Aquatic Animals

水生動物 第 2025 巻
令和 7 年 2 月

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

Seinen Chow[1, 2]*, Takashi Yanagimoto[3]

[1]Fisheries Technology Institute, 2-12-4 Fukuura, Yokohama, Kanagawa 236-8648, Japan, [2]Aquos Institute, 3-2153-79 Motohachioji, Hachioji, Tokyo 193-0826, Japan, [3]Fisheries Resources Institute, 2-12-4 Fukuura, Yokohama, Kanagawa 236-8648, Japan.

*Corresponding author, e-mail: kaiyoeell@gmail.com

**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 bindin 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* | *D. clarki* | Iki Is., Nagasaki | Feb. 2012 |
| DSV14 | *D. clarki* | Iki Is., Nagasaki | Feb. 2012 |
| DSV17 | *D. clarki* | Iki Is. Nagasaki | Feb. 2012 |
| DST1 | *D. setosum* | Yokosuka, Kanagawa | Dec. 2011 |
| DST3 | *D. setosum* | Yokosuka, Kanagawa | Dec. 2011 |
| DST7* | *D. setosum* | Yokosuka, Kanagawa | Dec. 2011 |
| DSV20 | *D. savignyi* | Ishigaki Is., Okinawa | Oct. 2013 |
| DSV22 | *D. savignyi* | Ishigaki Is., Okinawa | Oct. 2013 |
| DSV24* | *D. savignyi* | Ishigaki Is., Okinawa | Oct. 2013 |

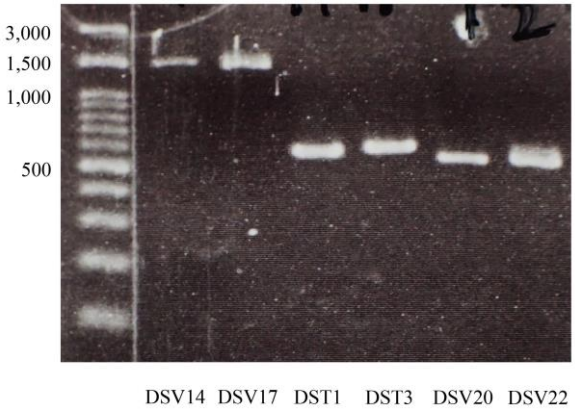


Fig. 1. Agarose gel electrophoresis profile of amplified 40S ribosomal protein S3 gene fragments of *Diadema clarki* (DSV14 and 17), *D. setosum* (DST1 and 3), and *D. savignyi* (DSV20 and 22). The leftmost lane is a DNA marker with the size (in bp) indicated at the left margin.

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

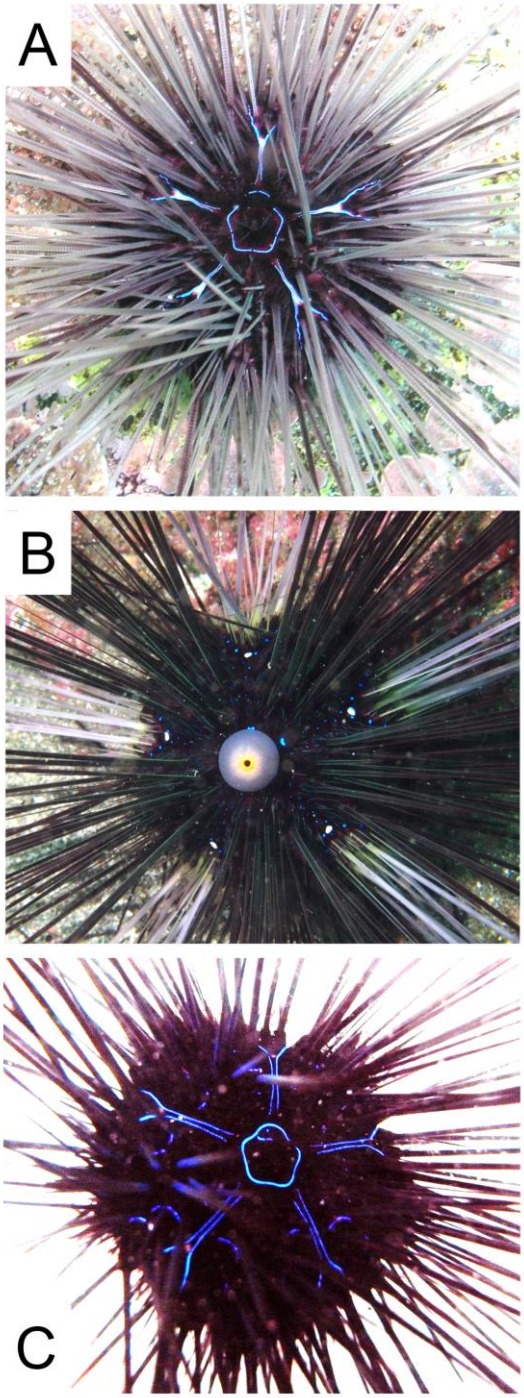


Fig. 2. Underwater aboral view of three *Diadema* species. A: *D. clarki* (DSV10), B: *D. setosum* (DST7), C: *D. savignyi* (DSV24). See Table 1 for sample information.

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

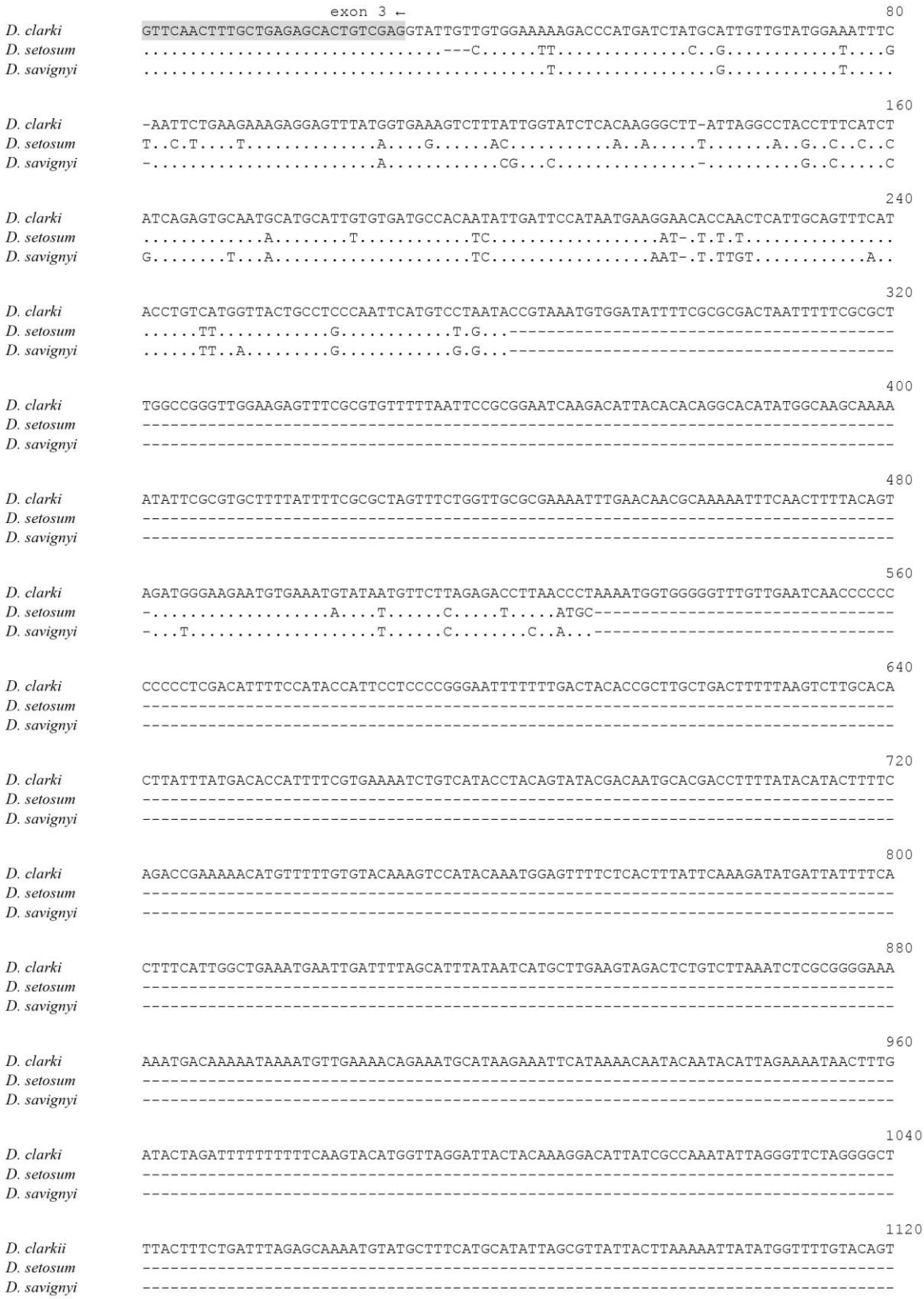


Fig. 3. Nucleotide sequence alignment of partial 40S ribosomal protein S3 gene of three *Diadema* species. Nucleotides identical with those of the top sequence are shown by dots and gap is shown by dash. Exon regions are shown in gray.


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.

## Acknowledgment

We thank Dr. Philippe Borsa, Institut de recherche pour le development, for reading and improving the manuscript. This work was supported by the Japan Fisheries Research and Education Agency.

## References

Chow, S., Kajigaya, Y., Kurogi, H., Niwa, K., Nanami, A., Kiyomoto, S. (2014). On the fourth *Diadema* species (*Diadema*-sp) from Japan. PLoS ONE 9: e102376.

Chow, S., Yanagimoto, T., Nakamura, Y. (2015). Universal primers for exon-priming intron-crossing (EPIC) PCR on ribosomal protein genes in marine animals. Int. Aquat. Res. 7: 245–250.

Chow, S., et al. (2016). DNA barcoding and morphological analyses revealed validity of *Diadema clarki* Ikeda, 1939 (Echinodermata, Echinoidea, Diadematidae). ZooKeys 585: 1−16.

Geyer, L. B., Zigler, K. S., Tiozzo, S., Lessios, H. A. (2020). Slow evolution under purifying selection in the gamete recognition protein bindin of the sea urchin *Diadema*. Sci. Rep. 10: 9834.

Lessios, H. A., Pearse, J. S. (1996). Hybridization and introgression between Indo-Pacific species of *Diadema*. Mar. Biol. 126: 715−723.

Lessios, H. A., Kessing, B. D., Pearse, J. S. (2001). Population structure and speciation in tropical seas: global phylogeography of the sea urchin *Diadema*. Evolution 55: 955−975.

Lessios, H. A. (2024). Introgression of the gamete recognition molecule, bindin, in the sea urchin *Diadema*. Integr. Comp. Biol. 64: 1578−1585.

Muthiga, N. A. (2003). Coexistence and reproductive isolation of the sympatric echinoids *Diadema savignyi* Michelin and *Diadema setosum* (Leske) on Kenyan coral reef. Mar. Biol. 143: 669−677.

Uehara, T., Asakura, H., Arasaki, Y. (1990). Fertilization blockage and hybridization among species of sea urchins. In: M. Hoshi, O. Yamashita (Eds.) Advances in Invertebrate Reproduction, Elsevier, Amsterdam, p. 305−310.

Received: 23 January 2025 | Accepted: 5 February 2025 | Published: 10 February 2025