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Phylogeny and development of marine model species: strongylocentrotid sea urchins

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SUMMARY The phylogenetic relationships of ten strongylocentrotid sea urchin species were determined using mitochondrial DNA sequences. This phylogeny provides a backdrop for the evolutionary history of one of the most studied groups of sea urchins. Our phylogeny indicates that a major revision of this group is in order. All else remaining unchanged, it supports the inclusion of three additional species into the genus *Strongylocentrotus* (*Hemicentrotus pulcherrimus*, *Allocentrotus fragilis*, and *Pseudocentrotus depressus*). All were once thought to be closely related to this genus, but subsequent revisions separated them into other taxonomic groupings. Most strongylocentrotid species are the result of a recent burst of speciation in the North Pacific that resulted in an ecological diversification. There has been a steady reduction in the complexity of larval skeletons during the expansion of this group. Gamete attributes like egg size, on the other hand, are not correlated with phylogenetic position. In addition, our results indicate that the rate of replacement substitutions is highly variable among phylogenetic lineages. The branches leading to *S. purpuratus* and *S. franciscanus* were three to six times longer than those leading to closely related species.

INTRODUCTION

Sea urchins in the family Strongylocentrotidae have been a model system for the study of development for well over a century. Their abundance, the large size and clarity of their eggs, and the ease with which experiments can be done on gametes made them a favorite target for early work on fertilization and embryogenesis (for review, see Ernst 1997). Work on the genome composition of animals was pioneered through study of thermal renaturation of repetitive and single-copy DNA in sea urchins (Angerer et al. 1976). Strongylocentrotids have been extensively studied developmentally (Strathmann 1987; Buznikov and Podmarev 1990; Biermann and Marks 2000; Kitamura et al. 2002), genetically (Roberts et al. 1985; Vawter and Brown 1986; Palumbi and Wilson 1990; Palumbi and Kessing 1991; Biermann 1998; Debenham et al. 2000), ecologically (Agatsuma 1998; Konar 2001), and morphologically (Jensen 1974; Strathmann 1979; Gagnon and Gilkinson 1994). Both in North America and in Japan, use of urchins in the genera *Strongylocentrotus* and *Hemicentrotus* has continued with the development of powerful molecular tools, allowing the study of patterns of cell fate and gene regulation during early development (Ogawa et al. 2000; Martin et al. 2001; Kitamura et al. 2002). The availability of whole-genome BAC libraries for *Strongylocentrotus purpuratus* (Cameron et al. 2000) has opened the door to complete sequencing of the genome of this species, which should be completed in 2003. Such information promises to provide an unparalleled opportunity to understand the evolution of development in early deuterostomes.

Understanding the evolution of development in these species depends on knowing their phylogenetic relationships. Because different researchers use different strongylocentrotid species in different genera, comparing results among research groups will be easier if a robust phylogenetic framework has been established. Morphological characters from adult tests (Clark 1912; Mortensen 1943; Jensen 1974) overlap, are confounded by ecological conditions, and have not elucidated phylogenetic relationships (R. Mooi, personal communication); this has limited the utility of comparative studies within this group.

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Although the detailed relationships of all strongylocentrotid species remained unknown, a number of molecular studies have determined partial affiliations. Thomas et al. (1989) presented a mitochondrial (mt)DNA phylogeny including five species based on amino acid substitutions in the ND5 protein coding region of the mitochondrial genome that suggests *S. franciscanus* is perhaps the most distant member of the genus *Strongylocentrotus*, followed by *S. intermedius*, then *S. purpuratus*, and finally *S. droebachiensis* and *S. pallidus*. A variety of genetic markers supports a large divergence from other strongylocentrotids for both *S. nudus* (Manchenko and Yakovlev 2001) and *S. franciscanus* (Angerer et al. 1976; Grula et al. 1982; Roberts et al. 1985; Vawter and Brown 1986; Springer et al. 1995; Gonzalez and Lessios 1999; Meeds et al. 2001). Palumbi and Wilson (1990) also found that *S. purpuratus* and *S. droebachiensis* are closely related using restriction fragment length polymorphisms but perhaps less so than *S. droebachiensis* and *S. pallidus* (Palumbi and Kessing 1991). The nuclear gene for sperm bindin does not resolve the branching order among the five most closely related species but shows that the genus *Hemicentrotus* falls inside *Strongylocentrotus* (Biermann 1998).

mtDNA is used in our study of these sea urchins because of the wealth of information already available on the mtDNA of sea urchins (Vawter and Brown 1986; Jacobs et al. 1988; Cantatore et al. 1989; Thomas et al. 1989; Palumbi and Kessing 1991). mtDNA has been widely used to reconstruct phylogenies, examine population structure, and date divergence times of species because it appears, at least within some taxa, that mtDNA evolves at a fairly constant rate (Brown et al. 1982; Hasegawa et al. 1985; Bermingham and Lessios 1993; Heyer et al. 2001; Marko and Moran 2002). However, in different mtDNA protein-coding regions, amino acid sequences evolve at different rates, presumably due to variation in the functional constraints on those regions (Brown et al. 1982; Jacobs et al. 1988; Kondo et al. 1993; Cummings et al. 1995; Heyer et al. 2001). If those constraints remain relatively constant through time, we would still have a suitable "molecular clock". A phylogenetic analysis of this group of sea urchins thus also offers an opportunity to look at rates of molecular evolution among closely related lineages.

We explore the phylogenetic relationships of the sea urchin species in the family Strongylocentrotidae. We use this phylogeny to examine the rates and patterns of evolution of gamete and larval characters and of the morphological traits used by previous taxonomists (Mortensen 1943) to define species and genera within the family. Our results show that this group of species represents a recent adaptive radiation of species that have become ecologically, developmentally, and reproductively differentiated and that at least two species in different monotypic genera belong phylogenetically within the genus *Strongylocentrotus*. Evolutionary relationships shown with mtDNA highlight the lability of egg size and confirm suspicions about species relationships based on larvae and biochemical data.

MATERIALS AND METHODS

Taxa

We collected mtDNA sequence data from nine sea urchin species in the family Strongylocentrotidae and from *Pseudocentrotus*

Table 1. Species of sea urchins sequenced in this study, the codes referred to in our figures, and collection locations. For former synonyms refer to Jensen (1974). Note that her distinction between *S. pulchellus* and *S. intermedius* is no longer accepted (Levin and Bakulin 1984; Tatarenko and Poltoraus 1988)

Genus	Species	Code used here	Location
Strongylocentrotus	*S. purpuratus*	P1	Jacobs et al. (1988)
		P2	Point Arena, CA
		P3	California
	S. franciscanus	F	Laguna, CA
	S. droebachiensis	D1	Friday Harbor, WA
		D2	Bodø, Norway
		D3	Tromsø, Norway
		D4	Breidafjordur, Iceland
	S. pallidus	L1	Friday Harbor, WA
		L2	Tromsø, Norway
		L3	Tromsø, Norway
	S. polyacanthus	Y	Kamchatka, Russia
	S. nudus	N	Hachinohe, Japan
	S. intermedius	I	Hachinohe, Japan
Allocentrotus	*A. fragilis*	A	La Jolla, CA
Hemicentrotus	*H. pulcherrimus*	H	Shimoda, Japan
Pseudocentrotus	*P. depressus*	S	Sagami Bay, Japan

Table 2. PCR Primers used and their positions in the sea urchin mitochondrial genome. Nucleotide positions are as in Jacobs et al. (1988) for *Strongylocentrotus purpuratus*

Primer Name	Primer 5′ Sequences 3′	Nucleotide Position
CO1c	TCGTCTGATCCGTCTTTGTCAC	6335
CO1g	CACTACGTTCTWTCAATRGG	6916
CO1a	AGTATAAGCGTCTGGGTAGTC	7108
t-ARGa	CGAAATCAGAGGTTCTCCTTAAAC	7380
CO2a	GGGGCTAACCATAGATTCATGCC	8312
8314+	GCTAACCATAGATTCATGCC	8314
8469+	TTAAGGAGTGCCACAACTAG	8469
8489 –	TTTACTGCCATYCANARAGG	8469
9064 –	ATTAGTGCKCTTGTTGTTCC	9064
ATP8a	TTAACTATAAAAAAGACCAC	8602
ATP6a	GTGCGCTTGGTGTTCCCTGTGG	9039

depressus, which had been suggested to be close to the strongylocentrotids (Matsuoka 1986, 1987; Tatarenko and Poltaraus 1993) (see Table 1 for species, codes, and collection sites). We decided to keep Jensen's (1974, 1981) and Mortensen's (1943) classification of the strongylocentrotids as a separate family (Strongylocentrotidae Gregory) in the order Camarodonta and not as a subfamily within the Echinometridae (Smith 1988), because they are genetically sufficiently distant from the Echinometridae to warrant family status (Tatarenko and Poltaraus 1991). Also, by sperm-activating peptide similarity, they are actually closer to the Toxopneustidae than to the Echinometridae (Suzuki and Yoshino 1992). We furthermore accept the genetic (Tatarenko and Poltaraus 1991) and morphological (Jensen 1974; Bazhin 1998) evidence that *Strongylocentrotus sachalinicus* and *S. echinoides* are synonymous with *S. pallidus* and *S. pulchellus* with *S. intermedius* (Levin and Bakulin 1984; Tatarenko and Poltoraus 1988; Bazhin 1998); that is, we believe our sampling of this group is comprehensive.

mtDNA sequences

Purified mtDNA from seven species (samples L1, D1, P2, N, F, H, and I) was obtained from the gonadal tissue and purified in a cesium chloride gradient (Palumbi and Wilson 1990). Polymerase chain reaction (PCR) and standard Sanger di-deoxy sequencing methods for obtaining sequences are described elsewhere (Kessing 1991; Palumbi and Kessing 1991). Genomic DNA from gonad tissue was extracted for our other samples (i.e., A, L2, L3, D2, D3, D4, S, and Y). Direct sequencing of PCR products was performed with these samples according to Khorana et al. (1994).

The complete mitochondrial genome has been sequenced from *Strongylocentrotus purpuratus* (Jacobs et al. 1988), which has enabled us to design specific oligonucleotide primers and PCR amplify two different mtDNA regions. The oligonucleotide primers used in the PCR and sequencing reactions are listed in Table 2, and their relative positions are diagrammed in Figure 1. A 742 base portion of the cytochrome oxidase subunit 1 (CO1) and a 688 base portion that includes part of the cytochrome oxidase subunit 2 (CO2) and ATPase subunit 6 (ATP6) regions, and all the lysine tRNA (lys-tRNA) and ATPase subunit 8 (ATP8) coding regions were sequenced for all samples (Fig. 1). Previous studies suggested that "sampling" the mtDNA genome with noncontiguous smaller PCR fragments is preferable to sampling one large fragment when trying to reconstruct the phylogenetic history of taxa (Cummings et al. 1995). The sequences have been submitted to GenBank (AY220988–AY221021).

In addition to the multiple individuals shown for *S. purpuratus*, *S. droebachiensis*, and *S. pallidus*, two *P. depressus* individuals and two *A. fragilis* individuals were sequenced and found to be invariant. A third individual of *A. fragilis* differed by only two point substitutions (data not shown). Our data for *Hemicentrotus* and *S. polyacanthus* were confirmed as well by partial sequences of additional individuals. Some species identifica-

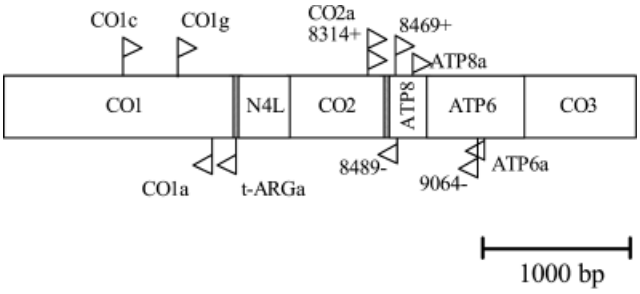

Fig. 1. The region of sea urchin mtDNA amplified and the primers used to sequence mtDNA in this study. Regions represented in the diagram are the cytochrome oxidase subunit 1 (CO1), arginine tRNA (hatched area), NADH dehydrogenase subunit 4L (N4L), cytochrome oxidase subunit 2 (CO2), lysine tRNA (hatched area), ATPase subunit 8 (ATP8), ATPase subunit 6 (ATP6), and the cytochrome oxidase subunit 3 (CO3). The primers and their direction are indicated by the flags. See Table 2 for primer sequences and their exact location in the *S. purpuratus* sequence published in Jacobs et al. (1988).

tions (samples L1, D1, P2, N, F, H, and I) were confirmed by M. Jensen using sea urchin tests. Additional mtDNA sequences for the ND5 protein-coding region have been published for five of our samples (Thomas et al. 1989), and we used them (i.e., with our D1, P2, I, F, and L1 samples) and the corresponding ND5 region of the published *S. purpuratus* sequence (P1 in Table 1, Jacobs et al. 1988) in our phylogenetic analyses. *Paracentrotus lividus* (Cantatore et al. 1989) and *Psammechinus miliaris* (un-published data) sequences were used as outgroups to root all initial phylogenetic trees. These genera are among the closest outgroups known within the Echinoida (Smith 1992; Smith et al. 1995).

Tree building and calculations of divergence

DNA sequences were aligned using the published *S. purpuratus* sequence (Jacobs et al. 1988) as a reference. The estimated divergences (based on changes at all sites) between all sequences were determined using a maximum-likelihood calculation. The corrected estimates of silent substitutions were calculated from fourfold degenerate codon positions only (K4 values) using the methods of Kimura (1983) to correct for multiple substitutions using a two-parameter model. Codons with more than one nucleotide substitution at different codon positions, incomplete codons, the lysine tRNA coding region, and the termination sequence for the CO2 protein-coding region were all eliminated from the analysis. These segments were eliminated because they lacked definable silent and amino acid replacement sites or because additional constraints may have influenced molecular evolution in those segments. In all we used 334 codons in our calculations. Although calculating substitutions in this way reduces the sample size, this method gives an estimate of the silent substitutions unconstrained by protein function and reflects mutation rates at positions that are as close to selectively neutral as possible (Kimura 1983). Replacement substitutions were calculated from all twofold degenerate and amino acid replacement codon positions as in Li et al. (1985).

We explored the topologies for the relationships of these species using the maximum-likelihood methods in PAUP* 4.0 (Swofford 1998). A great deal of effort has gone into developing and testing different phylogenetic tree estimation methods (Kuhner and Felsenstein 1994; Swofford et al. 1996; Nei 1997; Whelan et al. 2001). There are advantages and disadvantages to all methods, but there is a large consensus that maximum-likelihood methods outperform other methods under a wide variety of conditions—even when the assumptions of the model used to calculate the likelihoods are violated (Saitou and Imanishi 1989; Hasegawa et al. 1991; Kuhner and Felsenstein 1994; Huelsenbeck 1995). Maximum-likelihood methods also have the unique advantage among the tree-making algorithms of allowing statistical tests to be performed directly on the topologies generated. A log-likelihood analysis can be done to test whether different tree topologies are significantly better than others (Felsenstein 1993).

Testing for rate heterogeneity among sites and along mtDNA lineages

Maximum-likelihood methods are dependent on the DNA evolution models used to correct, or model, the substitutions in sequences. Rate variation among sites of protein coding sequences can adversely affect the efficiency of phylogenetic analyses (Kuhner and Felsenstein 1994). In an attempt to minimize this effect, a gamma correction was used in the maximum-likelihood PAUP* analysis (Swofford et al. 1996). We explored the pattern of substitutions at different sites in our data to help correct for rate variation among sites in our maximum-likelihood phylogenetic analysis.

Two distance-matrix methods were also used in this study: FITCH and KITSCH in the program PHYLIP 3.5 (Felsenstein 1993). FITCH is a method that allows rate variation among lineages, whereas KITSCH assumes a molecular clock when building phylogenetic trees. These two methods allow rate variation among lineages to be tested by comparing which tree the data fit better: a tree in which branches are variable in length or a tree in which branches are constrained to evolve at an equal rate (see below). An *F*-test comparison is applied to the sum of the squares values included in the output from the KITSCH and FITCH algorithms (Felsenstein 1993).

RESULTS

We sequenced 1073 bases of overlapping sequence in four protein-coding regions and the lysine tRNA coding region from the mtDNA of 10 temperate sea urchin species in the family Strongylocentrotidae (Table 1). With the additional published ND5 protein coding sequences (Thomas et al. 1989), we had a total of 1484 bases of overlapping sequences in our phylogenetic analysis. The sea urchin sequences that we determined were easily aligned with the published *S. purpuratus* sequence (Jacobs et al. 1988). The published *S. purpuratus* sequence contains two extra codons in the ATP6 protein-coding region at base positions 8913–8915 and 8925–8927 (Jacobs et al. 1988; Palumbi and Kessing 1991). These codons (a proline and a glutamine, respectively) were missing in our *S. purpuratus* sequences and in all other mtDNAs sequenced in this study. This discrepancy between sequences is discussed elsewhere (Palumbi and Kessing 1991) but may be due to an error in the published sequence. For purposes of phylogenetic analysis the published sequence was retained, but to be conservative all analyses of substitution patterns were limited to our *S. purpuratus* sequences.

Phylogenetic relationship of strongylocentrotid sea urchins

The phylogenetic relationships of the mtDNA from species of strongylocentrotid urchins are presented in Figure 2. This topology is the maximum-likelihood solution for an analysis of all sites, allowing the gamma and substitution parameters (using a six-parameter substitution model) to be optimized independently across all topologies when searching and maximizing topologies by PAUP* 4.0. Branch lengths depicted in Figure 2 are the maximum-likelihood estimates

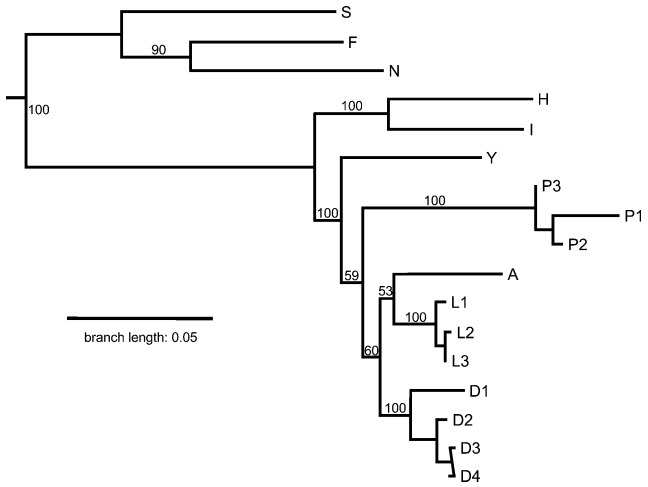

Fig. 2. The phylogenetic relationship of the 10 strongylocentrotid species based on all sites using a maximum-likelihood approach. The algorithm is a six-parameter (time-reversal) substitution model corrected for rate variation among sites using likelihood estimations of all parameters (PAUP* 4.0). Branch lengths are drawn to a relative scale: They are the maximum-likelihood estimates of the proportion of sites that have changed in the mtDNA sequence along that lineage. Numbers above branches are maximum likelihood bootstrap values. Species are abbreviated as in Table 1. The tree is outgroup rooted as described in the methods.

of sequence evolution along the branches (i.e., the proportion of sites that have changed along that mtDNA lineage).

There are two major clades in this group of sea urchins. One comprises the *Strongylocentrotus franciscanus*, *S. nudus*, and *Pseudocentrotus depressus* lineages and the other contains the other seven species in our study (Figs. 2 and 3). These clades are very distant, being on average 24% divergent between samples based on the maximum-likelihood corrected percent differences calculated from all sites (Table 3). The other clade includes all the other known species in the family Strongylocentrotidae. The western Pacific species *Strongylocentrotus intermedius* and *Hemicentrotus pulcherrimus* cluster together within this clade (bootstrap support 100%, Fig. 2), as do the eastern Pacific species *S. purpuratus*, *S. droebachiensis*, *S. pallidus*, and *Allocentrotus fragilis*. Between these two clades lies the Aleutian species *S. polyacanthus*. Our phylogenetic reconstruction strongly suggests that the genus *Strongylocentrotus* is paraphyletic. *Hemicentrotus pulcherrimus*, *Allocentrotus fragilis*, and *Pseudocentrotus depressus* are closely related to species in the genus *Strongylocentrotus*. All trees placing these three species outside the genus *Strongylocentrotus* (e.g., basal to this group) were worse based on log-likelihood analysis (using PAUP*), for *Allocentrotus* and *Hemicentrotus* significantly so. To confirm these results, multiple individual *Hemicentrotus*, *Pseudocentrotus*, and *Allocentrotus* were independently extracted and sequenced.

Multiple individuals from one species always grouped together (L1-L3, P1-P3, D1-D4 in Fig. 2). However, the topology clearly separates the *S. droebachiensis* samples collected from the eastern Atlantic (D2-D4) from the Pacific haplotype. The samples are 3% divergent based on the corrected percent difference seen between mtDNAs (Table 3).

Although maximum-likelihood trees grouped *A. fragilis*, *S. pallidus*, and *S. droebachiensis* as a sister-group to *S. purpuratus*, these relationships had low bootstrap support (Fig. 2). Based on a likelihood analysis (using PAUP*'s test), a four-way polytomy provided just as good an explanation of the data. Slight changes in the substitution model confirmed

Table 3. Comparison of maximum likelihood corrected percent substitutions at all sites among strongylocentrotid sea urchins. Species are abbreviated as in Table 1

	A	D1	D2	D3	D4	F	H	I	L1	L2	L3	N	P1	P2	P3	S
D1	6.8															
D2	5.4	2.7														
D3	6.0	3.1	0.7													
D4	5.7	3.1	0.7	0.2												
F	25.0	29.0	23.2	23.0	22.6											
H	10.8	11.4	11.0	11.5	11.5	24.4										
I	11.3	12.5	11.1	11.9	11.6	30.9	9.4									
L1	5.3	4.9	3.5	3.9	3.9	29.3	10.7	12.0								
L2	5.5	4.2	3.7	4.1	4.1	23.1	11.0	10.1	0.6							
L3	5.2	4.2	3.5	3.8	3.8	23.0	10.7	10.4	0.4	0.2						
N	26.6	23.4	25.9	25.1	25.1	11.8	26.5	21.0	24.8	25.4	25.3					
P1	12.7	13.4	10.5	10.1	10.4	40.2	16.5	19.2	12.9	9.9	10.2	30.0				
P2	11.6	10.8	9.2	9.0	9.3	35.1	15.8	16.9	10.1	9.0	9.3	27.4	2.5			
P3	10.2	7.9	7.7	7.5	7.8	25.0	14.6	13.7	7.6	7.6	7.9	26.8	2.1	1.0		
S	24.8	21.8	23.2	23.2	23.1	13.5	24.6	20.9	23.6	23.0	23.0	16.9	27.7	27.1	25.6	
Y	7.9	8.2	8.2	8.3	8.3	23.8	12.3	11.7	7.6	7.9	7.6	25.0	14.0	13.1	11.6	22.2

Table 4. Comparison of silent and replacement substitutions among sea urchin species. Values above the diagonal are silent substitutions (calculated from four-fold degenerate sites) and values below the diagonal are replacement substitutions (calculated as in Li et al. 1985). Species are abbreviated as in Table 1

	A	D1	D2	D3	D4	F	H	I	L1	L2	L3	N	P1	P2	P3	S	Y
A		19.3	15.1	16.7	16.7	70.4	31.3	29.8	13.6	15.3	14.4	75.0	27.3	25.6	24.0	62.1	29.4
D1	0.8		07.2	07.2	07.2	52.1	33.0	29.0	11.3	13.3	12.5	55.9	21.7	18.5	16.8	52.4	30.8
D2	0.8	0.5		01.2	01.2	54.2	30.0	29.9	09.4	10.9	10.2	60.9	21.2	18.0	16.7	57.7	32.3
D3	0.8	0.5	0.3		00.0	53.8	30.9	31.9	09.4	10.9	10.1	59.5	19.4	18.0	16.8	59.1	32.5
D4	0.8	0.5	0.3	0.0		53.8	30.9	31.9	09.4	10.9	10.1	59.5	19.4	18.0	16.8	59.1	32.5
F	3.1	5.9	3.5	3.5	3.5		60.7	61.7	54.6	56.9	56.9	31.8	58.1	61.4	55.0	40.5	68.7
H	2.1	1.8	2.1	2.1	2.1	3.1		19.3	30.7	33.0	31.9	61.0	34.7	35.0	35.6	53.0	37.8
I	1.8	2.8	1.6	1.6	1.6	5.8	2.3		26.6	27.8	28.9	53.2	30.7	29.2	32.1	53.0	37.3
L1	1.2	1.4	0.6	0.6	0.6	5.5	1.9	2.9		01.3	00.6	67.9	22.3	20.3	17.6	60.5	28.6
L2	0.9	0.6	0.4	0.4	0.4	3.1	1.7	1.7	0.3		00.6	67.9	20.3	18.8	17.6	62.5	30.9
L3	0.9	0.6	0.4	0.4	0.4	3.1	1.7	1.7	0.3	0.0		67.9	21.3	19.7	18.5	62.5	29.8
N	2.7	3.1	3.1	3.1	3.1	0.8	3.0	2.2	2.7	2.7	2.7		58.6	59.8	59.8	42.6	63.8
P1	3.2	4.2	3.0	3.0	3.0	7.9	4.1	5.2	4.2	5.1	1.8	3.1		02.3	02.5	55.9	41.4
P2	2.6	3.0	2.3	2.3	2.3	6.6	3.6	4.0	2.9	2.3	2.3	4.0	3.1		00.6	59.0	38.7
P3	2.1	1.8	1.8	1.8	1.8	4.4	3.2	2.3	1.9	3.9	0.5	1.9	1.9	3.6		61.7	40.4
S	3.0	3.0	3.2	3.2	3.2	2.1	3.6	2.7	3.1	2.1	4.4	2.8	2.8	2.5	5.2		72.3
Y	1.3	1.0	1.3	1.3	1.3	3.5	2.6	1.8	1.2	3.1	3.0	1.2	1.2	3.2	4.0	1.2	

this tetratomy: Under a Kimura two-parameter model or minimum evolution, for example, *S. purpuratus* groups with *S. droebachiensis* (not shown). The relatively long branches leading to *S. purpuratus* (Figs. 2 and 4) suggest additional caution in interpreting the phylogenetic relationships among these species.

Rate variation among lineages

Significant rate variation was detected along lineages of these sea urchins ($F = 8.7$; $df = 9, 36$; $P < 0.001$) using the distance data presented in Table 3. The long branch leading to the *S. purpuratus* individuals was particularly noticeable (Fig. 2), with a rate of mtDNA sequence evolution averaging about twice that seen in its closest relatives. To explore this phenomenon, the data were broken down into silent and replacement substitutions (data in Table 4) and the substitutions mapped out onto the topology from Figure 2 (Fig. 4). There was significant rate variation among lineages of these closely related sea urchin species (*F*-ratio test from the FITCH-KITSCH algorithms: $F = 6.9$; $df = 9, 36$; $P < 0.001$). However, this rate variation was much greater at replacement sites (Fig. 4; $F = 7.8$; $df = 9, 36$; $P < 0.001$). In particular, the *S. purpuratus* lineage evolved at the amino acid sequence level three to six times faster than its closest relatives (e.g., *A. fragilis*, *S. droebachiensis*, and *S. pallidus*). An accelerated amino acid replacement rate also appeared in the *S. franciscanus* lineage when compared with *S. nudus* (Fig. 4).

DISCUSSION

Phylogeny of strongylocentrotid sea urchins

Strongylocentrotid sea urchins fell into two distinct clades as measured by mtDNA sequence divergences. Both clades contained species that had been classified as belonging to the genus *Strongylocentrotus*, as well as to other genera. *Pseudocentrotus depressus*, *Hemicentrotus pulcherrimus*, and *Allocentrotus fragilis* all grouped with traditional members of the genus *Strongylocentrotus*.

Our placement of *Pseudocentrotus* within the family by mtDNA evidence was not entirely surprising; it was in fact proposed by Clark (1925) and Shigei (1974). Although this relationship was missed by a revision of this group based on morphology (Jensen 1974), Matsuoka (1986, 1987) suggested it based on genetic distances calculated using protein electrophoresis. It is corroborated by DNA–DNA hybridization (Tatarenko and Poltaraus 1993), the similarity of sperm-activating peptides (Suzuki and Yoshino 1992), and, arguably, the cross-fertilizability between *Pseudocentrotus* and *S. intermedius* (cited in Matsuoka 1980; Suzuki and Yoshino 1992). In our topology, *Pseudocentrotus depressus* was the most basal member in this family, but it clearly fell on the branch leading to *S. franciscanus* and *S. nudus*. Tree topologies placing *P. depressus* outside this branch were less likely, but not significantly so. Its separate generic name may nevertheless be justifiable on the basis of its many plesiomorphic traits. Mortensen (1943) placed *P. depressus* in the same family Strongylocentrotidae, but notes it is "beyond question that *depressus* has nothing to do with the true species

of *Strongylocentrotus* . . . (the) characters of the globiferous pedicellariae, spicules, gill-slits, as well as the characters of the larva show it decidedly . . . to belong to the family of the Toxopneustids." *Strongylocentrotus franciscanus* and *S. nudus*, too, are so distinct from the other strongylocentrotids on the basis of DNA-DNA hybridization and morphological traits (also see larval pedicellariae below) that it has been proposed to isolate them into the new genus *Mesocentrotus* (Tatarenko and Poltaraus 1993).

Hemicentrotus pulcherrimus was situated well within the genus *Strongylocentrotus* by this analysis. This species was once placed in the genus *Strongylocentrotus* by Mortensen in 1903, but he later included it in a monotypic genus *Hemicentrotus* because it had exactly three tube feet pore pairs per arch in test plates rather than three to four as in *S. intermedius* (Mortensen 1943; Jensen 1974). The low variance in pore pairs in *Hemicentrotus* was the major reason for Mortensen's naming of this genus. Other recent molecular studies substantiate our observation that *Hemicentrotus*, the closest relative of *Strongylocentrotus intermedius*, is a member of the genus *Strongylocentrotus* (Biermann 1998; Meeds et al. 2001). *Hemicentrotus* is the main echinoid target of developmental research in Japan, and a large number of studies have examined the early embryology and regulation of development in this species (Akasaka and Shimada 2001; Kitamura et al. 2002; Tokuoka et al. 2002). The close relationship between *Hemicentrotus* and the other major research vehicle, *S. purpuratus*, makes it likely that results from these two species will be more similar than results from *S. purpuratus* and *S. franciscanus*. The genetic distance between these latter species is about twice that of *S. purpuratus* and *Hemicentrotus*. However, interesting developmental differences can be found even among closely related species. For example, the second primary cleavage plane, along the aboral–oral axis, is offset by 45 degrees from the first two (animal–vegetal) cleavage planes in *S. purpuratus*, whereas the angle is not specified in *H. pulcherrimus* but instead randomly distributed among embryos (Raff 1999).

Interestingly, Mortensen (1943) remarked that *Allocentrotus* "is so very unlike that of any of the true species of *Strongylocentrotus* that the idea at once suggests itself that it must form a distinct genus." Contrary to Mortensen, Clark (1912) noted that the "pedicellariae, sphaeridia, and spicules do not appear to be in any way different from those of *droebachiensis*." Our analysis placed *Allocentrotus fragilis* very close within this group, and *Allocentrotus* shares larval characters with *S. purpuratus*, *S. pallidus*, and *S. droebachiensis* (Strathmann 1979). The placement of *S. polyacanthus* in close proximity to these other species in this clade seems consistent with morphological taxonomy as well. Clark (1912), in his descriptions of species in this genus, states that *S. polyacanthus* "is so near to *droebachiensis*, that it would be quite superfluous to give a detailed description . . . " (also see its placement with *S. droebachiensis* in Biermann 1998). Although *S. polyacanthus* is the most common echinoid in the Aleutians (Estes and Duggins 1995), it is not known from the Arctic or Atlantic Oceans.

Strongylocentrotus droebachiensis and *S. pallidus*, the only circumarctic species in the family, can be difficult to distinguish morphologically (Vader et al. 1986; Gagnon and Gilkinson 1994) but are clearly good species according to our data (see also Jensen 1974; Strathmann 1981; Falk-Petersen and Lønning 1983; Biermann 1998). The relationships of *S. droebachiensis* and *S. pallidus* individuals from the northeast Atlantic to their northern Pacific counterparts were strikingly different. *Strongylocentrotus pallidus* from Norway were closely related to the Pacific sample (differing by only 0.5% sequence divergence). This is consistent with the low mtDNA divergence seen between mtDNA sequences between the northwest Atlantic and north Pacific (0.1% between populations; Palumbi and Kessing 1991). However, the *S. droebachiensis* sequences from the eastern Atlantic (Norway and Iceland) were about 3% different from those in the western Atlantic (not shown) and northeast Pacific. This amount of sequence divergence is greater than that seen between some species of *Echinometra* sea urchins (Palumbi and Metz 1991) and suggests that the eastern Atlantic *S. droebachiensis* have been geographically isolated for a long time from those in the western Atlantic and north Pacific.

From ND5 sequence data only, Thomas et al. (1989) reported a congruent topology to ours for the five urchins they analyzed (i.e., *S. franciscanus*, *S. purpuratus*, *S. intermedius*, *S. droebachiensis*, and *S. pallidus*). The only discrepancy is that Thomas et al. (1989) placed *S. purpuratus* far outside the *S. droebachiensis* and *S. pallidus* bifurcation, whereas we consider these three species to be very closely related. Their analysis, however, was based on only part of the ND5 gene and examined only second base codon positions (all of which are amino acid replacement sites) using a maximum-parsimony algorithm (PAUP). Unfortunately, maximum parsimony is sensitive to rate variation among lineages and will often fail to find the "correct" tree when rate variation among lineages is extreme (Saitou and Imanishi 1989). Replacement substitutions (amino acid changes) are accelerated along the *S. purpuratus* lineage, and this alone could result in *S. purpuratus* being placed outside the trifurcation.

Rate variation among mtDNA lineages of sea urchins

Our phylogenetic analysis of the strongylocentrotid sea urchins provides evidence of marked rate variation among mtDNA lineages within closely related groups. Although silent substitution rates were not greatly different among these

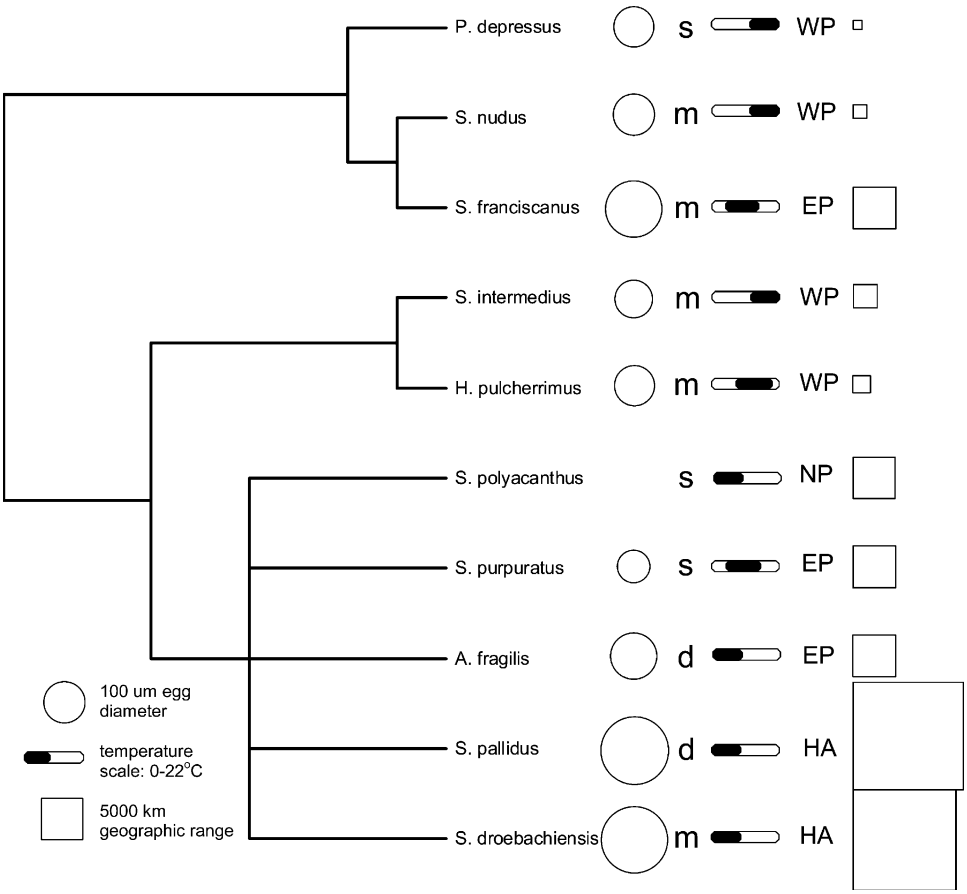

Fig. 3. Egg sizes, depth ranges, preferred development temperatures, and geographic ranges of strongylocentrotid sea urchins, mapped onto mtDNA cladogram (data from Jensen 1974; Strathmann 1979; Emlet et al. 1987; Strathmann 1987; Buznikov and Podmarev 1990; Emlet 1995; Bazhin 1998; Kasyanov et al. 1998; Park and Son 1998). Nodes with weak bootstrap support were collapsed. Adult depth range: s, shallow (0–50 m); m, medium (0–200 m); d, deep (50–2000 m). Distributions (all are Northern Hemisphere only): WP, West Pacific; EP, East Pacific; NP, Northwest Pacific; HA, holarctic. Note: Egg sizes shown for *S. pallidus* and *S. droebachiensis* are those reported for Washington State in the Pacific. Along the coast of Norway both species' egg diameters increase with latitude from 136 μm (Hagström and Lønning 1967) to over 200 μm (J. Marks, personal communication).

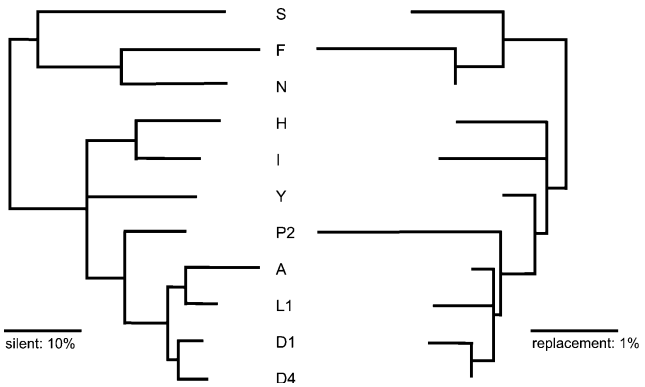

Fig. 4. Minimum-evolution estimation of the branch lengths for the topology from Figure 2, separated into silent and replacement changes. Left: silent substitutions (fourfold degenerate codon positions only), right: replacement substitutions. Species abbreviations as in Table 1.

species, the rate of replacement substitutions varied significantly among lineages. In particular, the *S. purpuratus* and *S. franciscanus* lineages evolved at a rate three to six times faster than their closest relatives, *S. droebachiensis*/*S. pallidus* and *S. nudus*, respectively. Such extreme rate variation is interesting in light of the close phylogenetic relationship of these urchins. One cause for rate speed-up may be a relaxation of the functional constraints on the amino acid composition of sea urchin mtDNA protein-coding regions. However, the average ratio of replacement-to-silent substitutions in these sequences, when *S. purpuratus* is compared with its closest relatives *A. fragilis*, *S. pallidus*, and *S. droebachiensis*, was 0.13. It was even more constrained, a ratio of 0.03, when *S. franciscanus* was compared with its closest relative *S. nudus*. This is comparable with functional constraints seen in other studies for mtDNA coding regions (Kondo et al. 1993), indicating strong selection against amino acid changes.

A similarly long branch leading to *S. purpuratus* in the nuclear gene for sperm bindin (Biermann 1998) suggests that the functional relaxation on sequences in the purple sea urchin was probably genome-wide and therefore caused by past demographic stochasticities (see below).

As a practical matter, inferring a phylogeny from replacement substitutions (suggested by Prager and Wilson 1988) or calibrating a local molecular clock could be very misleading in this family, because pairwise differences vary several-fold depending on the species comparison.

Ecological and developmental characters

Strongylocentrotid sea urchins are widely distributed throughout the northern oceans. Two of the crown group species, *Strongylocentrotus droebachiensis* and *S. pallidus*, are circumarctic. *Pseudocentrotus depressus*, *S. nudus*, *S. intermedius*, and *Hemicentrotus pulcherrimus* occur only in the western Pacific, whereas their close relatives, *S. franciscanus*, *S. purpuratus*, and *Allocentrotus fragilis*, are found only in the eastern Pacific (Jensen 1974; Emlet 1995; Bazhin 1998). *Strongylocentrotus pallidus* and *S. polyacanthus* are most abundant at high latitudes, with *S. polyacanthus* being restricted to the far north Pacific. It appears that the rapid diversification of the crown group, with the almost simultaneous appearance of five small short-spined species, may have led to the colonization of greater depths and higher latitudes. The species in the eastern Pacific that resulted from the more recent burst of speciation have divergent depth ranges, with *S. purpuratus* being found in the intertidal and shallow subtidal, *S. droebachiensis* being shallow to about 300 m deep, *S. pallidus* extending its range to at least 1000 m, and *Allocentrotus* being a strictly deep water species seldom found above 200 m (Jensen 1974; Strathmann 1979; Emlet et al. 1987; Emlet 1995). These species also tend to have larger eggs, and their embryonic and larval development is adapted to colder water temperatures (Fig. 3).

We estimate the rapid cladogenesis of seven species from a common ancestor to have taken place in the North Pacific during the late Miocene and Pliocene (Smith 1988). A global cooling event and sea-level drop (Herman and Hopkins 1980), dramatic oceanographic changes due to the opening of the Bering Strait 5–7 million years ago (Marincovich and Gladenkov 2001), and glacial climatic fluctuations (Vermeij 1991) could have led to local extinctions and the isolation of populations in refuges. Demographic and selective pressures could also have been exerted by the appearance of the modern sea otter, which probably originated in the east Pacific during the Pliocene (Willemsen 1992). Otter predation on herbivorous urchins likely allowed the diversification of kelp in the North Pacific during the late Cenozoic (Estes and Steinberg 1988). Predation (Estes and Duggins 1995) and glaciation pressures could have driven the evolution of sea urchins toward deeper water habitats and smaller adult sizes. The rapid evolution of gamete recognition molecules (Palumbi 1999), combined with enormous variance in reproductive success, can result in diverging fertilization guilds in spawners, especially when populations undergo bottlenecks. Sparser populations, in addition to potentially fostering reproductive isolation, may account for larger egg sizes to increase the target area for more dilute sperm (Levitan 1998). However, egg size is also known to increase with latitude—this trend is present but not significant in the strongylocentrotids (Emlet et al. 1987).

Egg sizes, sperm morphology (unpublished observation, Dan 1952) and egg jelly carbohydrates (Vilela-Silva et al. 2002) seem to vary independently of the taxonomic proximity indicated by our phylogeny. Egg sizes are known to differ within genera and to respond to selective pressures rapidly, certainly within 3 million years (Lessios 1990; Marko and Moran 2002). Egg jelly sulfated fucans, polysaccharides that mediate the species-specific induction of the sperm acrosome reaction, have different structures in *S. droebachiensis*, *S. pallidus*, *S. purpuratus*, and *S. franciscanus,* with those of *S. droebachiensis* being most distinct (Vilela-Silva et al. 2002). It is reasonable that the evolution of gamete recognition mechanisms is not constrained by phylogeny, especially when closely related species occur sympatrically.

Our phylogeny is in complete congruence with a gradual reduction of larval skeletal complexity toward the derived species in this family. (We have not found a picture of a larva of *S. polyacanthus*, however.) Although the larval skeleton at the four-arm pluteus stage resembles a basket-form in most sea urchin taxa (Wray 1992, Fig. 4), it is reduced to straight skeletal rods in the more recent strongylocentrotids. *Pseudocentrotus*–larvae have the plesiomorphic basked-shaped skeleton (Mortensen 1943; Tatarenko and Poltaraus 1993), which is straightened out but still has long ventral transverse rods in *Strongylocentrotus nudus* and *S. franciscanus* (Tatarenko and Poltaraus 1993). These rods are shorter in *Hemicentrotus* (Wray 1992) and are reduced to tiny processes in the remaining species (Strathmann 1979; Tatarenko and Poltaraus 1993). There has been a concomitant trend to reduce the thorniness of the distal ends of the body rods (Strathmann 1979; for *Hemicentrotus* see Mortensen 1921), although *S. droebachiensis* seems to maintain a relatively thorny appearance (Strathmann 1979). Another larval trait that sets the three basal species apart from *S. purpuratus*, *S. droebachiensis*, *S. pallidus*, *S. intermedius*, and *Hemicentrotus* is the presence of three aboral pedicellariae, which are found only on the rudiments of *Pseudocentrotus*, *S. franciscanus*, and *S. nudus* (Kawamura 1970; Strathmann 1987; Miller and Emlet 1999; R. Emlet, personal communication). These observations do not corroborate the homoplasious nature of larval morphological traits noted by Smith et al. (1995).

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