

## Intron size variation observed in a ribosomal protein gene among three *Diadema* species in the western North Pacific

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### Abstract

Exon-primed intron-crossing (EPIC) polymerase chain reaction (PCR) was applied to ribosomal protein genes of long-spined sea urchin species of the genus *Diadema*. Amplicon size differences among three species (*D. clarki*, *D. setosum*, and *D. savignyi*) were observed in 40S ribosomal protein S3, to which large indels in the intron were primarily responsible. This may be a simple and diagnostic nuclear DNA marker for hybridization study in *Diadema*.

**Key words:** EPIC-PCR; ribosomal protein gene; *Diadema*; intron size

Long-spined sea urchins of the genus *Diadema* are widespread and ecologically important species in tropical to temperate areas. Four *Diadema* species are known from the shallow coastal areas of the Japanese archipelago (Lessios et al. 2001; Chow et al. 2014, 2016), of which *Diadema setosum* is the most abundant and widespread species, ranging from subtropical areas such as the Ryukyu Islands to the temperate zone of mainland of Japan. *Diadema savignyi* and a rare *D. paucispinum* are scarce in the temperate zone, while *D. clarki* is abundant in the temperate zone. *Diadema setosum* and *D. savignyi* are sympatric in the Ryukyu Islands, as are *D. setosum* and *D. clarki* in the temperate zone. The co-occurrence of these three species is limited in the southern temperate zone (Chow et al. 2016). Lessios and Pearse (1996) first raised the question of hybridization in *Diadema* species. Although the temporal reproductive isolation between *D. setosum* and *D. savignyi* has been observed (Muthiga 2003), the temporal difference is not perfect. Natural hybrids between *D. setosum*, *D. savignyi*, and *D. paucispinum* were detected using allozyme assay (Lessios and Pearse 1996). Laboratory experiments showed that gametes of *D. setosum* and *D. savignyi* are

capable of fertilizing each other and of producing viable hybrids (Uehara et al. 1990). To further study genetic exchange and introgression between *Diadema* species, it is necessary to develop convenient nuclear DNA markers instead of the cumbersome and no longer available allozyme assay. However, the binding gene locus is currently the only nuclear DNA marker for hybridization study in *Diadema* species (Geyer et al. 2020; Lessios 2024).

Table 1. Nine individuals of three *Diadema* species collected in Japan and used in this study. Individuals marked with an asterisk were used for nucleotide sequencing.

ID	species	collection	
		locality	date
DSV10*	<i>D. clarki</i>	Iki Is., Nagasaki	Feb. 2012
DSV14	<i>D. clarki</i>	Iki Is., Nagasaki	Feb. 2012
DSV17	<i>D. clarki</i>	Iki Is. Nagasaki	Feb. 2012
DST1	<i>D. setosum</i>	Yokosuka, Kanagawa	Dec. 2011
DST3	<i>D. setosum</i>	Yokosuka, Kanagawa	Dec. 2011
DST7*	<i>D. setosum</i>	Yokosuka, Kanagawa	Dec. 2011
DSV20	<i>D. savignyi</i>	Ishigaki Is., Okinawa	Oct. 2013
DSV22	<i>D. savignyi</i>	Ishigaki Is., Okinawa	Oct. 2013
DSV24*	<i>D. savignyi</i>	Ishigaki Is., Okinawa	Oct. 2013

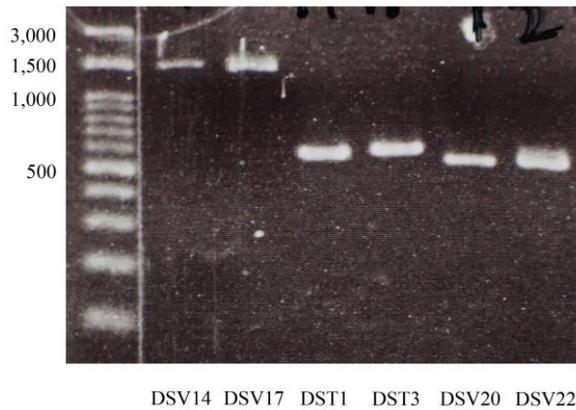


Fig. 1. Agarose gel electrophoresis profile of amplified 40S ribosomal protein S3 gene fragments of *Diadema clarki* (DSV14 and 17), *D. setosum* (DST1 and 3), and *D. savignyi* (DSV20 and 22). The leftmost lane is a DNA marker with the size (in bp) indicated at the left margin.

We performed PCR amplification for three *Diadema* species (*D. clarki*, *D. setosum*, and *D. savignyi*) (Table 1) using 19 primer pairs for 12 ribosomal protein genes (Chow et al. 2015). Species of these individuals used were identified using mitochondrial COI gene sequence analysis in previous study (Chow et al. 2014). PCR mixtures were preheated to 94 °C for 4 min, followed by 30 amplification cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 50 s), with a final extension at 72 °C for 7 min. Single fragment amplification and amplicon size differences between species were observed in one primer pair (RPS3ex3F and RPS3ex4R) targeting the 3rd intron of 40S ribosomal protein S3 gene (Fig. 1). Size of the amplicons estimated from gel electrophoresis was c.a. 1,500 bp for *D. clarki* (DSV14 and 17), c.a. 550 bp for *D. setosum* (DST1 and 3), and c.a. 500 bp for *D. savignyi* (DSV20 and 22). The PCR products from the other individuals of these species (Fig. 2) were cloned using a DynaExpress TA PCR Cloning Kit (BioDynamics Laboratory Inc.). Colony-direct PCR was performed using M13 primers, and the PCR products were treated with ExoSAP-IT (GE Healthcare) to remove PCR primers, purified and subjected to nucleotide sequence analysis using M13 primers and those used for the initial PCR

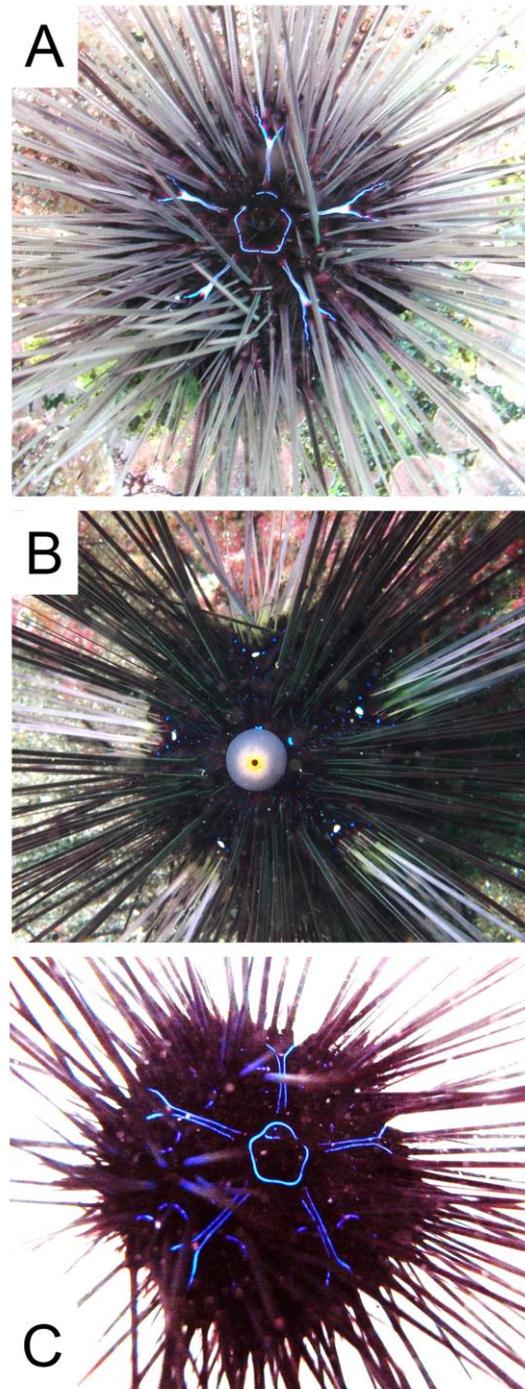


Fig. 2. Underwater aboral view of three *Diadema* species. A: *D. clarki* (DSV10), B: *D. setosum* (DST7), C: *D. savignyi* (DSV24). See Table 1 for sample information.

amplification. Nucleotide sequences determined are available in DDBJ/EMBL/GenBank database (LC861745–LC861747), and the alignment is presented in Fig. 3. The amplicon sequence consisted of the 3' region of exon 3, the entire intron 3, and the

exon 3 ←

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D. clarki      GTTCAACTTTGCTGAGAGCACTGTCGAGGTATTGTTGTGGAAAAAGACCCATGATCTATGCATTGTTGTATGAAATTTTC      80
D. setosum    .....C.....TT.....C..G.....T...G
D. savignyi   .....T.....G.....T.....

D. clarki      -AATTCTGAAGAAAGAGGAGTTTATGGTGAAAGTCTTTATTGGTATCTCACAGGGCTT-ATTAGGCCTACCTTTCATCT      160
D. setosum    T..C.T...T.....A...G.....AC.....A..A...T.....A..G..C..C..C
D. savignyi   -.....A.....CG..C.....-.....G..C.....C

D. clarki      ATCAGAGTGCAATGCATGCATTGTGTGATGCCACAATATTGATTCCATAATGAAGGAACACCAACTCATTGCAGTTTCAT      240
D. setosum    .....A.....T.....TC.....AT-.T.T.T.....
D. savignyi   G.....T..A.....TC.....AAT-.T.TGT.....A..

D. clarki      ACCTGTCATGGTTACTGCCTCCCAATTCATGTCCTAATACCGTAAATGTGGATATTTTCGCGCGACTAATTTTTCGCGCT      320
D. setosum    .....TT.....G.....T.G.....
D. savignyi   .....TT..A.....G.....G.G.....

D. clarki      TGGCCGGGTGGAAGAGTTTCGCGTGTTTTTAATCCGCGGAATCAAGACATTACACACAGGCACATATGGCAAGCAAAA      400
D. setosum    -----
D. savignyi   -----

D. clarki      ATATTCGCGTGCTTTTATTTTCGCGCTAGTTTCTGGTTGCGCGAAAATTTGAACAACGCAAAAATTTCAACTTTTACAGT      480
D. setosum    -----
D. savignyi   -----

D. clarki      AGATGGGAAGAATGTGAAATGTATAATGTTCTTAGAGACCTTAACCCTAAAATGGTGGGGTTTGTGAATCAACCCCCC      560
D. setosum    -.....A...T.....C...T...ATGC-----
D. savignyi   -..T.....T.....C.....C..A...-----

D. clarki      CCCCCTCGACATTTTCCATACCATTCCCTCCCGGAATTTTTTTGACTACACCGCTTGCTGACTTTTAAAGTCTTGACACA      640
D. setosum    -----
D. savignyi   -----

D. clarki      CTTATTTATGACACCATTTTCGTGAAAATCTGTCATACCTACAGTATACGACAATGCACGACCTTTTATACATACTTTTC      720
D. setosum    -----
D. savignyi   -----

D. clarki      AGACCGAAAAACATGTTTTTGTGTACAAAGTCCATACAAATGGAGTTTTTCTCACTTTATTCAAAGATATGATTATTTTCA      800
D. setosum    -----
D. savignyi   -----

D. clarki      CTTTCATTGGCTGAAATGAATTGATTTTAGCATTTATAATCATGCTTGAAGTAGACTCTGTCTTAAATCTCGCGGGGAAA      880
D. setosum    -----
D. savignyi   -----

D. clarki      AAATGACAAAAATAAAATGTTGAAAACAGAAATGCATAAGAAATTCATAAAAACAATACAATACATTAGAAAATAACTTTG      960
D. setosum    -----
D. savignyi   -----

D. clarki      ATACTAGATTTTTTTTTTCAAGTACATGGTTAGGATTACTACAAAGGACATTATCGCCAAATATTAGGGTTCTAGGGGCT      1040
D. setosum    -----
D. savignyi   -----

D. clarkii    TTACTTTCTGATTTAGAGCAAAATGTATGCTTTCATGCATATTAGCGTTATTACTTAAAAATTATATGGTTTTGTACAGT      1120
D. setosum    -----
D. savignyi   -----

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Fig. 3. Nucleotide sequence alignment of partial 40S ribosomal protein S3 gene of three *Diadema* species. Nucleotides identical with those of the top sequence are shown by dots and gap is shown by dash. Exon regions are shown in gray.

1200  
*D. clarki* TTGGTAGATTACGCCACAGGTAGTGTATGTGCCAATTTTCATGGCGATTGCGCTATCGACGCCAAGATCTCGGAGGGGTT  
*D. setosum* -----  
*D. savignyi* -----

1280  
*D. clarki* GTTTC AACCCCTCCCCGGCCACAGAACAGCCAAAAAGCCCGGCTGATTGCGGTTAAATGTGACCAGCAGAAGTCCTGA  
*D. setosum* -----T...A.....  
*D. savignyi* -----T.A.A.....

1360  
*D. clarki* CTTTTATCT-----AAATATAGTAAAGAGGATGAAAAGACATTTTCCATGAAT-----  
*D. setosum* ....T-.TATTAGAAATTGTTAAAAGTG..GT...C.....GG...C...-G.....TTATTA AAAAC  
*D. savignyi* T...T...TATTAGAAATTGTTAAAAGTG..GT.....A.--.GG-----

1440  
*D. clarki* -----TTATGACAACTAGCTTGGA  
*D. setosum* TGGCTTGAAAGACTGAGAAAAGTTACGTGTAGATGTTTTAACAG..CTC.CTTAGCAGTG.C.....G...GA.A.---  
*D. savignyi* -----TAGATGTTTTAACAG..C.CCCATAGCAGTG.CG.....G...GA.A.---

1501  
*D. clarki* AGACCCAGAAATGTGGAGGTCTTTTCAACTAACTTATCCTTGGGTTCTATGTCCGCAGCTC  
*D. setosum* ----.TG.....T.....  
*D. savignyi* ----.A.....T...A...T.....  
→ exon 4

Fig. 3. continued.

3' region of exon 4. The length of the nucleotide sequence excluding primer sequences was 1,408 bp for *D. clarki* (DSV10), 552 bp for *D. setosum* (DST7), and 496 bp for *D. savignyi* (DSV24), corresponding to the amplicon size estimated from gel electrophoresis. Large indels in the intron were primarily responsible for the difference in amplicon size between species, which may be a simple and diagnostic nuclear DNA marker for *Diadema*. The feasibility of this nuclear DNA marker for hybridization studies in *Diadema* needs to be verified with a much larger number of individuals.

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