Molecular identification of a new species of *Rhigonema* (Nematoda: Rhigonematidae) and phylogenetic relationships within the infraorder Rhigonematomorpha

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**Abstract**

**Background:** The infraorder Rhigonematomorpha comprises a group of obligate parasitic nematodes of millipedes (Arthropoda: Diplopoda). The current species identification of Rhigonematomorpha nematodes remains mainly based on morphological features, with molecular-based identification still in its infancy. Also, current knowledge of the phylogeny of Rhigonematomorpha is far from comprehensive.

**Methods:** The morphology of Rhigonematomorpha nematodes belonging to the genus *Rhigonema*, collected from the millipede *Spirobolus bungii* Brandt (Diplopoda: Spirobolida) in China, was studied in detail using light and scanning electron microscopy. Five different genetic markers, including the nuclear small ribosomal subunit (18S), internal transcribed spacer (ITS) and large ribosomal subunit (28S) regions and the mitochondrial *cox*\(^1\) and *cox*\(^2\) genes of these Rhigonematomorpha nematodes collected from China and *Rhigonema naylae* collected from Japan were sequenced and analyzed using Bayesian inference (BI) and Assemble Species by Automatic Partitioning (ASAP) methods. Phylogenetic analyses that included the most comprehensive taxa sampling of Rhigonematomorpha to date were also performed based on the 18S + 28S genes using maximum likelihood (ML) and BI methods.

**Results:** The specimens of *Rhigonema* collected from *S. bungii* in China were identified as a new species, *Rhigonema sinense* n. sp. Striking variability in tail morphology was observed among individuals of *R. sinense* n. sp. ASAP analyses based on the 28S, ITS, *cox*\(^1\) and *cox*\(^2\) sequences supported the species partition of *R. sinense* n. sp. and *R. naylae*, but showed no evidence that the different morphotypes of *R. sinense* n. sp. represent distinct genetic lineages. BI analyses also indicated that *R. sinense* n. sp. represents a separated species from *R. naylae* based on the *cox*\(^1\) and *cox*\(^2\) genes, but showed that *R. naylae* nested in samples of *R. sinense* n. sp. based on the ITS and 28S data. Phylogenetic results showed that the representatives of Rhigonematomorpha formed two large clades. The monophyly of the families Carnoyidae and Ichthyoccephalidae and the genus *Rhigonema* was rejected. The representatives of the family Ransomnematidae clustered together with the family Hethidae with strong support.

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Conclusions: A new species of Rhigonematormorpha, *R. sinense* n. sp. is described based on morphological and molecular evidence. ASAP analyses using 28S, ITS, *cox*1 and *cox*2 data indicate the striking variability in tail morphology of *R. sinense* n. sp. as intraspecific variation, and also suggest that partial 28S, ITS, *cox*1 and *cox*2 markers are effective for molecular identification of Rhigonematormorpha nematodes. The phylogenetic results support the traditional classification of Rhigonematormorpha into the two superfamilies Rhigonematoida and Ransomnematoida, and indicate that the families Carnoyidae and Ichthyocephalidae and the genus *Rhigonema* are non-monophyletic. The present phylogeny strongly supports resurrection of the family Brumptaemiliidae, and also indicates that the family Ransomnematidae is sister to the family Hethidae.

Keywords: Parasite, Nematoda, Rhigonematormorpha, DNA taxonomy, Genetic data, Species delimitation, Molecular phylogeny, Millipede

Background
Nematodes of the infraorder Rhigonematormorpha are obligate endoparasites of millipedes (Arthropoda: Diplopoda) with monoxenous life-cycles [1]. To date, over 200 nominal species of Rhigonematormorpha have been described worldwide [2, 3]. According to the current classifications, which are mainly based on morphological characters, Rhigonematormorpha is divided into six families assigned into two superfamilies, namely Rhigonematoida (Rhigonematidae, Ichthyocephalidae, Xystomatidae) and Ransomnematoida (Carnoyidae, Hethidae, Ransomnematidae) [1, 2, 4]. However, the monophyly and phylogenetic relationships of these six families are still under debate [3, 5–7].

The current species identification of Rhigonematormorpha nematodes remains mainly based on morphological features [8–13]. However, it is not easy to distinguish some congeners only using morphology due to their high similarities. Furthermore, the morphology-based method is not able to effectively delimit the phenotypic plasticity and discover sibling or cryptic species.

Several recent studies have provided some nuclear and mitochondrial (mt) DNA sequence data [i.e. the small subunit ribosomal DNA (18S), the large subunit ribosomal DNA (28S) and the mitochondrial cytochrome c oxidase subunit 1 (*cox*1) gene or the mitochondrial genome] that can be used for species identification or phylogeny of Rhigonematormorpha [6, 14–19]. However, the current genetic database for these nematodes remains very limited. In Rhigonematormorpha, only 21 nominal species have been genetically characterized [14–17, 19, 20], and most of the data available are represented by the 18S and 28S sequences, which are commonly used for molecular phylogeny of higher taxa within Nematoda [21–26]. Although the nuclear internal transcribed spacer (ITS) region and the mitochondrial *cox*1 and *cox*2 genes are widely used as powerful and practical genetic markers for revealing sibling or cryptic species, delimiting phenotypic variation and identifying species in the infraorders Ascaridomorpha, Spiruromorpha and Oxyuridomorpha [27–44], they have been scarcely employed in studies pertaining to Rhigonematormorpha species. Consequently, no current knowledge on the effectiveness of ITS, *cox*1 and *cox*2 as genetic markers for identification of Rhigonematormorpha nematodes is available.

In the present study, a large number of Rhigonematormorpha nematodes belonging to the genus *Rhigonema* (Rhigonematoida: Rhigonematidae) were collected from the millipede *Spirobolus bungii* Brandt (Diplopoda: Spirobolida) in Japan. Striking variability in the morphology of tail in both male and female specimens was observed among different individuals in the study material. In order to compare the suitability and efficacy of different nuclear and mitochondrial genetic markers for delimitation of the phenotypic variation of different individuals and discrimination of the morphologically similar Rhigonematormorpha congeners, the nuclear 18S, ITS and 28S regions and the mitochondrial *cox*1 and *cox*2 genes of the present specimens collected from China and *R. naylae* Morffe & Hasegawa, 2017 collected from *Parafontaria tonominea* (Polidesmida: Xystodesmidae) in Japan were sequenced and analyzed using Bayesian inference (BI) and Assemble Species by Automatic Partitioning (ASAP) methods. Furthermore, in order to test the monophyly and evaluate the evolutionary relationships of the six families within Rhigonematormorpha, we performed phylogenetic analyses, including the most comprehensive taxa sampling of Rhigonematormorpha to date, based on the 18S + 28S genes using maximum likelihood (ML) and BI.

Methods
Light and scanning electron microscopy
Nematodes were collected from the hindgut of the millipede *S. bungii* in Shijiazhuang, Hebei Province, China, and the specimens fixed and stored in 80% ethanol until study. For the light microscopy studies, nematodes were cleared in glycerin for examination using a Nikon® optical microscope (Nikon Corp., Tokyo, Japan). Photomicrographs were recorded using a Nikon® digital camera.
coupled to a Nikon® optical microscope (Nikon ECLIPSE Ni-U; Nikon Corp.). For scanning electron microscopy (SEM), specimens were re-fixed in a 4% formaldehyde solution, post-fixed in 1% OsO₄, dehydrated via an ethanol series (50%, 70%, 80%, 90%, 100%, 100%) and acetone (100%) and then critical point dried. Samples were coated with gold and examined using a Hitachi S–4800 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 20 kV. Measurements (range with mean in parentheses) are given in micrometers unless otherwise stated. Type specimens were deposited in College of Life Sciences, Hebei Normal University, Hebei Province, China.

Molecular procedures
The mid-body of 10 selected nematode specimens (4 males, 6 females) with a different morphology of the tail tip, all specimens collected from S. bungii in China, was used for molecular analysis (Table 1). Genomic DNA from each sample was extracted using a Column Genomic DNA Isolation Kit [Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China] according to the manufacturer’s instructions. The partial 18S region was amplified by PCR using the forward primer Nem_18S_F (5′-CGC GAA TRG CTC ATT ACA ACA GC-3′) and the reverse primer Nem_18S_R (5′-GGG CGG TAT CTG ATC GCCA AGG GCC AAG GG-3′) [45]. The partial 28S region was amplified by PCR using the forward primer D2a (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and the reverse primer D3b (5′-TCG GAA GGA ACC AGC TAC TA-3′) [46]. The ITS-1 region was amplified by PCR using the forward primer (5′-AGC GGG GAC TGC TGT TTC GAT ACC TTT CGG-3′) and the reverse primer (5′-GTT CGA CCC TCA GCC AGA CGT GCC AAG GGG-3′) designed in the present study. The ITS-2 region was amplified by PCR using the forward primer (5′-CTA CTC TTA GCG GTG GAT CAC TCG GCT CGT-3′) and the reverse primer (5′-TCT AGC ACC TTC TAT GGA CTG TAG GCC CGC-3′) designed in the present study. The partial cox1 region was amplified by PCR using the forward primer LCO (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and the reverse primer HCO (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) [47]. The partial cox2 region was amplified by PCR using the forward primer (5′-ATG AAA TTT CCA ATT TTG AGG CTT ATA GGG-3′) and the reverse primer (5′-ATA AAC TAA AAA GCT AAA AAT TAT TAA AAA-3′) designed in the present study.

Table 1  Specimens of Rhigonema sinense n. sp. selected for molecular analysis

| Samples (specimen no.) | Genbank accession numbers of partial 18S region | Genbank accession numbers of partial 28S region | Genbank accession numbers of ITS region | Genbank accession numbers of partial cox1 region | Genbank accession numbers of partial cox2 region | Morphotypes |
|------------------------|-----------------------------------------------|-----------------------------------------------|----------------------------------------|-----------------------------------------------|-----------------------------------------------|-------------|
| 1 female (49)          | ON936095                                      | ON936078                                      | ON936109                               | ON935732                                      | OP157155                                      | Without finger-like tail tip (Fig. 3g) |
| 1 female (43)          | ON938178                                      | ON936079                                      | ON936110                               | ON935729                                      | OP157154                                      | Without finger-like tail tip (Fig. 3g) |
| 1 female (69)          | ON938172                                      | ON936082                                      | ON936112                               | OP159049                                      | OP157157                                      | Long finger-like tail tip (Fig. 3e)     |
| 1 female (20)          | ON938182                                      | ON936083                                      | ON936106                               | OP153601                                      | OP157153                                      | Long finger-like tail tip (Fig. 3e)     |
| 1 female (68)          | ON936087                                      | ON936077                                      | ON936104                               | OP103756                                      | OP157162                                      | Short finger-like tail tip (Fig. 3f)    |
| 1 female (51)          | ON938174                                      | ON936080                                      | ON936111                               | ON935744                                      | OP157156                                      | Short finger-like tail tip (Fig. 3f)    |
| 1 male (71)            | ON936088                                      | ON936081                                      | ON936108                               | ON935613                                      | OP157158                                      | Long finger-like tail tip (Fig. 3c)     |
| 1 male (37)            | ON938171                                      | ON936086                                      | ON936107                               | OP103757                                      | OP157161                                      | Long finger-like tail tip (Fig. 3c)     |
| 1 male (67–1)          | ON937754                                      | ON936084                                      | ON936105                               | ON935751                                      | OP157159                                      | Short finger-like tail tip (Fig. 3h)    |
| 1 male (67–2)          | ON938173                                      | ON936085                                      | –                                      | OP103758                                      | OP157160                                      | Short finger-like tail tip (Fig. 3h)    |

cox1/2 Cytochrome c oxidase subunit 1/2, ITS internal transcribed spacer, 18S/28S small/large ribosomal subunit
using the forward primer Rhigo_COXII_F (5'-TCH ACY ACA ATA GGY ATA AAM CT-3') and the reverse primers Rhigo_COII_Rev (5'-GWT ATA TRG RTT GGT TYC ATA A-3'), as well as by Rhigo_COII_RevNtd (5'-GCT TYC ATA ATT TTA MTT RTA G-3') designed in the present study.

All PCR assays of nematodes collected from *S. bungii* in China were performed in a 50-μl volume containing PCR reaction buffer with 10 mM Tris HCl at pH 8.4, 50 mM KCl, 3.0 mM MgCl₂, 250 μM of each dNTP, 50 pmol of each primer and 1.5 U of Taq polymerase (Takara Bio Inc., Kusatsu, Shiga, Japan) in a thermocycler (model 2720; Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The cycling conditions for the different regions were:

Partial 18S region: an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C, 30 s (denaturation), 52 °C, 40 s (annealing) and 72 °C, 60 s (extension), with a final extension of 72 °C for 10 min.

Partial 28S region: an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C, 30 s (denaturation), 56 °C, 30 s (annealing) and 72 °C, 70 s (extension), with a final extension of 72 °C for 7 min.

Partial ITS region: an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C, 30 s (denaturation), 68 °C, 30 s (annealing) and 72 °C, 20 s (extension), with a final extension of 72 °C for 7 min.

Partial COX region: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C, 30 s (denaturation), 50 °C, 30 s (annealing) and 72 °C, 60 s (extension), with a final extension of 72 °C for 10 min.

Partial COX2 region: an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C, 30 s (denaturation), 46 °C, 30 s (annealing) and 72 °C, 60 s (extension), with a final extension of 72 °C for 10 min.

All PCR products were checked on GoldView-stained 1.5% agarose gels and purified with the Column PCR Product Purification Kit [Sangon Biotech (Shanghai) Co., Ltd.]. Sequencing for each sample was carried out on both strands. Sequences were aligned using ClustalW2. The DNA sequences obtained herein were compared (using the algorithm BLASTn) with those available in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). The 18S, 28S, ITS, cox1 and cox2 sequence data of specimens collected from China and *R. naylae* collected from Japan were deposited in the GenBank (http://www.ncbi.nlm.nih.gov).

**Species delimitation**

The BI and ASAP [48] methods were employed for species delimitation of *Rhigonema* spp. based on the 18S, 28S, ITS, cox1 and cox2 sequences, respectively. The BI trees were inferred using MrBayes 3.2.7 [49] under the JC model for each genetic marker (two parallel runs, 1,000,000 generations). *Rhigonema thyسانophora* (Rhigonematormorpha: Rhigonematormoidea) and *Krefftascaris sharpiloi* (Ascaridida: Ascaridoidea) were chosen as out-groups. The ASAP analyses were conducted using the ASAP online server (https://bioinfo.mnhn.fr/abi/public/asap) under the Kimura (K80) ts/tv model. The results of ASAP with the lowest scores were considered to be the optimal group number, with the exception of the optimal results recommended by ASAP.

**Phylogenetic analyses**

Phylogenetic analyses were performed based on the 18S + 28S sequence data using ML inference with IQTREE v2.1.2 [50] and BI with MrBayes 3.2.7 [49], respectively. *Oxyuris equi* (Oxyurida: Oxyuroidea) was chosen as the out-group. The in-group included 28 representatives of Rhigonematormorpha representing all six families belonging to the two superfamilies Rhigonematormoidea and Ransomnematoidae. Detailed information on the Rhigonematormorpha nematodes included in the present phylogenetic analyses is provided in Table 2.

The nucleotide sequences were aligned in batches using MAFFT v7.313 with the iterative refinement method of E-INS-I [51]; poorly aligned regions were excluded using BMGE v1.12 (h = 0.4) [52]. In addition, partially ambiguous bases were manually inspected and removed. Substitution models were compared and selected according to the Bayesian information criterion (BIC) by using ModelFinder [53]. The TIM3e + I + G4 model in ML inference
Zhang et al. Parasites & Vectors (2022) 15:427

and the SYM+I+G model in BI were identified as the optimal nucleotide substitution model, respectively. Reliabilities for ML inference were tested using 1000 bootstrap replications, and BIC analysis was run for \(5 \times 10^6\) Markov chain Monte Carlo (MCMC) generations.

In the ML tree, the bootstrap (BS) values \(\geq 90\) were considered to constitute strong nodal support, whereas BS values \(\geq 70\) and \(< 90\) were considered to constitute moderate nodal support. In the BI tree, the Bayesian posterior probabilities (BPP) values \(\geq 0.90\) were considered

**Table 2** Detailed information on Rhigonematomorpha nematodes with their genetic data included in the phylogenetic analyses

| Species               | Host                  | Locality | Accession numbers for 18S region | Accession numbers for 28S region | References |
|-----------------------|-----------------------|----------|---------------------------------|---------------------------------|------------|
| **Ingroup Rhigonematoidea** |                       |          |                                 |                                 |            |
| Rhigonema thysanophora | Euryurus sp.           | USA      | EF180067.1                       | MG195996.1                       | [70]       |
| Rhigonema naylae       | Parafontaria laminate | Japan    | KX844642.1                      | KX844643.1                       | [17]       |
| Rhigonema ingens       | Thyropygus sp.        | Vietnam  | JX069475.1                      | JX131616.1                       | [7]        |
| Rhigonema sp. 1179     | Apeuthes sp.          | Vietnam  | JX106453.1                      | JX155275.1                       | [7]        |
| Rhigonema sp. 1181     | Apeuthes sp.          | Vietnam  | JX106455.1                      | JX155276.1                       | [7]        |
| Rhigonema sinense      | Spirobolus bungii     | China    | ON936095                        | ON936078                         | Present study |
| Rhigonema sinense      | Spirobolus bungii     | China    | ON938178                        | ON936079                         | Present study |
| Rhigonema sinense      | Spirobolus bungii     | China    | ON938172                        | ON936082                         | Present study |
| Rhigonema sinense      | Spirobolus bungii     | China    | ON938182                        | ON936083                         | Present study |
| Rhigonema sinense      | Spirobolus bungii     | China    | ON938174                        | ON936080                         | Present study |
| Rhigonema sinense      | Spirobolus bungii     | China    | ON938173                        | ON936085                         | Present study |
| Rhigonema sinense      | Spirobolus bungii     | China    | ON938173                        | ON936085                         | Present study |
| Obainia sp. SVM-2017   | Archispirostreptus gigas | Tanzania | KU561101.1                     | KU561100.1                       | [64]       |
| Ichthyoecephaloides sumbatus | Spirobolus sp.       | Indonesia | JX101958.1                     | JX155273.1                       | [7]        |
| Xystrognathus phrissus | Apeuthes sp.          | Vietnam  | JX101957.1                      | JX155274.1                       | [7]        |
| Trachyglossoides sp.   | Spirobolus sp.        | Cuba     | MW030192.1                      | MW030188.1                       | Unpublished |
| **Ransomnematoidea**   |                       |          |                                 |                                 |            |
| Ransomnema bravoae     | Anadenobolus putealis | Mexico   | KY857887.1                      | KY857886.1                       | [3]        |
| Camoya mexicana        | Anadenobolus putealisLoomis | Mexico | KT236089.1                      | KT236088.1                       | [63]       |
| Camoya cepacapitatus   | Anadenobolus putealisLoomis | Mexico | KT236087.1                      | KT236086.1                       | [63]       |
| Camoya filipjevi      | Salpidobolus sp.       | Indonesia | JX982120.1                     | JX946703.1                       | [62]       |
| Camoya philippinensis  | Rhinoricidae sp.       | Philippines | KT957946.1                     | KT957945.1                       | [71]       |
| Catitena trachelomelgi | Thyropygus sp.        | Vietnam  | JX982117.1                      | JX419378.1                       | [5]        |
| Catitena fanspanis     | Pseudospirobolellidae sp. | Vietnam | JX982118.1                      | JX436470.1                       | [5]        |
| Brumptaemilius justini | Archispirostreptus gigas | Tanzania | JX999733.1                     | JX999732.1                       | [5]        |
| Insulanema longisculum | Apeuthes sp.          | Vietnam  | JX982119.1                      | JX436471.1                       | [5]        |
| Heth taybaci           | Harpagophoridae sp.    | Vietnam  | JX987085.1                      | JX946704.1                       | [5]        |
| Heth impaltriensis     | spirosreptidae sp.    | Philippines | KM226161.1                     | KM226162.1                       | [6]        |
| Heth turfensis         | Anadenobolus putealis | Mexico   | KY857883.1                      | KY857884.1                       | [3]        |
| Heth konoplevi         | Rhinoricidae sp.       | Philippines | KY854569.1                     | KY85470.1                        | [64]       |
| Heth initiens          | Rhinoricidae sp.       | Philippines | KY85471.1                      | KY85472.1                        | [64]       |
| Heth pivari            | Narceus gordanus      | USA      | MK182092.1                      | MK182091.1                       | [18]       |
| Heth gordae            | Anadenobolus putealis | Mexico   | KY857879.1                      | KY857880.1                       | [3]        |
| Heth sp. 1 HMM2018     | Anadenobolus putealis | Mexico   | KY857881.1                      | KY857882.1                       | [3]        |
| Heth sp. 1195          | Salpidobolus sp.       | Indonesia | JX987087.1                     | JX443483.1                       | [5]        |
| Heth sp. 1194          | Spirostreptida sp.    | Australia | JX987086.1                     | JX443484.1                       | [5]        |
| **Outgroup**           |                       |          |                                 |                                 |            |
| Oxyuris equi           | –                     | –        | KU180664.1                      | KU180675.1                       | [72]       |
to constitute strong nodal support, whereas BPP values ≥ 0.70 and < 0.90 were considered to constitute moderate nodal support. BS values ≥ 0.70 and BPP values ≥ 0.70 are shown in the phylogenetic trees.

Results
Order Spirurida Railliet 1914
Infraorder Rhigonematomorpha De Ley & Blaxter, 2002
Family Rhigonematidae Artigas 1930
Genus Rhigonema Cobb, 1898

Rhigonema sinense Zhang, Wang, Hasegawa, Nagae, Chen, Li & Li n. sp.

Type-host: Spirobolus bungii (Brandt) (Spirobolida: Spirobolidae).

Type-locality: Shijiazhuang, Hebei Province, China.

Site in host: Hindgut.

Type specimens: Holotype, male (HBNU-N-2022Ar008Z-L); allotype, female (HBNU-N-2022Ar009Z-L); paratypes: 16 males, 16 females (HBNU-N-2022Ar010Z-L); deposited in the College of Life Sciences, Hebei Normal University, Hebei Province, China.

Representative DNA sequences: Representative nuclear ribosomal and mitochondrial DNA sequences were deposited in the GenBank database under the accession numbers ON936087, ON936088, ON936095, ON937754, ON938171–ON938174, ON938178, ON938182 (18S), ON936077–ON936086 (28S), ON936104–ON936112 (ITS), OP159049, OP103756–OP103758, ON935601, ON935613, ON936077–ON936086 (28S), ON936104–ON936112 (ITS), OP159049, OP103756–OP103758, ON935601, ON935613, ON935729, ON935732, ON935744, ON935751 (cox1) and OP157153–OP157162 (cox2).

ZooBank registration: To comply with the regulations set out in article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub: 16047F5E-A719-4A63-9F8F-260AA4345341. The LSID for the new name Rhigonema sinense is urn:lsid:zoobank.org:act:D7722213-B0FD-450F-B891-619A97ECC90B.

Etymology: The specific name refers to its geographic origin (China), which represents the first new species of Rhigonematomorpha described in China.

Description
General
Small-sized, whitish nematodes with a maximum width at about mid-body. Cephalic region heavily cuticularized, consisting of well-developed cephalic cap and smooth cephalic collar (Figs. 1a, 2a). Cephalic cap bearing three apparent lips, dorsal lip with one pair of large cephalic papillae, subventral lips with a single large cephalic papilla each, amphidial apertures located laterally at junction of cephalic cap and cephalic collar (Figs. 1a, 2a); each lip with unconspicuous inner lip margins (Fig. 1a). Oral aperture simple, somewhat triangular (Figs. 1a, 2a). Cuticle posterior to cephalic region with dense, transverse rows of small spines (microtrichs); rows of spines gradually becoming distinctly sparser and smaller towards posterior region of body and disappearing at about the anterior 1/4 region of body (Figs. 1a, g, 2a, g–j). Esophagus divided into short chitinized pharynx with three flabellate pharyngeal plates (Fig. 2a), muscular cylindrical corpus (posterior part slightly wider than anterior part), unconspicuous isthmus and ovoid or nearly rounded posterior bulb (Fig. 3a, d). Nerve ring at about 1/2 of esophageal corpus (Fig. 3a). Excretory pore at about junction of corpus and posterior bulb of esophagus (Figs. 1g, 3a). Tail of both sexes conical, with polymorphic tip (Figs. 1b, e, 2c, 3c–h).

Male (based on 17 mature specimens)
Body 4.27–7.02 (mean 5.83) mm long; maximum width 251–444 (356) mm. Esophagus 397–477 (429) mm long, representing between 5.76% and 9.31% (7.77%) of body length; corpus 275–304 (293) mm long; size of bulb 98–138 (116) × 143–180 (160) mm. Nerve-ring and excretory pore 159–203 (180) mm and 295–343 (310) mm from cephalic cap, respectively. Posterior end of body distinctly curved ventrally. Spicules ventrally bent, similar and subequal in length, distal end somewhat blunt (Figs. 1b, 3c, g), surface of spicules ornamented with randomly scattered punctations, extending through most of its length and disappearing near the tip (Fig. 1b, h); right spicule 388–550 (451) mm long, representing between 6.28% and 11.7% (8.12%) of body length; left spicule 363–525 (419) mm long, representing between 5.65% and 9.96% (7.53%) of body length. Gubernaculum absent. Caudal papillae 11 pairs: 4 pairs of precloacal (1st–3rd pairs ventro-lateral, 4th pair ventral) (Fig. 1b, c); 7 pairs of postcloacal papillae (5 pairs ventro-lateral, 2 pairs lateral) (Fig. 1b, d, e). Single medio-ventral, pre-cloacal papillae present (Fig. 1b, e, f). Tail 125–205 (178) mm long, with short or long finger-like tip, representing between 2.44% and 4.28% (3.06%) of body length (Figs. 1b, e, 3c, h). Phasmids very small, between two postcloacal lateral papillae (Fig. 1e).

Female (based on 17 mature specimens)
Body 6.00–9.60 (7.51) mm long; maximum width 304–629 (472) mm. Esophagus 403–623 (522) mm long, representing between 5.57% and 9.74% (7.36%) of body length; corpus 295–363 (326) mm long; size of bulb 100–148 (131) × 145–210 (177) mm. Nerve-ring and excretory...
pore 155–246 (196) mm and 275–367 (329) mm from cephalic cap, respectively. Vulva slit-like, post-equatorial, anterior vulval lip with remarkable flap (Fig. 2d, e, 3b, d), between 3.49 and 5.93 (4.44) mm from cephalic cap, representing between 53.2% and 76.0% (59.7%) of body length. Type II genital tract according to Adamson [9], consisting of a muscular, anteriorly directed, thick-walled vagina (Fig. 3b, d). Ovaries reflexed, didelphic amphidelphic. Eggs oval, thick-shelled, with smooth surface, unembryonated, 60–113 (90) × 58–75 (64) mm (n = 17) (Figs. 2f, 3b, d). Tail 163–248 (203) mm long, with polymorphic tip, representing between 2.09% and 3.87% (2.69%) of body length (Figs. 2c, 3d–g). Phasmids very small, at about posterior 1/3 of tail (Fig. 2c).

**Molecular characterization**

**Partial 18S region**

Ten 18S sequences, of *R. sinense* n. sp., 877 bp in length, were obtained, with no nucleotide polymorphism detected. There is only one 18S sequence (KX844642.1) of *R. naylae* available in GenBank. Pairwise comparison of the 18S sequences of *R. naylae* obtained herein with that available in GenBank indicated 100% similarity. In the superfamily Rhigonematoidea, 18S sequences are also available in GenBank for *Rhigonema ingens* (JX069475.1), *Rhigonema thysanophora* (EF180067.1), *Xystrognathus phrissus* (IX101957.1), *Ichthyocephaloides sumbatus* (IX101958.1), *Obainia* sp. (KU561101.1) and *Trachyglossoides* sp. (MW030192.1).
Pairwise comparison of the 18S sequences of *R. sinense* n. sp. with those available in GeneBank showed nucleotide divergence of 0.34% (*R. ingens*) to 6.74% (*Trachyglossoides* sp.).

**Partial 28S region**
Ten 28S sequences of *R. sinense* n. sp., 767 bp in length, were obtained, representing four different genotypes, which exhibited 0.13–0.26% nucleotide divergence. A limited number of 28S sequences of *R. naylæ* are available in GenBank (KX844643, MT988354.1–MT988371.1). Pairwise comparison of the 28S sequences of *R. sinense* n. sp. with those available in GenBank showed 0.40–0.53% nucleotide divergence. In the superfamily Rhigonematoidea, 28S sequences are also available in GenBank for *R. ingens* (JX131616.1).
thysanophora (MG195996.1), X. phrissus (JX155274.1), *I. sumbatus* (JX155273.1), Obainia sp. (KU561100.1) and *Trachyglossoides* sp. (MW030188.1). Pairwise comparison of the 28S sequences of *R. sinense* n. sp. with those available in Genbank showed nucleotide divergence of 3.31% (*R. ingens*) to 15.8% (*Trachyglossoides* sp.).

**Partial ITS region**

Nine ITS sequences of *R. sinense* n. sp., 1190–1191 bp in length, were obtained, representing eight different genotypes, which exhibited 0.084–0.76% nucleotide divergence. In the superfamily Rhigonematoidea, no species with ITS sequences are available in GenBank. Consequently, we sequenced the ITS region of *R. naylae* based on specimens collected from *P. tonominea* in Japan. Pairwise comparison of the ITS sequences of *R. sinense* n. sp. with that of *R. naylae* obtained in this study showed 1.57–2.69% nucleotide divergence.

**Partial cox1 region**

Ten cox1 sequences of *R. sinense* n. sp. were obtained, all 670 bp in length, representing six different genotypes, which exhibited 0.15–0.60% nucleotide divergence. In the

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Fig. 3 Photomicrographs of *Rhigonema sinense* n. sp. collected from *Spirobolus bungii* (Spirobolida: Spirobolidae) in China. a Anterior part of male, lateral view. b region of vulva, lateral view. c, d body of female, lateral view. e–g tail of female, lateral view. h posterior end of male, lateral view. ep, Excretory pore; gc, glandular cell; nr, nerve ring.
superfamily Rhigonematoidea, cox1 sequences are available in GenBank only for *R. thysanophora* (NC_024020.1) and *Ru. karukerae* (MF509850.1). Pairwise comparison of the cox1 sequences of *R. sinense* n. sp. with those of *R. thysanophora* and *Ru. karukerae* showed 0.15–0.44% nucleotide divergence. In the 676 bp in length, representing three different genotypes, (Figs. 4, 5). However, BI analyses based on *R. naylae* sp. and *R. plagiolarum* obtained in this study displayed 14.3–14.9% nucleotide divergence.

**Partial cox2 region**

Ten cox2 sequences of *R. sinense* n. sp. were obtained, all 676 bp in length, representing three different genotypes, which exhibited 0.15–0.44% nucleotide divergence. In the superfamily Rhigonematoidea, cox2 sequences are available in GenBank only for *R. thysanophora* (NC_024020.1) and *Ru. karukerae* (MF509850.1). Pairwise comparison of the cox2 sequences of *R. sinense* n. sp. with those of *R. thysanophora* and *Ru. karukerae* displayed > 30% nucleotide divergence for both species. In the present study, we also sequenced the cox2 region of *R. naylae* based on specimens collected from *P. tonominea* in Japan; three cox2 sequences of *R. naylae* were obtained, all 530 bp in length, with no nucleotide polymorphism detected. Pairwise comparison of the cox2 sequences of *R. sinense* n. sp. with those of *R. naylae* displayed 13.0–13.3% nucleotide divergence.

**Species delimitation**

All ASAP analyses based on the 28S, ITS, cox1 and cox2 sequences supported the species partition of *R. sinense* n. sp. and *R. naylae* (Figs. 4, 5). However, BI analyses based on the ITS and 28S sequences displayed *R. naylae* nested in samples of *R. sinense* n. sp. (Fig. 5). Our results of ASAP and BI analyses based on the cox1 and cox2 genes were concordant, which clearly showed that *R. sinense* n. sp. represents a separated species from *R. naylae* (Fig. 5). Moreover, ASAP analyses based on the 28S, ITS, cox1 and cox2 sequences showed no evidence that the different morphotypes of *R. sinense* n. sp. represent distinct genetic lineages (Fig. 4). The results of BI and ASAP analyses based on the 18S gene both showed that *R. sinense* n. sp. and *R. naylae* formed a single group (Fig. 5).

**Phylogenetic analyses**

Phylogenetic trees constructed based on the 18S + 28S sequence data using ML and BI methods were nearly identical in topology, with both supporting the representatives of Rhigonematomorpha divided into two large clades (clade I and clade II) (Fig. 6). In the ML tree, clade I included species of the genera *Rhigonema*, *Ichthyocephaloides*, *Xystognathus*, *Obainia* and *Trachyglossoides*, which represent the superfamily Rhigonematoidea. In clade I, the genera *Ichthyocephaloides* and *Xystognathus* of the family Ichthyocephalidae did not cluster together (species of *Ichthyocephaloides* showed a sister relationship with *Trachyglossoides* sp. + *Rhigonema thysanophora* + *Rhigonema* sp. 1181, and *X. phrissus* clustered together with *Obainia* sp. SVM2017). The genus *Rhigonema* was not monophyletic as its representatives were present in some different and far lineages (Fig. 6).

Clade II was formed by species of the genera *Ransomnema*, *Carnoya*, *Brumptaemilius*, *Insulanema*, *Cattiena* and *Heth*, which represents the superfamily Ransomnematoidea. The representatives of the family Carnoyiidae were divided into two separated branches with strong support in both the ML and BI trees (Fig. 6). Species of the genera *Brumptaemilius*, *Insulanema* and *Cattiena* clustered together, and the genus *Carnoya* was sister to representatives of the families Ransomnematidae and Hethidae with weak support (Fig. 6). However, in the BI tree, species of *Brumptaemilius*, *Insulanema* and *Cattiena* grouped together, forming three polyphyletic branches with species of *Carnoya* and the representatives of the families Ransomnematidae and Hethidae (Fig. 6). The family Ransomnematidae (*Ransomnema bravoae*) was sister to the Hethidae (*Heth* spp.), with strong support in both ML and BI trees.

**Discussion**

The genus *Rhigonema* Cobb, 1898 is the largest group in the superfamily Rhigonematoidea, including approximately 90 nominal species that are mainly parasitic in millipedes in Africa, Asia, Australia and South and North America [4, 13, 17, 54–57]. Among the congeners, *R. sinense* n. sp. has its anterior 1/4 of body covered by spine, four pairs of precloacal papillae and seven pairs of postcloacal papillae, spicules subequal and not exceeding 0.60 mm in length, a relatively short tail tip in both sexes and the type 2 genital tract in females. Based on these characteristics, *R. sinense* n. sp. resembles the following *Rhigonema* species: *R. disparovis* Van Waerebeke, 1991, *R. fecundum* Hunt, 2002, *R. ingens* Hunt, 1998, *R. longicorpus* (Rao, 1973), *R. naylae* Morffe & Hasegawa, 2017, *R. neyrae* Singh, 1955, *R. ornatum* Majumdar, 1967, *R. oxydesmi* Hunt, 2002, *R. rigonanae* Hunt, 1999, *R. rostrellum* Hunt, 2002, *R. seychellarum* Adamson, 1987, *R. spiridonovi* Hunt, 1999 and *R. trichopeplum* Hunt & Moore, 1995 [9, 11, 12, 17, 54, 56, 58–61].

*Rhigonema sinense* n. sp. differs from *R. fecundum*, *R. oxydesmi*, *R. rigonanae*, *R. rostrellum*, *R. spiridonovi* and *R. trichopeplum* by having markedly longer spicules
(0.36–0.55 mm in the former species vs 0.22–0.34 mm in the latter six species). With three separated bilobed lips, *R. seychellarum* can be easily distinguished from *R. sinense* n. sp. that has three lips fused together. The new species is also different from *R. disparovis*, *R. longicorpus*, *R. neyrae*, *R. naylae* and *R. ornatum* by having a remarkable anterior vulval flap in females (vs anterior vulvar flap absent in the latter five species).

*Rhigonema sinense* n. sp. is most similar to *R. ingens* in morphometry and morphology; however, males of *R. ingens* are slightly longer (7.00–7.50 mm vs 4.27–7.02 mm in the new species). Moreover, we found the presence of 3.31% nucleotide divergence in the partial 28S gene between *R. sinense* n. sp. and *R. ingens*, which supported our present specimens representing a separated species from *R. ingens*.

Although some previous taxonomical studies provided 18S and/or 28S genetic data for diagnosis of species [5, 17, 18, 62–64], the molecular identification of Rhigonematomorpha nematodes remains in its beginning phase. BI and ASAP analyses based on the cox1 and cox2 sequences both supported species partition of *R. sinense* n. sp. and *R. naylae*. However, the results of BI inference and ASAP analyses of *R. sinense* n. sp. and *R. naylae* showed that the 18S gene, with its slow evolutionary rate, is unsuitable for species delimitation of Rhigonematomorpha nematodes.

The ITS sequences of *R. sinense* n. sp. and *R. naylae* were also provided in the present study. This is the first characterization of the ITS region for Rhigonematomorpha nematodes. Although the ASAP analyses based on both the ITS and 28S data supported the species partition of *R. sinense* n. sp. and *R. naylae*, BI showed that *R. naylae* nested in samples of *R. sinense* n. sp. The results of the BI inference and ASAP analyses performed in the present study provide more convincing evidence that *R. sinense* n. sp. represents a separated species from *R. seychellarum*. 

Fig. 4 Assemble Species by Automatic Partitioning (ASAP) analyses of Rhigonema sinense n. sp. and *R. naylae* based on five different nuclear and mitochondrial genetic markers. Asterisk indicates the optimal result recommended by ASAP. cox1/2, Cytochrome c oxidase subunit ½; ITS, internal transcribed spacer; OG, out-group; 18S/28S, small/large ribosomal subunit
Moreover, none of the different morphotypes of \textit{R. sinense} n. sp. formed a monophyletic/separated group in the BI or ASAP analyses. There is no evidence that the different morphotypes of \textit{R. sinense} n. sp. represent distinct genetic lineages. We considered the striking morphological variability in the tail tip of different individuals of \textit{R. sinense} n. sp. as intraspecific variation.
Current knowledge of the molecular phylogeny of Rhigonematomorpha remains very limited. Although some previous molecular phylogenetic studies made some attempts to solve the evolutionary relationships of Rhigonematomorpha and its related taxa (i.e. Ascarido-morpha, Spiruromorpha and Oxyuridomorpha), as well as the systematic status of some families or genera in the Rhigonematomorpha [3, 5, 6, 15, 16, 22–24, 65], the basic molecular phylogenetic framework for the Rhigonematomorpha is far from complete. The phylogenetic results of the present study are largely congruent with the traditional classifications of the Rhigonematomorpha [1, 2, 4], which support the division of this taxon into two superfamilies Rhigonematoidea and Ransomnematoidea. Our results are also in agreement with those of a previous molecular phylogenetic study based on 18S + 28S sequence data [3], but conflict with some molecular phylogenies using single 18S or 28S sequence data [5–7, 19].

The phylogenetic analyses performed in the present study indicate that the family Ichtyocephalidae and the genus Rhigonema in Rhigonematoidea are not monophyletic, which is consistent with the findings of previous studies [3, 5–7]. It is surprising that R. thy-sanophora + Rhigonema sp. were closely related with the family Xustromatidae (Trachyglossoides sp.), since species of Rhigonema, for example R. thy-sanophora, have a very different morphology of cephalic end and esophagus when compared with members of the Xustromatidae [66, 67]. The evolutionary relationships of the three families Rhigonematidae, Ichtyocephalidae and Xustromatidae in the Rhigonematoidea remain unsolved.

In the superfamily Ransomnematoidea, a previous study showed that the Ransomnematidae has a sister relationship with the Hethidae with weak support [3]. However, our phylogenetic results support the Ransomnematidae as having a sister relationship with the Hethidae with strong support in both the ML and BI trees. According to Poinar [68], the Carnoyidae includes only Carnoya and Rondonema. Subsequently, the genera Brumptaemilius, Clementeia, Raonema, Urucuia and Waerebekeia were transferred into the Carnoyidae [4]. Recently, two newly erected genera, Insulanema and Cat-tiena, were placed into the Carnoyidae [5, 69]. However, the phylogenetic results showed that the Carnoyidae,
with representatives of Carnoya, Brumptaemilius, Insulanema and Cattiena, is not a monophyletic group. We strongly support the resurrection of the family Brumptaemiliidae for Brumptaemilius, Insulanema and Cattiena. A more rigorous molecular phylogenetic study that includes broader representatives of the Rhigonematormpha using more nuclear and mitochondrial sequence data is need to further ascertain the phylogenetic relationships of different families.

Conclusions
A new species of Rhigonematormpha, \textit{R. sinense} n. sp., is described based on specimens collected from \textit{S. bungii} in China. ASAP analyses using 28S, ITS, \textit{cox}1 and \textit{cox}2 data support the species partition of \textit{R. sinense} n. sp. and \textit{R. naylae}, and also indicate the striking variability in tail morphology of \textit{R. sinense} n. sp. as intraspecific variation, in turn suggesting that the partial 28S, ITS, \textit{cox}1 and \textit{cox}2 regions are effective for molecular identification of Rhigonematormpha nematodes. Moreover, the molecular phylogenetic results of our study support the traditional classification of the infraorder Rhigonematormpha divided into two superfamilies, Rhigonematoidae and Ransomnematoidae, and also show that the families Carnoyidae, Ichthyocephalidae and the genus Rhigonema are non-monophyletic. The phylogeny reported here suggests that the Ransomnematidae is sister to the Hethiidae, and that the family Brumptaemiliidae should be resurrected. However, the evolutionary relationships of three families within Rhigonematoidae, namely Rhigonematidae, Ichthyocephalidae and Xustromatidae, remain unresolved.

Availability of data and materials
The nuclear and mitochondrial DNA sequences of \textit{Rhigonema sinense} n. sp. and \textit{R. naylae} obtained in the present study were deposited in GenBank database (sequences of \textit{R. sinense} under the accession numbers: ON936087, ON936088, ON936095, ON937754, ON938171–ON938174, ON938178, ON938182 (18S), ON936077–ON936086 (28S), ON936104–ON936112 (ITS), OP159049, OP103756–OP103758, ON935601, ON935613, ON935729, ON935732, ON935744, ON935751 (\textit{cox}1) and OP157153–OP157162 (\textit{cox}2); sequences of \textit{R. naylae} under the accession numbers: OP137258–137264 (ITS), OP113818–113820 (\textit{cox}1), OP162531-162532 (\textit{cox}2). Type specimens of \textit{R. sinense} n. sp. were deposited in the College of Life Sciences, Hebei Normal University, Hebei Province, China (under the accession numbers HBNU-N-2022Ar008Z-L, HBNU-N-2022Ar009Z-L and HBNU-N-2022Ar010Z-L).

Declarations
Ethics approval and consent to participate
This study was conducted under the protocol of Hebei Normal University. All applicable national and international guidelines for the protection and use of animals were followed.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Abbreviations
ASAP: Assemble Species by Automatic Partitioning; BI: Bayesian inference; BIC: Bayesian information criterion; \textit{cox}1: Cytochrome \textit{c} oxidase subunit 1; \textit{cox}2: Cytochrome \textit{c} oxidase subunit 2; ITS: Internal transcribed spacer; ML: Maximum likelihood; 18S: Small ribosomal subunit; 28S: Large ribosomal subunit; SEM: Scanning electron microscopy.

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Author contributions
YZ, LDW and LL contributed to the study design and identification of the nematode specimens. YZ, HK, SN and LL sequenced and analyzed genetic data. YZ and LL conducted the phylogenetic analyses and wrote the manuscript. LDW, LWL, HXC and LL collected specimens of \textit{R. sinense} n. sp. HK and SN collected specimens of \textit{R. naylae}. All authors read and approved the final manuscript.

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