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Phylogenetic Relationships within the Cyst-Forming Nematodes (Nematoda, Heteroderidae) Based on Analysis of Sequences from the ITS Regions of Ribosomal DNA

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The ITS1, ITS2, and 5.8S gene sequences of nuclear ribosomal DNA from 40 taxa of the family Heteroderidae (including the genera *Afenestrata, Cactodera, Heterodera, Globodera, Punctodera, Meloidodera, Cryphodera,* and *Thecavermiculatus*) were sequenced and analyzed. The ITS regions displayed high levels of sequence divergence within Heteroderinae and compared to outgroup taxa. Unlike recent findings in root knot nematodes, ITS sequence polymorphism does not appear to complicate phylogenetic analysis of cyst nematodes. Phylogenetic analyses with maximum-parsimony, minimum-evolution, and maximum-likelihood methods were performed with a range of computer alignments, including elision and culled alignments. All multiple alignments and phylogenetic methods yielded similar basic structure for phylogenetic relationships of Heteroderidae. The cyst-forming nematodes are represented by six main clades corresponding to morphological characters and host specialization, with certain clades assuming different positions depending on alignment procedure and/or method of phylogenetic inference. Hypotheses of monophyly of Punctoderinae and Heteroderinae are, respectively, strongly and moderately supported by the ITS data across most alignments. Close relationships were revealed between the Avenae and the Sacchari groups and between the Humuli group and the species *H. salixophila* within Heteroderinae. The Goettingiana group occupies a basal position within this subfamily. The validity of the genera *Afenestrata* and *Bidera* was tested and is discussed based on molecular data. We conclude that ITS sequence data are appropriate for studies of relationships within the different species groups and less so for recovery of more ancient speciations within Heteroderidae. © 2001 Academic Press

Key Words: **cyst-forming nematodes; coevolution; Heteroderidae; phylogeny; sequence alignments.**

INTRODUCTION

Cyst-forming nematodes (Heteroderidae) are highly derived and economically very important plant parasites. The members of this group are of special scientific interest within the order Tylenchida due to some remarkable and efficient parasitic adaptations. After root penetration, initiation of feeding by second-stage juveniles induce highly specialized nurse cells which are sustained throughout the life of the parasite. Three molts transform second-stage juveniles into vermiform migratory males or sedentary mature females with most of their swollen body exposed on the root surface. At the end of the life cycle, the female body turns into a hard-walled protective cyst filled with eggs. Some cyst-forming nematode species are highly pathogenic on major agricultural crops including cereals, root crops, and most legumes. Many of these species are distributed worldwide, in part because eggs in cysts remain viable under conditions of dispersal that would be fatal to most other organisms (Siddiqi, 1986; Baldwin and Mundo-Ocampo, 1991).

The family Heteroderidae contains 18 genera of which 6 belong to cyst-forming nematodes: *Heterodera,* and *Afenestrata* in the subfamily Heteroderinae and *Globodera, Punctodera, Cactodera,* and *Dolichodera* in the subfamily Punctoderinae. At present, from 90 to more than 100 cyst-forming nematode species are recognized by different authors; about two-thirds of these belong to the genus *Heterodera* (Siddiqi, 1986; Baldwin and Mundo-Ocampo, 1991; Evans and Rowe, 1998; Wouts and Baldwin, 1998).

Studies of phylogenetic relationships among nematodes are not only essential to taxonomy, but also allow a more complete understanding of the biology of nematodes as agricultural pests. Hypotheses of phylogenetic relationships within Heteroderidae based on traditional morphological characters, scanning electron

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microscopy surface morphology, cuticle ultrastructures, trophic specialization, cellular host response, and coevolution with hosts have been proposed by several authors (Krall and Krall, 1970, 1973, 1978; Wouts, 1972, 1973, 1985; Stone, 1975, 1979; Ferris, 1979, 1985, 1998; Krall, 1990; Baldwin, 1986, 1992; Baldwin and Schouest, 1990). Despite their use of similar characters and approaches, some differences were obtained in the phylogenetic reconstructions proposed by these authors. It is evident that homoplastic evolution of morphological and biological characters may hamper phylogenetic analyses. The use of molecular data for phylogenetic inference provides an independent approach that may shed new light on the evolution of such characters.

Comparative analysis of coding and noncoding regions of ribosomal DNA has become a popular tool for construction of phylogenetic trees of many organisms including nematodes. Recently, phylogenetic analyses based on the 18S rDNA sequences provided new insights into interrelationships within the phylum Nematoda and showed that homoplastic morphological characters may be widespread and that the present higher-level classification of the Nematoda needs revision (Aleshin *et al.,* 1998; Blaxter *et al.,* 1998). Partial sequences of the 18S rDNA gene and the D2–D3 expansion region of the 28S rDNA gene were used to examine evolutionary relationships among the root-lesion nematodes of the genus *Pratylenchus* and related genera, entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis,* and bacterial-feeding species of the genus *Acrobeloides* (Al-Banna *et al.,* 1997; Liu *et al.,* 1997; Duncan *et al.,* 1999; De Ley *et al.,* 1999).

The internal transcribed spacers (ITS1 and ITS2) situated between the small nuclear ribosomal subunit (the 18S gene) and the large subunit (the 28S gene) are considered appropriate genomic regions, both in terms of ease of isolation and sequencing and in terms of usefulness in resolving relationships at the species level. The ITS region of rDNA has recently been used to study phylogenetic relationships within the genera *Heterorhabditis* (Adams *et al.,* 1998) and *Meloidogyne* (Hugall *et al.,* 1999). The latter study discovered substantial sequence polymorphism within individual root knot nematodes, and the authors suggested that this might be due to hybrid origins of some *Meloidogyne* populations. The pioneering work on Heteroderidae of Ferris and co-authors (Ferris *et al.,* 1993, 1994, 1995; Ferris, 1998) demonstrated that nucleotide sequences of ITS1 and ITS2 are both useful and practical for phylogenetic analysis of the cyst-forming nematodes. They analyzed relationships among a limited number of species from the genera *Heterodera, Cactodera,* and *Globodera* and within the Goettingiana, Avenae, and Schachtii groups of *Heterodera.* In a recent paper, Ferris (1998) compared sequences from 15 taxa using parsimony analysis. The positions obtained for *G. virginiae, H. carotae,* and *H. bifenestra* in this phylogenetic tree were in conflict with morphological data. The same author pointed out two problems which emerged from the use of the rDNA sequences: (1) closely related sibling species, such as members of the Schachtii group, may have rDNA that is too similar to permit sorting on the basis of the ITS sequence data and (2) other, more phylogenetically distant, taxa may have rDNA that is too dissimilar to allow construction of a plausible alignment of the ITS sequence data, limiting the reliability of subsequent phylogenetic analysis.

In this study we present phylogenetic analyses of 40 species and subspecies of the family Heteroderidae. The species cover most of the known taxonomic and morphological diversity of this nematode group. Our analysis is based on 36 new sequences of complete ITS1 + 5.8S + ITS2 regions that we have analyzed with three phylogenetic procedures: maximum-parsimony (MP), minimum-evolution (ME), and maximum-likelihood (ML). The goals of this study were (1) to estimate the phylogenetic relationships between species, groups of species, and genera of the cyst-forming nematodes, (2) to consider the congruence of the molecular phylogenetic trees with morphological groupings and coevolution with host plants, and (3) to evaluate the influence of tree-building methods and alignment procedures on phylogenetic inference for cyst-forming nematodes.

Nucleotide insertions or deletions are found commonly in the ITS regions of the cyst-forming nematodes and no consensus exists for the alignment of such sequences. To approach this problem, we generated several alignments: computer alignments with different gap length and gap opening penalties and an elision alignment combining a range of alignments in a single matrix and culled alignment exclusion of the most variable fragments. All these alignments were compared by use of different tree-building methods. For analyses of sensitivity of tree topology we are interested in the branching order of clades and their statistical support within each analysis.

MATERIALS AND METHODS

Nematode Samples

Thirty-six new nucleotide sequences of the ITS regions of different nematode species and populations of the family Heteroderidae were determined during this study. Seven previously determined sequences of species were included in our phylogenetic analyses. A total of 28 valid *Heterodera* species, 4 *Globodera* species, and 1 species of each of the genera *Punctodera, Cactodera,* and *Afenestrata* were used in this study (Table 1). All were identified by their morphology and morphometrics. Two populations of the species *H. avenae* and *H. salixophila,* which revealed polymorphism during re-

TABLE 1

The Nematode Species and Populations from the Family Heteroderidae Used in This Study

Classification and species	GenBank Accession No.	Host plants (family)	Location	Source
Heteroderinae*				
Heterodera (28/62)**[a]				
H. arenaria	AF274396	*Ammophila arenaria* (Poaceae)	Linconshire, England	J. Rowe, UK
H. aucklandica	AF274398	*Microlaena stipoides* (Poaceae)	One Tree Hill, Auckland, New Zealand	W. Wouts, New Zealand
H. avenae	AF274395	Cereals (Poaceae)	Argentan, France	R. Rivoal, France
H. avenae	AF274397	Cereals (Poaceae)	India	J. Rowe, UK
H. bifenestra	AF274384	Grasses (Poaceae)	Sweden	J. Rowe, UK
H. bifenestra	AF274385	Grasses (Poaceae)	Luxembourg province, Belgium	S. A. Subbotin, Russia
H. cajani	AF274389	*Cajanus cajan* (Fabaceae)	India	J. Rowe, UK
H. carotae	AF274413	*Daucus* sp. (Umbelliferae)	Créances, France	M. Bossis, France
H. ciceri	AF274393	*Cicer* sp. (Fabaceae)	Syria	N. Vovlas, Italy
H. cruciferae	AF274411	*Brassica* sp. (Cruciferae)	Brielle, The Netherlands	B. Schoemaker, the Netherlands
H. cynodontis	AF274386	*Cynodon dactylon* (Poaceae)	Pakistan	F. Shahina, Pakistan
H. cyperi	AF274388	*Cyperus* sp. (Cyperaceae)	Spain	M. Romero, Spain
H. fici	AF274409	*Ficus carica* (Moraceae)	Sukhumi, Georgia	S. A. Subbotin, Russia
H. filipjevi	AF274399	Cereals (Poaceae)	Saratov, Russia	E. Osipova, Russia
H. glycines	AF274390	*Glycine max* (Fabaceae)	Arkansas, USA	R. Robbins, USA
H. goettingiana	AF274414	*Pisum* sp. (Fabaceae)	Germany	J. Rowe, UK
H. hordecalis	AF274401	Grasses (Poaceae)	Montrose, Scotland, UK	S. A. Subbotin, Russia
H. humuli	AF274408	*Humulus lupulus* (Cannabaceae)	Tsivilsk, Chuvashija, Russia	Yu. Danilova, Russia
H. iri	AF274400	Grasses (Poaceae)	Forfar, Scotland, UK	S. A. Subbotin, Russia
H. latipons	AF274402	Cereals (Poaceae)	Breda, Syria	U. Scholz, Germany
H. litoralis	AF274410	*Sarcocornia quinqueflora* (Chenopodiaceae)	Glen Innes, Auckland, New Zealand	W. Wouts, New Zealand
H. medicaginis	AF274391	*Medicago sativa* (Fabaceae)	Stavropol region, Russia	S. A. Subbotin, Russia
H. oryzicola	AF274387	*Oryza sativa* (Poaceae)	Kerala, India	J. Rowe, UK
H. riparia	AF274407	*Urtica dioica* (Urticaceae)	Germany	D. Sturhan, Germany
H. sacchari	AF274403	*Saccharum officinale* (Poaceae)	Ivory Coast	J. Rowe, UK
H. salixophila	AF274405	*Salix album* (Salicaceae)	Kherson, the Ukraine	S. A. Subbotin, Russia
H. salixophila	AF274406	*Salix* sp. (Salicaceae)	Nieuwpoort, Belgium	S. A. Subbotin, Russia
H. schachtii	AF274394	*Beta vulgaris* (Chenopodiaceae)	Germany	D. Sturhan, Germany
H. sorghi	AF274404	*Sorghum* sp. (Poaceae)	New Delhi, India	J. Rowe, UK
H. trifolii	AF274392	*Trifolium* sp. (Fabaceae)	UK	J. Rowe, UK
H. urticae	AF274412	*Urtica* sp. (Urticaceae)	Diksmuide, Belgium	S. A. Subbotin, Russia
Afenestrata (1/5)[a]				
A. orientalis		*Miscanthus purpurecens* (Poaceae)	Primorskii territory, Russia	Eroshenko *et al.* (unpublished)
Punctoderinae				
Globodera (4/8)[a]				
G. artemisiae	AF274415	*Artemisia* sp. (Asteraceae)	China	D. Peng, China
G. pallida		*Solanum tuberosum* (Solanaceae)	Risby, UK	Subbotin *et al.* (2000b)
G. rostochiensis		*Solanum tuberosum* (Solanaceae)	Moscow region, Russia	Subbotin *et al.* (2000b)
G. tabacum				
G. t. solanacearum		*Solanum dulcamara* (Solanaceae)	Connecticut, USA	Subbotin *et al.* (2000b)
G. t. tabacum		*Solanum dulcamara* (Solanaceae)	Virginia, USA	Subbotin *et al.* (2000b)
G. t. virginiae		*Solanum dulcamara* (Solanaceae)	Virginia, USA	Subbotin *et al.* (2000b)
Cactodera (1/9)[a]				
C. estonica	AF274417	*Polygonum* sp. (Polygonaceae)	The Netherlands	G. Karssen, The Netherlands
Punctodera (1/3)[a]				
P. punctata	AF274416	Grasses (Poaceae)	Luxembourg province, Belgium	S. A. Subbotin, Russia
Ataloderinae				
Thecavermiculatus (1/4)[b]				
T. crassicrustata		*Mertensia maritima* (Poaceae)	Kamchatka, Russia	Eroshenko *et al.* (unpublished)
Cryphoderinae				
Cryphodera (1/6)[c]				
C. brinkmani	AF274418	*Pinus thunbergii* (Pinaceae)	Yokokowa, Saitama-Ken, Japan	G. Karssen, The Netherlands
Meloidoderinae				
Meloidodera (1/9)[b]				
M. alni	AF274419	*Alnus* sp. (Betulaceae)	Luxembourg province, Belgium	S. A. Subbotin, Russia

* Classification of the family Heteroderidae according to Wouts (1985).

** The number of species sampled/total number of valid species in genera according to [a] Wouts and Baldwin (1998), [b] Evans and Rowe (1998), [c] Karssen and Van Aelst (1999).

striction analysis of the ITS regions in previous studies (Subbotin *et al.*, 1999, 2000a), and two populations of *H. bifenestra,* which were used by Ferris (1998) as an outgroup for phylogenetic analysis of the cyst-forming nematodes, were also included. Previously obtained sequences of *G. rostochiensis, G. pallida, G. tabacum*

solanacearum, G. t. virginiae, and *G. t. tabacum* (Subbotin *et al.,* 2000b) were added to the analysis. The non-cyst-forming sedentary nematode species *Thecavermiculatus crassicrustata* was used as an ingroup taxon to examine its position with respect to cyst-forming nematode genera. Sequences of *A. orientalis* and *T. crassicrustata* were obtained from an unpublished study (A. S. Eroshenko *et al.,* unpublished). The non-cyst-forming sedentary nematode species *Meloidodera alni* and *Cryphodera brinkmani* were sequenced and designated outgroup taxa (Table 1).

DNA Extraction, PCR, Cloning, and Sequencing

For each population, one to four cysts or females were transferred into 10 μl of double-distilled water in an Eppendorf tube and crushed with a microhomogenizer. Eight microliters of nematode lysis buffer (125 mM KCl, 25 mM Tris–Cl, pH 8.3, 3.75 mM $MgCl_2$, 2.5 mM dithiothreitol, 1.125% Tween 20, 0.025% gelatine) and 2 μl of proteinase K (600 μg/ml) were added. The tubes were incubated at 65°C (1 h) and 95°C (10 min) consecutively. After centrifugation (1 min; 16,000*g*) 10 μl of the resulting DNA suspension was added to the PCR mixture containing 10 μl of 10× *Taq* incubation buffer, 20 μl of 5× Q solution, 200 μM each dNTP (*Taq* PCR Core Kit, Qiagen, Germany), 1.5 μM each primer (synthesized by Eurogentec, Belgium), 0.8 U of *Taq* Polymerase (*Taq* PCR Core Kit, Qiagen), and double-distilled water to a final volume of 100 μl. The forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and the reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) were used in the PCR. The DNA amplification profile, carried out in a GeneE DNA thermal cycler (New Brunswick Scientific, Wezembeek-Oppem, Belgium), consisted of 4 min at 94°C; 35 cycles of 1 min at 94°C, 1.5 min at 55°C, and 2 min at 72°C; and 10 min at 72°C. After DNA amplification, 5 μl of the product was run on a 1% agarose gel. The remainder was stored at −20°C and then used for sequencing.

Amplified products were treated with shrimp alkaline phosphatase (1 U/μl; Amersham E70092Y) and exonuclease I (10 U/μl; Amersham, E70073Z) for 15 min at 37°C, followed by 15 min at 80°C to kill the enzymes. Sequences of *H. avenae, H. urticae, H. oryzicola, H. sorghi, H. sacchari, H. cruciferae, P. punctata, G. artemisiae,* and *M. alni* showed one or several ambiguous positions. Therefore, for these species, the nucleotide position was scored according to the IUB convention. DNA fragments were sequenced in both directions with TF1 (5′-GTAGGTGAACCTGCTGCTGG-3′), 28R1 (5′-TGATATGCTTAANTTCAGCGGGT-3′), and two additional internal forward and reverse primers MITSF (5′-ATGAAGAACGCAGC-3′) and MITSR (5′-AATGACCCTGAACC-3′) with BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, UK) according to the manufacturer's instructions. The following program was used for all sequencing reactions: 94°C for 30 s, 50°C for 15 s, and 60°C for 4 min. The resulting products were precipitated by addition of 50 μl of 95% ethanol and 2 μl of 3 M sodium acetate, pH 4.6, to each cycle sequencing reaction tube (20 μl). The pellet was rinsed with 250 μl of 70% ethanol, dried in a Speedvac concentrator, redissolved in loading buffer, and run on 48-cm 4% acrylamide sequencing gels with a Perkin–Elmer ABI Prism 377 DNA sequencer.

Initial sequences of *H. carotae, H. humuli, H. schachtii, H. ciceri, H. trifolii,* and *Cr. brinkmani* showed many ambiguous nucleotide positions for both direct forward- and reverse-primed sequencing. PCR products of these species were therefore cloned and resequenced. Low-yield PCR products of *H. bifenestra* and *A. orientalis* were also cloned and resequenced. Amplified products were excised from 1% TBE buffered agarose gels with the QIAquick Gel Extraction Kit (Qiagen), cloned into the pGEM-T vector, and transformed into JM109 High Efficiency Competent Cells (Promega Corp., Madison, WI). Several clones of each population were isolated by blue/white selection, submitted to PCR, and then cycle-sequenced. Only one sequence was used for analyses, because sequence variants from each species with polymorphism always cluster per species and do not affect tree topology with regard to interspecific relationships (data not shown).

Sequence Alignments

DNA sequences were edited with Chromas 1.45 (©1996–1998 Conor McCarthy), aligned with Clustal W 1.7 (Thompson *et al.,* 1994) and slightly modified manually with the DCSE v 3.4 for X-Windows (Linux; De Rijk and De Wachter, 1993) or GeneDOC 2.5.0 (Nicholas and Nicholas, 1997). Only sequences of ITS1, the 5.8S gene, and ITS2 were used for further analyses. The boundaries of ITS1 and ITS2 were determined by comparison of the aligned sequence with previously published sequences of *Globodera* (Bulman and Marshall, 1997; Blok *et al.,* 1998).

Several methods were used for generating alignments. Nine computer alignments were created, with gap open penalty parameters of 5, 15, and 30 and gap length penalty parameters of 3, 6.66, and 10. All of these alignments were subjected to minor manual editing followed by independent phylogenetic analysis (Table 2) and then combined into a single matrix for elision analysis (cf. Wheeler *et al.,* 1995; Liston *et al.,* 1999). A single culled alignment was also obtained, as follows. Two ingroup alignments for Heteroderinae and Punctoderinae, comprising, respectively, 1154 and 922 positions, resulted from aligning to each other and to *T. crassicrustata,* with Clustal W set to gap open/gap length penalty of 15/6.66 (default). Next, the two alignments were combined and aligned with outgroup species, resulting in a final alignment comprising 1185 positions. Subsequent phylogenetic analysis of the ITS regions was applied to an 853-character data set, after exclusion of most ambiguous positions. The original

TABLE 2

Alignment Parameters and Tree Statistics for 43 ITS Region Sequences of Species from the Family Heteroderidae

No. computer alignment	Gap open penalty	Gap length penalty	Aligned length	Informative characters	Constant characters	Tree length	Tree number	CI (w/o uninf)	HI (w/o unif)	RI	RC	*g*1
	Elision		10734	6130	3239	27050	6	0.4854	0.5146	0.7409	0.3845	−0.5955
	Culled		853	464	300	2003	2	0.4616	0.5384	0.7307	0.3597	−0.5841
1	5	3	1298	623	460	2425	12	0.5185	0.4815	0.7644	0.4366	−0.5624
2	5	6.66	1228	653	397	2638	6	0.5068	0.4932	0.7553	0.4134	−0.6257
3	5	10	1192	652	384	2773	12	0.4807	0.5193	0.7438	0.3860	−0.5310
4	15	3	1215	665	378	2875	6	0.4895	0.5105	0.7482	0.3953	−0.6220
5	15	6.66	1192	687	356	2967	6	0.4906	0.4906	0.7425	0.3892	−0.6013
6	15	10	1159	692	337	3183	6	0.4728	0.5272	0.7315	0.3663	−0.6182
7	30	3	1170	713	312	3279	6	0.4856	0.5144	0.7435	0.3841	−0.6765
8	30	6.66	1156	719	312	3317	12	0.4795	0.5205	0.7193	0.3641	−0.6778
9	30	10	1124	726	305	3498	12	0.4751	0.5249	0.7443	0.3681	−0.5629

sequences from this study are available in the GenBank database. Multiple alignments are available at http://allserv.rug.ac.be/~avierstr/ maintained by Andy Vierstraete (Department of Biology, Ghent University).

Sequence and Phylogenetic Analyses

All alignments were analyzed with maximum-parsimony, maximum-likelihood, and neighbor-joining (NJ) algorithms to resolve the interspecific and intergeneric phylogenetic relationships within Heteroderidae. *M. alni* and *Cr. brinkmani* were considered representatives of primitive genera in this family (Krall and Krall, 1978; Wouts 1985; Baldwin, 1992) and were used as outgroup taxa for phylogenetic analyses.

Equally weighted MP analyses were performed with PAUP* 4.0b4a (Swofford, 1998). Heuristic search settings were 100 replicates of random taxon addition, tree bisection–reconnection branch swapping, multiple trees retained, no steepest descent, and accelerated transformation. Gaps were treated as missing data. Bootstrap (BS) analysis with 1000 replicates was performed, to assess the support for each branch on the corresponding tree(s) obtained by heuristic search with simple addition sequences (Felsenstein, 1985). For elision analysis, mean and standard deviation of bootstrap results were calculated from the bootstrap support values obtained from individual alignments. Consistency index (CI) (Kluge and Farris, 1969), retention index (RI), rescaled consistency index (RC) (Farris, 1989), and the *g*1 statistic (Hillis and Huelsenbeck, 1992) were computed to estimate the amount of phylogenetic signal available for parsimony analysis. The *g*1 statistic, a measure of skewness of tree-length distribution, was computed by generation of 10,000 random trees with the RANDTREES option in PAUP. Pairwise divergences between taxa were computed as absolute distance values and as percentage mean distance values based on whole alignment, with adjustment for missing data.

For ML analysis, the appropriate substitution model of DNA evolution that best fitted the data set was determined by the likelihood ratio test (LRT) and the Akaike Information Criterion (AIC) with ModelTest 3.04 (Posada and Crandall, 1998). Alternative topologies were tested by the ML method of Kishino and Hasegawa (1989) as implemented in PAUP*.

Minimum-evolution analysis was performed by application of the selected ML substitution model to the NJ algorithm. Bootstrap analysis with 1000 replicates was conducted to assess the degree of support for each clade on the trees. The orders of corresponding plant hosts were mapped onto the strict consensus trees of MP, ME, and ML of the elision alignment with MacClade 3.07 (Maddison and Maddison, 1993). Trees were displayed with TreeView 1.6.1 (Page, 1996).

RESULTS

Analysis of DNA Sequence

The ITS region sequence length was shorter in species of Punctoderinae than in those of Heteroderinae and ranged from 898 bp (*C. estonica, G. rostochiensis*) to 910 bp (*G. artemisiae*) and from 931 bp (*H. oryzicola*) to 1021 bp (*H. cyperi*). The lengths of the nine alignments varied from 1124 to 1298 positions, and the elision alignment comprised 10,734 positions (Table 2). Sequence divergence for ingroup alignment ranged from 0.0 to 31.4% and from 0.3 to 14.7% within Heteroderinae and Punctoderinae, respectively, as observed by pairwise comparisons of the whole DNA alignment with adjustment for missing data.

A broad range of G + C content was observed in entire sequences of different species of Heteroderidae. A relatively higher value of G + C content was found in the ITS1 region than in the 5.8S gene or the ITS2 region. Although ITS2 was shorter than ITS1, sequence divergence between genera, and within *Heterodera* and *Globodera,* was higher in this region than

in ITS1. The length of the 5.8S gene was constant at 158 bp for all cyst-forming nematode species and *T. crassicrustata,* differing by a single insertion/deletion from outgroup taxa. Sequence variation in the 5.8S gene for the genus *Heterodera* was highest between *H. cynodontis* and *H. cajani* (6.9%). Within *Globodera* it reached only 1.3% and between *Heterodera* and *Globodera* it was 5.7%.

Phylogenetic Analyses

Elision alignment. MP analysis resulted in six equally parsimonious trees of 27,050 steps (CI = 0.4854, RI = 0.7409). The strict consensus of these six trees is presented in Fig. 1. Six main clades are supported within cyst-forming nematodes. The well-supported clade I included *Cactodera, Punctodera,* and *Globodera* species from the subfamily Punctoderinae. Within Heteroderinae, 28 species of *Heterodera* and 1 species of *Afenestrata* constituted five more clades: II, Goettingiana group; III, Humuli group + *H. salixophila;* IV, Avenae + Sacchari groups; V, Schachtii group; and VI, Cyperi group + *A. orientalis* + *H. cynodontis* + *H. bifenestra.* An additional clade (VII) consisted solely of *T. crassicrustata.* Monophyly of cyst-forming nematodes was moderately supported (BS = 84%), whereas monophyly of *Globodera* was weakly supported (BS = 69%).

Based on the results of the ModelTest LRT and AIC evaluations, ML analysis of the elision dataset was performed with the general time-reversible substitution model (GTR) with a gamma correction (Γ) for among-site rate variation and a correction for significant invariable sites (I), with the following values: R(a) = 0.9749, R(b) = 2.6468, R(c) = 1.5320, R(d) = 0.6423, R(e) = 2.5317, R(f) = 1.0000, proportion of invariable sites (p-inv) = 0.2041, and gamma shape parameter = 1.5658. This analysis yielded a tree (Ln likelihood = −127667.7399) differing from the MP trees in the positions of *T. crassicrustata* and clade I (Fig. 2, Table 3). A clade with *T. crassicrustata* + Punctoderinae and a monophyletic grouping of clades III and IV were highly supported in the ME tree. The Kishino–Hasegawa test of the ML with ME and MP trees detected significant differences among their topologies (Table 4).

Culled alignment. Parsimony analysis yielded two most parsimonious trees, the strict consensus tree being shown in Fig. 3. Relationships between main Heteroderidae clades were poorly resolved, although bootstrap support for each main clade varied from moderate to high, except for clade VI. The ML analysis was performed with the general time-reversible substitution model (GTR) corrected for among-site rate variation (Γ) and invariable sites (I), with the following values obtained from the ModelTest: R(a) = 0.9181, R(b) = 3.7942, R(c) = 1.8081, R(d) = 0.6139, R(e) = 3.4156, p-inv = 0.2534, and gamma shape parameter = 1.4533. The ML tree (Ln likelihood = −9848.51292) revealed paraphyly of Heteroderinae, whereas the ME analysis yielded an unresolved polytomy among Punctoderinae, the Goettingiana group, and other Heteroderinae clades. However, the topologies of all these trees were not significantly different (Table 4).

Computer alignments. The trends found in the phylogenetic analyses of the nine computer-generated alignments were in agreement with the above results (Table 3). Support for monophyly of the cyst-forming nematode group was higher in trees constructed by MP than in ML and ME analyses. The species of the subfamily Punctoderinae formed a strongly supported monophyletic group in all analyses. The basal position of *Cactodera* and its sister relationship with *Punctodera* were supported by most trees. *Globodera pallida, G. rostochiensis,* and subspecies of the *G. tabacum* complex formed a highly supported clade. Monophyly of *Globodera* was supported in most ME trees, whereas ML trees suggested paraphyly of this genus.

Strong or moderate support for the monophyly of Heteroderinae was found in most tree topologies. The highest support of monophyly was observed in trees obtained from ME analyses. All analyses placed *Afenestrata* within Heteroderinae, usually clustered with *H. cynodontis* and *H. bifenestra.* Except for clade VI, the five main clades within Heteroderinae were found in nearly all analyses. Incongruence between topologies usually corresponded to the weakest nodes across MP and ME analyses. Comparison of the ML tree from the culled alignment with all MP trees obtained from computer alignments by means of the Kishino–Hasegawa test yielded statistically significant differences for topologies based on computer alignments 5, 6, and 9. The MP tree obtained from computer alignment 7 was basically identical with MP trees obtained from the elision alignment.

The basal position of the Goettingiana group within Heteroderinae was moderately supported in all trees, except for analyses based on alignments 9 (MP, ME, and ML) and 7 (ME). Relationships between other main clades of Heteroderinae were not always well resolved. Within clade IV, sister taxon status of the Avenae and Sacchari groups was strongly or moderately supported in all trees except MP trees from alignment 8.

H. cajani always assumed a basal position within Clade V, with high bootstrap support. *H. glycines* and *H. medicaginis* were always strongly supported as sister species. *H. ciceri* and *H. trifolii* clustered together with moderate support; the position of *H. schachtii* was not well resolved.

Clade VI was highly to moderately supported in alignments 1, 2, and 8. In other trees either the relationships within this clade were poorly resolved or the Cyperi group formed a separate clade within Heteroderinae. Clade VI usually diverges after the Goettingi-

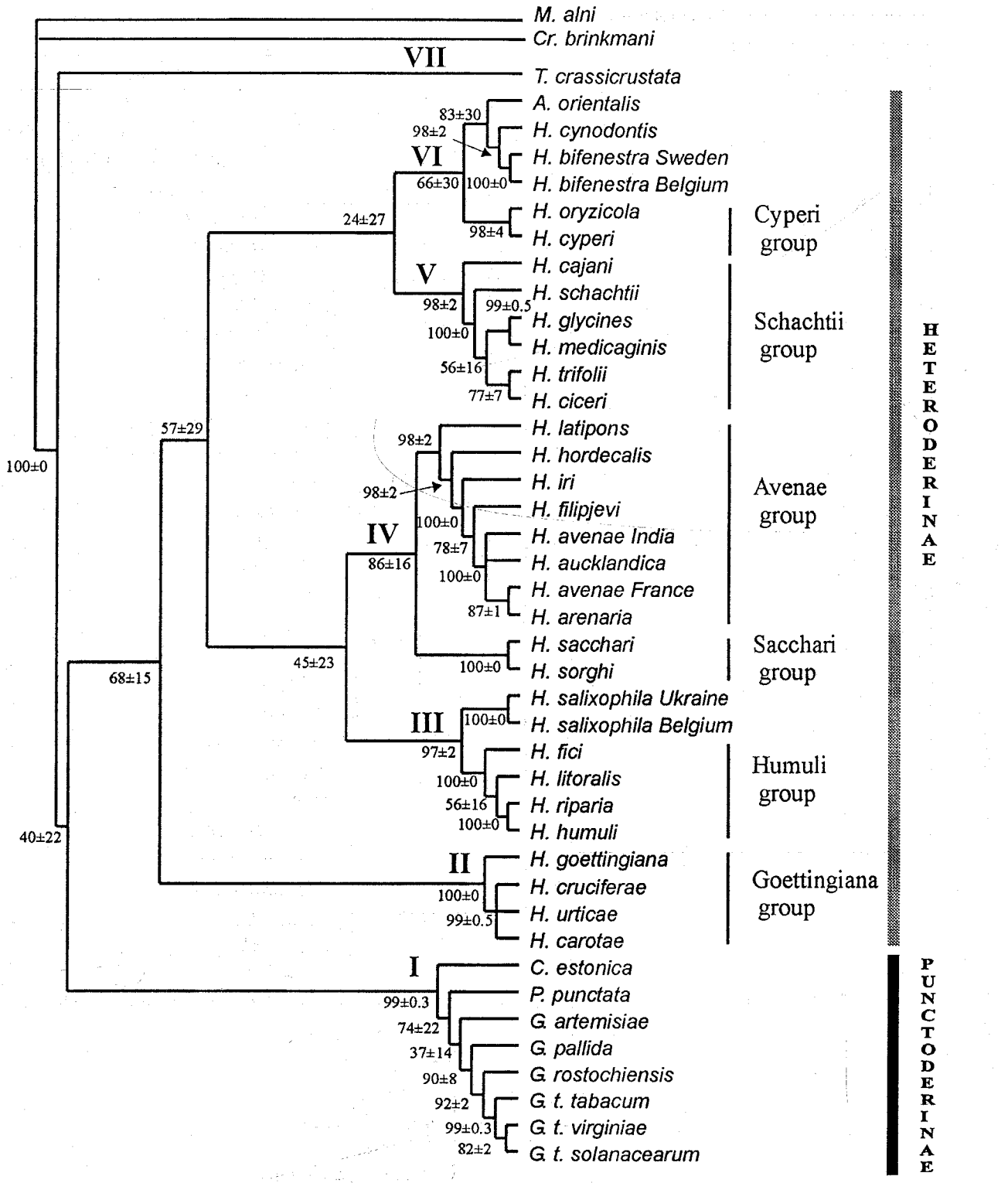

FIG. 1. Elision analysis of the ITS region sequence from 40 species and subspecies from the family Heteroderidae. Strict consensus of six equally parsimonious trees resulting from the elision of nine alignments. Mean and standard deviation of independent bootstrap analyses on the nine alignments are given on appropriate clades.

ana group, but it assumes a basal position in ME and MP trees based on alignment 9. Sister species relationships of *H. cyperi* and *H. oryzicola* were highly supported in all trees.

Hypothesis testing. Several hypotheses derived from ML analyses of the elision and culled alignments were evaluated by the Kishino–Hasegawa test (Table 4). Topologies constraining *A. orientalis* (to check the

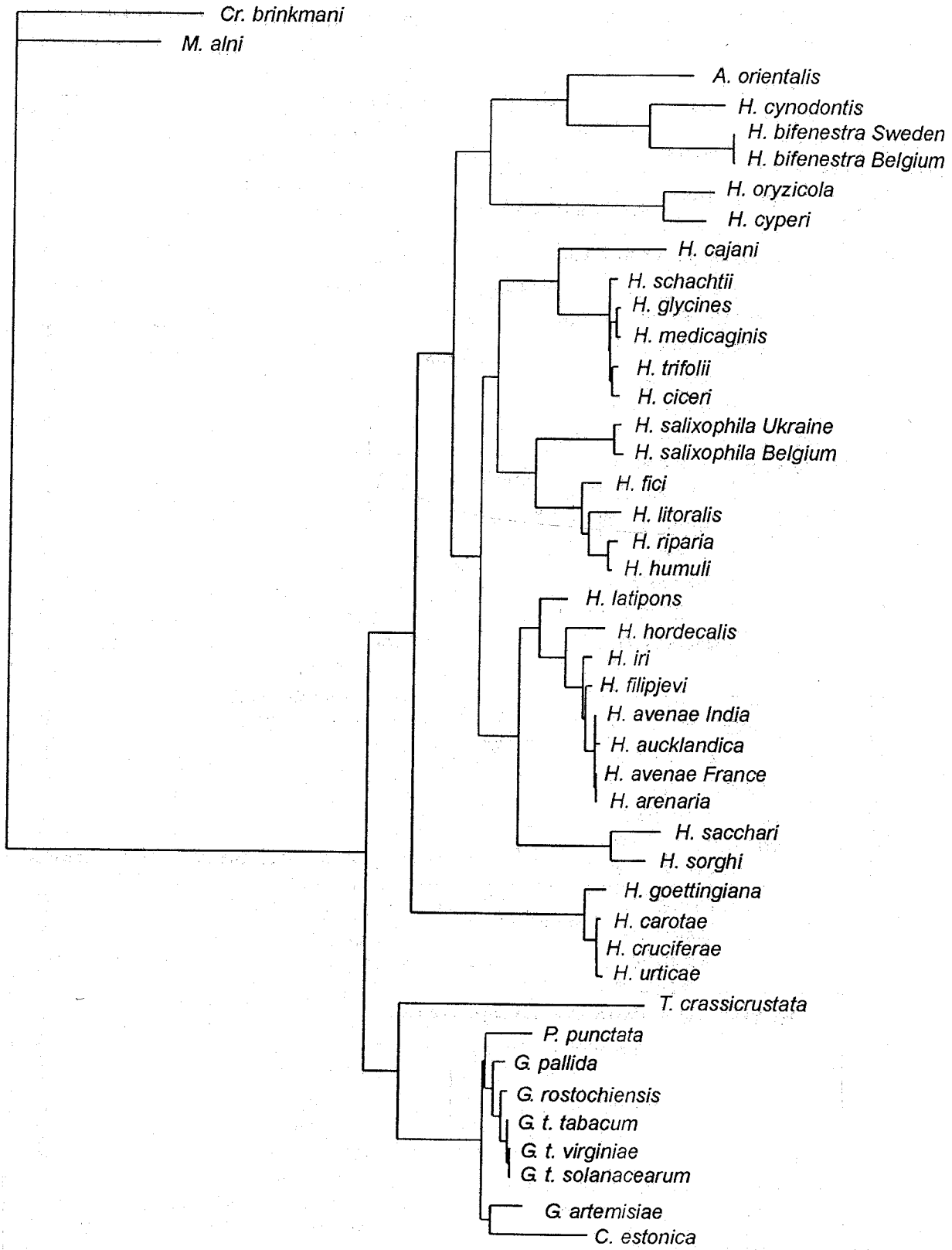

FIG. 2. Maximum-likelihood tree constructed from the elision alignment of the ITS sequence of 40 species and subspecies from the family Heteroderidae with GTR + Γ + I model of DNA sequence evolution (*Ln* likelihood = −127667.73996).

TABLE 3

Occurrence of Selected Clades in the Topologies Resulting from Maximum-Parsimony (MP), Minimum-Evolution (ME), and Maximum-Likelihood (ML) Analyses of Heteroderid ITS Sequences

	Elision			Culled			1			2			3			4			5			6			7			8			9		
	MP	ME	ML	MP	ME	ML	MP	ME	ML	MP	ME	ML	MP	ME	ML	MP	ME	ML	MP	ME	ML	MP	ME	ML	MP	ME	ML	MP	ME	ML	MP	ME	ML
Monophyly of cyst-forming nematode clades (Heteroderinae + Punctoderinae)	■			●			●						●									■	■	×	●						■	■	
Heteroderinae + *T. crassicrustata*									×							●	■	×	■														
Punctoderinae + *T. crassicrustata*		◆	×		◆	×		●		◆	◆	×		■	×					●	×					●	×	●	●	×			×
Monophyly of Heteroderinae clade	◆	◆	×	●	●		■	■	×	●	◆	×	●	■	×	■	■	×	■	◆	×	■	■	×	●	■	×	●	■	×	■	■	×
Paraphyly of Heteroderinae clade						×																											
Goettingiana group + other Heteroderinae	◆	◆	×	●	●		●	●	×	●	●	×	◆	◆	×	■	●	×	■	●	×	■	■	×	●		×	●	●	×			
Goettingiana group within Heteroderinae						×																				●					●	■	×
Humuli group + *H. salixophila*	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×
Avenae group + Sacchari group	◆	◆	×	■	◆	×	◆	◆	×	◆	◆	×	◆	◆	×	■	◆	×	■	◆	×	◆	◆	×	■	◆	×		◆	×	◆	◆	×
Sacchari group within other Heteroderinae																												●					
Cyperi group + *A. orientalis* + *H. cynodontis* + *H. bifenestra*	◆	◆	×		●		◆	◆	×	■	◆	×				●			●		×				●			●	■	×			
Cyperi group + *H. cynodontis* + *H. bifenestra*																						◆	◆	×							■	◆	×
Cyperi group within other Heteroderinae				●		×							●	●	×		●	×		■						●	×						
A. orientalis* + *H. cynodontis* + *H. bifenestra	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×	■	◆	×	◆	◆	×	◆	◆	×				◆	◆	×	◆	◆	×			
A. orientalis + Goettingiana group																						■	●	×									
A. orientalis + Cyperi group + *H. cynodontis* + *H. bifenestra*																															■	■	×
Monophyly of Punctoderinae clades	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×	◆	■	×	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×	◆	◆	×
Monophyly of *Globodera*	●	◆			●		●	■			●			●			■			●		●	●		●	■		●	●		●		
G. artemisiae + *P. punctata*				●																													
G. artemisiae + *P. punctata* + other *Globodera*						×			×	■						◆		×	■							×					●	●	
G. artemisiae + *C. estonica*			×									×	●		×						×			×					×			×

Note. Bootstrap support (%) for MP and ME trees: <70 (weak) ●; 70–90 (moderate) ■; >90 (strong) ◆.

TABLE 4

Comparison of the Likelihoods of Alternative Trees

	Elision				Culled			
	Best log likelihood	Δ lnL	SD	*P*	Best log likelihood	Δ lnL	SD	*P*
ML tree	−127667.73996	—	—	—	−9848.51292	—	—	—
MP trees	−127785.94668	118.20673	32.83653	0.0003*	−9851.98596	3.47305	10.28823	0.7358
ME tree	−127723.33141	55.59145	26.57420	0.0365*	−9860.21930	11.70638	10.93626	0.2847
Afenestrata + *Heterodera*[a]	−128102.42909	434.68914	44.85737	<0.0001*	−9901.37887	52.86596	15.88487	0.0009*
Avenae group + other heteroderids[b]	−127926.61973	258.87978	43.90586	<0.0001*	−9871.95430	23.44139	14.06753	0.0960
Avenae group + *H. bifenestra*[c]	−129909.14946	2241.40950	89.34631	<0.0001*	−10029.07902	180.56610	23.99201	<0.0001*
Sacchari group + Cyperi group + *A. orientalis* + *H. cynodontis* + *H. bifenestra*[d]	−128042.37297	374.63301	38.81976	<0.0001*	−9877.82666	29.31374	14.04401	0.0372*

[a] Validity of the genus *Afenestrata*.
[b] Validity of the genus *Bidera* (Avenae group).
[c] Grouping of species with bifenestrate cysts infecting grasses.
[d] Grouping of species with three incisures in juveniles.
* Asterisked values indicate significant difference at $P < 0.05$.

validity of the genus *Afenestrata*), grouping species with bifenestrate cysts (*H. bifenestra* with the Avenae group infecting grasses), or grouping species with three incisures in juveniles were significantly rejected. Placement of the Avenae group outside other Heteroderinae (to check the validity of the genus *Bidera*) was acceptable based only on the culled alignment.

DISCUSSION

Sensitivity of Phylogenies to the Employed Alignment and Tree-Construction Procedures

As has been previously emphasized, both the chosen alignment and the tree-building methods significantly influence the outcome of phylogenetic studies (Morrison and Ellis, 1997). In the particular case of ITS sequence data of cyst nematodes, our study clearly shows that the occurrence and position of clades can vary with the applied procedures. For example, the well-supported position of the Cyperi group within the clade comprising species with three incisures was generated from alignments with the lowest gap open and gap length penalties, and the position of *A. orientalis* was sensitive to alignment parameters, rather than to phylogenetic procedures. Increasing the gap length penalties decreased bootstrap support or disturbed the position of this species, which then clustered with the Goettingiana group or appeared as a sister taxon of the Cyperi group. In contrast, the monophyly of *Globodera* proved sensitive to the tree-building method. *Globodera* monophyly was revealed in most ME trees, although the ML trees showed sister relationships of *G. artemisiae* with other species belonging to the genera *Punctodera* or *Cactodera*.

In any phylogenetic study, difficulties arise during the alignment of regions with greater sequence divergence and more variable length. Inclusion of ambiguously aligned regions will increase noise levels and may support erroneous patterns of branching, whereas removal of such regions will lower signal levels and thus reduce attainable resolution (Gatesy *et al.*, 1993; Wheeler *et al.*, 1995). As expected, our culled alignment, excluding the most ambiguous fragments in our sequences, tended to lower the phylogenetic resolution within cyst-forming nematode groups. Wheeler *et al.* (1995) therefore advocated the less extreme approach of elision, whereby the combining of alternative alignments into a single extended alignment reduces the impact of erroneously aligned sites. In our study, the elision approach generally yielded higher bootstrap values. However, different tree-building methods still generated alternative topologies for some of the main Heteroderidae clades, reflecting underlying biases that cannot be avoided without more objective criteria for selecting a single "best" alignment.

Congruence of Molecular and Nonmolecular Phylogenetic Hypotheses for Heteroderidae

Outgroup relationships of cyst-forming nematodes. The non-cyst-forming nematodes *M. alni* (Meloidoderinae) and *Cr. brinkmani* (Cryphoderinae) were chosen as outgroups in our study. They are unanimously considered close to the ancestor of most species of the Heteroderidae (Krall and Krall, 1978; Ferris, 1979; Wouts 1985; Baldwin, 1986, 1992). Species from both these genera parasitize angiosperm and gymnosperms.

Two alternative views on the origin of cyst-forming nematodes were implied in different phylogenetic classifications of the Heteroderidae: monophyletic (Krall and Krall, 1978; Ferris, 1979; Baldwin, 1992) versus

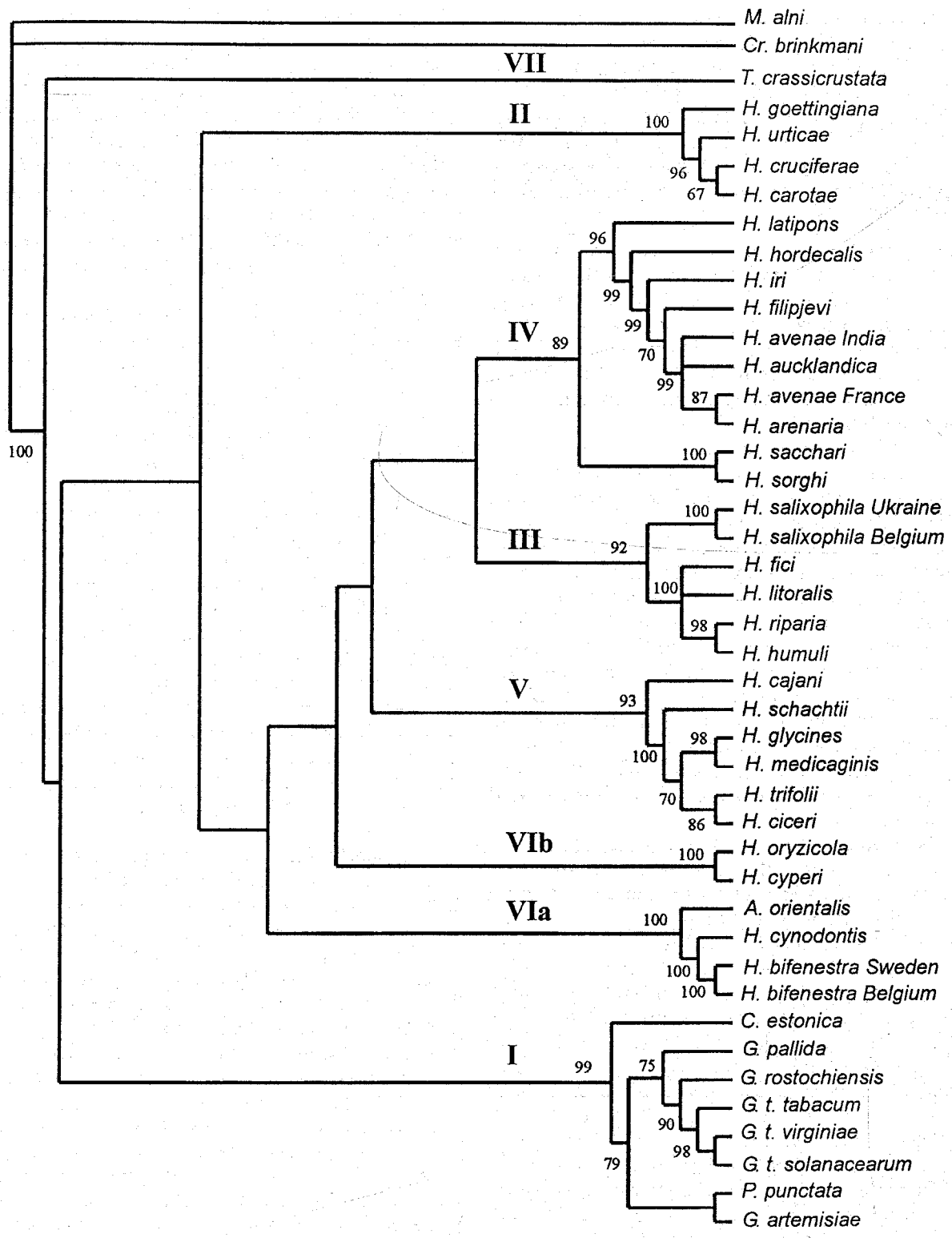

FIG. 3. Strict consensus of two most parsimonious trees (tree length = 2003) resulting from the culled alignment of the ITS sequences of 40 species and subspecies from the family Heteroderidae. Bootstrap numbers (70% or higher) are given on appropriate clades.

polyphyletic (Stone, 1979; Wouts, 1985). We tested these hypotheses by checking the position of the non-cyst-forming nematode *T. crassicrustata* (Ataloderinae) in our phylogenetic trees. Compared to both previously mentioned genera, *Thecavermiculatus* is considered more derived (Ferris, 1979; Wouts, 1985; Baldwin, 1992). Our phylogenetic analyses did not support any of these hypotheses. MP analyses of certain

alignments resulted in moderate to high statistical support of the position of *T. crassicrustata* variously placed outside or inside the cyst-forming nematode clade. Distance and ML analyses tended to support the polyphyletic origin of this group. Additional ITS sequence data for other species from the subfamily Ataloderinae may improve the evolutionary reconstruction and better resolve the position of the genus *Thecavermiculatus.*

Punctoderinae. Our analyses strongly support monophyly of this subfamily containing the genera *Cactodera, Punctodera,* and *Globodera.* According to phylogenetic hypotheses proposed by Wouts (1985), these three genera are considered very closely related, sharing a similar structure of the fenestration around the vulva (circumfenestrate type) and belonging to an exclusive evolutionary lineage.

The close relationships between *Cactodera* (*C. milleri* and *C. weissi*) and *Globodera* species parasitizing Solanaceae were demonstrated by Ferris (1998) based on the ITS rDNA sequence data. According to our data from most analyses, the genus *Cactodera* can be considered a basal taxon in Punctoderinae, confirming Ferris's (1979) hypothesis based on a cladistic morphological approach. Stone (1975) and Krall and Krall (1978) considered *Punctodera* a primitive genus originating independently from *Globodera* or *Cactodera.* Wouts (1985) placed *Punctodera* at the apex of the Punctoderinae and considered the genus more derived. The relatively high level of nucleotide sequence similarity among these three genera suggests that they may have diverged relatively recently and/or in rapid succession from one another.

Recognition of a monophyletic genus *Globodera* is not borne out by some of our analyses. Convergent evolution of *Globodera* species parasitizing plants from the unrelated families Solanaceae, Rosaceae, and Asteraceae was hypothesized by Krall and Krall (1978) without morphological evidence. Paraphyly of *Globodera* may simply reflect the general tendency of phylogenetic algorithms to produce unbalanced trees rather than the true evolutionary history of a group with high evolutionary rates. Genetic divergence within this genus reflects the species groupings based on geographical origin and host plants: (i) *G. artemisiae* infecting *Artemisia* in Europe and Asia, (ii) *G. pallida and G. rostochiensis* originating from the Andean regions of South America and parasitizing potato and other closely related species of the Solanaceae, and (iii) species of the *G. tabacum* complex being found mainly in the United States and parasitizing other members of the Solanaceae, notably tobacco (Evans and Rowe, 1998).

Heteroderinae. Taxonomists have divided cyst-forming nematodes from *Heterodera* into three groups based on vulval cone structures: the Schachtii, Goettingiana, and Avenae groups (Mulvey and Golden, 1983; Baldwin and Mundo-Ocampo, 1991). Three additional partly overlapping groups have also been proposed: the Humuli group (Mathews, 1971; Subbotin *et al.,* 1997), the Fici-humuli group (Ferris, 1979), and the Latipons group (Wouts and Sturhan, 1995). The combination of our molecular data with the morphology of vulval structures and the number of incisures in juvenile lateral fields supports the recognition of the Schachtii, Goettingiana, Avenae, and Humuli groups—albeit with a modified species composition—and the erection of two new groups: the Cyperi and the Sacchari groups.

There are several hypotheses about the earliest groups to arise within the genus *Heterodera:* amphimictic species *H. schachtii, H. glycines,* and perhaps *H. salixophila* (Krall and Krall, 1978), a *H. cruciferae*-like form parasitizing dicots (Stone, 1979); or the cyst-forming species currently placed in *Afenestrata* (Ferris, 1979; Wouts, 1985). Our phylogenetic analyses resolve some of the basal relationships within *Heterodera,* usually placing the Goettingiana group closest to the origins of the genus. Obviously, our molecular data lend significant support to Stone's (1979) hypothesis.

Molecular phylogeny of heteroderids allows us to evaluate the hypothesis of coevolution of heteroderids with their hosts as proposed by Krall and Krall (1978) and Stone (1979). Our data tend to support the idea that different heteroderid groups coevolved with hosts belonging to single or closely related families of plants (Fig. 4). Nevertheless, some species were secondarily able to colonize ecologically convergent plant species from unrelated families. Our data also suggest an early divergence between tropical and temperate heteroderid species.

The Goettingiana (or Cruciferae) group includes *H. goettingiana, H. carotae, H. urticae,* and *H. cruciferae.* Other species (*H. cyperi* and *H. cardiolata*), which were formerly considered members of this group (Baldwin and Mundo-Ocampo, 1991), should, according to our data, be considered representatives of different groups. The unexpected clustering of *H. carotae* (Michigan isolate, USA) with the Schachtii group in the phylogenetic cladogram based on the ITS sequences obtained by Ferris (1998) was perhaps due to incorrect identification of the population used.

The Cyperi group includes *H. cyperi* and *H. oryzicola.* These species infect grass-like plants (Cyperaceae) and grasses (Poacea, Oryzoideae), their juveniles have three incisures in the lateral field, bullae are poorly developed or altogether absent, and they are found in subtropical and tropical regions of the world. These two species are placed in a basal position in most of our phylogenetic trees. *A. orientalis* clustered with high support with *H. cynodontis* and *H. bifenestra* in most phylogenetic trees. The cysts of *Afenestrata* species have neither bullae nor underbridges and do not show fenestration. The latter characters, which are not shared with other cyst-forming genera, were argued to

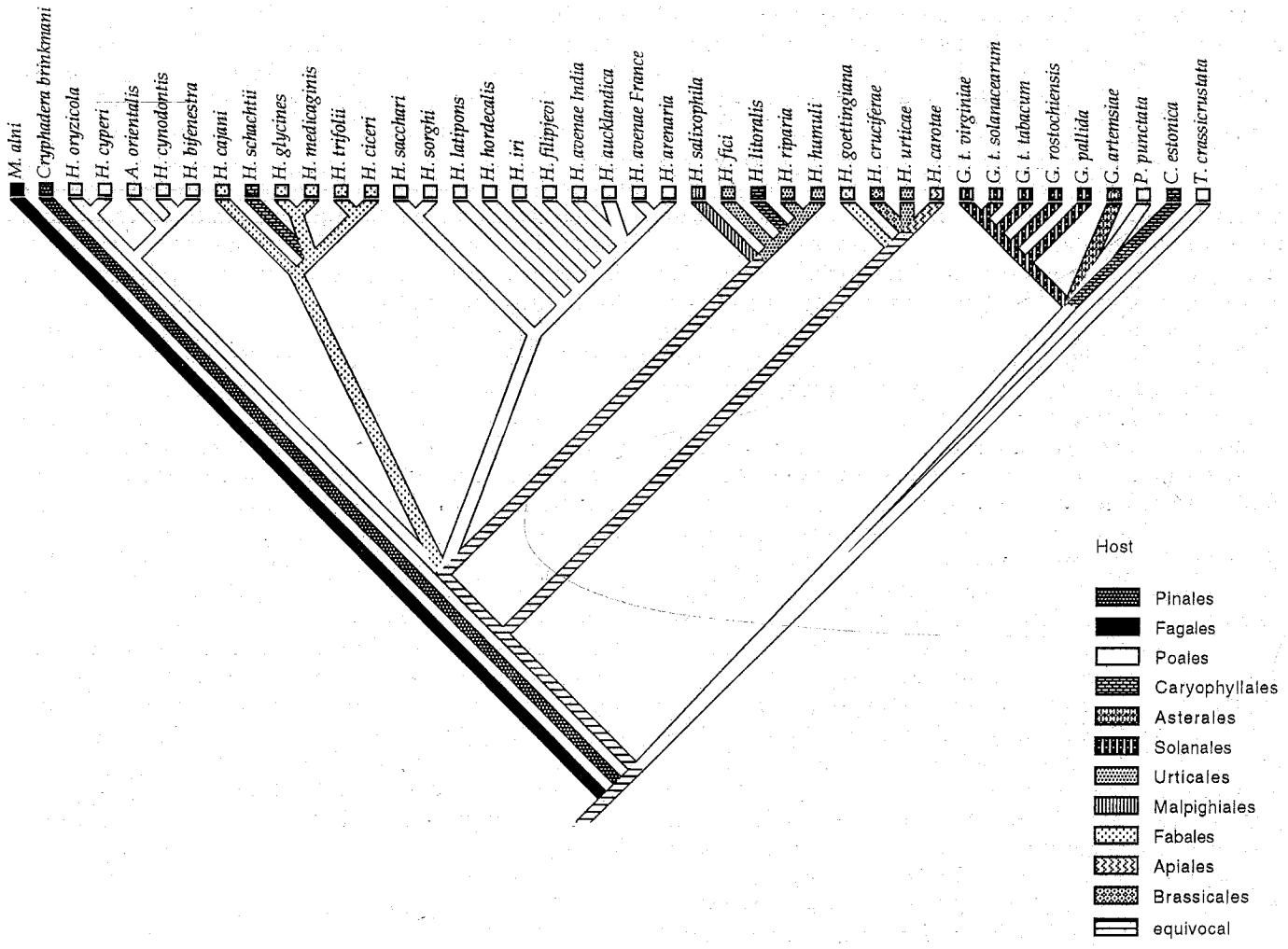

FIG. 4. Strict consensus of maximum-parsimony, minimum-evolution, and maximum-likelihood trees obtained from elision alignment with the mapping of host plant orders.

warrant a separate genus for these species, considered ancestral to the genus *Heterodera* (Baldwin and Bell, 1985; Wouts, 1985). Our molecular analyses do not provide obvious support for this hypothesis. Instead, they suggest the invalidity of *Afenestrata* and a close relationship of *A. orientalis* with *H. bifenestra* and *H. cynodontis,* all three species matching the above-mentioned characters of *H. cyperi* and *H. oryzicola.*

Our results support the competing view expressed by Wouts and Sturhan (1995), that *H. bifenestra* does not belong in the Avenae group because of the presence of only three incisures in the lateral field of the second-stage juveniles and is instead close to *H. cyperi, H. graminis,* and *H. mothi.* In our trees, *H. bifenestra* clustered robustly with *H. cynodontis,* which belongs to the *H. cardiolata* species complex. According to Ferris (1998), the ITS sequence of *H. bifenestra* is highly dissimilar to those of all other taxa in her phylogenetic analysis and this species was chosen by her as an outgroup for cyst-forming nematodes. Nevertheless, in our study this species was invariably placed within *Heterodera* with high statistical support, when non-cyst-forming nematode species were used as outgroup taxa.

Whereas the positions of the previous clades were well resolved in at least some trees, the branching orders among other major heteroderid clades remained much more uncertain. This unresolved polytomy suggests that these heteroderid groups may have undergone a relatively rapid radiation or an acceleration in evolitionary rate of the ITS region. Two of these groups (Schachtii group, Humuli group and *H. salixophila*) apparently coevolved with dicots while the others (Sacchari and Avenae groups) coevolved with monocots.

The tropical species *H. cajani,* distributed in India, Pakistan, and Egypt (Evans and Rowe, 1998) and attacking agricultural crops from the Fabaceae, shares a common ancestor with species from the *H. schachtii* group *sensu stricto.* We can therefore suggest that the Schachtii group primarily coevolved with plants from the family Fabaceae and secondarily colonized Chenopodiaceae (Fig. 4).

The Humuli group is characterized by a bifenestral condition of the vulva in most species (ambifenestrate in *H. fici*), few or absent bullae, a very weak underbridge, and a long vulval slit situated in a cleft between the thickened vulval lips (Subbotin *et al.,* 1997). Except for *H. litoralis,* this group clearly coevolved with plants from the Urticales. *H. fici,* a widely distributed subtropical and tropical species, apparently diverged prior to all temperate species. Sequence data confirm that the bifenestrate vulval region of the latter species is an apomorphic state. It is interesting that *H. salixophila,* previously considered a member of the Schachtii group by Baldwin and Mundo-Ocampo (1991), shares a common ancestor with the Humuli group. This relationship has not previously been proposed.

Another major clade includes two morphologically different groups which coevolved with monocots: Sacchari (ambifenestrate cysts, second-stage juveniles with three incisures) and Avenae (bifenestrate cysts, second-stage juveniles with four incisures). The close relationships between these groups is a novel finding of this study. Our molecular analyses also justify a synonymization of the genus *Bidera,* erected for species within the Avenae group, with *Heterodera,* as proposed by several authors (Mulvey and Golden, 1983; Wouts and Sturhan, 1995). Surprisingly, we did not find any nucleotide differences between European populations of *H. avenae* and *H. arenaria,* supporting the hypothesis that *H. arenaria* may be a polyploid of *H. avenae.*

Some inconsistencies between our molecular phylogeny and earlier-proposed phylogenetic hypotheses or groupings may be attributed to homoplastic evolution. For example, according to our data a bifenestral vulval cone developed independently at least three times during the evolution of cyst nematodes. Likewise, the presence of three incisures in the lateral field of second-stage juveniles of the cyst nematodes seems to have arisen twice independently. Finally, a short vulval slit developed independently in the Avenae group and in *H. bifenestra* within Heteroderinae.

Usefulness of ITS Sequence Data for Phylogenetic Studies of Heteroderidae

The analyses of ITS sequence data presented here provide new insights into the evolutionary relationships of cyst-forming nematode species. The obtained phylograms generally agree with some of the current morphological groupings of cyst-forming nematode species and with previous hypotheses of their phylogenetic relationships and coevolution with host plants. Although MP, ML, and ME analyses resulted in several alternative phylogenies, certain phylogenetic patterns were nevertheless substantiated, a number of species groups are supported as being monophyletic, and a number of inferences can be made about polarities of various morphological and host range characters.

Root knot nematodes of the genus *Meloidogyne* are ecologically and morphologically similar to cyst nematodes, and significant variation of ITS sequences was recently discovered in some *Meloidogyne* species (Hugall *et al.,* 1999). This variation conflicted with mitochondrial haplotype patterns and led Hugall *et al.* (1999) to suggest that multiple hybridization events may have occurred in *Meloidogyne.* Unlike Hugall *et al.* (1999), we set out to sample ITS extensively across a family by direct sequencing, rather than intensively sequencing cloned ITS amplicons from just a few species. Although less sensitive, direct sequencing does allow detection of polymorphism, and this occurred with a few of our heteroderid PCR products. For these few cases, the sequence variants obtained after cloning did not lead to their disparate placement in our phylogenetic trees. We assume that ITS microheterogeneity within cyst nematode species is less pervasive than that in some *Meloidogyne* species. It appears instead that the main constraint on the usefulness of heteroderid ITS sequence data consists of limited resolving power. Thus, greater confidence about some of the ancient speciations within the family must await even more extensive sampling of additional taxa and combined analyses of the ITS region with other molecular data sets. Such additional data may help us improve alignments by reducing the number of ambiguous positions, whereas tests of multilocus congruence will directly enhance our understanding of cyst-forming nematode phylogeny, at both the basal and the internal levels.

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