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Somatic DNA Modifications in the *Sp185/333* Gene Family in the Purple Sea Urchin Following Immune Challenge

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Introduction

The *Sp185/333* gene family is estimated to have 50(±10) members in the genome of *Strongylocentrotus purpuratus*, the California purple sea urchin [1]. The *Sp185/333* genes have two exons (Fig. 3): the first encodes the hydrophobic conserved leader while the second encodes the mature protein. The second exon is composed of a variety of repeats with 25-27 blocks of sequences called elements that are present in mosaic patterns resulting in 51 recognizable element patterns in 872 genes and messages [2]. Speculations on the underlying basis for the striking sequence diversity of the *Sp185/333* gene family have included gene conversion, duplication, deletion, recombination, and meiotic miss pairing [3]. The *Sp185/333* genes are significantly upregulated in response to marine bacteria and several pathogen associate molecular patterns (PAMPs) [4, 5]. They are expressed in coelomocytes, specifically subsets of the phagocytes [6]. *Sp185/333* genes from different tissues of different individuals have been cloned and sequenced [7]. Here we characterize the *Sp185/333* genetic repertoire of different tissues of the same sea urchin before and after challenge with heat-killed marine bacteria.

Methods

A new method for high-resolution detection of *Sp185/333* gene sizes and copy number ratios

The *Sp185/333* genes are characterized by having different sizes for different element patterns [4, 7]. However, the length differences may only be a few nucleotides, which can be difficult to detect by standard gel electrophoresis. In many cases there are a number of almost identical copies of the same *Sp185/333* gene in a genome which all result in a single band (amplicon). In order to improve our ability to identify different genes and gene copy numbers we have used a technique that is being regularly implemented to identify microsatellite length polymorphism in forensic biology and population genetics. We have used a fluorescent primer (R9-FAM) to amplify variable sequences of the *Sp185/333* genes and analyzed the amplicon length using ABI 3030 sequencer.

We tested the modified technique with amplicons from a previously sequenced BAC with a *Sp185/333* gene cluster [8] (**Fig. 1**). The results successfully predicted the number of genes as well as the number of gene copies (**Fig. 2**).

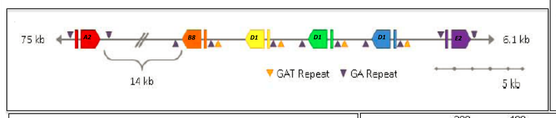
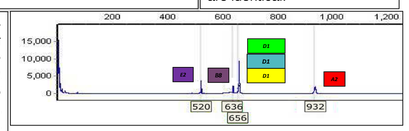


Fig. 1. (Left) The structure of *Sp185/333* gene cluster in sea urchin BAC clone R3-3033E12 (GenBank BK007096; [8]). Of the six genes, the three *D1* genes are identical.

Fig. 2. (Right) Fragment analysis of BAC clone R3-3033E12 predicts expected *Sp185/333* genes. Four gene sizes were identified reflecting the four gene sizes that were identified in this cluster (*E2, B8, D1* and *A2*; see **Fig. 1**). The peak height for amplicon 656 was approximately three times higher than the others, reflecting the three copies of this gene in this cluster. X axis; nucleotide length. Y axis; amplicon peak intensity.



Isolation, Extraction, Amplification, Analyze

a. Genomic DNA (gDNA) was extracted from sperm (S), Tube feet (T) and coelomocytes (C) of immunoquiescent sea urchin #13

b. Immunoquiescent sea urchin #13 was injected (challenged) with heat-killed *Vibrio diazo-trophicus* every day for three days (increasing amounts)

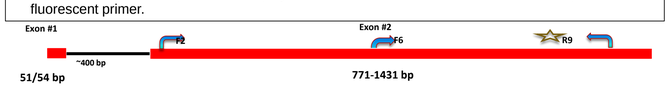
c. Coelomocytes were collected 14 days after challenge and gDNA was extracted using Qiagen kit with blood and tissue modified protocols

d. *Sp185/333* genes were amplified by PCR using F2/R9 and F6/R9-FAM primer sets with gDNA from sperm, tissues, pre- and post-challenge coelomocytes

e. Fluorescent amplicons were analyzed by fragment length analysis (ABI 3030 sequencer), revealing exact sizes and allele amounts for the *Sp185/333* genes

Primer Structure

Fig. 3. Exon structure of the *Sp185/333* genes. Two types of primer pairs were used in this study,
1. F2/R9: non-fluorescent primers to amplify most of the second exon.
2. F6/R9-FAM fluorescent primers to amplify the variable 3' end of the second exon. The represents a fluorescent primer.



Results

Different sea urchins have different genomic repertoires of the *Sp185/333* genes

To evaluate the genomic variability of the *Sp185/333* gene family in the sea urchin population, we extracted gDNA from seven different individuals. Similar to previous reports [1], no matches of *Sp185/333* amplification patterns were found among the animals (**Fig. 4**). There were variable differences in *Sp185/333* amplification patterns among all animals, which was revealed by fragment length analysis using sperm gDNA as the template.

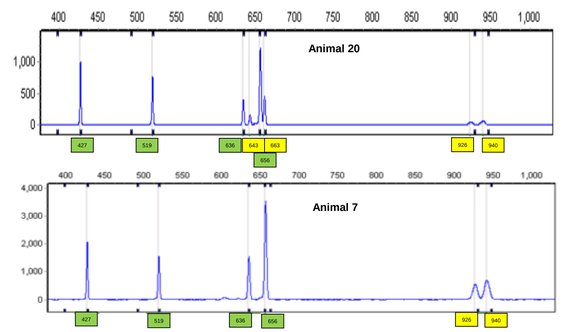


Fig. 4. (Above) *Sp185/333* gene repertoire of animal 20 (Top) compared to animal 7. There were six to nine *Sp185/333* amplicon sizes identified by fragment length analysis for each animal, of which only four were identical (labeled in green) while the other five were different in size (labeled in yellow).

A new gene size appeared in coelomocyte gDNA from sea urchin #13 after immune challenge

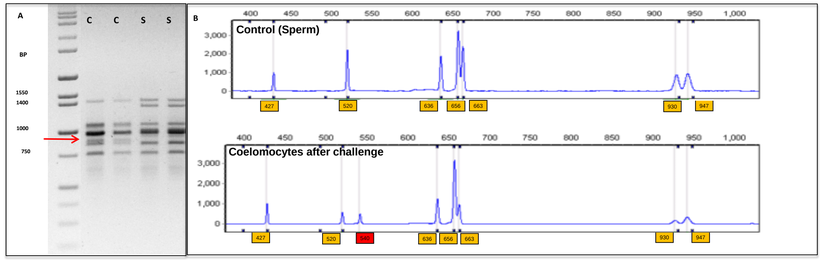
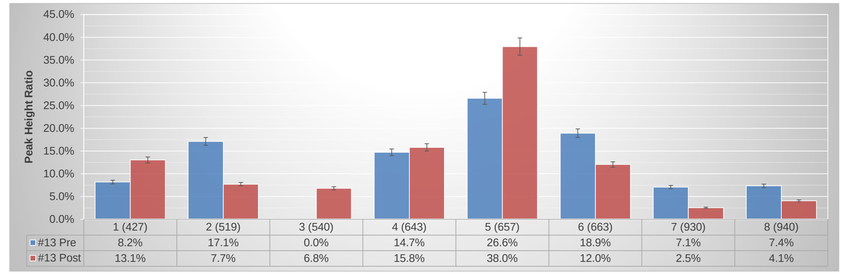


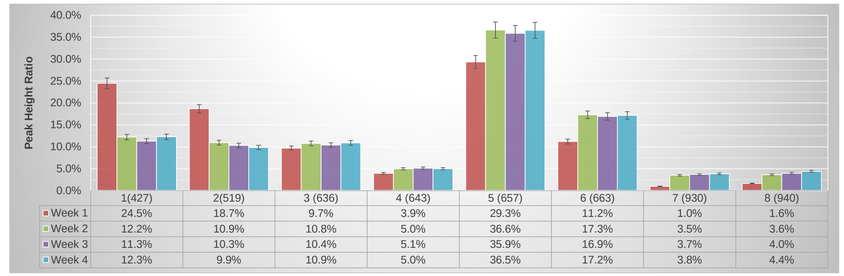
Fig. 5. A new *Sp185/333* gene size is present in coelomocytes after immune challenge. Animal #13 gDNA. **A.** Gel electrophoresis of F2/R9 *Sp185/333* amplicons shows the amplification patterns from sperm (S) and coelomocytes (C) after immune challenge. Red arrow indicates the new amplicon in coelomocytes that is not present in sperm. **B**. Fragment length analysis of R6/R9 fluorescent amplicons of the same samples. Sperm and coelomocytes share seven common amplicons. A new amplicon size (540 bp; indicated with red) is present in the coelomocyte sample after immune challenge. This was repeated 5-9 times with the same result.

Pre- and post-challenge coelomocyte populations have different *Sp185/333* gene copy number ratios



	1 (427)	2 (519)	3 (540)	4 (643)	5 (657)	6 (663)	7 (930)	8 (940)
#13 Pre	8.2%	17.1%	0.0%	14.7%	26.6%	18.9%	7.1%	7.4%
#13 Post	13.1%	7.7%	6.8%	15.8%	38.0%	12.0%	2.5%	4.1%

Figure 6 (Above). Allele Readout. ABI 3030 read intensities ratios and of Animal #13 pre- and post-challenge Amplicon read intensities were calculated based on the read peak heights. Intensity ratios were calculated for each of the pre-challenge ABI runs (n=8; in blue) and post-challenge runs (n=16; in red). The average peak height ratios represent the average percentage of the peak height from the total sum of all peak heights for each run. Error bars represent standard error values. Differences between pre- and post-challenged coelomocytes were found to be significant for each of the 8 amplicon sizes (ANOVA, p-values < 0.05) and for 16 amplicon sizes (MANOVA, p-values < 0.05)



	1(427)	2(519)	3 (636)	4 (643)	5 (657)	6 (663)	7 (930)	8 (940)
Week 1	24.5%	18.7%	9.7%	3.9%	29.3%	11.2%	1.0%	1.6%
Week 2	12.2%	10.9%	10.8%	5.0%	36.6%	17.3%	3.5%	3.6%
Week 3	11.3%	10.3%	10.4%	5.1%	35.9%	16.9%	3.7%	4.0%
Week 4	12.3%	9.9%	10.9%	5.0%	36.5%	17.2%	3.8%	4.4%

Figure 7 (Above). Allele Readout. ABI 3030 read intensities and ratios of Animal #20 week 1-4. Week 1 = week of challenge, week 2 = 1 week post challenge, week 3 = 2 weeks post challenge…. Similar to Figure 6, however, over 4 weeks instead of 2 weeks as was conducted on animal #13. Weeks 2,3,4 have a negligible difference between allelic ratio's (MANOVA, p-values >0.05). Weeks 1 and 2 have a drastic difference between allelic ratio's and have a significant difference between their results (AOVA, p-values < 0.05).

Discussion

The results shown in **Fig. 4** suggest that the *Sp185/333* genes have a very high genomic polymorphism and that the family may be very unstable.

After isolating gDNA from sperm, tube feet, and coelomocytes from sea urchin #13 at t=0 and t=2 weeks post injection with heat-killed *Vibrio diazotrophicus*, we unexpectedly identified a new amplicon size in coelomocytes after challenge (**Fig. 5**). This was repeated ~13 times using different samples of the same tissues, and all showed similar results. The new amplicon was confirmed as *Sp185/333* sequence after gel isolation.

Our fragment length analysis results for the sequenced *Sp185/333* BAC clone predicted accurately the ratios among the gene copy numbers (**Fig. 2**). Therefore, we used this method to estimate the proportions of different *Sp185/333* gene sizes in coelomocytes gDNA. In animal #13, significant changes in allele composition were identified (**Fig. 6**). These results correlate to differences in gene copy numbers; e.g. total cell populations may reflect different gene representations in single cells.

Not all animals displayed the same results. Certain animals, such as #1 and #6, did not show an immune response to the *Vibrio* challenge and did not generate a significant response between weeks 1-4, and thus no significant change in the *Sp185/333* gene sizes was detected. A larger amount of bacteria or possibly multiple/varying species of bacteria may be used in future analyses to ensure that the immunologically challenged animals become fully upregulated.

Future Directions

Further testing needs to be done on determining the threshold for what exactly is needed for the sea urchins to become fully challenged. Multiple pathogens may be used in further tests and possibly even live pathogens to test the limits on how far the sea urchin immune system can be pushed.

Acknowledgements

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