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New findings of *Heterorhabditis bacteriophora* and *Steinernema rarum* (Nematoda: Heterorhabditidae, Steinernematidae) in Córdoba, Argentina

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Abstract: A total of 152 soil samples collected from gardens of private properties at Córdoba city, Argentina, were evaluated for the presence of entomopathogenic nematodes (EPNs) and samples were analyzed for granulometry, relative humidity and pH. The *Galleria* bait method was used to isolate EPNs. The nematodes were characterized using both classic (morphometric characters) and molecular methods (analysis of internal transcribed spacer (ITS) and D2/D3 sequences of 28S genes). Phylogenetic analyses were performed to prove the identity of the studied nematodes. Recovery frequency of EPNs was 10.53%. Isolates belonging to the genera *Heterorhabditis* Poinar and *Steinernema* Travassos were detected. Two isolates were identified as *Steinernema rarum* (Doucet) Mamiya and another four as *Heterorhabditis bacteriophora* Poinar. No significant differences were found in edaphic characteristics among sites with presence or absence of EPNs. The known geographical distribution of *S. rarum* and *H. bacteriophora* is expanded, and the diversity of populations of the nematodes suitable to be used against insect pests of urban areas is increased.

Key words: entomopathogenic nematodes, detection, edaphic characteristics, urban zone.

Resumen: Nuevos hallazgos de *Heterorhabditis bacteriophora* y *Steinernema rarum* (Nematoda: Heterorhabditidae, Steinernematidae) en Córdoba, Argentina. Se evaluaron 152 muestras de suelo recolectadas en jardines de propiedades privadas de la ciudad de Córdoba, Argentina, para detectar la presencia de nematodos entomopatógenos (EPNs) y se analizaron granulometría, humedad relativa y pH de las muestras. Se utilizó el método de trampas de *Galleria*. Los nematodos se identificaron usando métodos clásicos (caracteres morfométricos) y moleculares (análisis de secuencias ribosomales de las regiones ITS y dominio D2 / D3 del gen 28S). Se realizaron análisis filogenéticos. La frecuencia de recuperación de EPNs fue de 10,53%. Se detectaron nematodos pertenecientes a los géneros *Heterorhabditis* Poinar y *Steinernema* Travassos. Dos aislados fueron identificados como *Steinernema rarum* (Doucet) Mamiya y cuatro como *Heterorhabditis bacteriophora* Poinar. No se encontraron diferencias significativas en las características edáficas de los sitios con presencia o ausencia de EPNs. Se amplía la distribución geográfica conocida de *S. rarum* y *H. bacteriophora* y se incrementa la diversidad de las poblaciones de EPNs aptos para usar contra plagas de insectos de zonas urbanas.

Palabras clave: nematodos entomopatógenos, detección, características edáficas, Heterorhabditidae, Steinernematidae, zonas urbanas.

INTRODUCTION

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae are obligate parasites mainly of soil-inhabiting insects and have great potential as biological control agents of several insect pests. They are found in a variety of habitats, and the species and isolates exhibit considerable variation in terms of host range, infectivity and conditions for survival (Hatting *et al.,* 2009; Lacey & Georgis, 2012). Soil, the natural habitat of EPNs, is a complex changing environment. The physicochemical characteristics of soil: texture, moisture content, pH, and organic matter, among others, affect the infective-stage juveniles (IJs) survival and EPNs distribution (Vänninen *et al.,* 1989). In addition to these factors, the presence of insect hosts and

natural enemies of EPNs affect their distribution and persistence (Baur & Kaya, 2001).

Search for EPNs have been conducted in temperate, subtropical and tropical regions, and they were detected in soils from woodlands, crops, prairies, thickets and roadsides in many parts of the world (Hominick, 2002; Hazir *et al.,* 2003; Adams *et al.,* 2006). The presence of these families of nematodes was also noted in urban parks in the city of Barcelona, Spain (Hoyos & García del Pino, 2004), in turf grass in Ohio, USA (Alumai *et al.,* 2006), and on golf courses in eastern Canada (Simard *et al.,* 2007). Despite EPNs have been isolated from diverse ecosystems, natural habitats are more likely uncontaminated by introduced nematodes and offer a better chance of finding native species, in contrast to human modified areas (Stock *et al.,* 1999; Stock & Gress, 2006). Though home gardens are not natural environments, they deserve attention as local sources of EPNs suitable as biological control agents. Because such studies have not been conducted in private properties of urban areas anywhere in the world, the aims of the present work are: 1 - to report the occurrence of EPNs in soils of Córdoba city, Argentina; 2 - to identify them through morphometry, molecular analysis and phylogenetic relationships; and 3- to evaluate the presence of EPNs in relation to the edaphic characteristics of the sites sampled: granulometry, pH and humidity of the soil.

METHODS

Collection and isolation of nematodes

Soil samples were collected from Córdoba city (Capital department, province of Córdoba, Argentina). It is situated in the central region of Argentina, between 360 and 480 m above sea level, at 64º 11' W - 31º 24' S. The soil sampling was carried out at 57 districts from northwest and south regions of the city (Fig. 1). The samples were taken in small urban gardens, with ornamental plants (i.e., roses, jasmine), bare soil and / or grasses.

The number of samples ranged from one to thirteen for each district. A total of 152 soil samples were collected.

Each soil sample of approximately 800 g was taken with an iron corer, and it was composed by five random sub-samples, taken at least 8-10 m apart, from the surface to a depth of 20 cm. All sub-samples were mixed together and placed in a polyethylene bag to prevent water loss. A small sub-sample was placed in a hermetic flask and analyzed for moisture values. For the laboratory tests, each soil sample was mixed, divided in two parts: one to detect EPNs and the other to determine soil pH and particle size. Both sub-samples were kept at 20°C. EPNs were recovered using the insect-baiting method (Bedding & Akhurst, 1975). The traps consisted of 6 cm diameter petri dishes covered with 80-mesh metal screen, containing five last-instar *Galleria mellonella* (L.) larvae. Two nematode traps were placed at the bottom of a 600 ml plastic container. Then, 0.5 kg of moistened soil from each sample was located into each container. To detect ambusher EPNs, another five free larvae were placed on the soil surface of each sample. Each plastic container was covered with a lid and kept at 22-25 ºC in complete darkness. After 10 days dead larvae were removed from the soil. Dead insects with signs of EPNs-infection, recognized by color change (usually red/purple for heterorhabditids; ochre/brown/black for steinernematids), were rinsed with sterile distilled water and individually placed in modified White traps (Kaya & Stock, 1997) for emergence of the IJs. Emerging nematodes were pooled for each sample and used to infect fresh *G. mellonella* larvae to confirm Koch's postulates of pathogenicity and to obtain nematodes for identification and establishment of cultures and to exclude saprophytic nematodes (Rio & Cameron, 2000).

Nematode identification

A preliminary morphological diagnosis of the recovered isolates (i.e., to check if the 1st generation nematodes were hermaphroditic or female and male individuals, and to observe the presence of bursa and spicule/gubernaculum morphology in 2nd generation males) was performed to sort them into similar species groups (López-Núñez *et al.*, 2008). Ten *G. mellonella* larvae were infected with 100 IJs/insect in Falcon multi-well tissue culture plates 3047, and dead hosts were dissected after three and six days, to obtain adults of 1st and 2nd generation, respectively. Adults from 1st and 2nd generation were separated according to their morphology, in addition to the development time.

Morphometrical observations followed the taxonomic criteria suggested by Stock & Kaya (1996) and Hominick *et al.* (1997). Briefly, 20 1st generation males and 20 IJs were randomly selected from different *G. mellonella* corpses. Nematodes were examined alive or heat-killed in a 60°C Ringer's solution. The heat-killed nematodes were placed in triethanolamine-for-

malin (TAF) fixative (Kaya & Stock, 1997) and processed to anhydrous glycerin for mounting (Seinhorst, 1959). Observations were made on live and mounted specimens using a Zeiss microscope. In general, and for all the nematode stages (1st generation males and IJs), the following characters were analyzed: total length, greatest width, distance from anterior end to excretory pore (EP), distance from anterior end to nerve ring, distance from anterior end to base of esophagus, tail length, width at anus, D% (EP of male divided by esophagus length x 100), and E% (EP of IJ divided by tail length x 100). According to their morphology and morphometric characters, isolates were assigned to different species groups using taxonomic criteria suggested by Hominick *et al.* (1997).

For molecular identification, sequence of internal transcribed spacer (ITS) rDNA and D2/D3 expansions of 28S genes were used. After DNA extraction from a single female following protocol I of Bruford *et al.* (1992), both genes were amplified using the set primers 18S-26S (Vrain *et al.,* 1992) for ITS and D2A-D3B (Spiridonov & Guzeeva, 2009) for 28S.

For both genes, amplification conditions were empirically adjusted from those described by Stock *et al.* (2001); PCR reaction was performed in a final 25 µl volume containing: 2 µl of DNA, 2.5 µl of 10x reaction buffer (500 mM Tris-HCl pH 9.0), 15 mM $MgCl_2$, and 150 mM $(NH_4)_2SO_4$), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 mM of each primer, and 0.75 U of Taq DNA Polymerase (Fermentas). The reaction started with denaturation at 94°C for 4 min, followed by 32 cycles of 30 sec at 94°C, 30 sec at 49°C (ITS) - 53ºC (28S), 60 sec at 72°C, and a final extension of 7 min at 72°C. PCR products were purified and sequenced in both directions with the corresponding same primers by Macrogen Inc. (Korea), the sequences were submitted to the NCBI database.

All obtained sequences were manually edited. The alignment was performed using the default settings in Clustal X 2.0 (Thompson *et al.,* 1997). Phylogenetic relationships among species in *Heterorhabditis* and *Steinernema* were estimated using a region of nuclear rDNA (that included the 18S 3'-terminus, ITS-1, ITS-2, 5.8S subunit and 28S 5'-terminus), and the D2-D3 domain of 28S rDNA gene segment. For phylogenetic analysis we included sequences from 10 species of genus *Heterorhabditis* and 11 of *Steinernema,* obtained from GenBank (accession numbers in figs. 1 and 2), used as references to confirm the morphological classification of the studied specimens. Bayesian inference (BI) method was used for both regions using MRBAYES 2.01 (Huelsenbeck & Ronquist, 2001). The sequence of *Caenorhabditis elegans* (X03680) was included as an outgroup in all analysis. To determine the most appropriate model of nucleotide substitution for each data set, sequences were analyzed with MrModeltest 2.3 (Nylander 2004). The models were selected using the Akaike criterion and the following corrections were employed for subsequent Bayesian inferences. For BI analysis, two independent runs were simultaneously performed on the data set, each one using one cold and three heated Markov chains. The program was run until the average standard deviation of split frequencies between the two independent runs was less than 0.01. After discarding 0.25 burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. A 50% majority rule consensus tree was generated; it was displayed using the TreeView (Win32) program.

Determination of granulometry, relative humidity and pH of soil samples

All EPN-positive samples and 30 randomly selected negative samples were analyzed following standard techniques. The soil classification was stated following criteria of Department of Agriculture of the United States (USDA Soil Survey Staff, 2014).

Statistical analysis

Presence/absence of EPNs and soil type were analyzed with Kruskal-Wallis Test. The combined influence of all considered factors (granulometry, relative humidity and pH of the soil) was also analyzed through a Multiple Correspondence Factor Analysis. Statistical analysis was performed using InfoStat v 1.1. Software (Di Rienzo *et al.,* 2011).

RESULTS

From a total of 152 soil samples, 16 (10.53%) were EPNs-positive. Occurrence of EPNs was detected in 13 out of the 57 sampled districts (22.8%).

Fifteen isolates were identified: 12 of them (80%) belonged to the genus *Heterorhabditis* (Heterorhabditidae) and three ones (20%) to the genus *Steinernema* (Steinernematidae). Considering the total samples, 7.89% were positive for heterorhabditids and 1.97% for steinernematids (Fig. 1).

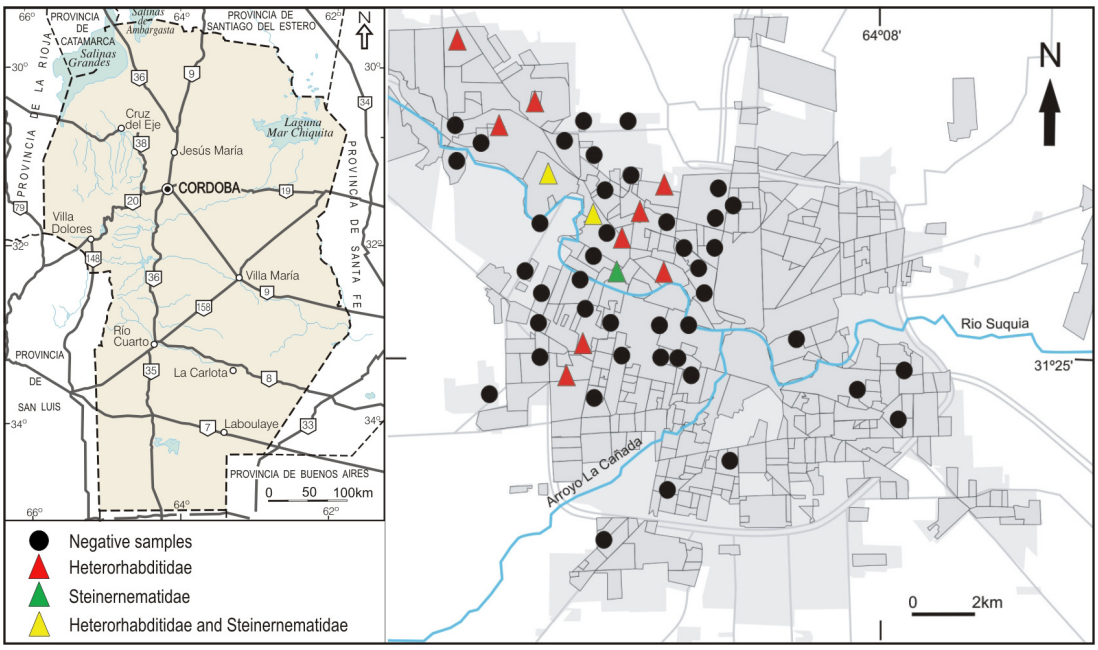

Fig. 1: Map of sampling sites for entomopathogenic nematodes in Córdoba city, Argentina.

We obtained sequences from six isolates for both molecular markers (GenBank accession numbers **KT378442 to KT378452). The phylogenetic** analysis based on ITS (Internal transcribed Spacer), and the partial sequence of the D2D3 from 28S, were successful to confirm nematode morphometrical identification. Four isolates named N4, N54, N82, and N116 were identified as *Heterorhabditis bacteriophora* Poinar, 1976. The ITS length was 1088 bp, that of partial 28S rDNA was 593 bp and 1681 bp to the combined fragment 28S+ITS. The best evolutionary model of nucleotide substitutions was GTR+G for ITS and SYM+I+G for 28S.

The BI analyses of combined fragment 28S+ITS (Fig. 2) placed these four nematodes with *H. bacteriophora*. This pattern was well supported by bootstrap resampling and posterior probabilities, providing further evidence for their specific identity. The 28S+ITS BI tree shows *H. georgiana* as sister-groups of *H. bacteriophora* (PP=1).

Phylogenetic inference of combined genes (Fig. 3) identified N7 and N105 isolates as *Steinernema rarum* (Doucet, 1986) Mamiya, 1988 with high support. The lengths of obtained fragments of this species was 1092 bp for ITS, 588 bp for parcial 28S rDNA, and the combined fragment was 1661 bp. The best evolutionary model of nucleotide substitutions for ITS and 28S was GTR+G. There was a single difference between the two sequences and the rest of samples for 28S gene.

The soil samples were classificated as sandy (Ar), loamy sand (AF) and sandy loam (FA). Most of the positive samples were AF. However, no significant differences were found between positive and negative samples in relation to the texture (Test Kruskal-Wallis, $p>0.05$).

As regards pH, positive samples corresponded to neutral to moderately basic soils (6.7-8.5) averaged in fairly basic floor (7.7), while negative samples were from moderately acid to strongly basic soils (5.6-8.9) with average in mildly basic soils (7.5). No significant differences were detected for pH of soils with presence and absence of entomopathogenic nematodes (Kruskal-Wallis test, $p> 0.05$).

Humidity of samples varied from 2.6 to 56%. The average humidity of the positive samples was 16.2%, while it was 16.6% for negative samples. There was no significant difference of mean soil humidity between positive and negative samples (Kruskal-Wallis test, $p> 0.05$). Notwithstanding, it was noted that the humidity range of the samples with presence of nematodes did not include extreme values.

All soil factors were analyzed together by a Multiple Correspondence Factorial Analysis. The first main shaft explains 15.7% of the variability

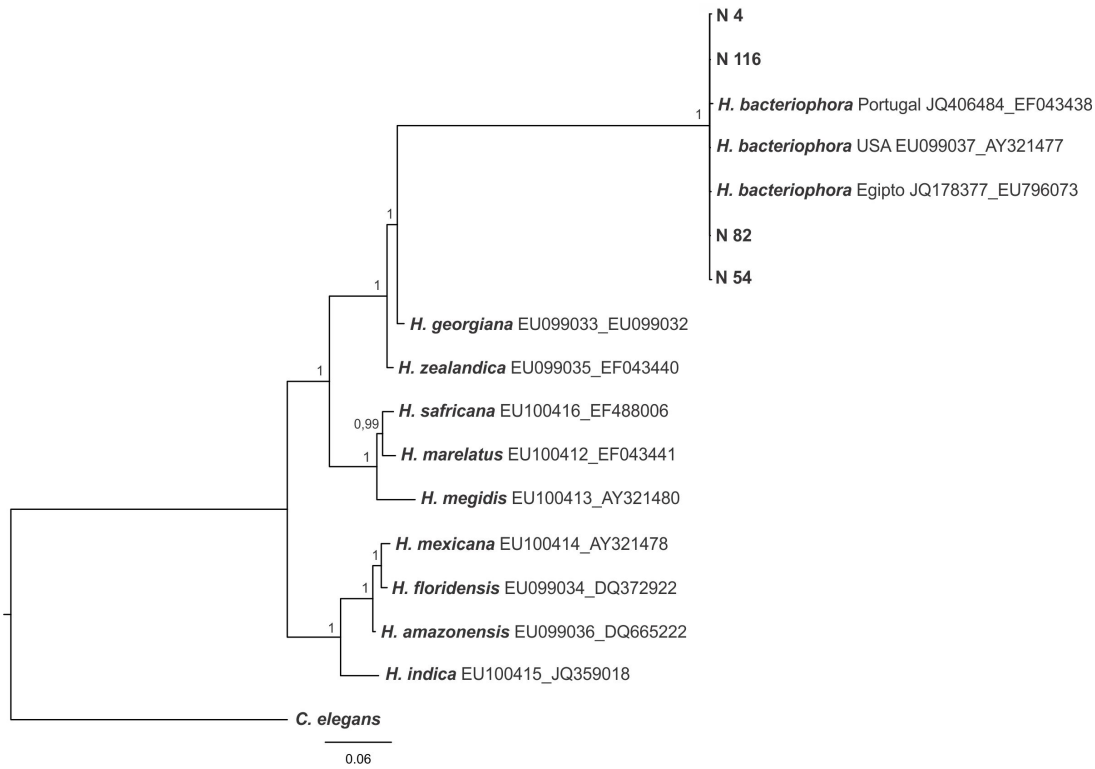

Fig. 2: Bayesian consensus tree of 28S+ITS rDNA combined sequences, showing phylogenetic relationships of *Heterorhabditis bacteriophora*. Running one million generations, under the GTR + G and SYM+I+G models. Posterior probabilities are given in nodes. Outgroup = *C. elegans*. GenBank accession numbers for 28S and ITS sequences are given in parentheses.

while the second axis explains 12.7%, so that the two main axes explain 28.4% of the total variability. Positive samples tended to be located in sandy loamy soils (AF) with pH values of 6.3 to 8.2 (slightly acid to moderately basic) with a humidity range of 13.3 to 24% (Fig. 4, Tables 1 and 2).

DISCUSSION

The isolates of entomopathogenic nematodes detected in this study belong to the species *H. bacteriophora* and *S. rarum*. *H. bacteriophora* is present on all continents except Antarctica (Hominick *et al.,* 1996). In South America, it was reported from Colombia, Costa Rica, Argentina, Brazil and México (Stock, 1995; Hominick, 2002; Uribe-Lorío *et al.,* 2005; Doucet & Doucet, 1997; Giayetto & Cichón, 2006; Doucet *et al.,* 2008; Barbosa-Negrisoli *et al.*, 2010; Zepeda-Jazo *et al.,* 2014). In Argentina, it is the only species of the genus *Heterorhabditis* detected to date and is more abundant than the species of the genus *Steinernema*.

In Argentina, *S. rarum* has been detected in the provinces of Córdoba (Doucet *et al.,* 2008), and Santa Fe (Del Valle *et al*., 2016). The two populations found in this study extend its distribution in our country. This species has a limited known distribution in the word, since it has been detected only in USA and Brazil (Shapiro-Ilan *et al.,* 2003; Barbosa-Negrisoli *et al.,* 2010). The previous findings of *S. rarum* (Doucet *et al*., 2008; Del Valle *et al*., 2016), and the data provided in this paper indicate that this species is the more abundant of the genus *Steinernema* in Argentina.

The topology of the trees obtained on the basis of 15 sequences (for *Heterorhabditis*) and 16 sequences (for *Steinernema*) was strongly supported.

The *bacteriophora*-group currently encompasses two species *Heterorhabditis georgiana* and *H. bacteriophora* (Hatting *et al.,* 2009). It is worth noting the lack of variability of the sequences for both genes "within" the species despite of different geographical origins.

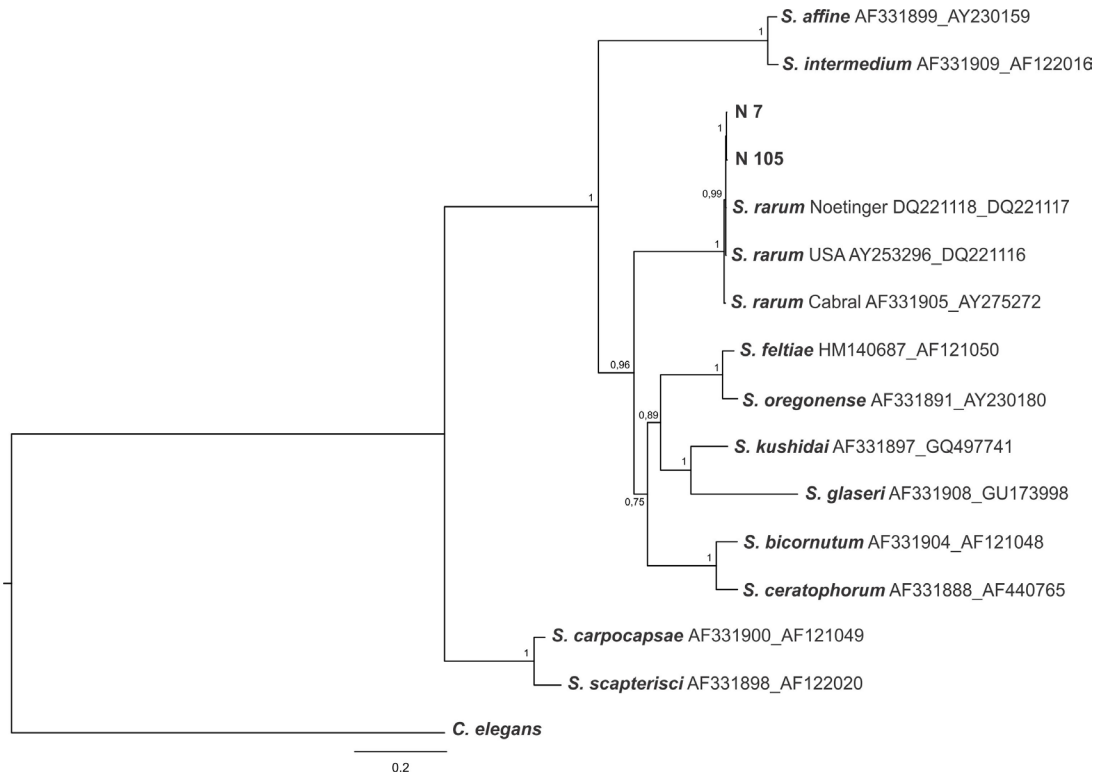

Fig. 3: Bayesian consensus tree of 28S+ITS rDNA combined sequences, showing phylogenetic relationships of *Steinernema rarum*. Running one million generations, under the GTR + G model. Posterior probabilities are given in nodes. Outgroup = *C. elegans*. GenBank accession numbers for 28S and ITS sequences are given in parentheses.

In *S. rarum*, all sequences were also placed clearly in a monophyletic group. The sequences of ITS and 28S regions of the *S. rarum* N105 and N7 from Córdoba city, were very similar to those of other isolates previously detected in the province: Noetinger (DQ221118-DQ221117) and Arroyo Cabral (AF331905-AY275272) and also to those from US strains (AY253226-DQ221116) (Nguyen *et al*., 2006). The sequences of Córdoba city isolates presented one autapomorphy for 28S gene. To explain this pattern of variation, we suggest analyzing more samples.

All soil sampled in this study presented granulometric characteristics with a high sand content in its composition, so no significant differences between positive and negative samples were detected with respect to the granulometry. The preference of the family Heterorhabditidae for sandy soils is well documented (Prasad *et al.,* 2001). Sandy soils have large pores and a low potential for moisture and they provide well ventilated environments where the nematodes have an efficient use of energy reserves and good mobility, increasing their survival (Croll & Matthews, 1977).

The optimum pH ranges for most soils with nematode ranging from 4 to 8 (Kaya, 1990). In this paper, the pH of the positive samples corresponded to neutral to moderately alkaline soils, so it did not affect the presence of EPNs. In Spain, the presence of these organisms was detected in moderately alkaline soils (García del Pino & Palomo, 1996). Other records indicate the occurrence of EPNs with pH values of 4.6 to 8 (Stock *et al.,* 1999), 4 to 8.1 (Miduturi *et al.,* 1996), 3.6 to 7.8 (Miduturi *et al.,* 1997), with prevalence of family Steinernematidae in acid soils. In this paper the two families were found in moderately alkaline soils, with averages of similar pH, 7.8 for Steinernematidae and 7.7 for Heterorhabditidae. No significant differences were found with regard to soil pH between positive and negative samples.

As for humidity, the range found in this study was within the range reported for the occurrence of EPNs in other environments, 10 to 30% (Glazer *et al.*, 1991). No significant differences of soil moisture were found between samples with presence or absence of EPNs.

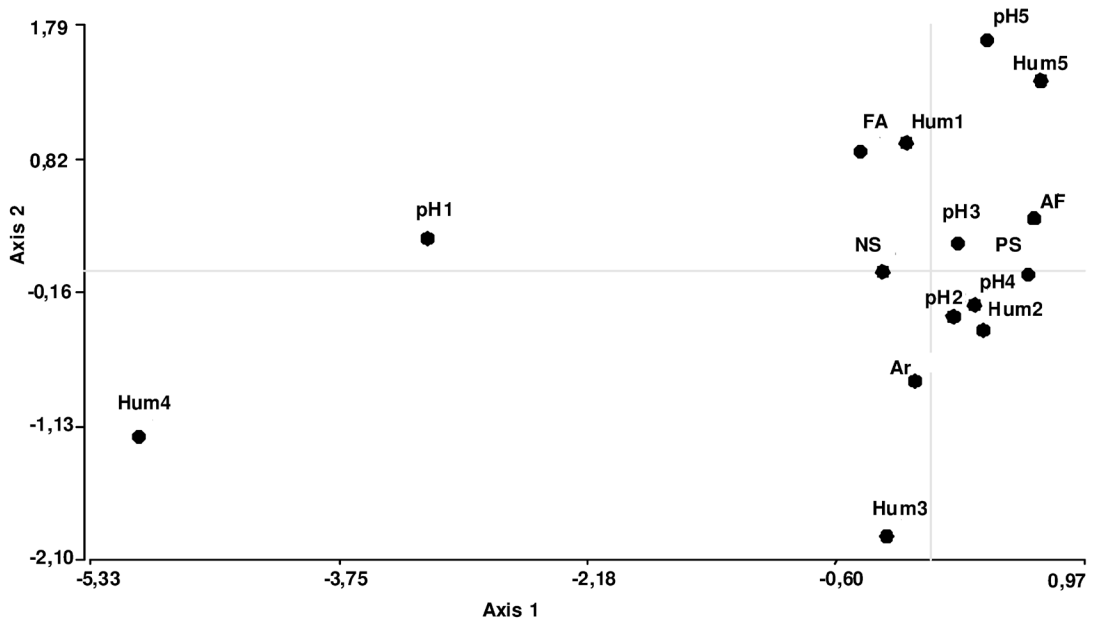

Fig. 4: Multiple Correspondence Factorial Analysis of the relationship among edaphic characteristics: pH, humidity, and soil texture (FA: sandy loam, AF: loamy sand, Ar: sandy) and positive and negative sites for dimensions 1 and 2.

Table 1: Distribution of frequencies to the pH.

Variable	*Class*	*Lower Limit*	*Upper Limit*	*Mean*	*Absolute frequency*	*Relative Frequency*
pH	*1*	*5.60*	*6.26*	*5.93*	*3*	*0.06*
pH	*2*	*6.26*	*6.92*	*6.59*	*4*	*0.09*
pH	*3*	*6.92*	*7.58*	*7.25*	*13*	*0.28*
pH	*4*	*7.58*	*8.24*	*7.91*	*25*	*0.53*
pH	*5*	*8.24*	*8.90*	*8.57*	*2*	*0.04*

Table 2: Distribution of frequencies to the humidity.

Variable	*Class*	*Lower Limit*	*Upper limit*	*Mean*	*Absolute frequency*	*Relative Frequency*
% Humidity	*1*	*2.60*	*13.29*	*7.95*	*18*	*0.38*
% Humidity	*2*	*13.29*	*23.98*	*18.64*	*24*	*0.51*
% Humidity	*3*	*23.98*	*34.68*	*29.33*	*4*	*0.09*
% Humidity	*4*	*34.68*	*45.37*	*40.02*	*0*	*0.00*
% Humidity	*5*	*45.37*	*56.06*	*50.71*	*1*	*0.02*

Up to date, only one work carried out in Spain documented the presence of both families Steinernematidae and Heterorhabditidae in urban gardens (Hoyos & García del Pino, 2004). In the present paper representatives of both families were detected in home gardens in the city of Córdoba (Argentina).

The lack of correlation between the presence of EPNs and soil characteristics may be due, as has been noted in other studies, to other factors not considered in this work, such as the particular distribution insect hosts, since it determines, in part, the permanence of infective forms on the soil (Vänninen *et al.*, 1989). In agricultural soils, it is known that the human impact is favourable to the presence of these organisms and population explosions of insect pests (Mráček & Webster, 1993). Coinciding with this work, in

surveys conducted in southern Argentina, no association was found between the distribution of EPNs and different environmental variables in the region (Giayetto & Cichón, 2006). That study was carried out in rural areas and not in urban areas.

The advantages of using native isolates against insect pests of particular location are well recognized. Indigenous EPN may be more suitable for inundative release against local insect pests because of their adaptation to local climate and other population regulators (Bedding, 1990). The occurrence of EPNs in soils of urban areas and the ability of these soils to allow their survival will enable the implementation of biological control actions against harmful insects in these environments.

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