Molecular Characterization and Phylogenetic Evaluation of the Hsp90 Gene from Selected Nematodes

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Abstract: While multiple genes are optimal for corroborating nematode phylogenies, only a few are commonly used. Here we examine the phylogenetic potential of the nuclear Hsp90 chaperone gene. We used degenerate primers to obtain partial Hsp90 sequences from several plant-parasitic and free-living nematodes. Hsp90 was single-copy in Heterodera glycines and Meloidogyne javanica, similar to the situation for Caenorhabditis elegans. The full-length H. glycines Hsp90 protein sequence showed homology to sequences from C. elegans and Brugia pahangi and to other eukaryotes, and contains several functionally important regions common to cytoplasmic Hsp90 proteins. The Hsp90 amino acid phylogeny supported the Coelomata hypothesis for metazoan evolution.

Phylogenetic trees, substitution scatter plots, and statistics for phylogenetic signal were made for Hsp90, 18S small subunit (SSU), and 28S large subunit (LSU) over a limited but broad sampling of nematode taxa. Only the LSU data set failed to recover any of the expected topology and showed extensive substitution saturation. In an intensive sampling of plant-parasitic nematode taxa, the Hsp90 tree topologies were generally congruent with rDNA results and alignments were unambiguous. Hsp90 sequences may help strengthen branch support or clarify tree topologies when other molecules show ambiguous alignments, greater branch-length heterogeneity, or codon bias in certain taxonomic groups.

Keywords: DNA, Hsp90, phylogenetics, protein, rDNA, saturation.

A number of molecules have been tested for their phylogenetic utility with varying degrees of success (Graybeal, 1994; Regier et al., 1998; Tang and Lewontin, 1999). The ribosomal genes are especially attractive for nematodes because their multi-copy nature facilitates amplification of the small quantity of DNA that may be available from single valuable specimens and they lack the introns that can sometimes complicate data analysis. However, length and sequence variability may require extensive optimization of sequence alignment procedures to ensure that the resulting trees are accurate (Morrison and Ellis, 1997). Heterogeneity in the ribosomal genes within individual nematodes has been reported (Bloch et al., 1998; Cherry et al., 1997; Subbotin, et al., 2000; Zijlstra et al., 1995). Such variation may, in some instances, confound phylogenetic analysis.

In spite of the need for additional molecules, only a few other nuclear genes have been employed in nematode phylogeny, including RNA polymerase II (Baldwin et al, 1997), major sperm protein (MSP) (Setterquist et al., 1996), and Hsp70 (Beckenbach et al., 1992; Hashmi et al., 1997). Both Hsp70 and Hsp90 heat shock protein genes have been used extensively for phylogenetic analysis in other animal and bacterial systems (Gupta, 1995, 1998; Krishna and Gloor, 2001; Landais et al., 2001; Pepin et al., 2001; Welch and Meselson, 2000, 2001a,b). A study of Hsp70 from sharks highlighted the limitations of using paralogous genes for phylogeny (Martin and Burg, 2002), but when true orthologs were distinguished from paralogs and then combined with morphological information, this gene family was demonstrated to be phylogenetically useful.

Hsp90 is an attractive candidate for use in nematode phylogenetics because it is likely present in a single copy and is not easily confused with distantly related genes. While many vertebrates (Pepin et al., 2001) and plants (Krishna and Gloor, 2001) have more than one nearly identical cytoplasmic Hsp90 gene, Birnby et al. (2000) reported a single Hsp90 gene in the Caenorhabditis elegans genome, located on chromosome V (cosmid C47E8.5). Other C. elegans genes that have been loosely classified as Hsp90 family members share limited structural homology with this sequence. The most closely related sequences include a putative endoplasmic reticulum heat shock protein GRP94 on chromosome IV (cosmid T05E11.3; 46% identical aa) and a TRAP1 homolog on chromosome III (cosmid R151.7; 42% identical aa, limited to the ATP-binding domain).

The only other nematode species for which a full-length Hsp90 gene has been described is the filarial nematode Brugia pahangi (Thompson et al., 2001). Although information regarding Hsp90 copy number in other nematodes is currently lacking, single copy Hsp90 genes have been demonstrated in several Drosophila species (Konstantopolou and Scouras, 1998) and in the Lepidoptera Bombyx mori and Spodoptera frugiperda (Landais et al., 2001).

Our goals in this study were to establish that Hsp90 sequences can be readily obtained from bulked nematode DNA or single nematode specimens and to determine the copy number of Hsp90 from selected plant-parasitic nematodes. Once we met these objectives, we set out to determine the extent of Hsp90 sequence variation at the protein and DNA levels and to test the phylogenetic utility of Hsp90 in comparison to the 28S LSU and 18S SSU ribosomal DNA genes commonly
used for nematode phylogeny. Here we examine the amount of variation and types of substitutions in a conserved partial region of the Hsp90 gene and compare the branching patterns in phylogenetic trees generated by various methods.

**Materials and Methods**

*Nematode isolates, culture methods, and DNA extracts: Meloidogyne javanica and M. arenaria* (both from North Carolina) were cultured on *Lycopersicum esculentum* cv. Big Boy. *Heterodera glycines* inbred strain OP50 (inbred from a North Carolina isolate) was maintained on *Glycine max*, cv. Lee 68. In these cases, nematode eggs were harvested by sugar flotation (Jenkins, 1964), snap-frozen in liquid nitrogen, and stored at –80 °C. Bulk nematode genomic DNA was prepared from *M. javanica*, *M. arenaria*, and *H. glycines* as described (Bird and Riddle, 1989). The following species used for single-nematode PCR were obtained from various locations in Maryland: *Anguina*, *Ditylenchus*, *Meloidogyne hapla*, and *Pratylenchus renatus*. Nematodes from other locations included *Acerobolides nanus* PS1959 (France), *Heterodera goldeni* (Egypt), *M. floridensis* (Florida), *M. sassaeri* (Delaware), *Oscheius myriophila* DF5020 (California), *P. penetrans* (Arkansas), *P. teres* (South Africa), and *Zelda punctata* PS1145 (California).

Extracts were prepared from single juveniles of the plant-parasitic or bacterial-feeding nematodes as previously described (Thomas et al., 1997). In some cases, extracts were made from a pool of two to nine juveniles to increase the amount of template DNA.

*Gene amplification, cloning, and sequencing:* Hsp90 was amplified using a modification of RAND-PCR (Skantar and Carta, 2000) and primers U831 [5’-AA(T/C)AA(A/G)AC(A/G)AAACC(A/C/G/T)T(T/C)TGAGAC-3’] and L1110 [5’-TC(A/G)CA(A/G)TT(G/A/C)TCCATGAT(A/G)AA(G/A/C)AC-3’]. Reactions were assembled in PCR tubes by first adding a cocktail containing dNTP's and primers. This mixture was overlaid with a drop of paraffin wax that was allowed to harden, separating it from a top layer that contained the DNA template and Eppendorf MasterCycler (Brinkmann, Westbury, NY) in reaction buffer supplied by the manufacturer. PCR reactions were performed in an Eppendorf MasterCycler (Brinkmann, Westbury, NY). Cycling conditions consisted of a preheat step of 94 °C for 10 minutes, to allow the layered components to mix in a hot start, followed by 35 cycles of 94 °C for 20 seconds, 65 °C for 5 seconds, 60 °C for 5 seconds, 55 °C for 5 seconds, 50 °C for 5 seconds, and 68 °C for 1 minute. A final step of 68 °C for 15 minutes allowed for complete extension of products. Each 25-µl PCR reaction was analyzed on 1.5% SeaKem GTG agarose (BMA, Rockland, ME) in 1X Tris-Acetate-EDTA (TAE). PCR products were visualized with UV illumination after ethidium bromide staining. Bands containing single PCR products were excised from the gel and, if necessary to increase yield, 2 µl was subjected to re-amplification as described above. Otherwise, excised PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), cloned into pCR2.1, and transformed into *Escherichia coli* TOP10 cells according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Plasmid DNA was prepared with a Wizard Plus miniprep kit (Promega, Madison, WI) and digested with EcoRI to verify the correct insert. Double stranded DNA was sequenced with either Big Dye 3.0 Terminator cycle sequencing reagents and analyzed with an ABI 310 Gene Analyser (Perkin Elmer Applied Biosystems, Foster City, CA) or with the CEQ DTCS Quick Start Kit and analyzed on the CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Sequence was determined on both strands from all clones using M13 forward and M13 reverse primers. Whether template was from bulked nematodes or single specimens, no less than two clones (typically four to six) were sequenced from each PCR reaction.

*Heterodera glycines* Hsp90 cDNA cloning: An *H. glycines* cDNA library in the Uni-ZAP vector was screened by PCR for the presence of the Hsp90 gene. Preparation of phage and mass excision of the pBluescript phagemid DNA containing nematode cDNA inserts were performed according to the manufacturer’s instructions. Excised phagemid DNA was used as template in PCR reactions at 30 ng per 25-µl reaction. Primer L1110 was paired with the M13 reverse primer and primer U831 was paired with the M13 forward primer to amplify the 5’ and 3’ ends of the *H. glycines* Hsp90 (Hg-Hsp90) cDNA, respectively. An additional 5’ end clone was obtained by pairing the primer INV-3, [5’-GAGCGTCAGAGGAGTTGAGG-3’] with the M13 reverse primer. All PCR products were TA-cloned as described above and sequenced.

*Southern hybridization:* Ten µg of *H. glycines* or *M. javanica* genomic DNA was digested with BamHI in 40-µl reactions overnight at 37 °C. The digests were electrophoresed overnight in 0.8% agarose/1X TBE, stained with ethidium bromide, and photographed. The DNA was transferred to nylon by standard methods (Ausubel et al., 2001). The Hsp90 probe was labeled with digoxigenin-UTP using established PCR conditions, and a chemiluminescent Southern hybridization was performed using the Genius kit (Roche Applied Science, Indianapolis, IN) as described by the manufacturer. The probe was hybridized overnight at 65 °C. The blot was washed twice for 5 minutes at room temperature in 2X SSC/1% SDS and followed by two 15-minute washes at 65 °C in 0.1X SSC/0.1% SDS. Hybridized products were detected with the chemiluminescent substrate CDP-Star and visualized by autoradiography.

*Hsp90 amino acid sequence alignments:* The deduced amino acid sequence of Hg-Hsp90 was determined in Sequencher 4.1 (Gene Codes, Ann Arbor, MI) using

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**Hsp90 Sequence Phylogenetics:** Skantar, Carta 467

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standard codon tables. The organisms and GenBank accession numbers for full-length Hsp90 amino acid sequences are listed in Table 1. Hsp90 amino acid sequence alignments were performed with ClustalW (vers. 1.4) (Thompson et al., 1994) and manually adjusted in GeneDoc (Nicholas et al., 1997).

Nucleic acid sequence alignments: The phylogenetic properties of Hsp90 were examined in comparison to LSU and SSU rDNA genes commonly used in nematode systematics. Six nematode taxa were selected to broadly represent expected nematode clades (Blaxter, 2001): *B. malayi*, *Strongyloides ratti*, *C. elegans*, *M. javanica* or *M. incognita* (SSU), *H. glycines*, and either *P. teres* (Hsp90, LSU) or the closely related *Nacobbus aberrans* (SSU) (Carta et al., 2001). DNA sequence alignments were generated for the Hsp90 gene (213 bp), the LSU D3 expansion segment (178 nucleotides excluding gaps), and the SSU gene (including 832 bp, corresponding to positions 1015 through 1846 in the *C. elegans* reference sequence, Accession # X03680). Accession numbers for DNA sequences (from this study or GenBank) from these taxa are listed in Table 2. *Schistosoma mansoni* was selected as the closest outgroup for which all genes were available. Based upon large SSU rDNA datasets (Aguinaldo et al., 1997), *D. melanogaster* also was tested as an outgroup in the Bayesian analysis. Both outgroups were used for rooting trees. Introns in the Hsp90 partial genomic sequences were determined by comparing the three-frame conceptual translations to the *C. elegans* Hsp90 sequence; these regions were removed prior to further analysis. Sequences obtained from GenBank were trimmed as necessary to remove regions that did not match in all aligned taxa.

Alignments were made in ClustalW with default parameters and subsequently checked for consistency of conserved positions among sequences. Manual adjustments to remove all gaps from the ribosomal DNA alignments were performed in GeneDoc (Nicholas et al., 1997). Our objective was not to produce a definitive phylogenetic estimate using relatively sparse data from each of the three genes but to demonstrate their general substitution properties relative to one another, using unambiguous alignments. However, due to the sensitivity of tree topology to alternative alignments for SSU rDNA (Morrison and Ellis, 1997), various other trees were constructed for comparison with the conservative, non-gapped alignment and the topology abstracted from a large SSU Tree (Blaxter et al., 1998). These alignments and their corresponding trees (not shown) were made with and without gaps for both Clustal default and minimal gap extension penalty alignments. Some alignments included an extra segment represented by an additional 679 bp in the raw alignment for six taxa that was missing from *H. glycines*. For higher-density taxon sampling of nematodes, we generated partial Hsp90 sequences were used from GenBank were trimmed as necessary to remove regions that did not match in all aligned taxa. Some alignments included an extra segment represented by an additional 679 bp in the raw alignment for six taxa that was missing from *H. glycines*.

For higher-density taxon sampling of nematodes, we generated partial Hsp90 sequences were used from GenBank were trimmed as necessary to remove regions that did not match in all aligned taxa.

### Table 1. Hsp90 protein sequences included in this study.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Group, common name</th>
<th>Accession No.</th>
</tr>
</thead>
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<tr>
<td><em>Escherichia coli</em></td>
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<td>NP_286214</td>
</tr>
<tr>
<td><em>J. coli</em></td>
<td>capsulatus</td>
<td>AAA33383</td>
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<tr>
<td><em>Aspergillus niger</em></td>
<td>fungi</td>
<td>CAA27229</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>fungi</td>
<td>CAA56931</td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td>fungi</td>
<td>CAC28765</td>
</tr>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td>Plasmodium falciparum</td>
<td>AAA27842</td>
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<tr>
<td><em>Caenorhabditis elegans</em></td>
<td>nematode</td>
<td>Z75530</td>
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<td><em>H. glycines</em></td>
<td>nematode</td>
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<tr>
<td><em>Heterodera glycines</em></td>
<td>nematode</td>
<td>X03680</td>
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<tr>
<td><em>Meloidogyne javanica</em></td>
<td>nematode</td>
<td>X03680</td>
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</tbody>
</table>

### Table 2. Nucleotide sequences used for seven taxon comparisons of Hsp90, 28S, and 18S.

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Hsp90</th>
<th>28S</th>
<th>18S</th>
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<tr>
<td><em>Brugia malayi</em></td>
<td>AW17995</td>
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<td><em>Carnsobaditis elegans</em></td>
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<td><em>Heterodera glycines</em></td>
<td>AF449485</td>
<td>AF133304</td>
<td>AF216579</td>
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<td><em>Meloidogyne javanica</em></td>
<td>AF201338</td>
<td>U47359</td>
<td>U81578</td>
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<tr>
<td><em>Pratylenchus teres</em></td>
<td>AF457583</td>
<td>AF96353</td>
<td>AF422190</td>
</tr>
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<td><em>Strongyloides ratti</em></td>
<td>BG895447</td>
<td>AF48342</td>
<td>U81581</td>
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<tr>
<td><em>Schistosoma mansoni</em></td>
<td>J04017</td>
<td>Z46503</td>
<td>M62562</td>
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</table>

- Unless noted otherwise, sequences obtained from GenBank.
- This study.
- Outgroup taxon

* This study.
4). Retention Index describes the proportion of synapomorphy from the data that is retained on a tree, with larger values representing more consistency of the characters in the cladogram. Unlike the CI, empty information from autapomorphies is not included. A test for data structure was also generated in PAUP, with g1 skewness scores from a million random trees compared to a table of critical values (Hillis and Huelsenbeck, 1992). For Hsp90, SSU, and LSU, an entropy-based statistical test for saturation of alignment positions (Xia et al., 2003) was made with DAMBE (Xia and Beck, 1992). For Hsp90, SSU, and LSU, an entropy-based statistical test for saturation of alignment positions (Xia et al., 2003) was made with DAMBE (Xia and Beck, 1992).

Table 3. Nematode Hsp90 sequences used for clade-specific analyses.*

<table>
<thead>
<tr>
<th>Species name</th>
<th>Accession #</th>
<th>Species name</th>
<th>Accession #</th>
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<tbody>
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<td>Anclyostoma sp.</td>
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<td>Ascaris lumbricoides</td>
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<td>M. javanica</td>
<td>AF201338</td>
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<tr>
<td>Brugia malayi</td>
<td>BU568768</td>
<td>M. saussieri</td>
<td>AF457581</td>
</tr>
<tr>
<td>B. pahangi</td>
<td>AW179950</td>
<td>Oecherus myriophila</td>
<td>AY605515</td>
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<td>Caenorhabditis elegans</td>
<td>Z75530</td>
<td>Pratylenchus crenatus</td>
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<td>P. penetrans</td>
<td>AY605513</td>
</tr>
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<td>AY605512</td>
<td>P. teres</td>
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<td>BG895447</td>
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<td>S. stercoralis</td>
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<td>M. arenava</td>
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<td>M. floridus</td>
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</table>

* Unless noted otherwise, sequences obtained from Genbank.

b Described in another study.

c This study.

Table 4. Properties from ModelTest performed for equivalent seven-taxa nucleotide alignments of Hsp90, 18S SSU, and 28S LSU, and for a 16-taxon alignment of Hsp90.

<table>
<thead>
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<th>Parameter</th>
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<th>LSU r tDNA (seven taxa)</th>
<th>Hsp90 (16 taxa)</th>
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<td>TIM + γ</td>
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<td>nst</td>
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<td>6</td>
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<td>Prop. invariant sites</td>
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<td>Ti/Tv</td>
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<tr>
<td>-lnL</td>
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<td>0.50</td>
<td>0.63</td>
<td>0.39</td>
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AIC = Akaike Information Criteria; CI = Consistency Index; GTR = General Time Reversible Model (Tavaré, 1986); γ = gamma distribution; hLRT = hierarchical Likelihood Ratio Test; I = Invariable sites; K80, K81 = Models of Kimura (Kimura, 1980; Kimura, 1981); L = Likelihood; nst = number of substitution parameters; RI = Retention Index; SYM = Symmetrical Model (Zharkikh, 1994); Ti/Tv = ratio of transitions to transversions; TIM = Transitional model (rAC = rGT ≠ rAT = rCG = rAG ≠ GT, Posada and Crandall, 2001); TrNef = Tamura-Nei model, equal base frequencies (Tamura and Nei, 1993).
idly than traditional maximum likelihood methods. Beginning with a random tree, MCMC was run at least twice, for 250,000 to $10^6$ cycles with each 100th cycle sampled. Branch lengths were recorded to allow generation of a consensus phylogram. The first 10% of samples were excluded as burn in and examined for convergence before creating 50% majority-rule consensus trees from the remaining trees in PAUP. The numbers reported on the consensus trees represent the probability a clade was supported among sampled trees, analogous to bootstrap support using ML parameters (Huelsenbeck et al., 2000).

**Results**

Amplification and cloning of Hsp90 from plant-parasitic nematodes: Degenerate PCR primers U831 and L1110 were used previously in a ramped-annealing PCR reaction (RAN-PCR) to amplify the Hsp90 gene from *H. glycines*. These primers and PCR conditions were designed to specifically amplify the gene for cytoplasmic Hsp90, thus reducing the chance of obtaining unwanted Hsp90 paralogs. Using genomic DNA from either bulked nematodes or crude single-nematode DNA extracts for template, we generated Hsp90 PCR products from a broad range of plant-parasitic and free-living species (Tables 2 and 3). Each PCR reaction typically generated a single major band in the size range predicted from the *C. elegans* Hsp90 gene (not shown). Excision and purification of Hsp90 PCR products from agarose gels were routinely performed to remove any minor reaction products or primer dimers prior to cloning and sequencing.

To determine whether *H. glycines* Hsp90 was present in a single genomic location, the 350-bp *Hg*-Hsp90 PCR product was hybridized to restriction enzyme-digested *H. glycines* genomic DNA. The probe detected a single band in the Hind III-digested DNA and two bands in the Eco RI-digested DNA (Fig. 1A). DNA sequencing revealed a single Eco RI site in the middle of the *Hg*-Hsp90 PCR probe, so two bands would be expected in the Southern hybridization to Eco RI-digested DNA. Sequencing also showed a Hind III site located 39 bp from the 3′ end of the amplified probe. Thus, the lack of detectable hybridization to a second band in the Hind III digested lane is not surprising and may have been due to insufficient pairing between the probe and the blotted restriction products. The *Hg*-Hsp90 probe also hybridized to a blot of Bam HI-digested *M. javanica* genomic DNA (Fig. 1B). A single band was observed, consistent with the presence of a single copy of Hsp90 in this species. Taken together, these results mirror the single Hsp90 gene that was found in *C. elegans*, whose entire genome sequence is known.

To isolate the Hsp90 cDNA, an excised *H. glycines* second-stage juvenile cDNA library was used as PCR template for PCR. In these reactions, the U831 and L1110 primers were paired separately with either the M13 forward or reverse vector primers. The 5′ and 3′ PCR products were cloned and sequenced, and the contiguous cDNA sequence was reconstructed. Upon sequencing the 5′ end clone obtained from the pairing of L1110 with M13 reverse, we found that this cDNA lacked the initiator methionine codon. We then performed an additional PCR amplification of the library cDNA’s, using the internal *Hg*-Hsp90 primer (INV-3) paired with the M13 reverse primer. The PCR product of this reaction was cloned and sequenced, revealing the genuine 5′ end of the cDNA. Attempts to obtain the 5′ end by pairing INV-3 or other internal primers with an SL1 spliced leader primer were unsuccessful, suggesting that unlike *C. elegans* daf-21, *Hg*-Hsp90 does not undergo trans-splicing (not shown).

*Hg*-Hsp90 sequence analysis: BLAST analysis of the *Hg-*Hsp90 amino acid sequence revealed a high level of similarity to other members of this protein family. The alignment with two other available full-length nematode Hsp90 protein sequences and representative
Hsp90 sequences from other organisms is shown in Figure 2. Identities between \textit{Hg}-Hsp90 and other organisms were 83\% for \textit{B. pahangi}, 82\% for \textit{C. elegans}, 74\% for \textit{Mus musculus}, 73\% for \textit{D. melanogaster}, 67\% for \textit{Arabidopsis thaliana}, and 65\% for \textit{Dictyostelium discoideum}. The sequence also showed a high level of identity to the Hsp90 protein sequences from pigs, humans, and other vertebrates (not shown).

Several structural features common to the Hsp90 class of proteins are noteworthy. \textit{Heterodera glycines} contained all five signature sequences that are common to all eukaryotic Hsp90 proteins (Fig. 2, domains I-V). The MEEVD motif, which is uniquely found at the C-terminus of cytoplasmic Hsp90 proteins, was nearly invariant in all sequences examined, including \textit{H. glycines} (Fig. 2, domain VI). In addition to these highly conserved regions, \textit{Hg}-Hsp90 also contained three commonly observed variable domains (Fig. 2). The extreme N-terminus was highly variable in all organisms, with mouse exhibiting a PEETQ motif that is unique to vertebrate Hsp90's. A second variable region called the charged-linker was located between residues 214–257.

### Fig. 2. Amino acid alignment of the \textit{Heterodera glycines} Hsp90 amino acid sequence with the available Hsp90 sequences from 2 other nematodes and 4 other eukaryotes. HGL = \textit{Heterodera glycines}; BPA = \textit{Brugia pahangi}; CEL = \textit{Caenorhabditis elegans}; MMU = \textit{Mus musculus}; DME = \textit{Drosophila melanogaster}; ATH = \textit{Arabidopsis thaliana}. Amino acid residue numbers are indicated next to each sequence. Residues identical to \textit{H. glycines} are indicated by periods; gaps are indicated by hyphens. Five Hsp90 signature sequences (IV) and the cytoplasmic Hsp90 sequence motif (VI = MEEVD) are overlined; the position of amino acids that are identical between \textit{H. glycines} and one or both of the other nematode species (but not in the other organisms) are shaded in light gray. The position of conserved amino acids used for the design of primers U831 and L1110 are indicated by arrows and boxed in black. The \textit{C. elegans} mutant strain, \textit{daf-21(p673)} contains an E to K mutation in a highly conserved residue at position 293, noted by an asterisk above the sequence.
In *H. glycines*, this 45 amino acid domain was 14 residues longer than the corresponding region from *C. elegans*, five residues longer than *B. pahangi*, but comparable in length to *D. melanogaster* and *M. musculus*. This region was followed by a highly conserved domain, which contains the amino acid residues used for design of the degenerate PCR primers, U831 and L1110 (boxed in black). A third highly variable region was located between residues 679–707, just upstream of the C-terminal MEEVD motif found only in cytoplasmic Hsp90 sequences. The one known *C. elegans* Hsp90 mutation, daf-21(p673), appears at position 293, buried within a 38 amino acid region that was highly conserved across all taxa. Amino acids that were identical in *H. glycines* and one or more of the other nematodes, but not in the other species, were dispersed throughout the sequence (Fig. 2, shaded in gray).

### Amino acid phylogeny

There was sufficient phylogenetic information in the full protein sequence to provide strong support for most branches (Fig. 3). The nematode clade branched basal to the insects. The clade including insects plus vertebrates was supported at 70%. Although *S. mansoni* would have been a desirable outgroup to the nematodes, a full-length amino acid sequence was not available for comparison.

### Nucleic acid phylogeny

To determine the phylogenetic value of Hsp90 relative to the LSU and SSU rDNA genes traditionally used for nematodes, substitution parameters resulting from ModelTest were determined for each molecule and subjected to MP and ME analyses. Hsp90, LSU, and SSU each exhibited unique data set patterns derived from MP analyses of the seven-taxon alignments (trees not shown), Bayesian probability trees (Fig. 4), and a scatter plot of sequence variations (Fig. 5). Each data set resulted in a single bootstrapped consensus tree. MP analysis results are shown...
at the bottom of Table 4. In the LSU and SSU alignments, about 20% of the characters consist of gaps for one or more taxon, compared to only 1% for Hsp90 (due to a single codon insertion in outgroup *S. mansoni*). The MP tree length for Hsp90 showed 87% support for a clade including *C. elegans* as an outgroup to the three plant parasites. The SSU tree had 81% bootstrap support for *Brugia* as an outgroup taxon to the three plant parasites. This topology was also present in the alternative alignments tested. The LSU tree showed 65% support for *C. elegans* as an outgroup to *M. javanica* + *P. teres*, and 73% support for *H. glycines* as an outgroup to *B. malayi* + *S. ratti*. Outgroup comparisons revealed that *S. mansoni* provided shorter branch length and higher support values for in-group clades than did *D. melanogaster*. Because the *Schistosoma* results were consistent with those expected from intensively sampled SSU nematode trees (Blaxter et al., 1998), this taxon was used as the outgroup for trees shown in Figure 4. The Hsp90 tree topology (Fig. 4a) was consistent with SSU rDNA-based trees (Blaxter et al., 1998), except for the reversal in position of *Strongyloides ratti* and *C. elegans*. A similar topology was generated with *D. melanogaster* as the outgroup, but with much lower branch support for the clade of *C. elegans, H. glycines, P. teres*, and *M. javanica* (64% vs. 99%) (tree not shown). The SSU tree (Fig. 4b) was constructed with model TIM + γ (Table 4), resulting in a clade of (*B. malayi* + *S. ratti* + *C. elegans, 100%) and (H. glycines + (N. aberrans + M. incognita) 100%). The LSU tree (Fig. 4c) was constructed with K80 model. For LSU, an unusually long branch for *H. glycines* persistently joined the more divergent animal parasites at the tree base, although the support among various tree searches was not strong. This pattern occurred even when a heuristic ML tree with the expected topology was presented to MrBayes, although support values for the unexpected grouping of *H. glycines* + *B. malayi* + *S. ratti* were notably reduced (73% to 64%). Other than this difference, the support values from duplicate MrBayes runs generally varied by as little as two to four units for any branch.

The gl parsimony tree scores for the three genes each were derived from a distribution of a million tree lengths that indicated significant signal in these data sets. GI scores (expressed as the observed score relative to a set critical value; Hillis and Huelsenbeck, 1992) were: Hsp90 = −0.52 < −0.45; SSU = −0.83 < −0.49; 28S LSU = −1.036 < −0.46 (p < 0.01 for all). We also employed a more robust, entropy-based statistical test that takes into account tree topology, sequence length, and the number of taxa to arrive at critical values for determining phylogenetic signal (Xia et al., 2003). This method of determining substitution saturation (Xia et al., 2003) showed significant signal for two of the seven-taxon data sets from Figure 4, i.e., Hsp90 (Iss = 0.30 < 0.73 for symmetrical tree, < 0.63 for asymmetrical tree, p < 0.0001) and SSU (Iss = 0.35 < 0.78 for symmetrical tree, < 0.69 for asymmetrical tree, p < 0.0001) but not for LSU (Iss = 0.70 < 0.73 insignificant for symmetrical tree, p = 0.22, and significantly > 0.63 for asymmetrical tree, p = 0.01). Significant signal was present in the 16-taxon plant parasite Hsp90 alignment for Figure 6 (Iss = 0.37 < 0.68 for symmetrical tree, p < 0.0001, and < 0.49 for asymmetrical tree, p = 0.01).

Saturation scatter plots with curve fitting of transitions and transversions for all taxon pairs were created for each data set using DAMBE (data not shown). Information from these plots was abstracted in Figure 5, which shows a snapshot of possible signal saturation in Hsp90, SSU, or LSU, in pairwise comparisons of the taxa represented in Figure 4. The data in Figure 5 are expressed as the number of faster-accumulating transitions and rarer transversions as a function of distance. For all Hsp90 taxon pairs except for *Brugia-Strongyloides*, the number of transitions was greater than the number of transversions, which may be attributed to the relatively high AT content of *Strongyloides* (60% to 62%) compared to the average for other taxa (50% to 55%).
The inverse pattern of more transversions appeared uniformly in the LSU rDNA cluster, with the single exception of the least divergent *Meloidogyne-Pratylenchus* pair (Fig. 5, point 7). The SSU data points showed an intermediate pattern, where sequences became more divergent as a function of distance, and transversions approached and exceeded transitions in the four most divergent pairs. The distance between the outgroup and the most basal nematode taxa, between numbered data points 1 and 2, was at least twice as great in SSU compared to Hsp90. The Bayesian inference phylogram for the Hsp90 matrix was obtained for a select group of plant-parasite taxa and rooted with outgroup *Acrobeloides nanus*, with branch support shown for ME distance bootstrap values, MP bootstrap values, and MB posterior probabilities (Fig. 6). A range of variation in simple sequence divergence between species included a 1% difference between *M. javanica* and *M. incognita*, 8% between *M. hapla* and *M. javanica*, 14% between *P. penetrans* and *P. crenatus*, and 16% between *H. goldenii* and *H. glycines*. The ME values were always higher than MP values but lower than MB probabilities, except for the 11% higher value of 100% for the clade of *M. javanica* + *M. floridensis*, + *M. incognita* + *M. arenaria*. The MP tree resolved *Ditylenchus* and *Anguina* (92%) and all
seven Meloidogyne (82%), with M. hapla (53%) outside four other Meloidogyne spp. (98%). In this Hsp90 alignment, there were 77 parsimony informative characters (36% of total) where 78% of these characters were in third codon positions, plus 17 variable but uninformative characters (8%), 119 constant characters (56%), and no gaps. The best evolutionary model for this alignment was GTR + γ (Table 4). In the Bayesian tree, the position of the Pratylenchus teres branch was equivocal between the position shown and a position basal to other Pratylenchus and Heterodera spp. Among the sparsely sampled animal parasites, trees including S. ratti and S. stercoralis (not shown) never joined a clade with a partial sequence of Parasenoryloides trichosuri, whether they were rooted with Brugia spp. or were placed at the root of other taxa from clade V (Blaxter et al., 1998).

**Discussion**

There is a clear need for multiple genes of different types to construct reliable phylogenies (Baldauf et al., 2000; Kroken and Taylor, 2001; Sidow and Thomas, 1994). The nuclear gene Hsp90 possesses sufficient variation in its DNA and protein sequences to be useful for nematode phylogeny at different taxonomic levels. The Southern blots detected single Hsp90 genes in both *H. glycines* and *M. javanica*, a result we expect would be true for most, if not all, nematodes. While it would be impractical to perform the Southern analysis for every species, it is noteworthy that the U831 and L1110 primers specifically amplified single PCR products representing only the cytoplasmic form of Hsp90 from all nematodes examined thus far. These results are consistent with the single-copy nature of Hsp90 in *C. elegans*. That our primers specifically amplified only the gene for the cytoplasmic form of Hsp90 allowed us to avoid the complications of paralogy that can sometimes occur with multiple gene families. As more nematode Hsp90 genes are obtained and knowledge of taxon-specific codon usage increases, PCR primers and cycling conditions can be tailored to improve the amplification of Hsp90 from specific taxa of interest. The degenerate primers presented here serve as a useful entry point for sampling Hsp90 from diverse species, particularly when codon usage information is unknown.

**Hsp90 protein sequence analysis:** The *Hg-Hsp90* amino acid sequence exhibits a high level of overall conservation with the sequences from two other nematodes for which full-length sequences were available, *C. elegans* and *B. pahangi*, and lower levels of identity to other organisms. The high degree of sequence conservation in Hsp90 is advantageous for phylogeny because sequence alignments are straightforward. We have located positions of amino acid variation that may represent signature sequences for nematode Hsp90, several of which occur in or near functionally significant domains (reviewed in Buchner, 1999; Caplan, 1999; Pearl and Prodromou, 2000; and Prodromou et al., 1997).

As expected, *H. glycines* contains all of the consensus sequences previously defined by Gupta (1995, 1998). The high levels of conservation observed in regions I-IV reflect the involvement of these regions in ATP binding to Hsp90, which is essential for the protein’s chaperone function (Caemel and Kahn, 1991). Hsp90-mediated regulation of steroid hormone receptor folding and activation has been widely established (Pratt, 1997), and region V is known to be critical for receptor binding (Carrello et al., 1999). The presence of a steroid-binding domain in nematode Hsp90 is fascinating given that *C. elegans daf-12*, which encodes a nuclear hormone receptor (Antebi et al., 2000), coexists in the dauer pathway with *daf-21/Hsp90*. Although an interaction between *C. elegans daf-21/Hsp90* and *daf-12* has not been directly addressed experimentally, the known biochemical features of both molecules would be consistent with Hsp90 regulation of DAF-12-mediated effects on nematode development and lifespan.
The C-terminal MEEVD sequence (region VI) in *H. glycines* also agreed with the consensus defined by Gupta (1995). This domain is required for the binding of tetratricopeptide- or TPR-containing co-chaperones to Hsp90 (Russell et al., 1999) and distinguishes cytoplasmic Hsp90 from the endoplasmic reticulum-specific paralogs identified from many eukaryotes. Examination of the region just upstream of MEEVD reveals several additional residues that appear to be unique to nematodes (Fig. 2, gray-shaded amino acids). Several putative TPR-containing co-chaperones of Hsp90 can be identified from publicly available nematode EST's or from the completed *C. elegans* genome. Thus, the extended “GAEE/DASRMEEVD” motif may add specificity to the interaction of such co-chaperones with nematode Hsp90's.

**Protein phylogeny:** The Hsp90 tree (Fig. 3) shows nematodes branching outside insects, in line with the majority of other protein-coding genes that support a traditional Coelomata hypothesis (Wägele et al., 1999) over the newer Ecdysozoa hypothesis for metazoan evolution (Aguinaldo et al., 1997). In a comparison of 36 orthologous protein-coding genes from *Homo sapiens*, *D. melanogaster*, *C. elegans*, and *Saccharomyces cerevisiae*, 24 of them supported trees where insects were a sister group to humans, whereas 11 supported a clade containing both insects and nematodes (Mushegain et al., 1998). When even more genes were analyzed in similar four-taxon comparisons, trees with the highest confidence levels favored the Coelomata hypothesis at a 10:1 ratio over the Ecdysozoa (Blair et al., 2002). A more recent study of more than 500 protein sequences, alone and concatenated, also supported Coelomata (Wolf et al., 2004). While these conflicting hypotheses are still controversial (Mallatt et al., 2003), it is important to note that Hsp90 is one of those genes supporting Coelomata. This difference may increase its value in robustly comparing phylogenetic hypotheses against other genes supporting Ecdysozoa. These include actin, tubulin, and ribosomal genes, which are known to have lineage-specific biases worth testing with a more uniformly evolving character as seen in the full amino acid alignment of Hsp90 (Steichmann and Cavalier-Smith, 2003). Hsp90 protein sequences have proven especially informative in a study comparing four alveolate genes, and supported groupings of animals with fungi (opisthokonts) and plants with red algae (bikonts) more strongly than did Hsp70, despite sparser taxon sampling (Fast et al., 2002). Hsp90 protein comparisons also provided strong support for the monophyly of bikonts with Apsuozo, thus contradicting a less strongly supported position in rRNA trees (Steichmann and Cavalier-Smith, 2003).

**Hsp90, SSU, and LSU comparisons:** When phylogenetic signal is overwhelmed by multiple changes at each nucleotide alignment site, saturation of the data occurs. With increasing taxon divergence, saturation can be seen when relatively high ti/tv drops below 1 and approaches 0.4 (Hillis, 1991). Saturation in a data set can adversely affect likelihood parameter estimates and the final tree, and long-branch attraction of random sequence similarity may increase (Philippe, 2000). While the gI skewness statistic for the seven-taxon alignments indicated the presence of phylogenetic signal for all three molecules, a robust new statistical method for measuring substitution saturation detected significant signal in the Hsp90 and SSU data sets, but not in the LSU data set.

Comparison of the trees and associated scatter plots for the three genes (Figs. 4, 5) shows interesting nucleotide substitution patterns that reflect this range of saturation. The portions of all three genes we examined are small relative to their total sizes. Because fractional data sets combined with sparse taxon sampling can have a large influence on tree topology, these data sets should not be used to infer which molecule is likely to provide the truest tree. However, the data are useful for comparing substitution patterns. In the SSU data, as distance between taxon pairs increases, the number of transversions approached the relative number of transitions. The LSU data were more saturated than those of either Hsp90 or SSU, as reflected in trees with relatively poor phylogenetic resolution and the apparent long-branch attraction of *H. glycines* to the animal parasites at the base of the tree. This is not surprising because the D3 region of LSU region is more commonly used with intermediate-to-shallow phylogenetic distances than the deeper divergences resolved by longer segments of SSU rDNA (Baldwin et al., 1997; Larson, 1991). A similar pattern (Fig. 5) of increased saturation in LSU over SSU rDNA data, and greater divergence among taxa for SSU than for LSU, was previously reported for fish mitochondrial mt-rRNA genes (Bakke and Johansen, 2002). Tree topologies from our Hsp90 and SSU data sets (Fig. 4a,b), both of which were less saturated than LSU, were generally consistent with the patterns seen in trees based upon broader taxon sampling of SSU (Blaxter, 2001; Blaxter et al., 1998).

One possible tree topology for the same taxa as in Figure 4 but derived from prior SSU data sets (Blaxter et al., 1998; DeLey et al., 2002) has a succession of taxa with *B. malayi*, *C. elegans*, and *S. ratti*, followed by the plant parasites *H. glycines*, *P. teres* or *N. aberrans*, and *M. javanica* or *M. incognita*. Both rDNA data sets gave a single parsimony tree in a simple search, but the topology of both trees required major topological rearrangement to approximate this expected tree. These differences probably resulted from saturated positions in this portion of the SSU from the basal taxa of *Brugia*, *Caenorhabditis*, and *Strongyloides*. The partial LSU parsimony tree had *H. glycines* and *C. elegans* reversed from their positions in the SSU trees. The Hsp90 data set was slightly less decisive, giving two trees in a simple heuristic search with surprisingly good topology. Only the
Caenorhabditis Hsp90 sequence was homoplastic within the plant parasites. The only difference in these trees involved whether Pratylenchus and Meloidogyne were joined in a clade. Likelihood methods that went beyond the observed data to model hidden substitutions were required to improve the resolution of trees from these data sets; thus, the more resolved MB trees are shown in Figure 4 rather than MP trees.

The three tree-building methods used in the Hsp90 trees (Fig. 6) showed no major conflicts in branching order, but MrBayes trees always showed higher branch support than distance or parsimony trees as observed in other studies (Mallatt et al., 2003). Similarly, maximum likelihood methods applied to an Hsp90 amino acid phylogeny of basal eukaryotes also reflected other gene trees better than either distance or parsimony methods (Stechmann and Cavalier-Smith, 2003). The Hsp90 tree topology (Fig. 4a) was nearly congruent with the expected SSU tree (Blaxter et al., 1998), except that C. elegans and Strongyloides branches were reversed. In this case, Bayesian likelihood could not overcome the questionable grouping of Brugia + Strongyloides (Fig. 4a). The topology of plant-parasitic taxa within an Hsp90-based tree (Fig. 6) is generally congruent with the previous 12-species SSU tree, which showed that Globodera was not the closest lineage to Meloidogyne (DeLey et al., 2002). Similarly, the Hsp90 tree placed Heterodera spp. closer to Pratylenchus than to Meloidogyne. In the SSU trees, M. chitwoodi and M. hapla were basal to the M. incognita group. (DeLey et al., 2002) similar to Hsp90 (Fig. 6). However, the placement of M. javanica differed in SSU and Hsp90 trees. In SSU, M. javanica joined M. incognita, with M. arenaria outside (DeLey et al., 2002). In the Hsp90 tree, M. javanica was basal to the group of M. incognita and M. arenaria. Meloidogyne sasseri was basal to the other Meloidogyne species. At the species level, the partial Hsp90 sequence did not resolve M. incognita, M. arenaria, and M. floridensis. However, a longer Hsp90 sequence or its intron sequence variation should be useful for this purpose (not shown). Heterodera goldeni was morphologically similar to H. graminiphila (Handoo and Ibrahim, 2002), which also has affinity to H. cypseri (Mulvey and Golden, 1983). Examination of ITS rDNA supported six major species clades within Heterodieridae and showed that the Cyperi group is most similar to the Schachtii group, including H. glycines (Subbotin et al., 2001). Therefore, the two Heterodera species in our Hsp90 tree appear to represent the two closely related clades from ITS trees. The lack of resolution of P. teres relative to the other two Pratylenchus spp. and Heterodera may be expected to improve with more taxa.

The Hsp90 sequence divergence between other nematode species was generally lower than mitochondrial gene differences reported for the same taxa. For instance, the 8% difference in Hsp90 between M. hapla and M. javanica was lower than the 20% difference seen in the mitochondrial (mt) COII to cytochrome b interval (Blouin et al., 1998; Hugall et al., 1997). The 6% difference between C. elegans and C. briggsae was lower than the 10% difference in COII (Thomas and Wilson, 1991). The mean AT content of Hsp90 for 27 nematode taxa was 54%, lower than the approximately 80% AT composition for nematode mt DNA (Blouin et al., 1998; Hugall et al., 1997). Because extremes in base composition can reduce the phylogenetic utility of sequence variations, it appears that Hsp90 is less prone to this limitation than are mitochondrial genes.

The greater sequence divergence in Hsp90 between P. crenatus and P. teres (12%) compared to LSU rDNA (8%) (Carta et al., 2001) favors the utility of Hsp90 for resolving these species when more taxa are included. At the population level, two morphologically distinct populations of P. teres from South Africa had identical LSU D3 rDNA (Carta et al., 2002) and identical partial Hsp90 sequences (not shown). Also, two isolates of M. hapla with different pathogenicity (Eastern Shore, MD, and Maui, HI) were nearly identical over a longer (1078 bp) partial Hsp90 sequence, with two variable intron positions and three silent changes at third codon positions (not shown). The full length of Hsp90 may contain sufficient variation to be useful for the molecular discrimination of nematode populations.

The relatively large data set of Figure 6 showed clock-like behavior, where terminal taxa were equally distant from the root when evaluated by the AIC of ModelTest (Table 4). Other Hsp90 data sets with different taxa gave similar results (not shown). This finding is consistent with the clock-like behavior of a large amino acid alignment of eukaryotes (Stechmann and Cavalier-Smith, 2003).

The few basal animal parasite and free-living taxa for which this segment of Hsp90 sequence was available (e.g., Trichinella spiralis, Brugia spp., Ascaris lumbricoides, Ancylostoma caninum, Oscheius myriphila, and C. elegans) were too sparse and divergent to provide reasonable tree topologies with more distant taxa. However, from various data subsets we examined it was clear that S. stercoralis and S. ratti were not present in a clade with P. trichosuri (data not shown). This is consistent with P. trichosuri branching outside Strongyloides spp. in SSU trees (Dorris et al., 2002).

**Conclusion:** When variable-length intron sequences are removed, the alignment of single-copy Hsp90 sequences is relatively simple. We also found relatively uniform branch lengths in Hsp90 across nematode taxa, similar to that reported in a broader eukaryotic amino acid phylogeny (Stechmann and Cavalier-Smith, 2003). This uniformity has been attributed to the central role of Hsp90 in regulating many developmentally important proteins. Compared to the rDNA pattern, the smaller divergences of Hsp90 ingroups to outgroups may reduce the opportunity for long-branch attraction. In other organisms, as many as 100 molecules
per taxonomic sampling have been used to improve phylogenetic estimation (Bapteste et al., 2002). The fact that a relatively small part of Hsp90 demonstrates sufficient signal and produces trees generally congruent with previous trees supports the use of this gene to augment nematode phylogenetic hypotheses that are currently based upon only a few molecules.

**LITERATURE CITED**


