Phylogenetic Relationship of *Bombyx mori* Protein Disulfide Isomerase

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Protein Disulfide Isomerase, *Bombyx mori*

A cDNA that encodes protein disulfide isomerase was isolated from *Bombyx mori* (bPDI), in which an open reading frame of 494 amino acids contained two PDI-typical thioredoxin active sites of WCGHCK and an ER retention signal of the KDEL motif at its C-terminal. The bPDI protein shared less than 55% of the amino acid sequence homology with other reported PDIs. bPDI is most genetically similar to the *D. melanogaster* PDI. The most serious evolutional diversity was observed between the metazoa and nematoda through PDI evolutional processing. Although bPDI shows a relatively low amino acid homology with other PDIs, in which both sites of the two thioredoxin active sites and the endoplasmic reticulum (ER) retention signal are completely conserved, it was successfully recognized by anti-rat PDI antibodies. This suggests that bPDI may have the activity of a protein isomerase and a chaperone.

Introduction

Protein disulfide bond formation is a rate-limiting step in protein folding and is catalyzed by enzymes belonging to the protein disulfide oxidoreductase superfamily, including PDI in eukarya and DsbA in bacteria (Noiva, 1994). PDI catalyzes disulfide oxidation and isomerizes incorrect disulfides on newly synthesized polypeptides undergoing correct folding in the oxidizing ER environments. It is now accepted that PDI is a multifunctional protein that is involved in the folding, assembly, and posttranslational modification of many proteins in addition to actin filament polymerization, gene expression, cell-cell interaction and the regulation of the receptor function (Frand *et al*., 2000). PDIs are known to share a strong DNA sequence homology and their expression is found abundantly in many cell types. The typical PDI structure has led to a five-domain model for enzyme activity, in which two protein-thiol oxidoreductase active site sequences of WCGHCK are shown in both the C-/N-terminal regions, and an ER retention signal of KDEL in the C-terminal. This is evidence that the PDI is located/retained in the ER lumen and functions as an ER chaperone (Ciaffi *et al*., 2001; Noiva, 1999; Warsame *et al*., 2001).

The bPDI protein (a PDI homologue from *Bombyx mori*) in this study also has two thiol oxidoreductase sites and a KDEL motif in the C-terminal. The cDNAs encoding the PDI family have been isolated from a number of organisms or tissues. However, only one of the PDI cDNA sequences has been reported from *Drosophila melanogaster* and limited information on the PDI in insects is available (McKay *et al*., 1995). It has already demonstrated that bPDI expression depends on ER stress and hormones in the Bm5 cell line derived from *Bombyx mori*. While both the thiol oxidoreductase sites and the ER retention signal are very well conserved, a high sequence variation was shown among the known PDIs. In addition, most recent studies have demonstrated that coexpressed or overexpressed PDI increased the folding and secretion of heterologous proteins from the cells used (Ailor and Betenbaugh, 1999; Hsu *et al*., 1996; Ritchie *et al*., 1999). To establish that the forced expression of PDI in a cell is effective in enhancing the folding and secretion of het-

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erologous proteins, it is essential that further understanding of the phylogenetic relationships of bPDI among the PDIs be obtained. With this in mind, results are reported of the evolutionary position among eukaryotic PDIs and a cDNA encoding the *Bombyx mori* homologue PDI. The cloned cDNA was successfully translated, and the expressed bPDI also recognized by anti-rat PDI antibodies.

Materials and Methods

Experimental insects and cells

Silkworms, *Bombyx mori* (Jam 306), were reared on an artificial diet at 24–27 °C with 70–90% humidity. The culture cell line Bm5 derived from *B. mori* ovary was cultured at 27 °C in a TC-100 medium (Sigma Chemical, St. Louis, MO) with 10% fetal bovine serum (GIBCO Life Technologies, Gland Island, NY) using the standard method.

Differential screening for bPDI gene

We constructed a cDNA library from the Bm5 cells inhibited N-linking glycosylation of the protein for 5 h with tunicamycin (5 μg/ml) using an Uni-ZAP XR vector kit (Stratagene, La Jolla, CA), and 768 randomly selected cDNA fragments were duplicated and fixed on the nitrocellulose membranes, respectively. Other cDNA fragments derived from both kinds of poly(A^+) RNAs, normal and tunicamycin-treated were labeled with [α-^{32}P]dATP and used as molecular probes. Two membrane sheets were hybridized in an ExpressedHyb Hybridization Solution (Clontech, Palo Alto, CA). The selected cDNA fragments were cloned and amplified. The resulting positive cDNA fragments were partially sequenced. One of these sequences shares a high homology with the PDI gene family, and was fully sequenced after obtaining a full cDNA sample by the 3′-RACE (Rapid Amplification of cDNA Ends) PCR method.

DNA sequencing and sequence analysis

The selected cDNA clones were partially sequenced using T3 and T7 primers by an automatic sequencer (Perkin Elmer, Watsonville, CA, ABI 377). The double-stranded DNA was prepared using the Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, WI). Each DNA sample (300–500 ng) was mixed with primer (3.2 pmole) and a Termination Reaction Mix (Perkin Elmer), and sequenced following 25 cycles of PCR conditions (96 °C for 30 sec, 50 °C for 15 sec, and 60 °C for 4 min). The resulting PCR products were separated on a 4.5% denatured polyacryamide gel and analyzed by DNA Sequencing Analysis Software (Perkin Elmer). Both strands of the cDNA clones were sequenced. The amino acid sequence was deduced from the cDNA sequence, and the homology with other species was analyzed through a GenBank database search. The sequence identity and homology search was achieved using BLAST (Basic Local Alignment Search Tool). The multiple alignments of sequences was carried out using the Clustal X program, and then modified manually to correct for misalignments as determined by the results of pairwise alignments and by a visual inspection. The phylogenetic analysis based on the PDI amino acid sequences was carried out using the maximum parsimony method of the PAUP (Phylogenetic Analysis Using Parsimony).

Construction of expression vector for bPDI and Western blot analysis

The cDNA encoding bPDI was amplified using a sense primer (5′-CGGGATCCCGGAAATGCGTGTTTTAATTTTCACG-3′; underline indicates the initial codon) and an antisense primer (5′-GAAGGCCTTCTAACTCGTCTTTGGCAGGC-3′; underline indicates the stop codon that was modified from the original TAA). The PCR products were ligated once to a TA cloning vector, pGEM-T (Promega). The pGEM-bPDI was digested with *Bam*H I/*Stu* I and subcloned into the baculovirus vector, pBAC1-bPDI. After incubation for 15 min, a mixture pBAC1-bPDI/BacVector-3000 Triple Cut Virus DNA (Novagen, Madison, WI) with Eufectin (Novagen) was inoculated at 27 °C for 5 h at the Sf-9 cell line. The infected cells were collected after 3 days and digested with lysis buffer (6.25 mM Tris (hydroxymethyl)aminomethane-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% DTT). Electrophoresis was performed in 7.5% polyacrylamide gels. Protein transfer to a PVDF membrane

was carried out in a semi-dry system from Bio-Rad. The PVDF membrane was quenched for 30 min in 150 mM NaCl, 10 mM Tris (hydroxymethyl) aminomethane-HCl, pH 7.5 containing 2% non-fat dry milk. Immunological detection was done by first incubating the PVDF membrane for 1 h at room temperature with the primary antiserum in TBS containing 0.05% Tween 20. Subsequently, the PVDF membrane was incubated for 1 h with peroxidase-conjugated secondary immunoglobulins. The PVDF membrane was washed intensively between each step in 150 mM NaCl, 10 mM Tris (hydroxymethyl) aminomethane-HCl, pH 7.5, 2% non-fat dry milk.

Results and Discussion

A *Bombyx mori* PDI homologue (bPDI) was isolated from the culture cell line Bm5, which was treated with tunicamycin using a differential screening method, and both strands sequenced the cDNA clone (Hoog, 1991). The sequence data of the bPDI was submitted to Genbank under the accession number AF325211. Although a cDNA encoding bPDI shows a high sequence variation compared with known PDI cDNAs, the bPDI protein has two well conserved thiol oxidoreductase sites and a KDEL motif in the C-terminal, which is similar to the other PDIs. To confirm whether or not the cDNA encoding bPDI translates correctly *in vivo* by bPDI, the recombinant baculovirus (vAc-bPDI) was translated in the culture insect Sf-9 cell line. As shown in Fig. 1, the cDNA encoding the *Bombyx mori* PDI homologue was successfully translated in bPDI (line 3 in panel A), which was also recognized by anti-rat PDI antibodies (line 3 in panel B). No band estimated PDI appeared between the wild type cells and the cells infected with the wild type baculovirus (line 1, 2). The result suggests that although the bPDI-cDNA isolated in this study shows a low DNA sequence homology among the known PDIs, the pPDI protein shares the same antigen domains with another PDI that is recognized by anti-rat PDI antibodies. In addition, the bPDI-cDNA encodes a typical PDI. Furthermore, bPDI has a similar 3-D structure to other known PDIs and may play a similar functional role in the insect cells, although the exact 3-D structure has not yet been determined.

Eukaryotic PDIs typically are composed of five functional domains, a-b-b'-a'-c. Both the a and a' domains (approximately 110 amino acid residues each), which are well conserved between species, contain one of the thioredoxin active sites (WCGHCK), respectively (Kemmink *et al.*, 1997). The b and b' domains are comparatively less conserved between species, but the domain b' has a similar binding property to other proteins (Kemmink *et al.*, 1999; Klappa *et al.*, 1998). The domain c (about 30 amino acid residues) has a calcium-binding site and ER retentonal motifs (KDEL) (Darby *et al.*, 1998; Koivunen *et al.*, 1999). A multiple sequence alignment was carried out with the bPDI and PDIs reported, using the Clustal X program (Thompson *et al.*, 1997), the bPDI amino acid sequence was compared with other eukaryotic PDIs (Fig. 2). An overview of the multiple sequence alignment showed that while both domains a and a' including thioredoxin active sites share a relatively high homology between the PDIs, the other domains do not. Considering that the total amino acid sequence homology is not conserved between the PDIs, it is interesting that PDIs appear to be essential for cell survival and proliferation. This is strongly suggested based on the results shown in Fig. 1, which indicates that the sequence diversity between the PDIs should not prevent the activation of both the disulfide isomerase and the chaperone unless the active sites are different but shares common antigen domains.

The pairwise matrix of the amino acid sequence divergence was calculated by the neighbor-joining

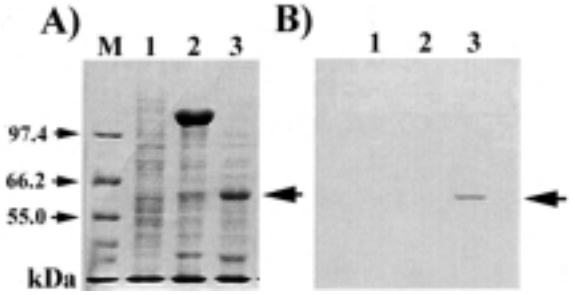
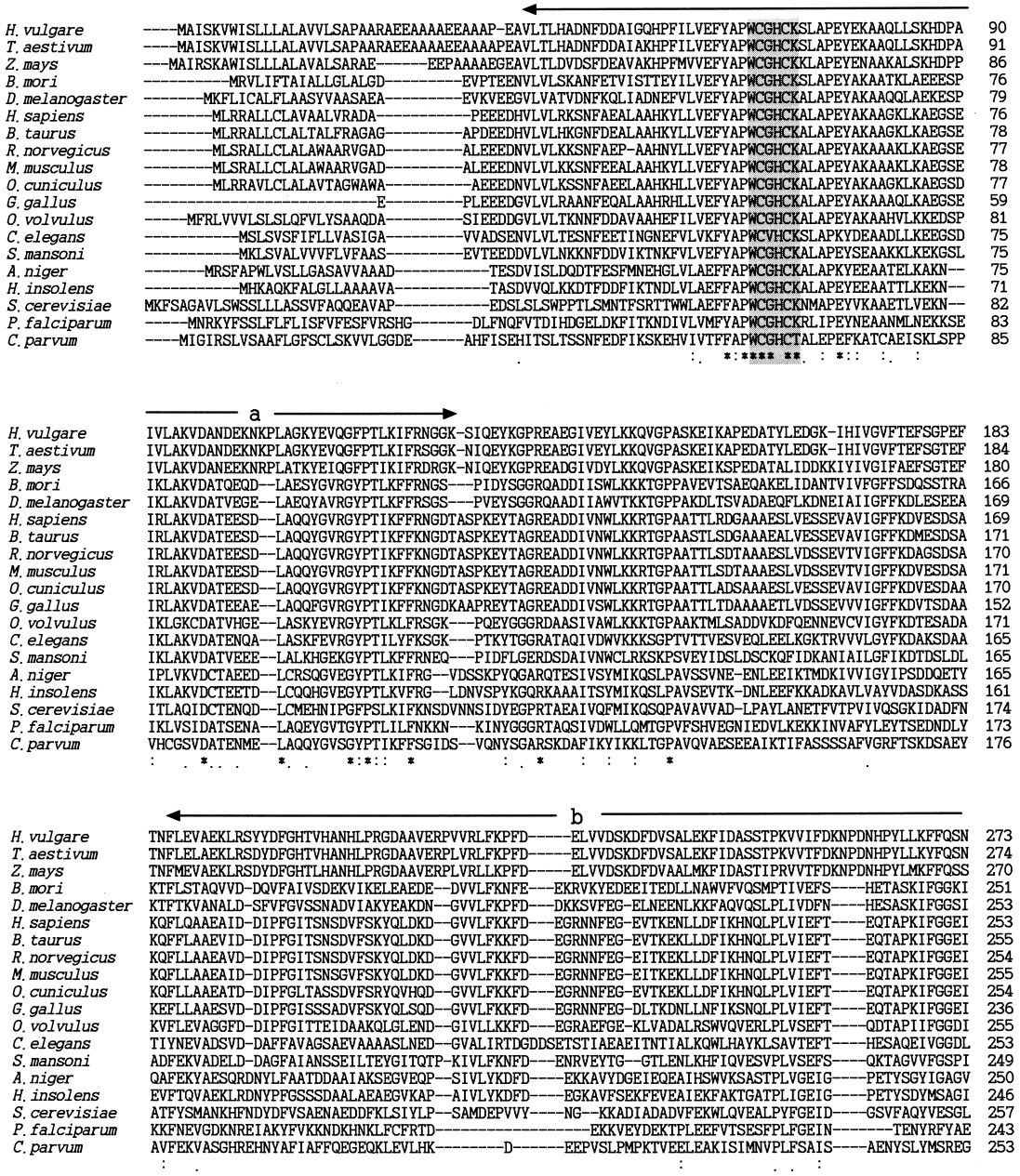


Fig. 1. Western blot analysis of bPDI. Sf-9 cells were infected with a recombinant virus (vAc-bPDI) and harvested 72 h after infection (line 3). Lane 1; protein extracted from normal cells, Lane 2; protein extracted from the cells infected with wild type baculovirus. Panel A and B, are the results of SDS-PAGE stained with Coomassie Blue and Western blot analysis, respectively. An arrow indicates the estimated PDI and the rabbit anti-rat PDI antibodies that were was used as a second antibody in panel B.



method with the results shown in Table I (Saitou and Nei, 1987). The sequence divergences of the PDIs ranged from 22% to 55% within the PDIs compared in this study, while among of them, the bPDI sequence of bPDI shares the lowest divergence, 55%, with *Drosophila melanogaster* PDI. On the other hand, the sequence divergences between plants, vertebral animals and bPDI ranged from 27%-29% and 48%-49%, respectively. Based on the results shown in Table I, and the pairwise matrix of amino acid sequence divergence, the maximum parsimonious tree constructed with 1000 bootstrap replicates is shown in Fig. 3. bPDI was most closely related to *D. melanogaster* PDI with a low genetic distance value of 920 and branched independently off in the early stages of

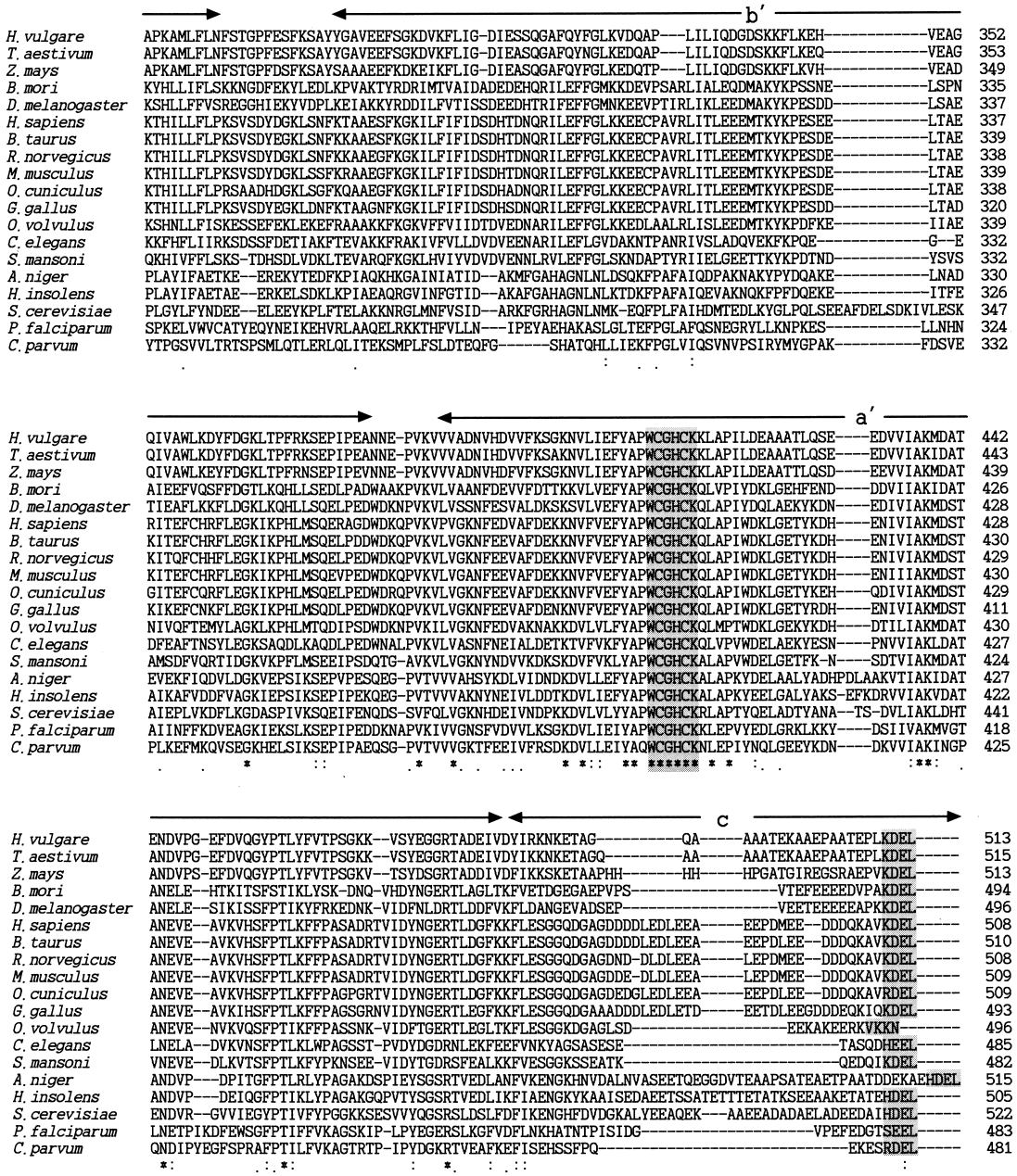


Fig. 2. Multiple sequence alignment of bPDI. Each amino acid sequence is represented by the standard single letter code. The two thioredoxin active sites (WCGHCK) as well as the ER retention signal (KDEL) are shaded. The arrows above the sequences indicate the five typical PDI domains a-b-b′-a′-c. Identical residues (*), conserved substitutions (:) and semi-conserved substitutions (.) between 19 sequences are indicated below the sequences. *Hordeum vulgare* (L33250; Chen and Hayes, 1994), *Triticum aestivum* (U11496; Shimoni *et al*., 1995), *Zea mays* (L39014; Li and Larkins, 1996), *Bombyx mori* (AF325211), *Drosophila melanogaster* (U18973; McKay *et al*., 1995), *Homo sapiens* (J02783; Cheng *et al*., 1987), *Bos taurus* (M17596; Yamauchi *et al*., 1987), *Rattus norvegicus* (X02918; Edman *et al*., 1985), *Mus musculus* (J05185; Mazzarella *et al*., 1990), *Oryctolagus cuniculus* (J05602; Fliegel *et al*., 1990), *Gallus gallus* (X13110; Parkkonen *et al*., 1988), *Onchocerca volvulus* (U12440; Wilson *et al*., 1994), *Caenorhabditis elegans* (U95074; Veijola *et al*., 1996), *Schistosoma mansoni* (Z22933; Finken *et al*., 1994), *Aspergillus niger* (X98797; Ngiam *et al*., 1997), *Humicola insolens* (S74296; Kajino *et al*., 1994), *Saccharomyces cerevisiae* (M62815; LaMantia *et al*., 1991), *Plasmodium falciparum* (AJ250363; Florent *et al*., 2000), *Cryptosporidium parvum* (U48261; Blunt *et al*., 1996).

Table I. Comparison of pairwise identities of *B. mori* protein disulfide isomerase (bPDI) gene and the known eukaryotic PDI genes. Sequences were adjusted to optimize the alignment of the conserved residues and the percentage of aligned identities determined.

Organism	Gene sequence similarity of protein disulfide isomerase (PDI)																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
H. vulgare	–																		
T. aestivum	95	–																	
Z. mays	77	78	–																
B. mori	29	28	27	–															
D. melanogaster	31	30	31	55	–														
H. sapiens	28	28	29	48	51	–													
B.taurus	28	28	28	49	52	94	–												
R. norvegicus	28	28	28	49	51	91	91	–											
M. musculus	28	29	28	49	51	92	93	96	–										
O. cuniculus	28	28	28	48	51	89	87	88	88	–									
G. gallus	29	29	28	48	52	84	84	83	83	82	–								
O. volvulus	29	29	28	46	51	56	26	55	55	54	55	–							
C. elegans	25	25	25	40	40	39	39	41	40	41	40	42	–						
S. mansoni	27	26	26	40	40	41	41	41	41	42	41	45	36	–					
A. niger	26	29	25	28	28	26	26	27	28	27	28	26	26	28	–				
H. insolens	29	30	28	29	29	29	30	29	29	29	30	27	26	30	58	–			
S. cerevisiae	24	24	21	22	23	24	25	25	25	26	25	23	22	24	33	35	–		
P. falciparum	24	24	23	25	27	26	27	25	25	26	25	28	26	26	24	27	17	–	
C. parvum	22	23	23	23	24	26	25	25	25	26	25	24	23	24	27	28	18	28	–

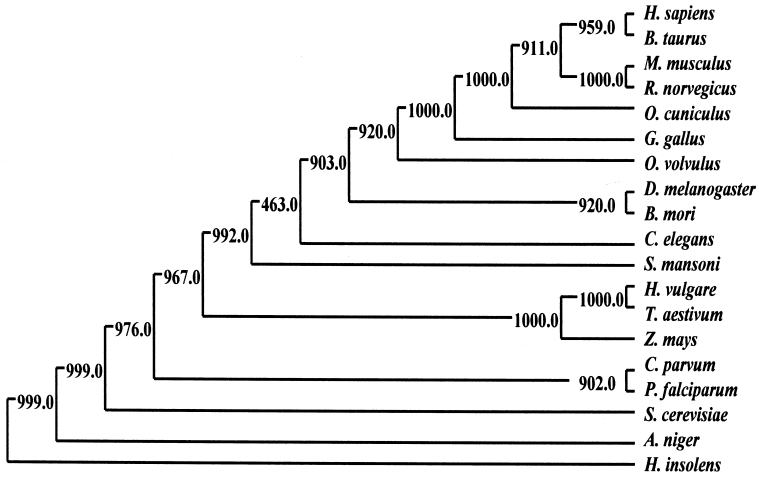


Fig. 3. Fig. 3. Phylogenetic status of bPDI. The evolutional tree was obtained by the neighbor-joining method based on the amino acid alignments. The numbers on each branch indicate the percentage of the most parsimonious trees which were found in 1000 bootstrap replications performed with PAUP.

PDI evolution, separating from nematoda, *C. elegans* PDI (Veijola *et al*., 1996). It may be suggested that the evolutional critical point of the PDIs occurred between metazoa (*S. mansoni*) and nematoda (*C. elegans)* since a rapid genetic distance value of 464 appears, which is the lowest among the PDIs.

In summary, bPDI isolated from a silkworm, *Bombyx mori,* shared an amino acid sequence homology of less than 55% with the reported PDIs. However the protein that was overexpressed by the baculovirus was recognized with anti-rat PDI antibodies. Although bPDI shows a comparatively low amino acid homology, in which the two redox

active sites (WCGHCK) are completely conserved, it was recognized by the anti-rat PDI antibodies. bPDI is genetically most related to *D. melanogaster* PDI and the critical point in PDIs evolution processing occurred between metazoa and nematoda when the lowest genetic distance value of 464 appeared among the PDIs. Further understanding of the phylogenetic relationship of bPDI among the known PDIs should provide important knowledge of the production of heterologous proteins in eukaryotic environments, in which frequently heterologous proteins form insoluble aggregates or are improperly folded in the ER.

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