Positive natural selection has driven the evolution of the Hsp70s in *Diguetia* spiders

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Hsp70s are a ubiquitous family of highly conserved proteins. Hsp70s are chaperones and have important roles in both protein folding and thermotolerance. It has been widely assumed that Hsp70 sequence evolution is governed by the strong functional constraints imposed by its crucial cellular functions. In this study of cytosolic heat-inducible Hsp70s from three spider families, we have found clear evidence of positive natural selection altering Hsp70s in desert-dwelling and heat-loving Diguettidae spiders. These spiders are a small family restricted to deserts. They display heat-tolerant behaviours not seen in their closest relatives, the Pholcidae and Plectreuridae.

Keywords: Hsp70; positive selection; molecular evolution

1. INTRODUCTION

Temperature stress challenges most organisms. Thermotolerance is often a crucial factor in determining species ranges and thus can strongly influence biogeography (Somero 2005). It is well established that high temperatures can induce the heat-shock response leading to the production of heat shock proteins (Hsp) that confer thermotolerance. To date, much of the research on adaptation to extreme temperatures has focused on differences in Hsp gene expression and in Hsp protein levels (Garbuz *et al.* 2003; Lerman & Feder 2004). However, there are clear limits on this type of adaptation including the detrimental effects of Hsp70 overexpression (Krebs & Feder 1997).

The Hsp70s are among the most highly conserved proteins known with little variation across highly diverged organisms and to date, there have been no reports of positive selection among any Hsp70s. Studies of *Drosophila* Hsp70s reveal that they are essentially invariant (98–100% identical within and between species; Bettencourt & Feder 2002). This lack of variation has been attributed to the crucial cellular functions of Hsp70s and the evolutionary constraints that these functions have imposed. Hsp70s are molecular chaperones that assist in the folding of other proteins and prevent the misfolding and aggregation of other proteins (Hartl & Hayer-Hartl 2002). Constitutively

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expressed Hsp70s have crucial roles during protein translation, translocation and folding in the unstressed cell. Heat-induced Hsp70s confer thermal tolerance during high-temperature stress (Mayer & Bukau 2005).

The little-studied spider family, Diguettidae, consists of several species whose main habitats are the Sonoran and Mojave deserts (Gertsch 1958; Boulton & Polis 1999). The closest relatives of the Diguettidae are the Pholcidae and Plectreuridae. The plectreurids are a small family of two genera found in the southwestern US. The pholcids (the best-known pholcid is the daddy-long-legs spider, *Phocis phalangioides*) are a very large family of spiders with a worldwide distribution. The distributions of all three families overlap in the Mojave and Sonoran deserts. This region is extremely hot and dry. The plectreurids and pholcids are heat avoiding (i.e. nocturnal and shelter seeking; Gertsch 1958; Coddington & Levi 1991; Platnick *et al.* 1991). However, the diguetids are not known to be heat avoiding (Nuessly & Goeden 1984). In fact, individuals of *Diguetia imperiosa* were observed capturing prey in aerial webs at midday when temperatures reached 47°C (Bentzen 1973). In addition, *Diguetia mojavea* silk retreats are predominately located on the southern sun-exposed sides of plants. Day and night temperatures in these deserts vary greatly. During the summer months, highs can reach 50°C while lows at night can be 20°C. Nocturnal species such as the pholcids and plectreurids rarely, if ever, experience the high temperatures that the diguetids experience every day. Thus, in comparison to their close relatives, diguetid spiders do not avoid long periods of exposure to intense heat. Based on this knowledge and the knowledge that Hsp70s can be crucial for thermotolerance, we hypothesized that diguetids may possess divergent Hsp70s.

2. MATERIAL AND METHODS

More detailed descriptions of the methods are provided in the electronic supplementary material.

(a) DNA extraction, PCR and DNA sequencing

Species from each family (Pholcidae, Plectreuridae and Diguettidae) were collected (figure 1a). Genomic DNA was extracted from single spiders using Qiagen DNeasy kits. Approximately 900 bp of the mitochondrial gene cytochrome oxidase I (*COI*) were amplified using *ExTaq* (Takara, Madison, WI). Cytosolic Hsp70 genes were obtained using the EasyA high-fidelity enzyme (Stratagene, La Jolla, CA) using degenerate primer pair sets located in conserved areas. PCR products were purified and then sequenced.

(b) Phylogenetic and evolutionary analyses

Phylogenetic trees were generated using MRBAYES v. 3.1 (Ronquist & Huelsenbeck 2003). In each analysis, parameters for each partition were set as unlinked and four chains were run simultaneously for two to three million generations with trees saved every 100 generations. The initial 40% of trees were excluded as 'burnin'. Remaining trees were used to compute the consensus tree and sum the parameters.

Using the codeml module of the PAML package (Yang 1997), we analysed three types of selection model: branch models, site models and branch-site models (Anisimova *et al.* 2002; Yang & Nielsen 2002; Yang *et al.* 2005). Likelihood ratio tests (LRTs) were used to determine the best-fit model.

The rate shift analysis program was used (Knudsen & Miyamoto 2001) to examine the rates and properties of functional divergence among Hsp70s. The program GENECONV v. 1.81 (Sawyer 1999) was used to evaluate gene conversion among the spider Hsp70 genes and none was found.

3. RESULTS

Based on our analysis of *COI* sequence data (figure 1b), it is clear that the pholcids, plectreurids and diguetids are each monophyletic and together they form a

Figure 1. Collection locations and phylogenetic relationships. (a) Collection locations. Circles indicate diguetid sites, boxes indicate plectreuids and triangles indicate pholcids. (b) Phylogenetic tree based on *COI* sequence data. Spider species from four different families were used as outgroups. Posterior probability for each node is located above or directly next to that node. Scale bar reflects 0.10 substitutions per site.

monophyletic group. Phylogenetic analysis of spider Hsp70 amino acids sequences with other known Hsp70s clearly shows that we have identified members of the cytosolic-inducible Hsp70 family (figure 2a). We confirmed using RT-PCR that the spider Hsp70 genes are induced by heat and are not constitutively expressed (electronic supplementary material).

In our analysis of Hsp70s, we found that within species and spider families, all ω values (the ratio of dN or non-synonymous to dS or synonymous substitutions) are below 0.10, consistent with strong purifying selection to maintain their function. However, within the diguetids, ω values are double (0.097 compared with 0.040) those found in the other two spider families. Analysis of branch-based models revealed that the ω value for the diguetid branch is

significantly different from those for the pholcid and plectreuid branches (table 1a; figure 2b). We also tested and rejected the hypothesis that the ω values are significantly different between the pholcids and plectreuids. Therefore, while purifying selection is acting on the Hsp70s within each lineage, the diguetid Hsp70s have a higher rate of evolutionary change.

Given these results, we then asked if there were specific codons under positive selection among these spiders. We first examined the hypothesis that natural selection is acting on specific codons across all the spiders studied by testing three alternative models (table 1a, M0 versus M3; M1a versus M2a; M7 versus M8). In each case, we rejected the hypothesis that there are codons under positive selection across the entire spider Hsp70 phylogeny.

Figure 2. Evolutionary relationships of Hsp70s. Posterior probabilities are located above or near each node. Owing to space limitations, some values are abbreviated: 1, 1.00 and 0.5, 0.50. (a) Phylogenetic analysis of Hsp70 amino acid sequences. Mitochondria-localized Hsp70s from *Drosophila melanogaster* and *Caenorhabditis elegans* are outgroups. An arrow indicates the monophyletic group of heat-inducible spider Hsp70s. (b) Phylogenetic analysis of Hsp70 DNA sequences. Branches for each of the spider families are labelled: a, pholcids; b, plectreurids; c, diguetids. Scale bar reflects 0.10 substitutions per site.

Next we considered the possibility that positive selection has occurred in only one spider lineage by performing branch-site analyses. We found strong support for the hypothesis that positive selection has

occurred only within the diguetid lineage (table 1a; figure 2b). Branch-site analyses identified nine codons under positive selection (table 1b). Two of these sites are in the ATPase domain, three sites

Table 1. Likelihood ratio test statistics and positively selected amino acid residues *Diguertia* heat-inducible Hsp70s.

(a) likelihood ratio test statistics				
		likelihood ratio	d.f.	p value
branch only	one ω value versus two ω values $\omega_c \neq \omega_o$	5.686	1	0.017
	two ω values versus three ω values $\omega_c \neq \omega_b \neq \omega_o$	0.159	1	0.690
sites only	one rate class versus three rate classes M0 versus M3 (K=3)	1029.989	4	<0.001
	no positive selection sites versus positive selection M1a versus M2a	0.00	2	1.00
	beta distribution versus beta with positive selection M7 versus M8	2.910	2	0.233
branch-site	no positive selection versus positive selection only on <i>Diguertia</i> branch			
	M1a versus MA (modified)	25.387	2	<0.001
	MA (fixed) versus MA (modified)	12.122	1	<0.001
	M3 (K=2) versus MB	23.655	2	<0.001
(b) positively selected sites				
model		positively selected sites		
MA (fixed)		90 Q, 289 S, 602 L, 620 M, 630 A		
MA (modified)		90 Q, 289 S, 602 L, 620 M, 630 A		
MB		90 Q, 289 S, 414 R, 571 Q, 602 L, 620 M, 628 Q, 629 Y, 630 A		

occur in the peptide-binding domain and the more variable C-terminal domain contains four sites. It can be difficult at times to distinguish between relaxed- and positive selection. However, it is highly unlikely that these Hsp70 genes are pseudogenes, as they contain no premature or internal stop codons, and have no deletions or insertions. Further evidence against continued relaxed selection among diguetid Hsp70s is the fact that these sites are not evolving rapidly within the diguetids.

The pattern found here is then one of the purifying selection within lineages and positive selection between the diguetids and the two other spider families. This is indicative of past episodes of adaptive evolution to drive Hsp70 evolution followed by selection to maintain these altered Hsp70 sequences.

Adaptive evolution in diguetid Hsp70s is further supported by the evidence of evolutionary rate shifts (table 2). Most of the sites inferred to be under positive selection in the diguetid branch are associated with conserved amino acids in diguetids that are radically different from conserved sites in the plectreurids and pholcids. Additionally, a number of amino acids conserved across the plectreurids and pholcids show increases in the rates of change across diguetid paralogues. Interestingly, many of the amino acid changes and rate shifts detected here occur in clusters.

4. DISCUSSION

We propose that early in the diversification of the *Diguertia*, these spiders became specialized to the desert habitat. During this time, positive natural selection acting on the Hsp70s drove amino acid changes in these proteins. The evidence for purifying selection within *Diguertia* indicates that this positive selection is not continuing but rather that once evolved, the 'better' combination of amino acid residues was then maintained via purifying selection.

Our findings raise the question of why positive natural selection has acted on diguetid spiders while there is no evidence of positive natural selection acting to alter Hsp70 sequences in other species with high thermotolerance. It is notable that while there are demonstrable differences among *Drosophila* species for thermotolerance, no evidence of positive natural selection has been reported. The heat-inducible cytosolic Hsp70s in *Drosophila* and other species (e.g. nematodes) are known to undergo gene conversion (Bettencourt & Feder 2002; Nikolaidis & Nei 2004). In contrast, our analysis found no evidence of gene conversion among any of the spider Hsp70s. This lack of gene conversion in spider Hsp70s is significant because it indicates that each gene is free to evolve independently. Natural selection can then act on multiple heat-inducible Hsp70 proteins at the same time and can explore a greater portion of protein sequence space. We propose that with multiple independently evolving Hsp70s, it was possible for some diguetid Hsp70s to acquire new amino acids under positive natural selection, while at least one of the Hsp70 homologues could retain the ancestral function under purifying selection. It is important to note here that the differences in the evolutionary dynamics of the spider and *Drosophila* Hsp70 genes are most probably not due to disparities in evolutionary time-scale. The sequence divergence among the *COI* diguetid genes is comparable to the *COI* divergence (less than 10%) between *Drosophila simulans* and *Drosophila melanogaster*.

Functional comparisons of Hsp70s from divergent organisms indicate that even minor sequence variation among Hsp70s can result in altered functional characteristics (Mayer & Bukau 2005). Thus, it is quite possible that the differences found in the diguetid Hsp70s reflect functional differences between diguetids and other spiders. Further, the distribution of residues under positive selection in the C-terminal

Table 2. Sites detected with rate shifts in *Diguetia* cytosolic heat-induced Hsp70s. (The site numbers refer to positions in the amino acid alignment. Type I sites are constant in one phylogenetic group but vary in the other. Type II sites have different functional properties in each phylogenetic group. The groups were D, *Diguetia*; P, pholcids and plectreurids. The sites listed have significant LRTs (0.05 level).)

site	rate shift	constant group	site	rate shift	constant group
ATP-binding domain			peptide-binding domain		
8	I	D	378	I	D
35	I	D	380	I	P
39	I	D	414	II	
63	I	D	471	I and II	P
72	I	D	482	II	
81	I	D	514	I	P
82	II		515	I	D
90	II		518	I	P
92	I	D	522	I	D
93	I	D	526	II	
97	I and II	D	527	I	D
119	I	D	528	I	D
123	I	P	529	I	P
176	I	D	551	II	
207	II		552	I	P
208	I	P	553	II	
226	II		557	II	
227	I	D	564	I	P
242	I	D	568	I	P
247	I	P	569	II	
265	II		570	I	P
275	I	P	571	II	
289	II		573	I	D
310	I	D	582	I	D
342	I	D	584	I	D
349	I	P	596	I	P
			598	II	
			600	I	P

region suggests that functional differences may also exist between diguetid Hsp70s.

In summary, in contrast to their closest relatives, the diguetid spiders do not avoid exposure to heat and have undergone positive selection on the Hsp70s. This combination strongly suggests that selection for increased thermotolerance has driven the evolution of the Hsp70s in these spiders.

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