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Molecular and Morphological Characterization of the Corn Cyst Nematode, *Heterodera zeae*, from Greece

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Abstract: The corn cyst nematode *Heterodera zeae* was detected in soil from an organic maize field in northern Greece. In greenhouse studies, reproduction of *H. zeae* was detected on maize plants (*Zeae mays*) using soil high in organic matter; the field was under winter fallow at the time of sampling. Maize plants were grown in a greenhouse with soil from the affected field used as inoculum. Females appeared after six weeks incubation, and abundant cysts were present after 12 weeks. Morphological and molecular diagnosis confirmed the presence of *H. zeae* in the field. Cysts were identified on the basis of cyst shape and characteristics of the cyst terminal cone, including nature of fenestration, presence of bullae, cyst wall pattern, and fenestral diameter. Second-stage juveniles were identified by body and stylet length, the shape of stylet knobs, shape and length of the tail and hyaline tail terminus, and by the number of lateral lines. Molecular analysis included amplification of the ribosomal internal transcribed spacer regions (ITS 1&2 rDNA) 28S large ribosomal subunit (LSU) D2-D3 expansion segment, and partial 18S small ribosomal subunit (SSU). Restriction fragment length polymorphism (RFLP) of ITS rDNA exhibited several unique enzyme patterns that may be diagnostically useful for *H. zeae*. These findings are in agreement with prior analysis of *H. zeae* populations from the U.S. and India. Phylogenetic relationships inferred from ITS rDNA are congruent with previous analyses that placed *H. zeae* in a clade with *H. turcomanica, H. salixophila* and species of the *Humuli* group. Phylogenetic trees based upon heat shock protein (Hsp90) coding sequence were in general agreement with a prior study using the same marker. This study represents the first record of *H. zeae* in Greece and the second report of this nematode in Europe.

Key words: Corn cyst nematode, *Heterodera zeae*, ITS rDNA, restriction fragment length polymorphism, maize.

The corn cyst nematode *Heterodera zeae* Koshy, Swarup & Sethi, 1971 was originally described on maize in India (Koshy et al., 1971). It was later reported to have wide distribution in that country and was also found in Egypt and Pakistan (Swarup and Sosa-Moss, 1990), Nepal (Sharma et al., 2001), and Thailand (Chinnasri et al., 1995). Within the U.S., *H. zeae* was first found in Maryland, primarily in heavy silt-clay soils at fairly low densities (Sardanelli et al., 1981). It has since been reported in Virginia (Eisenback et al., 1993). Within Europe, this nematode has been found in Portugal (Correia and Abrantes, 2005).

Substantial damage to maize due to *H. zeae* has been reported in India (Srivastava and Sethi, 1984). High soil temperatures in India and Egypt may account for the economic importance of *H. zeae* on maize in those countries (Hashmi et al., 1993). In Greece, there is limited information on nematodes attacking cereals, and the only cyst nematode reported so far has been *H. avenae* on wheat (Hirschmann et al., 1966; Kyrou, 1976). In May 2009, a soil sample from Paleochori Kavallas (Northern Greece) was investigated for the presence of nematodes. The sample was from a field with soil high in organic composition that was annually cultured with maize in summer but was under winter fallow at the time of sampling. In previous years, the maize plants in this field had exhibited symptoms of stunting and wilting.

The soil samples contained abundant cysts of a *Heterodera* species (Fig.1A). Soil from the field site was used to fill 1.5 L pots, which were planted with maize (*Zea mays*) and grown in a greenhouse. Females appeared after six weeks incubation, and abundant cysts were present after 12 weeks (Fig.1B,C). Morphological and molecular approaches to confirm the identity of *Heterodera zeae* and to investigate its phylogenetic relationships with other *Heterodera* species are described herein.

Materials & Methods

Cysts were obtained from soil samples taken from the affected field. Second stage juveniles (J2s) for morphological observations were recovered from cysts kept in water in watch glasses. Juveniles were fixed in 3% formaldehyde and processed to glycerine by the formalin-glycerine method (Hooper, 1970; Golden, 1990). Cysts were fixed for 12 hours in 3% formaldehyde solution. Light microscopic images of cyst vulval cones and juveniles were taken with an automatic 35 mm camera attached to a Leica Leitz DMRB compound microscope having an interference contrast system. Measurements were made with an ocular micrometer on this same compound microscope.

Molecular characterization of the *H. zeae* population included analysis of ribosomal (rDNA) and protein-coding genes. Single J2 were mechanically disrupted with sharp forceps tips in 20 µl nematode extraction buffer (Thomas et al., 1997) and stored at -80°C. To prepare DNA extracts, frozen nematodes were thawed, 1 µl proteinase K (from 2 mg/ml stock solution) was added, and the tubes were incubated at 60°C for 60 min, followed by 95°C for 15 min to deactivate the proteinase K. Two or

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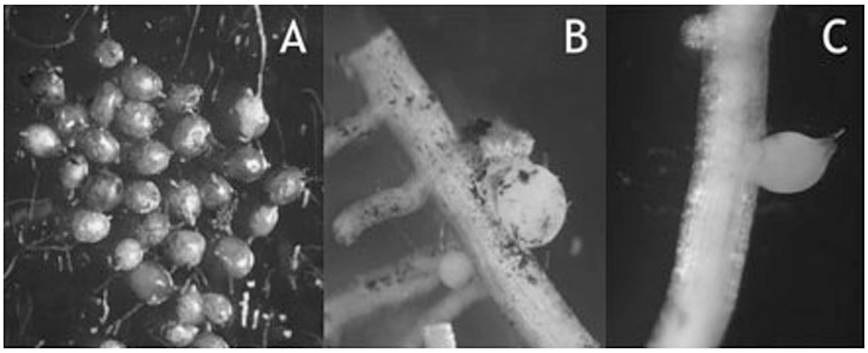

FIG. 1. *Heterodera zeae* from Greece. A) Cysts. B,C) females attached to maize roots.

five microliters of extract were used for each PCR reaction.

PCR amplification and cloning: The internal transcribed spacer (ITS rDNA) region, which includes ITS1, 5.8S rRNA coding sequence, and ITS2 was amplified using 0.2 µM each of primers TW81 [5'-GTTTCCGTAGGT GAACCTGC-3'] and AB28 [5'-ATATGCTTAAGTTCA GCGGGT-3']. Reactions also contained 2-3 µl nematode DNA extract, 200 µM dNTP, 1U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), and supplied enzyme reaction buffer in a total volume of 25 or 50 µl. Cycling included one step of 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec, finished with one cycle at 72°C for 5 min. Amplification of the 28S large ribosomal subunit (LSU) D2-D3 expansion segment included the primers D2A [5'-ACAAGTACCGTGAGGGAAAGTT-3'] and D3B [5'-TCGGAAGGAACCAGCTACTA-3'] and were amplified as previously described (De Ley et al., 2005; Ye et al., 2007). Partial 18S (small subunit: SSU) sequence was amplified with the primers 18s1.2 [5'-GGCGATCAGA TACCGCCCTAGTT-3'] and 18sr2b [5'-TACAAAGGG CAGGGACGTAAT-3']. Cycling conditions were: 1 cycle of 94°C for 2 min, followed by 40 cycles of 94°C for 20 sec, 59°C for 30 sec, and 72°C for 30 sec, and finishing with 1 cycle of 72°C for 5 min. Heat shock protein 90 (Hsp90) fragments were amplified with degenerate primers U288 [5'-GAYACVGGVATYGGNATGACYAA-3'] and L1110 [5'-TCRCARTTVTCCATGATRAAVAC-3'] as previously described (Skantar and Carta, 2004). All PCR products were analyzed by electrophoresis on 2% agarose with 1X SB (sodium borate-EDTA) buffer. Gels were stained with ethidium bromide and visualized using the U:Genius gel documentation system (Syngene, Frederick, MD).

PCR products from ITS rDNA and Hsp90 were each cloned using the Strataclone PCR Cloning Kit (Agilent, Santa Clara, CA) according to manufacturer's instructions. Plasmid clone DNA was prepared with the QiaPrep Spin Miniprep Kit (Qiagen, Valencia, CA) and digested with Eco RI to verify the presence of insert. Sequencing was performed at the University of Maryland Center for Biosystems Research. Two new ITS sequences were obtained from a single J2 (GU145613 and GU145614), and three from a second J2 (GU145615, GU145616, and GU145611). Direct sequencing of PCR amplicons was used to obtain the 28S sequence (GU145612) and the 18S rDNA sequence (HQ724313). New Hsp90 sequences obtained from clones were given accession numbers JQ316172-JQ316193.

For PCR RFLP, ITS amplifications were performed as above in 50 µl reactions with all components increased two-fold. Seven microliters of each amplicon were analyzed on an agarose gel and the rest was purified using the Qiaquick PCR Purification Kit (Qiagen). DNA was quantified on a NanoDrop ND-8000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Each restriction digest included enzyme, supplied 10x reaction buffer diluted to 1x, and 200 ng PCR product in a volume of 20 µl. Digests were incubated at the recommended temperature for each enzyme (Table 1) for a minimum of two hours. Products were separated on 2% agarose/SB as above.

Phylogenetic analysis: Raw sequences were processed in Sequencher 5.0 (Genecodes, Inc., Ann Arbor, MI); alignments were created and manually trimmed using Geneious Pro 5.5.6 (Drummond et al., 2011). ITS sequences of the new isolate of *H. zeae* were aligned with *H. zeae* ITS 1-5.8S sequences from India and the U.S. Maximum Parsimony (MP) analysis was conducted in PAUP* version 4.0b10 (Swofford, 2002), with *H. goettingiana* used as the outgroup. For MP we used a heuristic search with ten replicates of random taxon addition and TBR branch swapping to find the most parsimonious tree; gaps were treated as missing data. Support for branches was determined with 1000 bootstrap replicates, a heuristic search, and simple sequence addition.

Full-length ITS1 - 5.8S - ITS2 and selected *Heterodera* sequences from GenBank were aligned using the plugin for MUSCLE (Edgar, 2004) integrated within Geneious, with further refinements of the alignment either done manually or via Geneious algorithms. A trimmed alignment of 1124 characters was used for subsequent analyses, with *Globodera pallida* specified as the outgroup. Maximum

TABLE 1. Restriction fragment sizes predicted or observed after digestion of ITS rDNA amplicons from *Heterodera zeae*.

	Restriction fragment sizes observed after digestion of ITS rDNA amplicons						
Enzyme →	Alu I 541, 501, 303, 233, 180	Ava I 930, 114	Bsr I 1024, 957, 891, 822, 224, 157, 99, 80	Hha I 396, 246, 114, 89, 73, 59	Bst NI 781, 619, 282, 139, 91,73	Rsa I 938, 854, 807, 146, 75	Scr FI 773, 615, 280, 139
Clone #	Restriction fragment sizes predicted from sequence data						
2301	307/308, 236, 182	918, 115	647, 234, 80, 72	387, 245, 112, 89, 70, 56, 55, 19	610, 281, 142	1012, 21	610, 281, 142
2302	307/308, 236, 182	918, 115	647, 234, 80, 72	388, 245, 112, 89, 70, 56, 55, 19	611, 281, 142	1012, 21	611, 281, 142
2303	307/308, 236, 182	918, 115	647, 234, 80, 72	389, 245, 89, 70, 56, 55, 19	610, 281, 142	1012, 21	610, 281, 142
2304	544, 308, 183	920, 115	649, 166, 140, 80	388, 245, 89, 70, 56, 55, 19	753, 282	792, 149, 73, 21	753, 282
2305	544, 308, 183	919, 72, 43	648, 166, 140, 80	387, 245, 89, 70, 56, 55, 19	752, 282	791, 149, 73, 21	752, 282

Likelihood (ML) analysis was performed using RAxML 7.2.8 Black Box (Stamatakis, 2006; Stamatakis et al., 2008) at the CIPRES Science Gateway (Miller et al., 2010; www.phylo.org). The data was analyzed according to the model GTR (general time reversible) with joint branch length optimization and 1000 rapid bootstrap inferences following a thorough ML search. Free model parameters were estimated by RAxML with 25 per site rate categories. Likelihood of the final tree was evaluated under GAMMA, with model parameters estimated to 0.10 Log Likelihood units. Bayesian Inference (Huelsenbeck and Ronquist, 2001) was performed via the CIPRES Gateway under the model GTR + I + G with random starting tree and four chains run for 1,000,000 generations with sampling every 500 chains. Two runs were performed for each analysis. Log-likelihood stabilized after approximately 1000 generations. Burnin equal to 500 samples was discarded and the data evaluated for convergence. The remaining samples were used for further analysis to generate a 50% majority rule consensus tree, with posterior probabilities listed on the appropriate clades.

Hsp90 sequences were aligned in Geneious. Two Hsp90 alignments were analyzed: a 216 bp alignment that allowed inclusion of shorter Hsp90 sequences available from GenBank; and a longer 813 bp alignment that included all five *H. zeae* Hsp90 clone sequences but fewer taxa. For both alignments, ML analysis was performed using RAxML as above, except that the data included two distinct model/data partitions (with the first and second codon positions treated together and the third position separately), with joint branch length optimization and 1000 rapid bootstrap inferences following a thorough ML search. Free model parameters were estimated as described above. Bayesian inference was performed via the CIPRES Gateway, with the data partitioned according to codon position and each set of parameters estimated separately. The analysis was run for 1,000,000 generations with sampling every 1000 chains. Two runs were performed for each analysis. Log-likelihood stabilized after approximately 1000 generations. Burnin equal to 2000 samples was discarded and the data evaluated for convergence. Remaining data were used to generate a 50% majority rule consensus tree, with posterior probabilities listed on the appropriate clades.

RESULTS AND DISCUSSION

Morphological characterization: Morphological characters used for identification of cysts (Fig. 2A-E) included cyst shape and characteristics of the cyst terminal cone including nature of fenestration, presence of bullae, cyst wall pattern, and the fenestral diameter (Koshy et al., 1971; Golden & Mulvey, 1983; Golden, 1986). Cysts (n = 5) were lemon shaped and light brown in color (Fig. 1A). The cuticle was thin walled and the cyst wall had a zigzag pattern. The vulval cone was ambifenestrate with horseshoe-shaped semifenestra. The cysts were characterized by body length including neck (range = 700 to 767 μm, mean = 739.4, st. dev. = 98.4), body width (400 to 550, 472.2, 63.5), L/W ratio = (1.2 to 1.4, 1.3, 0.1), neck length (50 to 100, 75.0, 17.6) and width (50 to 65, 56.0, 5.4), fenestra length (50 to 55 μm, 52.5, 2.5) and width (32 to 35, 33.6, 1.5), underbridge length (40 to 50, 45.0, 5.0), and width (11 to 15, 12.8, 2.0), vulval slit (36 to 40, 38.6, 2.3), and bullae located at two levels in vulval cone; level one immediately below (anterior) the underbridge and having four fingerlike bullae projecting inward; level two a mass of randomly located long, heavy bullae.

The morphological characters critical for identification of second-stage juveniles were body and stylet length, shape of stylet knobs, shape and length of tail and hyaline tail terminus, and the number of lines in

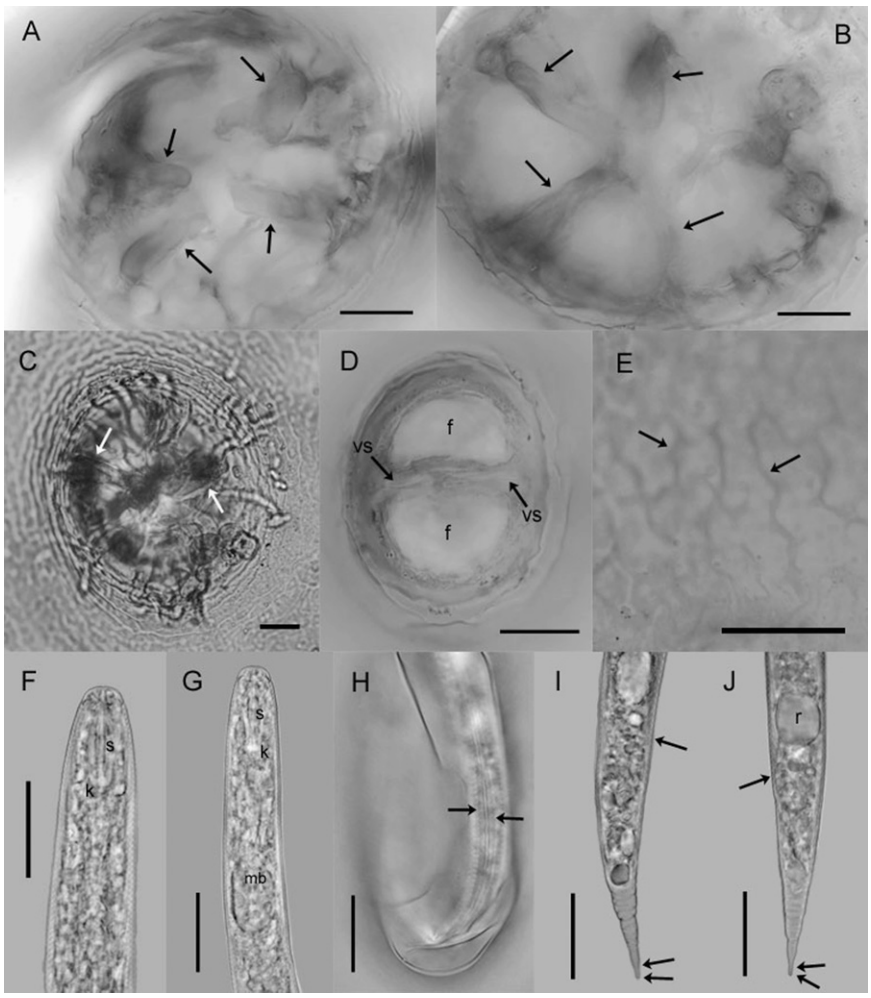

FIG. 2. Morphological features of cysts and second-stage juveniles for *Heterodera zeae* from Greece. A-C) Vulval areas of cyst cones showing finger-like projections (arrows) and other bullae. D) Vulval area of cyst cone showing ambifenestrate fenestra (f) and vulval slit (vs) along vulval bridge. E) Cyst wall showing zigzag patterns (arrows) in cuticle. F,G) Anterior regions of juveniles showing stylets (s), anchor-shaped stylet knobs (k), and median bulb (mb). H) Juvenile lateral field containing 4 lines (arrows). I,J) Posterior regions of juveniles showing anal areas (single arrows), rectum (r), and acutely rounded tail termini (double arrows). All scale bars are 20 μm.

lateral field (Koshy et al., 1971; Golden and Mulvey, 1983; Golden, 1986). Measurements of J2 (n = 10) included length of body (range = 367 to 400 μm, mean = 387, st. dev. = 9.6), stylet (19.7 to 20.0, 19.9, 0.1) with anchor-shaped basal knobs, tail (42.5 to 46.0, 42.9, 2.3), and hyaline tail terminal (20.0 to 22.5, 20.9, 1.2). The lateral field had four distinct lines. Tail short, conically tapering, with acutely rounded terminus, hyaline terminal about one-half tail length. Shapes of the tail, tail terminus, and stylet knobs were consistent with *H. zeae* type and other material as previously described (Golden and Mulvey, 1983) (Fig. 2F-J).

Molecular characterization: Because ITS heterogeneity within individual nematodes had been previously reported for *H. zeae* (Szalanski et al. 1997), multiple clones of ITS rDNA from the Greece population were sequenced. Intraclonal variation was 0.39 - 3.09%. These sequences fell into two distinct types as determined from restriction fragment sizes predicted from virtual clone digests (Table 1). One group included clones 2301, 2302, and 2303; the other group contained clones 2304 and 2305. Except for clone 2303, the sequence types segregated according to the individual J2 from which they came. Both J2 used for the PCR came from a single cyst. Therefore, it is likely that examination of clones from multiple J2 from distinct cysts would reveal the presence of additional sequence variation within individual J2, as was shown previously for populations from India and the USA (Szalanski et al., 1997).

ITS heterogeneity was also known from previous RFLP analysis of *H. zeae* populations from India (Umarao et al., 2008), as revealed in restriction patterns generated by several enzymes Alu I, Ava I, Msp I, Hha I, and Hinf I. Because only the ITS1 region was used for the Szalanski et al. (1997) RFLP study, a direct comparison with fragment sizes (Fig. 3) and that study was not possible. Cyst nematode RFLP data compiled by Subbotin et al. (2010b) did not include patterns for *H. zeae*, but comparison of the present data with that work shows patterns from several enzymes that appear to be unique to the species.

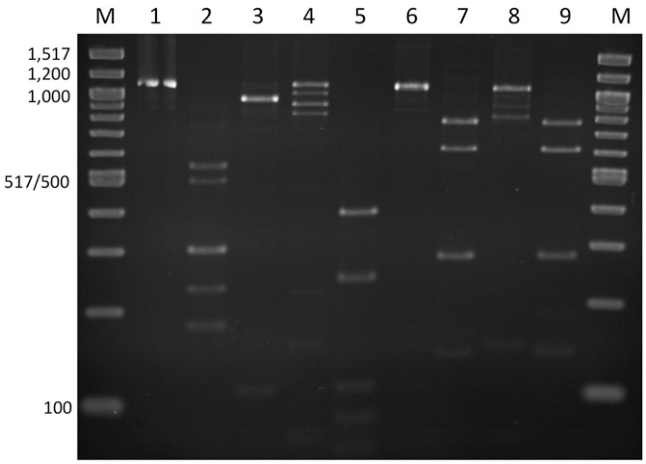

FIG. 3. PCR-RFLP profile of ITS rDNA from *Heterodera zeae* from Greece. Lane 1- undigested ITS1&2 PCR product; PCR products digested with resriction enzymes: lane 2 - Alu I; 3 - Ava I; 4 - Bsr I; 5 - Hha I; 6 - Pml I; 7- Bst NI; 8 - Rsa I; 9 - Scr FI; M- 100 bp ladder (New England BioLabs); sizes in bp to left of markers.

Sequences previously deposited in GenBank include only ITS1 - 5.8S. In the area overlapping with our sequences, identity ranged from 94 to 98%. This alignment included 675 characters of which 182 were parsimony informative. Sequences grouped into two clades that were independent of geographic location or individual specimen (Fig. 4).

Previous phylogenetic studies have placed *H. zeae* in a lineage near the *Humuli* group, with *H. turcomanica* and *H. salixophila* (Subbotin et al., 2010a). rDNA sequences extending from ITS1 through ITS2 were selected for comparison to *H. zeae* based on previously published phylogenetic trees. Taxa included representatives of the *Avenae, Humuli, Sacchari, Schachtii, Cyperi, Afenestrata,* and *Goettingiana* lineages. Two new *H. zeae* sequences and 24 known sequences were used for the phylogenetic analysis. An alignment containing 1124 characters was analyzed by Bayesian inference (Fig. 5). Tree structure and support values were congruent with Subbotin et al. (2010a; Volume 8A, Fig. 27), and placed *H. zeae* between *H. turcomanica* and *H. salixophila* with high posterior probability (PP).

Genomic sequences containing the central portion of the protein-coding Hsp90 gene were between 1140 and 1145 bp in length and contained five introns. Sequence divergence among *H. zeae* Hsp90 clones (not including degenerate primer ends) was ≤2.4%. Initial alignments of Hsp90 genomic sequences contained some large gaps that were difficult to resolve and gave rise to poorly supported trees (not shown). Therefore, introns were manually trimmed out and cDNA sequences aligned in Geneious, using *Globodera pallida* as the outgroup.

Alternative alignment methods were tested for Hsp90 in order to improve upon the low to moderate branch

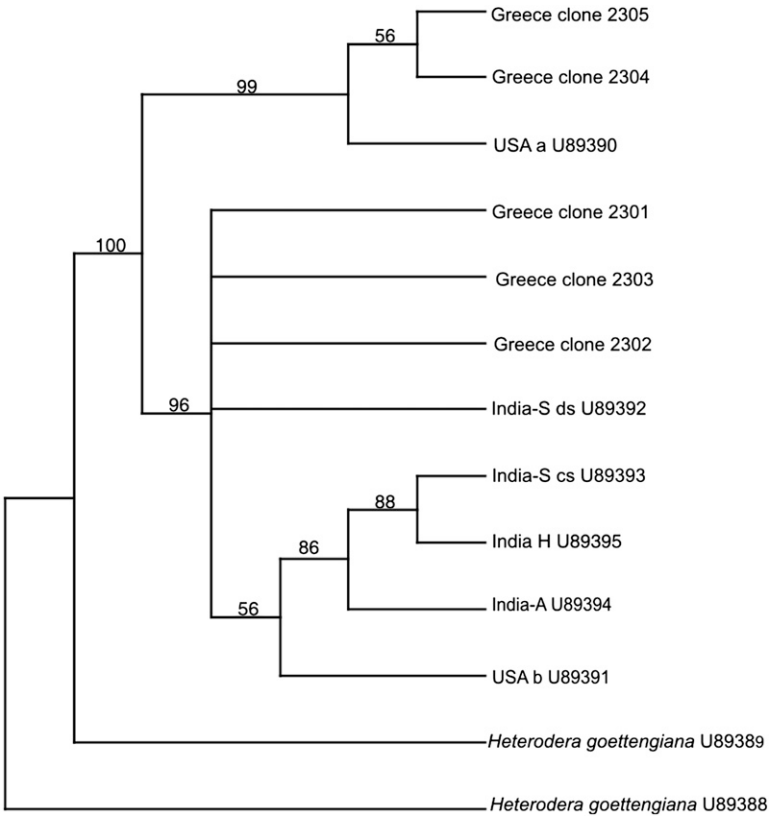

FIG. 4. Maximum parsimony tree based on alignment of *Heterodera zeae* ITS1 - 5.8S rDNA sequences.

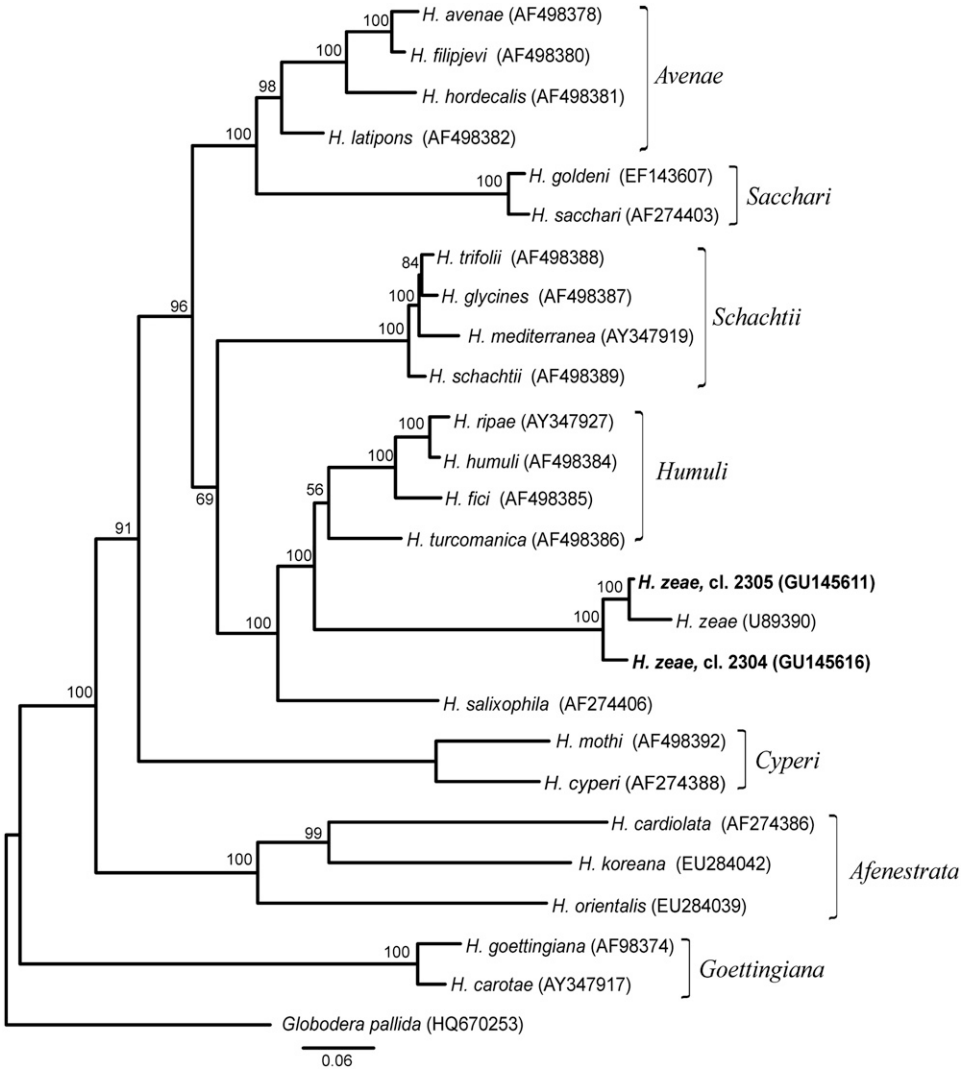

FIG. 5. Phylogenetic relationships among *Heterodera* species based on Bayesian 50% majority rule consensus tree from two runs as inferred from an 1124 bp alignment of 26 ITS1 - 5.8S - ITS2 rDNA sequences. Representative taxa from the major lineages included in the tree are: *Avenae, Sacchari, Schachtii, Humuli, Cyperi, Afenestrata,* and *Goettingiana. Globodera pallida* was used as the outgroup. Posterior probabilities are shown for appropriate clades. New sequences are marked in bold type.

support values in initial trees. The translation alignment feature within Geneious, that takes into account the alignment of projected amino acid sequences, did lead to modest increases in PP or bootstrap values on some branches. The charged linker region contains runs of positively and negatively charged amino acids (K, D, E) with similar codons that can sometimes be misaligned by software. These areas were checked by eye and adjusted as needed. Analysis of the protein sequence alignments revealed a lack of sufficient variation to distinguish many species, and was unable to improve upon the resolution of cDNA-based trees (not shown). The cDNA alignment used for the tree in Fig. 6 consisted of 813 bp with 24 sequences from six species. Pairwise sequence divergence among *H.* zeae sequences was ≤1.3%; and for *H. glycines*, ≤0.6%. Divergence between species ranged from 2.3 - 33.5%.

Bayesian inference and maximum likelihood analyses gave rise to similar trees with high support values for most branches (Fig. 6). *Heterodera carotae* showed the greatest divergence from the other species, due in part to significant gaps in the central charged linker region (located from base 292 - 580 in the alignment). However, exclusion of the gapped region from the phylogenetic analysis did not change the position of *H. carotae* in the tree nor significantly improve the support values; instead, structure for some branches was lost and the relationships were poorly resolved (not shown). *Heterodera glycines* and *H. schachtii* grouped together, consistent with their known position in the *Schachtii* lineage; the placement of *H. avenae* adjacent to this clade is also consistent with known relationships.

Using shorter but overlapping *Heterodera* Hsp90 sequences from GenBank, a second cDNA alignment of

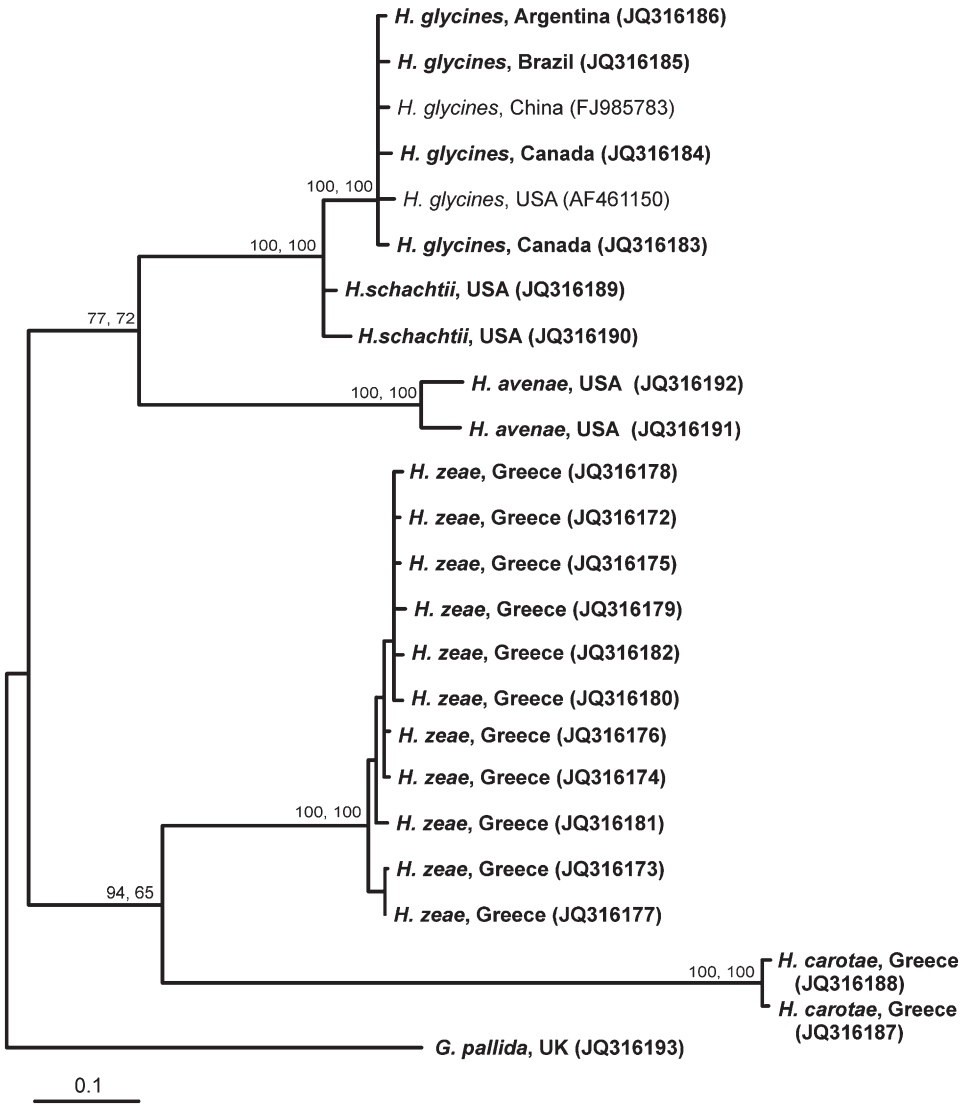

Fig. 6. Bayesian 50% majority rule consensus tree as inferred from an alignment of 813 bp of Hsp90 coding sequence from five species of *Heterodera* with *G. pallida* as the outgroup. Numbers on branches include posterior probabilities from two BI runs followed by bootstrap support values resulting from RAxML analysis. New sequences are marked in bold type.

216 bp (excluding two introns) was made with 20 sequences from 11 species. This included the region of Hsp90 immediately downstream of the charged linker region. Pairwise sequence divergence ranged from 0.9 - 22.1%. Bayesian interference and maximum likelihood trees based on this alignment are shown in Fig. 7. The basic topology of this Hps90 tree is congruent with another one that included many of the same sequences (Mundo-Ocampo et al., 2008). In our tree, *H. orientalis* grouped with *H. carotae*, although the support values were fairly low. The position of *H. zeae* outside of this clade still leaves the relationship of *H. zeae* somewhat unclear; examination of Hsp90 from *H. turcomanica* and *H. salixophila* could help to resolve this issue. These Hsp90 trees provide a good starting point upon which more taxa can be added, adding to the utility of this DNA marker for resolving phylogenetic relationships of the Heteroderinae.

DNA sequence for the 28S rRNA D2-D3 expansion segment from this *H. zeae* population (GenBank #GU145612) was 99% identical to accession number DQ328695, the sequence representing *H. zeae* S470 from the U.S. (Subbotin et al., 2006). A published tree based on 28S rRNA showed *H. zeae* as a sister group to *H. litoralis*, but with a weak posterior probability (PP= 60) (Subbotin et al., 2010a, Fig. 26). The availability of more 28S sequences from closely related *Heterodera* species would further strengthen phylogenies based on this marker. The 18S sequence of *H. zeae* was 99% identical to sequences from *Heterodera* cf. *iri* (AY912046), *H.* cf. *medicaginis* (AY912047, AY912048), *H. avenae* (FJ040403), and *H. mani* (EU669916). No further conclusions based on 18S could be drawn, as it should be noted that 18S sequence representation in GenBank is somewhat limited. Moreover, the extent of variation in the 3' end of 18S may be inadequate to infer relationships at the species level.

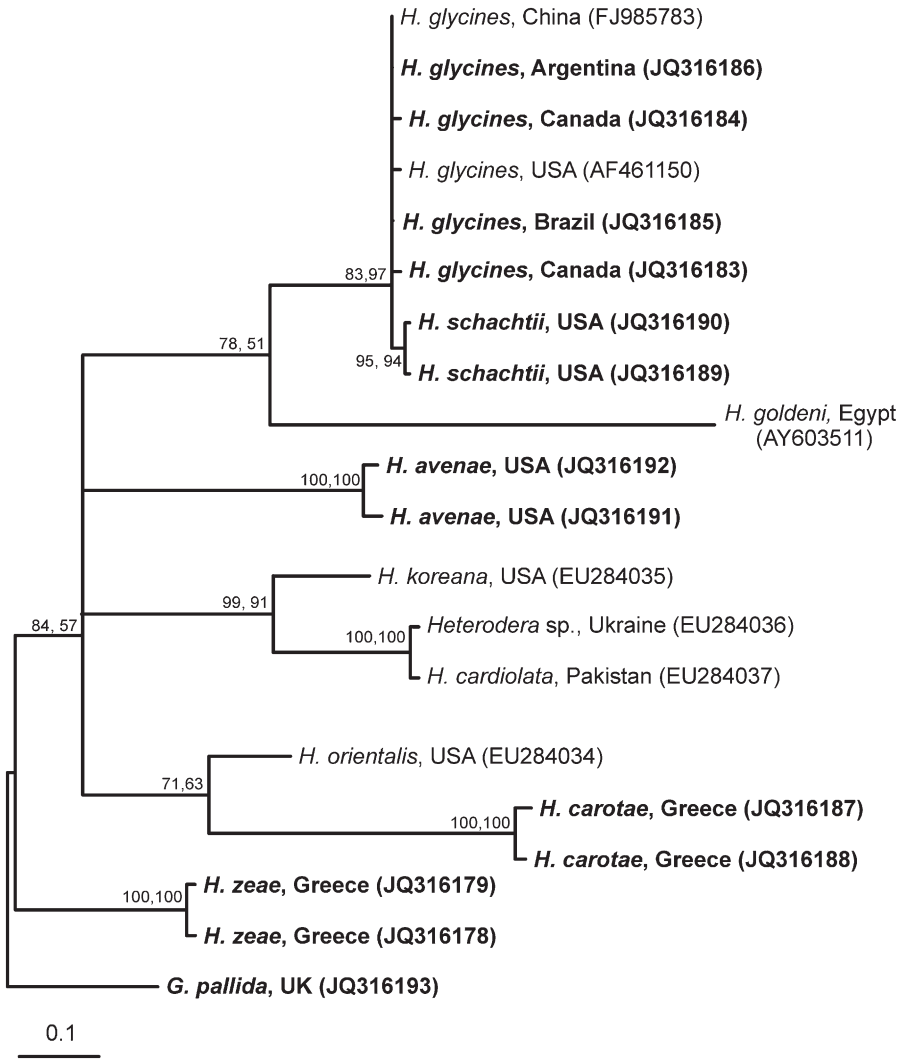

FIG. 7. Bayesian 50% majority rule consensus tree as inferred from an alignment of 216 bp of Hsp90 coding sequence from 10 species of *Heterodera* with *G. pallida* as the outgroup. Numbers on branches include posterior probabilities from two BI runs followed by bootstrap support values resulting from RAxML analysis. New sequences are marked in bold type.

The morphology of cysts and second-stage juveniles and molecular analysis of J2s established the species identity as *Heterodera zeae* Koshy, Swarup & Sethi, 1971. Detection of *H. zeae* in the organic maize field in northern Greece represents the first record for the occurrence of this species in Greece and the second report of this nematode in Europe. However, it is not known for certain whether the symptoms observed in maize at the site of this collection in Greece can be attributed to this species. Further studies are required to prove whether nematode presence is associated with economic losses to maize in Greece.

ITS sequences from the present *H. zeae* population were distinct from cyst nematodes of the *Avenae, Afenestrata, Cyperi,* and *Sacchari* lineages that are also known to affect monocots (Subbotin et al., 2001; 2010a). Therefore, ITS-based restriction enzyme profiles may be useful for discriminating *H. zeae* from other cyst nematodes associated with the Poaceae. A previous study of cyst nematodes in the Mediterranean basin included RFLPs for a population of *H. fici* on *Ficus carica* as well as RFLPs and sequence data from *H. ripae* on *Urtica dioica* (Madani et al., 2004). The present study thus adds to the catalog of molecular information needed to facilitate identification of nematodes in this geographic region and provides new valuable molecular data for *H. zeae*.

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