Phylogenetic Relationships Based on Ribosomal DNA Data for Four Species of Cyst Nematodes from Italy and One from Syria[1]

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Abstract: Phylogenetic analysis of new ribosomal DNA (rDNA) data for *Heterodera mediterranea, H. hordecalis, H. carotae,* and *H. fici* from Italy and *H. ciceri* from Syria, along with published data for other species, showed high bootstrap support for the following relationships: *(((((H. carotae H. cruciferae) H. goettingiana) (((H. trifolii H. ciceri) H. mediterranea) ((H. avenae H. latipons) H. fici))) (Cactodera betulae H. hordecalis)) (Globodera rostochiensis G. pallida)).* The rDNA sequence data were for the two internal transcribed spacers (ITS1 and ITS2) plus the 5.8S gene between them. These inferred relationships support the classic ''Goettingiana Group'' of *H. carotae, H. cruciferae,* and *H. goettingiana.* A clade comprised of *Cactodera betulae* and *H. hordecalis* is only distantly related to the other species in the analysis.

Key words: *Heterodera, Heterodera carotae, Heterodera ciceri, Heterodera fici, Heterodera hordecalis, Heterodera mediterranea,* ITS1, ITS2, nematode, phylogenetic analysis, ribosomal DNA, 5.8S gene.

Introduction

Molecular data are frequently used along with classical data for systematic inference in plant-parasitic cyst nematodes. We report such data for five species of cyst nematodes—four collected in Italy and one in Syria. The species collected in Italy include *Heterodera mediterranea* Vovlas, Inserra and Stone, *H. hordecalis* Andersson, *H. carotae* Jones, and *H. fici* Kirjanova. *Heterodera ciceri* Vovlas, Greco and Di Vito was collected in Syria. Previously published molecular data for eight additional species were included in the comparative and phylogenetic analyses (Ferris et al., 1993, 1994, 1995, 1998, 1999). These species include: *H. cruciferae* Franklin, *H. goettingiana* Liebscher, *H. trifolii* Goffert, *H. avenae* Wollenweber, *H. latipons* Franklin, *Cactodera betulae* Hirschmann and Riggs, *Globodera rostochiensis* (Wollenweber) Behrens, and *G. pallida* (Stone) Behrens.

Based on classical morphology and host data, the species collected in Italy and Syria have been placed in several of the recognized species groups of cyst nematodes of the genus *Heterodera. Heterodera hordecalis,* which grows on cereals and grasses in Europe (Andersson, 1974), has been compared with *H. latipons* and placed in the avenae group of *Heterodera* species (Andersson, 1974; Baldwin and Mundo-Ocampo, 1991). *Heterodera fici,* a worldwide parasite of *Ficus spp.* (Evans and Rowe, 1998), has been variously placed with members of the schachtii group (Baldwin and Mundo-Ocampo, 1991; Mulvey, 1972) or in the avenae group (Mathews, 1971; Stone, 1975). *Heterodera ciceri,* with a distribution restricted to Syria, is a parasite of plants from Fabaceae (Evans and Rowe, 1998) and has been considered to be a member of the schachtii group (Baldwin and Mundo-Ocampo, 1991; Vovlas et al., 1985). *Heterodera carotae* has been reported in Europe,

India, the former Soviet Union, and the United States (Baldwin and Mundo-Ocampo, 1991), growing on carrots; on the basis of morphological characters, it is usually placed in the Goettingiana group (Baldwin and Mundo-Ocampo, 1991; Ferris et al., 1993; Mathews, 1971; Mulvey, 1972; Stone, 1975). *Heterodera mediterranea* has been detected on roots of olive trees and *Pistacia spp.* (Vovlas and Inserra, 1983) in Italy and Spain (Castillo et al., 1999). Although the hosts of *H. mediterranea* are woody plants, this species also has been placed in the goettingiana group (Baldwin and Mundo-Ocampo, 1991; Vovlas et al., 1981) on the basis of morphological characteristics of the second-stage juvenile lip and cyst vulval cone. The objectives of this study were to infer evolutionary relationships among the five species of genus *Heterodera* collected in Italy and Syria, and to evaluate their current placement in three species groups of the genus.

Materials and Methods

Cysts of *Heterodera mediterranea* were collected in Brindisi, southern Italy, from the roots of lentisc, *Pistacia lentiscus. Heterodera hordecalis* cysts from quack grass, *Agropyron repens* on coastal dunes, Foggia, and *Heterodera carotae* from carrots in sandy soil (*Daucus carota*) were all collected in Zapponeta, southern Italy. Specimens of *H. fici* were from a greenhouse culture of fig, *Ficus carica,* from Italy. *Heterodera ciceri* cysts were collected from roots of chickpea, *Cicer arietinum,* from crop regions in Aleppo, Syria. Nematode specimens (Table 1) were preserved in 70% alcohol. For each nematode isolate, several DNA preparations were made from one to four cysts. Nematodes were homogenized in 25 µl TE buffer (pH 7.5), and total genomic DNA was extracted using InstaGene Matrix (Bio-Rad, Hercules, CA). Primers used for amplification of a ribosomal DNA fragment that spanned the two internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene as well as the PCR reaction parameters were as previously described (Ferris et al., 1993). The amplified fragment was cloned in pGEM-T vector (Promega, Madison, WI) and transformed into *Escherichia coli* strain JM109. Plasmid prepa-

TABLE 1.   Source of isolate and host data for species used in study.

Species name	Source of isolate	Host
Heterodera avenae Wollenweber, 1924	Oregon, USA	Oats
H. carotae Jones, 1950	Zapponeta, southern Italy	Carrot
H. ciceri Vovlas, Greco & Di Vito, 1985	Aleppo, Syria	Chickpea
H. cruciferae Franklin, 1945	California, USA	Carrot
H. fici Kirjanova, 1954	Italy	Fig
H. goettingiana Liebscher, 1892	Washington, USA	Peas
H. hordecalis Andersson, 1974	Zapponeta, southern Italy	Quack grass
H. latipons Franklin, 1969	Gilat, Israel	Oats
H. mediterranea Vovlas, Inserra & Stone, 1981	Brindisi, southern Italy	Lentisc
H. trifolii Goffert, 1932	Indiana, USA	Clover
Cactodera betulae Hirschmann & Riggs, 1969	Arkansas, USA	Birch
Globodera pallida (Wollenweber) Behrens, 1975	Caddishead, UK	Potatoes
G. rostochiensis (Stone) Behrens, 1975	Feltwell, UK	Potatoes

rations were made using the Wizard plus mini prep system (Promega) from bacterial colonies containing inserts of the expected size as assessed by PCR amplification. Sequencing of the plasmid preparations was done using automatic sequencers (ALFexpress, Pharmacia Biotech, and LI-COR) at the Purdue High Definition Genomics Center. Both strands of DNA from several clones (3–8) were sequenced for each nematode isolate, and the resulting sequences have been deposited in GenBank (AY045754–AY045758).

Sequences were aligned using the computer program PILEUP in the Sequence Analysis Software package of the Genetics Computer Group (GCG) version 9.1 (Devereaux et al., 1984) and Clustal W version 1.7 (Thompson et al., 1994) with default penalty values (gap weight = 50 and 15, gap length = 3 and 6, respectively). Uncorrected (''p'') distances and total character differences between sequence pairs were calculated using PAUP* 4.0b4a (Swofford, 1998). Phylogenetic analysis was carried out using PAUP* 4.0b4a under parsimony and maximum likelihood optimality criteria. Maximum parsimony analysis was performed using the branch-and-bound search with gaps treated as missing, and as the 5th character state. Support for individual branches was evaluated using the bootstrap method with heuristic search and 500 replicates. Maximum likelihood analysis was performed using the Hasagawa-Kishino-Yano (HKY) model of sequence evolution (Hasagawa et al., 1985) and a heuristic search with the tree bisection and reconnection branch swapping method. The HKY model is one of the models commonly used for maximum likelihood tree inference based on DNA data of all kinds, and it implements an instantaneous rate matrix that allows for unequal equilibrium base frequencies and different rates for transitions and transversions (Swofford et al., 1996). Parameters of the model used (proportion of invariable sites, transition:transversion ratio and gamma distribution shape parameter α) included both default values and those estimated from the data set. Trees were rooted using *Globodera pallida* and *G. rostochiensis* as an outgroup to show the relationships among the group of nominal *Heterodera* species.

RESULTS

Multiple-sequence alignment generated by PILEUP (Fig. 1) had 1,013 characters, of which 410 were constant, 75 variable parsimony-uninformative, and 528 parsimony-informative. Clustal W alignment was comprised of 1,051 characters in total, with 469 constant, 140 variable parsimony-uninformative, and 442 parsimony-informative characters. Trees inferred from the two different alignments had the same topology under both parsimony and maximum likelihood criteria; therefore, only phylogenies resulting from the GCG pileup alignment are represented and discussed further. Most of the sequence variability was within the ITS1 and ITS2 regions, as was expected and previously reported (Ferris et al., 1999), while the 5.8S gene had a high degree of nucleotide conservation. DNA sequences of *H. hordecalis* and *Cactodera betulae* had the lowest p-distance value of 0.004 and only four base pair differences (Table 2). *Heterodera ciceri* and *H. trifolii* also had low p-distance value and only six base pair differences. *Heterodera mediterranea* was most similar to *H. ciceri* and *H. trifolii*, with total character differences of 34 and 37, respectively. The sequence of *H. carotae* was most similar to *H. cruciferae*, with 24 base pair differences and a distance of 0.025. The sequence of *H. fici* was most similar to that of *H. avenae*, with p-distance value of 0.249 and a total of 227 character differences.

Phylogenetic analysis under parsimony optimality criterion with gaps treated as missing character states resulted in one best tree with a length of 1,245 steps with the following relationships: (((((*H. carotae H. cruciferae*) *H. goettingiana*) (((*H. trifolii H. ciceri*) *H. mediterranea*) ((*H. avenae H. latipons*) *H. fici*))) (*C. betulae H. hordecalis*)) (*Globodera rostochiensis G. pallida*)). Maximum parsimony analysis with gaps coded as the 5th character state resulted in a tree with the same topology. A majority of the clades were supported by bootstrap values of 96 and higher (Fig. 2). An exception was the placement of *H. fici* as a sister taxon to a clade comprising two species from the avenae group, *Heterodera avenae* and *H. latipons*, which had a bootstrap support of 71.

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            10                  30                  50
HOR  GTGAACCTGC TGCTGGATCA TTACCCAAGT GAT-CTCATT CACCAGTTAC C--TGCTGTC
BET  .......... .......... .......... .......... .......... ..........
ROS  .......... .......... .......... ...A.CA... .....CC... ..........
PAL  .......... .......... .......... ...A.CA... .....CC... .........T
CAR  .......... .......... .......... ...T.CA... .....CC..T .TG......T
CRU  .......... .......... .......... ...T.CA... .....CC..T .TG......T
PEA  .......... .......... .......... ...T.CA... .....CC..T .TG......T
TRI  .......... .......... .......... ...T.CT... .....CC... ..........
CIC  .......... .......... .......... ...T.CT... .....CC... ..........
MED  .......... .......... .......... ...T.CT... .....CC... ..........
LAT  .......... .......... .......... ...T.C-... .....TC... ..........
HAV  .......... .......... .......... ...T.C-... .....TC... .TG.......
FIC  .......... .......... .......... ...T.C-... .....TC... ..........
            70                  90                  110
HOR  TGGCTTAGTG TTTGTGGGCG TGCACCATTC GCCTCCGTTT GCTATTGA-- -CGGACCAGA
BET  .......... .......... .......... .......... .......... ..........
ROS  CA.T.G...C AG.......- AA.....CAT .......... .T.G...... ......ACAT
PAL  CA.T.G...C AG.......- AA.....CAT .......... .T.G...... ......ACAT
CAR  CATG..G.CT .G..C-..GC AC......AT ....T..C.. ..AG.A.-.. CG.ACTT...
CRU  CATG..G.CT .G..C-..GC AC......AT ....T..C.. ..AG.A.-.. CG.ACTT...
PEA  CATG..G.CT .G..C-..GC AC......AT ....T..C.. ..AG.A.-.. CG.ACTT...
TRI  CT.T.GG.CT AGC.-TT.GC AC.....AAT ...C....CC ...GA---.. ....CA...G
CIC  CT.T.GG.CT AGC.-TT.GC AC.....AAT ...C....CC ...GA---.. ....CA...G
MED  CT.T.GG.CT AGC.-TT.GC AC.....AAT ..GC....CC ...GA---.. .......A..G
LAT  CA.T.G-.AC ..GTGTC.GC AT......AT ...C.T..C. ...G....GA CG..CA.G.T
HAV  CA.T.G.AAC G...CTT.GC AC.....CAT ...C....C. ...G----.. .AC.CA.G..
FIC  CA.T.G.CCT GGC..T..GC AC.G.ATA.G C...GTC.G. TGGTAGC.GA CG.ACA....
            130                 150                 170
HOR  CCCAGTGGAT TTCGGGTTGG CACAACGCAC TGAGCTGTAA GGACAGCGGC CTTGGAAGCA
BET  .......... .......... .......... .......... .......... .....G.A..
ROS  G..C.CT.TG .AT...C... ....TT.AC. AACAA....C .......-.. .C..TGC...
PAL  G..C.CTATG ..T...C... ....TT.AT. AACAA....T .......-.. .C..TGC...
CAR  ..GTAC.A.. ..G.T--CT. ACGC..TGG. ...CGCTA.T ...TG..C.. .C..TGCAAC
CRU  ..GTAC.A.. ..G.T--CTC ACGC..TGG. ...CG.TA.T ...TG..C.. .C..TGC.AC
PEA  ..GTACAAG. ..G.T--CT. ACGC..TTG. ...CGCTA.T .A.T...C.. .C..TGC.AC
TRI  T.GTTC.AGA .GACTTG... ACGCT--GC. GA.CA.TACG ..G....T.. ..CACG...C
CIC  T.GTTC.AGA .GACTTG... ACGCT--GC. CA.CA.TACG ..G....T.. ..CACG...C
MED  T.GTTC.AGA AGACCTG... ACGCT--GCT CA.CA.TACG ..G....T.- ..CACG...C
LAT  ..GGTC.AG- .GGTCTG... ACAC-G.G.. AACA...A.C ........TTA .---CCT..G
HAV  ..GGTC.AGA .GGTCTG... GCAC-G.G.. AACA...AGT ....G..TA. .C..CG....
FIC  ..GTTG.AT. .GGTCTG..A GCGCTG.G.A GAC....AGT ..G....T-- ---.CTGCG.
            190                 210                 230
HOR  TGACTGTTGG ---------- ---------- GGTGCTGCTT TACGTGTCGG TGTCAGTCCT
BET  .......... .......... .......... .......... .......... ..........
ROS  ...G...... .......... .......... ....------ ..ACC.AT.T ..GTG.C...
PAL  ...G...... .......... .......... ....------ ..ACC.AT.T ..GTG.C..A
CAR  .CT.GT.G.. GTGTTTACGC CTCTACGGTT ..A....G.A ...CA...AC A.CTTACA.G
CRU  .CT.GT.G.. GTGTTTACGC CTCTACGGTT ..A....G.A ...CG...TA CAG.TTA..G
PEA  .CT.GT.G.. GTGTTTACGC ..CTACGGTT ..A....G.A ...CA.-.AC ..CTTACA.G
TRI  A----TGCTT TTGGGGTGCT TCCATACGTT ..A....G.G ...CG-CTCA GTG.T.CA.A
CIC  A----TGCTT TTGGGGTGCT TCCATACGTT ..A....G.G ...CG-CTCA GTG.T.CA.A
MED  A----TGCTT TTGGGGTGCT TCCCTACGTT ..G....G.A ...CG-CTCA GTG.T.CA.A
LAT  A.CAGAGCTT TGGGGTGTTT TC..AATGTT ..A....GCA ...TGACTC. .TG.T.AG.A
HAV  CTCTGTGCTT TGGGGTGTTC TCCGACGATG .TGCT..G.A ...TGACTC. .TG.T.AG.A
FIC  GC.TGAG..T TGGGGTGCTT TCCATACGTT .......G.A .G.TG.CTCC .TG.C.CA.A
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Fig. 1. Multiple-sequence alignment of rDNA: 3′ end of 18S gene (italics, 1–23), ITS1 (24–630), 5.8S gene (italics, 631–794), and ITS2 (795–1013), with periods representing identity and hyphens gaps. All base notations are for the nontranscribed strand. *Cactodera betulae* (BET), *Heterodera hordecalis* (HOR), *Globodera pallida* (PAL), *G. rostochiensis* (ROS), *H. cruciferae* (CRU), *H. carotae* (CAR), *H. goettingiana* (PEA), *H. ciceri* (CIC), *H. trifolii* (TRI), *H. mediterranea* (MED), *H. avenae* (HAV), *H. latipons* (LAT), *H. fici* (FIC).

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              250                 270                 290
HOR  CCAGCGAGAA AGCCAGTGGT TTGGCTATGG A---GGTGTT GACGTGGGTA GACCGGTTGC
BET  .......... .......... .......... .......... .......... ..........
ROS  ATG.T...CC GA.G.T..C. GCT.TCG.C. G....TC.C. ---------- ----------
PAL  ATG.T..CTC GA.G.T..C. G.T.TCG.C. G....TC.C. ---------- ----------
CAR  .ATAAA..CC T.AGGT...C .GC.TCTA.C TTGT.TAC.G .TG...A..G .T...C.-TG
CRU  .AT-AA..CC T.AGGT...C .GC.TCTAC. TTGT.TGC.G .TG...A..G .T...C.---
PEA  .AT.AA..CC T.AGGT.T.G C...CG.C.AC GTGT.CGC.G .TG...A..G .G.T.C.CTG
TRI  TGT.AA..CC T.TGGA...C .GCTG.G... CAAT.TGTCG .TG.---CGG .C.G-C.C..
CIC  TGT.AA..CC T.TGGA...C .GCTG.G... CAAT.TGTCG .TG.---CGG .C.G-C.C..
MED  TGT.AA..CC T.TGG....C .GTTG.G... CAAT.TGT.G .TG.---CGG .C.GCC.C..
LAT  AGT.AA..CC T.AGGT...C .GCTG.GA.. CAAAT.A... .GT.---.CG ...T.C.C..
HAV  AAGTGA.--. ....T.A... ......GC.C .AGTC.A... .GT.---.CG .....C....
FIC  AGTAAA..CC GTTGGT...C .GT.TGGC-- -.ATT.A.C. .GT.---.CG .....CGG..
              310                 330                 350
HOR  TGTTGCCGGA CGCCGCGCTG ACACGTGGGT GGTACGTTCA CAGGGCCAAA TAACGGCTGT
BET  .......... .......... .......... .......... .......... ..........
ROS  ---------- ------..AC CA...GA..A A.C...CC.. ......ACCC ........A.
PAL  ---------- ------..AC CA...GA... ..C...CC.. ......ACCC ..........
CAR  C..CC.TCCT ...T..A.CA ..CTAGT.TG C.G..ACG.C GCAC.AGC.C ..........
CRU  -.GCC.TCCT ...T..A.CA ..CTAGT.TG C.G..ACG.C .GCACGGC.C ..........
PEA  C.G.C.TCCT ...T..A.C. ..CTTG..TA C.G..ACG.C .GCACGGC.C ..........
TRI  .TGGTTG.TT ...T....CA ..GG.G.ATG CACG.TC-GT GG..CGAC-C ..........
CIC  .TGGTTG.TT ...T..A.CA ..GG.G.ATG CACG.TC-GT GG..CGAC-C ..........
MED  .TGGTTG.TT ...T..A.CA G.GG.G.ATA CACG.TC-GT GG..CGAC-C ..........
LAT  -TGGTTG.TT T..T..A.CA ..GGAG..AG CACG.TC-GT TG..TGAC-C A.........
HAV  -TGGTTG.TT T..T.TATC. T.GTTG.ACG A.C...C.GT TG..TAAC-C C.........
FIC  -TGGTTG.TT T..T..AGCA ..GGAG..AG CACG.TC--G T.....A.-C A.....A.A.
              370                 390                 410
HOR  GCTGGCGTCT GTGCGTCGTT GAGCGGTTGT TGCACCTTGC ACA-CTGATG AGCTGGTTCG
BET  .......... .......... .......... .......... .......... ..........
ROS  .......... ....--.... .......... ...G...C.. .G.TA..C.A .CA...AGT.
PAL  .......... .......... .......... ...CTTGC.. GG.TA..C.. .CA...AAT.
CAR  .......... .A....T... ...T...... ..TG..AA.. GTGTG---CA T....AC.TT
CRU  .......... .A....T... ...T...... ..TG..AA.. GTGTG---CA T....AC.TT
PEA  .......... AA....T... ...T...... ..TG..AG.. GT.TG---CA T.....C.TT
TRI  .......... .......... .......... ..TGGTAG.. .....-.A.CA CA...AC.G.
CIC  .......... .......... .......... ..TGGTAG.. .....-.A.CA CA...AC.G.
MED  .......... .......... .......... ..TGG.GG.. .....-.A.CA CA...ACCG.
LAT  .......... .......... .......... ..TG..AA.- -..TA...CA CA....CCT.
HAV  .....T.... .......... .......... ..TG...G.. ...TG...CA CA....CCT.
FIC  .....T.... .......... .......... ..TG...A.T G...-.T.CA GA...ACCG.
              430                 450                 470
HOR  GA------CT TGTTGTCCCT GTCTTACAGA CCGTAACTCG TGGCGTGTCA GTGTGTTTGT
BET  .......... .......... .......... .......... .......... ..........
ROS  T......G.. GC.AC...A. ..TG...GTG .....C..T. C...A....- -..C.C....
PAL  T.....GG.. GC.AT...A. ..TG...GTG .....C.-.. C...A....- -..C.C....
CAR  .GAAGT..GG .TC.TC.ATA .......GT. .........A. C..--...GT ...C.C....
CRU  .GAAGT..GG .TC.TC.ATA .......GT. .........A. C..--...GT ...C.C....
PEA  .GAAGT..GG .TC.TC.TTA .C.....GT. .........A. C..--...GT ...C.C....
TRI  .GATGGTGG. .TCGT....A .......GTG .........A. C..--..-TG T.TGTGC.TG
CIC  .GATGGTGG. .TCGT....A .......GTG .........A. C..--..-TG T.TGTGC.TG
MED  .GATGGTGG. .TCGTC.... .......GTG .........A. C..--...TG T.AGTGC.TG
LAT  ..ATATTGG. ...GT...TG .......GTG ......TAA. C..--...GT CG...C...C
HAV  .GAAAGTGG. .C.-T...TG .C.....GA. .........A. C..--...GT C....CC...
FIC  .GACTGTGG. GA.GTC.T.G .C.....GTG .......... ...--.C.GT .....C...G
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FIG. 1. Continued.

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          490              510              530
HOR  GCGCTACGTC CGTGGCCGTG ATGAGACGAC GTGTTAGGAC CCGTGC--TT GCCTTTGCAC
BET  .T........ .......... .......... .......... .......... ..........
ROS  .T........ .......... .......... .......... ......CT.G ..A..G....
PAL  .T........ ....A..... .......... .......... ......C..G ..A..G....
CAR  .T........ .......AC. ......TAC. ........G. ......T.CG CTGCAG....
CRU  .T........ .......AC. .......AC. ........G. ......T.CG CTGCAG....
PEA  .T........ .......AC. .......AC. ........G. ......T.CG CTGCAG....
TRI  CT........ .......... .......... .C.G....G. ......TTGG C--C.A....
CIC  CT........ .......... .......... .C.G....G. ......TTGG C--C.A....
MED  CT...G.... .......... .......... .C.G....G. ......TTGG C--C.A....
LAT  .T........ .......... .......... ...G....G. ......TT.G CTGA.A....
HAV  .T........ .......... .......... ...G....G. ......TA.G CTTC.-....
FIC  .T...-GT... .......... .......... ...G...TG. ..T...TTAG CGTGAGCA.T
          550              570              590
HOR  GTGGTTTAAG ACTCAATGAG TGCTTGCCTG CACCGCCAGC TTTTTTCTCA TTTTCAATTA
BET  .......... .......... .......... .......... .......... ..........
ROS  .......... ...TG..... ----....C. ..G.A.GCCA GC....TC.C A...TT...T
PAL  .......... ...TG..... ----....C. ..G.A.GCCA GC....TCTC A...TT..A.
CAR  ....C..... ...T...... ...AC.GA.T GCA.CG.CAG CG...AT.AT C.A.T---..
CRU  ....C..... ...T...... ...AC.GA.T GCA.CG.CAG CG...AT.AT C.A.T---..
PEA  ....C..... ...T...... ...AC.GA.T GCA.CG.CAG C-...AT.AT ..A.T---..
TRI  ....C..... .......... ..TCA..TC. GG.AC.GCCA GC....TCTT ....TTCA.T
CIC  ....C..... .......... ..TCA..TC. GG.AC.GCCA GC....TCTT ....TTCA.T
MED  ....C..... .......... ..TCA..TC. GG.AC.GCCA GC....TCTT ....TTCA.T
LAT  ....C..... ...T...... ..TCA..TA. GCA.CG-CAG .G..C.T.TT CA..T.T..T
HAV  ....C..... .......... ..TCC..TA. GCA.CG.CAG .G....T.TT CA..T.--.T
FIC  .......... ...T...... ....A..-GT GTA.CG.CAG CAA..CTCTT ..........
          610              630              650
HOR  TTTTTTCAAT GCAACGCTGT TGCTGTGAAA CAAATTCTAG TCTTATCGGT GGATCACTCG
BET  .......... .......... .......... .......... .......... ..........
ROS  A.....T-.. ....TT.GA. ....----.. A.T....... .......... ..........
PAL  A.....T... ....TTTGA. ....----.. A.T....... .......... ..........
CAR  ......T.-- -..TTCA.TG AATGAAC..G T.T....... C......... ..........
CRU  ......T.-- -..TTCA.TG AATGAAC..G T.T....... C......... ..........
PEA  ......T.-- -..TTCTATG AATGAAC..G T.T....... C......... ..........
TRI  A.....TT-- -T.CACT.C. GTTGAA...T G-........ .......... ..........
CIC  A.....TT-- -T.CACT.C. GTTGAA...T G-........ .......... ..........
MED  A.....TT-- -T.TACT.C. GTTGAA...T G-........ .......... ..........
LAT  ......A.-- -..CTT.AT. GCTGAA.... G......... .......... ..........
HAV  .....GAC-- -..CTT..T. GTTGAA.... G......... .......... ..........
FIC  ......T.-- -..CACT.CC GTG..AAG.. .......... .......... ..........
          670              690              710
HOR  GCTCGTGGAT CGATGAAGAA CACAGCCAAC TGCGATAATT AGTGTGAACT GCAGAAACCT
BET  .......... .......... .G........ .......... .......... ..........
ROS  .......... .......... .G........ .......... .......... ..........
PAL  .......... .......... .G........ .......... .......... ..........
CAR  .......... .......... .G........ .......... .......... ..........
CRU  .......... .......... -G........ .......... .......... ..........
PEA  .......... .......... -G........ .......... .......... ..........
TRI  .......... .......... .G........ .......... ....C..... ..........
CIC  .......... .......... .G........ .......... ....C..... ..........
MED  .......... .......... .G........ ........A. ....C..... ..........
LAT  .......... .......... .G........ .......... ....C..... ..........
HAV  .......... .......... .G........ .......... ....C..... ..........
FIC  .......... .......... .G........ .......... ....C..... ..........
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Fig. 1. Continued

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          730              750              770
HOR  TGAACACAGA ACTTTCGAAT GCACATTGCG CCATTGGAGT GACATCCATT GGCACGCCTG
BET  .......... .......... .......... .......... .......... ..........
ROS  .......... .......... .......... .......... .......... ..........
PAL  .......... .......... .......... .......... .......... ..........
CAR  .......T.. .......... .......... .......... T.T....... -.........
CRU  .......T.. .......... .......... .......... T.T....... -.........
PEA  .......T.. .......... .......... .......... T.T....... -.........
TRI  ........A. ..A....... .......... .......... T......... ..........
CIC  .......A. ..A....... .......... .......... T......... ..........
MED  .......A. ..A....... .......... .......... T......... -.........
LAT  .......A. .......... .......... .......... T......... -.........
HAV  .......A. .......... .......... .......... T......... ..........
FIC  .......TA. ..A....... .......... .......... T......... ..........
          790              810              830
HOR  GTTCAGGGTC GTAACCAAAA AACGCACTGC TGTGTGTGCG TGTTCTTGTT TGTGGGATCA
BET  .......... .......... .......... .......... .......... ..........
ROS  .......... .......... ..T....... ---A...... ..---..T.A .T..CT.AG.
PAL  .......... .......... ..T....... ---A...... ..---..T.A .T..CT.AG.
CAR  .......... .......T.. ........A.. ----.TATGC GTG.T.AAC. C..T......
CRU  .......... .......T-. .......A.. ----.TATGC GTG.T.AAC. C..T......
PEA  .......... .......T-. .......A.. ----.TATGC GTG.TA.AC. C..A......
TRI  .......... ..T....T.. ..T....... ----.TGTGC GT.G..--.C GTG..ATCAT
CIC  .......... ..T....T.. ..T....... ----.TGTGC GT.G..--.C GTG..ATCAT
MED  .......... ..T....T.. ..T.T..A.. ----.TGTGC GT.G..--.C GTG..ATCAT
LAT  .......... ..T.T..T.. ..-CG..... ----...... ....A.GT.G GTGA.ATCAT
HAV  .......... ..T.T..T.. ..-....... ---.....C ....A.GT.G GTGA.ATCAT
FIC  .......... ..T.T..... ..T....... ---....... ....G..C.C GTG..ATCAT
          850              870              890
HOR  TGCCGTTAGG CGTGTTCTCG CATACT-GAC AATGCTACGT GGCGTAGCGA TGGGTATGCT
BET  .......... .......... .......... .......... .......... ..........
ROS  .A.GC..C.. ........T. ......ATTG ...CG....C T.T......T ...ACG.CG.
PAL  .A.GC..C.. ........T. .....CATTG ...CG....C T.T....T.T ...ACG.CG.
CAR  ...G.AC.C. ........TT .GGTG.GACT T...TG--TC .CG.GT.G-- .TT-GG..TG
CRU  ...G.AC.C. ........TT .GGTG.GACT T...TGCTTC .CG.GT.G-- .TT.GG..TG
PEA  ...G.AC.C. ........TT .G.TG.GACT T...TGCTTC .TA.G-.G-- .TT.AG..TG
TRI  GTA.T.GT-A ........TA .G.TACTTG. TCA---G.TC ...TGT.G.G .TTTGG.TG.
CIC  GTA.T.GT-A ........TA .G.TACTTG. TCA---G.TC ...TGT.G.G .TTTGG..TG
MED  GTA.T.GT-A ........TA .G.TACTTG. TTA---G.TC ...TGT.G.G .TTTGG..TC
LAT  GT.T.C.T.A ........T. .GCTA.TTCA TTGAA.G.CC ...TGT.GAT ..T.GT--TC
HAV  GT.G.CAT.A ........T. .GCTA.TCTT G.AAA.G.TC ...CGT.GAG ..T.GT..TG
FIC  GT.T...T.A ........T. .G..A.GTT- ----G.G.CC ...TGT.G... .TT.GT.ATG
          910              930              950
HOR  GGCGTGGAAC TGTGTTGTTG TTATTCCACG TTTTACACAC CGTAACTCGG GCGTATCCCA
BET  .......... .......... ...T...... .......... .......... ..........
ROS  ....C.A..A ........CA ..----.G.. C......G.. .....T.TA. ..ACGC..TT
PAL  ....C.A..A ........CT ..----.G.. C......G.. .....T.TA. ..ACGC..TT
CAR  CTG.C.TG.T CT--G.A.GT ...A.-.G.. C.....GTG. .....T.TA. ...C.C..AT
CRU  CTG.C.TG.T CT--G.A.GT ...A.-.G.. C.....GTG. .....T.TA. ...C.C..AT
PEA  CTG.C.TG.T CT--G.A.GT ...A.-.... .C....GT.. .....T.... ...C.C..AT
TRI  .TG.C.CG.A CT...G...C .A...-.G.. ......GG.. .......... ...C.C.AGT
CIC  CTG.C.CG.A CT...G...C .A...-.G.. ......GG.. .......... ...C.C.AGT
MED  .TG.C.CG.A CT...G...C .A...-.G.. ......GG.. ...G...... ...C.C.AAT
LAT  .TA.C.CG.A AT...CAAGT ..T..-TCGC G.....GG.. .......T.. ...C.A.TGT
HAV  TTG.C.CG.A AC...CAGGT .CT..-.G.. ......GGT. ........A.. A..C.A.TGC
FIC  CTA.C.CG.A AC...CAG.T .AT..-.G.. .......... .......... CGTAGC.AAT
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FIG. 1. Continued.

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                   970                    990                  1010
       HOR  TTGCTTCGCG GCGATAGCGA ATGCCTGGCA AATTGGCATT GGACCTCGAA TTT
       BET  ...-...... .......... .......... .......... .......... ...
       ROS  CGTTCA.AT. CGATAGCT.. ......C..C ...A...... T.CAA.T... CA.
       PAL  CGTTCA.AT. CGATAGCT.. ......C..C ...A...... T.CAA.T... CA.
       CAR  .CC...GC-- .TTG.GCA.. G...T....T GG.A.C-.C. CAGTGCTTT. AC.
       CRU  .CC...GC-- .TTG.GCA.. G...T....T GG.A.CG.C. CAGTGCTTT. AC.
       PEA  ACT...GC-- .TTG.GCA.. G...T....T GG.A.CG.C. CAGTGCTTT. AC.
       TRI  CCTTCG.AT. CT.TGGCG.. G........T T.C....... CCTG..TTG. ...
       CIC  CCTTCG.AT. CT.TGGCG.. G........T T.C....... CCTG..TTG. ...
       MED  .CTTCG.AT. TT.TGGCG.. G........T T.C....... CCTT..TTG. ...
       LAT  .CT.CAT.TA TGCT.G.GCG GAA.G.C..T GG.A...... CCT.T.TTG. A..
       HAV  .C..CAT.T. TGCTGG.T.G .ACTG.C..T GG.A...... CCGTG.TTG. A..
       FIC  .CA.A.GCAT ..TGCG.T.G .ATG.CT.GC TTG....G.. CCGT..TTG. AC.
```

Fig. 1. Continued.

Maximum likelihood analysis with default parameters of the Hasagawa-Kishino-Yano model of sequence evolution and heuristic search resulted in a tree (Fig. 3) with −ln likelihood of 6,722.82. Maximum likelihood estimation of model parameters resulted in transition: transversion ratio ti/tv = 0.86, proportion of invariable sites 0.31, and gamma distribution shape parameter, α = 4.15. Empirical base frequencies were A = 0.19, C = 0.23, G = 0.28, and T = 0.3. The best tree inferred under the maximum likelihood criterion with empirical base frequencies and estimated parameters had −ln likelihood of 6,436.88 and the same topology as the tree inferred with default parameters. Topologies of the most parsimonious tree and the best tree under the maximum likelihood criterion were the same. Topological congruence of trees derived by different methods or from different kinds of data is an accepted method of evaluating reliability of phylogenetic inference (Kim, 1993).

DISCUSSION

Phylogenetic relationships inferred from the rDNA sequence support the classic goettingiana group of *Het-erodera* species. Both the *H. cruciferae* + *H. carotae* (Italy) clade and its sister relationship with *H. goettingiana* are supported by a bootstrap value of 100%. Reasons for the different sequence from *H. carotae* from Michigan in the United States, reported in Ferris et al. (1993), are not clear but could be the result of either laboratory error or a mixed population. The results of the present research show convincingly that *H. carotae* belongs to the goettingiana group of *Heterodera* species. *Heterodera ciceri,* as was previously indicated (Baldwin and Mundo-Ocampo, 1991; Vovlas et al., 1985), is a member of the schachtii group and closely related to *H. trifolii.* High bootstrap values support the evolutionary relatedness of *H. mediterranea* to other species in the schachtii group, despite a reported morphological similarity to members of the goettingiana group (Baldwin and Mundo-Ocampo, 1991; Vovlas et al., 1981). Although placement of *H. fici* as a sister species to the *H. avenae* + *H. latipons* clade is supported with a lower bootstrap value, congruence between maximum parsimony and maximum likelihood trees indicates validity of this relationship; therefore, *H. fici* is probably a member of the avenae group, as was suggested (Mathews, 1971;

TABLE 2. Matrix of uncorrected (''p'') distance and total character differences.[a]

	BET	HOR	PAL	ROS	CRU	CAR	PEA	CIC	TRI	MED	HAV	LAT	FIC
BET	—	4	227	221	340	341	338	344	342	349	324	339	344
HOR	0.004	—	230	224	342	345	340	347	345	352	326	340	346
PAL	0.249	0.252	—	29	324	324	324	297	293	303	307	303	322
ROS	0.243	0.246	0.032	—	324	324	325	293	289	297	301	296	319
CRU	0.361	0.363	0.364	0.365	—	24	62	292	297	302	312	306	333
CAR	0.361	0.365	0.365	0.365	0.025	—	61	300	305	310	316	314	333
PEA	0.358	0.360	0.365	0.367	0.064	0.063	—	297	302	305	318	317	342
CIC	0.367	0.370	0.336	0.332	0.309	0.317	0.314	—	6	34	237	211	253
TRI	0.365	0.368	0.331	0.327	0.314	0.322	0.320	0.006	—	37	241	212	256
MED	0.372	0.375	0.343	0.337	0.319	0.327	0.322	0.035	0.038	—	244	219	260
HAV	0.342	0.344	0.342	0.337	0.325	0.328	0.331	0.247	0.251	0.254	—	139	239
LAT	0.359	0.360	0.338	0.332	0.321	0.329	0.333	0.221	0.222	0.230	0.144	—	227
FIC	0.366	0.367	0.362	0.359	0.351	0.351	0.361	0.266	0.269	0.273	0.249	0.236	—

[a] Uncorrected (''p'') distance values are below the diagonal, and total character differences (gaps not included) are above the diagonal.
  Cactodera betulae (BET), *Heterodera hordecalis* (HOR), *Globodera pallida* (PAL), *G. rostochiensis* (ROS), *H. cruciferae* (CRU), *H. carotae* (CAR), *H. goettingiana* (PEA), *H. ciceri* (CIC), *H. trifolii* (TRI), *H. mediterranea* (MED), *H. avenae* (HAV), *H. latipons* (LAT), *H. fici* (FIC).
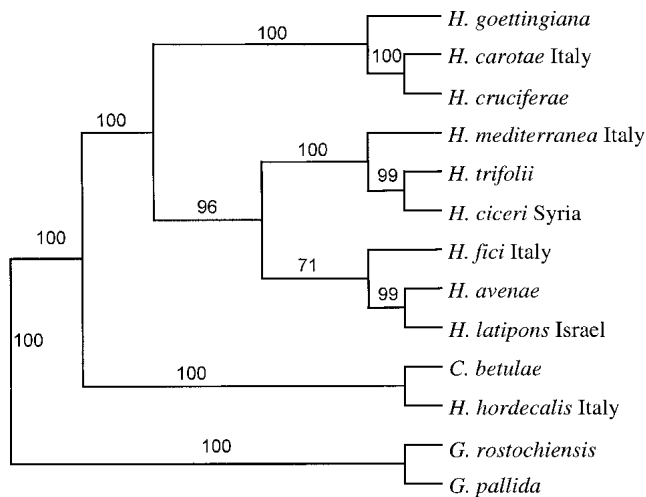
FIG. 2.   Most parsimonious tree of length 1,245 steps for *Cactodera, Globodera,* and *Heterodera* species. Bootstrap support values for monophyletic groups are indicated above branches.

Stone, 1975). An unexpected result of the analyses is the placement of *H. hordecalis* from Italy, which appears to be only distantly related to other *Heterodera* species in this analysis. Instead, the evolutionary relationship based on rDNA data indicates with high bootstrap support a sister taxon relationship to *C. betulae.* As discussed previously (Ferris et al., 1998), the placement of *C. betulae* in the genus *Cactodera* is uncertain, as is the relationship of this species to species of *Heterodera* and *Globodera.* In all of our analyses that included other nominal *Cactodera* species (data not shown), *C. betulae* and *H. hordecalis* form a sister group only distantly related to other nominal *Cactodera.* Based on our current study, *C. betulae* and the *H. hordecalis* specimens from Italy that we examined, both belong to a genus distinct
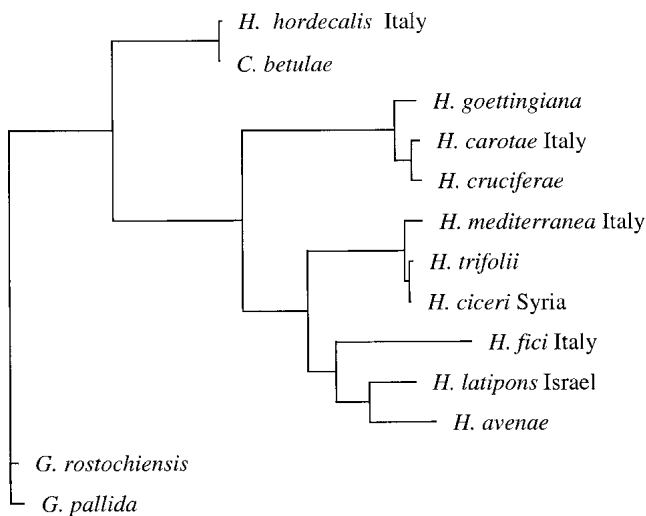
0.1 substitutions/site

FIG. 3.   Best maximum likelihood tree (-ln likelihood = 6,722.82), inferred using HKY model of sequence evolution for *Cactodera, Globodera,* and *Heterodera* species. Analysis with default and estimated parameters resulted in trees with the same topology.

from either *Cactodera* or *Heterodera.* More data are needed to confirm this phylogenetic relationship.

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