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Taxonomic studies on *Ulva pertusa* (Ulvophyceae). II. Preliminary isozyme analysis

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Preliminary comparisons of isozyme banding patterns were carried out between *Ulva pertusa* Kjellman and *U. arasakii* Chihara, and between *U. pertusa* and "stalked-*Ulva*". Several electrophoretic differences were detected for enzyme-species including GDH, IDH and GOT between specimens of *U. pertusa* and *U. arasakii*. Otherwise, no differences for any of assayed enzymes were detected between specimens of *U. pertusa* and "stalked-*Ulva*" at the same population in Ebisujima (Shimoda, Shizuoka Pref.).

Overall electrophoretic patterns among specimens of *U. pertusa* from fourteen localities from Hokkaido to Yamaguchi were almost identical in spite of their wide morphological variability. However, a variation in GDH was found for the typical form of *U. pertusa* in populations on the eastern side of Ebisujima. Other variations in GDH and IDH were recognized in the populations of Kabushima (Hachinohe, Aomori Pref.).

Cross experiments of the gametes from two electroforms of GDH from Ebisujima revealed that they are interfertile.

*Key Index Words: crossing test—genetic variability—isozyme analysis—"stalked-*Ulva*"—taxonomy—* Ulva arasakii—Ulva pertusa.

The plants of the genus *Ulva* (Ulvales, Ulvophyceae) are among the most common coastal seaweeds in Japan. Classification of the numerous species assigned to this genus is based on gross morphology of the thallus. However, shape, size and thickness of *Ulva* thalli generally vary with age and habitat. Specimens of *U. pertusa* show significant morphological variation, ranging from an *U. conglobata*-type to an *U. arasakii*-type. The previously reported "stalked-*Ulva*" represents an additional morphological form assigned to this species (Kamiya *et al.* 1993).

Genetic data of isozyme analysis have been applied to taxonomic studies of red algae (Lindstrom and South 1989), dinoflagellates (Hayhome *et al.* 1987), charophytes (Grant and Procter 1980), and desmids (Francke and Coesel 1985; Coesel and Menken 1986, 1988; Jurgenson and Biebel 1989). In the Ulvales, Innes and Yarish (1984) and Innes (1987, 1988) serveyed intraspecific genetic variability in *Enteromorpha linza* (L.) J. Agardh. Relatively fewer isozyme studies have been conducted on algae compared to higher plants, and the techniques suitable for algae tissue have not been satisfactorily established. Consequently, the number of enzymes examined in most studies of algae has been insufficient, making precise genetic interpretation difficult. In addition, the intra- and interspecific taxonomy of many algal species investigated has not been established by means of crossing tests or morphology and genetic analysis.

In this paper, the preliminary interspecific comparison of isozyme patterns between *U. pertusa* and *U. arasakii* growing sympatrically was carried out. It was followed by intraspecific comparison of *U. pertusa* among allopatric populations, and between typical

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U. pertusa and "stalked-*Ulva*". The effectiveness of isozyme analysis for the taxonomy of these algae is discussed.

Materials and Methods

Collection and preservation of algae. Specimens showing the typical appearances of both *Ulva pertusa* and "stalked-*Ulva*" were collected from populations in Ebisujima, Shimoda, Izu Peninsula, (Kamiya *et al.* 1993) from March, 1989 to May, 1991. At the same time, specimens of the typical *U. pertusa* were collected from various localities in Hokkaido and Honshu (Fig. 1) for comparison. Specimens of *U. arasakii* were collected from Ooarai (Ibaraki Pref.) and Kimigahama (Chiba Pref.) as well.

Living specimens from which surface sea water was removed were placed into individ-

Morphologically typical *Ulva pertusa*

1. Monbetsu (Monbetsu-shi, Hokkaido)
2. Ougonmisaki (Rumoi-shi, Hokkaido)
3. Zenibako (Otaru-shi, Hokkaido)
4. Haruka (Otaru-shi, Hokkaido)
5. Aomori (Aomori-shi, Aomori)
6. Kabushima (Hachinohe-shi, Aomori)
7. Areshima (Shizugawa-cho, Miyagi)
8. Ooarai (Ooarai-machi, Ibaraki)
9. Kimigahama (Choushi-shi, Chiba)
10. Kominato (Amatsukominato-machi, Chiba)
11. Ebisujima (Shimoda-shi, Shizuoka)
12. Nabeta (Shimoda-shi, Shizuoka)
13. Tokiwa (Ube-shi, Yamaguchi)

Ulva arasakii

8. Ooarai (Ooarai-machi, Ibaraki)
9. Kimigahama (Choushi-shi, Chiba)

"stalked-*Ulva*"

11. Ebisujima (Shimoda-shi, Shizuoka)

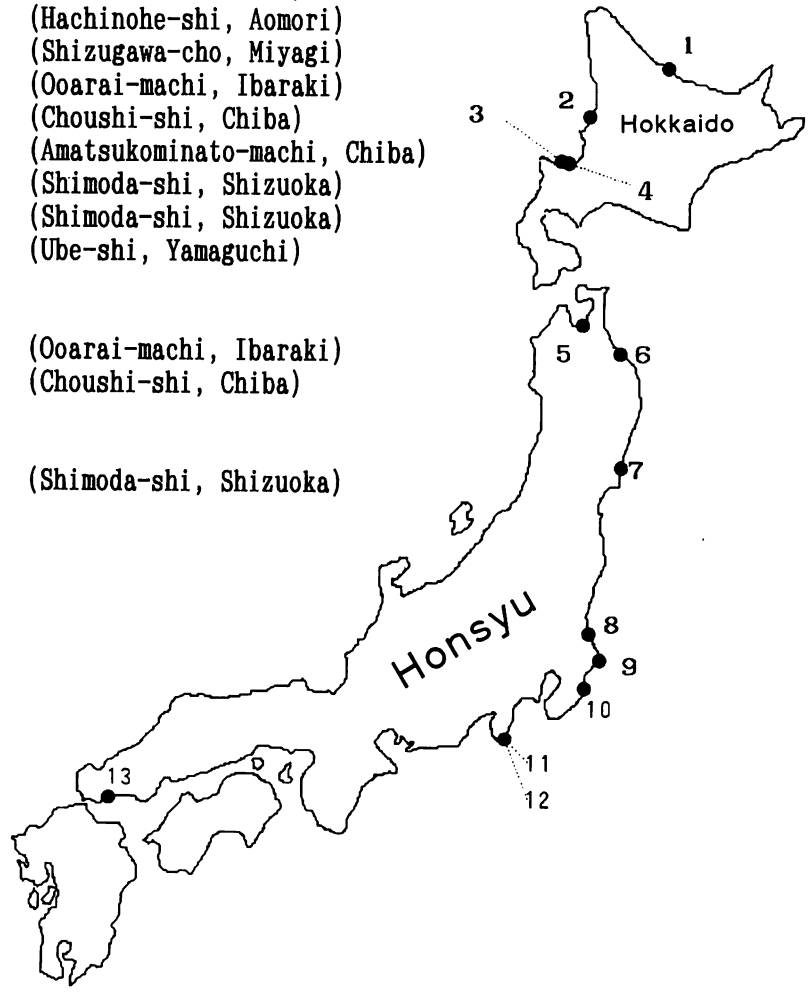

Fig. 1. Localities of *Ulva pertusa* and related taxa collected in Japan.

ual vinyl bags, maintained at approximately 10°C and transported rapidly to University of Tsukuba.

Motile cells released from matured specimens were observed to identify gametophyte and sporophyte thalli. Gametes determined to have compatible mating types were used for the crossing tests. For isozyme analysis, fresh specimens were ground in liquid nitrogen, and stored at −80°C until being assayed.

Isozyme analysis. Crude extracts were obtained by grinding material (at least 500 mg wet weight) in 1.0 m*l* of cold extraction buffer; 0.5 M Tris-HCl buffer, pH 8.0, containing 70 mM 2-mercaptoethanol, 26 mM sodium metabisulfite, 0.5 mM EDTA, 5 mM sodium ascorbate, 0.1% Tween 80 and 4% soluble polyvinylpyrrolidone with an average molecular weight of 40,000. Extracts were centrifuged at 6,200 G for 10 min. The supernatants were immediately filtered through a simplified gel filtration method (Kato 1987) for purifying isozyme samples.

For electrophoresis, starch gel (12.8% w/v) made up with the system 5 and 10 buffers of Soltis *et al.* (1983) was used. Samples were absorbed onto rectangular wickes of Advantec 51B chromatography paper, inserted into a slice made across the gel ca. 5 cm from the cathode. Electrophoresis was done at 4°C and 150 volts (constant voltage) for 3–5 hours, until the bromphenol blue marker, which was inserted in the gel with the samples, had migrated 10 cm from the origin.

The following fifteen enzymes were preliminarily tested: esterase (EST), glutamate dehydrogenase (GDH), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH), hexokinase (HK), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), triphosphate isomerase (TPI), 6-phosphogluconate dehydrogenase (6-PGD), acid phosphatase (ACPH), glutamate oxaloacetate transaminase (GOT), superoxide dismutase (SOD), and shikimate dehydrogenase (SKDH). The protocol of staining was followed by Soltis *et al.* (1983) with some modifications (Table 1).

Table 1. The staining protocol of enzymes employed in this study.

Enzyme	Reactant/Stain
GDH	100 m*l* 0.1 M Tris-HCl, pH 8.0
	2.94 g L-glutamic acid
	20 mg NAD
	10 mg MTT
	2 mg PMS
IDH	100 m*l* 0.1 M Tris-HCl, pH 8.0
	100 mg isocitric acid, trisodium salt
	5 m*l* 1.0 M $MgCl_2$
	20 mg NADP
	10 mg MTT
	2 mg PMS
GOT	100 m*l* 0.1 M Tris-HCl, pH 8.0
	100 mg L-aspartic acid
	100 mg α-ketoglutaric acid
	5 mg pyridoxal-5'-phosphate
	90 mg Fast blue BB salt
G6PDH	100 m*l* 0.1 M Tris-HCl, pH 8.0
	200 mg glucose-6-phosphate
	20 mg NADP
	10 mg MTT
	2 mg PMS

Abbreviations: ATP, adenosine-5'-triphosphate; EDTA, disodium ethylenediamine tetra-acetic acid; MTT, (3-[4, 5-dimethylthiazol-2-1]-2, 5-diphenyltetrazolium bromide; NAD, β-nicotinamide adenine dinucleotide; NADP, β-nicotinamide adenine dinucleotide phosphate; PMS, phenazine methosulphate.

Crossing tests. Crossing tests were carried out between gametes from different electroforms of morphologically typical *U. pertusa* at Ebisujima. The technique was outlined in the previous paper (Kamiya *et al.* 1993).

Results

Preliminary observations using typical *Ulva pertusa* (Fig. 2) for the fifteen enzymes listed above showed that stable bands could be obtained from four enzymes; IDH, GDH, G6PDH, and GOT.

U. arasakii (Fig. 3) was provided to analyze its isozymes in comparison with those of *U. pertusa*. Clear differences were recognized for three of four enzyme species examined (Fig. 5), whereas no difference was found for G6PDH between two entities (Table 2).

When the banding patterns of morphologically typical *U. pertusa* and "stalked-*Ulva*"

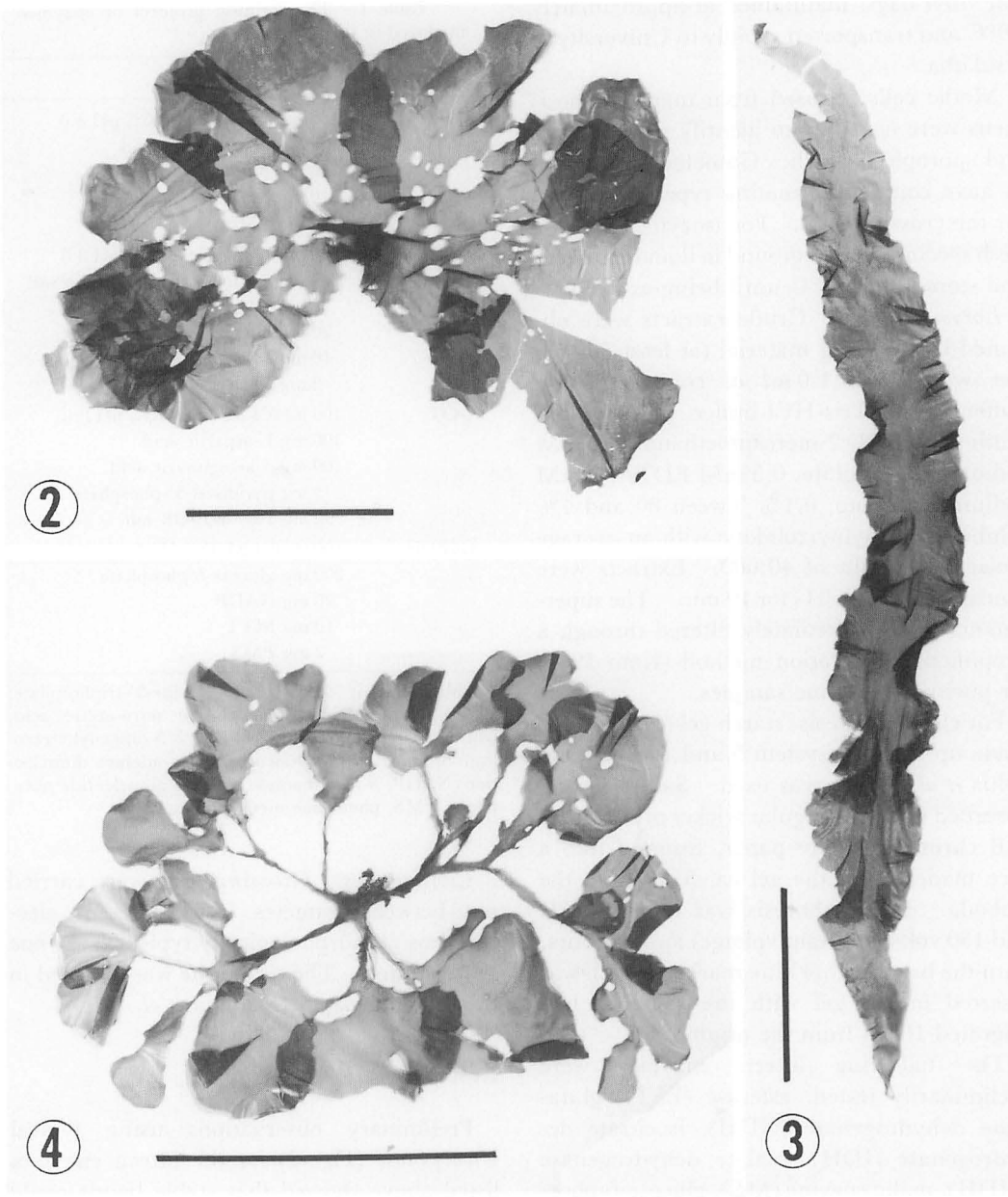

Fig. 2. Specimen of typical *Ulva pertusa* collected at Ebisujima in December, 1989. Scale bar=5.0 cm.
Fig. 3. Specimen of typical *Ulva arasakii* collected at Kimigahama (Chiba Pref.) in May, 1990. Scale bar=5.0 cm.
Fig. 4. Specimen of "stalked-*Ulva*" collected at southern population in Ebisujima in December, 1989. Scale bar=5.0 cm.

(Fig. 4) in Ebisujima were compared, no specific banding patterns were found (Fig. 6). Otherwise, two banding patterns for GDH were found in typical *U. pertusa* from different populations in Ebisujima (Fig. 7). Specimens of the populations on the eastern side of this island (sites A, B and C in Fig. 1 of Kamiya *et al.*, 1993) exhibited one banding pattern, labelled "GDH-B", whereas specimens of the other sites had a banding pattern,

Table 2. The summarized results of electrophoretic patterns of *U. pertusa*, *U. arasakii* and "stalked-*Ulva*". Letters designate the electrophoretic forms in each enzyme recognized within each population.

Populations	Enzyme species			
	IDH	GDH	GOT	G6PDH
U. pertusa				
Ebisujima	A	A, B	A	A
Kabushima	A, B	A, C	A	A
Other populations	A	A	A	A
U. arasakii	B'*	C'*	B	A
"Stalked-*Ulva*"	A	A	A	A

* Migration distance of these electromorphs not compared directly with specimens from Kabushima.

"GDH-A", common to populations of all other Japanese localities investigated (Fig. 1). The specimens showing "GDH-B" were similar to other populations for other enzymes examined (Table 2), and could not be distinguished morphologically.

Other variations of GDH and IDH were also found in the population of typical *U. pertusa* from Kabushima (Fig. 7). Some specimens showed unusual banding patterns for GDH and IDH (labelled "GDH-C" and "IDH-B") while the others in the same populations showed the pattern of "GDH-A" and "IDH-A" which were widely spread. No characteristics of gross morphology were associated with this genetic variation.

No difference in the electrophoretic behavior specific to sporophytic and gametophytic

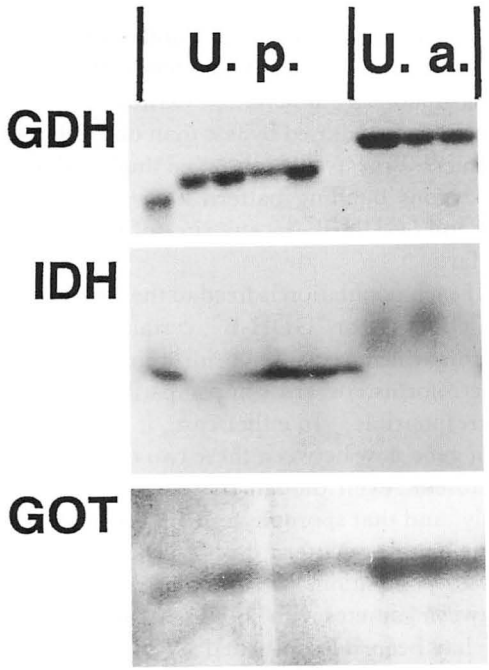

Fig. 5. Representative zymogram patterns for *Ulva pertusa* and *U. arasakii*. U.p., *U. pertusa*. U.a., *U. arasakii*.

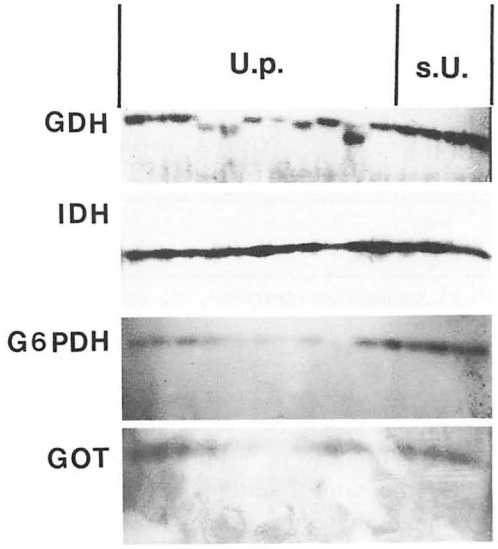

Fig. 6. Representative zymogram patterns for *Ulva pertusa* and "stalked-*Ulva*" in Ebisujima. U.p., *U. pertusa*. s.U., "stalked-*Ulva*".

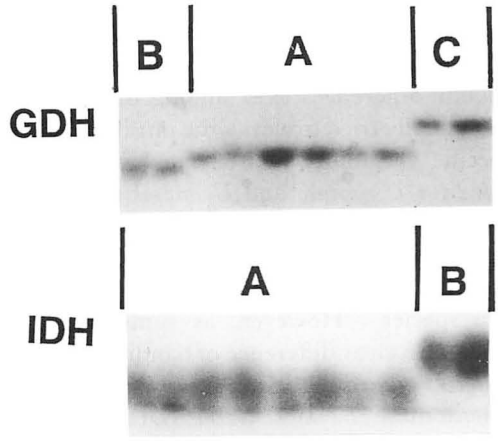

Fig. 7. Representative zymogram patterns for *Ulva pertusa* in Kabushima. Letters designate electrophoretic forms (see Table 2).

Table 3. The results of test crosses between typical specimens of *Ulva pertusa* showing "GDH-A" and "GDH-B" banding patterns. Alphabets designate populations (see Kamiya *et al.* 1993). Numbers represent specimens examined.

"GDH-B" specimens	"GDH-A" specimens					
	B-1	B-2	B-5	B-6	C-1	C-2
B-3	+	+	−	−	+	+
B-4	+	−	−	−	+	+

+: conjugation observed. −: conjugation not observed.

plants was observed in this study. Eight sporophytic specimens of typical *U. pertusa* from Ebisujima were tested, and all of them showed the same banding pattern found in the gametophytes, i. e. they were homozygous for "GDH-A" or "GDH-B" genotypes.

On the basis of isozyme data obtained in this study, crossing tests were performed between specimens representing different electroforms of *U. pertusa* from Ebisujima showing "GDH-A" and "GDH-B" respectively. Conjugations were observed among gametes of compatible mating types, except for the cases that experimental errors happened (Table 3).

Discussion

Previously, we reported that *Ulva pertusa* is morphologically variable, and that petiolate forms are included within this taxon (Kamiya *et al.* 1993). Molecular taxonomic analysis with isozymes was initiated to determine if genetic differences exist among these forms. We initially tried to clarify the intra- and interspecific variability of isozyme banding patterns for *U. pertusa* and *U. arasakii*. Results indicate the presence of main bands for four enzyme-species mentioned above. The banding pattern of G6PDH was common for the two species. However, as summarized in Table 2, a clear difference of banding patterns was recognized between these two species for at least three enzymes: GDH, IDH and GOT. In contrast, intraspecific variability was found in only one enzyme, GDH, from two populations of *U. pertusa*.

For all of the examined enzyme-species, no differences in isozyme banding patterns were detected between specimens of "stalked-*Ulva*" and typical *U. pertusa* growing at the same locality. These results suggest that the two forms are not genetically isolated.

Kamiya *et al.* (1993) showed clear morphological differences between typical *U. pertusa* and "stalked-*Ulva*". However, the presence of numerous intermediate forms made assignment of individual specimens to either species problematic. Also, sexual reproduction was shown between gametes from typical *U. pertusa* and "stalked-*Ulva*" under laboratory culture condition. Consequently, it was concluded that "stalked-*Ulva*" could not be treated as an autonomous entity of *U. pertusa*. Isozyme data from the present study supports this previous thesis (Kamiya *et al.* 1993). In addition, it indicates that isozyme analysis is effective for observing genetic structure of algae such as "stalked-*Ulva*", and possible to use for evaluating these intraspecific relationships.

It is noteworthy that two electrophoretic forms of typical *U. pertusa*, "GDH-A" and "GDH-B", were found in Ebisujima. Through interpopulational comparison of isozymes in this island, it was found that clear electrophoretic difference existed between specimens separated by less than one hundred meters. We have not observed thalli with heterozygous banding pattern between "GDH-A" and "GDH-B" in any sites of this island, so far.

If each population is fixed to the single form of "GDH-A" or "GDH-B", certain ecological factors which prevent hybridization of the two electroforms, or cross incompatibility could be responsible. In either case, it is supposed that gene flow between these two electroforms is absent, even though they grow sympatrically, and that sporophytes with the heterozygous banding pattern do not occur in any of these populations. Although conjugation between gametes with "GDH-A" and "GDH-B" has been demonstrated by crossing tests under laboratory culture condition, the viability of these hybrids has not been evaluated. Efforts continue to identify heterozygous

sporophytes from the natural populations from Ebisujima. However, probability of success is slight because populations of *U. pertusa* are considered to have a high gametophyte/sporophyte ratio since gametophytes can be reproduced by their own parthenogenesis and through zoospore formation of the sporophytes, whereas the sporophytes can be reproduced only by conjugation of the gametophytes.

Innes (1987) described genetic differentiation for *Enteromorpha linza* among localities separated by less than a few hundred meters. He suggested that the genetic differentiation was maintained by some environmental factors such as difference of salinity. It is possible in our case that the difference of environmental factors such as the amount of solar radiation relates to the genetic differentiation between these populations. The eastern population with "GDH-B" is considerably shaded by the steep cliff and canopies of trees in contrast to the southern population inhabiting broad flat rock platform with no shadings. Further examination is necessary to study their physiological differences induced by the environmental factors.

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土井考爾*・神谷充伸*・原　慶明*・千原光雄**：アナアオサ（アオサ藻綱）の分類学的研究．II．アイソザイムの分析

アナアオサ，ナガアオサ，及び通称エツキアオサのアイソザイムを解析し比較した。アナアオサとナガアオサの間では，4種類のアイソザイムのうち3種類にバンドパターンの明瞭な差異が認められたが，エツキアオサはアナアオサと同じパターンを示した。一方，全国14地点のアナアオサ集団間では，静岡県下田市恵比須島および青森県八戸市蕪島を除き，バンドパターンは共通であった。恵比須島のアナアオサの集団を調査したところ，典型的なバンドパターンを示す個体と共に，他のどの集団にも見られないバンドパターンを示す個体がみられた。両パターンを示すタイプの個体間では，配偶子が接合し，正常に交雑が起こることが確認された。(*305 茨城県つくば市天王台1-1-1　筑波大学生物科学系，**150 東京都渋谷区広尾4-1-3　日本赤十字看護大学)

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