Phylogenetic Relationships Among Selected Heteroderoidea Based on 18S and ITS Ribosomal DNA[1]

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Abstract: In a study of relationships among selected cyst-forming and noncyst-forming species of Heteroderoidea, combined sequences comprised of DNA from part of the conserved 18S ribosomal RNA gene (rDNA) plus the complete ITS rDNA segment were more similar to analyses based on the ITS data alone than to analyses based on the 18S data alone. One of the two noncyst-forming species, *Ekphymatodera thomasoni*, grouped with cyst-forming species of Heteroderoidea. *Bilobodera flexa*, also a noncyst-forming species, was separated from all the other taxa by a long branch. *Afenestrata koreana*, with a weakly sclerotized cyst, grouped closely with *H. bifenestra*. These observations suggest that phylogenetic analyses using molecular data may aid in our understanding of the evolution of cyst formation in nematodes, including the possibility of secondary loss. The usefulness of molecular phylogenetic analyses in nematodes may depend more on the particular selection of taxa than on mere addition of data from additional genes.

Key words: *Afenestrata koreana, Bilobodera flexa, Cactodera betulae, Ekphymatodera thomasoni, Globodera, Heterodera bifenestra,* ITS1, ITS2, nematode, phylogenetic analysis, ribosomal DNA, 5.8S gene, 18S gene.

Early studies to determine heteroderoid relationships as revealed by ribosomal DNA (rDNA) showed that *Heterodera bifenestra* and *Cactodera betulae* had marked differences from other groups of widely studied species of cyst nematodes, *Heterodera, Globodera,* and *Cactodera* (Ferris, 1998). More recently, relationships of species of the cyst-forming genus *Afenestrata* to other heteroderoids have been studied (Bert et al., 2002; Sabo, 2002; Subbotin et al., 2001). We present here new rDNA data for five heteroderoid taxa (listed in bold on Table 1), including one species each of the noncyst-forming genera *Ekphymatodera* and *Bilobodera, viz., E. thomasoni,* and *B. flexa.*

In phylogenetic studies using DNA sequence data it is always desirable to use as many taxa and genes as is feasible. The problem of whether it is better to use more genes and fewer taxa, or more taxa and fewer genes, is vigorously debated in the literature (Hillis et al., 2003; Rosenberg and Kumar, 2003; Rydin and Källersjö, 2002). Often simulation data are used to support arguments. In a recent exchange, Rosenberg and Kumar (2003) concluded that their trees were more accurately reconstructed when more data were included for fewer taxa than vice versa. Hillis et al. (2003) pointed out that enough taxa need to be present to make the phylogenetic problem tractable. If the study is confined to few taxa that diverged long ago, the taxa are separated by long branches. Using more data for these same taxa would not, by itself, improve the likelihood of a correct solution. In such cases, only increased taxon sampling will be of benefit. A "scattered genome" approach will not lead to more accurate trees despite the inclusion of data from many genes.

Initially, we obtained and analyzed the 18S and ITS rDNA sequence data separately for the small group of five nematode taxa named above, plus *Caenorhaditis elegans* as the outgroup. Following this, we increased the number of species to 11 (Table 1) plus *C. elegans* and analyzed the separate and combined 18S and ITS rDNA data for the larger set of species.

Materials and Methods

Isolates and treatment of samples: The isolates used, the collectors, and host data are listed in Table 1. *Ekphymatodera thomasoni* originally collected as juveniles from soil in Yosemite National Park, California, in May 1999 by M. Mundo-Ocampo, J. Baldwin, V. Ferris, and J. Ferris was subsequently cultured on *Juncus effusus* by Mundo-Ocampo to produce cysts that were picked from roots and used for the molecular research. The ITS rDNA sequence data for *Cactodera betulae* and *Heterodera bifenestra* have been discussed earlier (Ferris, 1998; Ferris et al., 1998). New data were collected for 18S rDNA for all isolates except *C. elegans,* for which data from GenBank were used (Acc. # X03680). Methods for handling the nematodes and obtaining rDNA were similar to those previously described (Ferris et al., 1993, 1994, 1995). For each nematode isolate, DNA preparations were made from single cysts. A cyst was homogenized with a Radnoti glass homogenizer (Thomas Scientific, Swedesboro, NJ) in 25 µl TE buffer (pH 7.5). Homogenate was either used immediately or frozen for later use. Total genomic DNA was extracted using InstaGene Matrix (Bio-Rad, Hercules, CA) according to manufacturer's recommendations. Primers for amplification of the 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). Primers for the approximately 600-bp fragment of the 18S primer were: (forward) 5′AGGGCAAGTC-

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

Species	Collector	Locality	Host
Afenestrata koreana	R. N. Inserra	Florida, USA	fishpole bamboo
Ekphymatodera thomasoni	M. Mundo, J. Baldwin, V. Ferris, J. Ferris	Yosemite National Park, USA	Juncus effusus
Bilobodera flexa	S. Sharma	Patancheru, India	Allmania nodiflora
Cactodera betulae	R. Riggs	Arkansas, USA	River birch
Heterodera bifenestra	A. Ireholm	Sweden	barley
H. avenae	H. mojtahedi	Idaho, USA	wheat
H. filipjevi	A. Ireholm	Sweden	oats
H. fici	N. Vovlas	Italy	fig
H. mediterranea	N. Vovlas	Brindisi, Italy	lentisc
H. cruciferae	E. P. Caswell-Chen	California, USA	cabbage
Globodera rostochiensis	A. Stone	Feltwell, UK	potato

Bold = initial set of species.

TGGTGCCAGC3′ and (reverse) 5′TTTCAGCTTTG-CAACCATAC3′. The amplified fragment was cloned in pGEM-T vector (Promega, Madison, WI) and transformed into *Escherichia coli* strain JM109. Plasmid preparations were made using the Wizard Plus Minipreps system (Promega) from bacterial colonies containing inserts of the expected size as assessed by PCR amplification. Sequencing of the plasmid preparations was done using (at various times) automatic sequencers (ALFexpress, Pharmacia Biotech, and LI-COR) at the Purdue Genomics Facility. Both strands of DNA from two to four clones were sequenced for each nematode isolate.

Phylogenetic Analysis: 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) with default penalty values (gap weight = 50, gap length = 3) and then manually adjusted. Phylogenetic analysis was carried out using PAUP* 4.0b10 (Swofford, 2000) under parsimony and maximum likelihood optimality criteria. Maximum parsimony analysis was performed using the branch-and-bound search with gaps treated as missing. Support for individual branches was evaluated using the bootstrap method with heuristic search and 100 replicates. The model of sequence evolution that best fits our data sets was selected using Modeltest, version 3.06 (Posada and Crandall, 1998, 2001). The selected model and its estimated parameters were implemented in the maximum likelihood inference using PAUP* 4.0b10 with heuristic search. Trees were rooted using *C. elegans* as the outgroup.

RESULTS

Sequence length for the 18S data varied among the species from 585 bp in *B. flexa* to 593 bp in *C. betulae*. All 18S sequences from this study have been deposited in GenBank (AY566809–AY566819). The ITS rDNA analyzed in the study varied in length from 954 bp in *G. rostochiensis* to 1054 bp in *H. bifenestra*. New sequences, obtained from *A. koreana, H. bifenestra, C. betulae, E. thomasoni,* and *B. flexa* have been deposited in GenBank (AY569016–AY569020).

For the 18S data, multiple sequence alignment generated by PILEUP had 603 characters, of which 451 were constant; 130 variable, parsimony-uninformative; and 22 parsimony-informative characters. For the ITS data, the multiple sequence alignment generated 1069 characters of which 158 were constant, 505 were parsimony-uninformative, and 407 parsimony-informative. Alignments and resulting trees have been deposited in TreeBASE, study number SN1807.

Six Taxa, 18S data only (Fig. 1): Afenestrata koreana grouped with *Heterodera bifenestra,* with *Cactodera betulae* linked to those two sister species. *Ekphymatodera thomasoni* linked to those three species. *Bilobodera flexa* was the most distant taxon. Because the trees and bootstrap values under maximum parsimony and maximum likelihood (default values) were nearly identical, the model of sequence evolution chosen in Modeltest was not used in the bootstrap test for the maximum likelihood analysis.
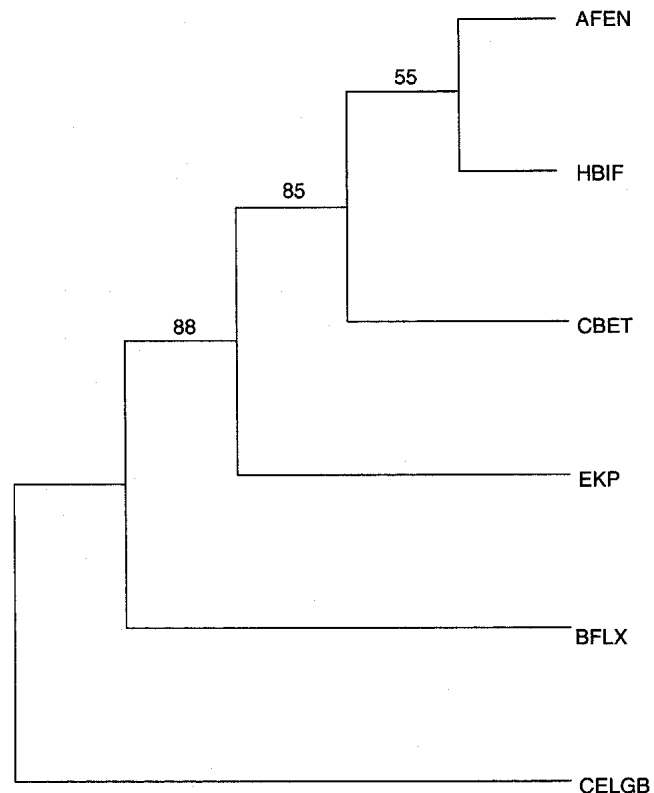

FIG. 1. Maximum parsimony tree inferred from 18S rDNA aligned sequences for six taxa (five heteroderoid species + *C. elegans*). Bootstrap values shown above branches. AFEN = *Afenestrata koreana,* HBIF = *Heterodera bifenestra,* CBET = *Cactodera betulae,* EKP = *Ekphymatodera thomasoni,* BFLX = *Bilobodera flexa,* CELGB = *Caenorhabditis elegans* (sequence from GenBank).

Six taxa, ITS data only (Fig. 2): Afenestrata koreana and *H. bifenestra* were sister species as well as *C. betulae* and *E. thomasoni.* In the maximum parsimony tree *B. flexa* was the most distant species. The maximum likelihood tree (not shown) performed with the model of sequence evolution (HKY85) chosen in the Modeltest analysis had a similar topology as the maximum parsimony tree, except that *B. flexa* grouped with *C. betulae* and *E. thomasoni.*

Six taxa, 18S plus ITS data combined (Fig. 3): In this analysis, *A. koreana* and *H. bifenestra* again were sister species as in the previous analyses. *Cactodera betulae* and *E. thomasoni* formed a sister group with 81% bootstrap support on the maximum parsimony tree, and *B. flexa* was the most distant taxon. The maximum likelihood tree with default values was identical to the maximum parsimony tree in topology and with nearly identical bootstrap values.

12 taxa, 18S plus ITS data combined (Fig. 4): All but one of the additional six species grouped in a separate clade. For the six original taxa in the study group, the maximum parsimony tree and the maximum likelihood tree with default values (not shown) had the same topology and similar bootstrap values. The maximum likelihood tree with Modeltest values (not shown) was less resolved. In the tree shown (Fig. 4) *A. koreana* and *H. bifenestra* comprised a sister group as in all of the other analyses. In this larger analysis of combined data, *C. betulae* grouped most closely with *Globodera rostochien-*

FIG. 3. Maximum parsimony tree inferred from 18S plus ITS rDNA aligned sequences for six taxa (five heteroderoid species + *C. elegans*). Bootstrap values shown above branches. Abbreviations as in Fig. 1.

sis; and *E. thomasoni* linked to these sister species. The most distant species was *B. flexa.*

DISCUSSION

Recent large-scale investigations of phylogenetic relationships among nematodes have used DNA sequence data from the 18S gene region of ribosomal DNA (rDNA) (Blaxter et al., 1998) or sequence from the ITS regions of rDNA (Subbotin et al., 2001). In a recent study that used beta-tubulin data from heteroderoid species to test a phylogenetic tree based on ITS rDNA, Sabo (2002) found that on the ITS rDNA tree, the non-cyst-forming species *Ekphymatodera thomasoni* and *Bilobodera flexa* were basal to other groupings on the tree. Additional molecular data for these genera are not yet available. The 18S gene is considered to be more highly conserved than the ITS regions of rDNA; therefore, we selected it to investigate relationships between these non-cyst-forming species and several previously studied cyst-forming species in *Heterodera* and *Globodera.* We also obtained sequence data from both rDNA regions for the two species, *Cactodera betulae* and *Afenestrata koreana,* which were not included in the study by Subbotin et al. (2001).

The combined data with the initial study group of six taxa showed the same relationships as the combined data using the larger group of taxa in the maximum parsimony analysis. The relationships showed by this
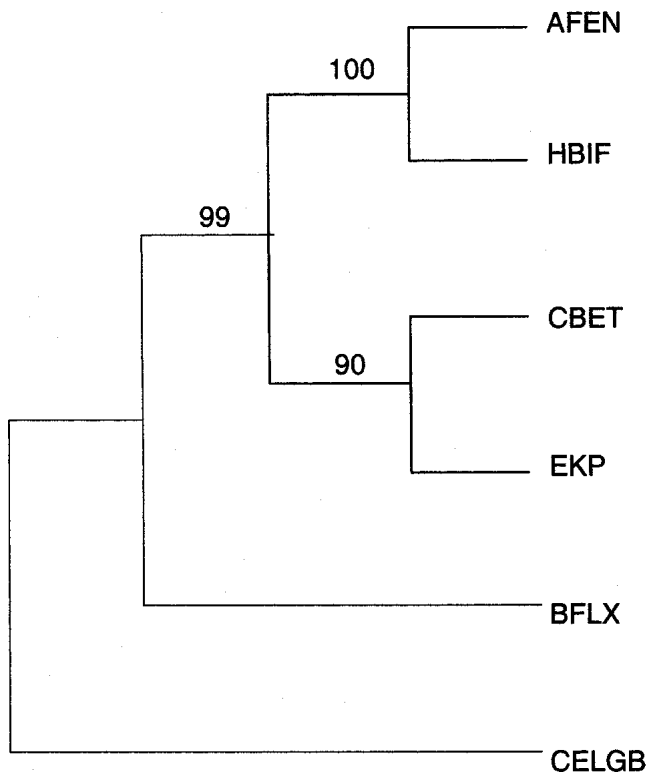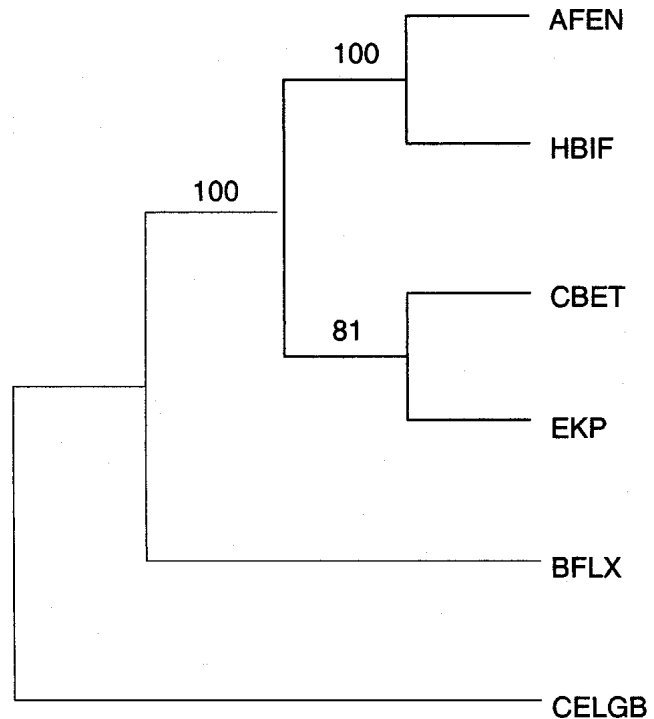

FIG. 2. Maximum parsimony tree inferred from ITS rDNA aligned sequences for six taxa (five heteroderoid species + *C. elegans*). Bootstrap values shown above branches. Abbreviations as in Fig. 1.

tree with combined 18S and ITS data were more similar to those of the smaller analysis (six taxa) based on ITS data than to those based on data from the 18S gene alone. In the six-taxon tree based on 18S only, *E. thomasoni* is a sister species to a three-taxon cluster of *A. koreana, H. bifenestra,* and *C. betulae,* whereas on the other trees *E. thomasoni* and *C. betulae* are phylogenetically closer together. The data set from the ITS region was larger than that from the 18S region (about 1,000 bp for the ITS vs. about 600 bp for 18S). Because the ITS data were more variable than the conserved 18S data, the ITS rDNA region also had many more parsimony-informative sites. The inclusion of *C. elegans* as the outgroup in the analysis exacerbated these differences between the parsimony-informative sites vs. constant sites.

In an earlier study of the phylogeny of cyst nematodes (Ferris, 1998) the relationship of *H. bifenestra* to the rest of the taxa included could not be determined based on ITS rDNA data, and Ferris (1998) suggested that in time the relationship might be clarified by the inclusion of additional sequence data, as yet unknown. Subsequently, a study by Sabo (2002) revealed that *H. bifenestra* grouped with *Afenestrata koreana* based on ITS rDNA. Likewise, Subbotin et al. (2001) showed that *H. bifenestra* grouped with *A. orientalis* in their study, also based on ITS rDNA.

Recently, Bert et al. (2002) showed that gonad morphology of *A. koreana* clearly differed from that of species of *Heterodera* and *Globodera* that they examined. Based on this and other differences, these authors questioned the conclusions of recent phylogenetic analyses that suggested that *Afenestrata* is a sister taxon to *Heterodera.* We note, however, that morphology of sister taxa might reasonably be expected to differ. Therefore, pending morphological examination of additional taxa, the distinctive features of the *A. koreana* gonad are best interpreted as autapomorphies within the context of Heteroderinae. This interpretation does not preclude a sister relationship of *Afenestrata* spp. with *H. bifenestra* as suggested by the present analyses.

In recent phylogenies, particularly those based on rDNA, *Heterodera* species comprise a number of disparate groups. In both Subbotin et al. (2001) and Sabo (2002) the Goettingiana group of *Heterodera* are even more distant from other *Heteroderoidea* species than is the *H. bifenestra/Afenestrata* clade. The historical fact that many cyst nematode species have been assigned to *Heterodera* should not impede our search for understanding the phylogeny of the larger group. An increasingly useful approach, recently applied to both plants and animals, is to track morphological and other differences on a well-corroborated molecular phylogeny of the species. This approach can reveal much about the evolution and development of morphological and behavioral differences, and even suggest useful re-

search to support or falsify a particular phylogenetic inference (Sabo, 2002).

Of the two non-cyst-forming species, *E thomasoni* grouped with cyst-forming heteroderoids, whereas *B. flexa* was separated from them by a long branch in all the analyses. The relationships of *B. flexa* to other heteroderoids will have to await sequence from a wider spectrum of Heteroderoidea. The cysts in species of *Afenestrata* differ from the typical *Heterodera* cysts by being less sclerotized and by not withstanding desiccation well (Inserra et al., 1999; Vovlas et al., 1992). All of these observations may have implications for our understanding of the evolution of cyst formation including the possibility of secondary loss of the cyst in the heteroderoids.

With nematodes, as with other kinds of organisms, the nature of added taxa is more important than the sheer number of taxa or genes analyzed, as suggested by Hillis et al. (2003). Although some components of the groupings had low bootstrap support in the analysis of 12 taxa, their general relationships were consistent with other published analyses that included additional taxa closely related to these five species and showed better bootstrap support at most nodes (Sabo, 2002).
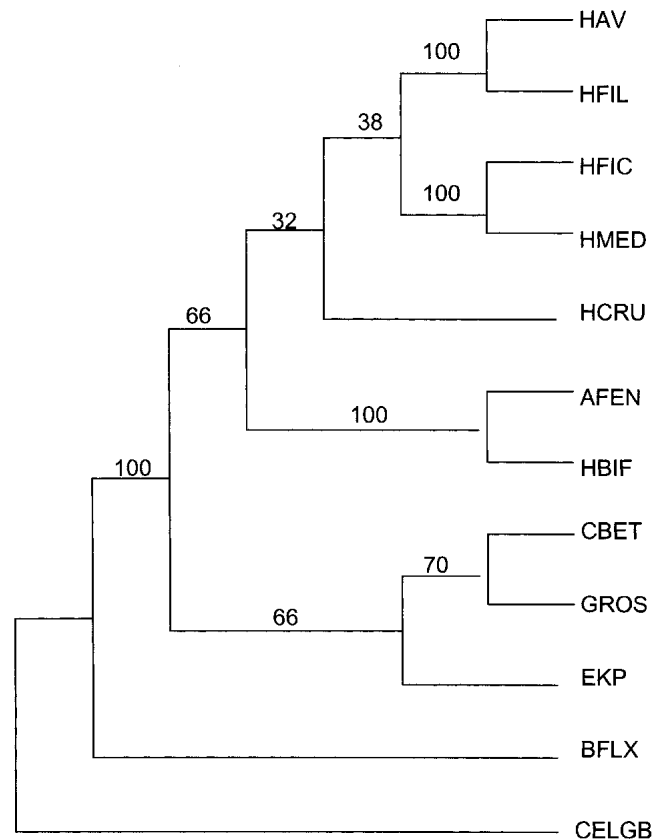

FIG. 4. Maximum parsimony tree inferred from 18S plus ITS rDNA aligned sequences combined for 12 taxa (all species on Table 1 plus *C. elegans*). Bootstrap values shown above branches. Abbreviations as in Fig. 1, plus HAV = *Heterodera avenae,* HFIL = *H. filipjevi,* HFIC = *H. fici,* HMED = *H. mediterranea,* HCRU = *H. cruciferae,* GROS = *Globodera rostochiensis.*

The placement of *H. cruciferae* on this tree is problematical, owing primarily to the fact that species more closely related to *H. cruciferae* were not included in the analysis. The inclusion of both *C. betulae* and *E. thomasoni* in our study was probably fortuitous. The assignment of *C. betulae* to *Cactodera* has been questioned previously, based on comparisons of rDNA ITS molecular data. Analyses of earlier data suggested low similarities with other species of *Cactodera,* as well as as with *Globodera* and *Punctodera,* although *C. betulae* was phylogenetically closer to these species with round or spheroid cysts than with other *Heterodera* (Ferris et al., 1998). The inclusion of both *A. koreana* and *H. bifenestra* was also fortuitous although we did not ascertain this in advance based on the morphological descriptions of the taxa. Of the long-studied and well-known taxa, *G. rostochiensis* was the only one that clustered with our group of less-studied taxa, grouping with *C. betulae* and *E. thomasoni,* as we might have predicted based on our earlier work with *C. betulae.*

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