Zymogram Differences among Five Species of the Abalones from the Coasts of Japan^{*1}

Kiyoshi SASAKI^{*2}, Kenji KANAZAWA^{*2}, and Kazuo FUJINO^{*2}

(Received May 19, 1980)

Comparison of zymograms of myogen and 13 enzymes exhibited that the presence or absence of the six loci, among 26 loci examined, encoding sorbitol dehydrogenase, adenylate kinase, malate dehydrogenase, malic enzyme, tetrazolium oxidase, and phosphoglucose isomerase can be the key characters for identifying the five species of the abalones: *Haliotis discus hannai*, *H. discus*, *H. gigantea*, *H. sieboldii*, *H. japonica*, and their hybrids sampled from the coasts of Japan. No difference, however, was seen between *H. discus hannai* and *H. gigantea* with these characters. While, a marked difference was found between specimens of *H. japonica* collected from Aki, Kochi and those from Yuki, Tokushima with regard to presence or absence of at least one locus, suggesting a necessity of further systematic work in the future. On the basis of observations on hybrids of wild origin, possible overlapping of spawning seasons of *H. discus* and *H. discus hannai* was noted in the waters of Oga, Akita and Yuki, Tokushima sampled so far.

FUJINO *et al.*¹⁾ stated that the presence or absence of the three loci, encoding sorbitol dehydrogenase, adenylate kinase, and malate dehydrogenase, can be the key characters for identifying *Haliotis discus*, *H. discus hannai*, and their hybrids, that can not be distinguished clearly each other by the morphological traits. Application of the key characters disclosed the sympatric distribution of the three groups of the abalones, two parental varieties and their hybrids in the Japanese waters sampled. The present paper describes the further study on differences in electrophoretic patterns with more enzyme loci between the five species and/or varieties of the abalones, including the two varieties reported before, from the coasts of Japan.

Materials and Methods

Abalone Specimens

A total of 295 specimens of the five species of the abalones, *Haliotis discus* REEVE, *H. discus hannai* Ito, *H. gigantea* Gmelin, *H. sieboldii* REEVE, *H. japonica* REEVE, and the hybrids were sampled from the west coasts of Hokkaido and Honshu (the mainland of Japan) and the east coast of Honshu as shown in Table 1. Tissues of shell-muscle and/or digestive diverticula either from fresh or frozen specimens were used for electrophoretic analyses. Shell of each animal was measured for morphometric

comparisons.

Electrophoresis

Tissue extract, prepared according to FUJINO,²⁾ or drip from frozen specimen was used for starch-gel electrophoreses for myogen and 13 enzymes. Myogen, esterase, leucine-aminopeptidase, tetrazolium oxidase, malate dehydrogenase, phosphoglucose isomerase, and malic enzyme were analysed according to FUJINO *et al.*^{3,4)} Fumarase, aldehyde oxidase, adenylate kinase, sorbitol dehydrogenase, and phosphoglucosmutase were analysed according to FUJINO.¹⁾ Alpha-glycerophosphate dehydrogenase was analysed according to FUJINO.⁵⁾ Hexokinase was analysed according to AYALA *et al.*⁶⁾ as follows. 1) Bridge buffer: A solution containing 0.097 M Tris aminomethane, 0.047 M H_3BO_3 , and 0.001 M Na_2EDTA (pH 9.0), 2) Gel buffer: Same as the bridge buffer, 3) Staining mixture: Prepare 100 ml/ solution of 0.1 M Tris-HCl (pH 7.1) with distilled water. To this, dissolve 20 mg NBT, 25 mg NADP, 25 mg ATP, 20 mg $MgCl_2$, 900 mg glucose, and 80 units G6pdh. Immediately before use, add 5 mg PMS to the above mixture. 4) Procedure: Twelve percent starch-gel was prepared. Electrophoresis was performed in refrigerator at 4°C with 7 volts electric current per linear cm of gel for 4 h. The gel was stained at 37°C overnight in dark.

^{*1} Genetic Studies on the Pacific Abalone—V.

^{*2} Kitasato University School of Fisheries Sciences, Sanriku, Iwate 022-01 (佐々木喜代志・金沢堅二・藤野和男: 北里大学水産学部).

Table 1. Abalone specimens used for zymogram comparisons

Species and localities	Number of specimens examined	Origin of animals	Month and year sampled
<i>H. discus</i> REEVE			
Oga, Akita	4	wild	Jun. 1979
Kisakata, Akita	4	wild	Aug. 1977
Yuki, Tokushima	2	wild	Sep. 1979
<i>H. discus hannai</i> INO			
Rebun Is., Hokkaido	10	wild	Aug. 1977
Okushiri Is., Hokkaido	10	wild	Aug. 1978
Oga, Akita	14	wild	Jun. 1979
Kisakata, Akita	3	wild	Aug. 1977
" "	11	wild	Aug. 1979
Fukura, Yamagata	15	wild	Jul. 1979
Tsuruoka, Yamagata	9	wild	Jul. 1979
Hekura Is., Ishikawa	4	wild	Aug. 1979
Notoshima, Ishikawa	21	artificial	Aug. 1979
Sanriku, Iwate	25	wild	Nov. 1977
Nakaminato, Ibaraki	20	wild	Jun. 1977
Chikura, Chiba	9	artificial	Jun. 1977
Yuki, Tokushima	13	wild	Sep. 1979
Aki, Kochi	7	wild	Aug. 1978
Hybrids			
Oga, Akita* ¹	5	wild	Jun. 1979
Chikura, Chiba* ¹	28	artificial	Jun. 1977
Yuki, Tokushima* ¹	13	wild	Sep. 1979
Yuki, Tokushima* ²	3	wild	Sep. 1979
Awa Is., Niigata* ²	8	wild	Jul. 1979
<i>H. gigantea</i> GMELIN			
Tsuruoka, Yamagata	5	artificial	Jul. 1979
Awa Is., Niigata	6	wild	Jul. 1979
Murakami, Niigata	2	artificial	Aug. 1979
Notoshima, Ishikawa	5	artificial	Aug. 1979
Yuki, Tokushima	4	wild	Sep. 1979
<i>H. sieboldii</i> REEVE			
Hekura Is., Ishikawa	5	wild	Aug. 1979
<i>H. japonica</i> REEVE			
Yuki, Tokushima	20	wild	Sep. 1979
Aki, Kochi	10	wild	Aug. 1978

*¹ Hybrids between *H. discus hannai* INO and *H. discus* REEVE.

*² Hybrids more likely between *H. discus* REEVE and *H. gigantea* GMELIN.

Identification of Species

Several specimens from different species were electrophoresed on a gel. Specificity for each species was identified from zymograms in terms of presence or absence and/or mobility of each zone (locus) relative to other species. Morphometric characteristics were observed for each animal as a reference for species identification.

Results and Discussions

Key Characters for Species Identification

Among more than 26 loci encoding myogen and

13 enzymes, distinct differences between species were observed at least in seven loci encoding six enzymes: sorbitol dehydrogenase (*Sdh*), adenylate kinase (*Adk*), malate dehydrogenase (*Mdh*), malic enzyme (*Me*), tetrazolium oxidase (*To*), and phosphoglucose isomerase (*Pgi*) as described below (see Fig. 1).

Sdh Activity of *Sdh* was seen at a single locus common to the three species, *H. discus hannai*, *H. gigantea*, and *H. sieboldii* neither with inter-specific difference nor intraspecific variation. No *Sdh* activity has been observed in *H. discus* as reported before.¹⁾ *H. japonica* revealed a single

Fig. 1. Zymograms for six enzymes in some selected specimens from *H. discus hannai* Ino (A) *H. discus* REEVE (B), *H. gigantea* GMELIN (C), *H. sieboldii* REEVE (D), and *H. japonica* REEVE (E).

zone that migrated faster than that in the three species noted above. Then the zymogram of *Sdh* can be used to distinguish *H. discus* and *H. japonica* from the other three species.

Adk *Adk* activities were seen at the three loci, coded as M, S₁, and S₂, commonly in all the five species, indicating some intraspecific variations at the M and S₂ loci. In *H. discus*, as reported before,¹⁾ observed was an additional locus F, that is missing in the other four species. Then the presence of the F locus can be the key character to identify *H. discus* among the five species studied.

Mdh *Mdh* activities were seen at the two loci, coded as F and S, commonly in the four species other than *H. japonica*, indicating intraspecific variations in *H. discus hannai* with dimeric structure. An additional locus F was observed in *H. discus* only as reported before,¹⁾ while being missed in all the other species. *H. japonica* revealed *Mdh* activity at a single locus between the F and S loci, indicating intraspecific variations with monomeric structure. Then the presence of the F locus can be the key character for identifying *H. discus*, while the absence of both the F and S loci for *H. japonica* among the five species studied.

Me *Me* activity was seen at a single locus in the three species, *H. discus hannai*, *H. discus*, and *H. gigantea*, suggesting some intraspecific variations. All the five specimens of *H. sieboldii* analysed revealed a pair of two bands without variation. One of the two bands migrated slower than any of those observed in the three species noted above. *H. japonica* exhibited some intraspecific variations with a single or double bands of differential mobilities from those in the other species. Zymogram of heterozygotes suggested monomeric structure. Then the zymograms of *Me* could be used as a referential key to identify either *H. sieboldii* and/or *H. japonica*.

To *To* activities were seen at the three loci, coded as F, M, and S, commonly in the four species other than *H. japonica*, indicating intraspecific variations at M and S loci in some species. Presence of hybrid band in heterozygote suggested a dimeric structure both at the two loci. Approximate one half of the specimens of *H. japonica* examined exhibited activity of *To* at F locus but no activity in the rest. Variations were seen at S locus with monomeric structure that migrated slower than that in the other species. Then the

Table 2. Differences with regard to presence (+) or absence (—) of the *To·F* and *Pgi·F* loci among the specimens of *H. japonica* sampled from Yuki, Tokushima and Aki, Kochi

Localities sampled	Locus of:					Number of specimens examined
	<i>Sdh</i>	<i>Adk·F</i>	<i>Mdh·F'</i>	<i>To·F</i>	<i>Pgi·F</i>	
Yuki, Tokushima	+	—	—	+	—	9
" "	+	—	—	—	—	11
Aki, Kochi	+	—	—	—	+	10

Table 3. Key loci for identifying five species of the abalones

Species	<i>H. discus</i>	<i>H. gigantea</i>	<i>H. sieboldii</i>	<i>H. japonica</i>
<i>H. discus hannai</i>	<i>Sdh</i> <i>Adk·F</i> <i>Mdh·F'</i> <i>Pgi·F</i>	None	<i>Me</i>	<i>Sdh</i> <i>Mdh·F'</i> , <i>Mdh·F</i> , <i>Mdh·S</i> <i>To·F</i> , <i>To·S</i> <i>Pgi·F</i>
<i>H. discus</i>		<i>Sdh</i> <i>Adk·F</i> <i>Mdh·F'</i> <i>Pgi·F</i>	<i>Sdh</i> <i>Adk·F</i> <i>Mdh·F'</i> <i>Me·S</i> <i>Pgi·F</i>	<i>Sdh</i> <i>Adk·F</i> <i>Mdh·F'</i> , <i>Mdh·F</i> , <i>Mdh·S</i> <i>To·F</i> , <i>To·S</i>
<i>H. gigantea</i>			<i>Me</i>	<i>Sdh</i> <i>Mdh·F'</i> , <i>Mdh·F</i> , <i>Mdh·S</i> <i>To·F</i> , <i>To·S</i> <i>Pgi·F</i>
<i>H. sieboldii</i>				<i>Sdh</i> <i>Mdh·F</i> , <i>Mdh·S</i> <i>Me</i> <i>To·F</i> , <i>To·M</i> , <i>To·S</i> <i>Pgi·F</i>

shorter distance of migration of S locus can be the key character to distinguish *H. japonica* from the other four species. Variations of presence or absence of the F locus within *H. japonica* need to be studied further (Table 2).

Pgi *Pgi* activities were seen at the two loci, coded as F and S, in *H. discus* consistently but in some limited numbers of specimens from *H. japonica*. The F locus was absent in the other three species. Intraspecific variations were observed at the S locus in some species. Then the presence of F locus can be the key character to distinguish *H. discus* from the other three species: *H. discus hannai*, *H. gigantea*, and *H. sieboldii*. In *H. japonica*, F locus was present in all the specimens from Aki, Kochi but absent in all the specimens from Yuki, Tokushima (Table 2). Further studies must be conducted to clarify the discrepancy between the specimens sampled from the two localities in future.

In consequence, presence or absence of nine

loci encoding six enzymes and the zymogram characteristics can be used to identify the five species of the abalones as summarized in Table 3.

Occurrence of Hybrids

FUJINO *et al.*¹⁾ described the four categories of hybrids between *H. discus* and *H. discus hannai*, three categories of wild origin and another from artificial crossing, as characterized by the presence or absence of the three loci encoding *Sdh*, *Adk·F*, and *Mdh·F'*. Application of an additional locus of *Pgi·F* exhibited four kinds of the polymorphic hybrids in the specimens collected from Yuki, Tokushima as shown in Table 4. Occurrence of such polymorphic hybrids may stimulate one's interest in any differential performances such as fertility, hatchability, survival rate, growth rate etc. among them.

Fig. 2 compares shell length-shell weight relationships between *H. discus hannai* and *H. gigantea*. The regression was calculated from the measure-

Table 4. Polymorphic hybrids between *H. discus hannai* and *H. discus* as demonstrated by presence (+) or absence (—) of the four loci encoding *Sdh*, *Adh·F*, *Mdh·F'*, and *Pgi·F**¹

Parental species or hybrids	Locus of:				Number of specimens examined
	<i>Sdh</i>	<i>Adh·F</i>	<i>Mdh·F'</i>	<i>Pgi·F</i>	
<i>H. discus hannai</i>	+	—	—	—	169
<i>H. discus</i>	—	+	+	+	10
Hybrids* ²					
Oga, Akita	+	+	+	+	5
Yuki, Tokushima	+	+	+	+	8
" "	+	+	+	—	2
" "	+	+	—	—	2
" "	+	—	+	—	1
Chikura, Chiba	—	—	—	—	28

*¹ All the specimens examined are identical to those presented in Table 2 in FUJINO *et al.*¹⁾.

*² Hybrid specimens from Akita and Tokushima were of wild origin, but those from Chiba crossed artificially at the hatchery.

Fig. 2. Comparison of shell weight-shell length relationships between *H. discus hannai* INO and *H. gigantea* GMELIN. Small dots, open circles, double circles, and solid circles represent individual animals of *H. discus hannai*, *H. gigantea*, the specimens from Awa Island, and the hybrid specimens from Awa Island respectively. The regression was calculated for *H. discus hannai* from individual data of the specimens collected from Oga, Kisakata, Fukura, and Tsuruoka, as indicated in this figure. One double circle (no. 5), one solid circle (no. 6), and the two crosses represent the specimens, of which the photographs are shown in the lower part of Fig. 3.

ments of 42 specimens of *H. discus hannai* taken from Oga, Kisakata, Fukura, and Tsuruoka. It can be stated from this figure that shells of *H. gigantea* are significantly heavier than those of *H. discus hannai*. Eight hybrid specimens from Awa Island, Niigata (see Table 1) exhibited enzyme activities at all the four key loci of *Sdh*, *Adh·F*, *Mdh·F'* and *Pgi·F* without variation. According to the above relationships, all these eight specimens (represented by solid circles in the Fig. 2) are more likely those between *H. discus* and *H. gigantea* rather than *H. discus* and *H. discus hannai*. Among the three hybrid specimens from Yuki, Tokushima

(see Table 1), two specimens nos 6 and 7 exhibited activities of enzymes at all the four key loci of *Sdh*, *Adh·F*, *Mdh·F'*, and *Pgi·F*, but the specimen no. 3 did not at *Adh·F* and *Mdh·F'*, indicating polymorphic hybrids similarly to that noted in the foregoing paragraph. These three hybrid specimens were all interpreted as those between *H. discus* and *H. gigantea* according to the above shell length-shell weight relationships. The two specimens of artificial origin from Murakami, Niigata were more likely hybrid between *H. discus hannai* and *H. gigantea* by the same reason (Fig. 3).

Fig. 3. Dorsal and anterior views of shells of the abalones. From left to right, the upper three are the specimens of *H. gigantea* from Yuki, Tokushima, *H. sieboldii* from Hekura Island, Ishikawa and *H. japonica* from Aki, Kochi, and the lower three are the specimens nos. 5 (*H. gigantea*) and 6 (probable hybrid between *H. gigantea* and *H. discus*) from Awa Island, Niigata, and that crossed (probable hybrid between *H. gigantea* and *H. discus hannai*) artificially and reared at the Niigata Prefectural Fisheries Experimental Station, Murakami, Niigata.

Possible Conditions for Occurrence of Natural Hybrids

No detailed observation has been reported on intervals of breeding seasons both for *H. discus* and *H. discus hannai* in the waters of Oga, Akita and Yuki, Tokushima. The occurrence of hybrids of wild origin strongly suggested that spawning seasons of the parental species should overlap each other to some extent, resulting in inter-species hybridization. It might be interesting to note that the hybrids were monomorphic in the specimens from Oga but polymorphic from Yuki with regard to presence or absence of the key loci of the parental species, of which the reasons are not known yet.

Possible differential performances such as viability and growth rate among polymorphic hybrids need to be studied from the points of view of ecological genetics as well as breeding sciences in future.

Needs for More Key Loci

Lack of key locus to distinguish between *H.*

discus hannai and *H. gigantea* and the discrepancy with regard to presence or absence of the loci among the specimens of *H. japonica* sampled from Aki, Kochi and Yuki, Tokushima suggested a necessity of further works on additional key loci.

References

- 1) K. FUJINO, K. SASAKI, and N. P. WILKINS: *Bull. Japan. Soc. Sci. Fish.*, **46**, 543-548 (1980).
- 2) K. FUJINO and N. NAGAYA: *Bull. Japan. Soc. Sci. Fish.*, **43**, 983-988 (1977).
- 3) K. FUJINO: *Bull. Japan. Soc. Sci. Fish.*, **44**, 357-361 (1978).
- 4) K. FUJINO: *The Proc. of the XVIth Intern. Conf. Anim. Bl. Gr. Bioch. Polymor.*, Aug. 14-18, 1978, Leningrad, USSR.
- 5) K. FUJINO and M. MATSUYA: *Fish Genetics and Breeding Science*, **5**, 13-17 (1980).
- 6) F. J. AYALA, J. R. POWELL, M. L. TRACEY, C. A. MOURAO, and S. PEREZSALAS: *Genetics*, **70**, 113-139 (1972).