

## A natural hybrid limpet between *Cellana nigrolineata* and *Cellana grata grata* (Gastropoda: Nacellidae) from Shikoku, Japan

Kōji Yokogawa\*

13-5 Higashihama, Tadotsu-cho, Nakatado-gun, Kagawa 764-0016, Japan

**Abstract.** A limpet specimen from Shikoku, Japan, having an appearance intermediate between *Cellana nigrolineata* and *C. grata grata*, were conchologically and genetically examined. The hybrid-like specimen had intermediate proportions between the two species at most of shell regions, and principal component analysis of the conchological data placed the specimen in the border between the two species. Genetic examination of isozyme analysis showed complete or almost complete replacements of alleles between the two species at the *AAT-1\**, *AAT-2\**, *ACP-3\**, *AH-2\**, *AK-1\**, *FBALD\**, *G3PDH\**, *GPI\**, *IDDH\**, *IDHP-1\**, *LAP-3\**, *LDH-1\**, *MDH-1\**, *MPI\**, and *PGDH\** loci, where the hybrid-like specimen were wholly heterozygous for the major alleles of both species. Also, average values of individual genetic distance between the hybrid-like specimen and the *C. nigrolineata* and *C. grata grata* specimens resulted in 1.178 and 1.146, respectively, being almost equivalent. The results of the conchological and genetic examinations concluded that the hybrid-like specimen is a F<sub>1</sub> hybrid between *C. nigrolineata* and *C. grata grata*.

**Key words:** limpet, *Cellana nigrolineata*, *Cellana grata grata*, hybrid, shell, genetics, isozyme

### Introduction

Nacellid limpets of *Cellana nigrolineata* (Reeve, 1839) (Fig. 1D–F) and *Cellana grata grata* (Gould, 1859) (taxonomic view provisionally followed Habe, 1975) (Fig. 1G–I) commonly occur in coastal rocky areas around Japan and the Korean Peninsula (Sasaki, 2000). During field sampling for the author's study series on nacellids (Yokogawa, 2014a, b), a specimen with an appearance that was intermediate between these two species (Fig. 1A–C) was collected from Naruto, Shikoku, Japan. Therefore, the present study compared this hybrid-like specimen conchologically and genetically with *C. nigrolineata* and *C. grata grata*.

### Materials and methods

**Specimens examined.** The hybrid-like specimen (Fig. 1) was collected alive from the rocky coast of Naruto, Tokushima Pref., Japan (34°10'38"N, 134°38'26"E), on 15th December 1997. A shell (dried) and soft part (ethanol-preserved) of the specimen was deposited in the Natural History Museum and Institute, Chiba (catalogue no. CBM-ZM 164554). Control materials of *Cellana nigrolineata* (64 specimens) and *C. grata grata* (45 specimens) were collected from the rocky coast of Hiwasaura, Tokushima Pref., Japan (33°43'59"N, 134°32'27"E), on 21st November 1997. The former were the same specimens which were included in materials of the former study (Yokogawa, 2014a).

**Conchological examinations.** A shell of each individual was removed and several portions (Fig. 2) and shell weight (SW) were measured. The projec-

---

\*Corresponding author: gargariscus@ybb.ne.jp

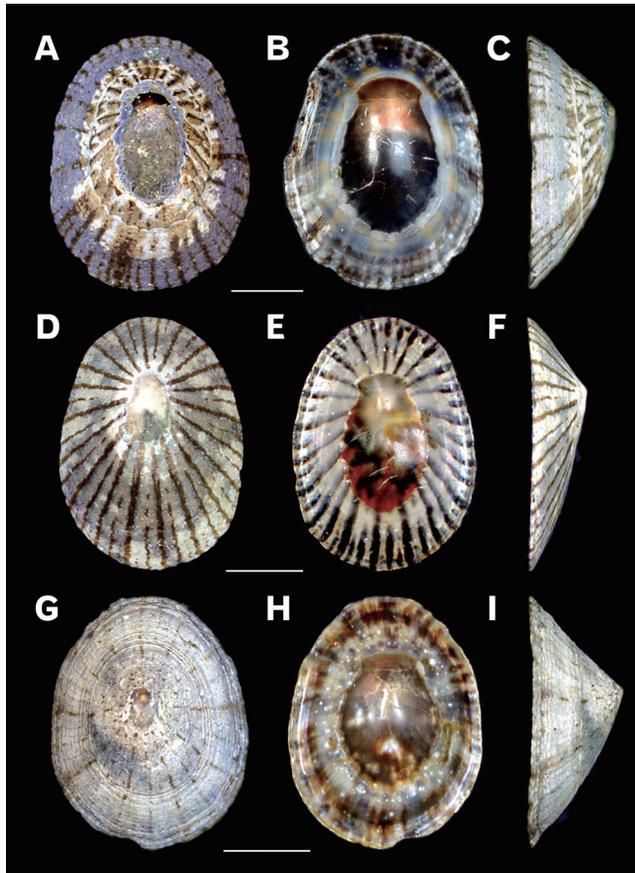


Fig. 1. Views of *Cellana nigrolineata* (Radially striped form) (D–F), *C. grata grata* (G–I), and the hybrid-like specimen between the two species (CBM-ZM 164554) (A–C). A, D, G – Apical views; B, E, H – Ventral views; C, F, I – Lateral views. Bars indicate 10 mm.

tive pre-apex length (PPRAL), projective post-apex length (PPOAL), and apex angle (AA) were calculated using the trigonometric functions (Yokogawa, 2014b). Because some specimens from *C. nigrolineata* and the hybrid-like specimen have radial stripes (RS) on their shell surface, the stripes were wholly counted.

Relationships between shell length (SL) and the shell portion dimensions were expressed by plotting graphs. Because most of the shell proportions and RS counts change with growth in nacellid shells (Yokogawa, 2014b), SL and other dimensions as well as RS counts were transformed into natural logarithms. However, because PPRAL / PPOAL proportion was

stable in *C. nigrolineata* and *C. grata grata* (Yokogawa, 2014b), they were not transformed.

In order to evaluate the conchological data analytically, principal component analysis by the usual method (Arima & Ishimura, 1997) was introduced. For the analysis, although all measured items shown in Fig. 2 plus shell weight were adopted as variates, shell length (SL) was deleted because it was the sum of PPRAL and PPOAL (Fig. 2). In addition, the measured dimension values were transformed into natural logarithms because most of the shell proportions change with growth in nacellid shells (Yokogawa, 2014b).

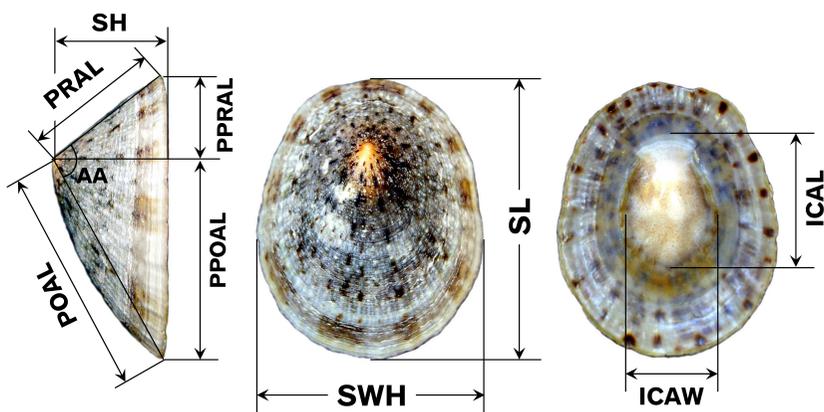


Fig. 2. Measured portions of shell. SL – Shell length, SWH – Shell width, SH – Shell height, PRAL – Pre-apex length, POAL – Post-apex length, PPRAL – Projective pre-apex length, PPOAL – Projective post-apex length, AA – Apex angle (degrees), ICAL – Internal colored area length, ICAW – Internal colored area width.

Table 1. Shell dimensions of the hybrid-like specimen (CBM-ZM 164554) (abbreviations are referred to Fig. 1 and the text).

|              |       |
|--------------|-------|
| SL (mm)      | 34.5  |
| SD (mm)      | 26.8  |
| SH (mm)      | 10.6  |
| PRAL (mm)    | 15.0  |
| PROL (mm)    | 27.0  |
| PPRAL (mm)   | 9.9   |
| PPROL (mm)   | 24.6  |
| ICAL (mm)    | 21.0  |
| ICAW (mm)    | 13.3  |
| AA (degrees) | 107.0 |
| SW (g)       | 2.7   |

**Genetic examinations.** Isozymes detected by horizontal starch-gel electrophoresis were adopted as genetic markers. The isozyme analysis was performed for all (64) specimens in *C. nigrolineata* and for 33 of 45 specimens in *C. grata grata*. Soft parts of the collected specimens were preserved in a freezer at  $-80\text{ }^{\circ}\text{C}$  prior to analysis. Using the methodologies of Yokogawa (1999), electrophoresis was performed to examine isozymes. Loci in esterases (*EST-1\**, *EST-2\**, *EST-3\** and *EST-5\**) detected in the former study (Yokogawa, 2014a) were omitted for the analysis because homology of loci could not

be identified from multi-banded electrophoretograms between *C. nigrolineata* and *C. grata grata*. Furthermore, the *LDH-2\** locus detected in *C. nigrolineata* (Yokogawa, 2014a) was also omitted because it was not detected in *C. grata grata*.

The gene nomenclature followed Shaklee *et al.* (1990), and the alleles were symbolized as relative mobility percentages compared with the most dominant alleles in *C. nigrolineata* (*\*100* in the anodal zone, *\*-100* in the cathodal zone) at each locus. Genetic relatedness between individuals ( $R_{xy}$ ) by samples was calculated following Queller & Goodnight (1989), and converted into the distance between individuals ( $Di$ ) following Noguchi *et al.* (2003) ( $Di = 1 - R_{xy}$ ).

## Results

**Conchological characters.** Measured and calculated shell dimensions of the hybrid-like specimen are shown in Table 1. Relationships between shell length and other dimensions and proportions showed divergent distribution patterns between *Cellana nigrolineata* and *C. grata grata* for the characters shown in Fig. 3A–E, and plots of the hybrid-like

specimen fell midway between the plot clusters of the two species.

In principal component analysis, the first and sec-

ond principal components explained 78.6 and 14.7% (total 93.3%) of the total variance, respectively.

Regarding distribution graphs plotted with PC-1 and

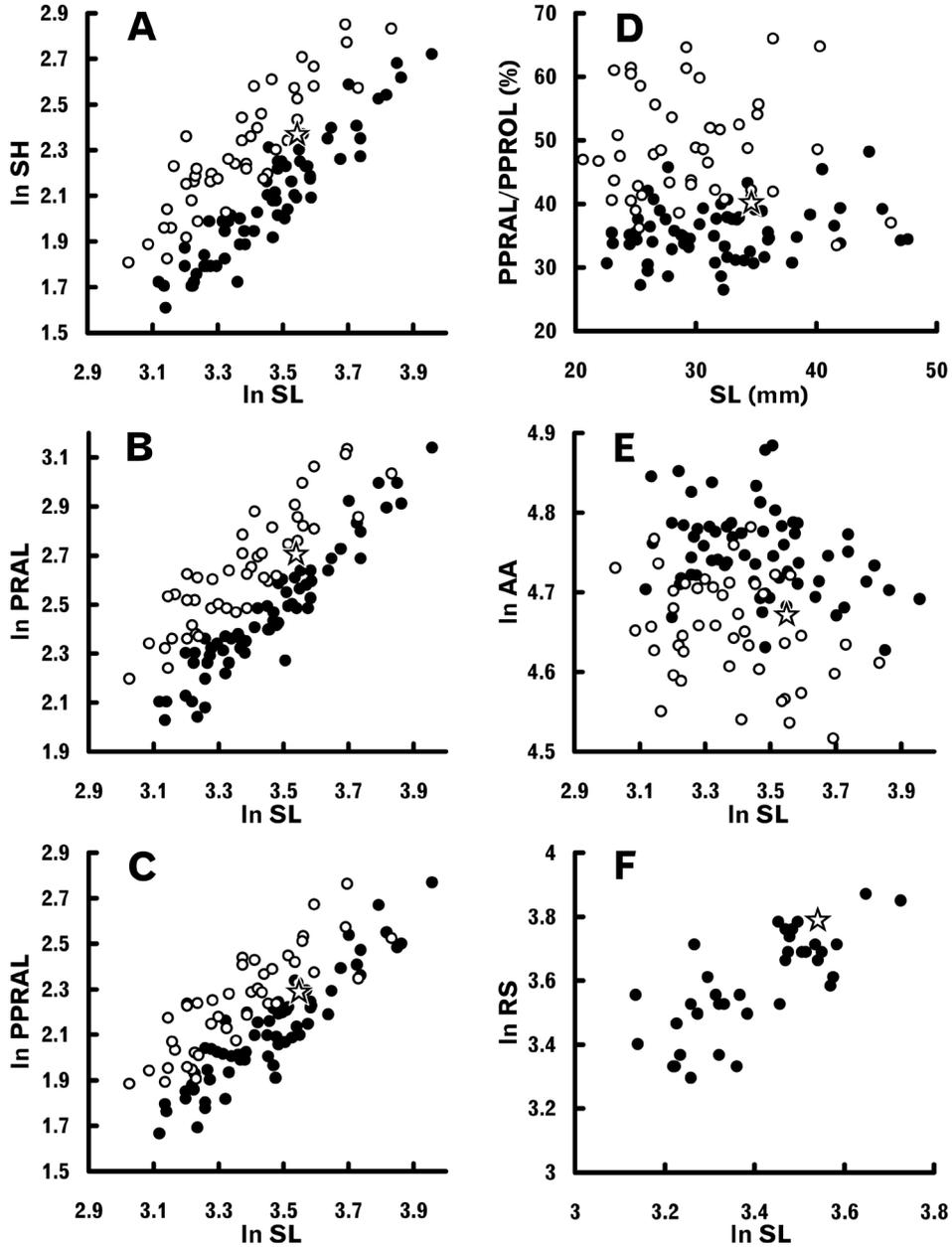


Fig. 3. Relationships between shell length (SL) and shell portion dimensions in *Cellana nigrolineata*, *C. grata grata* and the hybrid-like specimen (CBM-ZM 164554). Abbreviations other than radial stripes (RS) are referred to Fig. 2. Dark circles – *C. nigrolineata*, Open circles – *C. grata grata*, Star mark – CBM-ZM 164554.

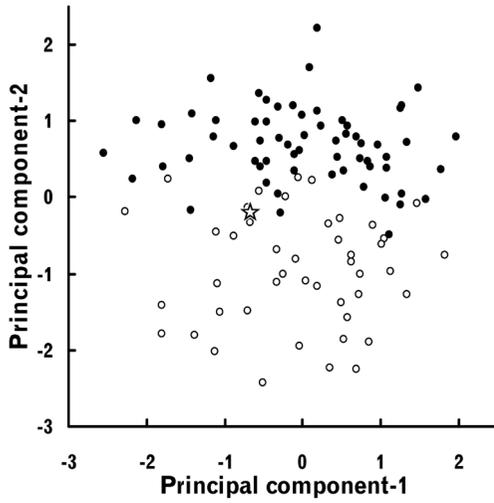


Fig. 4. Result of principal component analysis for *Cellana nigrolineata*, *C. grata grata* and the hybrid-like specimen (CBM-ZM 164554): distribution graph plotted with principal component scores 1 and 2 for conchological data. Dark circles – *C. nigrolineata*. Open circles – *C. grata grata*, Star mark – CBM-ZM 164554.

PC-2 scores, plots of *C. nigrolineata* and *C. grata grata* specimens were clustered almost separately from each other, and the plot of the hybrid-like specimen fell around the border between the two clusters (Fig. 4). These facts indicated that the shell morphology of the hybrid-like specimen was quite intermediate between the two species.

On the other hand, the plot of the radial stripe counts of the hybrid-like specimen fell within the plot cluster of *C. nigrolineata* (Fig. 3F). Furthermore, the internal colored area of the hybrid-like specimen was dark brown with light orange (Fig. 1B), which is peculiar to *C. grata grata* (Yokogawa, 2014b).

**Genetic characters.** The isozyme analyses detected 32 loci common to *C. nigrolineata* and *C. grata grata*; among these, alleles had completely or almost completely replaced each other between the two species at 15 loci: *AAT-1\**, *AAT-2\**, *ACP-3\**, *AH-2\**, *AK-1\**, *FBALD\**, *G3PDH\**, *GPI\**, *IDDH\**, *IDHP-1\**, *LAP-3\**, *LDH-1\**, *MDH-1\**, *MPI\** and

Table 2. Allelic frequencies of *Cellana nigrolineata* and *C. grata grata*, and genotypes of the hybrid-like specimen (CBM-ZM 164554).

| Locus          | Allele | Allelic frequency      |                       | Genotype of CBM-ZM 164554 |
|----------------|--------|------------------------|-----------------------|---------------------------|
|                |        | <i>C. nigrolineata</i> | <i>C. grata grata</i> |                           |
| <i>AAT-1*</i>  | *170   |                        | 0.015                 | *100/*130                 |
|                | *130   | 0.078                  | 0.985                 |                           |
|                | *100   | 0.914                  |                       |                           |
|                | *85    | 0.008                  |                       |                           |
| <i>AAT-2*</i>  | *30    | 0.016                  | 1.000                 | *-100/*-30                |
|                | *-100  | 0.984                  |                       |                           |
| <i>ACP-1*</i>  | *100   | 1.000                  | 1.000                 | *100/*100                 |
| <i>ACP-2*</i>  | *175   |                        | 0.419                 | *50/*100                  |
|                | *160   | 0.040                  | 0.016                 |                           |
|                | *100   | 0.540                  | 0.371                 |                           |
| <i>ACP-3*</i>  | *50    | 0.421                  | 0.194                 | *-100/*-85                |
|                | *-85   |                        | 1.000                 |                           |
|                | *-100  | 0.883                  |                       |                           |
| <i>AH-1*</i>   | *-135  | 0.117                  |                       | *100/*100                 |
|                | *100   | 1.000                  | 1.000                 |                           |
| <i>AH-2*</i>   | *-65   |                        | 1.000                 | *-100/*-65                |
|                | *-100  | 1.000                  |                       |                           |
| <i>AK-1*</i>   | *115   |                        | 1.000                 | *100/*115                 |
|                | *100   | 1.000                  |                       |                           |
| <i>AK-2*</i>   | *100   | 1.000                  | 1.000                 | *100/*100                 |
|                | *-100  | 1.000                  | 1.000                 |                           |
| <i>AK-3*</i>   | *-100  | 1.000                  | 1.000                 | *-100/*-100               |
|                | *100   | 0.813                  | 0.875                 |                           |
| <i>ALP*</i>    | *-150  | 0.156                  | 0.094                 | *-150/*-100               |
|                | *-200  | 0.031                  | 0.031                 |                           |
|                | *-100  | 0.983                  | 1.000                 |                           |
|                | *-200  | 0.017                  |                       |                           |
| <i>FBALD*</i>  | *175   |                        | 0.227                 | *100/*135                 |
|                | *135   |                        | 0.773                 |                           |
|                | *100   | 1.000                  |                       |                           |
| <i>G3PDH*</i>  | *100   | 0.984                  |                       | *65/*100                  |
|                | *65    | 0.016                  | 0.955                 |                           |
|                | *50    |                        | 0.045                 |                           |
| <i>GPI*</i>    | *100   | 1.000                  |                       | *75/*100                  |
|                | *75    |                        | 1.000                 |                           |
| <i>IDDH*</i>   | *200   |                        | 0.929                 | *100/*200                 |
|                | *180   | 0.347                  |                       |                           |
| <i>IDHP-1*</i> | *100   | 0.621                  | 0.071                 | *50/*100                  |
|                | *0     | 0.032                  |                       |                           |
|                | *100   | 1.000                  |                       |                           |
| <i>IDHP-2*</i> | *50    | 1.000                  |                       | *100/*100                 |
|                | *140   | 0.008                  |                       |                           |
| <i>IDHP-3*</i> | *100   | 0.992                  | 1.000                 | *100/*100                 |
|                | *100   | 1.000                  | 0.985                 |                           |
|                | *50    |                        | 0.015                 |                           |
| <i>LAP-1*</i>  | *120   |                        | 0.045                 | *100/*120                 |
|                | *100   | 1.000                  | 0.955                 |                           |
| <i>LAP-2*</i>  | *100   | 0.786                  | 0.929                 | *100/*100                 |
|                | *80    | 0.214                  | 0.071                 |                           |
| <i>LAP-3*</i>  | *100   | 1.000                  |                       | *60/*100                  |
|                | *60    |                        | 1.000                 |                           |
| <i>LDH-1*</i>  | *100   | 0.992                  |                       | *80/*100                  |
|                | *80    | 0.008                  | 0.985                 |                           |
|                | *60    |                        | 0.015                 |                           |
| <i>LDH-3*</i>  | *100   | 1.000                  | 1.000                 | *100/*100                 |
|                | *120   | 0.023                  | 0.182                 |                           |
| <i>MDH-1*</i>  | *100   | 0.977                  |                       | *85/*100                  |
|                | *85    |                        | 0.803                 |                           |
|                | *75    |                        | 0.015                 |                           |
|                | *100   | 1.000                  | 1.000                 |                           |
| <i>MDH-2*</i>  | *100   | 1.000                  | 1.000                 | *100/*100                 |
|                | *200   |                        | 0.030                 |                           |
| <i>MEP-1*</i>  | *150   |                        | 0.030                 | *100/*150                 |
|                | *100   | 1.000                  | 0.939                 |                           |
|                | *0     |                        | 0.348                 |                           |
| <i>MEP-2*</i>  | *-100  | 0.945                  | 0.621                 | *-150/*-100               |
|                | *-150  | 0.047                  | 0.030                 |                           |
|                | *-200  | 0.008                  |                       |                           |
|                | *130   | 0.039                  |                       |                           |
| <i>MPI*</i>    | *100   | 0.617                  |                       | *15/*35                   |
|                | *35    | 0.344                  |                       |                           |
|                | *15    |                        | 1.000                 |                           |
| <i>PGDH*</i>   | *-10   |                        | 0.924                 | *-100/*-10*               |
|                | *-100  | 0.992                  | 0.076                 |                           |
|                | *-175  | 0.008                  |                       |                           |
| <i>PGM-1*</i>  | *120   |                        | 0.045                 | *110/*120                 |
|                | *110   | 0.016                  | 0.227                 |                           |
|                | *100   | 0.381                  | 0.455                 |                           |
|                | *85    | 0.484                  | 0.197                 |                           |
|                | *75    | 0.071                  | 0.045                 |                           |
| <i>PGM-2*</i>  | *65    | 0.048                  | 0.030                 | *100/*100                 |
|                | *110   | 0.080                  | 0.047                 |                           |
|                | *100   | 0.900                  | 0.922                 |                           |
|                | *90    | 0.020                  | 0.031                 |                           |
|                |        |                        |                       |                           |

*PGDH\** (Table 2). At these loci, the hybrid-like specimen was wholly heterozygous for the major alleles of both species (Table 2, Fig. 5). Average values of the individual genetic distance (*Di*) between the hybrid-like specimen and the *C. nigrolineata* and *C. grata grata* specimens resulted in 1.178 and 1.146, respectively, being almost equivalent to each other.

### Discussion

The results of the above conchological and genetic examinations conclude that the hybrid-like specimen is a *F*<sub>1</sub> hybrid between *C. nigrolineata* and *C. grata*

*grata*. In shell mollusks, although such natural hybrids have been reported for trochids [*Tectus triserialis* × *T. pyramis* (Yokogawa, 1990)], pectinids [*Pecten albicans* × *P. excavatus* (Hirata, 1953)] and venerids [*Gomphina melanaegis* × *G. veneriformis* (Tanaka, 1979); *Meretrix lusoria* × *M. petechialis* (Kawase, 2002)], these reports were based only on conchological intermediateness between their parental species. Identification of such hybrids should involve genetic examinations including isozyme analysis, which also revealed hybrids in pinnids (between two forms of *Atrina pectinata*) (Yokogawa, 1996), as well as the present study. The isozyme which is coded by nuclear genomic information is one of the

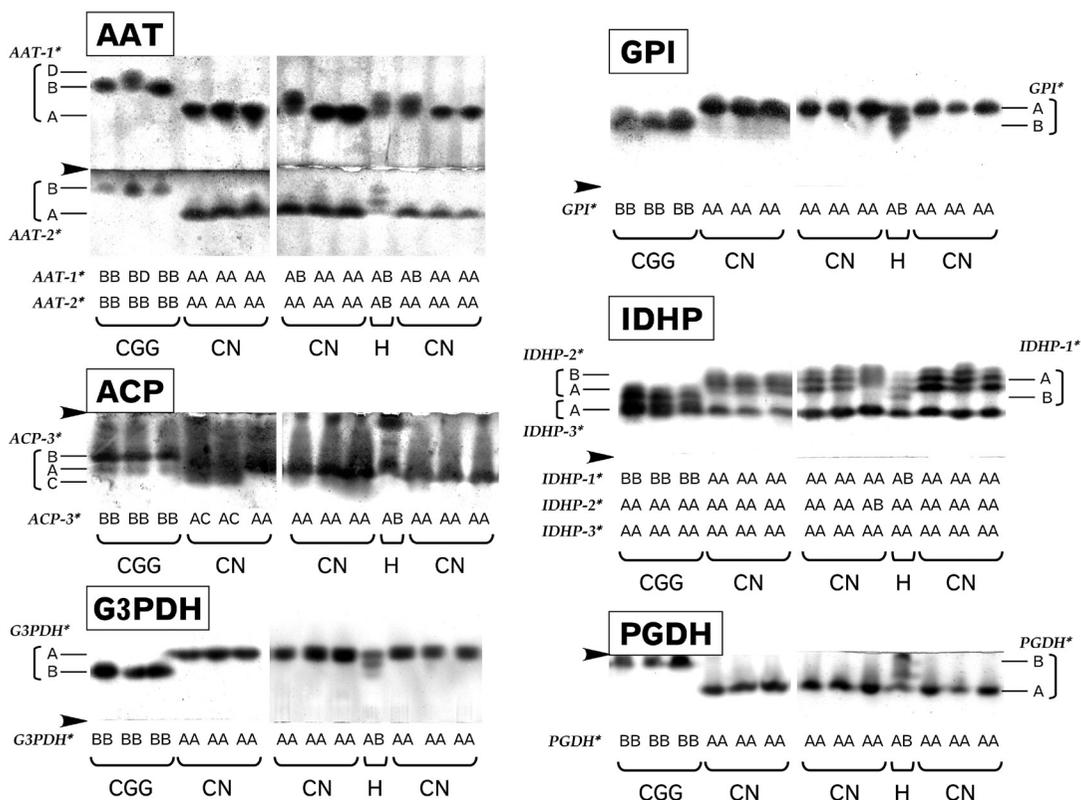


Fig. 5. Selected electrophoretograms of significant loci in *Cellana nigrolineata* (CN), *C. grata grata* (CGG) and the hybrid-like specimen (CBM-ZM 164554) (H). Upper case letter signs represent alleles: A, B – Major alleles in *C. nigrolineata* and *C. grata grata*, respectively; C, D – Minor alleles in *C. nigrolineata* and *C. grata grata*, respectively. Arrows indicate origins of electrophoresis.

best genetic markers to identify hybrids.

The hybrid limpet in this study had radial stripes (RS) (Fig. 1A–C), which are present in some *C. nigrolineata* individuals (Yokogawa, 2014a, b) (Fig. 1D–F) and absent in *C. grata grata* (Fig. 1G–I). Because the plot of the RS counts of the hybrid corresponded well with the plot cluster of *C. nigrolineata* (Fig. 3F), this character is not considered to be controlled by genes of *C. grata grata*. On the other hand, the color pattern of the internal colored area of the hybrid was peculiar to *C. grata grata* (Fig. 1H). These facts infer that a few phenotypic characters are inherited dominantly, although most conchological characteristics of the hybrid are intermediate between the parental species.

The nacellid limpets have a primitive breeding ecology, *i.e.*, parental matured males and females release eggs and sperms into seawater, where they encounter each other and fertilize (Amio, 1963). Spawning seasons of the parental species of *C. nigrolineata* and *C. grata grata* have been reported to be synchronized (during June–August) in Shimonoseki (34°10′30″ N, 130°54′22″ E) (Amio, 1963). Although spawning seasons of shells differ by locality, even within identical species (Sumikawa, 1963), similar latitudes of Naruto, where the hybrid was collected, and Shimonoseki imply at least some overlap of spawning seasons of the parental species in Naruto, which could allow a natural hybrid to occur.

In this relation, hybrids between haliotid abalones (*Haliotis discus* × *H. madaka*, *H. discus* × *H. gigantea*, *H. madaka* × *H. gigantea*) have been artificially created (Koike *et al.*, 1988) despite very few reports of their natural hybrids. The absence of the natural hybrids may be caused by slight differences in spawning seasons of the parental species from one another (Amio, 1963), inferring that differences in spawning seasons can be a great barrier for hybridization. Similar breeding isolation by difference in

spawning seasons has been reported for other true limpets of the genus *Collisella* (Sasaki & Okutani, 1994).

Although fertility of the F<sub>1</sub> hybrid limpet is uncertain, it is unlikely that it makes F<sub>2</sub> and higher generations. This is because there are many divergent loci, in which alleles have been replaced completely between *C. nigrolineata* and *C. grata grata* (Table 1), indicating no evidence of gene flow from the hybrids and higher generations. Contrastingly in the case of the pinnids (see above), probability of gene flow was suggested by presence of no loci, in which alleles had been replaced completely between parental species of hybrids (Yokogawa, 1996).

Although hybrids are commonly known from various creature taxa that breed by coupling (Mallet, 2005), those originated from aquatic species that have the same breeding ecology with the parental limpets may be actually rare. As such hybrids may have been overlooked, the present case has significant implications that taxonomy needs to be aware of the presence of hybrids.

### Acknowledgements

The author is grateful to Dr. Yukio Ueta, Fisheries Research Institute, Tokushima Agriculture, Forestry and Fisheries Technology Support Center, who gave some support in collecting the control specimens. Dr. Taiji Kurozumi, Natural History Museum and Institute, Chiba, registered the hybrid specimen. Mr. Shigefumi Yano, Mitoyo City, Kagawa Prefecture, provided some literature. Finally, I wish to thank Ms. Dawn L. McKendry, Middleburg, Florida, US, for grammatically checking the manuscript.

### References

Amio, M. 1963. A comparative embryology of marine gastropods, with ecological considerations.

- Jour. Shimonoseki Univ. Fish.*, **12**: 229–358. (in Japanese with English summary)
- Arima, S. & Ishimura, S. 1997. *A Story of Multivariate Analysis*. Tokyo-to-sho Co., Tokyo, Japan. (in Japanese)
- Hirata, K. 1953. Analysis of features of three species belonging to genus *Pecten*, *P. albicans* (Schröter), *P. excavatus* Anton and *P. puncticulatus* Dunker. *Sci. Rep. Kagoshima Univ.*, **2**: 133–164.
- Mallet, J. 2005. Hybridization as an invasion of the genome. *Tre. Ecol. Evol.* **20**: 229–237.
- Koike, Y., Sun, Z. & Takashima, F. 1988. On the feeding and growth of juvenile hybrid abalones. *Suisanzoshoku*, **36**: 231–235. (in Japanese with English abstract)
- Kawase, M. 2002. Macrobenthic organisms in the estuary of the Yahagi River. *Yahagi River Res.* (6): 81–98. (in Japanese with English summary)
- Noguchi, D., Dong, S. & Taniguchi, N. 2003. Assignment of individuals for amphidromous and landlocked populations of ayu, *Plecoglossus altivelis*, by relatedness estimator. *Suisanzoshoku*, **51**: 219–224. (in Japanese with English abstract)
- Queller, D. C. & Goodnight, K. F. 1989. Estimating relatedness using genetic markers. *Evolution*, **43**: 258–275.
- Sasaki, T. 2000. Family Nacellidae. In Okutani, T. (Ed.), *Marine Mollusks in Japan*. 24–25, Tokai Univ. Press, Tokyo, Japan. (in Japanese)
- Sasaki, T. & Okutani, T. 1994. An analysis on *Collisella heroldi* complex (Gastropoda: Lottiidae) with description of three new species. *Venus (Japan. Jour. Malac.)*, **53**: 251–285. (in Japanese with English abstract)
- Shaklee, J. B., Allendorf, F. W., Morizot, D. C. & Whitt, G. S. 1990. Gene nomenclature for protein-coding loci in fish. *Trans. Amer. Fish. Soc.*, **119**: 2–15.
- Sumikawa, S. 1963. Comparative physiological and ecological studies on the useful gastropods and lamellibranch. *Sci. Hum. Life*, **6**: 11–33. (in Japanese with English summary)
- Tanaka, Y. 1979. An intermediate form between two venerids, *Gomphina melanaegis* Römer and *G. veneriformis* (Lamarck), in the western Japan sea shores. *Venus (Japan. Jour. Malac.)*, **38**: 61–65. (in Japanese with English abstract)
- Yokogawa, K. 1990. An Individual of genus *Tectus* (Trochidae), obtained in waters of Iriomote Island, with intermediate characters between *T. triserialis* and *T. pyramis*. *Chiribotan*, **21**: 56–58. (in Japanese)
- Yokogawa, K. 1996. Genetic divergence in two forms of pen shell *Atrina pectinata*. *Venus (Japan. Jour. Malac.)*, **55**: 25–39. (in Japanese with English abstract)
- Yokogawa, K. 1999. Morphological and genetic characteristics in two forms of gibbose conch *Gibberulus gibberulus gibbosus*. *Suisanzoshoku*, **47**: 363–368.
- Yokogawa, K. 2014a. Conchological and genetic characteristics in three forms of the true limpet *Cellana nigrolineata* (Gastropoda: Nacellidae) mainly around Shikoku, Japan. *Fish Genet. Breed. Sci.*, **43**: 35–44.
- Yokogawa, K. 2014b. Growth-related conchological changes in Japanese true limpets of the Family Nacellidae. *Venus*, **72**: 29–48.

(Received June 5, 2015; Accepted September 10, 2015)