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# *Heterorhabditis sonorensis* n. sp. (Nematoda: Heterorhabditidae), a natural pathogen of the seasonal cicada *Diceroprocta ornea* (Walker) (Homoptera: Cicadidae) in the Sonoran desert

S. Patricia Stock [a,*], Benjamin Rivera-Orduño [b], Yolanda Flores-Lara [a,b]

[a] Department of Entomology, University of Arizona, Tucson, AZ 85721-0036, USA
[b] Universidad de Sonora, Caborca, Sonora, Mexico

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ABSTRACT

A new *Heterorhabditis* species was isolated from nymphal stages of the seasonal cicada *Diceroprocta ornea* (Walker) in an asparagus field in the state of Sonora, Mexico. Concomitantly, another isolate of the same nematode species was also collected from an oak woodland habitat in the Chiricahua mountain range in southeastern Arizona. Morphological and molecular studies together with cross-hybridization tests indicate these two isolates are conspecific and represent a new undescribed *Heterorhabditis* sp. This new species is distinguished from other species in this genus by a combination of several qualitative and quantitative morphological traits. Key diagnostic features include: presence of a pronounced post-anal swelling in the hermaphrodite; male with nine pairs of bursal rays, with pairs 4 and 7 bent outwards and one pair of papillae placed on the cloacal opening, value of *D*% (average: 79); infective juveniles with a well developed cuticular tooth, long tail (average: 105 μm) and values of *D*% (average: 90) and *E*% (average: 99). In addition to these diagnostic characters, cross-hybridization tests between the new species with *H. bacteriophora* and *H. mexicana* yielded no fertile progeny. Comparison of ITS rDNA sequences with other available sequences of described species depicted the two isolates as a new species. Phylogenetic analysis of these sequence data placed *H. sonorensis* n. sp. as a member of the *indica*-group.


## 1. Introduction

The sate of Sonora, Mexico is one of the most important asparagus (*Asparagus officinalis*) growing areas in this country (López, 2002; Navarro, 2002). At present, several insect pests and plant pathogens such as white grubs, *Phyllophaga vetulla* (Coleoptera: Scarabaeidae), seasonal cicadas *Diceroprocta ornea* (Walker) and *D. semicincta* Davis (Homoptera: Cicadidae), root-knot nematode, *Meloidogyne incognita* (Kofoid and White) (Nematoda: Meloidogynidae) and, the plant pathogenic fungus *Fusarium oxysporum* f.sp. asparagi Cohen, 1946 (Mycota: Pyrenomycetes) have been reported to affect this perennial crop. Of all the above mentioned pests and pathogens, damage caused by *Diceroprocta* spp. nymphs is considered to be the most detrimental to asparagus, since wounds caused on the crowns and roots by these insects become portals of entry of plant pathogens and parasites (Espinoza, 1997; Navarro, 2002).

The increasing demand for organic and pesticide-free asparagus produce has put pressure to growers in this region for non-chemical alternatives for control of insect pests of this crop. In this respect, a survey for native entomopathogens was initiated in the spring of 2003. As a result, an entomopathogenic nematode, *Heterorhabditis* sp. was recovered from infested fourth-instar nymphs of *D. ornea*. Only 5% of the collected cicadas (total 250 nymphs) in the field were infected with this entomopathogenic nematode (Rivera-Orduño pers. comm.) Examination of morphological traits together with DNA sequence analysis (ITS rDNA sequences) and cross-hybridization tests indicated this species is a new and undescribed member of *Heterorhabditis*. Herein, we described this nematode and assess its evolutionary relationships with other members of this genus. This new species represents the fourth *Heterorhabditis* species reported in Mexico. Other previously encountered *Heterorhabditis* species in this country are: *H. bacteriophora* Poinar, *H. indica* Poinar, Karunakar and David and, *H. mexicana* Nguyen, Gozel, Koppenhöfer and Adams.

## 2. Materials and methods

### 2.1. Nematode isolation

*Heterorhabditis* sp. (Caborca strain) was isolated from nymphs of the seasonal cicada *Dicerorpocta ornea* (Homoptera: Cicadidiae) in an asparagus field in Caborca (Sonora, Mexico) (latitude N

* Corresponding author. Fax: +1 520 621 1150.
*E-mail address:* spstock@ag.arizona.edu (S.P. Stock).

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30°41′50″, longitude 112°09′29″). Parasitized insects had the typical 'burgundy' coloration of *Heterorhabditis*-infected hosts. Cadavers recovered in the field were rinsed in distilled water and placed in a modified White trap according to procedures described by Kaya and Stock (1997). Attempts to use *D. ornea* for *in vivo* rearing of the nematode failed, as nymphs of this insect are extremely sensitive to soil moisture changes and died either in transit to the laboratory or soon thereafter. Consequently, *in vivo* multiplication of the new *Heterorhabditis* species was done with last instar larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) to confirm Koch's postulates for pathogenicity and to establish cultures in the laboratory. Procedures followed Kaya and Stock (1997).

In this study we also considered another *Heterorhaditis* isolate (CH-35), collected from oak-juniper woodlands in the Chiricahua mountain range in Arizona, USA, which based on comparison of ITS rDNA sequences, it was depicted as conspecific to the new species. Details of the collection and isolation of CH-35 strain were provided by Stock and Gress (2006).

### 2.2. Morphological characterization

For morphological studies, first generation hermaphrodites and second generation adults were obtained by dissecting infested cadavers on days 4–5 and 7–8 after initial infection. Third-stage infective juveniles were obtained upon emergence and during the first two days. A total of 20 specimens from each life stage were considered for evaluation of qualitative and quantitative data. Nematodes were examined live and/or heat-killed in 60 °C Ringer's solution. Heat-killed nematodes were placed in triethanolamine-formalin (TAF) fixative (Kaya and Stock, 1997) and processed to anhydrous glycerine for mounting (Seinhorst, 1959). Measurements and digital light microscopy images were made from live and mounted specimens using an Olympus IX51 microscope equipped with differential interference contrast optics and a digital video image system. Specimen measurements were made using AnalySIS Image software (Soft Imaging System Corp. CA, USA).

The following abbreviations for morphological features are used in the text or tables: ABW = anal body width, *D*% = EP/ES × 100, *E*% = EP/TL × 100, EP = distance from anterior end to excretory pore, ES = distance from anterior end to esophagus base, GL = gonad length, GS = GuL/SpL, GuL = gubernaculum length, *H* = length of hyaline portion of the tail; *H*% = *H*/TL × 100, MBW = maximum body width, NR = nerve ring position, StL = stoma length, StW = stoma width, SpL = spicule length, SW = SpL/WA, TBL = total body length, TL = tail length. TRF = testis reflexion, *V*% = TBL/length to vagina × 100, WA = width at anus/cloaca.

For electron microscopy observations, adult males and third-stage infective juveniles were rinsed three times in M9 buffer at five minutes per rinse. All nematodes were relaxed and heat-killed as above, then fixed in 8% glutaraldehyde buffered in cacodylate at pH 7.33 overnight. Fixed nematodes were rinsed in distilled water three times, post-fixed in $OsO_4$ for 1 h, rinsed again in distilled water and serially dehydrated at 15 min intervals in ethanol (McClure and Stowel, 1978). Specimens were then critical point dried in liquid $CO_2$, mounted on SEM stubs, coated twice with gold and scanned using a Philips XL series microscope at 20 kV accelerating voltage.

### 2.3. Molecular characterization

Nucleic acid preparations used for PCR amplifications of the new *Heterorhabditis* sp. were extracted from pools of 10–50 third-stage infective juveniles. Nucleic acids were extracted from the digestion supernatant using phenol–chloroform enrichment, ethanol/ammonium acetate precipitation (Ausbel, 1989). The resulting pellet was washed with 70% ethanol, resuspended in TE buffer (pH 8.0), treated with 50 μg of RNAse A (1 h at 37 °C), and DNA recovered following reprecipitation with ethanol. DNA was quantified by spectrophotometry, and 100–200 ng used per PCR reaction.

PCR was used to amplify a region within the 5′-end of the nuclear internal transcribed spacer (ITS) region. Typical PCR reactions included 0.5 μM of each primer, 200 μM deoxynucleoside triphosphates, and $MgCl_2$ concentration of 2 mM in a total reaction volume of 25 μl. PCR conditions, e.g., annealing temperature and $MgCl_2$ concentration, were adjusted empirically as needed to optimize reaction specificity for individual isolates. Typical conditions included denaturation at 94 °C for 3 min, followed by 33 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1.15 min, followed by a post-amplification extension at 72 °C for 5 min.

PCR primers considered were 93, forward (5′-TTAGTT TCTTTTCCTCCGCT), and 94, reverse (5′-TTGAACCGGGTAAAAGTCG) as described by Stock et al. (2001). One microlitre of each PCR product was used for agarose gel electrophoresis (1.3% agarose in 1X TBE buffer) to confirm amplicon size and yield. PCR products were prepared for direct sequencing using enzymatic treatment with EXOSAP-IT (product info). Sequencing reactions were performed using dye-terminator cycle sequencing chemistry and reaction products were separated and detected using an ABI 3730 automated DNA Sequencer. Sequences for each species were completely double-stranded for accuracy using the PCR primers and two internal sequencing primers. The forward internal primer was #264 (5′-GGTTTTTCATCGATACGCG), and the reverse internal primer #389 (5′-TGCAGACGCTTAGAGTGGTG).

### 2.4. Sequence alignment and phylogenetic analysis

Contig assembly and sequence conflict resolution were performed with the aid of EditSeq (DNAStar software, Lasergene Corp., Madison, WI, USA). Sequence regions corresponding to the PCR amplification primers were removed prior to multiple sequence alignment and phylogenetic analysis, because primer incorporation during amplification masks potential mismatches (substitutions) that may occur in PCR priming sites. Sequences were aligned initially using CLUSTAL X v1.53b (Thompson et al., 1997), and the resulting output was adjusted manually to improve homology statements.

ITS sequences of *H. sonorensis* n. sp. were deposited in the GenBank under accession numbers FJ477730 and FJ477731.

Sequence data were analyzed by unweighted maximum parsimony (MP) using PAUP* v 4.0b3a (Swofford, 2002). Unrecoded gaps were treated as missing data. Tree searches for these ITS datasets were performed using heuristic methods with TBR (tree-bisection-reconnection) branch-swapping, and a minimum of 1000 replicates of random stepwise addition. Reported consistency indices (C.I.) do not include uninformative characters. Bootstrap parsimony analyses were performed using heuristic searches (simple stepwise addition, TBR branch-swapping, MULPARS) and 2000 pseudoreplicates.

### 2.5. Cross-hybridisation tests

Cross-breeding tests were carried out on lipid agar plates according to (Dix et al., 1992). Isolates Caborca and CH35 of the new *Heterorhabditis* species were crossed with each other and with *H. bacteriophora* (isolate NC1) and *H. mexicana* (isolate MX4). One female and one male of the appropriate strains were placed on the agar plates (35 mm diam.) and incubated at 25 °C. Controls consisted of one males and one female of the same isolate. There were 10 plates per isolate tested. Progeny production was checked daily and for a period of three consecutive days according to Stock and Kaya (1996).