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Heterodera elachista the Japanese cyst nematode parasitizing corn in Northern Italy: integrative diagnosis and bionomics

Francesca De Luca[1], Nicola Vovlas[1], Giuseppe Lucarelli[2], Alberto Troccoli[1], Vincenzo Radicci[1], Elena Fanelli[1], Carolina Cantalapiedra-Navarrete[3], Juan E. Palomares-Rius[4], and Pablo Castillo[3]

[1] *Istituto per la Protezione delle Piante (IPP), Consiglio Nazionale delle Ricerche (CNR), U.O.S. di Bari, Via G. Amendola 122/D, 70126 Bari, Italy*
[2] *Horto Service, Via S.Pietro, 3, 70016 Noicattaro (BA), Italy*
[3] *Instituto de Agricultura Sostenible (IAS), Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 4084, 14080 Córdoba, Campus de Excelencia Internacional Agroalimentario, ceiA3, Spain*
[4] *Department of Forest Pathology, Forestry and Forest Products Research Institute (FFPRI), Tsukuba 305-8687, Ibaraki, Japan*

Received: ______/Accepted ________.

*Author for correspondence: P. Castillo
E-mail: p.castillo@csic.es
Fax: +34957499252

Short Title: Heterodera elachista *on corn in Europe*

Abstract The Japanese cyst nematode *Heterodera elachista* was detected parasitizing corn cv Rixxer in Bosco Mesola (Ferrara Province) in Northern Italy. The only previous report of this nematode was in Asia (Japan, China and Iran) attacking upland rice; being this work the first report of this cyst nematode in Europe, and confirmed corn as a new host plant for this species. Integrative morphological and molecular data for this species were obtained using D2-D3 expansion regions of 28S rDNA, ITS1-rDNA, the partial 18S rDNA, the protein-coding mitochondrial gene, cytochrome oxidase c subunit I (*COI*), and the heat-shock protein 90 (*hsp90*). *Heterodera elachista* identified in Northern Italy was morphologically and molecularly clearly separated from other cyst nematodes attacking corn (*viz*. *H. avenae*, *H. filipjevi*, *H. delvii*, *H. oryzae*, *H. sacchari*, *H. sorghi*, *H. zeae*, *Punctodera chalcoensis*, and *Vittadera zeaphila*) and rice (*H. oryzae*, *H. sacchari*). The phylogenetic relationships of *H. elachista* from Northern Italy with other cyst-nematodes using rDNA and mtDNA showed a separation of the genus *Heterodera* in various morphospecies groups based on vulval cone structures. The development and parasitic habit of *H. elachista* on naturally infected corn cv Rixxer confirmed a typical susceptible reaction, including multinucleate syncytial cells in parenchymatic cells. Under greenhouse conditions, *H. elachista* successfully reproduced on two crops widely used in Northern Italy, such as corn (cv PR 33) and rice (cv Baldo). Considering the limited host-range of this nematode, that include two of the three world's most important crops, special attention is needed for avoiding the dispersal of this nematode into new areas, by movement of soil on equipment, water, and contaminated containers infested soil, or agricultural practices.

Keywords: Cytochrome c oxidase subunit 1 (*COI*), heat shock protein 90 (*hsp90*), Heteroderidae, Histopathology, mtDNA, New geographic record, New host plant, Phylogeny, rDNA.

Introduction

Wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), and rice (*Oryza sativa* L.) constitute the three world's most important crops that are grown in more than 220, 170, and 160 million ha, respectively (FAOSTAT, 2011). In Italy, corn is widely cultivated (more than 990,000 ha) throughout the country (FAOSTAT, 2011), with the major growing areas being in the northern regions, where is commonly sown in late spring and harvested in early autumn. Under these environmental conditions, corn is exposed to several diseases, particularly those caused by soil-borne plant pathogens, including a wide range of plant-parasitic nematodes (PPN). However, the parasitism of corn by PPN is frequently considered weak as the extensive fibrous root system of corn compensates the root impairment caused by the nematode that usually leads to unnoticeable yield losses, unless nematode population density is high or infection occurred together with crop stress due to environmental conditions that resulted in crop losses (Windham, 1998). Nevertheless, some PPN have been documented causing yield losses to corn, including root-knot- (*Meloidogyne* spp.), root-lesion- (*Pratylenchus* spp.), sting- (*Belonolaimus* spp.), needle- (*Longidorus* spp.), stubby-root-nematodes (*Trichodorus* spp., *Paratrichodorus* spp.), and cyst-nematodes (*Heterodera* spp., *Punctodera* spp.) (Norton, 1984; Windham, 1998; Castillo and Vovlas, 2007).

Damage caused by cyst-nematodes to corn plants is characterized by stunting in irregular patches, sometimes chlorotic and narrow leaves, and many short lateral roots giving the appearance of a bottle brush (Norton, 1984; Windham, 1998;). Nine species of cyst-nematodes are known to attack corn, *viz. Heterodera avenae* Wollenweber, 1924, *H. delvii* Jairajpuri, Khan, Setty & Govindu, 1979, *H. filipjevi* (Madzhidov, 1981) Stelter, 1984, *H. oryzae* Luc & Berdon, 1961, *H. sacchari* Luc & Merny, 1963, *H. sorghi* Jain, Sethi, Swarup, & Srivasta, 1982, *H. zeae* Koshy, Swarup & Sethi, 1971, *Punctodera chalcoensis* Stone, Sosa Moss & Mulvey, 1976, and *Vittatidera zeaphila* Bernard, Handoo, Powers, Donald & Heinz, 2010 (Bernard et al., 2010).

The suitability of a plant as a host for PPN species is defined as its capacity to sustain the nematode feeding and reproduction on it. Host suitability may be expressed objectively as the ratio of the number of nematode units recovered at the end of a nematode-infection assay, namely the final nematode population density (*Pf*), to the number of nematode units used to inoculate a plant, namely the initial population density (*Pi*) (Lewis, 1987). Since cyst-nematodes species are often host-specific, accurate and precise identification and estimation of their population density in soil are crucial for designing effective control

measures within the context of sustainability and integrated pest management practices, because of the long persistent dormant stage (cyst filled with eggs). In fact, it is well established that the extent of crop growth suppression is influenced by the nematode species. The corn cyst nematode *H. zeae* is an important pathogen of corn in Egypt, Pakistan, India, Nepal, Thailand, and some areas of USA (Subbotin et al., 2010). In Europe, this nematode has been detected in Portugal (Correia and Abrantes, 2005) and Greece (Skantar et al., 2012). The host-suitability of cyst-nematodes can be determined by assessing the nematode reproduction on roots of crops after artificial inoculations (Subbotin et al., 2010).

In Italy, corn has been parasitized by race 1 of the root-knot nematode *Meloidogyne incognita* (Di Vito et al., 1980), but no report exists of cyst nematodes in this crop. In October 2012, a soil and root sample from a commercial field of corn (cv Rixxer) at Bosco Mesola in Ferrara province (Northern Italy) was analysed for the presence of PPN. In the soil sample feeder roots revealed a severe infection by cysts of a *Heterodera* species that were also present in the soil (14 eggs and second-stage juveniles/ml of soil). The abundance of cyst-nematode infected roots suggested a highly specialized nematode-plant interaction, which required a detailed morphological and molecular comparative study of this sample in order to get an accurate identification of the nematode species.

The objectives of the present study were: (i) to identify the cyst-nematode species attacking corn in Northern Italy; (ii) to characterise molecularly the cyst-nematode population using the D2-D3 expansion segments of 28S rDNA, ITS1, partial 18S rRNA, cytochrome oxidase c subunit 1 (*COI*) and heat-shock protein 90 (*hsp90*) gene sequences; (iii) to explore the phylogenetic relationships of this cyst-nematode population within Heteroderidae; (iv) to provide morpho-biological information on the host-parasite relationships of this nematode species in corn-nematode-feeding sites; and due to the limited host-range known for this species, (v) to determine the host-suitability of the Italian cyst-nematode population in other corn cvs or species commonly cropped in that area, such as corn cv PR 33 and rice cv Baldo, under glasshouse conditions.

Materials and methods

Morphological identification

Samples of infected corn roots together with rhizosphere and bulk soil were taken from a commercial field in Bosco Mesola (Ferrara Province) in Northern Italy. Males and second-

stage juveniles (J2s) were extracted from soil by magnesium sulphate ($MgSO_4$) centrifugal flotation method (Coolen 1979), while cysts were extracted from soil by the routine sieving-decanting method (Fenwick, 1940). Cysts were also recovered from infected root tissues, processed and mounted in glycerin (Hooper, 1986). Glycerin infiltrated specimens were examined by light microscopy for diagnosis. Morphological differentiation was based on mature cysts, males and J2s as described by Subbotin et al. (2010). Mature cysts were characterized by cyst shape and features of the cyst terminal cone such as nature of fenestration, bridge structure, presence/absence of bullae, cyst wall pattern, and the shape and size of semifenestra in the fenestra area, based on observations of at least 20 specimens. Observations of bridge and semifenestra relied on ≈100-by-100-μm section of the vulval cone of the posterior end of a cyst with intact fenestral area, excised and prepared for observation (Subbotin et al., 2010).

Specimens for microscopic observation were killed by gentle heat, fixed in a solution of 4% of formaldehyde + 1% propionic acid, and processed to glycerol according to Seinhorst's method (Hooper, 1986). Photographs were taken with a Leica DFC 425 system mounted on a Leitz Wetzlar optical microscope, on specimens mounted on water agar temporary slides (Troccoli, 2002), whereas measurements and drawings were made at the *camera lucida* on glycerine infiltrated specimens. Abbreviations used in Table 1 are defined in Siddiqi (2000).

Molecular identification

For molecular analyses, two young females were temporarily mounted in a drop of 1M NaCl containing glass beads and after taking measurements and photomicrographs of diagnostic characters the slides were dismantled and DNA extracted. Nematode DNA was extracted from single individual female nematodes and PCR assays were conducted as described by Castillo et al. (2003). The D2-D3 expansion segments of 28S rDNA was amplified using the D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (Castillo et al. 2003). The ITS1 region was amplified using forward primer TW81 (5΄ GTTTCCGTAGGTGAACCTGC-3΄) and reverse primer AB28 (5΄-ATATGCTTAAGTTCAGCGGGT-3΄) as described in Subbotin et al. (2001); and the partial 18S was amplified using primers A (5'-AAAGATTAAGCCATGCATG-3'), 13R (5'-GGGCATCACAGACCTGTTA-3') and 18P-SSU_R_81 (5' TGATCCWKCYGCAGGTTCAC-3') (Boutsika et al. 2004). The portion of

the *COI* gene was amplified as described by Derycke et al. (2005) using forward primer JB3 (5'- TTTTTTGGGCATCCTGAGGTTTAT -3') and reverse primer JB4 (5'- TAAAGAAAGAACATAATGAAAATG -3'). Finally, the portion of the *hsp90* gene was amplified using primersU831 (5'- AAYAARACMAAGCCNTYTGGAC - 3') and L1110 (5' – TCRCARTTVTCCATGATRAAVAC – 3') (Skantar and Carta, 2005).

PCR products were purified after amplification using ExoSAP-IT (Affymetrix, USB products, High Wycombe, UK). For partial *hsp90* and *COI* genes the PCR products were cloned into the pGEM-T Easy vector (Promega Corporation) and transformed into *Escherichia coli* JM 109 High Efficiency Cells (Promega Corporation) according to the manufacturers' instructions. Several *E. coli* colonies were studied using PCR analysis with the primers used in their respective PCR. The plasmid DNA containing the insert from the positive *E. coli* colonies or PCR products were quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct sequencing in both directions using the primers referred above. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyser; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA), at the Servicio Centralizado de Apoyo a la Investigación (SCAI), University of Córdoba sequencing facilities (Córdoba, Spain). The newly obtained sequences were submitted to the GenBank database under accession numbers KC618462-KC618475.

Phylogenetic analysis

D2D3 and ITS1 rDNA and sequences of different Heteroderinae from GenBank were used for phylogenetic reconstruction and *Cryphodera brinkmani* Karssen & Van Aelst, 1999 (JQ965677, DQ328705, JQ965678 and AF274418, respectively) as outgroup taxa, while for the partial 18S *Radopholus similis* (Cobb, 1893) Thorne, 1949 (AJ966502) was used as outgroup. *COI* and *hsp90* genes were studied with a more widely taxon sampling from GenBank, using *Meloidogyne hispanica* Hirschmann, 1986 (JX683713) and *Bursaphelenchus doui* Braasch, Gu, Burgermeister & Zhang, 2005 (GU373910) as outgroups, respectively. The newly obtained and published sequences for each gene were aligned using Clustal W (Thompson et al. 1994) with default parameters. Sequence alignments were manually edited using BioEdit (Hall et al. 1999). Phylogenetic analyses of the sequence data sets were performed with Maximum Likelihood (ML) using PAUP * 4b10

(Swofford 2003) and Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). The best fitted model of DNA evolution was obtained using jModelTest v. 2 (Darriba et al., 2012) with the Akaike Information Criterion (AIC). The Akaike-supported model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then used in phylogenetic analyses. BI analysis for D2-D3 expansion segments of 28S rDNA, ITS1 and partial 18S regions under the TIM3+I+G, TVM+I+G and TIM3ef+I+G models, respectively, were initiated with a random starting tree and run with the four Metropolis-coupled Markov chain Monte Carlo (MCMC) for 1×10^6 generations, 2×10^6 generations, and 2×10^6 generations, respectively. While for *COI* and *hsp90* genes a partitioned (site specific rate) rate model was specified for 2×10^6 generations. Models for *COI* and *hsp90* genes were 012110+I+G+F and TIM1ef+I+G, respectively. The MCMC were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding 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 clades. Trees were visualised using TreeView (Page 1996). In ML analysis the estimation of the support for each node was obtained by bootstrap analysis with 100 replicates and fast step replicates.

Histopathology

For histopathological observations naturally infected roots of corn cv Rixxer were gently washed free of adhering soil and debris. Individual infected and healthy root pieces were fixed in FAA (formaldehyde-acetic acid- alcohol) for a minimum of 48 h, dehydrated in tertiary butyl alcohol series (70-85-90-100%) and embedded in Histosec® embedding paraffin melting point 56-58 °C (Merck, Darmstadt, Germany). Embedded tissues were sectioned transversely and longitudinally at 10-12 µm with a rotary microtome, mounted on glass slides, stained with safranin and fast green (Johansen, 1940), mounted permanently in a 40% xylene solution of a polymethacrylic ester (Synocril 9122X, Cray Valley Products, NJ) and observed with an optical microscope. Images were taken with a Leica DFC 425 system.

Host-suitability test

In order to test the suitability of corn and rice cvs widely used in Italy as host for the Italian population of *H. elachista*, the inoculum was obtained from naturally corn-infected roots and soil from Bosco Mesola (Ferrara Province), which were harshly washed free of adhering cysts and soil, and cysts were extracted from soil by the routine sieving-decanting method (Fenwick, 1940). Extracted cysts retained on the 250-µm-pore sieve were counted and crushed to estimate their eggs and J2 content (Seinhorst and den Ouden, 1966). Numbers of cysts in the appropriate proportion to give a population density of 3,000 eggs and J2 per plant, were mixed thoroughly with autoclaved (121 ºC, 1 h, twice) sandy soil mixture (pH 7.2, sand > 99%, silt < 1%, clay < 1%, organic matter = 0.75%). Corn (cv PR 33) and rice (cv Baldo) seeds, were surface-disinfested with 2% NaOCl for 3 min and germinated on sterile, moistened filter paper in petri plates at 25ºC in the darkness for 48 to 72 h. Germinated seeds, selected for uniformity, were sown into 15-cm-diameter clay pots (one seed per pot) filled with 0.7 liters of the autoclaved soil mixture infested or not with nematodes. The pots were arranged on benches in a greenhouse at 25 ± 2 °C in a randomized complete block design with eight replications for each crop. Plants in pots were watered as needed and fertilized once a week with 100 ml of a 0.1%, 20-5-32 + micronutrients hydro-sol fertilizer solution (Haifa Chemicals Ltd., Haifa, Israel). Seventy six days after inoculation, plants were removed from pots and roots washed free of adhering soil, and the nematode population were recorded. Final nematode population density in the soil and roots of each pot was determined by processing the 700 ml of soil by Fenwick's method (Fenwick, 1940). Eggs and J2 in the soil and roots were counted by crushing cysts content (Seinhorst and den Ouden, 1966) and final nematode population density (*Pf*) in each pot was determined. The reproduction rate ($Rf = Pf/Pi$) was also calculated for the initial nematode population density.

Results

Field symptoms and morphological identification

Corn plants cv. Rixxer in commercial fields at Bosco Mesola (Ferrara Province) infected by the nematode showed severe decline with stunting, a patched distribution of field, and heavily infected roots with a high proliferation of short lateral roots (Fig. 1). Roots of infected plants had mature cysts and adult females visible on the root surface, and in the soil

(Fig. 1). Population density in naturally infested soil ranged from 12 to 14 eggs plus J2s per 100 cm^3 of soil. Cysts contained an average of 47 eggs each (range 20 to 74).

Mature cysts of *Heterodera* sp. from corn were of moderate- to small-size, light to dark brown in colour (Fig. 1), mainly lemon-shaped, ambifenestrate vulval cone with semifenestrae almost as wide as long (Fig. 1J,K), narrow vulval bridge and weak underbridge, and prominent dark brown bullae (Fig. 1L). Second-stage juveniles vermiform, slender and ventrally curved after heat fixation. Lip region hemispherical with three annuli, slightly offset from the rest of body. Stylet well developed with rounded basal knobs. Lateral field about one-fourth body diam. wide, with three incisures (Fig. 1H). Tail long, tapering uniformly to a finely rounded terminus with large hyaline region (Fig. 1G). The occurrence of males was very rare, because we did not observe any males at the time of sampling we speculated that the species may be parthenogenetic; only two specimens were detected (about 0.1% with respect to females). These specimens were used for morphological and molecular analysis, respectively. Males were recorded in the original description of the species, but they were not found in the Chinese and Iranian populations. Male measurements (in μm): L = 740; a = 34.3; b = 6.7; b' = 5.6; c = 105.7; c' = 0.6; stylet = 19.5; DGO = 3.5; excretory pore = 103; testis = 269; T = 36.4; spicules = 23; gubernaculum = 9.5. Male was characterized by a lip region hemispherical, offset from the rest of body and bearing four annuli. Stylet well developed with rounded basal knobs, sloping posteriorly (Fig. 2B), and spicules ventrally curved.

Figs. 1-2

Histopathology

Naturally nematode-infected roots of corn cv Rixxer usually showed the posterior portion of nematode females of the Japanese cyst nematode protruding from the root surface, but some females were also observed with the entire body embedded in the root tissues (Fig. 3). Our observations on comparative histological sections of healthy and *H. elachista*-infected corn roots confirmed that the nematodes successfully established permanent feeding sites which caused cellular alterations in the root cortex, endodermis, pericycle, and vascular cylinder by inducing the typical multinucleate cell syncytial-formations, feeding sites characteristic for *Heterodera* spp. and due to expansion of nematode female bodies (Fig. 3H). Syncytial cytoplasm was granular and dense, contained several variously sized vacuoles, hypertrophied nuclei and nucleoli (Fig. 3). These feeding sites include extensive

multinucleate syncytial with granular dense cytoplasm, thickened cell walls near the nematode lip region and many interrupted sectors between walls of components of the syncytium. No hyperplasia of tissues adjacent to syncytium was observed.

Fig. 3

Molecular characterization and phylogenetic relationships of *Heterodera elachista*

The amplification of D2-D3 expansion segments of 28S rDNA, ITS, partial 18S, partial *hsp90* and the partial *COI* regions yielded a single fragment of approximately 700 bp, 750 bp, 1600 bp, 350 bp and 400 bp, respectively, based on gel electrophoresis. D2-D3 expansion segments of 28S rDNA of *H. elachista* parasitizing corn in Northern Italy (KC618462 and KC618463) matched well (99% similarity) with the D2-D3 sequences of *H. elachista* deposited in GenBank (HM560843, JN202918-JN202922), differing from 1 to 3 nucleotides (779/782 identities); the next close Heteroderidae were unpublished species from China with accession numbers JX081320-JX081322 and JX566451 with 97% and 96% similarities, respectively. Intraspecific variability between the two female individuals and one male sequenced (KC618462-KC618465) was only 2 nucleotides in both sides of the shorter sequence (0.3-0.4% differences, 2-4 bp in 770-926 bp). ITS1 from *H. elachista* from Italy (KC618466-KC618469) matched well (98-99% similarity) with the ITS1 sequences of *H. elachista* deposited in the GenBank (JN202913-JN202917, JN864882-JN864884, HM560778-HM560779, JN257081-JN257082 and AF498391), followed by *H. cyperi* (AF274388) and *H. mothi* (AF498392) by 85% and 83% similarities, respectively. Both individuals sequenced showed 3 differences in this sequence (0.3% differences, 3 bp in 1172 bp) and the studied clones showed 1 mutation and 2 deletions between them (1172 nucleotides in length). The partial 18S (KC618470 and KC618471) was 98% similar to *Heterodera schachtii* Schmidt, 1871 (EU306355) (1603/1631 bp identities and 5 indels), followed by *H. avenae* (FJ040403) and *Heterodera betae* Wouts, Rumpenhorst & Sturhan, 2001 (FJ040404) with 98% similarities. Intraspecific variability between the two individuals sequenced was 4 nucleotides (0.2% differences; 4 bp in 1722 bp). The partial *hsp90* gene was similar to *H. zeae* (JQ316174) with 75% similarity (277/367 bp identities and 27 indels), followed by *Pratylenchus teres* Khan & Singh, 1974 (AF457584) and *H. avenae* (JQ316191) with similarities of 75% and 74%, respectively. Intraspecific variability between the two clones sequenced were 5 nucleotides (1.5% differences; 5 bp in 324 bp) and both sequences showed two introns with a low similarity to other nematodes. Finally,

the partial *COI* was 82% similar to *H. glycines* (HM640930) (311/378 identities and 0 indels) followed by *H. cardiolata* (HM640929) and *P. chalcoensis* (HM640928) with 82% and 80% similarities, respectively. Non intraspecific variability between the two clones sequenced was observed.

Phylogenetic relationships among heteroderid nematodes were inferred from analyses of D2-D3 expansion segments of 28S rDNA, ITS1, partial 18S, partial hsp90 and partial COI. Trees using BI and ML are given in Figures 4-8. D2-D3 expansion segments of 28S rDNA and ITS1 showed a separation of the genus *Heterodera* in different groups based on vulval cone structures, *viz. Afenestrata*, *Avenae*, *Cyperi*, *Goettingiana*, *Humili*, *Sacchari*, and *Schachtii* (Subbotin et al., 2010). In D2-D3 phylogenetic tree (Fig. 4), *Cyperi* group formed a homogenous sister clade with *Avenae* and *Schachtii* groups, except for *H. cardiolata (= H. cynodontis*, DQ328698) which clustered with species of *Afenestrata* group. Similarly, in ITS1 tree (Fig. 5) *Cyperi* group formed a homogenous sister clade with *Schachtii, Humuli, Sacchari,* and *Avenae* groups, except for *H. cardiolata (= H. cynodontis*, AF274386) which clustered with *H. bifenestra* Cooper, 1955 (AY569020) and *H. orientalis* (Kazachenko, 1989) Mundo-Ocampo, Troccoli, Subbotin, Cid del Prado, Baldwin & Inserra, 2008 (EU284041).

Figs. 4-8

Host-suitability test

Both corn cv PR 33 and rice cv Baldo were infected and allowed reproduction of *H. elachista* from Italy. Observations of nematode-infected roots of corn and rice showed the posterior portion of abundant nematode females of the Japanese cyst nematode protruding from the root surface. Reproduction rates of *H. elachista* were 12.77 and 12.22 times *Pi* on corn and rice, respectively.

Discussion

The primary objective of this research was to provide an unequivocal diagnosis and bionomics of a cyst-forming nematode attacking corn in northern Italy. Morphology and morphometrics of cysts, second-stage juveniles and male (Table 1) fit closely with those of the original description of the Japanese cyst nematode, *H. elachista,* from upland rice in Japan (Ohshima, 1974), as well with subsequent reports in upland rice in Iran (Tanha Maafi et al., 2004), and in hilly rice fields in China (Ding et al., 2012), except for minor

intraspecific variations (Table 1). Because of these morphological traits the population of *H. elachista* infecting corn in Northern Italy belongs to the *Cyperi* group characterized by J2 with three incisures in the lateral field, vulval cones of cysts with bullae poorly developed or altogether absent, weak or absent underbridge. *Cyperi* group includes species infecting monocot, and distributed in tropical and subtropical regions of the world, *viz*. *Heterodera canadensis* Mulvey, 1979, *Heterodera cardiolata* Kirjanova & Ivanova, 1969, *Heterodera cyperi* Golden, Rau & Cobb, 1962, *H. elachista*, *Heterodera graminis* Stynes, 1971, *Heterodera graminophila* Golden & Birchfield, 1972, *Heterodera longicolla* Golden & Dickerson, 1973, *Heterodera mothi* Khan & Husain, 1965, *H. oryzicola* Rao & Jayaprakas, 1978, *Heterodera pakistanensis* Maqbool & Shahina, 1986, *Heterodera phragmitidis* Kazachenko, 1986, and *Heterodera raskii* Basnet & Jayaprakash, 1984, from which morphologically differs by several diagnostic features (Nobbs et al., 1992; Subbotin et al., 2010). *Heterodera elachista* infecting corn in Northern Italy is morphologically close to other cyst-nematodes attacking rice (*H. oryzae* and *H. sacchari*) but differs in some morphological characters including, considerably smaller cysts, lack of finger-like projections on the underbridge, and a shorter J2 body length (Nobbs et al., 1992; Subbotin et al., 2010). Furthermore, *H. elachista* from Northern Italy is morphologically clearly separated from other cyst nematodes attacking corn (*viz. H. avenae*, *H. oryzae*, *H. zeae*, *P. chalcoensis*, and *V. zeaphila*) by the structure and morphology of the vulval cone, as well as morphometrics of cysts, J2 and males in general (Subbotin et al., 2010). In particular, these morphological features clearly distinguished this species from the corn cyst nematode, *H. zeae*, by ambifenestrate vulval cone, significantly larger cysts, lateral fields of second-stage juveniles with four incisures and a longer tail and hyaline region (Subbotin et al., 2010).

The histopathological changes induced by *H. elachista* on corn are quite similar to those induced by other cyst nematodes on corn, including *Punctodera chalcoensis* on cv Criollo Chalqueño in Mexico (Suarez et al., 1985) or *H. avenae* on variety Pride 5 (Johnson and Fushtey, 1966). Syncytia in corn tissues were initiated in cortical cells from where they eventually extended into the vascular tissues, as reported for other cyst nematodes (i.e. *H. glycines* Ichinohe, 1952 in soybean, Endo, 1964). The development and parasitic habit of *H. elachista* on corn confirmed a typical susceptible reaction (Subbotin et al., 2010). Feeding sites are metabolic sinks sequestering nutrients from the host plants and limiting water and nutrient translocation from infected roots to above ground plant tissues (Hussey and Williamson, 1997). Results also suggested that these infections support successful cyst nematode reproduction that increases the soil inoculum for successive growing seasons.

The partial 18S, partial *hsp90* and partial *COI* sequences were obtained for the first time for this species in this study. Consequently, species identification based on sequencing of rDNA regions (D2-D3 and ITS1) was congruent with morphological and morphometrical data of this species in other studies (Tanha Maafi et al., 2003; Ding et al., 2012), and resulted useful for species identification. Additionally to these molecular markers, we have showed that differences in partial *COI* and *hsp90* genes could separate this species form other closely related. While the partial 18S showed few differences when comparing with other species. However, the partial *hsp90* genes has two introns in the sequence and these regions displayed a strong variability to the sequences in comparison to other PPN species, but it makes the alignment for phylogenetic analysis more difficult, and for this reason, only coding regions were used in this case. Phylogenetic relationships inferred from analyses of D2-D3 expansion segments of 28S rDNA and ITS1 showed a separation of the genus *Heterodera* in different groups. However, these clades are better supported in ITS1 than in D2-D3. Consequently, additional research is needed to clarify if the mentioned accessions of *H. cardiolata* are misidentifications or if the *Cyperi* group showed in fact a polyphyletic origin. Partial 18S showed similar clades and groups, but only terminal clades were supported by BI and ML (Fig. 6). This could be because of the low number of differences among species within this gene, as well as the different sizes in the sequences obtained from GenBank. In order to find if a phylogenetical signal was present in this gene, a permutation test ($P \leq 0.01$) was performed. This grouping is in accordance to other studies using rDNA sequences (Zhuo et al., 2013; Subbotin et al., 2001; 2010). Partial hsp90 gene is also a good marker for species identification, but the position of some species is not congruent with the general phylogeny using rDNA markers (Subbotin et al., 2006; Holterman et al., 2008) or phylogeny of partial COI in this study (Fig. 8). The position of *H. elachista* using this gene is not congruent with the majority of *Heterodera* spp. This incongruence is difficult to explain and their support values are low (Fig. 8). In our case, this gene alignment showed only a small variation in the aminoacid sequence in the species analysed, being the majority of mutation synonymous and in the third nucleotide in the codon position. Additionally, the performed permutation test showed a small, but a significant phylogenetic signal is in this gene. However, when species with more variability in the sequence and well supported in our phylogeny were removed from the analysis (*Meloidogyne* and *Ditylenchus* spp.) the permutation test could not be performed, which could be affected by random synonymous mutations the permutation test was unable to be performed, which could be a reason of random synonymous mutations. Consequently, molecular characterization of the Italian cyst

nematode infecting corn clearly separated this species (*H. elachista*) from the corn cyst nematode (*H. zeae*) as showed by differences in D2-D3, ITS1, and hsp90 genes (Figs. 4, 5, 8).

The results of the host-suitability test indicate that both rice and corn are good hosts for this nematode, and enlarge the host-range of this cyst nematode which had only been reported previously in rice in Japan, Iran and China (Ohshima, 1974; Tanha Maafi et al., 2004; Ding et al., 2012).

Conclusion

The dispersal of plant-parasitic nematodes and particularly cyst-forming nematodes is an essential component for spread of these pathogens and may occur within a field or across continents. As concluding remarks we would like to point out that the spread of this nematode to rice or corn growing areas in Europe should be avoided, since may constitute a serious potential risk to the European rice and corn production. Furthermore, additional research is needed in order to clarify if the occurrence of this nematode in corn in northern Italy may constitute a disease focus. Also, information about the genetic structure of this European and the three Asiatic populations of *H. elachista* is needed, which may offer insights into the evolutionary potential of this pathogen, as well as to provide intra-and inter-population genetic variability in order to elucidate the origin and/or dispersal followed by successful reproduction (gene flow) at various spatial scales.

In summary, the present study enlarges the knowledge of the geographical distribution and the molecular characterization of the Japanese cyst nematode *H. elachista*, and provides additional molecular markers for precise and unequivocal diagnosis of this species (partial *COI* and partial *hsp90*), which may help for effective quarantine inspection and appropriate application of exclusion principles. Furthermore, taking into consideration the limited host-range of this nematode, including two of the three world's most important crops and the high risk of its dispersal into new areas, special attention is needed for avoiding the distribution of this nematode by movement of soil on equipment and plant parts, water, and contaminated containers infested soil, agricultural practices or any other indirect mechanism.

Acknowledgements

Authors thank Dr. Valerio Finetti of Bayer Crop Science Srl., Milan, Italy; Mr. Antonio Carella of Horto Service, Noicattaro (BA), Italy, and Dr. J.A. Navas-Cortés (IAS-CSIC) for critical reading of the manuscript before submission. The present work was supported with funds provided by the Italian Ministry of Economy and Finance to the National Research Council for the project "Innovazione e Sviluppo del Mezzogiorno-Conoscenze Integrate per Sostenibilità ed Innovazione del Made in Italy Agroalimentare-Legge n. 191/2009, and partially by grant AGR-136 from 'Consejería de Economía, Innvovación y Ciencia' from Junta de Andalucía and the European Social Fund.

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Figure legend

Fig. 1. Root infection and light microscopy micrographs of *Heterodera elachista* detected in Italy. A, B: root segments of corn with cysts protruding from the root surface. C: Newly formed and mature lemon shape cysts. D: Juveniles and a mature cyst. E-H: entire body, anterior and posterior body portions, and detail view of lateral field of second-stage juvenile, respectively. I: terminal vulval cone. J –L: fenestral cone features at different focus. M: Detail of cyst contained embrionated.(Scale bars: A,B = 1 mm, C,E,I-M = 50 µm; D = 200 µm; F-H = 20 µm).

Fig. 2. Light micrographs of male morphological features. A: entire body. B: lip region. C: tail region. (Scale bars: A = 50µm; B,C = 20 µm).

Fig. 3. Histopahological changes induced by *Heterodera elachista* in corn roots. A: Cross section of uninfected root , showing healthy cortical and stelar vascular tissues. B –E: Cross sections of infected roots showing large cortical syncytia (CS). F, G : Cross sections of infected roots showing stellar syncytia (SS). H: Longitudinal section showing a senescent stelar syncytium associated with a mature cyst with its entire body embedded in the root tissues.(Scale bars: A,B,C = 100 µm¸D –H = 50 µm). (Abbreviations used: c = cortex; en = endodermis, x = xylem, p = pith, cs = cortical syncytium, ss = stelar syncytium; n = nematode)

Fig. 4. Phylogenetic relationships within the genus *Heterodera*. Bayesian 50% majority rule consensus trees as inferred from D2-D3 expansion segments of 28S rRNA gene sequence alignment under the TIM3 + I + G model. Posterior probabilities more than 65% are given for appropriate clades (in bold letters); bootstrap values greater than 50% are given on appropriate clades in ML analysis. Newly obtained sequence in this study are in bold.

Fig. 5. Phylogenetic relationships within the genus *Heterodera*. Bayesian 50% majority rule consensus trees as inferred from ITS1 rRNA gene sequence alignment under the TVM + I + G model. Posterior probabilities more than 65% are given for appropriate clades (in bold letters); bootstrap values greater than 50% are given on appropriate clades in ML analysis. Newly obtained sequence in this study are in bold.

Fig. 6. Phylogenetic relationships within Heteroderidae. Bayesian 50% majority rule consensus trees as inferred from partial 18S rRNA gene sequence alignment under the TIM3ef + I + G model. Posterior probabilities more than 65% are given for appropriate clades (in bold letters); bootstrap values greater than 50% are given on appropriate clades in ML analysis. Newly obtained sequence in this study are in bold.

Fig. 7. The 50% majority rule consensus tree from Bayesian analysis generated from the partial *COI* gene dataset with 012110 + I + G + F model. Posterior probabilities more than 65% are given for appropriate clades; bootstrap values greater than 50% are given on appropriate clades in ML analysis. Newly obtained sequence in this study are in bold.

Fig. 8. The 50% majority rule consensus tree from Bayesian analysis generated from the *hsp90* gene dataset with TIM1ef + I + G model. Posterior probabilities more than 65% are given for appropriate clades; bootstrap values greater than 50% are given on appropriate clades in ML analysis. Newly obtained sequence in this study are in bold.

Table 1. Selected morphometrics of the Italian population of *Heterodera elachista* in comparison to previous records (all measurements in μm).

Population	Italy (Bosco Mesola, Italy)	Japan (Ohshima, 1974)	Japan (Nobbs et al, 1992)	Tonekabon. Iran (Tanha Maafi et al., 2004)	Hunan, China (Ding et al., 2012)
Cyst(n)	10	-	50	39	20
Length (excl. neck)	404 ± 66.6 (278-484)	-	446.3 ± 30.3 (328-557)	431 ± 48 (340-530)	338.9 ± 63.7 (354-586)
Width	253 ± 34.8 (207-321)	-	322.6 ± 26.0 (229-449)	311 ± 48 (25-540)	354.5 ± 60.1 (283-495)
Length/width	1.6 ± 0.2 (1.3-2.0)	-	1.4 ± 0.1 (1.1-1.9)	1.4 ± 0.2 (1-1-1.9)	1.4 ± 0.2 (1.1-1.9)
Vulval areas (n)	-	10	10	10	-
Fenestral length	42 ± 2.7 (38-47)	28.5 (25-37)	29.3 ± 2.0 (23-36)	40 ± 6.7 (30-50)	37.4 ± 5.0 (30-50)
Fenestral width	35 ± 4.4 (23-39)	29.8 (25-37)	32.0 ± 2.5 (26-39)	31.2 ± 4.7 (26-42)	35.1 ± 7.1 (25-47.5)
Vulval bridge width	-	-	5.8 ± 2.0 (2-16)	-	-
Underbridge length	-	78.3 (75-90)	72.0 ±2.9 (65-78)	85	83.4 ± 8.2 (70-95)
Underbridge width	-	8.9(8-12)	13.5 ± 3.2 (10-23)	-	-
Vulval slit length	30 ± 2.1 (26-33)	36 (30-43)	37.5 ± 2.8 (26-46)	40 ± 3.8 (32-45)	40.3 ± 9.1 (30.3-55.5)
Vulva-anus distance	-	39.4 (30-46)	-	-	-
Juveniles (n)	10	25	20	10	20
L	436 ± 19 (411-470)	367 (330-405)	402 ±10.0 (377-450)	391 ± 11 (372-410)	461.6 ± 34.5 (404-525)
a	22.4 ± 0.9 (21.2-24.9)	22.6(20.3-24.1)	24.6 ± 0.9 (21.3-28.1)	22. 5 ± 1.0(20.8-24)	22. 5 ± 1.0(20.8-24)
b		4.2 (4.0-4.6)	4.9 ± 0.3 (3.8-6.3)	4.3 ± 0.3 (3.9-4.9)	4.3 ± 0.3 (3. 9-4.9)
c	7.4 ± 0.3 (7.0-7.9)	7.2 (6.7-8.1)	7.0 ± 0.4 (5.4-8.4)	6.7 ± 0.3 (5.9-7.2)	6.7 ± 0.3 (5.9-7.2)
c’	4.7 ± 0.3 (4.4-5.1)				
Stylet (S)	20 ± 0.5 (19.5-20.8)	18.6 (18-19.5)	19.2 ± 0.8 (16-21)	20 ± 0.7 (18-21)	22.5 ± 1.1 (20-25)
Lip region height	-	3.3 (3.0-3.6)	-	3	-
Lip region diam.	-	7.7 (7.2-8)	-	7.8 ± 0.4 (7-8)	-
DGO	-	5.5 (4.5-6)	-	5.2 ± 0.8 (4-7)	-
Anterior end to median bulb valve	-	56.4 (52-60)	-	59 ± 3.6 (52-67)	-
Anterior end to excretory pore	-	-	-	84 ± 2.8 (79-88)	-
Pharynx length	-	88.8 (84-97)	82.8 ± 3.9 (66-99)	-	-
Body diam. at mid-body	19 ± 0.6 (18.3-20.2)	16.6 (15.5-18)	-	17.4 ± 0.8 (16-19)	-
Body diam. at anal level	12.4 ± 0.5 (11.3-13.0)	10.4 (9-11)	11.4 ± 0.8 (10-15)	10.2 ± 0.6 (9-11)	-
Tail length	58.8 ± 1.7 (56.3-61.2)	52.7 (44-57)	57.8 ± 2.9 (47-70)		67.3 ± 6.9 (60-87.5)
Tail hyaline region (H)	33.1 ± 2.5 (29.0-37.7)	31.4 (26-36)	-	32 ± 3.0(25-39)	37.5 ± 6.4 (30-50)
H/S	-	1.7 (1.4-1.9)	-	1.6 ± 0.1 (1.3-1.9)	-
L/MB	-	-	-	6.7 ± 0.4 (5 .6-7.4)	-