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Molecular cloning and expression of the full-length tropomyosin gene from *Trichinella spiralis*

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Abstract

A clone, designated as TsTM, was selected from the cDNA library of newborn larvae (NBL) of *Trichinella spiralis* through immunoscreening against infected sera. The clone contained a cDNA transcript of 855 bp in length with a single open reading frame, which encoded 285-amino acids (33 kDa in the estimated molecular weight). A sequence analysis revealed that the clone TsTM encoded the full-length of tropomyosin gene. The phylogenetic analysis of the tropomyosin gene was in good agreement with the classical taxonomical position of *T. spiralis*. The fusion proteins encoded by the clone TsTM were produced in an *Escherichia coli* expression system and affinity purified, and the antibody was raised against the protein for the following studies. The antibody against the fusion protein positively bound to the hypodermal muscle layer in immunolocalization analysis, and the 35 kDa band in crude extracts of muscle larvae but not in excretory and secretory (ES) products on Western blots. The antigenicity of the clone TsTM was recognized by host mice but exhibited little species specificity.

Introduction

Trichinella and trichinellosis have been extensively studied at the molecular level. Examples include parasite identification based on genetic polymorphism (Pozio *et al.*, 1992; Wu *et al.*, 2000; Zarlenga & Higgins, 2001), and bioactive substances of excretory and secretory (ES) products with serine proteinase inhibitor activity (Nagano *et al.*, 2001a), DNA binding protein (Lindh *et al.*, 1998) and a type of heat shock protein (Vayssier *et al.*, 1999). During the screening of the cDNA library of *Trichinella*, we have cloned some interesting genes, for instance, the myosin heavy chain gene, the serine proteinase inhibitor gene (Nagano *et al.*, 2001a) and the 21 kDa ES products gene (Nagano *et al.*, 2001b). Recombinant vaccine against *Trichinella* and recombinant antigen for immunodiagnosis of trichinellosis are now under extensive investigation. In the present study, we cloned a full length gene of tropomyosin, which enabled us to produce the fusion protein and reveal its molecular and immunological profiles. This is the first report on a *Trichinella* tropomyosin gene which may lead to a better understanding of this protein in helminth parasites.

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Materials and methods

Parasites

Muscle larvae of *T. spiralis* ISS 413 were obtained by a conventional digestion method (Despomier, 1975). Newborn larvae (NBL) were obtained as described by Takada & Tada (1988), and frozen at −80 °C until use.

Crude extracts and ES products

First stage larvae (L1) of *Trichinella* were isolated using the digestion method from muscles of mice 2 months post-infection. Crude saline extracts of L1 and ES

products were prepared using conventional methods (Wakelin *et al.*, 1994; Wu *et al.*, 1998).

Preparation of antisera

Polyclonal antibodies against crude extracts of the NBL were collected from BALB/c mice injected subcutaneously with 30 μg of the crude extracts and Freund's complete adjuvant followed by two booster injections of 15 μg of the crude extracts mixed with Freund's incomplete adjuvant at 2-week intervals.

Infected sera of the parasites and polyclonal antibodies against ES products were prepared by conventional methods (Nagano *et al.*, 2001a,b).

cDNA library construction and immunoscreening

Construction of a cDNA library from *T. spiralis* NBL was performed according to our previous method (Nagano *et al.*, 1999). In brief, frozen NBL were homogenized in an extraction buffer, and mRNA was isolated by affinity chromatography using oligo (dT) cellulose. The NBL were subjected to cDNA library construction using a Timesaver cDNA synthesis kit (Amersham Pharmacia Biotech, Tokyo, Japan), the λ ZAP II vector (Stratagene, La Jolla, California, USA), and Gigapack Gold III packaging extract (Stratagene).

Escherichia coli XL1-Blue MRF′ strain infected with the λ ZAP II vector was cultured on NZY agar plates, and it was then overlaid with a nitrocellulose membrane soaked with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The membrane was reacted with polyclonal antibodies against *T. spiralis* NBL and a second antibody, tagged with alkaline phosphatase, was developed with 5-bromo-4-chloro-3-indolyl-p-toluidine salt (BCIP) and nitroblue tetrazolium (NBT). Positive clones (named as clones TsTM) were picked up and rescreened three times.

DNA sequence and homology search

The clone TsTM was subjected to sequencing according to our previously described method (Nagano *et al.*, 1999). The DNA sequences were assembled and analysed by using DNASIS software (Hitachi Software Engineering, Tokyo, Japan). The NCBI BLAST network service at the National Center for Biotechnology Information was used to search for the sequence homology.

Production of fusion proteins

The TsTM clone was subcloned into the expression vector (pTrcHis, Invitorogen Corporation, Carlsbad, California, USA), transfected to *E. coli* according to our previous method (Nagano *et al.*, 1999) and grown in a bacterial culture medium. IPTG was added to induce protein production. The induced cells were disrupted by ultrasonication (28 kHz, 20 W, 3 s) three times. The resultant sonicate was centrifuged (5000 *g*, 10 min) and the supernatant subjected to affinity purification (HisTrap, Amersham Pharmacia Biotech, Tokyo, Japan).

Immunocytochemistry

Antibodies to the fusion protein were produced in mice for the immunocytochemistry and Western blot analysis. Muscles from mice after 42 days from *Trichinella* infection were fixed with 4% paraformaldehyde. The cryosections were treated with the first antibodies (1/100 dilution of antibody against the TsTM fusion protein or control normal mouse serum) for 30 min at 25°C. After washing, sections were treated with the second antibody (biotinylated anti-mouse IgG) for 30 min at room temperature, washed three times, treated with avidin coupled peroxidase for 10 min at 25°C, and reacted with diaminobendizone solution for peroxidase staining. Positive reactivity was associated with a purplish red colour.

Western blot analysis of the fusion protein TsTM

The fusion protein TsTM was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and then blotted on to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in phosphate buffered saline (PBS) and reacted with the first antibodies (1/100 dilution) including anti-NBL antibodies, infected sera, antibodies against ES products of muscle larvae (to examine whether the TsTM is a part of the ES products or not), or control uninfected mouse sera for 60 min at 37°C. After washing, the membranes were reacted with alkaline phosphatase-conjugated anti-mouse IgG for 30 min at 37°C. The alkaline phosphatase was developed using BCIP and NBT.

Western blot analysis of antibody against the fusion protein TsTM

Four types of samples (crude extracts of *T. spiralis*, ES products of *T. spiralis*, crude extracts of *T. pseudospiralis* and ES products of *T. pseudospiralis*) were checked for their reactivity against the anti-TsTM fusion protein antibody. Samples (10 ng of protein) were subjected to SDS–PAGE and blotted to nitrocellulose membrane in the same way as described above, and immunostained with the anti-TsTM antibodies.

Antigenic property of fusion protein TsTM

The antigenic property of the fusion protein was assessed using the following approach. A panel of crude extracts of adult parasites of *Paragonimus miyazakii*, *P. westermani*, *Fasciola* sp., *Clonorchis sinensis*, *Schistosoma japonicum*, *Toxocara canis*, *Ascaris suum*, *Strongyloides ratti*, *Angiostrongylus cantonensis*, *Gnathostoma doloresi*, *Trichuris vulpis*, *Anisakis* sp., *Brugia pahangi*, *Dirofilaria immitis*, *Diphyllobothrium latum*, *Taenia solium* (*Cysticercus cellulosae*) and *Spirometra erinacei* (plerocercoid) was reacted against the anti-fusion protein TsTM antibody, or the uninfected mouse sera as a control, by a dot ELISA. The reaction was visualized by the method described for Western blots.

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Kinetics of antibody response against the fusion protein TsTM

BALB/c mice were inoculated orally with 300 infective *T. spiralis* larvae. Sera from infected mice were collected before infection for a control serum and at 1, 2, 4, 8 and 16 weeks after infection. Antibody titre against fusion protein TsTM was assessed by means of dot ELISA as described above.

Results

DNA and amino acid sequences

The clone TsTM consisted of 855 bp with a single open reading frame and encoded 285 amino acid residues (GenBank accession number AF419300). The molecular weight of the predicted protein was 33229 Da. An NCBI BLAST search revealed significant homology to tropomyosin of a number of nematodes, including *Caenorhabditis elegans*, *Onchocerca volvulus*, *Anisakis simplex* and *Acanthocheilonema viteae* (fig. 1). The TsTM seemed to be the full-length gene of tropomyosin.

Tropomyosin of *C. elegans* had a 72% homology to TsTM in the DNA sequence and 83% in the deduced amino acid sequence. *Onchocerca volvulus*, *Anisakis simplex* and *Acanthocheilonema viteae* had a 76 to 78% homology in the DNA sequence and 83 to 84% in the deduced amino

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TsTM     1:MDAIKKKMQAMKIEKDNAMDRADAAEEKARQQQERVEKLEEELRDTQKKMMQVENELDKA
TpTM     1:............................................................
O.vol    1:.................L.........V..MT.KL.RI.............T..D.V..
A.sim    1:.................L.........V..MTDKL.RI.............T..D....
A.vit    1:...............T.L......K..V..MTDKL.RI.............T..D.N..
           *************** * ****** ** **     *  ************ ** * **

TsTM    61:QEELTGANAQLEEKEKKVQEAEAEVAALNRRIQLLEEDFERAEERLIIATEKLGEASQTA
TpTM    61:.....................................................K......E......
O.vol   61:..D.SV..TN..D.....................MT....EL.......K...D..E..TH..
A.sim   61:..D.ST..SN.....................MT....EL.......KL..A..E..TH..
A.vit   61:..D.AV..TN..D......K.....PP....MT....EL.......K...D..EK.TH..
           ** *  **  ** ****** *****  ****  ****  *******  ** **  *  **

TsTM   121:DESERVRKVMENRSLQDEERVYQLEAQLKEAQLLAEEADRKYDEVARKLAMVEADLERAE
TpTM   121:............................................................
O.vol  121:..............F.....ANTV.S.E.....................................
A.sim  121:..............F.....ANTV.S......M.................T..........
A.vit  121:...D..........F.....ANTV.S.....................................
           *** ********** *****    * * **** ***************** **********

TsTM   181:ERAEAGENKIVELEEELRVVGNNLKSLEVSEEKALQREDSYEEQIRLLTQRLKEAETRAE
TpTM   181:............................................................
O.vol  181:........E.....................................Q....TVSV.........
A.sim  181:....T.....................................................TVSA.........
A.vit  181:.........................F.....................TVSA.........
           **** *** ***************** ***************** ****     **********

TsTM   241:FAERSVQKLQKEVDRLEDELVHEKEKYKAISEELDQTFQELSGY
TpTM   241:.............................................
O.vol  241:...........K.............R..N...............
A.sim  241:.........................R..S...............
A.vit  241:.....................GR..N...........F..
           *********** ************  ** ************ **
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Fig. 1. Comparison of amino acid sequence of the clone TsTM and its comparable genes from other nematodes including *Trichinella pseudospiralis* (TpTM), *Onchocerca volvulus* (O. vol), *Anisakis simplex* (A. sim), *Acanthocheilonema viteae* (A. vit). The asterisks indicate amino acids which are identical to those of TpTM, O. vol, A. sim, A. vit and TsTM. Tropomyosin of *O. volvulus* had a 78% homology in the DNA sequence and 83% in the deduced amino acid sequence. *Anisakis simplex* had a 76% homology in the DNA sequence and 84% in the deduced amino acid sequence. *Acanthocheilonema viteae* had a 76% homology in the DNA sequence and 84% in the deduced amino acid sequence.

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acid sequence (see fig. 1 caption). Human tropomyosin had only a 64% homology in the DNA sequence and 60% in the deduced amino acid sequence. A comparable study with *T. pseudospiralis* (GenBank accession number AF453521) revealed that the two species of *Trichinella* had a 97% homology in the DNA sequence and a 99% homology in the deduced amino acid sequence (T. Nakada, I. Nagano & Y. Takahashi, unpublished data).

Western blots

The fusion protein TsTM migrated at 35 kDa on SDS–PAGE. On Western blots, the fusion protein was immunostained positively with *T. spiralis* infected sera (lane 1 in fig. 2) or *T. pseudospiralis* infected sera (lane 3 in fig. 2), but not with the anti-ES product of *T. spiralis* muscle larvae antibody (lane 2 in fig. 2) or the anti-ES product of *T. pseudospiralis* muscle larvae antibody (lane 4 in fig. 2), which also acted as a negative control. These anti-ES product antibodies stained ES products of each parasite (photograph not shown).

A Coomassie blue SDS–PAGE analysis revealed that crude extracts and ES products of L1 larvae were composed of many bands with different molecular weights (photograph not shown). The antibody against the TsTM fusion protein reacted positively with a single band of 35 kDa of crude extracts of *T. spiralis* (lane 1 in fig. 3) and *T. pseudospiralis* (lane 3 in fig. 3) but not against any components within the ES products from the two species (lanes 2 and 4 in fig. 3).

Immunolocalization

As shown in fig. 4A–B, positive immunostaining was observed in the internal structures of the *T. spiralis* muscle larvae. The staining consisted of spots and lining which seemed to correspond to the location of the hypodermal muscle layer. The stichosome, intestinal tract and genital primordial cells did not stain and control sera failed to stain any structures, which suggested specificity of the staining.

Antigenic property

The multiple dot ELISA showed positive reactivity of the anti-fusion protein TsTM antibody against crude extracts of *Toxocara canis*, *Strongyloides ratti*, *Angiostrongylus cantonensis*, *Gnathostoma doloresi*, *Trichuris vulpis*, *Anisakis* spp., *Brugia pahangi*, *Dirofilaria immitis*, *Ascaris suum* (fig. 5), and no reactivity against crude extracts of *Paragonimus miyazakii*, *P. westermani*, *Fasciola* sp., *Clonorchis sinensis*, *Schistosoma japonicum*, *Diphyllobothrium latum*, *Taenia solium* and *Spirometra erinacei*. Some nematode samples (*Trichinella spiralis*, *Strongyloides ratti*, *Angiostrongylus cantonensis*, *Trichuris vulpis* and *Dirofilaria immitis*) showed positive reactivity, probably due to cross reaction.

Antibody response

Antibodies against the fusion protein were detected by means of dot ELISA 2 weeks after the infection and the titre remained positive throughout the experiment (fig. 6).

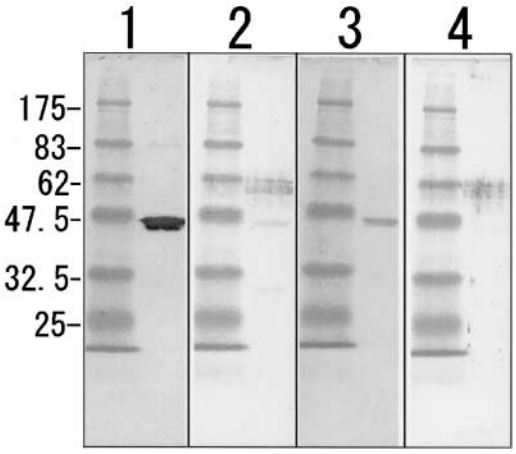

Fig. 2. Western blot analysis of the fusion protein TsTM against infected sera of *Trichinella spiralis* (lane 1), infected sera of *T. pseudospiralis* (lane 3), anti ES product of *T. spiralis* antibody (lane 2) and anti ES product of *T. pseudospiralis* antibody (lane 4). The left side of each lane is molecular marker and the right side is the test sample. The numbers on the left side are molecular weights.

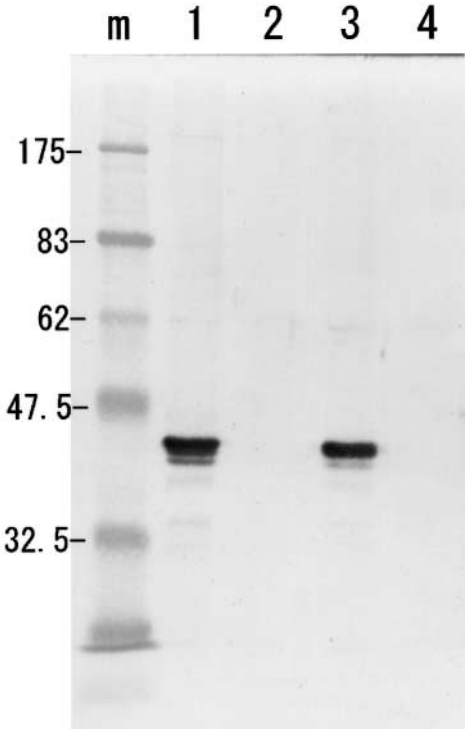

Fig. 3. Western blot analysis of reactivity of anti-TsTM antibody against *Trichinella spiralis* crude extracts (lane 1), *T. pseudospiralis* crude extracts (lane 3), ES products of *T. spiralis* (lane 2) and ES products of *T. pseudospiralis* (lane 4). The numbers are molecular weights of marker (m).

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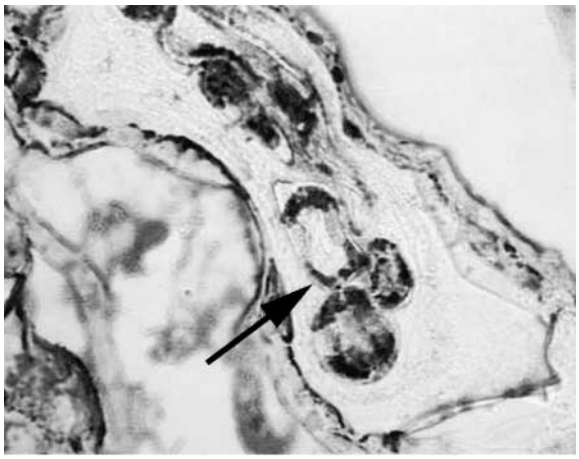
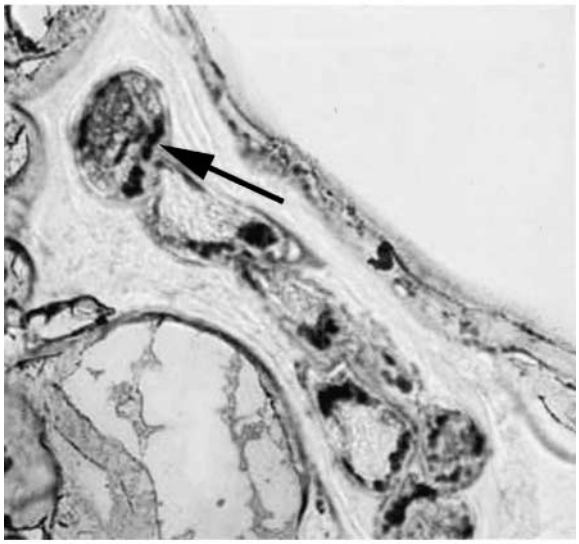


A



B

Fig. 4. Immunocytochemical staining of muscle larvae of *Trichinella spiralis* with antibody against TsTM fusion protein (A, B). The positive staining is shown by the arrow.

Phylogenetic analysis of the tropomyosin gene and TsTM gene

The amino acid sequences of tropomyosin and TsTM were aligned using CLUSTAL X ver. 1.81 (Thompson *et al.*, 1997). A phylogenetic tree (fig. 7) was constructed by means of the neighbour-joining method using the tools (PROTDIST, NEIGBOR, SEQBOOT and CONSENSE of PHYLIP ver. 3.5c) following instructions on the web site (http://evolution.genetics.washington.edu/phylip.html). Phylogenetic analysis of the tropomyosin gene and TsTM gene revealed that *Trichinella* falls within a group of nematodes which includes *Acanthocheilonema*

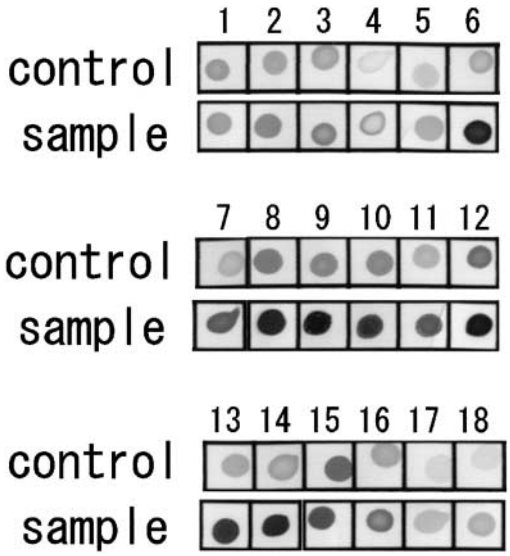


Fig. 5. Dot-ELISA analysis of reactivity of anti-TsTM fusion protein antibody against a panel of crude extracts. 1, *Paragonimus miyazakii*. 2, *Paragonimus westermani*. 3, *Fasciola* sp. 4, *Clonorchis sinensis*. 5, *Schistosoma japonicum*. 6, *Toxocara canis*. 7, *Ascaris suum*. 8, *Trichinella spiralis*. 9, *Strongyloides ratti*. 10, *Angiostrongylus cantonensis*. 11, *Gnathostoma doloresi*. 12, *Trichuris vulpis*. 13, *Anisakis* spp. 14, *Brugia pahangi*. 15, *Dirofilaria immitis*. 16, *Diphyllobothrium latum*. 17, *Taenia solium* (*Cysticercus cellulosae*). 18, *Spirometra erinacei* (plerocercoid).

viteae, *Caenorhabditis elegans*, *Anisakis simplex*, *Onchocerca volvulus* and *Trichostrongylus colubriformis*, and this group is distinct from vertebrates, trematodes and insects (fig. 7).

Discussion

In the present study, we investigated the clone TsTM from *Trichinella spiralis* NBL, which is likely to be the full length of the tropomyosin gene based on sequence homology (about 80% homology), its expected molecular weight (33 kDa) and the localization of the molecule within the parasite (i.e. in the muscle layer). Western blots and immunolocalization studies showed that there was no evidence of the molecule being present in the ES products.

From a clinical point of view, tropomyosin is attracting much attention as a pan-allergen. It is a cross-reacting allergen among invertebrates (Edgerton *et al.*, 2000;

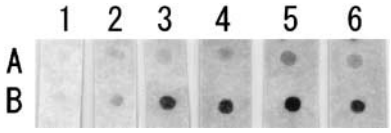


Fig. 6. Dot-ELISA analysis of kinetics of antibody against fusion protein TsTM (A) and crude extracts (B). Sera were collected from mice before infection (lane 1), 1 week (lane 2), 2 weeks (lane 3), 4 weeks (lane 4), 8 weeks (lane 5) and 16 weeks after infection (lane 6). Antibody against fusion protein TsTM is detected from 2 weeks post-infection. Antibody against crude extracts is detected from 1 week post-infection.

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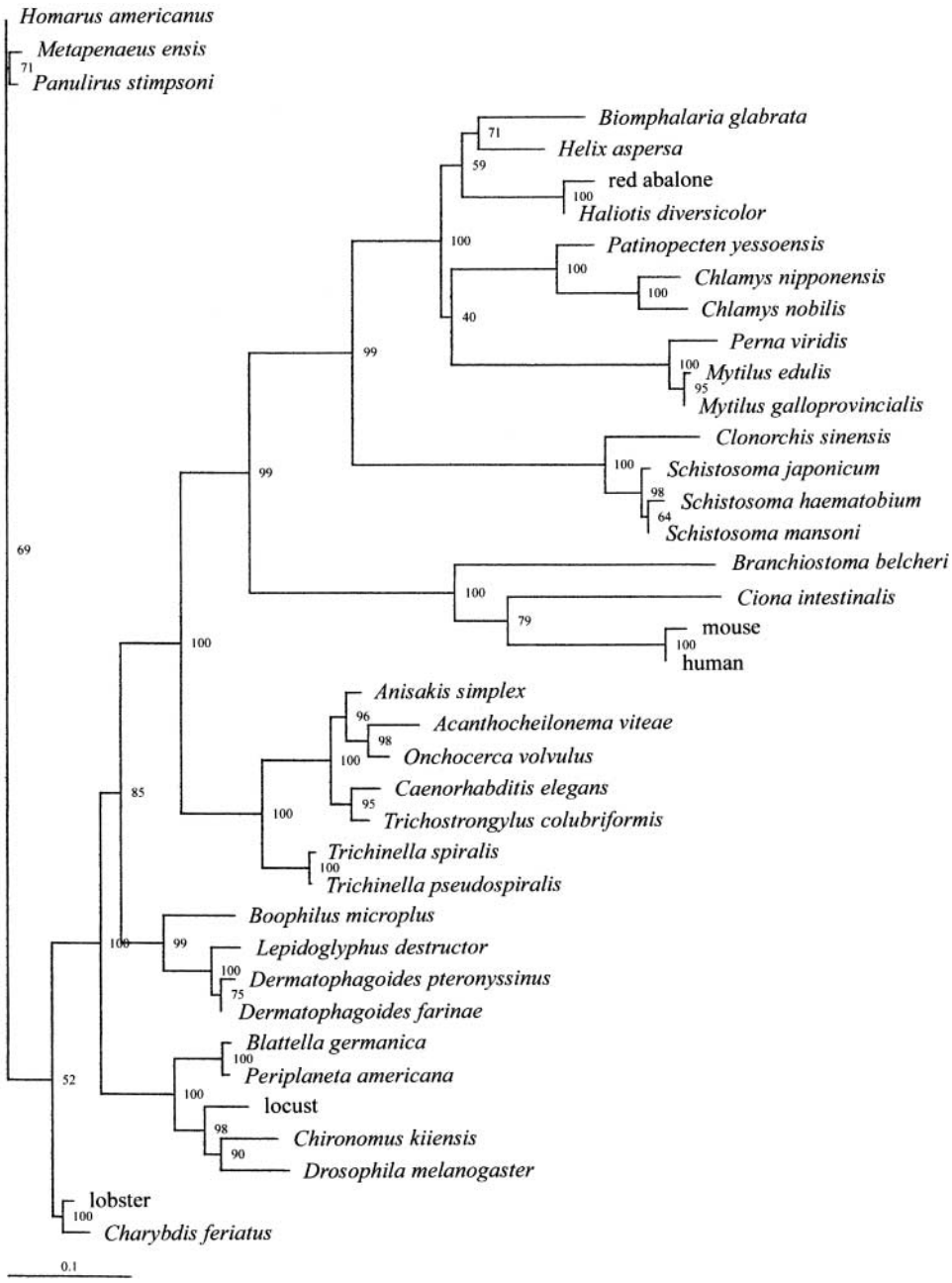

Fig. 7. Phylogenetic analysis of tropomyosin gene from GenBank. Numbers at branches indicate the number of times that a branch was detected out of 100 bootstrap replicates. The bar indicates 0.1 amino acid substitutions per site.

Kagamiyama & Hayashi, 2000; Perez-Perez *et al.*, 2000), and it elicits hypersensitivity reactions in atopic patients. Shrimp, which is well known as an allergic food, has tropomyosin with six IgE binding sites. Also tropomyosin from many origins may be antigenic, and the DNA sequence of this structural protein is highly conserved. Indeed, we found that some regions of the nucleotide sequence of tropomyosin of *Trichinella* are conserved, particulaly among nematodes (fig. 1).

Tropomyosin, shared by every cell, is an essential protein for parasites and their hosts, and it is antigenic during the infection. Therefore its antigenic property and possible immunodiagnostic use were also assessed. These kinds of fusion proteins of parasites have been constructed as a candidate for an immunodiagnostic antigen with some success (Zarlenga & Gamble, 1990; Chandrashekar *et al.*, 1991; Zarlenga *et al.*, 1994). Indeed, tropomyosin produced in the present study carried an

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antigenic epitope, which is recognized by hosts during experimental infection. Mice infected with *Trichinella* produced antibodies at detectable levels against tropomyosin during the early phase of infection. However, the fusion protein seems to lack species specificity because tropomyosin reacted against sera from all patients examined, including those with helminth infections (nematode, cestode and/or trematode), and the antibody against tropomyosin reacted against crude extracts from a variety of helminths (fig. 5). Nonetheless, based on the apparent specificity in mice, tropomyosin has some potential as an immunodiagnostic antigen especially in detecting helminth infections at an early stage.

Acknowledgements

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