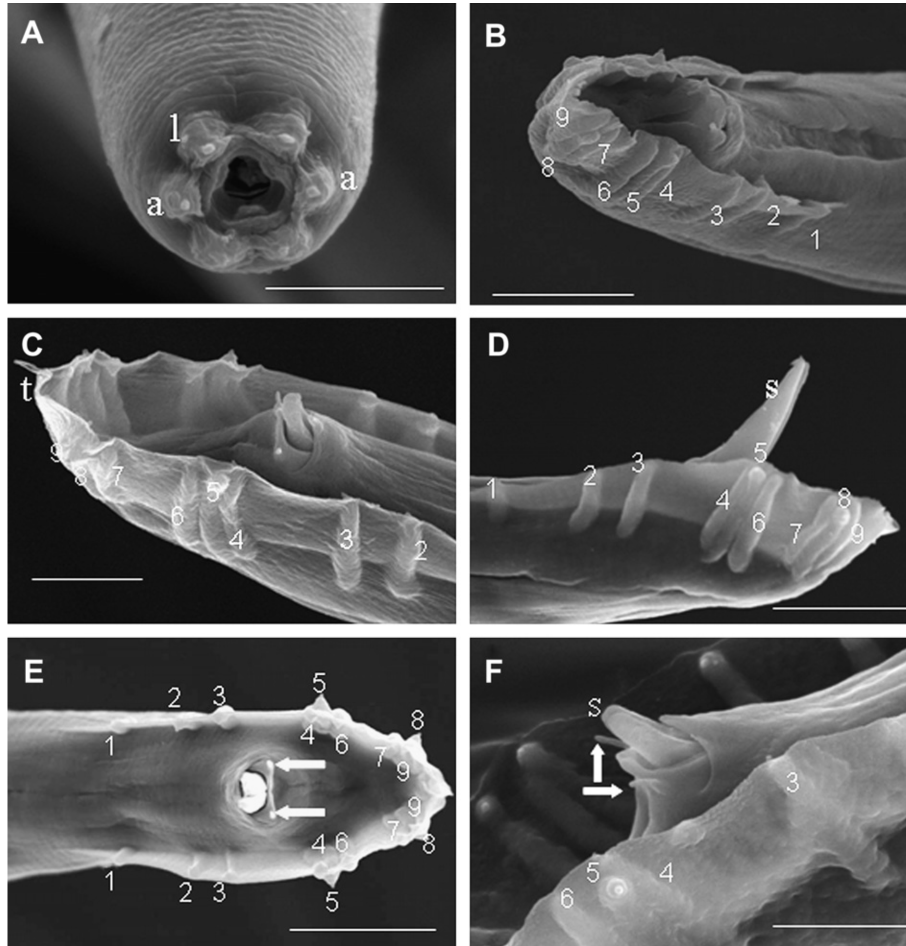


Fig. 1. SEM photographs of males of *Heterorhabditidoides chongmingensis* gen. nov., sp. nov. (A) Head of a male showing six labial papillae (1) and amphidial opening (a). (B–F) Posterior region in male showing different views showing bursal papillae (1, 2, 3, 4, 5, 6, 7, 8 and 9) in shape and position, small papillae on posterior edge of cloaca (arrows), tail tip (t), and spicules(s). Scale bars: (A) 10 μm, (B) 20 μm, (C) 10 μm, (D) 20 μm, (E) and (F) 10 μm.

pore usually posterior to basal bulb (Fig. 2A and C). Lateral fields with three longitudinal ridges. Testis monorchic, reflexed. Vas well developed. Spicules paired, separate, symmetrical, slightly curved ventrally (Fig. 2D and E). Head of spicules with rounded anterior end. Gubernaculum boat-shaped in lateral view, about 50% length of spicule, curved ventrally. Bursa peloderan. Cuticula of bursa extended on both sides and surrounding male cloaca. The papillae formula of bursa is 1, 2, 3 and 3 with constant papillae number in the terminal group. From anterior to posterior, pair one is well anterior to the cloaca, its tips reach beyond the bursal rim. The distance between pairs 2 and 3 are close (Fig. 1B, C, D and E), the two papillae standing immediately anterior to cloaca and reaching beyond the bursal rim. Pairs 4, 5 and 6 papillae form a group, these papillae are situated just posterior to the cloaca, with pair 5 curved outward (laterally viewed) (Fig. 1D). Pairs 7, 8 and 9 form a group, these papillae are located posterior to the cloaca (Fig. 1D and E). The number and distribution of papillae in the terminal group are constant. In addition to bursal papillae, a pair of smaller papillae was observed on the posterior edge of the cloacal opening (Fig. 1E and F). Tail small, pointed.

*3.1.1.2. Hermaphroditic females.* Hermaphroditic females are C-shaped fixed with TAF, body robust, always with many eggs in mature adults. Cuticle smooth under light microscope, but finely annulated with SEM. Head region tapering anteriorly. The labial region has six prominent lips, two pore-like amphids, cephalic papillae separated. Mouth is trigonal in face view (Fig. 3A and B). Stoma tubular-shaped, isthmus short and distinguishable (Fig. 4A and B). Nerve ring surrounding isthmus located anterior to isthmus. Valve of basal bulb prominent. Gonads didelphic, amphidelphic. Vulva with a transverse slit, situated on a protruding area, ususlly posterior to mid-body ($V = 50.2$–$54.4\%$), without cuticular flaps to protected (Fig. 3C), vagina short. Tail longer than anal body width, conoid or post-anal swelling with pointed terminus (Figs. 3D, 3E, 3F and 4E). Phasmids, 1–6, protruding, can be observed distinguishable on SEM. Post-anal swelling, well distinguished.

*3.1.1.3. Amphimictic females.* Amphimictic female similar to hermaphroditic female but smaller. Reproductive system amphidelphic. Vulva rarely protruding, not functional for

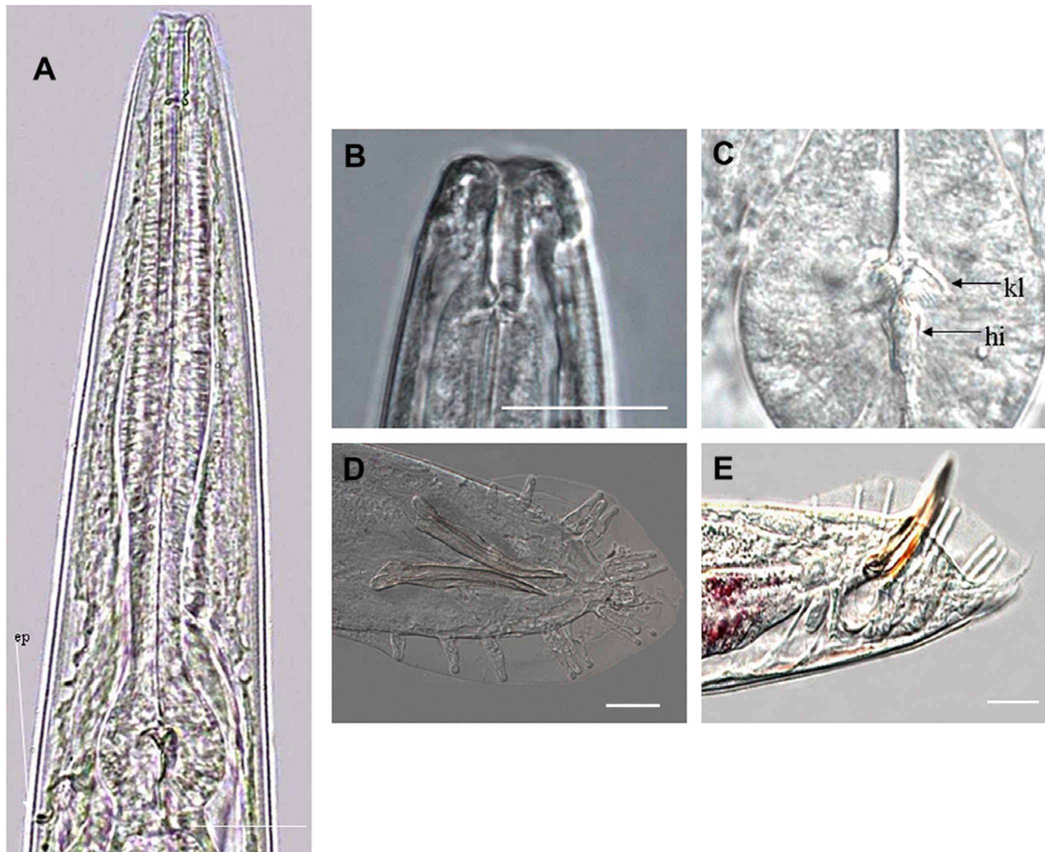


Fig. 2. Male nematode under light microscope. (A and B) Anterior of male, excretory pore (ep) and excretory duct of male nematode. (C) Terminal bulb with valvular apparatus (kl) and bouble haustrulum (hi). (D) and (E) Male tails with bursal papillae in different views showing. Scale bars: (A)–(E) 20 μm.

egg deposition (Fig. 4C and D), covered with copulation plug after mating.

*3.1.1.4. Infective juveniles.* Body elongate, sheath (second-stage cuticle) present immediately after harvesting, but many IJs lose their sheath in storage. Labial region with six labial papillae, without cephalic papillae. Two amphids, symmetrical arranged, labial-shaped. Head without prominent dorsal tooth (Fig. 5A and B). Excretory pore posterior to basal bulb. Exsheathed IJs body annulated, with two longitudinal ridges that form a very deep groove in lateral fields (Fig. 5C).Tail long, pointed (Fig. 5D). Excretory duct pronounced. Phasmid not observed. Pointed terminus tail elongate. Tail length with sheath is about twice as long as the tail without sheath.

### 3.1.2. Type host and locality

Natural host unknown. The nematode was collected by baiting with *G. mellonella* larvae from soil sample in Chongming Island, Shanghai, PR China.

### 3.1.3. Type material

Paratypes (males, hermaphroditic females, amphimictic females and infective juveniles) were deposited in the United States Department of Agriculture Nematode Collection (USDANC), Beltsville Maryland. Slides T-5644p–T-5667p: T-5644p–T-5649p (males), T-5650p–T-5655p (hermaphroditic females), T-5656p–T-5661p (amphimitic females), T-5662p–T-5667p (infective juveniles). Additional specimens deposited in the laboratory of Department of Zoology, College of Life Sciences of Nanjing Agricultural University, Nanjing, PR China.

### 3.1.4. Etymology

*Heterorhabditidoides chongmingensis* gen. nov., sp. nov. The specific epithet derives from the Greek “heteros” = different, “rhabdos” = rod, “oides” = similar, resembling.

### 3.1.5. Molecular characterization

The ITS regions of rDNA including complete ITS1, ITS2, and 5.8S rDNA subunit and the 18S rDNA were used as molecular markers to differentiate *H. chongmingensis* gen. nov., sp. nov. from other described Heterorhabditidae, and Rhabditidae. The length of 18S rDNA and ITS sequences of the new nematode were 1535 bp (410A, 412T, 313C, 400G) and 809 bp (149A, 262T, 182C and 216G), respectively. The phylogenetic trees reconstruction based on 18S rDNA and ITS sequence data indicate that *H. chongmingensis* gen. nov., sp. nov. forms a monophyletic group and has a closer relationship with the genus

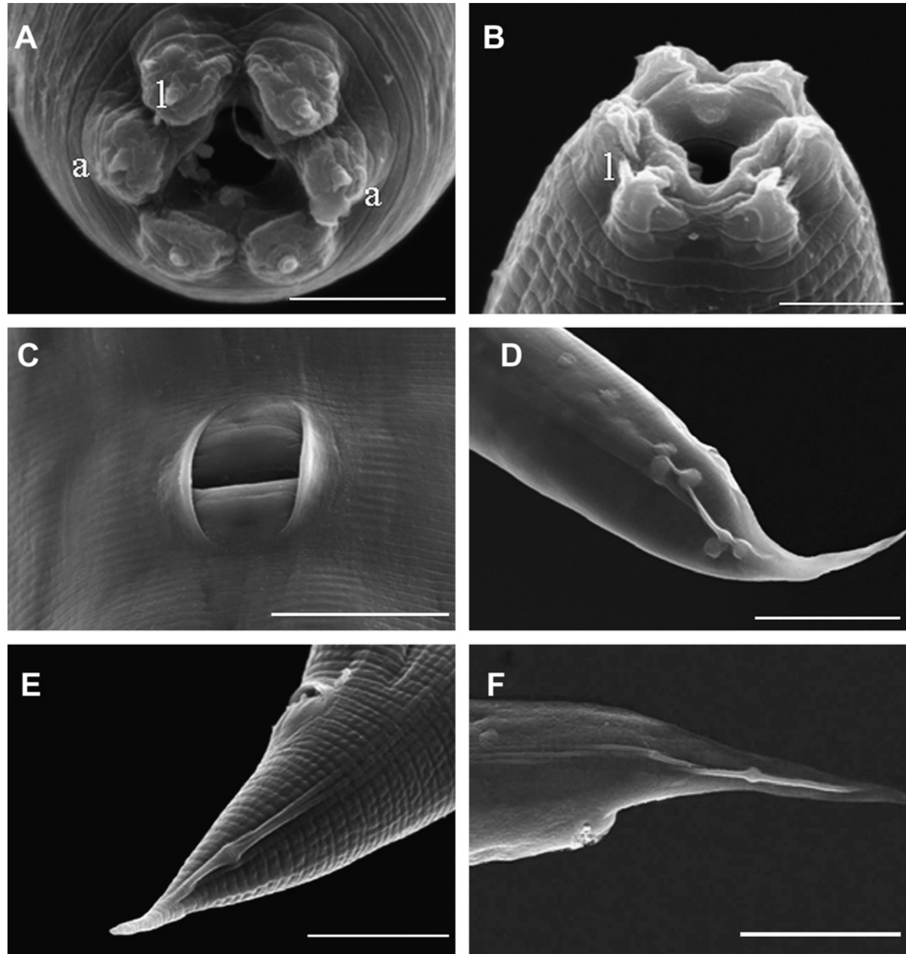


Fig. 3. (A and B) SEM of face view of hermaphroditic and amphimictic female showing six lips protruding into stoma with six labial papillae (1) on top and amphidial opening (a). (C) Vulva pattern. (D and E) Tail of two different tail shapes of hermaphroditic female. (F) Tail of amphimictic female. Scale bars: (A and B) 5 μm, (C–E) 20 μm, (F) 30 μm.

of *Oscheius*, *Rhabditis* and *Pellioditis* of the family Rhabditidae (Figs. 6 and 7).

The ITS rDNA region of *H. chongmingensis* gen. nov., sp. nov. is characterized by the length (809 bp, ITS1 = 418 bp, ITS2 = 237 bp). Compared with the ITS sequences of all other species in the family Heterorhabdit-idae, the sequence length of the novel species is longer than *Heterorhabditis* of the family Heterorhabditidae, shorter than *Caenorhabditis elegans* of the family Rhabditidae (Table 3). The sequence length of ITS1 is longer than that of *Pellioditis* of the family Rhabditidae, but shorter than that of *C. elegans*, The nucleotide composition of the ITS regions show that the percentage of A of *H. chongmingensis* gen. nov., sp. nov. is the least, whereas the percentage of C and T are higher than those of *Heterorhabditis* and *Caenorhabditis* (Table 3). The percentage of G is lower than *Caenorhabditis* but higher than *Heterorhabditis*.

### 3.2. Diagnosis

Infective juveniles, EP = 90 (80–105) μm, ES = 104 (92–120) μm, tail length = 111 (89–159) μm, and $a$ = 19.1 (15–21). For males, the head has six conical lips, well developed, separate, each with a terminal papilla, two pore-like amphids on the lateral side of lips on two conical lips. The papillae formula of bursa is 1, 2, 3 and 3 with constant papillae number in the terminal group, spicules length averaging 51 (37–68) μm, head of the spicule tip is rounded, anterior end blunt, and gubernaculum shape. Females of the new species has a very different vulvae pattern and without annulate to surrounded.Vulva of female with a transverse slit, situated on a protruding area, ususlly posterior to mid-body ($V$ = 50.2–54.4%), without cuticular flaps to protected, vagina short. Tail longer than anal body width, conoid or post-anal swelling with pointed terminus. Head of the infective juveniles without prominent dorsal tooth, anterior part of body without tessellate pattern, which can be distinguished from Heterorhabditidae. Different characteristics of the new nematode from the *Pellioditis*, *Caenorhabditis*, *Phasmarhabditis*, *Dolichorhabditis*, *Oscheius* in body length, annulated cuticle, stoma size, cuticularized cheilorhabdions, esophagus collar; swollen median bulb, and symbiotic *Serratia* bacteria in its intestine (Figs. 1, 2, 4 and 10; Tables 2 and 4). These characters

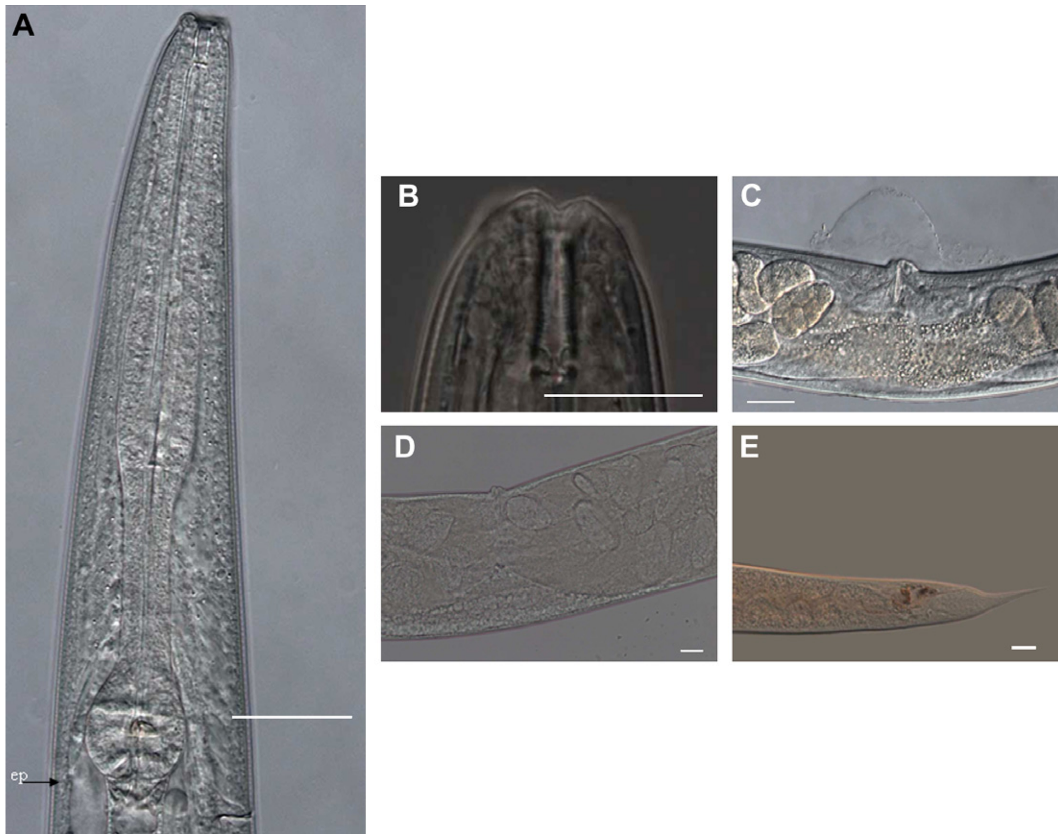


Fig. 4. Female under light microscope. (A) Anterior of female, excretory pore (ep) and excretory duct. (B) Anterior of female. (C) and (D) lateral view of vulva of amphimictic and hermaphroditic female, note that vulva of hermaphroditic female slightly protruding and vulva of amphimictic female covered with exudates or copulation plug after mating. (E) Tail of hermaphroditic female. Scale bars: (A) 40 μm, (B) 20 μm, (C) 40 μm, (D) 21 μm, (E) 20 μm.

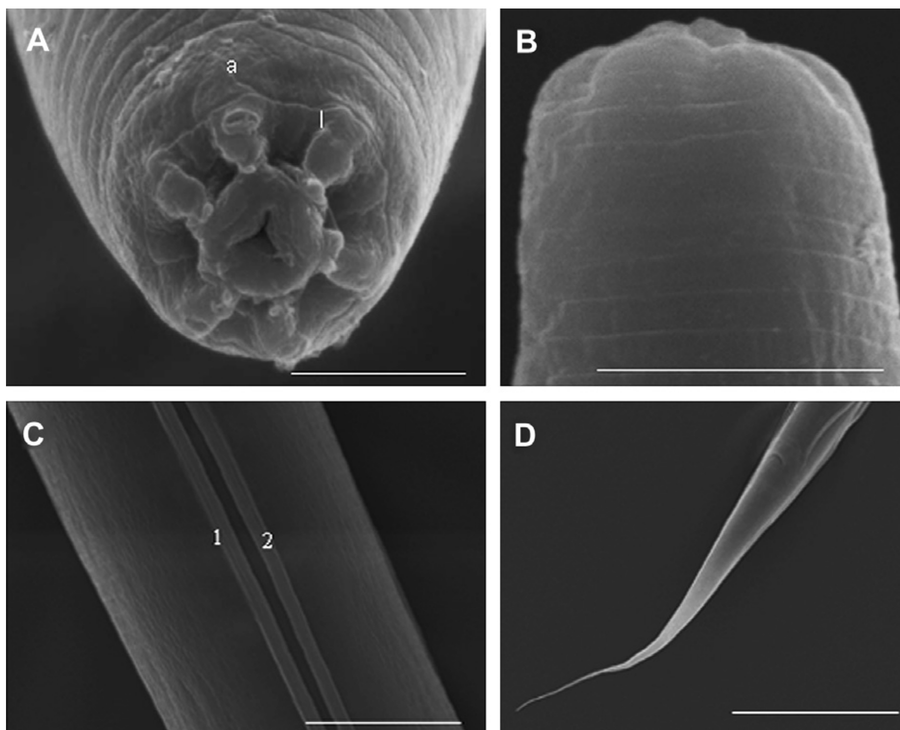


Fig. 5. (A) Head of a infective juvenile (IJ) showing six labial papillae (1) and amphidial opening (a). (B) SEM of IJ showing annules. (C) Two ridges and forms a very deep groove in lateral fields of IJ. (D) Tail of a infective juvenile. Scale bars: (A) and (B) 5 μm, (C) 10 μm, (D) 30 μm.

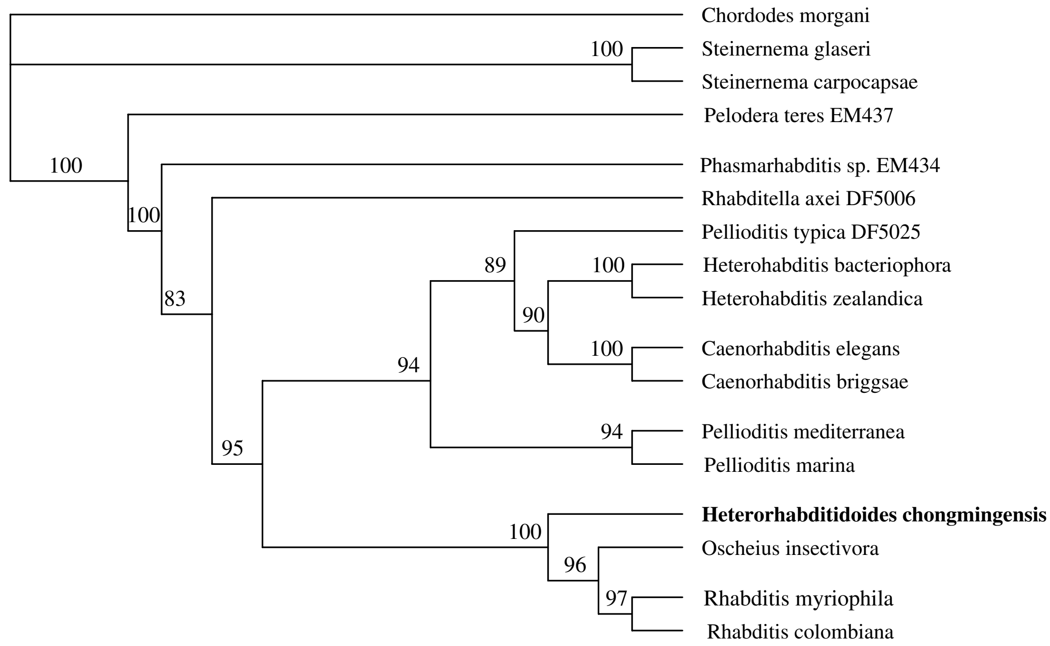


Fig. 6. Phylogenetic relationships of *Heterorhabditidoides chongmingensis* gen. nov., sp. nov. and other closely related nematodes reconstructed by bayesian inference method based on 18S rDNA sequences data. MCMC posterior probabilities are shown above internal nodes.

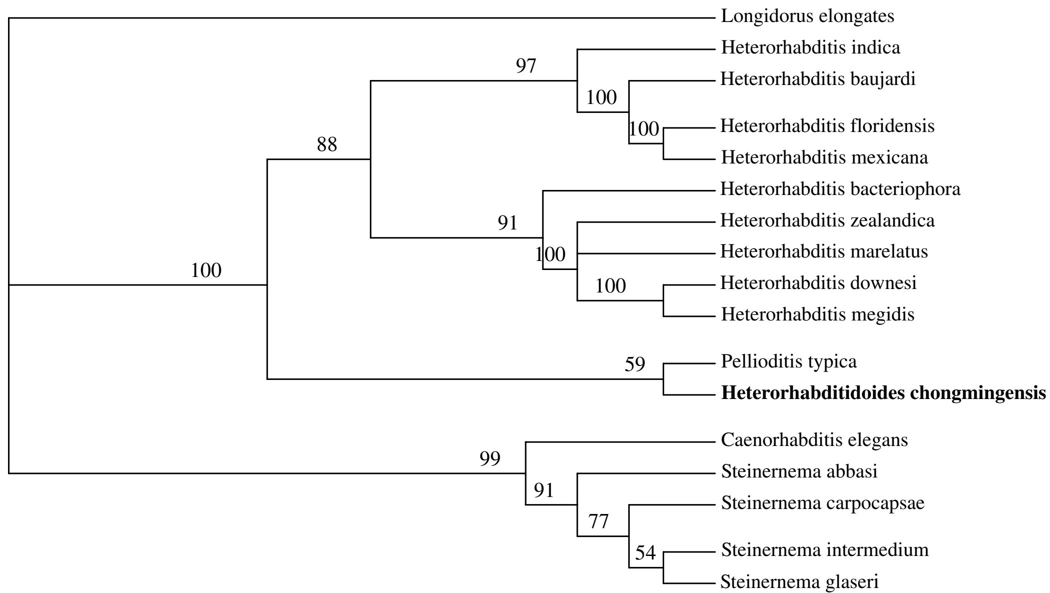


Fig. 7. Phylogenetic relationships of *Heterorhabditidoides chongmingensis* gen. nov., sp. nov. and other closely related members of EPNs reconstructed by bayesian inference method based on ITS rDNA sequences data. MCMC posterior probabilities are shown above internal nodes.

Table 3
Sequence length (base pairs = bp) and composition of ITS regions of nine species of *Heterorhabditis*, *Pellioditis typica*, *Caenorhabditis elegans* of subfamily of Pelodera and of *Heterorhabditidoides chongmingensis* gen. nov., sp. nov. (*H. chongmingensis*)

| Species | ITS1 | 5.8S | ITS2 | A | C | G | T |
|---|---|---|---|---|---|---|---|
| *Heterorhabditis* (740–771 bp) | 371–396 | 154 | 211–228 | 0.21–0.26 | 0.19–0.22 | 0.25–0.27 | 0.29–0.31 |
| *Pellioditis typica* (557 bp)[a] | 381 | [b] | [b] | [b] | [b] | [b] | [b] |
| *Caenorhabditis elegans* (1001 bp) | 464 | 153 | 384 | 0.23 | 0.22 | 0.29 | 0.25 |
| *H. chongmingensis* (809 bp) | 418 | 154 | 237 | 0.18 | 0.23 | 0.28 | 0.32 |

[a] This sequence is not as complete as in other species.
[b] Not available.

Table 4
Characteristics of five genus *Caenorhabditis*, *Phasmarhabditis*, *Pellioditis*, *Dolichorhabditis* of Subfamily Peloderinae; *Heterorhabditis* of Heterorhabditidae

| Genus | *Heterorhabditidoides* | *Pellioditis* | *Oscheius* | *Caenorhabditis* | *Phasmarhabditis* | *Dolichorhabditis* | *Heterorhabditis* |
|---|---|---|---|---|---|---|---|
| Size of nematodes | Body length, 0.8–2.2 mm | Body length, 0.5–3.0 mm | Body length, 1.2–3.2 mm | | Large nematodes, 0.8–3.40 mm | | |
| Head | Cuticle annulated, head slightly rounded | Head not or slightly offset | | Head continuous with body | | | Head truncate to slightly rounded |
| Stoma | Stoma about 1–1.5 times as long as head width, stomal with thick walls, cuticularized | Stoma narrow, long, about 1.5–2 head width | Stoma usually short | Stoma narrow, just a little longer than head width | Stoma about 1.2–2 times as long as head width | Stoma well developed, 1–2 head width | Stoma wide but shallow |
| Cheilorhabdions | Cheilorhabdions cuticularized | Cheilorhabdions not cuticularized | Cheilorhabdions not cuticularized | Cheilorhabdions not cuticularized | Cheilorhabdions cuticularized | Cheilorhabdions not cuticularized | Cheilorhabdions present, forming a ring, in lateral view resembling two refractile elongate structures |
| Metarhabdions | | Metarhabdions each with 3–5 small warts | Metarhabdions each with three small denticles or warts | Metarhabdions each with two fine bristle-like denticles | Metarhabdions each with three denticles | Metarhabdions each with two fine bristle-like denticles | |
| Esophagus | Esophagus collar present and long; median bulb present | Esophagus collar present; esophagus as illustrated | Esophageal collar present, short, corpus usually cylindrical; basal bulb strong | Esophagus with median bulb, basal bulb prominent | Esophageal collar present, median bulb present | Esophagus collar present corpus cylindrical or slightly swollen | Esophagus without metacorpus |
| Female gonads | Female gonads paired | Female gonads paired | Female gonads paired. Cervical duct, elongated female rectum | Female gonads paired | Female gonads paired | Female gonads paired | Female gonads paired |
| Sipcules | Spicules not fused, Slightly curved | Spicules not fused | Spicules not fused | Sipcules not fused | Spicules not fused, slightly curved | Spicules not fused | Spicules paired, separate, slightly curved ventrally, spicule head short, offset from lamina by a constriction |
| Bursa | Bursa peloderan, well developed with nine pairs of ribs, tail variable, conoid, sharply pointed | Bursa peloderan, well developed with nine pairs of ribs, tail variable, conoid, rounded, long, spicate | Bursa open, leptoderan with nine pairs of ribs, tail conical, pointed | Bursa peloderan, anteriorly closed with wavy edges, nine pairs of ribs, two of them precloacal, female tail long | Bursa peloderan, with nine pairs of ribs. female tail conoid or spikate. phasmids large, papiliform, protruding | Bursa peloderan, large with nine pairs of ribs, female tail conoid | Bursa peloderan, with nine pairs of genital papillae, tail pointed, longer than anal body width, post-anal swelling usually present |
| Presence of pathogenic bacteria | *Serratia* | | | | | | *Photorhabdus* |

Data of *Caenorhabditis*, *Phasmarhabditis*, *Oscheius* were taken by Andrássy, 1976; *Pellioditis* and *Dolichorhabditis* was taken by Andrássy, 1983; *Heterorhabditis* was taken by Poinar, 1976.

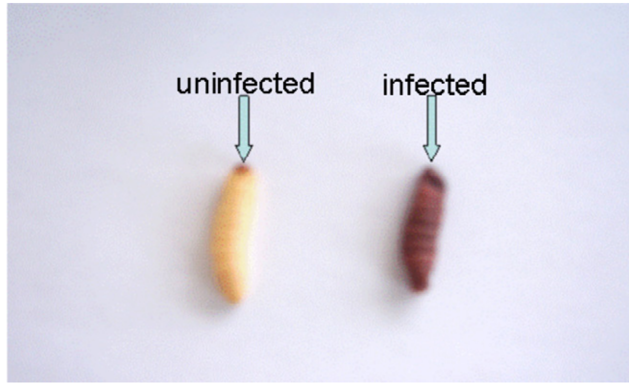


Fig. 8. *Galleria mellonella* larvae uninfected and infected by *Heterorhabditidoides chongmingensis* gen. nov., sp. nov. with its symbiotic bacteria DZ0503SBS1.

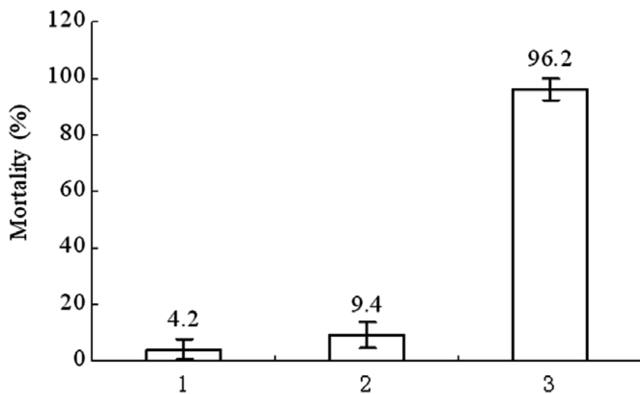


Fig. 9. Mortality of *Galleria mellonella* larvae injected with IJs of *Heterorhabditidoides chongmingensis* gen. nov. after 7 days. 1, Control; 2, axenic IJs; 3, native IJs. 20 larvae/each, means ± SD (range).

revealed that the new species is belong to a new genus can be further distinguished from Heterorhabditidae and all the other genera of Rhabditidae by its molecular characteristics of 18S rDNA and the ITS regions of ribosomal DNA (Figs. 6 and 7; Table 3).

### 3.3. Pathogen relationship test between the nematode and the insect host

A suspension of more than 50,000 IJs with the bacterial symbiont in their intestines placed in contact with 50 *G. mellonella* larvae (25 °C) infected the insects and the *G. mellonella* became red in color (Fig. 8), after infecting *G. mellonella*, the nematodes reproduced well in the *G. mellonella*. Axenic IJs infected the insects poorly, only a few *G. mellonella* died and did so without nematodes in the *G. mellonella* after 7–10 days.*G. mellonella* larvae injected with axenic IJs, after 5–7 days, showed no mortality. Dissection of the 25 injected insects showed that all had no nematodes, the results indicated that axenic IJs cannot induce mortality (Fig. 9), and that without the bacterial symbiont the axenic IJs cannot reproduced and survive.

### 3.4. Isolation and identification of the bacterial symbiont

Two phenotypic bacterial colonies (three species of bacteria) were obtained, of which four strains, DZ0503SBS1 (SBS1), DZ0503SBS2 (SBS2), DZ0503SBS3 (SBS3), DZ0503SBS4 (SBS4) were selected for further identification. Strain SBS1 produced a red pigment on NBTA and LB plates and luminesced under the fluorescence microscope (Fig. 10A, C and D), strain SBS2 produced a blue

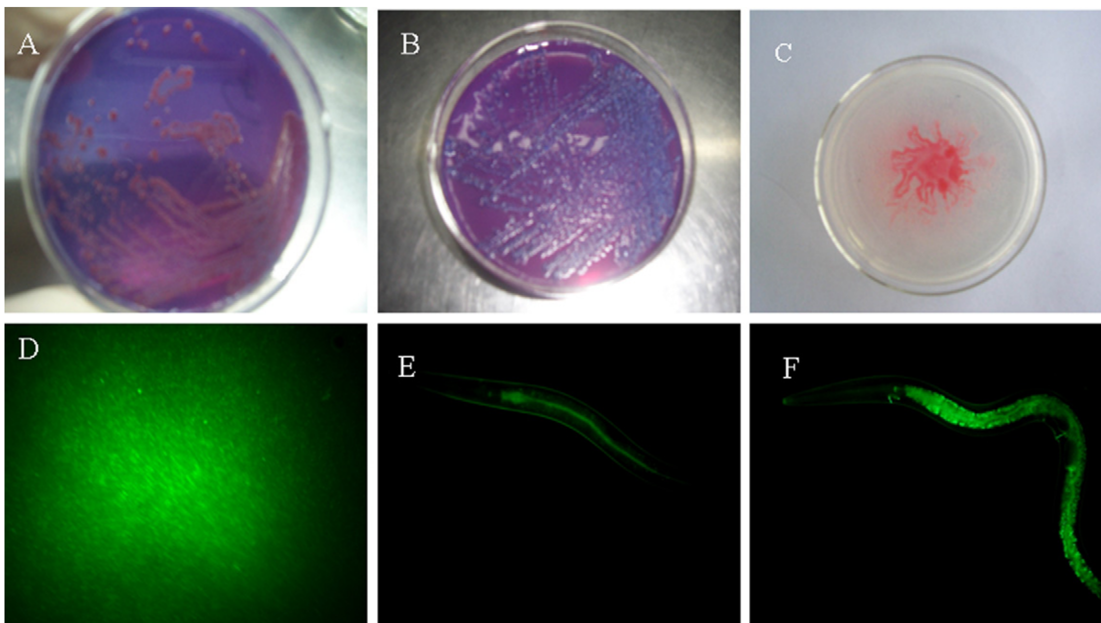


Fig. 10. The symbiotic bacteria of *Heterorhabditidoides chongmingensis* gen. nov., sp. nov. (A) A red pigment of strain DZ0503SBS1 on NBTA plate. (B) A blue pigment on NBTA plate. (C) A red pigment on LB plate and its motility. (D) Produce fluorescence under the fluorescence microscope. (E) and (F) Location of the symbiotic bacteria of *Heterorhabditidoides chongmingensis* gen. nov., sp. nov. under the fluorescence microscope.

pigment on the NBTA plate (Fig. 10B). 16S rDNA sequences data analysis showed that strains SBS1 and SBS2 have identical 16S rDNA sequences and were considered as two clones of the same species. The almost complete 16S rRNA gene sequence (1500 bp in length) of the symbiotic bacteria and related sequences selected from the GenBank following BLAST searches were used to draw the phylogenetic tree. Phylogenetic analyses and sequences BLAST searches showed that the symbiotic bacteria belongs to the genus *Serratia*, and the relatedness level of DNA–DNA hybridization between the bacteria species and its closer relatives, and its phenotypic characteristics indicates that DZ0503SBS1 (SBS1) should be a new species of the genus *Serratia* and described as *Serratia nematodiphila* sp. nov. (Zhang et al. 2008, in revision). SBS3 and SBS4 were characterized to the genus *Proteus* and *Acinetobacter*, respectively.

### 3.5. The relationship between the new nematode and its bacteria (bacterial strain SBS1, SBS2, SBS3, SBS4)

The bacterial symbiont is essential for *H. chongmingensis* gen. nov., sp. nov. as a pathogen of insects. IJs (with bacterial symbiont in their intestines) infected the insects and the *G. mellonella* died and became red in color and after infecting *G. mellonella*, the nematodes reproduce well in the *G. mellonella*, the mean number of nematodes produced was 1130 (845–1474). Injected axenic IJs (without the bacterial symbiont in their intestines) into *G. mellonella*, after 5–7 days, no mortality was recorded, dissection of the 25 injected insects showed that none had nematodes. The results indicate that axenic IJs cannot induce mortality, and without bacterial symbiont the axenic IJs cannot reproduced and survive.

*Galleria mellonella* larvae were injected with 50–1000 cells of the isolated bacterial symbiont SBS1. Injection of about 50 cells was necessary to reach the $LD_{50}$. The bacterial symbiont was lethal to *G. mellonella*; all of the insects ceased feeding by 16 h after injection with bacterial symbiont SBS1, and all the *G. mellonella* larvae were dead by 48 h post-injection.

Deprivation of the bacterial symbiont SBS1 from *H. chongmingensis* gen. nov., sp. nov. resulted in retarded IJs growth and development (Table 5). This indicated that

Table 5
Effect of bacterial symbiont elimination on adult nematodes emergence time

| Condition | Native IJs (with symbiont) | Axetic IJs (without symbiont) |
|---|---|---|
| $dH_2O$ | None | None |
| LB | 3 days | None |
| SH filtration | 3 days | 3 days |
| SH bacteria cell | 3 days | 3 days |
| SH fermentation | 3 days | 3 days |

SH, bacterial symbiont DZ0503SBS1 from *Heterorhabditidoides chongmingensis* gen. nov., sp. nov.; LB, Luria–Bertani.

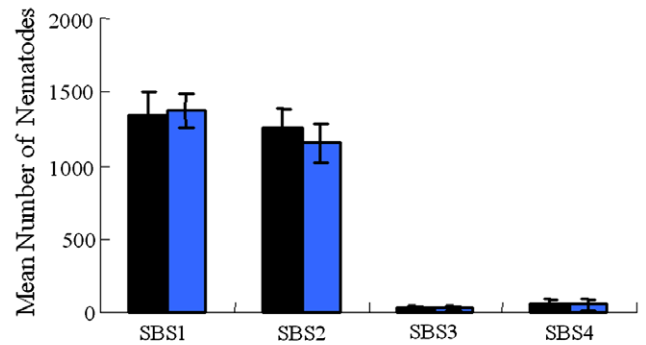


Fig. 11. The reproduction of *Heterorhabditidoides chongmingensis* gen. nov., sp. nov. with cell and supernatants of bacteria strains, DZ0503SBS1 (SBS1), DZ0503SBS2 (SBS2), DZ0503SBS3 (SBS3), DZ0503SBS4 (SBS4). Black lane: with cell of bacteria, blue lane: with supernatants of bacteria.

the symbiotic bacteria SBS1 is essential for survival and reproduction of *H. chongmingensis* gen. nov., sp. nov. The developmental time of the normal IJs and axenic IJs were not significantly different from each other after placed being in the fermentation of the bacterial symbiont, whereas the adult emergence rate, in the case of axenic IJs, was reduced in comparison with that of normal IJs. SBS3 and SBS4 can also cause the death of *G. mellonella* larvae, but after dead, the color of *G. mellonella* was not red. Comparative 25 infective juveniles of *H. chongmingensis* gen. nov., sp. nov. IJs growth, development and reproduction with SBS1, SBS2, SBS3 and SBS4 in 5 days. SBS3 and SBS4 almost did not have any effect on the growth and development of IJs. (Fig. 11).

### 3.6. Anterior midgut as specialized symbiotic organ

Under the light and fluorescence microscopes, anatomically, *H. chongmingensis* gen. nov., sp. nov. is very similar to other heterorhabditid species. In IJs their alimentary tract is complete. Their gut is normally organized, which allows the ingestion and regurgitation of the symbiont colonizing the gut. In the developmental course, however, in adult of *H. chongmingensis* gen. nov., sp. nov., the symbiont is located mostly in the intestine, and the other tissues surrounding the intestine had very little symbiont. The anterior midgut of *H. chongmingensis* gen. nov., sp. nov. was transformed into a voluminous organ for harboring a huge amount of the symbiont in the lumen (Fig. 10E and F).

## 4. Discussion

In recent years, phylogenetic analyses of nematodes using small-subunit ribosomal RNA (SSU rRNA) sequences have produced interesting hypotheses that have refined understanding of nematode evolution (Blaxter et al., 1998; Nadler and Hudspeth, 1998; Blaxter, 2001). 18S rRNA gene and ITS sequences data revealed the phylogenetic position of the new nematode species. Phyloge-

netic tree of 18S rDNA and ITS rDNA showed that the new nematode is a monophyletic group, and is a closer relative of the genus *Oscheius*, *Rhabditis* and *Pellioditis* in the family Rhabditidae. Molecular data combined with the morphological data indicates that the nematode described in this paper is a new species of a new genus belong to the family Rhabditidae. All the previously described EPN belong to the two families of Heterorhabditidae and Steinernematidae, which comprise the genus *Heterorhabditis*, *Steinernema* and *Neosteinernema*, respectively. This is the first time to discover the entomopathogenic nematode in the family Rhabditidae.

*Heterorhabditidoides chongmingensis* gen. nov., sp. nov. can develop completely to the adult in the host. Observations indicate that IJs of this new species penetrate their insect host through the spiracles. Infective juveniles were found in the body cavity of *G. mellonella* dissected 3–4 days after exposure to infective juveniles. In addition, dissections of *G. mellonella* performed 4–5 days after exposure to the nematodes revealed the presence of hermaphroditic females in the insect hemocoel, which suggested that nematodes may also gain access to the insect host via the cuticle.

The presence of pathogenic bacteria vectored by nematodes is a characteristic of nematodes in the Steinernematidae, Heterorhabditidae and, within Rhabditidae, in *Phasmarhabditis hermaphrodita* (Schneider). *Photorhabdus* and *Xenorhabdus* are two genera of the superfamily Enterobacteriaceae that are carried symbiotically in the gut of nematodes of the genus *Heterorhabditis* and *Steinernema*, respectively. Third-stage infective juveniles of these nematodes carry bacterial cells in their intestine (Bird and Akhurst, 1983; Kaya and Gaugler, 1993). Once the dauer juveniles reach the host hemocoel, the bacteria are released, thereby causing death of the host through septicemia (Poinar, 1990). Phenotypic characters and phylogenetic analysis based on 16S rDNA sequence data indicated that the symbiotic bacterium (SBS1) of *H. chongmingensis* gen. nov., sp. nov. belong to genus *Serratia* (superfamily Enterobacteriaceae). The new nematode carried the symbiotic bacterium not only in the ventricular portion of the intestine in the third-stage IJs and in the adult. The bacterial symbiont can be found in the hemolymph of insects infected by the *H. chongmingensis* gen. nov., sp. nov. The bacteria in the IJs of *H. chongmingensis* gen. nov., sp. nov. plays an important role in the insect host death and is essential for the development of *H. chongmingensis* gen. nov., sp. nov. Gouge and Snyder (2006) have reported *S. marcescens* as being in temporal association with EPN (Rhabditida: Steinernematidae and Heterorhabditidae) when the entomopathogenic nematodes cultured in the lab. In this study, the bacterial symbiont SBS1 was isolated from the intestine of IJs and the adult of *H. chongmingensis* gen. nov., sp. nov., which acquired a symbiotic lifestyle. The bacterial symbiont is different from *Photorhabdus* and *Xenorhabdus*, the other two bacterial symbiont of EPN.

The mechanism whereby the symbiotic bacteria support the growth and reproduction of the *H. chongmingensis* gen. nov., sp. nov. is intriguing but totally unknown and is a major focus of future research in our laboratory.

## Acknowledgments

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