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β-tubulin Paralogs Provide a Qualitative Test for a Phylogeny of Cyst Nematodes[1]

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Abstract: Evolutionary relationships among cyst nematodes based on predicted β-tubulin amino acid and DNA sequence data were compared with phylogenies inferred from ribosomal DNA (ITS1, 5.8S gene, ITS2). The β-tubulin amino acid data were highly conserved and not useful for phylogenetic inference at the taxonomic level of genus and species. Phylogenetic trees based on β-tubulin DNA sequence data were better resolved, but the relationships at lower taxonomic levels could not be inferred with confidence. Sequences from single species often appeared in more than one monophyletic clade, indicating the presence of β-tubulin paralogs (confirmed by Southern blot analysis). For a subset of taxa, good congruence between the two data sets was revealed by the presence of the same putative β-tubulin gene paralogs in monophyletic groups on the rDNA tree, corroborating the taxon relationships inferred from ribosomal DNA data.

Key words: β-tubulin, cyst nematodes, paralogous genes, ribosomal DNA.

Inferences of evolutionary relationships and subsequent taxonomic decisions in cyst nematodes were traditionally based on morphological and host data. Recently, the focus has shifted to molecular data, especially ribosomal DNA (rDNA) sequence data (Ferris, 1998). Subbotin et al. (2001) presented relationships for a wide range of taxa from this group inferred from rDNA data. Although rDNA is the most widely used nuclear genomic region for phylogenetic inference for nematodes (Blaxter et al., 1998) and other organisms, many authors (e.g., Johnson and Whiting, 2002) have pointed out the need for information from additional genes to determine evolutionary relationships with greater confidence. Often the sequence data of conserved protein genes are suggested as desirable possibilities.

Palumbi (1996) included β-tubulin among the conserved protein genes with broad applicability for inferring phylogenies at lower taxonomic levels. β-tubulin data have been used for phylogenetic inference at the kingdom level, using protein sequence data (e.g., Baldauf et al., 2000), and at lower taxonomic levels, e.g., species level, using DNA sequence data (Scheffer and Lewis, 2001). As for many other protein genes in eukaryotes, β-tubulin was recently discovered to comprise a small gene family in *Caenorhabditis elegans* (Gogonea et al., 1999). If similar but distinct members of such a gene family are recovered with the same PCR primers, they must be treated as distinct entities for phylogenetic inference. This is obvious when protein orthologs and paralogs of a gene family are distinct; and it is expected that a species tree will be mirrored in a subtree representing each paralog.

Martin and Burg (2002) showed the perils for phylogenetic analysis of unrecognized paralogy in conserved protein genes in sharks. Although orthology of genes from different taxa is often assumed, genes from one taxon may actually be paralogous to those of another taxon. They found that if orthology were assumed for heat shock protein 70 (HSP70) in sharks, species phylogenies based on morphological data were not concordant with those based on HSP70 data for the same group of sharks. Page and Holmes (1998) suggested that genes can be viewed as "tracking" species and that reconciled trees can be used when faced with paralogous genes. For efficient data collection, it is desirable to be able to use the same primer set across a broad range of phylogenetically distinct taxa under study. The set of primers we used yielded β-tubulin sequence from phylogenetically diverse plant-parasitic cyst nematode taxa. The objective of this study was to find a way to use our new information about β-tubulin gene evolution for testing cyst nematode phylogenies based on rDNA data.

Materials and Methods

Specimens: Nematode specimens from which β-tubulin sequence was obtained included 45 taxa listed in Table 1. A subset of 31 taxa (marked with an asterisk in Table 1) for which beta-tubulin and rDNA data were both available were used to test congruence among the data sets. All specimens were isolated from roots or soil around the roots of the host plants and preserved in 70% ethanol.

DNA extraction, amplification, and sequencing: For each nematode isolate, DNA preparations were made from single cysts. These nematodes were homogenized in 25 µl TE buffer (pH 7.5), and total genomic DNA was extracted using InstaGene Matrix (Bio-Rad, Hercules, CA). The β-tubulin gene region spanning amino-acid positions 213 to 308 (based on the position numbers in the *C. elegans* β-tubulin genes) was amplified from all the study taxa (Table 1) using the forward primer 5′CTTTACGACATTTGTTTCCGCAC 3′ and the de-

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TABLE 1. Source of isolate and host data for species used in the study[a]

Species name	Source of isolate	Host
*Afenestrata koreana**	Florida	fishpole bamboo
*Bilobodera flexa**	India	*Allmania nodiflora*
Cactodera betulae bl.	Arkansas	black locust
*C. betulae sl.**	Arkansas	birch
*C. estonica**	Estonia	*Polygonum neglectum*
C. milleri	Indiana	lambsquarter
*C. salina**	Mexico	*Salicornia bigelovii*
C. weissi	Indiana	smart weed
*Ekphymatodera thomasoni**	California	*Juncus effusus*
*Globodera artemisiae**	Estonia	*Artemisia rubripes*
*G. millefolii**	Estonia	*Achillea millefolium*
*G. pallida**	UK	potatoes
*G. rostochiensis**	UK	potatoes
Globodera sp.	Mexico	*Solanum dulcamara*
*G. tabacum**	Connecticut	tobacco
G. virginiae B	Virginia	*Solanum dulcamara*
G. virginiae C	Virginia	*Solanum dulcamara*
Globodera X75	Mexico	*Solanum dulcamara*
*Heterodera avenae**	Idaho	wheat
H. avenae	Australia	wheat
*H. avenae ES**	Estonia	oats
*H. avenae ES sl.**	Estonia	oats
*H. cajani**	India	pigeon pea
*H. carotae IT**	Italy	carrots
H. carotae MI	Michigan	carrots
*H. ciceri**	Syria	chickpea
*H. cruciferae**	US	cabbage
H. fici	Italy	fig
*H. glycines**	Arkansas	*Glycines max*
*H. goettingiana**	Washington	peas
*H. hordecalis ES**	Estonia	*Bromus inermis*
*H. hordecalis IT**	Italy	quack grass
*H. iri**	UK	grasses
*H. latipons IS**	Israel	oats
H. latipons R	Russia	false wheat
*H. mani**	UK	wheat
*H. mediterranea**	Italy	lentisc
H. mothi	India	*Cyperus rotundus*
*H. schachtii**	Michigan	sugar beat
*H. trifolii ES**	Estonia	clover
*H. trifolii IT**	Indiana	clover
H. zeae	Maryland	corn
H. zeae	India	corn
*Punctodera chalcoensis**	Mexico	corn
*P. punctata**	Estonia	*Poa pratensis*

[a] A subset of species used to investigate congruence among ribosomal and β-tubulin data sets is marked by an asterisk.

generate reverse primer 5′GCGGGTCACAKGCGGCCATCATG 3′. These primers worked well for our entire spectrum of cyst nematode species and were chosen from a series of primer pairs we designed based on comparisons of published sequence of β-tubulin for *C. elegans* and *Brugia pahangi*. All the primer pairs were from an area of the *C. elegans* sequence that spanned the region between exons 3 and 6. Each set we tested included an intron because non-coding introns between conserved exons offer the best potential for the high rates of sequence evolution useful for phylogenetic analysis.

The amplified fragment was cloned into 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 carried out using (at various times) automatic sequencers (ALFexpress, Pharmacia Biotech; and LI-COR) at the Purdue Genomics Center. Both strands of DNA from at least 3 to 5 clones were sequenced for each nematode isolate. In an attempt to amplify all β-tubulin genes from a single species, we sequenced 10 to 15 clones for selected taxa. Sequences from different clones from the same taxon that consistently clustered together on a neighbor-joining tree (data not shown) were assumed to represent either polymorphisms or sequencing errors, and only one sequence—the sequence that predominated—was used to represent a cluster. A ribosomal DNA fragment that spanned the two internal transcribed spacers (ITS1 and ITS2) and the intervening 5.8S gene was amplified using primers that were previously described (Ferris et al., 1993).

The resulting sequences for β-tubulin for all 45 taxa were deposited in GenBank (accession numbers AY689374–AY689435), and previously unpublished rDNA sequences (*Heterodera trifolii* Estonia, *Cactodera estonica, H. hordecalis* Estonia, and *H. mani*) were also deposited (accession numbers AY692354–AY692357).

Southern blot analysis: Genomic DNA from 100 mg of *H. glycines* cysts was isolated using the DNeasy Tissue Kit (QIAGEN) and cleaned by ethanol precipitation. Restriction digestion reaction of 1 μg of genomic DNA was carried out using 25 μl of buffer H (Promega), 2.5 μl BSA, 20 μl EcoRI enzyme (Promega), and 195 μl water overnight at 37 °C and electrophoresed on a 1.0% agarose 1 × TBE gel, with a 1-kb DNA ladder. Preparation of the gel for blotting was done at room temperature in three steps: depurination, 5-minute wash with 0.25 M HCl, denaturation, two 15-minute washes with 0.6 M NaCl, 0.4 M NaOH, neutralization, two 15-minute washes with 0.5 M Tris, 1.5 M NaCl, pH 7.5. Transfer of DNA fragments from an electrophoresis gel to a nylon membrane was done overnight by upward capillary transfer using a filter paper wick and 10× SSC as a transfer buffer. After rinsing the blot in 2× SSC for 10 minutes at room temperature, the DNA was UV crosslinked to the membrane. Plasmid preps with determined sequence from *Heterodera glycines* were used for probes. DNA fragments were PCR amplified from plasmid preps and cleaned using the QuickStep PCR purification kit (BioEdge System). For the probe ~0.2 μg DNA was radioactively labeled using the Rediprime II DNA Labeling System (Amersham Pharmacia Biotech). Prehybridization of the membrane was carried out in a hybridization oven at 65 °C for 14 to 16 hours in 5× SSPE, 7.5% SDS solution. Hybridization was carried out in the same prehybridization solution with added denatured labeled probe mixed with 100 μl of 5 mg/ml

denatured sheared salmon sperm DNA at 65 °C for 14 to 16 hours. After hybridization the membrane was washed twice for 15 minutes at room temperature in 2× SSC, 0.1% SDS solution, followed by two 30-minute washes at 65 °C in 1× SSC, 0.1% SDS solution. The membrane was placed on a phosphoimager screen overnight and the image collected.

Phylogenetic analysis: Sequences were aligned using the computer program Clustal X (Thompson et al., 1997) with default penalty values (gap open = 15, gap extend = 6.66), and then manually adjusted using MacClade 4.0 (Maddison and Maddison, 2000). The amino acid sequence data set was derived from aligned DNA sequences by conceptual intron splicing and amino acid translation using MacClade 4.0.

Phylogenetic analysis was carried out using PAUP* 4.0b10 (Swofford, 2001) under parsimony and maximum likelihood optimality criteria, and using the neighbor-joining algorithm in PAUP*. To investigate relationships among cyst nematode β-tubulin paralogs and other invertebrate β-tubulin genes, a neighbor-joining tree in PAUP* was inferred from the amino acid sequence data for representative taxa for cyst nematode β-tubulin paralogs with none, one, and two introns plus the data from GenBank for invertebrate taxa. The neighbor-joining algorithm was used to minimize the long branch attraction problem of parsimony (Felsenstein, 1978). Maximum parsimony analysis of our β-tubulin and rDNA data was performed using the heuristic search with gaps treated as missing characters. The model of sequence evolution that best fit our data set (HKY + G) was chosen based on the likelihood ratio tests as implemented in Modeltest, version 3.06 (Posada and Crandall, 1998), and the molecular clock tested using the likelihood ratio test with default values, as implemented in PAUP* (Huelsenbeck and Rannala, 1997). The selected model and its estimated parameters were implemented in the maximum likelihood inference using PAUP* with a heuristic search. Support for individual branches was evaluated using the bootstrap method with a heuristic search and 500 or 100 replicates for parismony and maximum likelihood analysis, respectively.

To investigate congruence between trees inferred from β-tubulin and from rDNA, and to assess the evolution of the β-tubulin gene family in cyst nematodes, we initially used GeneTree software with default values (Page and Cotton, 2000) to search for a species tree that could best explain β-tubulin gene evolution while minimizing the number of duplications, losses, or deep coalescence events. A second approach was to use the presence of sequences from a single taxon in several clades to sort monophyletic groups on the β-tubulin tree into putative gene family members and trace this information using MacClade 4.0 (Maddison and Maddison, 2000) on the tree inferred from rDNA data.

Results

β-*tubulin gene organization:* Differences in the number of introns plus the appearance of the same nematode taxon in multiple clades suggested the presence of β-tubulin paralogs among our sequences. Four distinct bands detected by southern blot analysis of *H. glycines* DNA (Fig. 1) provided confirmation. β-tubulin genes with one intron in the sequenced region were present in 43 out of 45 taxa. Four taxa, *Heterodera fici, H. zeae, H. carotae,* and *Cactodera weissi,* had sequences with two introns. Of these four taxa, *H. carotae* and *H. zeae* also had sequence with one intron, whereas for *H. fici* and *C. weissi,* a two-intron sequence was the only β-tubulin gene detected. Two *H. latipons* isolates had β-tubulin sequence with no introns in addition to a sequence with one intron. The DNA sequences with no introns had a two-base-pair deletion corresponding to amino acid position 15 and a one-base-pair deletion corresponding to amino acid position 57, possibly the result of a frame shift that might indicate a dysfunctional pseudogene. Sequence length of the β-tubulin DNA fragments varied from 251 base pairs in the *H. latipons* sequence with no introns to 382 base pairs for the *Cactodera weissi* sequence with two introns. Average length for sequences with no introns was 251 with one intron 298, and with two introns 348 base pairs.

β-*tubulin, DNA data:* From the original 45 taxa of cyst nematodes examined, 62 distinct sequences of β-tubulin were identified, with several taxa represented by more than one sequence, indicating the presence of paralogs. Multiple sequence alignment yielded 415

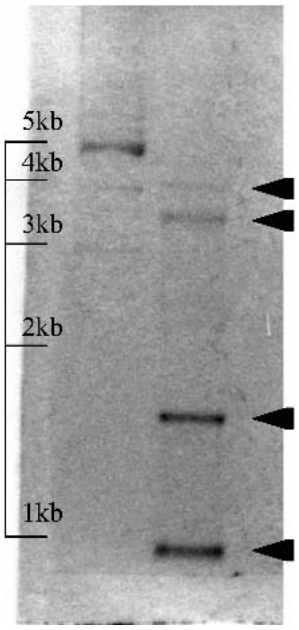

Fig. 1. Southern blot analysis, lane 1: 1.0 kb Molecular Ladder Ruler, lane 2: *Heterodera glycines* genomic DNA digested with EcoRI and hybridized with probe for the β-tubulin one intron paralog of *H. glycines*. The four distinct bands (marked with arrows) indicate multiple β-tubulin paralogous genes.

aligned base pairs, with 184 parsimony informative sites. The average mean uncorrected p distance among the one-intron sequences was 0.189 (right column in Table 2), and the average uncorrected distance for the complete data set was 0.207. Phylogenetic analysis with maximum parsimony as the optimality criterion resulted in 24,300 equally parsimonious trees that differed mostly in groupings at the lower taxonomic level, apparent on the bootstrap tree (Fig. 2).

To simplify, the complex β-tubulin gene data set was reduced to 43 sequences from 31 taxa (all sequences with one intron and marked with an asterisk in Table 1). These sequences were used to test evolutionary relationships inferred from rDNA sequence data for the same 31 taxa. Multiple sequence alignment of 331 characters revealed that 35% of the parsimony informative sites were contributed by intron sequence (Table 3), and two-thirds by exons, of which most (54% out of 65%) of the phylogenetic signal came from the third codon position. Parsimony analysis resulted in 106 equally parsimonious trees with the length of 691 steps. The groups at the lower taxonomic levels had weak bootstrap support (Fig. 3). For maximum likelihood, the selected model of sequence evolution was Hasegawa-Kishino-Yano (HKY + G + I) with empirical base pair frequencies, transition-transversion ratio of 2.11, proportion of invariable sites of 0.43, gamma distribution shape parameter of 1.07, and molecular clock rejected. The maximum likelihood tree (not shown) had a similar topology to the tree inferred using parsimony, with a difference regarding the support for monophyly of putative group 4, which was below the 50% cutoff limit.

β-*tubulin, amino acid data:* The translated amino acid sequence data set for the 45 cyst nematode taxa comprised 82 amino acids corresponding to the region spanning positions 221 to 303 in the *C. elegans* β-tubulin gene. The average uncorrected amino acid distance (left column, Table 2) for the complete data set was 0.063. The number of parsimony informative sites was 26 in the total data set and 12 in the data set comprising sequences with one intron. Phylogenetic analysis under maximum parsimony optimality criteria resulted in a highly unresolved tree (data not shown), owing to the nearly identical amino acid sequence data.

The inferred amino acid sequence data from our cyst nematode paralogs with one and two introns grouped closely to the β-tubulin genes from the nematode genera, *Brugia, Onchocerca,* and *Dirofilaria,* all of which are animal parasites, and with β-tubulin genes from chromosome III (tbb-3, tbb-4, tbb-5) of *C. elegans.* β-tubulin sequences with no introns from *H. latipons* were distantly related to the other cyst nematode sequences and grouped more closely to the *C. elegans* tbb-1 gene, based on the inferred amino acid sequence data (data not shown).

TABLE 2. Uncorrected p distances for the complete β-tubulin DNA data set as calculated by PAUP*.

	Amino acid distances	DNA distances
Mean	0.063	0.207
One intron[a]	0.039	0.189
No intron[b]	0.279	0.334
Two introns[c]	0.144	0.261

[a] Mean distance among one intron sequence.
[b] Mean distance between no intron sequences and the rest of the data set.
[c] Mean distance between two intron sequences and the rest of the data set.

Testing rDNA phylogenies using β-*tubulin data:* The rDNA multiple sequence alignment (Table 4) comprised 1063 characters with most of the phylogenetically informative sites contributed by the internal transcribed spacer (ITS) regions, ITS1 contributing 67% and ITS2 30% of the parsimony informative sites. Maximum parsimony analysis resulted in six equally parsimonious trees (all hits in one island) with the length of 1979 steps. Most of the clades were supported with bootstrap values higher than 85% (Fig. 4). *Globodera millefoli, G. artemisiae,* and *Cactodera sauna* form a group, but their relationships within this group could not be inferred because these taxa have very similar rDNA data, as previously discussed by Ferris et al. (1999). The same can be said for the interrelationships among the two *H. trifolii* populations and *H. ciceri.* For maximum likelihood, the model of sequence evolution selected using ModelTest software was the Hasegawa-Kishino-Yano (HKY + G) model with empirical base frequences, transition-transversion ratio of 1.66, gamma distribution parameter of 0.65, and molecular clock rejected. The maximum likelihood tree and the tree inferred using parsimony had the identical topology with slightly different bootstrap support values (data not shown).

A problem arose for us in using the Reconciliation methods (Martin and Berg, 2002, Page and Cotton, 2000) because of the limited number of differences among our paralogs. Our search, using GeneTree software, to reconcile the β-tubulin tree (Fig. 3) with the rDNA tree (Fig. 4) resulted in a tree different from the starting ribosomal DNA tree (according to the Kishino-Hasegawa test as implemented in PAUP*, significance level 0.05) and not in agreement with any current hypotheses based on morphology or other data previously published. We therefore tried a second, qualitative approach for testing congruence between the two data sets. Unique copies of *Heterodera goettingiana* genes were present in four of the six clades (2, 4, 5, and 6) (Fig. 3); *Heterodera glycines* was present in clades 2, 3, and 6; whereas other taxa were present in only one or two clades. Using this information, six monophyletic groups representing putative gene family members were delimited (Fig. 3). We hypothesized that the rDNA tree was the true species tree because it generally agreed with previous hypotheses of the evolutionary relationships within the group based on molecular and morphological data. We then assessed the evolution of

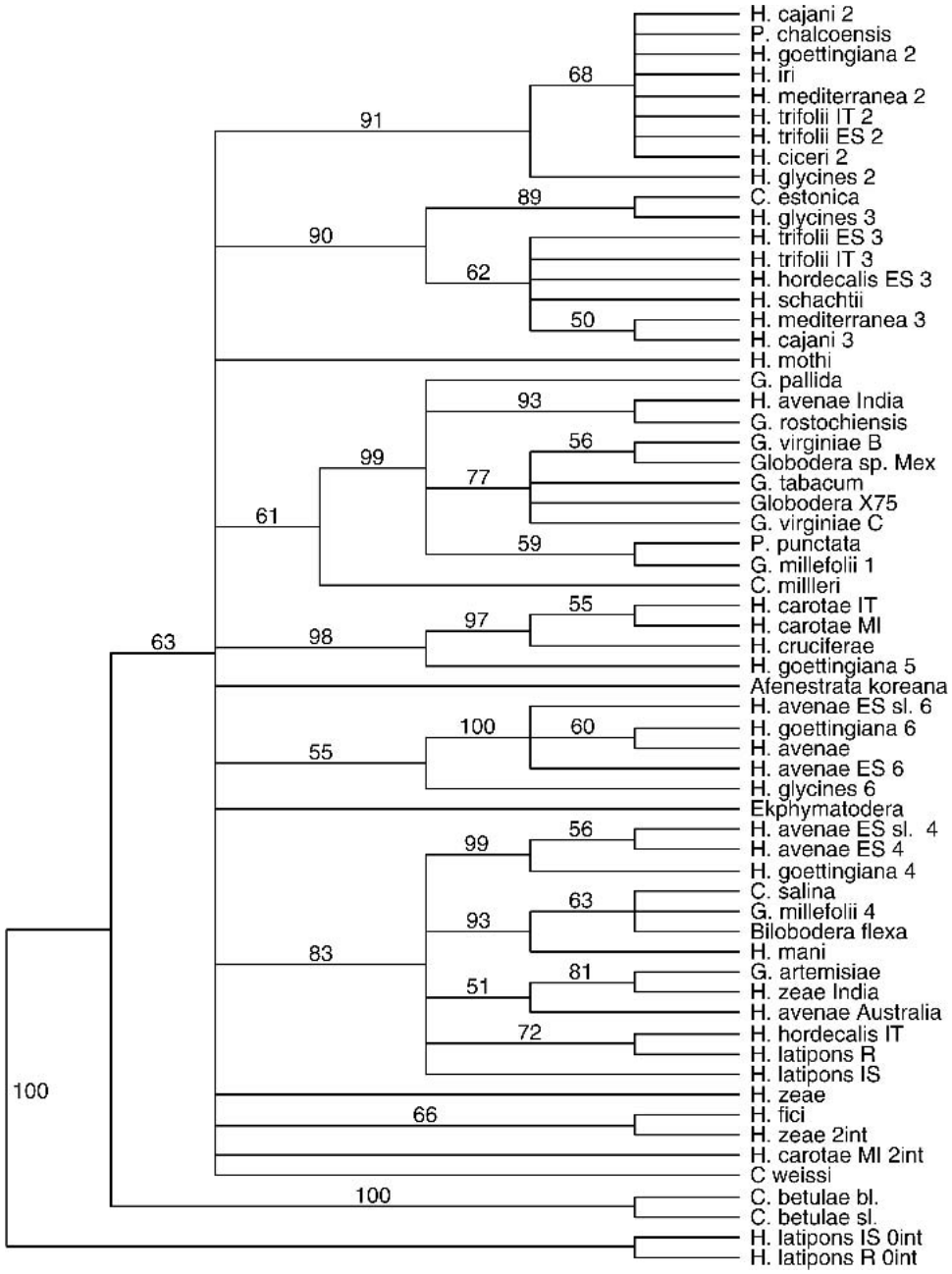

FIG. 2. Maximum parsimony tree inferred from the complete β-tubulin DNA data set with bootstrap support of monophyletic clades indicated above the branches. Numbers following species names refer to groupings assigned in Fig. 4. H = *Heterodera,* C = Cactodera, G = *Globodera,* P = *Punctodera.*

the β-tubulin gene family by tracing the putative β-tubulin family members on the rDNA species tree using MacClade 4.0 (Maddison and Maddison, 2000) and manually adding information for polymorphic characters for species that had more than one putative β-tubulin paralogous gene. Three different species groups in the genus *Heterodera* (Avenae, Schachtii, and Goettingiana groups), well established in the literature and each monophyletic on our species tree, had different putative β-tubulin family members present (Fig. 5). Species of *Heterodera* placed in the Avenae group based on ribosomal data had either putative gene family

TABLE 3. Number of total and parsimony informative characters in intron and in the first, second, and third position in the codon for the trimmed β-tubulin DNA data set. Percentage of total parsimony informative characters is given in brackets.

Gene region	Total char.	Inf. char.
Intron	79	48 (35%)
1st	84	11 (8%)
2nd	84	4 (3%)
3rd	84	74 (54%)
Total	331	137

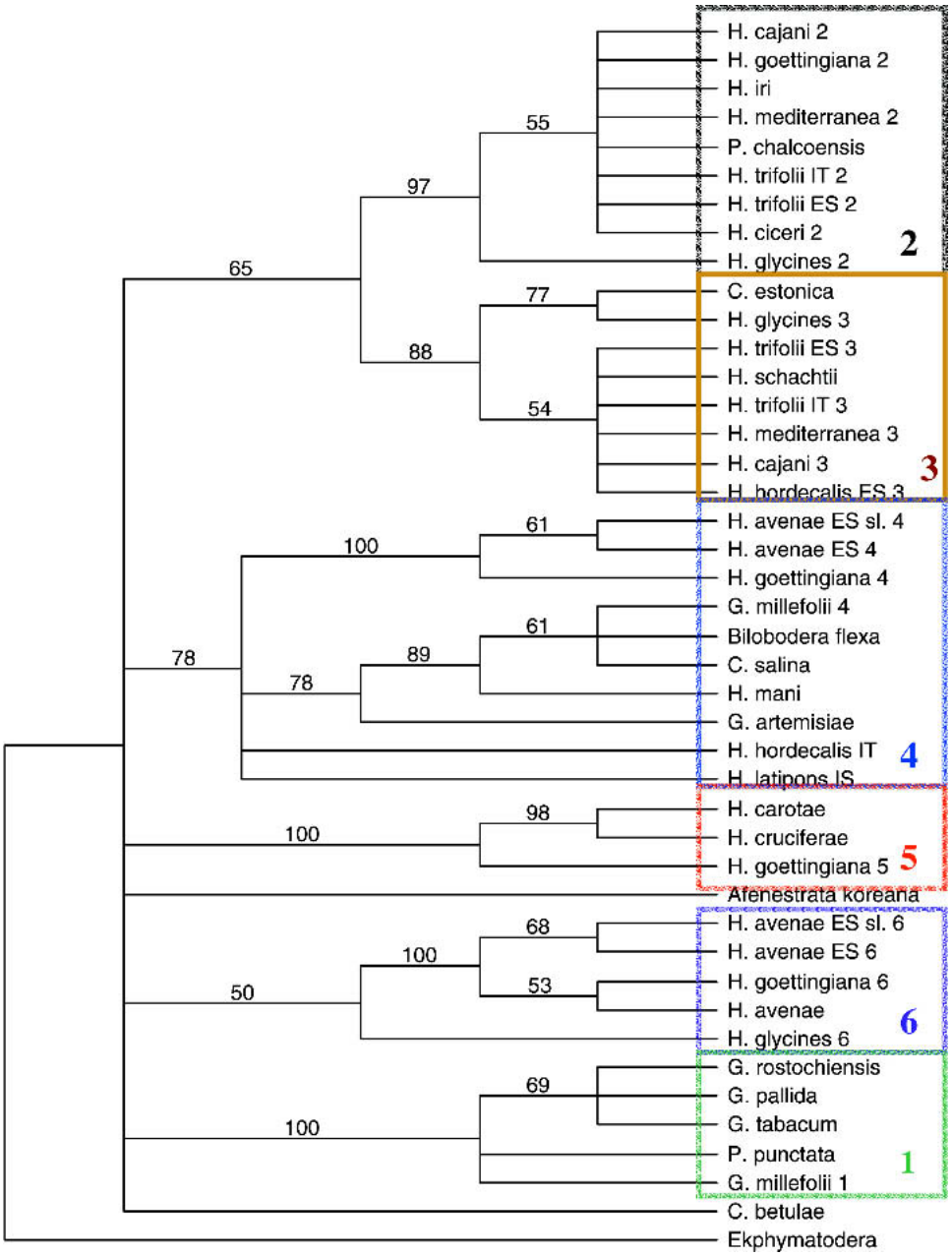

FIG. 3. Maximum parsimony tree inferred from the trimmed β-tubulin data set with bootstrap support of monophyletic clades indicated above the branches. Six putative β-tubulin paralogs, corresponding to clades, are indicated by different colors and numbers. H = *Heterodera,* C = *Cactodera,* G = *Globodera,* P = *Punctodera.*

TABLE 4. Number of total and parsimony informative characters in ITS1, ITS2, and 5.8S regions for the ribosomal DNA data set. Percentage of total parsimony informative characters is given in brackets.

Gene region	Total char.	Inf. char.
ITS1	659	351 (67%)
5.8S	166	15 (3%)
ITS2	238	156 (30%)
Total	1,063	522

member 4 (*H. hordecalis* population from Italy, *H. mani,* and *H. latipons*) or putative gene 6 (*H. avenae,* population from the United States) or both genes 4 and 6 (two *H. avenae* populations from Estonia). Thus, they appear to be a united monophyletic group based on these commonalities. The exceptions were the *H. hordecalis* population from Estonia, with gene number 3 and *H. iri* with gene number 2. *Heterodera* species placed in the Schachtii group were united in having either putative gene 3 (*H. schachtii* and *C. estonica*) or gene 2 (*H. ciceri*) or both putative genes 3 and 2 (two populations of *H. trifolii, H. glycines, H. mediterranea,* and *H. cajani*). *Heterodera cruciferae* and *H. carotae,* which belong to the

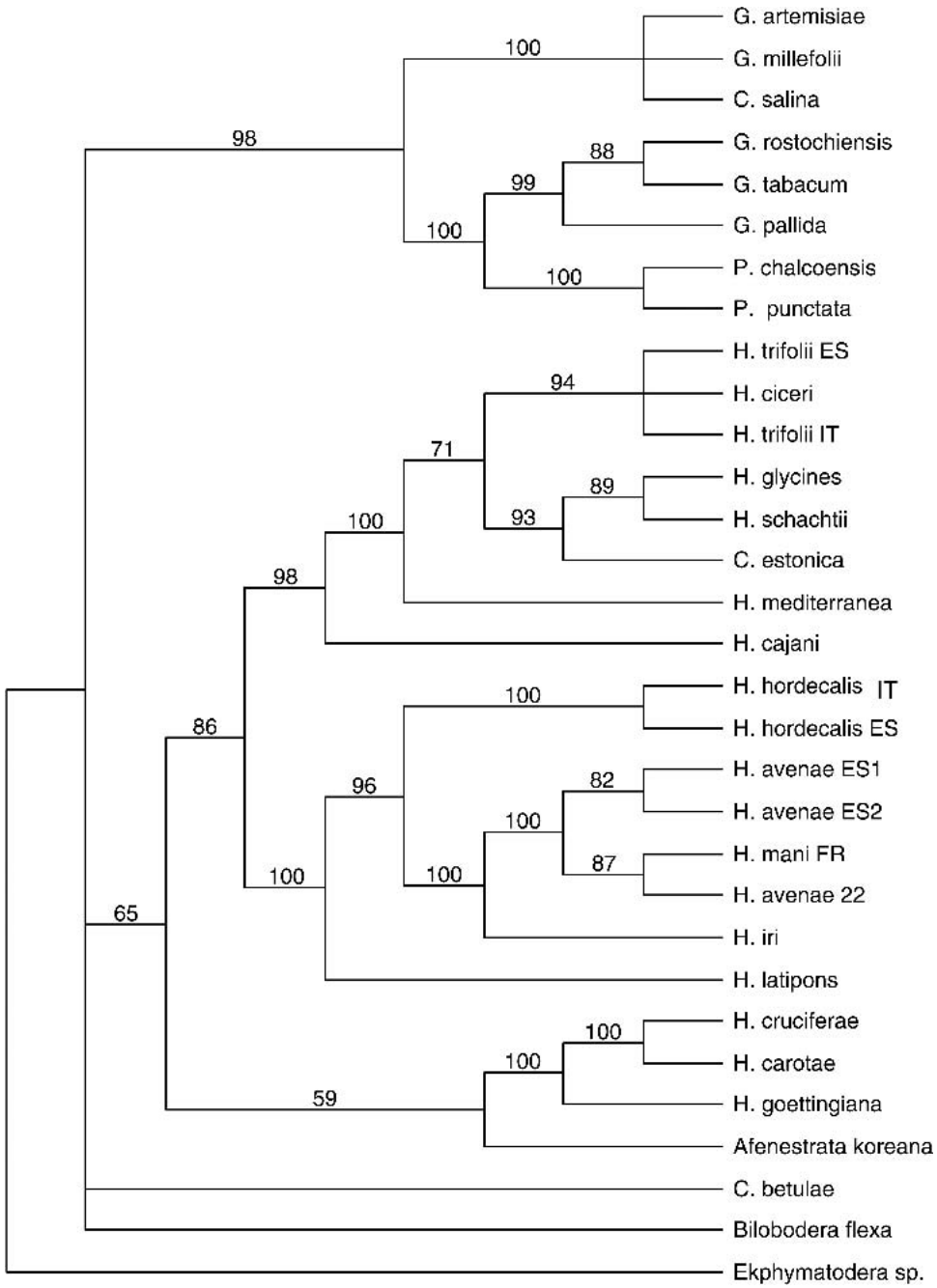

FIG. 4. Maximum parsimony tree inferred from ribosomal DNA data with bootstrap support of monophyletic clades indicated above the branches. H = *Heterodera,* C = *Cactodera,* G = *Globodera,* P = *Punctodera.*

Goettingiana species group, had putative gene 5, whereas *H. goettingiana* had four different paralogs, 2, 4, 5, and 6. A group of species not in *Heterodera* (*Globodera* spp., *Punctodera* spp., and *Cactodera salina*) had either putative gene number 1 (*G. rostochiensis, G. tabacum, G. pallida,* and *P. punctata*) or gene number 4 (*G. artemisiae* and *Cactodera salina*). *Globodera millefolii* had both genes 1 and 4. An exception was *P. chalcoensis,* which apparently had only gene number 2. Ambiguous groupings of *Afenestrata koreana, C. betulae,* and *Ekphymatodera thomasoni* on the β-tubulin tree prevented us from assigning putative gene family members to these taxa. In summary, five taxonomic subgroups in Figure 5 were distinct because each has its own unique set of paralogs.

DISCUSSION

The occurrence of β-tubulin as a gene family in cyst nematodes is in agreement with recent data from other nematodes, where the number of paralogs ranges from one in *Trichuris trichiura* (Bennett et al., 1999) to six in *C. elegans* (Gogonea et al., 1999). The presence of paralogs that differ regarding the number of introns was reported in animal-parasitic nematodes (Bennett et al.,

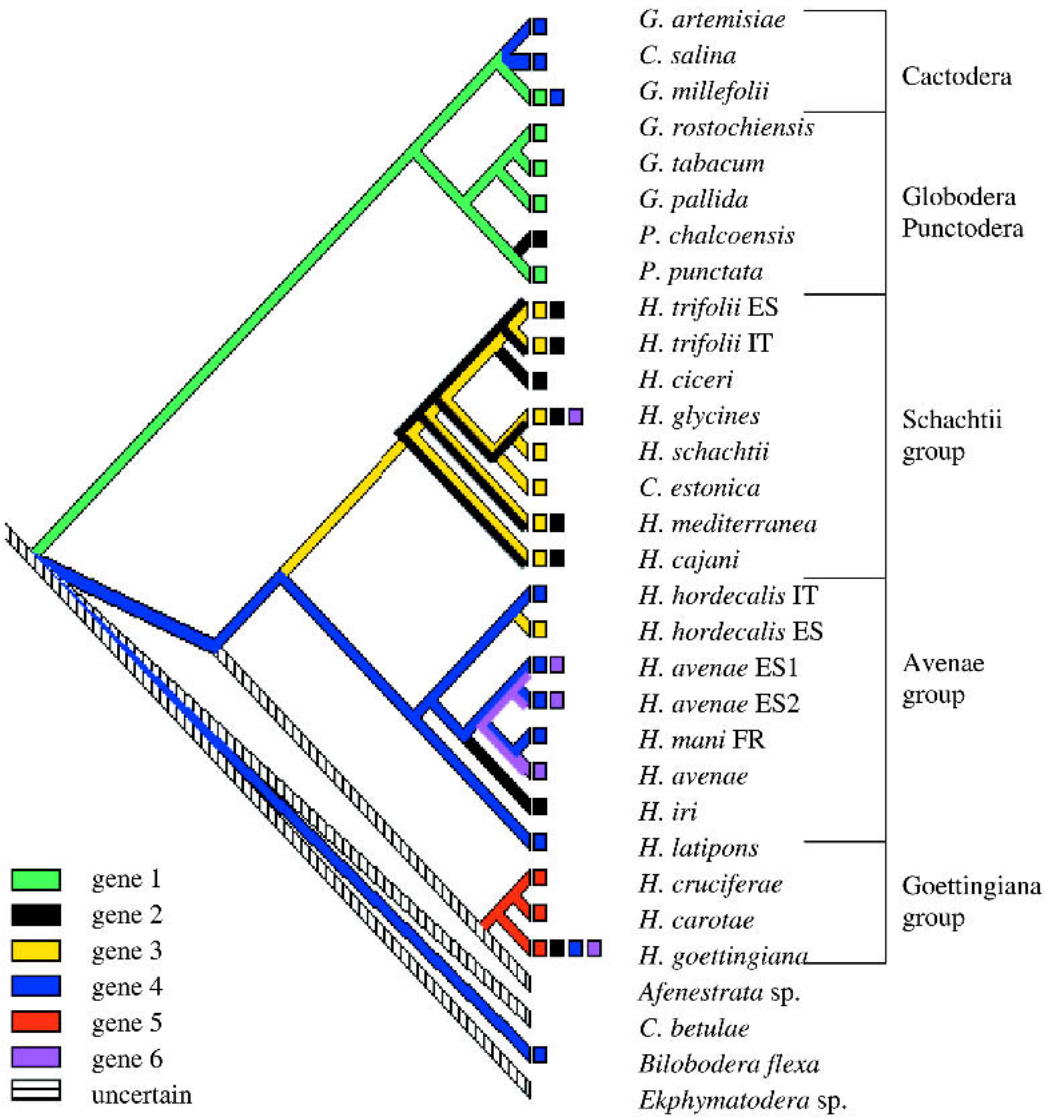

FIG. 5. Six putative β-tubulin genes (numbered 1–6) traced in color on the tree inferred from rDNA data. H = *Heterodera,* C = *Cactodera,* G = *Globodera,* P = *Punctodera.*

1999), which indicates that intron loss is common for the β-tubulin genes in the phylum Nematoda. Our inability to amplify all putative paralogs from all species under study might be due to sequence divergence in the primer regions in some of the paralogs or, more likely, to the loss of β-tubulin gene paralogs, which has been documented in animal-parasitic nematodes (Roos et al., 1995) as a resistance mechanism to antihelminth agents.

The data set based on the amino acid composition of β-tubulin resulted in a highly unresolved bootstrap tree owing to low phylogenetic signal as indicated by the small mean distance and a low number of informative sites resulting from conserved amino acid sequence. Amino acid data will be more useful for investigating higher-taxonomic level phylogenies as has been shown by published β-tubulin phylogenies (e.g., Baldauf et al., 2000). For our β-tubulin DNA sequence data set, a large number of equally parsimonious trees resulted from ambiguous groupings among species at the tips of the tree branches, although the bootstrap summary tree showed far greater resolution than the amino acid tree.

Relationships inferred from our rDNA sequence data set are similar to those obtained by Ferris et al. (1994, 1995, 1998, 1999), Eroschenko et al. (2001), Sabo (2002), Sabo et al. (2001, 2002), and Subbotin et al. (2001), which also were based on rDNA. *Heterodera mani,* not included in published cladograms, was closely related to our *H. avenae* population from Idaho. Relationships of the rDNA sequences of three other species, *H. mediterranea, Punctodera chalcoensis,* and *Cactodera betulae,* were previously reported by Ferris et al. (1998), Sabo (2002), and Sabo et al. (2001, 2002). Placement of the sequences of *Cactodera estonica,* which grouped with *Heterodera* species, was unexpected. We included the rDNA data from this species as a part of the analysis because it also grouped with *Heterodera* species from the Schachtii group in the β-tubulin gene tree. This population may belong to a *Heterodera* species morphologically similar to the *Cactodera* species.

When Martin and Burg (2002) recognized that paralogs were present, they used GeneTree software (Page and Cotton, 2000) and successfully reconciled their species trees, based on morphological data, with those derived from HSP70 paralogs. Our attempts to resolve the nematode β-tubulin gene evolution using GeneTree were not successful. This outcome was likely due to the low support for some of the groupings on the β-tubulin tree, possibly because some of the multiple paralogous genes in the ancestral state were lost or not sampled in some lineages under study.

Congruence of our two data sets was revealed by the presence of the same putative β-tubulin gene paralogs in monophyletic groups on the ribosomal DNA tree. Relationships among the species within the same *Heterodera* species group cannot be inferred with confidence from the β-tubulin data alone; therefore, it is difficult to assess congruence at the lower taxonomic levels. Even so, the traced (color) patterns are consistent enough to show that the β-tubulin paralogs we obtained with the same PCR primers, although highly conserved, contained sufficient nucleic acid sequence variation to corroborate the species tree based on rDNA. This was surprising in view of the similarity in the amino acid sequences and the limited amount of DNA sequence available (ca. 300 nucleotides). Perhaps our qualitative ''tracking'' approach was better able to recognize the complex patterns in the tree than were the statistical methods. We suggest that the presence of multiple paralogs, as in *H. goettingiana,* is the ancestral condition for cyst nematodes, and that some paralogs were lost in a mosaic fashion as the nematode groups evolved. We further suggest that other highly conserved protein genes in other animal and plant groups might also comprise cryptic paralogs and that information obtained from DNA sequence data could be used as corroborative data for existing phylogenetic trees, which are often based on rDNA data alone.

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