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Morphological, Molecular and Ecological Characterization of a Native Isolate of *Steinernema Feltiae* (Rhabditida: Steinernematidae) From Southern Chile

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Abstract

Background: *Steinernema feltiae* is an entomopathogenic nematode used in biological control programs with a global distribution. It has been shown that populations of *S. feltiae* may have phenotypic plasticity derived from local adaptation and may vary in different traits, such as location and penetration of the host. This is the first time that a Chilean isolate has been described in detail, taking into account morphological, molecular and ecological characteristics.

Methods: *S. fealtiae* was detected a few years ago in southern regions of Chile in the town Lican Ray and was morphologically and morphometrically described, along with a molecular description based on the ITS and D2-D3 regions. Some ecological characteristics were determined, including the temperature requirements for completion of its life cycle and the effect of three water levels in soil for optimal reproduction.

Results: Morphometric characteristics of different life stages show large intraspecific variability in averages and ranks compared with isolates from different geographical origins. The molecular data also show intraspecific variability with respect to other isolates. The lower, optimal and higher temperatures found to limit the infestation and reproduction of *Galleria mellonella* were 10, 20 and 30 °C respectively, and emergence from the host larvae occurred approximately ten days after inoculation. Differences were observed in offspring emerging from the host, and the 120 infective juveniles (IJ)/larvae dose was the most prolific at 20 °C. The water content of the soil did not affect the number of infective juvenile invaders, penetration efficacy (%), and time of emergence of the IJ or offspring per larvae, but it caused a delay in achieving full mortality at the permanent wilting point with respect to saturation and field capacity.

Conclusions: Data obtained in this study are valuable since they outline some environmental requirements of this strain to perform optimally in the event of being used as a soil pest bioantagonist.

1. Background

Entomopathogenic nematodes (EPNs) are lethal insect parasites that belong to the families Steinernematidae and Heterorhabditidae and are associated with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively. The search for new alternatives for pest management has promoted new surveys and research in the area of biological control. The potential of EPNs for the control of insects continues to be one of the most studied alternatives, as reflected, for example, in the rate of new descriptions (1). This surge in interest stems from the need to evaluate more intensively their potential to replace some chemical insecticides for the control of soil borne pests. These nematodes appear to be good insect control agents considering their easy mass production, wide host range (2) and relative safety with respect to nontarget organisms and the environment (3). On the other hand, antimicrobial and insecticidal compounds, particularly from bacterial symbiotes have also received special attention (4, 5).

A better understanding of the biology and ecology of EPN species can improve the effectiveness of biological control. It has been shown that populations of the same species from different locations may have phenotypic plasticity derived from local adaptation and may vary in different traits, such as location and penetration of the host (6). These behaviors are influenced by abiotic and biotic factors as well as by intrinsic nematode characteristics (7). Knowledge of key intrinsic and extrinsic factors affecting the infection process can improve the management of EPNs for biological control (8).

The search for and study of EPNs in Chile is a relatively new discipline compared with others, such as, for example, the incidence of plant-parasitic nematodes in economically important crops. Chile is a country of extremes in the Americas, with a high diversity of ecosystems; Chile is isolated from other countries, surrounded by the Andes Mountains, the Pacific Ocean, the Atacama Desert and the Antarctic, and has a length of 4,329 km. All of this suggests a rich fauna with EPNs adapted to different conditions (9). A previous survey detected several EPNs, including three new species: *Heterorhabditis atacamensis* (10), *Steinernema unicornum* (11) and *S. australe* (12). The presence of *S. feltiae* was also reported from several locations in the southern zone, with 39 isolates associated with different habitats including grassland, coastal sites and a blueberry plantation, although most isolates were from natural forests or woodland (9). Those *S. feltiae* isolates were identified by molecular assessment of the internal transcribed spacer (ITS) rDNA region.

During a posterior survey, another isolate of *S. feltiae* was found in the southern zone of the country; however, there is no complete description of any isolate of this species from Chile, including most of its environmental requirements. For this reason, the aim of this study was to characterize the morphological and molecular characteristics of this new isolate, isolate its symbiotic bacteria and assess some of its environmental requirements under different soil and climate conditions.

2. Material And Methods

2.1 Nematode isolate and symbiotic bacterium culture

The *S. feltiae* isolate Lican Ray (LR) was recovered from soil obtained from an oak forest near the city Lican Ray (39º 28´12´´S; 72º 7´12´´W) by baiting the soil with late instar waxmoth larvae, *Galleria mellonella,* in glass flasks with volumes of 500 cm^3 (13). The flasks were closed and kept at 20 °C for 96 h, after which dead larvae were moved to modified White traps (14), and emerging infective juveniles (IJ) were removed in a drop of water and used to infect fresh waxmoth larvae to increase the population. Emergent IJ were then stored in tap water at 10 °C.

To retrieve symbiotic bacteria, a pool of IJ was surface-sterilized in 2% NaClO for 3 min, washed thoroughly with sterile water and crushed to release the bacteria. One aliquot of the homogenate was streaked onto plates with nutrient agar supplemented with 0.004% (w/v) triphenyltetrazolium chloride and 0.025% (w/v) bromothymol blue, pH 7 (NBTA plates) (15, 16). On this solid culture medium, *Xenorhabdus* colonies exhibit a typical green blue color, which allows them to be distinguished from

potential contaminants (17). After 48 h of incubation at 28 °C, colonies corresponding to the symbiotic phenotype were isolated and preserved at -80 °C in nutrient broth supplemented with 20% glycerol.

2.2 Morphological and morphometric studies

First and second adult generations and IJ were collected at random from infected insect larvae (14). Males and females were collected on the fourth and eighth day after inoculation of *G. mellonella* for the first and second generations, respectively, while IJ were collected within two days after emergence. For descriptive purposes, 25 specimens for each stage were fixed in TAF and processed to glycerin by Seinhorst´s rapid method (18). Morphological and morphometric parameters suggested by Hominick *et al*. (19) were analyzed using an Axiocam MRC in a Zeiss Axioimager A.1 light microscope.

For scanning electron microscopy, adults were obtained from dead *G. mellonella* larvae and washed three times in buffer M9, the same as the IJ recovered from White traps. All nematodes were relaxed in 60 °C water, fixed in 8% glutaraldehyde and mounted according to the methodology of Koppenhöfer and Stock (20). Scanning was performed using a Philips XL microscope with an SES DS-130 at 20 kV accelerating voltage.

2.3 Molecular characterization

2.3.1 Nematode isolate

DNA was extracted from single females using the extraction method of Williams *et al*. (21). Single nematodes were collected in PCR tubes with WLB buffer containing 10 mg/ml proteinase K and frozen for at least 10 min at –80° C. The tubes were quickly placed in a water bath at 65° C and then incubated at this temperature for 90 min to allow digestion by proteinase K. Finally, proteinase K was inactivated by heating to 95° C for 15 min, and the tubes were centrifuged to separate the supernatant.

PCR was performed to amplify the large ribosomal subunit (LSU) 28S rDNA using forward primer no. 391 (5´-AGCGGAGGAAAAGAAACTAA-3´) (22) and D3B (5´-TCGGAAGGAACCAGCTACTA-3´) reverse primer (23). One fragment of rDNA that includes the internal transcribed spacer ITS-1, the 5.8S subunit and ITS-2 was PCR amplified using the primer pair 93 (5´-TTGAACCGGGTAAAAGTCG-3´) and 94 (5´-TTAGTTTCTTTTCCTCCGCT-3´) (24). In both PCRs, a volume of 2 µl DNA was used as template in a 50 µl reaction mix that contained 0.5 µM of each primer, 200 µM dNTP and 1 unit of Taq DNA Polymerase Recombinant (Invitrogen) along with 1.5 mM $MgCl_2$ final concentration. Amplifications were performed in a BIOER-LifePro Thermal Cycler. To amplify the LSU fragment, the PCR mix was denatured at 94° C for 3 min, followed by 33 cycles of 94° C for 30 sec, 52° C for 30 sec and 72° C for 1 min, and a final extension of 7 min at 72° C. A similar PCR program was used for the ITS region, adjusting the annealing temperature to 60° C. The amplified fragments were separated by electrophoresis on 1% agarose (w/v) gels using 1X TBE buffer at 100 V for 1 h, then purified using an E.Z.N.A. Gel Extraction Kit (OMEGA Bio-tek). PCR products were sequenced (Macrogen, USA) using internal primers. Forward 502 (5´-CAAGTACCGTGAGGGAAAGTTGC-3´) and reverse 503 (5´-CCTTGGTCCGTGTTTCAAGACG-3´) primers

were used for 28S; forward 533 (5´-CAAGTCTTATCGGTGGATCAC-3´) and reverse 534 (5´-GCAATTCACGCCAAATAACGG-3´) were used for ITS fragment (25).

2.3.2 Symbiotic bacteria

DNA was extracted from 1.5 ml of overnight Miller's LB Broth (10 g/L Tryptone, 10 g/L NaCl, 5 g/L Yeast Extract) culture, using a GenElute Bacterial Genomic DNA kit (Sigma, Sigma-Aldrich). Universal primers that amplify nearly the full-length 16S rDNA from many bacterial genera were used: 27f (5'-AGAGTTTGATCATGGCTCAG-3') and 1492r (5'-TACGGTTACCTTGTTACGACTT-3') (26). The reaction was carried out in a final volume of 30 µl containing 1 µl DNA, 1 µM of each primer, 200 µM dNTP and 1 unit Taq DNA Polymerase Recombinant (Invitrogen). PCR parameters consisted of an initial denaturation at 94° C for 3 min, 35 cycles of 94° C for 50 s, annealing at 58° C for 50 s and 72° C extension for 50 s followed by a final extension at 72° C for 7 min. PCR products were visualized, purified and sequenced as previously mentioned.

2.3.3 Phylogenetic analysis

The DNA sequences of the isolate Lican Ray were compared with those present in GenBank by means of the basic local alignment search tool (BLAST) of the National Center for Biotechnology Information (NCBI). The 28S and ITS sequences and corresponding reference nucleotide sequences of *Steinernema* spp., including "feltiae group", available in GenBank were aligned with the default parameters of Clustal W (27). The alignments were manually edited using BioEdit (28). The 16S rRNA sequence of the symbiotic bacteria was aligned to corresponding sequences of *Xenorhabdus* spp.

Phylogenetic relationships were determined by using maximum likelihood (ML) based on the Tamura-Nei model (29) via the program Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA 6) (30). Trees were constructed by clustering of associated taxa based on 1,000 replicates in a bootstrap test. The newly obtained sequences were submitted to the NCBI GenBank database under accession numbers indicated in bold on the phylogenetic trees. The dataset was also analyzed using Bayesian inference (BI) with MrBayes 3.1.2 (31). The best fitted model of DNA evolution was obtained using jModelTest 0.1.1 (32) with the Akaike information criterion. The GTR+G model (ITS and 28S) and GTR+G+I models (16S) were selected. Two independent runs were performed simultaneously on the data, each one using one cold and three heated chains. After 5 million generations, the average standard deviation of split frequencies between the two independent runs at completion were 0.005 (ITS and 16S) and 0.006 (28S). After discarding 25% of burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate nodes. Trees were visualized using TreeView (33). The newly obtained sequences were submitted to the NCBI GenBank database under the accession numbers indicated in bold on the phylogenetic trees.

2.4 Determination of some optimal parameters

The effects of temperature, lethal concentration and different water levels in the substrate on the mortality of *G. mellonella* larvae, penetration rate and reproduction of the nematode were determined. These data were useful to determine the main characteristics of the life cycle under optimal conditions.

2.4.1 Temperature

The optimal temperature for insect mortality (days after inoculation, DAI), penetration rate, time to emerge from the insect cadaver (emergence days) and offspring production was determined. One hundred IJ were applied in 0.2 mL of water per *G. mellonella* larvae in Petri dishes (3.5 cm diam) with a filter paper on the bottom. They were covered with a plastic bag to maintain humidity and stored in an incubator at different temperatures (5, 10, 15, 20, 25 and 30 ºC). Each treatment had five replicates, each consisting of a group of four plates, distributed in a random design. Insects were checked for five days to determine mortality. The level of IJ invaders was measured by dissecting cadaver one day after death and counting the number of specimens inside. Offspring were determined by counting the total number of emerged IJ from dead larvae in a modified White trap. Nematodes were recovered and stored at 10 ºC in Falcon tubes every day until no more were detected.

2.4.2 Lethal concentration

The effect of the different inoculum densities was determined for the same parameters as assessed previously as well as the penetration efficacy (%); this effect was evaluated by applying 0.2 mL of water containing 0, 10, 20, 40, 80 and 240 IJ per Petri dish containing one *G. mellonella* larvae, thus employing the same conditions used previously. Each treatment had five replicates, with each replicate consisting of a group of four plates, and the replicates were distributed in a random design. Plates were stored as indicated above at 20 °C, the optimal temperature determined by the previous assay. Penetration efficacy (%) was calculated according to the formula of Kaya and Stock (14), which relates number of nematodes recovered from dissection to those inoculated. Recovered nematodes were maintained as indicated above.

2.4.3 Water content

Substrates containing three water levels were assessed to determine IJ movement at the permanent wilting point, field capacity and saturation. The substrate consisted of a steamed mixture of uniform proportions of sand, agricultural soil and organic matter, with a content of clay, silt and sand of 13.2, 22.9 and 63.9%, respectively. The texture was sandy loam. Petri dishes (3.5 cm diam, with a filter paper) containing 4.5 g of the different substrates were inoculated with 120 IJ/0.2 mL of water. This nematode density was selected according to the previous assay. After 30 min, one *G. mellonella* larva was placed in each petri dish, kept in the conditions indicated previously and incubated at 20 °C. Each treatment had five replicates, each consisting of a group of four plates, distributed in a random design. The same parameters determined before were used to evaluate water content.

The three experiments were repeated twice under the same conditions.

2.5 Data analysis

For all the assays, mortality was corrected according to Abbot´s formula (34). The control percentage data was arcsine transformed (angular transformation), and normality and variance homogeneity were verified prior to performing an ANOVA using the program Minitab V 15. The other variables were analyzed with no transformations. A Tukey test was performed in case the ANOVA showed significant differences at $p<0.05$.

To determine lethal concentration (LC), mortality at 48 h was considered. Data were analyzed using a probit test using the Probit Program V 1.5, calculating the CL_{50} and CL_{90}.

3. Results

3.1 Morphological and morphometric studies (Table 1, Figs. 1-3)

Male, first generation. Body C- or J-shaped posteriorly when heat-killed (Fig. 1A). Cuticle with fine, annular striation under SEM but smooth under light microscope; lateral fields and phasmids inconspicuous. Anterior end slightly rounded, continuous with the body. Six prominent lips, each lip bearing a labial papilla. Four cephalic papillae, also notorious (Fig. 3 B, D). Small amphidial opening, behind to lateral lip papillae. Deirids conspicuous, located in the first third, after than excretory pore. Stoma short and wide, inconspicuous sclerotized walls. Esophagous muscular with cylindrical procorpus, metacorpus slightly swollen, isthmus fairly notorious, ending in a pyriform basal bulb. Nerve ring surrounding the isthmus or the anterior end of basal bulb. Excretory pore anterior to the nerve ring, around first third of the isthmus (Fig. 1C). Simple testis, reflexed. Vas deferens with inconspicuous walls. Spicules paired, symmetrical, curved, ocher brown color (Fig. 1G); head (manubrium) oblong, shaft (calomus) notorious, velum present. Gubernaculum curved, approximately 2/3 of spicule's length; boat-shaped in lateral view, anterior end curved ventrally (Fig. 1G); in ventral view, corpus with two projections. Tail conoid, tail terminus with a mucron (Fig. 1E). One single, midventral, precloacal papilla, and 11 pairs of papillae. Six pairs are precloacal, subventral, one pair lateral precloacal, one pair adanal, two pairs subterminal subventral, and one pair post cloacal, lateral (Fig. 1F).

Male, second generation. Similar to the first-generation male, but more slender and smaller in body length and other morphometric characters. Deirids not observed. Mucron on tail terminus present and longer than that in the first generation.

Females, first generation. Body robust, habitus C-shaped (Fig. 2A). Cuticle, lips, stoma and esophageal region as in males. Excretory pore at mid of metacorpus (Fig. 2B). Reproductive system didelphic-amphidelphic, ovary reflexed dorsally. Vulva a transverse slit at midbody region, protuberant, with a double epiptygma (Fig. 2D). Vagina short, leading into paired uteri. Tail conoid with ventral postanal swelling (Fig. 2E). Mucron absent.

Females, second generation. Similar to first generation females (Fig. 2C), but smaller in size. Vulva located slightly back compared to the first generation females; symmetric and protuberant lips with a double epiptygma. Relation excretory pore/tail length bigger than in the first generation. Tail conoid, with a slight postanal swelling (Fig. 2F).

Third juvenile stage

Body slender, habitus straight. Cuticle with fine transverse striae. Head continuous with body contour, slightly truncate (Fig. 3A), not annulated. Labial papillae not observed, amphidial opening like a pore at the level of four distinct cephalic papillae (Fig. 3B). Oral aperture and anus closed. Lateral fields with 8 notorious ridges at midbody region (Fig. 3C). Long esophagous, narrow, procorpus slightly expanded, narrowing in isthmus and base bulb pyriform (Fig. 3A). Excretory pore at mid-esophagous level, isthmus surrounded by nerve ring. Deirids not observed. Cardia present. Small bacterium receptacle in the anterior part of intestine. Tail conoid, tapering gradually (Fig. 3D, E), hyaline portion equivalent to 36% of tail length (Fig. 3D).

3.2 Molecular characterization

For the ITS region a fragment of 859 bp was obtained for the Chilean *S. feltiae* isolate. This sequence was tested in BLAST with data deposited in GenBank, showing approximately 97-99% similarity with sequences of the same species. The majority-rule consensus tree of the Bayesian inference showed a well-supported group (100% bootstrap) that comprised the LR isolate (MK504438) and known sequences of *S. feltiae* from different countries, including one from Chillan, Chile (MK504439) sequenced in the present work as reference (Fig. 4). For 28S, a fragment of 894 bp was obtained (MK509752) showing 99% similarity with published sequences of *S. feltiae* and other species from the glaseri group. The phylogenetic relationships revealed a clade (100% bootstrap) that included sequences of *S. feltiae* from different geographical origins including Chillan, Chile (MK509780) and from *S. jollieti, S. puntauvense, S. litorale, S. ichnusae, S weiseri and S. silvaticum* (Fig. 5). Based on the BLAST search and phylogenetic analysis of 16S rDNA, the symbiotic bacterium of *S. feltiae* LR is *X. bovienii* (BLAST similarities 99%). The Bayesian inference showed that the sequence obtained (MK504451) formed a well-supported group with sequences of the same species deposited in the GenBank (Fig. 6). ML analysis produced trees with the same topology for all the genes considered.

3.3 Biological aspects

The life cycle of the isolate was similar to those described for other *Steinernema* species. The dauer larvae killed *G. mellonella* larvae between the first and second day after inoculation (25 °C). Male and females of the first generation were present on the third or fourth day. On the fifth day the first IJ were seen in the insect larvae. A second adult generation occurred between the seventh and eighth day. Massive exit of infective juveniles from insect body was observed on the tenth day.

3.4 Determination of some optimal parameters

3.4.1 Temperature

The effect on *G mellonella* larval mortality inoculated with IJ of *S. feltiae* LR are shown in Table 2. On the second day, most larvae stored at 20 °C or higher were immobile and starting to change color at 5 °C, no mortality was recorded, while at 10 °C, a few dead larvae were observed five DAI. Mortality increased dramatically with higher temperatures, reaching 100% at 15, 20 and 25 ºC third DAI. The last two temperatures seemed to be optimal for reaching the highest mortality in the shortest time, with a 90-97.5% of mortality on the second DAI. The highest temperature (30 °C) had an effect on mortality, reaching the maximum value on the fifth DAI.

The number of invader IJ per *G. mellonella* larvae at different temperatures is shown in Table 3. At low temperatures (5 and 10 °C), nematode presence was not detected by dissection. IJ penetration was higher at 15 and 20 °C (approximately 25% of JI inoculated) and significantly decreased as temperature increased. Females with eggs were observed on the second day after larval death at 15, 20 and 25 °C. The time for emergence of IJ from insect cadavers was optimal for medium temperatures (15 and 20 °C), coming out of the insect around the tenth DAI (Table 3). At lowest (5 and 10 °C) and highest (10 °C) temperatures neither IJ emergence nor offspring were seen. The greatest production occurred at 20 °C. The maximum IJ recovery was during the fifth DAI, and the emergence lasted for 30 days (data not shown). It was found that 20 °C was the optimal temperature for parasitism and reproduction of *S. feltiae* LR, so this temperature was used for the following assays.

3.4.2 Lethal Concentration

Results for mortality of insect larvae for 4 days after inoculation are presented in Table 4, showing that mortality increased as the inoculum and DAI increased. On the third day, 100% mortality was reached for all nematode densities; however, on the second day, over 80 IJ were enough to achieve maximum control. The CL_{50} and CL_{90} determined for the second DAI were 7.2 and 40.4 IJ/larvae, respectively.

The number of nematodes entering the host increased with density (Table 5); however, efficacy of penetration, ranging from 24% to 49.6%, did not show differences. Independent of IJ inoculum density, no differences were seen in the necessary time for new IJ to start to leave the insect cadaver, varying between 11 and 13.5 days. Differences were observed in the offspring emerging from the host, where doses of 120 IJ/larvae were the most prolific. This nematode concentration was used to investigate effects of soil water content.

3.4.3 Water content

The effect of water content on the mortality of *G. mellonella* over five days is shown in Table 6. On the second and third days, differences were observed between water levels; however, fourth day mortality was statistically the same for all treatments, thus indicating a delay in achieving full mortality (day 5). Water content did not affect the number of IJ invaders or penetration efficacy (Table 7). The time of emergence of the IJ and offspring per larva showed no differences between treatments.

4. Discussion

Despite the fact that *S. feltiae* had already been isolated from Chilean soils (9), this is the first time that an isolate has been described in detail, taking into account morphological, molecular and ecological characteristics. Morphometric characteristics from the different life stages are analyzed, showing great intraspecific variability in the averages and ranks between isolates from different geographical origins (35, 36, 37, 38, 39, 40, 41). In Chile, Edgington *et al*. (9) collected several *S. feltiae* isolates from 6 zones and based on ITS region, classified the isolates into two subgroups (I and II) based on the ITS region. Morphometric characters from D030 (subgroup I) showed smaller sizes than isolate D087 (subgroup II). The isolate Lican Ray was isolated from zone 4 and showed intermediate sizes for IJ.

Considering the high morphometrical variability of *S. feltiae*, molecular data are fairly useful (36); however, a marker such as the ITS region, which has proven useful in resolving phylogenetic relationships in EPN, has exhibited intraspecific and intraindividual variability for this species as well as others in the *feltiae* and *glaseri* group, (42). According to these authors, considering the frequency of intraindividual variability, sequencing of the D2-D3 region of 28S appears to be necessary to confirm species status. This region is too conserved among some species of the *feltiae* group, as was observed when analyzing the phylogenetic relationships in the present work, while other similar species such as *S. weiseri* and *S. puntauvense* were observed within the clade corresponding to *S. feltiae* with 99% genetic similarity. On the other hand, analysis of the 16S rRNA gene allowed for the identification of *X. bovienii*, the symbiotic bacterium of *S. feltiae* (6).

Based on climatic conditions from the place of origin, with median temperatures of 17.7 °C and 8 °C in summer and winter, respectively, *S. feltiae* LR could be an isolate more adapted to cold conditions; however, below 10 °C, the IJ were not able to infest *G. mellonella*, and 15–25 °C was the optimum temperature range for infestation and reproduction. This finding could mean that the specie's parasitic activities under natural conditions are higher in spring-summer. The optimal temperature ranges vary with the species and isolate. Studies performed by Umana (43) with seven isolates of *S. feltiae* obtained from Chile (9) showed that at 20 °C, *G. mellonella* death occurs at 48–72 h, one day later than with the LR isolate, and no differences were found among the seven isolates. At 20 °C, the life cycle was complete in 13–14 days, which was three to four days later than the isolate LR at the same temperature.

S. feltiae is a common species reported in many places in the world; this species is known to be adapted to cold, able to infect hosts in a temperature range between 8 and 28 °C and can produce offspring between 8 and 25 °C (44, 45, 46, 47).

Sáenz (48) considered that the mortality of *S. feltiae* is not dependent on the number of juveniles penetrating the host, but Fan *et al.* (49) have estimated that mortality increases with the rate, as occurred in this study. According to our results, the number of nematodes able to enter the host increased with the number of IJ inoculated, but the efficacy of penetration was similar; this observation was also reported by Fan *et al* (49) with *S. feltiae*, who obtained 100% mortality with a range of initial populations from 53 to 114 IJ per *G. mellonella* larvae. The penetration capacity was variable, and 20–50% of applied IJ

penetrated the host, based on several variables. According to Lewis *et al.* (7), there is a minimum number required to overcome the insect's defenses and a maximum due to a high competence between them. In our study, with 240 IJ per larvae the reproductive rate was minimum. It is also interesting that with 40.4 IJ / larvae, there was no increase in mortality on the second day after inoculation, thus showing that after a threshold, mortality does not increase. Koppenhöfer and Kaya (50) observed that an increase in density of *S. glaseri* in soil affected the penetration of and reproduction in *G. mellonella* larvae. They observed that the highest number of new J3 occurred between 20.7 and 58.0 IJ per larvae and that no reproduction was observed with over 184.4 specimens.

Moisture is other important soil factor for survival and infectivity of NEP since they need a water film around them for movement (51, 52, 53). The amount of water in soil may affect movement, penetration and other factors depending on the species and their physiological adaptations. The optimal ranges are variable across different species (52, 54). Infectivity and reproduction of *S. feltiae* LR were optimal when water content was near field capacity, similar to observations by Koppenhöfer and Fuzy (52) working with *S. scarabei* in sandy loam and loamy soils. Susurluk *et al*. (55) reported that the ideal water content for *S. feltiae* penetration of hosts was 10%, with drastic decreases when water content increased to 20%. Gungor *et al*. (54), working with *Steinernema anatoliense*, found similar results, reporting an optimum of 10% of water content.

Conclusions

Data obtained in this study are valuable since they outline some environmental requirements of this strain to perform optimally in the event of being used as a soil pest bioantagonist.

Abbreviations

IJ: infective juveniles

ITS: internal transcribed spacer

EPN: entomopathogenic nematodes

LR: Lican Ray

NCBI: National Center for Biotechnology Information

LSU: large ribosomal subunit

ML: maximum likelihood

BI: Bayesian inference

PP: posterior probabilities

DAI: days after inoculation

LC_{50}: lethal concentration 50

LC_{50}: lethal concentration 90

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Data supporting the conclusions of this article are included within the article. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Contribution

PF participated in data analysis and performed morphological and molecular descriptions. AA assisted with the in vitro assays. GL and PL performed the statistical analysis and contributed to writing the manuscript, respectively. SP participated in the data molecular analysis. EA designed the study and participated in the manuscript writing. All authors read and approved the final manuscript.

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Tables

Table 1
Morphometrics of *Steinernema feltiae* isolate Lican Ray, Chile. Measurements are in µm, except the indexes, in form: mean ± standard deviation (range).

Character	Males		Females		Third juvenile stage
	1° Generation	2° Generation	1° Generation	2° Generation	
n	25	25	25	25	25
L	1,428 ± 86	866 ± 75	5,318 ± 907	3,269 ± 286	807 ± 21
	(1,309-1,578)	(731-1,004)	(3,856-7,327)	(2,890-3,930)	(779–841)
MBW	95 ± 7	53 ± 5	189 ± 27	154 ± 11	31 ± 4
	(85–116)	(44–64)	(156–243)	(131–173)	(27–41)
ES	143 ± 10	115 ± 9	167 ± 17	166 ± 15	119 ± 7
	(122–177)	(99–138)	(141–200)	(136–200)	(104–130)
EP	101 ± 9	72 ± 7	90 ± 17	85 ± 14	55 ± 3
	(80–118)	(61–86)	(46–118)	(67–117)	(50–60)
NR	113 ± 11	85 ± 7	117 ± 16	114 ± 13	87 ± 5
	(90–144)	(71–101)	(93–149)	(86–140)	(80–98)
TL	39 ± 3	35 ± 3	55 ± 6	48 ± 7	74 ± 3
	(35–47)	(31–40)	(42–64)	(37–62)	(68–78)
ML	5 ± 1	11 ± 2			
	(4–8)	(8–14)			
ABD	40 ± 4	32 ± 3	65 ± 15	47 ± 6	16 ± 1
	(35–48)	(25–39)	(42–91)	(37–60)	(14–20)
SpL	68 ± 5	57 ± 6			
	(58–78)	(42–65)			
GuL	46 ± 3	36 ± 4			
	(40–55)	(29–45)			
SW	1.7 ± 0.2	1.8 ± 0.3			

L: total body length; MBW: maximum body width; ES: esophagus length; EP: anterior end to excretory pore; NR: anterior end to nerve ring; TL: tail length; ML: mucron length; ABD: anal body diameter; SpL: spicule length; GuL: gubernaculum length; H: hyaline portion; V: position of vulva (%); D%= (EP/ES)x100; E%=(EP/TL)x100; SW = SpL/ABD; GS = GuL/SpL; H%=(H/TL)x100; a = L/MBW; b = L/ES; c = L/TL.

Character	Males		Females		Third juvenile stage
	1° Generation	2° Generation	1° Generation	2° Generation	
	(1.3–2.1)	(1.3–2.4)			
GS	0.7 ± 0.05	0.6 ± 0.1			
	(0.6–0.8)	(0.5–0.7)			
V			50 ± 2	53 ± 3	
			(46–55)	(49–58)	
a					26 ± 3
					(19–31)

Table 1. Continued.

Character	Males		Females		Third juvenile stage
	1° Generation	2° Generation	1° Generation	2° Generation	
b					7 ± 0.4
					(6–8)
c					11 ± 0,4
					(10–12)
D%	71 ± 7	62 ± 6	54 ± 10	51 ± 7	46 ± 4
	(56–84)	(52–79)	(33–67)	(39–62)	(40–55)
E%	261 ± 32	207 ± 27	165 ± 36	180 ± 44	75 ± 5
	(202–326)	(162–252)	(76–232)	(118–263)	(67–83)
H					26 ± 4
					(17–33)
H%					36 ± 6
					(23–45)

L: total body length; MBW: maximum body width; ES: esophagus length; EP: anterior end to excretory pore; NR: anterior end to nerve ring; TL: tail length; ML: mucron length; ABD: anal body diameter; SpL: spicule length; GuL: gubernaculum length; H: hyaline portion; V: position of vulva (%); D%= (EP/ES)x100; E%=(EP/TL)x100; SW = SpL/ABD; GS = GuL/SpL; H%=(H/TL)x100; a = L/MBW; b = L/ES; c = L/TL.

Table 2
Percentage of mortality of *Galleria mellonella* larvae at different temperatures during the five days after inoculation with infective juveniles of *Steinernema feltiae* from Lican Ray, Chile.

Temperature (ºC)	Days after inoculation				
	1	2	3	4	5
5	0 a	0 a	0 a	0 a	0 a
10	0 a	0 a	0 a	0 a	10 a
15	0 a	0 a	100 c	100 b	100 b
20	0 a	97.5 c	100 c	100 b	100 b
25	0 a	90 c	100 c	100 b	100 b
30	12.5 b	60 b	90 b	95 b	100 b

Means (n = 20) in columns followed by the same letter do not differ according to Tukey´s multiple range test ($p < 0.05$).

Table 3
Number of invader infective juveniles (IJ) per larvae insect, time to emerge from the cadaver and offspring production (IJ/larvae) after inoculation at different temperatures.

Temperature (ºC)	Invader IJ	Emergence days	Offspring
5	nd	nd	nd
10	nd	nd	nd
15	26.6 ± 11.4 c	17.3 ± 1.4 c	72884.9 ± 26417 b
20	26.4 ± 11.2 c	10.6 ± 1.4 b	102807.3 ± 23256 c
25	12.4 ± 7.2 b	9.1 ± 0.7 a	52107.8 ± 15452 a
30	3.1 ± 2.6 a	nd	nd
nd: nematodes no detected.			

Means (n = 20) in columns followed by the same letter do not differ according to Tukey´s multiple range test ($p < 0.05$).

Table 4
Percentage of mortality of *G. mellonella* larvae during the four days after inoculation with different infective juvenile (IJ) dosis at 20º C.

doses (IJ/larvae)	Days after inoculation			
	1	2	3	4
10	0 a	70 a	100 a	100 a
20	0 a	70 a	100 a	100 a
40	0 a	80 ab	100 a	100 a
80	0 a	100 b	100 a	100 a
120	0 a	100 b	100 a	100 a
240	20 b	100 b	100 a	100 a

Means (n = 20) in columns followed by the same letter do not differ according to Tukey´s multiple range test ($p < 0.05$).

Table 5
Number of invader infective juveniles (IJ) per larvae insect, percentage of penetration efficacy, emergence days and offspring at 20º C and different doses of *Steinernema feltiae* isolate Lican Ray from Chile.

Doses (IJ/larvae)	Invader IJ	Penetration efficacy	Emergence days	Offspring
10	2.4 ± 1.7 a	24 ± 16.7 a	13.5 ± 5.7 a	64919.3 ± 41294.8 a
20	6.6 ± 1.8 b	33 ± 9.1 a	12 ± 1.7 a	79130.2 ± 16801.7 ab
40	10 ± 5.2 bc	25 ± 13.3 a	11.2 ± 0.5 a	95787.8 ± 18570.1 abc
80	20.8 ± 3.9 d	26.4 ± 4.8 a	11.4 ± 0.6 a	90316.2 ± 8542.4 abc
120	40.8 ± 17.3 de	34 ± 14.3 a	11 ± 0.7 a	106152 ± 13569.3 c
240	118.8 ± 46.8 f	49.6 ± 19.6 a	11.2 ± 1.5 a	86240 ± 67172 abc

Means (n = 5) in columns followed by the same letter do not differ according to Tukey´s multiple range test ($p < 0.05$).

Table 6

Assessment of mortality of *Galleria mellonella* larvae during 5 days after inoculation with *Steinernema feltiae* isolate Lican Ray infective juveniles (IJ) in soil with different water contents.

Water content	Days after IJ inoculation				
	1	2	3	4	5
Permanent wilting point	0 a	73 a	93 a	98 a	100 a
Field capacity	2.5 a	93 b	100 b	100 a	100 a
Saturation	2.5 a	100 b	100 b	100 a	100 a

Means (n = 20) in columns followed by the same letter do not differ according to Tukey´s multiple range test ($p < 0.05$).

Table 7

Incidence of water content in number of invader infective juveniles (IJ) per larvae insect, percentage of penetration efficacy (PE), emergence days and offspring of *Steinernema feltiae* isolate Lica Ray from Chile.

Water content	Invader IJ	Penetration efficacy	Emergence days	Offspring
Permanent wilting point	24.9 ± 11.5 a	20.8 ± 11.5 a	10.1 ± 1.6 a	75180.1 ± 37210.5 a
Field capacity	36.8 ± 21.4 a	30.8 ± 21.4 a	10.5 ± 0.7 a	81518.3 ± 24150.1 a
Saturation	38.4 ± 18.2 a	38.4 ± 18.2 a	10.1 ± 0.2 a	70975.5 ± 23629.5 a
Means (n = 20) in columns followed by the same letter do not differ according to Tukey´s multiple range test ($p < 0.05$).				

Figures

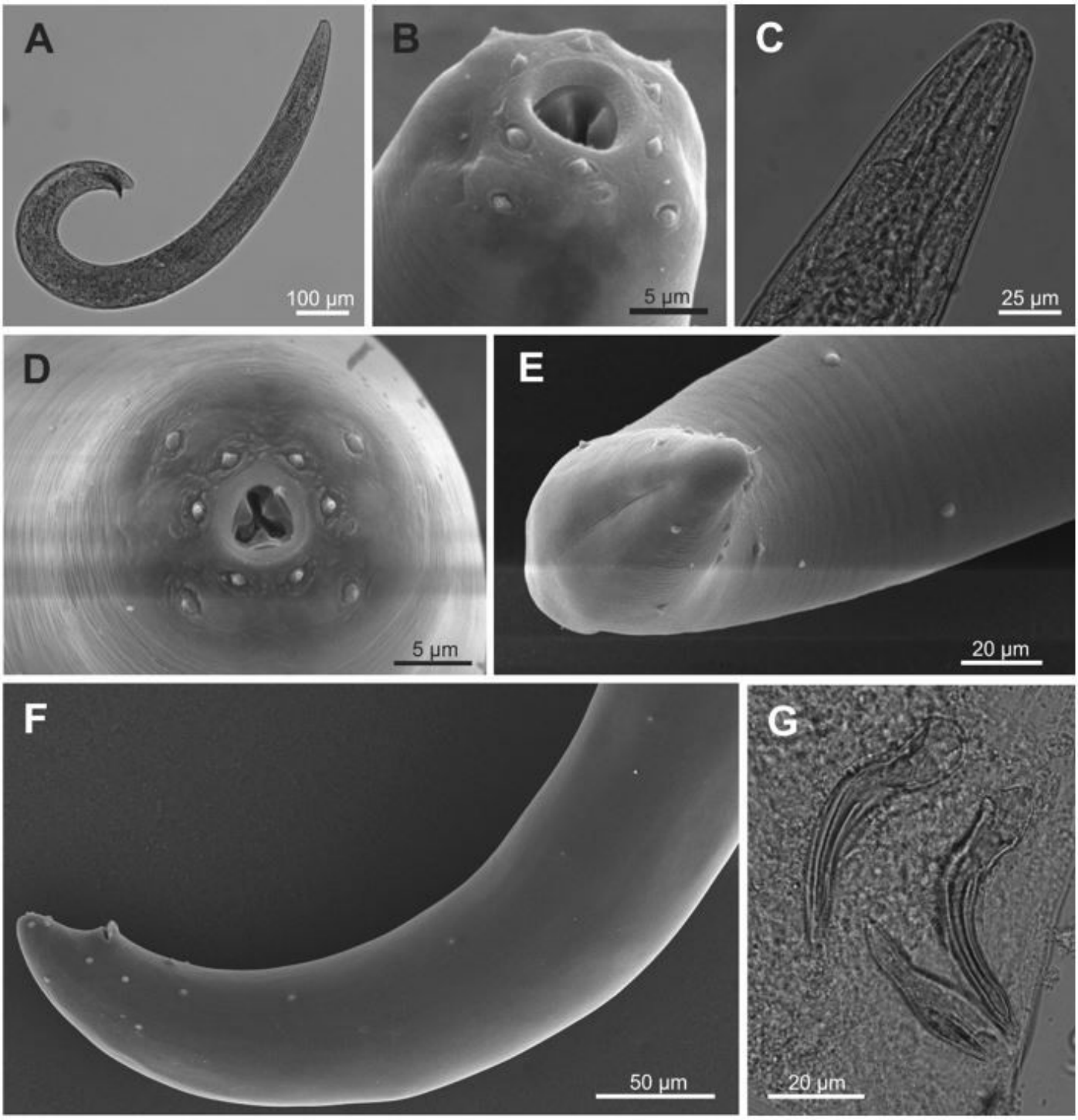

Figure 1

Steinernema feltiae isolate from Lican Ray, Chile. Male, first generation. A: entire body; B, D: cephalic region, showing lips, labial papillae and cephalic papillae and amphids; C: anterior region, showing excretory pore location; E: tail with mucron; F: posterior region, showing genital papillae; F. Spicules and gubernaculum.

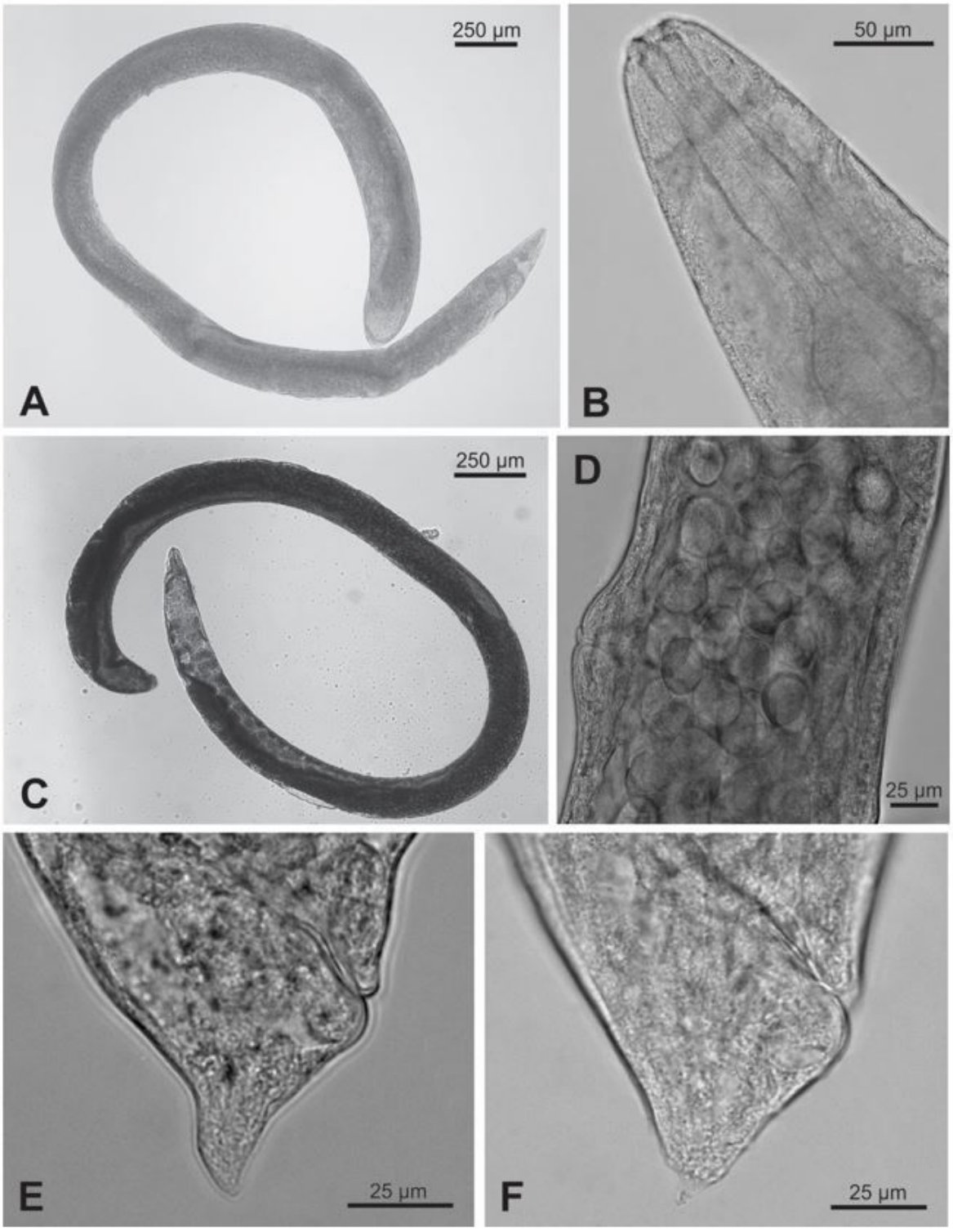

Figure 2

Steinernema feltiae isolate from Lican Ray, Chile. Female. First generation. A: entire body; B: anterior region, showing oesophagus and excretory pore. Second generation. C: entire body. First generation. D: vulvar region; E: tail. Second generation. F: Tail.

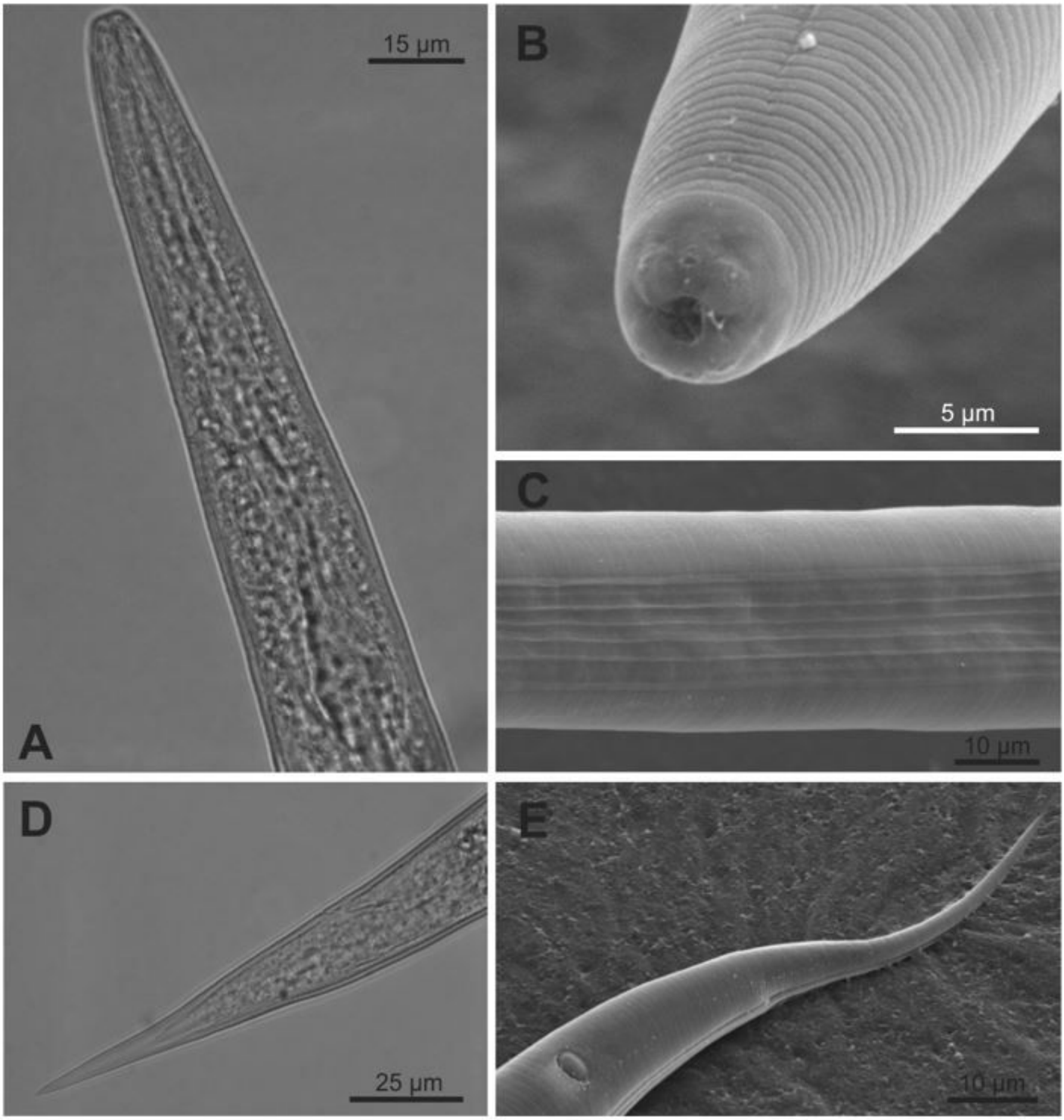

Figure 3

Steinernema feltiae isolate from Lican Ray, Chile. Third juvenile stage. A: anterior region, showing oesophagus; B: anterior region, showing cephalic papilla and amphid; C: lateral field at midbody; D: Hyaline portion; E: Tail.

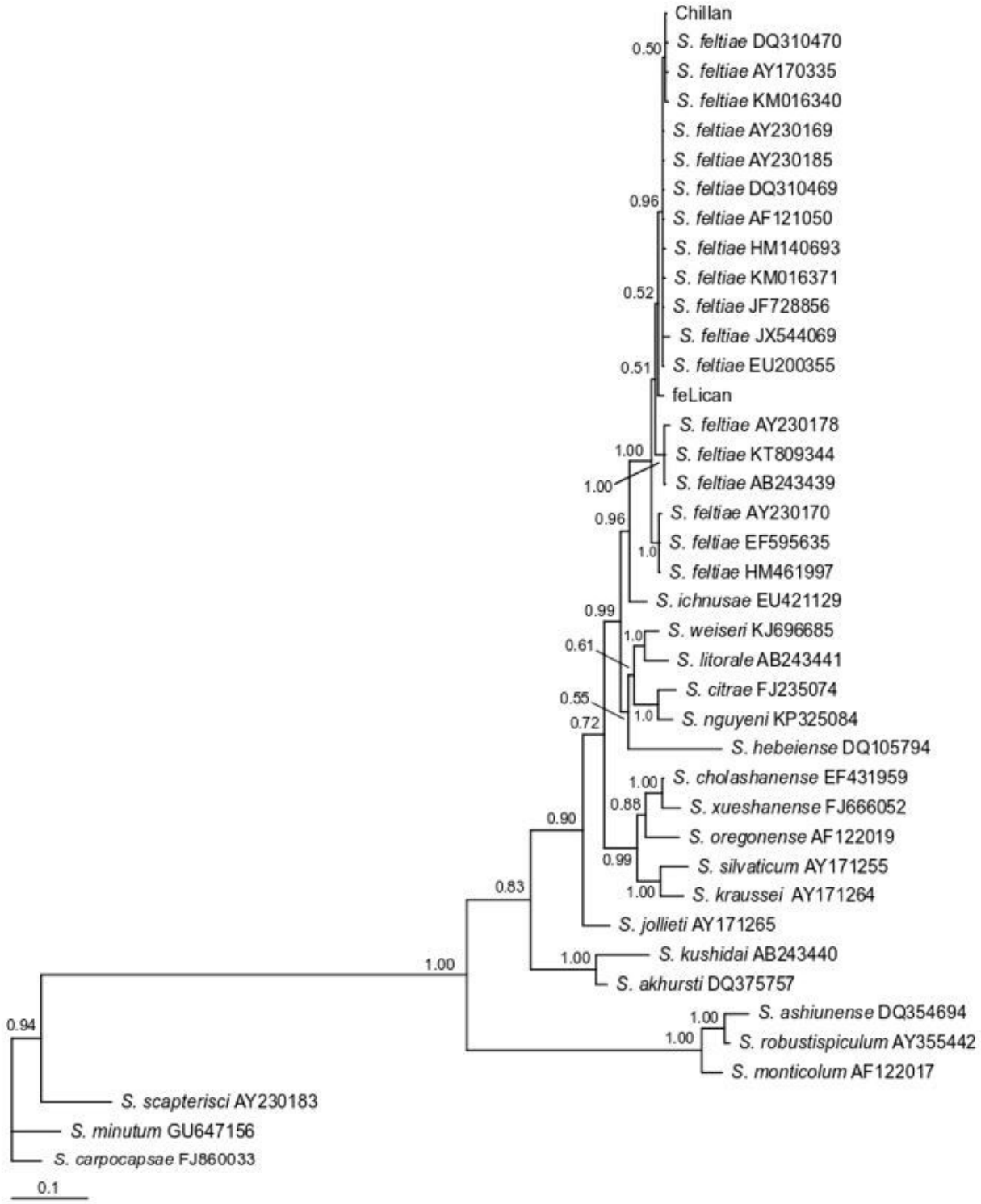

Figure 4

Phylogenetic relationships of the ITS rRNA sequences of Steinernema spp. The 50% majority rule consensus tree from Bayesian analysis generated with the GTR+G model. Posterior probabilities are given in the nodes. Newly obtained sequences are in bold letters.

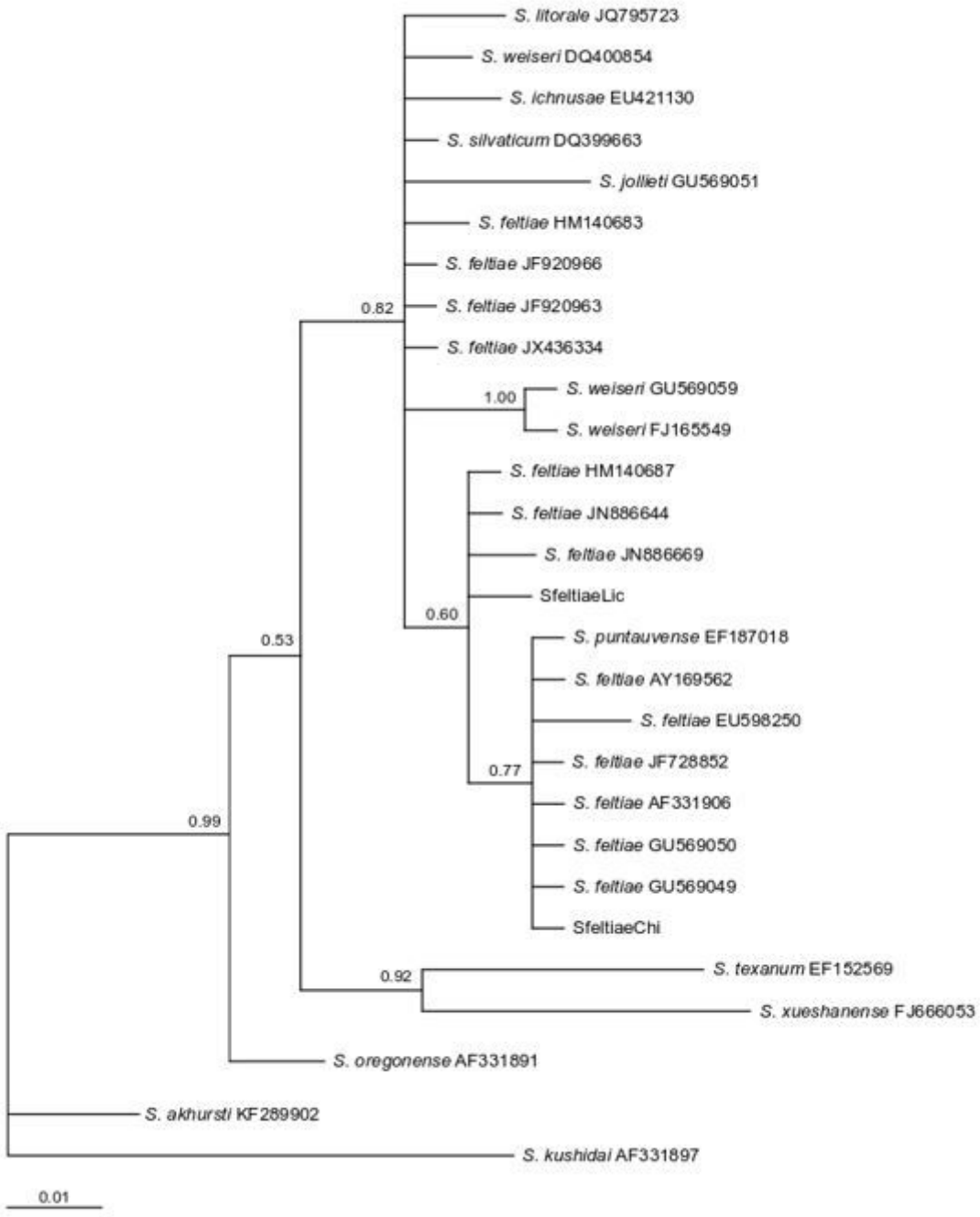

Figure 5

Phylogenetic relationships of the 28S rRNA sequences of Steinernema spp. The 50% majority rule consensus tree from Bayesian analysis generated with the GTR+G model. Posterior probabilities are given in the nodes. Newly obtained sequences are in bold letters.

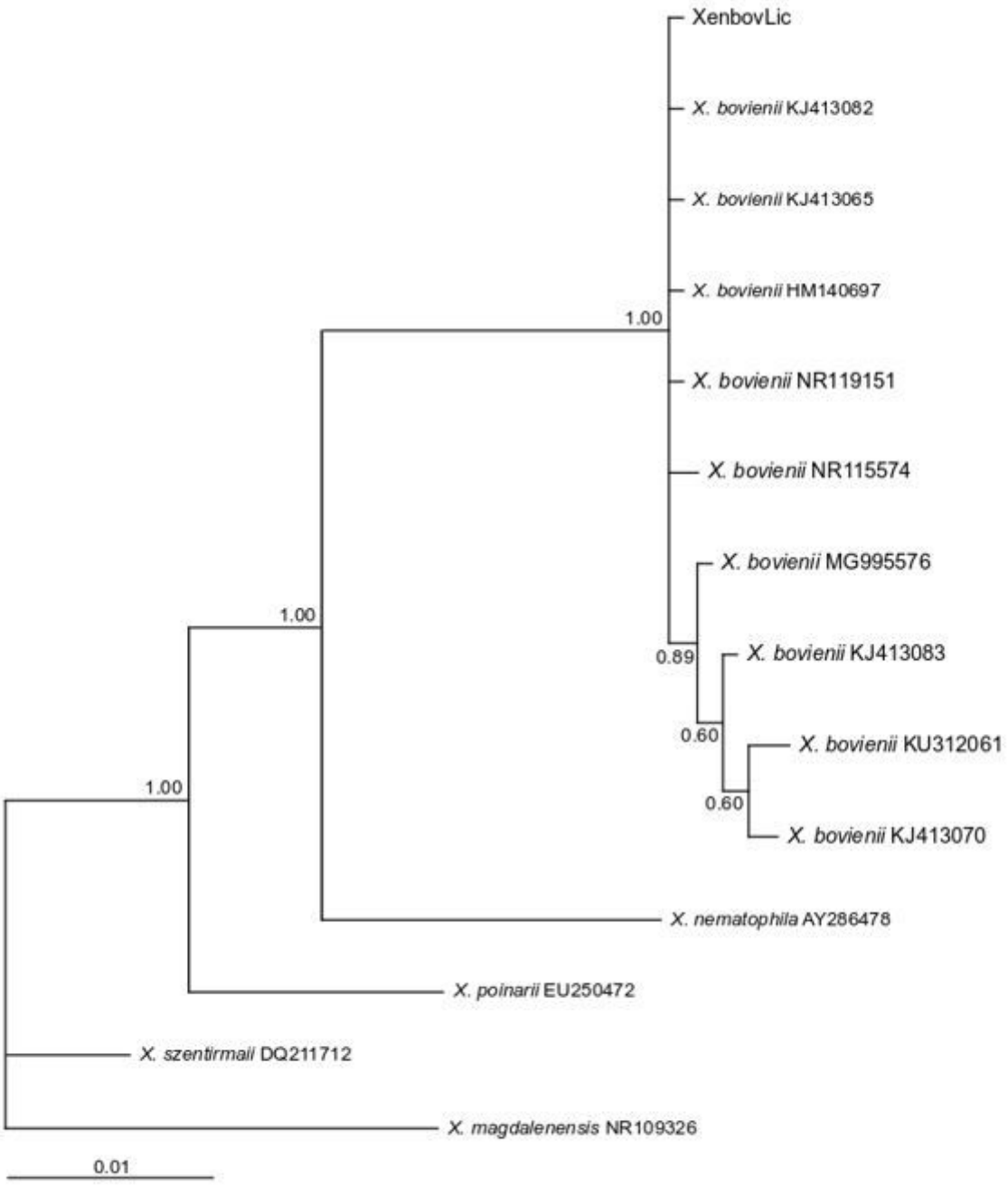

Figure 6

Phylogenetic relationships of the 16S rRNA sequences of Xenorhabdus spp. The 50% majority rule consensus tree from Bayesian analysis generated with the GTR+G+I model. Posterior probabilities are given in the nodes. Newly obtained sequences are in bold letters.