Biological Journal of the Linnean Society (1997), *62*: 581–592. With 3 figures

Cloning of Hsp70 genes from the marine sponges *Sycon raphanus* (Calcarea) and *Rhabdocalyptus dawsoni* (Hexactinellida). An approach to solve the phylogeny of sponges

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Received 1 July 1997; accepted for publication 12 August 1997

The phylogenetic relationships among the three classes of the Porifera—Demospongiae, Calcarea and Hexactinellida—are still unresolved, despite the use of molecular analyses of rRNA. To determine whether phylogenetic resolution of these classes is possible based on genes coding for specific proteins, in the present study the genes for the 70 kDa heat shock protein [Hsp70] were isolated from *Rhabdocalyptus dawsoni* [Hexactinellida] and from *Sycon raphanus* [Calcarea], and compared to that previously isolated from the demosponge *Geodia cydonium*. The gene from *R. dawsoni* is 2021 bp long and encodes a predicted Hsp70 of M_r 77,697; the protein comprises the characteristic sites of eukaryotic, cytoplasmic Hsp70 polypeptides. The Hsp70 isolated from cDNA from *S. raphanus* is 2326 bp long. It encodes a potential polypeptide of M_r 85,927 and belongs to the same class of Hsp70s. All three sponge sequences for Hsp70 were found to be highly identical to both human and plant Hsp70s. The degree of identity at the amino acid (aa) level between the sponge sequences and the human sequence for Hsp70 is 77%–84% and at the nucleotide (nt) level, between 69% and 75%. Resolution of the phylogenetic relationship between the three classes of sponges based on the Hsp70 was not possible due to the high degree of identity [similarity] of their respective aa sequences, which ranged from 80% [90%] to 82% [91%]. The evolutionary rates—k_{aa}-values—calculated for the sponge Hsp70 molecules, are low, reflecting the strong functional contraints placed upon these polypeptides. These values range from 0.125×10^{-9} for *G. cydonium* and *R. dawsoni* to 0.087×10^{-9} for *S. raphanus*. Higher values have previously been reported for the *G. cydonium* galectin molecule [k_{aa}-value of 1.7×10^{-9}] and the receptor tyrosine kinase [1.24×10^{-9}] from the same animal. The occurrence of at least one double mutation, in the codon for the aa Ser in the conserved regions of the Hsp70 sequences, also suggests that these molecules are subjected to strong functional constraints.

ADDITIONAL KEY WORDS:—Porifera – heat shock protein – molecular systematics – evolutionary rate.

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0024–4066/97/120581 + 12 $25.00/0/bj970169

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INTRODUCTION

The Porifera (sponges) are animals that lack specialized organ systems. Fossil records suggest that they are the oldest multicellular animals on earth (Steiner *et al.*, 1993). However, the exact relationship of Porifera to other diploblastic animals is difficult to decipher based on morphological, biochemical, cytological and physiological evidence (reviewed in: Corliss, 1984; Margulis & Schwartz, 1995; Kumar & Rzhetsky, 1996). Attempts to use partial and whole rRNA nucleotide [nt] sequences to resolve these early relationships have been unsuccessful (Rodrigo *et al.*, 1994). Even by using an improved statistical analysis of the nt sequences from nuclear small-subunit rRNA it has not been possible to resolve the dichotomy of diploblasts and triploblasts, nor is the relationship among the diploblastic phyla any clearer (Kumar & Rzhetsky, 1996).

The first statistically significant evidence that the Porifera arose from an ancestor common to the other phyla of the Metazoa comes from analysis of genes coding for proteins which are important for multicellularity, such as adhesion molecules and receptors (Müller *et al.*, 1994a; Gamulin *et al.*, 1994, Müller, 1995, 1997). Sequences from cDNA coding for lectin (Pfeifer *et al.*, 1993), integrin (Pancer *et al.*, 1997) and receptor tyrosine kinase (Schäcke *et al.*, 1994 a–c) have all shown a striking identity to the corresponding sequences from higher metazoans.

The Porifera, which the above analysis identifies as the earliest group of metazoans, has been divided into two subphyla based on dramatic cytological differences (Reiswig & Mackie, 1983). All members of the class Hexactinellida have syncytial tissues and are now grouped in the subphylum Symplasma, whereas sponges in the other classes are cellular, and hence are placed in the subphylum Cellularia. Molecular phylogenetic analysis of partial sequences of rRNA, however, provides weak support for a grouping of the hexactinellida with the Demospongiae, and suggests that the Calcarea evolved separately (West & Powers, 1993; Cavalier-Smith *et al.*, 1996). Suggestion that hexactinellids and demosponges share larval similarities also support that hypothesis (Böger, 1988).

The aim of the present study is to analyse a gene encoding a housekeeping protein, the 70 kDa heat shock protein (Hsp70), in an attempt to resolve the differences between the Hexactinellida, the Demospongiae and the Calcarea. The

expression of genes that encode heat shock proteins rises greatly after a change of the ambient temperature. Hsp70, the most abundant Hsp, is localized in the cytoplasm and in the endoplasmic reticulum. These proteins share approximately 50% sequence identity to Hsp70 from prokaryotes, and to Hsp70 in organelles of prokaryotic origin (reviewed in Boorstein *et al.*, 1994).

MATERIAL AND METHODS

Materials

Restriction endonucleases and other enzymes for recombinant DNA techniques and vectors were obtained from Stratagene (Heidelberg; Germany), Boehringer Mannheim (Mannheim; Germany), Epicentre Technologies (Madison, WI; USA), and USB (Cleveland, OH; USA).

Sponges

Specimens of *Rhabdocalyptus dawsoni* (Lambe, 1892) (Porifera; Hexactinellida; Hexasterophora; Lyssacinosida; Rossellidae) were collected from Saanich Inlet and Barkley Sound, British Columbia (Canada) by scuba diving. The cleaned tissue was dissociated through 150 μm Nitex mesh and allowed to aggregate for 2 days. Aggregates were cleaned in millipore-filtered sea water, frozen in liquid nitrogen and stored at −80°C. *Sycon raphanus* (Schmidt, 1862) (Porifera, Calcarea, Calcarona, Leucosoleniida, Sycettidae) was collected near Rovinj (Croatia). The material was immediately frozen in liquid nitrogen until use.

Cloning of Hsp70 from R. dawsoni

The gene encoding Hsp70 was isolated using the polymerase chain reaction procedure (PCR) (Ausubel *et al.*, 1995) from genomic DNA from the hexactinellid sponge *R. dawsoni.*

Oligonucleotides were designed against the conserved regions present in the Hsp70 sequence previously prepared from the demosponge *Geodia cydonium* (Koziol *et al.*, 1996) as follows: (1) DHSP.F1 [forward-1; corresponding to aa 29–36 in the *G. cydonium* Hsp70 sequence (Koziol *et al.*, 1996)]: 5′-ATCGCCAAY-GACCARGGBAAC-3′ (where Y=C/T; R=A/G; B=C/G/T); (2) DHSP.F2 [forward-2; aa 52–59] 5′-ATYGGHGATGCBGCYAAGAAYCA-3′ (H=A/C/T); (3) DHSP.R3 [reverse-3; aa 441-448]: 5′-YTCKCCYTCGTASACCTGGA-3′ (K=G/T; S=C/G); and (4) DHSP.R4 [reverse-4; aa 366-373]: 5′-RT-AMGCVACAGCYTCRTCWG-3′ (R=A/G; M=A/C; V=A/C/G; W=A/T). These oligonucleotides were used as degenerate primers. PCR reaction mixtures of 50 μl included: 10 pmoles of each primer, 200 μM of each nt, 2.5 μl of *R. dawsoni* DNA and 2.5 units of *Taq* DNA polymerase (Boehringer Mannheim). PCR amplifications were run on a GeneAmp 9600 thermal cycler (Perkin Elmer) with the following cycling parameters: 3 min at 95°C, 35 cycles of 95°C for 45 sec, 58°C for

45 sec, 74°C for 3 min, and 74°C for 10 min. The amplification products (about 1–1.2 kb long) were cloned into pGEM-T (Promega) and sequenced.

To complete the gene 5′-RACE (Frohman, 1993) as well as 3′-RACE (Frohman *et al.*, 1988) were performed. For this procedure the kits '5′-' and '3′ RACE-systems for Rapid Amplification of cDNA Ends' (GIBCO-BRL, Eggenstein; Germany) were used. Five primers for the 5′ RACE within the nt sequence 647-665 [final; reverse], 611–630 [reverse], 440–458 [reverse], 407–425 [reverse] and 337–356 [reverse] as well as three primers for the 3′ RACE nt 1114–1132 [forward], 950–968 [forward] and 926-944 [forward] were used.

Cloning of Hsp70 from S. raphanus

A cDNA library was prepared from *S. raphanus* in lambda ZAP Express (Stratagene). cDNA-inserts were integrated in ZAP Express between the *Eco*RI and *Xho*I restriction sites and packaged *in vitro* using MaxPlax Packaging Extract (Epicentre Technologies). The resulting number of independent clones for *S. raphanus* was 8.7×10^6.

The cDNA encoding sponge Hsp70 from *S. raphanus* was cloned by PCR from the *S. raphanus* cDNA library using the degenerate reverse primer 5′-CCTGCRT-CYWTIGTDGCTTG-3′ (where I = inosine; Y = C/T; R = A/G; W = A/T and D = A/G/T) in conjunction with the ZAPII 5′-end vector-specific primer T3. The PCR reaction mixtures of 50 µl included 10 pmoles of the degenerate primer and 5 pmoles of the primer T3 (Stratagene), 200 µM of each nt, 1 µl of the cDNA library (approximately 10^9 pfu) and 2.5 units of *Taq* DNA polymerase (Boehringer Mannheim). PCR amplifications were run with the following cycling parameters: an initial denaturation of 3 min at 95°C, then 35 cycles of 95°C for 45 sec, 60°c for 1 min, 74°C for 1 min, and a final extension step at 74°C for 10 min. after electrophoresis one band of 0.2 kb was observed in the ethidium bromide-stained gel. Amplification products were purified through a QIAquick Spin column (QIAGEN), cloned in bulk into pGEM-T (Promega). The sponge cDNA coding for Hsp70 from *S. raphanus* homologue was designated *HSP70*-SYCON. Primers corresponding to *HSP70*-SYCON were designed to complete both termini of the cDNA, by using the T3- and T7-vector primers.

Sequence analysis

Prediction of sites and signatures was performed with programs available in PC/GENE (1995). Homology searches were done via the E-mail servers at the European Bioinformatics Institute, Hinxton Hall, UK (blitz@ebi.ac.uk and fasta@ebi.ac.uk) and the National Centre for Biotechnology Information, National Institutes of Health, MD, USA (blast@ncbi.nlm.nih.gov). Phylogenetic trees were constructed on the basis of aa sequence alignment by neighbour-joining, applying the 'Neighbor' program from the PHYLIP package (Saitou & Nei, 1987; Felsenstein, 1993). The degree of support for internal branches was further assessed by bootstrapping (Felsenstein, 1993). The distance matrix was calculated as described (Dayhoff *et al.*, 1978). The graphical output of the bootstrap figure was produced by the program

Treeview (Roderic D.M. Page, University of Glasgow UK; http://taxonomy.zoology.gla.ac.uk/software.html#Treeviewing). Multiple alignment—the default options were used—was performed with CLUSTAL W version 1.6 (Thompson *et al.*, 1994), and the graphic presentation was composed with GeneDoc (Nicholas & Nicholas, 1996).

RESULTS AND DISCUSSION

Cloning of the gene encoding the Hsp70 from R. dawsoni

The gene encoding the Hsp70 from the hexactinellid sponge *R. dawsoni* was isolated from genomic DNA using PCR and degenerate primers. The clone, designated *HSP70-RHABDO*, is 2021 bp long and has an open reading frame (ORF) of 1974 bp (Fig. 1). The complete nt sequence is deposited in the EMBL data base under Y13926. The sequence from *R. dawsoni* has no intron, indicating that it belongs to the heat-inducible members of the Hsp70 family and not to the constitutively expressed heat shock cognate protein 70 (Günther & Walter, 1994).

The predicted translation product of 658 aa, named HSP70_RD (Figs 1, 2), has a mass of 77,697 and an isoelectric point [pl] of 5.5. The following sites on HSP70_RD are characteristic for Hsp70 polypeptides (PC/GENE, 1995): the Hsp70 family signature-1 IDLGTTYS (aa 6–13) and -2 DLGGGTFD (aa 196–203), as well as the two potential 'bipartite nuclear targeting sequences' KRKHKKDLTTNKRALRR (aa 243–259) and KRALRRLRTACERAKRT (aa 254–270). The ATP/GTP-binding site motif A (P-loop), AEAYLGKT, is located at aa 128–135. The eukaryotic Hsp70 signature R-A-R/K-F-E-E/D-L/M (Rensing & Maier, 1994) is also present in this sponge sequence (aa 296–302).

Cloding of the cDNA encoding the Hsp70 from S. raphanus

The Hsp70 from *S. raphanus, HSP70*-SYCON, isolated from a cDNA library, is 2326 bp long and has an ORF of 1968 bp. The complete nt sequence is deposited in the EMBL data based under Y15109. Whereas in *R. dawsoni* the stop codon reads UGA, in both *S. raphanus* and *G. cydonium* it reads UAG (data not shown).

The predicted polypeptide of 656 aa, named HSP70_SR (Fig. 2), has a M_r of 85,927 and a pl of 5.6, as well as the characteristic sites; the Hsp70 family signature-1 [IDLGTTYS] (aa 6–13) and -2 [DLGGGTFD] (aa 196–203), the 'bipartite nuclear targeting sequences' at aa 243–259 and aa 254–270 and the eukaryotic Hsp70 signature (aa 301–307). the putative ATP/GTP-binding site motif A, is found between aa 128 and aa135.

Conservation of the Hsp70 polypeptides

For phylogenetic analysis Hsp70 sequences were truncated due to their different chain lengths. The new Hsp70 sequences of *S. raphanus* (selected sequence aa 3–508) and of *R. dawsoni* (aa 3–508) were found to be highly similar to the previously

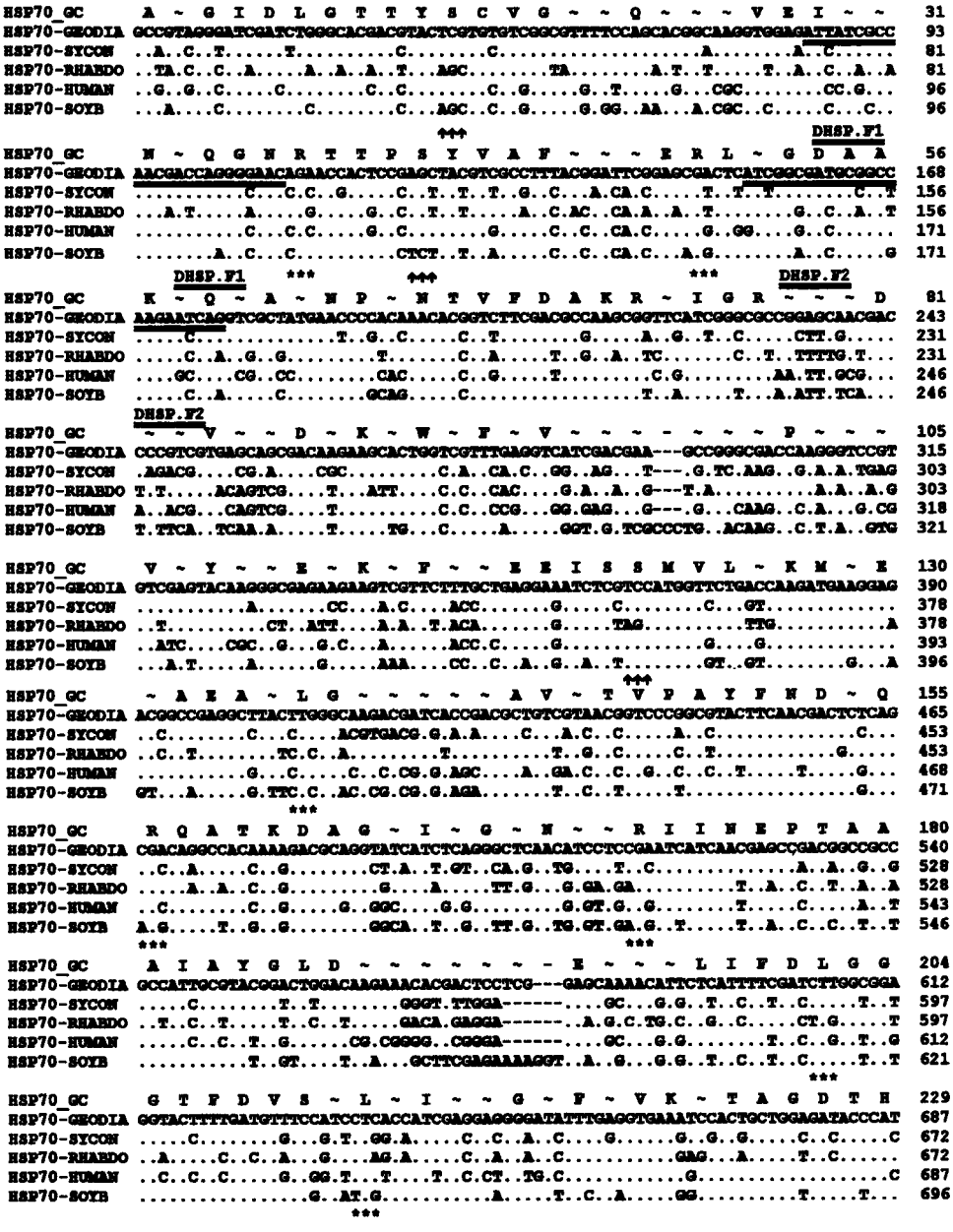

Figure 1. Alignment of the nt sequences of the Hsp70 from the demosponge *G. cydonium* (HSP70-GEODIA), the calcareous sponge *S. raphanus* (HSP70-SYCON), and the hexactinellid sponge *R. dawsoni* (HSP70-RHABDO), with the Hsp70s from human (HSP70-HUMAN, P17066; Leung *et al.*, 1990) and soy bean *Glycine max* (HSP70-SOYB, X62799; Roberts & Key, 1991). Identical nts [.] as well as gaps [-] are indicated. Above the nt sequences the deduced aa sequence of the *G. cydonium* Hsp70 is given; the highly conserved aa in the *G. cydonium* sequence which are present also in the four further Hsp70s are given in the single letter code; the variable aa are marked [~]. The positions of nt triplets encoding a given aa with more than one nt difference to the *G. cydonium* sequence, are specialy marked [*** or ↑↑↑]; in three positions the codons for the aa Ser belong to two codon families [↑↑↑]. The locations of the two first primers—DHSP.F1 and -F2- are double underlined. For the alignment shown here the sequences were truncated at the 5′-terminus [starting with aa 6, with respect to the *G. cydonium* squence] and at the 3′-terminus [aa 513]. The numbers indicate the nt position of the sequences beginning with the start ATG triplet of the ORF.

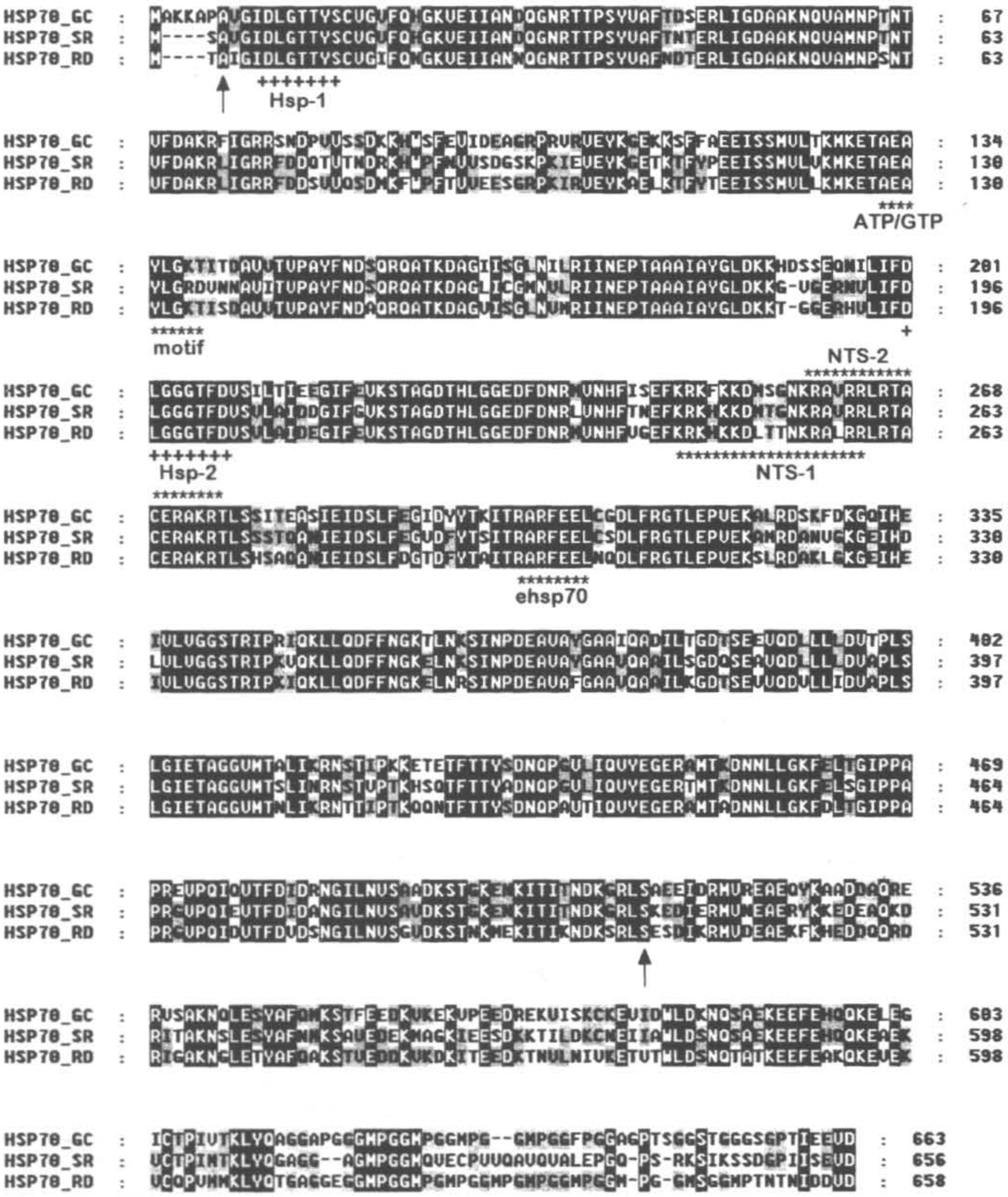

Figure 2. Alignment of the deduced aa sequences of the Hsp70 from *G. cydonium* (HSP70_GC, accession number X94985; Koziol *et al.*, 1996), *S. raphanus* (HSP70_SR) and *R. dawsoni* (HSP70_RD). Identical aa residues in all sequences are shown in inverted type, while residues conserved in two of the sequences are shaded. The sites characteristic for metazoan Hsp70s, the heat shock HSP70 protein family signature 1 and 2 [Hsp-1 and -2 (+ + + + + +)], the ATP/GTP-binding site motif, nuclear targeting sequences [NTS-1 and -2] and the eukaryotic Hsp70 [ehsp70] signature are marked (*******). The arrows delimit the part of the sequences used for the tree construction in Fig. 3.

reported sponge sequence from *G. cydonium* (Koziol *et al.*, 1996; aa 7–513) as well as to other metazoan Hsp70s, e.g. human (accession no. P17066, Leung *et al.*, 1990; aa 8–513), and plant Hsp70s, e.g. the soybean *Glycine max* (P26413, Roberts & Key, 1991; aa 8–516). The percentage of identical [similar] aa to the human sequence was as follows: *S. raphanus* 84% [93%], *R. dawsoni* 78% [89%], *G. cydonium* 78% [90%], and for the soybean *G. max.*, 77% [87%]. Among the sponges alone, the percentage of identical [similar] aa ranges from 80% [90%] to 82% [91%].

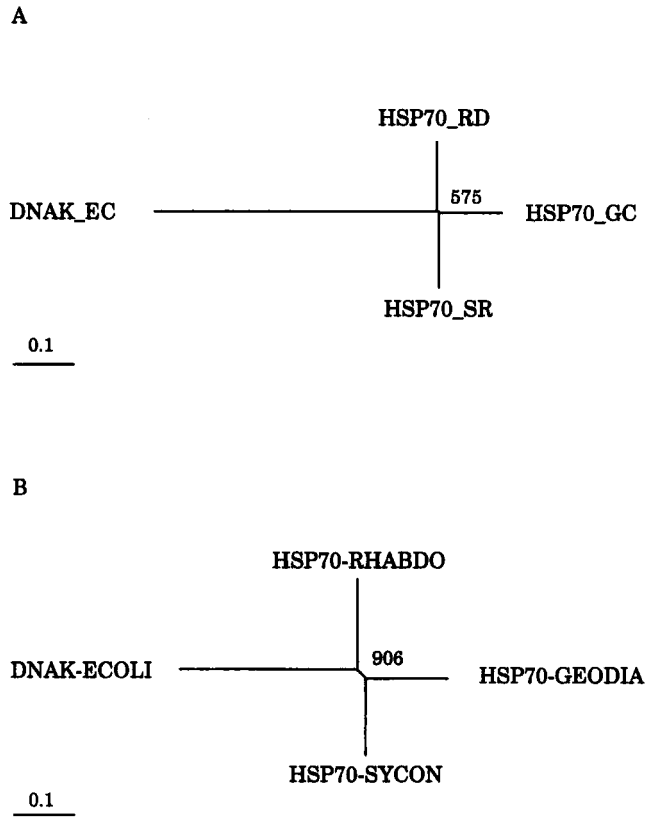

Figure 3. Phylogenetic tree using Hsp70 from the sponge sequences and the bacterial DNAK molecule inferred by neighbour-joining as described in Material and Methods. The numbers at the nodes refer to the level of confidence as determined by bootstrap analysis [1000 bootstrap replicates]. Scale bar indicates an evolutionary distance of 0.1 aa (in A) or 0.1 nt (in B) substitutions per position in the sequence. A comparison of the truncated (see text) aa sequences from the sponges *G. cydonium* (HSP70_GC]. *S. raphanus* [HSP70_SR], and *R. dawsoni* [HSP70_RD], and the bacterium *E. coli* [DNAK_EC]. B, tree calculated by comparing the corresponding truncated sponge nt sequences from *G. cydonium* [HSP70-Geodia], *S. raphanus* [HSP70-Sycon], and *R. dawsoni* [HSP70-Rhabdo] as well as from *E. coli* [DNAK-ECOLI].

A high level of identity to the human sequence was also found with the corresponding truncated nt sequences: *S. raphanus* 75%, *R. dawsoni* 69%, *G. cydonium* 73%, and *G. max* 70%. Among the sponges the corresponding values are between 71% and 73%.

For the construction of a phylogenetic tree the three sponge Hsp70 sequences were compared with the Hsp70 sequence from the bacterium *Escherichia coli* (DNAK_ECOLI, Ohki *et al.*, 1986). At the aa level the bacterial Hsp70 (accession no. P04475) showed 50–52% identity and 67–69% similarity to the sponge sequences, while at the nt level the *E. coli* sequence (D10765) showed 51–52% identity to the sponge sequences. The unrooted trees, from comparisons of both aa (Fig. 3A) and nt (Fig. 3B) sequences, reflect that the sponge Hsp70 molecules are closely related. The branches with the sponge sequences show that the hexactinellid sponge is somewhat closer to *E. coli*; however, this suggestion is not statistically significant and must be

further proven by analysis of nt and aa sequences of proteins which display a lower degree of identity.

Evolutionary rates in sponges

The evolutionary rates—expressed as k_{aa}-values—vary between different proteins (Zuckerkandl & Pauling, 1965; Kimura, 1983; Li *et al.*, 1987). As shown by Kimura (1983), k_{aa}-values vary from 8.3×10^{-9} [fibrinopeptides] to 0.01×10^{-9} [histon H4], with an average k_{aa}-value of 1×10^{-9}, meaning 1 aa substitution per site on 10^9 years. In a previous study it was calculated that the galectin protein from the sponge *G. cydonium* (Pfeifer *et al.*, 1993) has a k_{aa}-value of 1.7×10^{-9} (Hirabayashi & Kasai, 1993). A k_{aa}-value of 1.24×10^{-9} was calculated for the receptor tyrosine kinase (Schäcke *et al.*, 1994d) from the same animal.

The Hsp70s from the three sponges have comparably low evolutionary rates with k_{aa}-values between 0.125×10^{-9} for *G. cydonium* and *R. dawsoni* and 0.087×10^{-9} for *S. raphanus* [the calculation has been performed according to Kimura (1983)]. The slow rates of evolution of Hsp70 molecules reflect the strong functional constraints placed upon these polypeptides.

This conclusion is supported by the observation that within the conserved domains in the Hsp70 molecules double mutations have occurred without changing the aa. Evidence for this process can be clearly seen in the aa for Ser (Fig. 1). Of the 11 Ser residues present in each of the conserved domains, six are coded by codons either belonging to the four-codon family TCY or to the two-codon family AGN, while the remaining five Ser residues are encoded by one codon family only. A switch between these two codon families by single consecutive and silent mutations is not possible. The Ser residues at positions 17 and 40—with respect to the sequence from *G. cydonium*—are conserved in all sequences belonging to the Hsp70 multigene family (Boorstein *et al.*, 1994), while the others at positions 86, 364, 402 and 529 are restricted to the eukaryotic cytoplasmic Hsp70 only. Hence we can conclude that the mutations at Ser positions 17 and 40 are the result of double mutations, while the other four positions coding for Ser might have changed the codon families by single mutations under a transitional change of the aa. For the sponge sequences alone, the codons of the Ser residue at positions 17 and 86 in *R. dawsoni* are only from the two-codon family, while in *G. cydonium* the codons for Ser at 364 and 519 are only from the four-codon family. It is interesting to note that in the deduced aa sequence of the polyubiquitin gene of *G. cydonium* (Müller *et al.*, 1994b), three Ser residues at position of 65 within the monoubiquitin segments are also coded by codons from the four-codon family, while the other three Ser codons are from the two-codon family.

Two other aa, Arg and Leu, that are encoded by codons belonging to a four-codon and a two-codon family, are present in the conserved domains. Positions 13, arg, and 12, Leu, are used by codons belonging to different families (Fig. 1). However, in this case transitions between the two codon families can be explained by single consecutive and silent mutations without changing the aa.

CONCLUSION

The Hsp70 polypeptides determined from gene/cDNA isolated from sponges from the classes Hexactinellida, Calcarea and Demospongiae, belong to the group

of cytoplasmic Hsp70 due to their characteristic C-termini (V/I)(E/S/D)(E/D)VD (Boorstein *et al.*, 1994). The sequences are highly identical both at the aa [80–82%] and at the nt [71–73%] level. The strong functional constraints which are put upon these polypeptides, which results in such high conservation, can also be deducted from the presence of double mutations which have occurred at the codons for the aa Ser. Alignment of the sponge sequences with the sequence from bacterium *E. coli*, which is only distantly related to the Hsp70 (reviewed in Georgopoulos *et al.*, 1994), shows that the sponge sequences cluster together. This analysis has shown that comparison of genes coding for proteins which are highly conserved cannot resolve the relationships between the classes of the Porifera, the earliest metazoan phylum. For this purpose it will be necessary to examine proteins, such as the protein kinase C, which have been demonstrated to have a higher rate of evolutionary change.

ACKNOWLEDGEMENTS

The sequences reported here are deposited in the EMBL data base: *Rhabdocalyptus dawsoni* Hsp70 [*RDHSP70*] Y10529 and *Sycon raphanus* Hsp70 [*SRHSP70*] Y10530.

Supported by a grant from the Deutsche Forschungsgemeinschaft [Mü 348/14-1], and the International Human Frontier Science Program [RG-333/96-M].

REFERENCES

Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seidmann JG, Struhl K. 1995. *Current Protocols in Molecular Biology*. New York: John Wiley.

Blake MS, Johnston KH, Russel-Jones GJ, Gotschlich EC. 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated antibody on Western blots. *Analytical Biochemistry* **136:** 175–179.

Böger H. 1988. Versuch über das phylogenetische System der Porifera. *Meyniana* **40**, 143–154.

Boorstein WR, Ziegelhoffer T, Craig EA. 1994. Molecular evolution of the HSP70 multigene family. *Journal of Molecular Evolution* **38:** 1–17.

Cavalier-Smith T, Allsopp MTEP, Chao Ee, Boury-Esnault N, Vacelet J. 1996. Sponge phylogeny, animal monophyly, and the origin of the nervous system: 18S rRNA evidence. *Canadian Journal of Zoology* **74:** 2031–2045.

Corliss CO. 1984. The kingdom Protista and its 45 phyla. *Biosystems* **17:** 87–126.

Dayhoff MO, Schwartz RM, Orcutt BC. 1978. A model of evolutionary change in protein. In: Dayhoff MO ed. *Atlas of Protein Sequence and Structure* Vol. 5 suppl. 3. Washington, DC., Nat. Biomed. Res. Foundation, 345–352.

Felsenstein J. 1993. *PHYLIP, ver. 3.5*. University of Washington, Seattle.

Frohman MA, Dush M, Martin GR. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proceedings of the National Academy of Sciences USA* **85:** 8998–9002.

Frohman MA. 1993. Rapid amplification of complementary DNA ends for generation of full length complementary DNAs: Thermal RACE. *Methods in Enzymology* **218:** 340–456.

Gamulin V, Rinkevich B, Schäcke H, Kruse M, Müller IM, Müller WEG. 1994. Cell adhesion receptors and nuclear receptors are highly conserved from the lowest Metazoa [marine sponges] to vertebrates. *Biological Chemistry* **375:** 583–588.

Georgopoulos C, Liberek K, Zylicz M, Ang D. 1994. Properties of the heat shock proteins of *Escherichia coli* and the autoregulation of the heat shock response. In: Morimoto RI, Tissères A, Georgopoulos. C, eds. *The Biology of Heat Shock Proteins and Molecular Chaperones* Cold Spring Harbor: Spring Harbor Laboratory Press, 209–249.

Günther E, Walter L. 1994. Genetic aspects of the hsp70 multigene family in vertebrates. *Experientia* **50:** 987–995.

Hirabayashi J, Kasai K. 1993. The family of metazoan metal-independent β-galactoside-binding lectins: structure, function and molecular evolution. *Glycobiology* **3:** 297–304.

Kimura M. 1983. *The Neutral Theory of Molecular Evolution.* Cambridge: Cambridge University Press.

Knoll AH. 1994. Proterozoic and Early Cambrian protists: Evidence for accelerating evolutionary tempo. *Proceedings of the National Academy of Sciences USA* **91:** 6743–6750.

Koziol C, Wagner-Hülsmann C, Cetkovic H, Gamulin V, Kruse M, Pancer Z, Schäcke H, Müller WEG. 1996. Cloning of the heat-inducible biomarker, the cDNA encoding the 70-kDa heat shock protein, from the marine sponge *Geodia cydonium:* Response to natural stressors. *Marine Ecology Progress Series* **136:** 153–161.

Kumar S, Rzhetsky A. 1996. Evolutionary relationships of eukaryotic kingdoms. *Journal of Molecular Evolution* **42:** 183–193.

Leung TKC, Rajendran MY, Monfries C, Hall C, Lim L. 1990. The human heat-shock protein family: Expression of a novel heat-inducible HSP70 (HSP70B') and isolation of its cDNA and genomic DNA. *Biochemical Journal* **267:** 125–132.

Li WH, Tanimura M, Sharp PM. 1987. An evaluation of the molecular clock hypothesis using mammalian DNA sequences. *Journal of Molecular Evolution* **25:** 330–342.

Lindquist S, Craig EA. 1988. The heat-shock proteins. *Annual Review of Genetics* **22:** 631–677.

Margulis L, Schwartz KV. 1995. *Five kingdoms.* New York: Freeman.

Müller WEG, Müller IM, Gamulin V. 1994a. On the monophyletic evolution of the Metazoa. *Brazilian Journal of Medical and Biological Research* **27:** 2083–2096.

Müller WEG, Schröder HC, Müller IM, Gamulin V. 1994b. Phylogenetic relationship of ubiquitin repeats of the polyubiquitin gene from the marine sponge *Geodia cydonium. Journal of Molecular Evolution* **39:** 369–377.

Müller WEG. 1995. Molecular phylogeny of metazoa [animals]: monophyletic origin. *Naturwissenschaften* **82:** 321–329.

Müller WEG. 1997. Molecular phylogeny of Eumetazoa: experimental evidence for monophyly of animals based on genes in sponges [Porifera]. *Progress in Molecular Subcellular Biology* **19:** 98–132.

Nicholas KB, Nicholas HB Jr. 1996. *Genedoc version 1.1.004.*

Ohki M, Tamura F, Nishimura S, Uchida H. 1986. Nt sequence of the *Escherichia coli* DnaK gene and purification of the gene product. *Journal of Biological Chemistry* **261:** 1778–1781.

Pancer Z, Kruse M, Müller I, Müller WEG. 1997. On the origin of adhesion receptors of metazoa: cloning of the integrin α subunit cDNA from the sponge *Geodia cydonium. Molecular Biology and Evolution* **14:** 391–398.

PC/GENE 1995. *Data Banks CD-ROM; Release 14.0.* Mountain View, CA: IntelliGenetics, Inc.

Pfeifer K, Haasemann M, Gamulin V, Bretting H, Fahrenholz F, Müller WEG. 1993. S-type lectins occur also in invertebrates: high conservation of the carbohydrate recognition domain in the lectin genes from the marine sponge *Geodia cydonium. Glycobiology* **3:** 179–184.

Reiswig HM, Mackie GO. 1983. Studies on hexactinellid sponges. III The taxonomic status of the Hexactinellida within the Porifera. *Philosophical Transactions of the Royal Society, London* **B 301:** 419–428.

Rensing SA, Maier UG. 1994. Phylogenetic analysis of the stress-70 protein family. *Journal of Molecular Evolution* **39:** 80–86.

Roberts JK, Key JL. 1991. Isolation and characterisation of a soybean HSP70 gene *Plant Molecular Biology* **16:** 671–683.

Rodrigo AG, Bergquist PR, Bergquist PL, Reeves RA. 1994. 1994. Are sponges animals? An investigation into the vagaries of phylogenetic interference. In: van Soest RWM, van Kempen TMG, Braekman JC, eds. *Sponges in Time and Space.* Rotterdam: Balkema Press, 47–54.

Saitou N, Nei M. 1987. The neighbor-joining methods: a new method for constructing phylogenetic trees. *Molecular Biology and Evolution* **4:** 406–425.

Schäcke H, Müller WEG, Gamulin V, Rinkevich B. 1994a. The Ig superfamily includes members from the lowest invertebrates to the highest vertebrates. *Immunology Today* **15:** 497–498.

Schäcke H, Rinkevich B, Gamulin V, Müller IM, Müller WEG. 1994b. Immunoglobulin-like domain is present in the extracellular part of the receptor tyrosine kinase from the marine sponge *Geodia cydonium. Journal of Molecular Recognition* **7:** 272–276.

Schäcke H, Schröder HC, Gamulin V, Rinkevich B, Müller IM, Müller WEG. 1994c. Molecular cloning of a receptor tyrosine kinase from the marine sponge *Geodia cydonium:* a new member of the receptor tyrosine kinase class II family in invertebrates. *Molecular Membrane Biology* **11:** 101–107.

Schäcke H, Müller IM, Müller WEG. 1994d. Tyrosine kinase from the marine sponge *Geodia cydonium:* The oldest member belonging to the receptor tyrosine kinase class II family. In: Müller, WEG ed. *Use of Aquatic Invertebrates as Tools for Monitoring of Environomental Hazards.* Stuttgart, New York: Gustav Fischer, 201–211.

Steiner M. Mehl D, Reitner J, Erdtmann BD. 1993. Oldest entirely preserved sponges and other fossils from the Lowermost Cambrian and a new facies reconstruction of the Yangtze Platform (China). *Berliner Geowissenschaftliche Abhandlungen (E)* **9:** 293–329.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673–4680.

Welch WJ. 1992. Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiological Reviews* **72:** 1063–1081.

West L, Powers D. 1993. Molecular phylogenetic position of hexactinellid sponge in relation to the Protista and Demospongiae. *Molecular Marine Biology & Biotechnology* **2:** 71–75.

Willmer P. 1994. *Invertebrate Relationships: Pattern in Animal Evolution.* Cambridge: Cambridge University Press.

Zuckerkandl E, Pauling L. 1965. Evolutionary divergence and convergence in proteins. In: Bryson V, Vogel HJ, eds. *Evolving Genes and Proteins.* New York: Academic Press, 97–166.