

Phylogenetic Relationships Based on Ribosomal DNA Data for Four Species of Cyst Nematodes from Italy and One from Syria¹

A. SABO,² N. VOVLAS,³ AND V. R. FERRIS⁴

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 Zaponeta, 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 JMI09. Plasmid prepa-

Received for publication 26 February 2001.

¹ Funded in part by National Science Foundation grant DEB 975740 to V. R. Ferris.

² Graduate Assistant and ⁴Professor, Department of Entomology, Purdue University, West Lafayette, IN 47907-1158.

³ Istituto di Nematologia Agraria, C.N.R., Via Amendola 165/A, 71426 Bari, Italy.

E-mail: anisabo@purdue.edu

This paper was edited by B. C. Hyman.

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

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

	10		30		50	
HOR	<i>GTGAACCTGC</i>	<i>TGCTGGATCA</i>	<i>TTACCCAAGT</i>	GAT-CTCATT	CACCAGTTAC	C--TGCTGTC
BET
ROSA.CA...CC...
PALA.CA...CC...T
CART.CA...CC..T	.TG.....T
CRUT.CA...CC..T	.TG.....T
PEAT.CA...CC..T	.TG.....T
TRIT.CT...CC...
CICT.CT...CC...
MEDT.CT...CC...
LATT.C-...TC...
HAVT.C-...TC...	.TG.....
FICT.C-...TC...
	70		90		110	
HOR	<i>TGGCTTAGTG</i>	<i>TTTGTGGGCG</i>	<i>TGCACCATTC</i>	<i>GCCTCCGTTT</i>	<i>GCTATTGA--</i>	<i>-CGGACCAGA</i>
BET
ROS	CA.T.G...C	AG.....-AA.....CATT.G.....ACAT
PAL	CA.T.G...C	AG.....-AA.....CATT.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	<i>CCCAGTGGAT</i>	<i>TTCGGGTTGG</i>	<i>CACAACGCAC</i>	<i>TGAGCTGTAA</i>	<i>GGACAGCGGC</i>	<i>CTTGGAAGCA</i>
BETG.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.CTTA	---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	<i>TGACTGTTGG</i>	-----	-----	<i>GGTGCTGCTT</i>	<i>TACGTGTCGG</i>	<i>TGTCAGTCCT</i>
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	TGGGGTGTTT	TCCGACGATG	.TGCT..G.A	..TGACTC.	..TG.T.AG.A
FIC	GC.TGAG..T	TGGGGTGCTT	TCCATACGTTG.A	.G.TG.CTCC	..TG.C.CA.A

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).

	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...C	CAAT.TGTCG	.TG.---CGG	.C.G-C.C..
CIC	TGT.AA..CC	T.TGGA...C	.GCTG.G...C	CAAT.TGTCG	.TG.---CGG	.C.G-C.C..
MED	TGT.AA..CC	T.TGG...C	.GTTG.G...C	CAAT.TGT.G	.TG.---CGG	.C.GCC.C..
LAT	AGT.AA..CC	T.AGGT...C	.GCTG.GA..	CAAAT.A...C	.GT.---.CG	...T.C.C..
HAV	AAGTGA.--T.A...GC.C	.AGTC.A...C	.GT.---.CGC....
FIC	AGTAAA..CC	GTTGGT...C	.GT.TGGC--	-.ATT.A.C	.GT.---.CGCGG..
	310		330		350	
HOR	TGTTGCCGGA	CGCCGCGCTG	ACACGTGGGT	GGTACGTTCA	CAGGGCCAAA	TAACGGCTGT
BET
ROS	-----	-----AC	CA...GA..A	A.C...CC..ACCCA.
PAL	-----	-----AC	CA...GA...C	..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	.TGTTG.TT	...T...CA	..GG.G.ATG	CACG.TC-GT	GG..CGAC-C
CIC	.TGTTG.TT	...T..A.CA	..GG.G.ATG	CACG.TC-GT	GG..CGAC-C
MED	.TGTTG.TT	...T..A.CA	G.GG.G.ATA	CACG.TC-GT	GG..CGAC-C
LAT	-TGTTG.TT	T..T..A.CA	..GGAG..AG	CACG.TC-GT	TG..TGAC-C	A.....
HAV	-TGTTG.TT	T..T.TATC	T.GTTG.ACG	A.C...C.GT	TG..TAAC-C	C.....
FIC	-TGTTG.TT	T..T..AGCA	..GGAG..AG	CACG.TC--G	T....A.-C	A.....A.A.
	370		390		410	
HOR	GCTGGCGTCT	GTGCGTCGTT	GAGCGTTTGT	TGCACCTTGC	ACA-CTGATG	AGCTGTTTCG
BET
ROSG...C..	.G.TA..C.A	.CA...AGT.
PALCTTGC..	GG.TA..C..	.CA...AAT.
CARA...T...	...T.....	..TG..AA..	GTGTG---CA	T...AC.TT
CRUA...T...	...T.....	..TG..AA..	GTGTG---CA	T...AC.TT
PEA	AA...T...	...T.....	..TG..AG..	GT.TG---CA	T...C.TT
TRITGGTAG..-A.CA	CA...AC.G
CICTGGTAG..-A.CA	CA...AC.G
MEDTGGTAG..-A.CA	CA...ACC.G
LATTG..AA..	..TA...CA	CA...CCT.
HAVT....TG...G..	..TG...CA	CA...CCT.
FICT....TG...A.T	G...-T.CA	GA...ACCG.
	430		450		470	
HOR	GA-----CT	TGTTGTCCCT	GTCTTACAGA	CCGTAACCTCG	TGGCGTGTC	GTGTGTTTGT
BET
ROS	T.....G..	GC.AC...A	..TG...GTGC..T	C...A....-	-.C.C....
PAL	T.....GG..	GC.AT...A	..TG...GTGC.-..	C...A....-	-.C.C....
CAR	.GAAGT..GG	.TC.TC.ATAGT.A	C.---.GT	..C.C....
CRU	.GAAGT..GG	.TC.TC.ATAGT.A	C.---.GT	..C.C....
PEA	.GAAGT..GG	.TC.TC.TTA	.C.....GT.A	C.---.GT	..C.C....
TRI	.GATGGTGG.	.TCGT...AGTGA	C.---.TG	T.TGTGC.TG
CIC	.GATGGTGG.	.TCGT...AGTGA	C.---.TG	T.TGTGC.TG
MED	.GATGGTGG.	.TCGT...AGTGA	C.---.TG	T.AGTGC.TG
LAT	.ATATTGG.	...GT...TGGTGTAA	C.---.GT	CG...C...C
HAV	.GAAAGTGG.	.C.-T...TG	.C.....GAA	C.---.GT	C...CC...
FIC	.GACTGTGG.	GA.GTC.T.G	.C.....GTG-.C.GTC...G

Fig. 1. Continued.

	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	GTGGTTTAAAG	ACTCAATGAG	TGCTTGCCTG	CACCGCCAGC	TTTTTTCTCA	TTTTCAATTA
BET
ROSTG....	----...C.	..G.A.GCCA	GC...TC.C	A...TT...T
PALTG....	----...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
FICT.....	...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.....
CART.--	-.TTCA.TG	AATGAAC..G	T.T.....	C.....
CRUT.--	-.TTCA.TG	AATGAAC..G	T.T.....	C.....
PEAT.--	-.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-.....
LATA.--	-.CTT.AT.	GCTGAA...G	G.....
HAVGAC--	-.CTT..T.	GTTGAA...G	G.....
FICT.--	-.CACT.CC	GTG..AAG..
	670		690		710	
HOR	GCTCGTGGAT	CGATGAAGAA	CACAGCCAAC	TGCGATAATT	AGTGTGAACT	GCAGAAACCT
BETG.....
ROSG.....
PALG.....
CARG.....
CRU	-G.....
PEA	-G.....
TRIG.....C.....
CICG.....C.....
MEDG.....A.	...C.....
LATG.....C.....
HAVG.....C.....
FICG.....C.....

FIG. 1. Continued

	730		750		770	
HOR	TGAACACAGA	ACTTTTGAAT	GCACATTGCG	CCATTGGAGT	GACATCCATT	GGCACGCCTG
BET
ROS
PAL
CART..	T.T.....	-.....
CRUT..	T.T.....	-.....
PEAT..	T.T.....	-.....
TRIA..	..A.....	T.....
CICA..	..A.....	T.....
MEDA..	..A.....	T.....	-.....
LATA..	T.....	-.....
HAVA..	T.....
FICTA..	..A.....	T.....
	790		810		830	
HOR	G TTCAGGGTC	GTAACCAAAA	AACGCACTGC	TGTGTGTGCG	TGTTCTTGTT	TGTGGGATCA
BET
ROST.....	---A.....	..---.T.A	.T..CT.AG.
PALT.....	---A.....	..---.T.A	.T..CT.AG.
CART..A..	----.TATGC	GTG.T.AAC	C..T.....
CRUT-A..	----.TATGC	GTG.T.AAC	C..T.....
PEAT-A..	----.TATGC	GTG.TA.AC	C..A.....
TRIT...T..	..T.....	----.TGTGC	GT.G.--.C	GTG..ATCAT
CICT...T..	..T.....	----.TGTGC	GT.G.--.C	GTG..ATCAT
MEDT...T..	..T.T..A..	----.TGTGC	GT.G.--.C	GTG..ATCAT
LATT.T..T..	..-CG....	----.....	...A.GT.G	GTGA.ATCAT
HAVT.T..T..	..-.....	----.....C	...A.GT.G	GTGA.ATCAT
FICT.T.....	..T.....	----.....	...G..C.C	GTG..ATCAT
	850		870		890	
HOR	TGCCGTTAGG	CGTGTCTCTCG	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-ATA	.G.TACTTG	TCA--G.TC	...TGT.G.G	.TTTGG.TG.
CIC	GTA.T.GT-ATA	.G.TACTTG	TCA--G.TC	...TGT.G.G	.TTTGG..TG
MED	GTA.T.GT-ATA	.G.TACTTG	TTA--G.TC	...TGT.G.G	.TTTGG..TC
LAT	GT.T.C.T.AT..	.GCTA.TTCA	TTGAA.G.CC	...TGT.GAT	..T.GT--TC
HAV	GT.G.CAT.AT..	.GCTA.TCTT	G.AAA.G.TC	...CGT.GAG	..T.GT..TG
FIC	GT.T...T.AT..	.G..A.GTT-	----G.G.CC	...TGT.G..	.TT.GT.ATG
	910		930		950	
HOR	GGCGTGGAAC	TGTGTTGTTG	TTATTCCACG	TTTTACACAC	CGTAACTCGG	GCGTATCCCA
BETT.....
ROSC.A..ACA	..----G..	C.....G..T.TA.	..ACGC..TT
PALC.A..ACT	..----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.-.G..	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

FIG. 1. Continued.

	970		990		1010
HOR	TTGCTTCGCG	GCGATAGCGA	ATGCCTGGCA	AATTGGCATT	GGACCTCGAA TTT
BET	...T.....
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	.CCTTCG.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, $\alpha = 4.15$. 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-*

eroder 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).

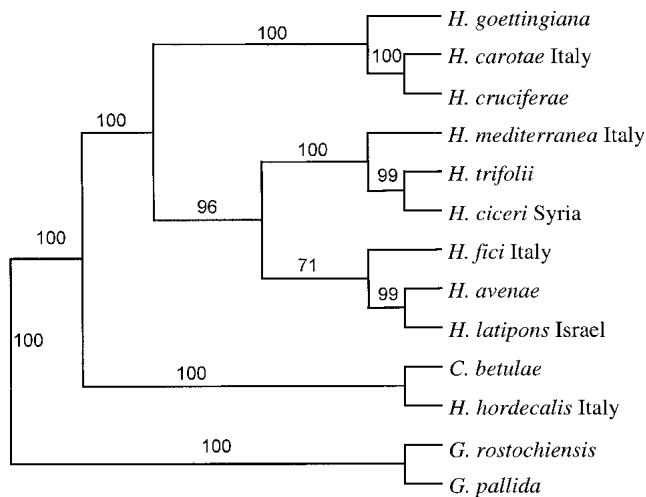
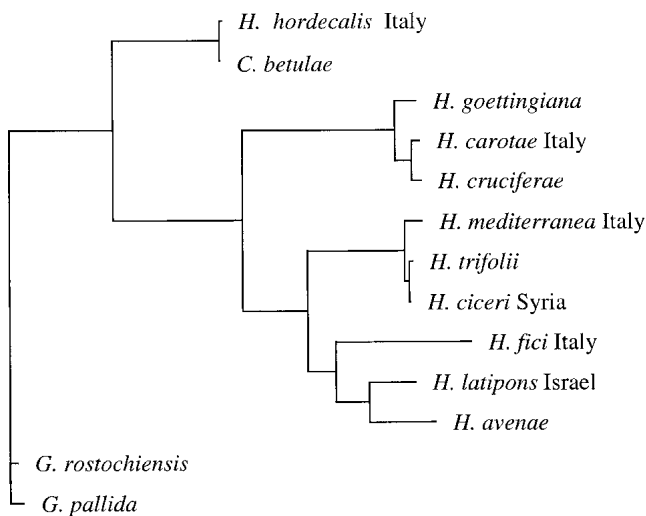


FIG. 2. Most parsimonious tree of length 1,245 steps for *Cactodera*, *Globodera*, and *Heterodera* species. Bootstrap support values for monophyletic groups are indicated above branches.

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.

LITERATURE CITED

Andersson, S. 1974. *Heterodera hordecalis* n. sp. (Nematoda: Heteroderidae), a cyst nematode of cereals and grasses in southern Sweden. *Nematologica* 20:445-454.

Baldwin, J. G., and M. Mundo-Ocampo. 1991. Heteroderinae, cyst and non-cyst-forming nematodes. Pp. 275-362 in W. R. Nickle, ed. *Manual of agricultural nematology*. New York: Marcel Dekker.

Castillo, P., N. Vovlas, A. I. Nico, and R. M. Jimenez-Diaz. 1999. Infection of olive trees by *Heterodera mediterranea* in southern Spain. *Plant Disease* 83:710-713.

Devereaux, J. R., P. Haerberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for VAX. *Nucleic Acids Research* 12:387-395.

Evans, K., and J. A. Rowe. 1998. Distribution and economic importance. Pp. 1-30 in S. B. Sharma, ed. *The cyst nematodes*. Dordrecht: Kluwer Academic Publishers.

Ferris, V. R., J. M. Ferris, and J. Faghihi. 1993. Variation in spacer ribosomal DNA in some cyst-forming species of plant-parasitic nematodes. *Fundamental and Applied Nematology* 16:177-184.

Ferris, V. R., J. M. Ferris, J. Faghihi, and A. Ireholm. 1994. Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein patterns and ribosomal DNA. *Journal of Nematology* 26:144-151.

Ferris, V. R., E. Krall, J. M. Ferris, and J. Faghihi. 1999. Phylogenetic relationships of *Globodera millefolii*, *G. artemisiae*, and *Cactodera salina* based on ITS region of ribosomal DNA. *Journal of Nematology* 31:498-507.

Ferris, V. R., L. I. Miller, J. Faghihi, and J. M. Ferris. 1995. Ribosomal DNA comparisons of *Globodera* from two continents. *Journal of Nematology* 27:273-283.

Ferris, V. R., R. D. Riggs, A. Sabo, J. Faghihi, and J. M. Ferris. 1998. Relationships of *Cactodera betulae* to other cyst nematodes based on ribosomal DNA. *Phytopathology* 88:S28.

Ferris, V. R., S. A. Subbotin, A. Ireholm, Y. Spiegel, J. Faghihi, and J. M. Ferris. 1999. Ribosomal DNA sequence analysis of *Heterodera filipjevi* and *H. latipons* isolates from Russia and comparisons with other nematode isolates. *Russian Journal of Nematology* 7:121-125.

Hasagawa, M., H. Kishino, and T. Yano. 1985. Dating the human-ape split by molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 22:160-174.

Kim, J. 1993. Improving the accuracy of phylogenetic estimation by combining different methods. *Systematic Biology* 42:331-340.

Mathews, H. J. P. 1971. Morphology of the nettle cyst nematode *Heterodera urticae* Cooper, 1955. *Nematologica* 16:503-510.

Mulvey, R. H. 1972. Identification of *Heterodera* cysts by terminal and cone top structures. *Canadian Journal of Zoology* 58:1697-1702.

Stone, A. R. 1975. Head morphology of second-stage juveniles of some Heteroderidae (Nematoda: Tylenchoidea). *Nematologica* 21:81-88.

Swofford, D. L. 1998. PAUP*. Phylogenetic analysis using parsimony (* and other methods), version 4. Sunderland, MA: Sinauer Associates.

Swofford, D. L., G. J. Olsen, P. J. Waddell, and D. M. Hillis. 1996. Phylogenetic inference. Pp. 407-514 in D. M. Hillis, C. Moritz, and B. K. Mable, eds. *Molecular systematics*, 2nd ed. Sunderland, MA: Sinauer Associates.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple-sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. *Nucleic Acids Research* 22:4673-4680.

Vovlas, N., N. Greco, and M. Di Vito. 1985. *Heterodera ciceri* sp. n. (Nematoda: Heteroderidae) on *Cicer arietinum* L. from northern Syria. *Nematologia Mediterranea* 13:239-252.

Vovlas, N., and R. N. Inserra. 1983. Biology of *Heterodera mediterranea*. *Journal of Nematology* 15:571-576.

Vovlas, N., R. N. Inserra, and A. R. Stone. 1981. *Heterodera mediterranea* n. sp. (Nematoda: Heteroderidae) on *Pistacia lentiscus* in southern Italy. *Nematologica* 27:129-138.