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Molecular Evidence for the Existence of Four Sibling Species within the Sea-Urchin, *Echinometra mathaei* in Japanese Waters and their Evolutionary Relationships

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ABSTRACT—Four different types of the sea-urchin, *Echinometra mathaei* (Blainville) which are distinguishable by several characters such as color pattern of spines are observed abundantly on Okinawan reef flats, southern Japan. Their taxonomic, genetic and evolutionary relationships were examined by enzyme electrophoresis. The allozyme studies demonstrated that the four types of sea-urchins designated as Types A, B, C and D do not share gene pools with each other in spite of their sympatric distribution. They were fixed for different alleles at 7 genetic loci in a total of 28 genetic loci scored. This clearly shows no gene flow between the four types, and is a strong evidence for that they are reproductively isolated and genetically distinct species. The Nei's genetic distances between the four types were significantly higher than those between conspecific local populations, and comparable to those between incipient species or very closely related species in many other animal groups. We therefore propose that these four types of sea-urchins should be classified as distinct and separate species of the genus *Echinometra*. The molecular phylogenetic tree constructed on the basis of the Nei's genetic distances revealed the close affinities between Types A and C and between Types B and D. It also showed a large genetic differentiation between Types A and B. The phylogenetic tree suggested that the four types speciated in relatively recent geological age of the middle Pleistocene. The speciation process of the four types is also discussed.

INTRODUCTION

Various species of sea-urchin are found along the coast of Okinawa Island, southern Japan, but *Echinometra mathaei* (Blainville) which is widely distributed from central Japan to south Australia [1], is one of the most abundant species. It has been well known that *E. mathaei* generally shows extensive morphological variations in the shape of test and color pattern of spines, etc. [1]. *Echinometra mathaei* specimens found along the Okinawan coast were not exceptions; Tsuchiya and Nishihira [2] reported that two different types of *E. mathaei* are observed on Okinawan reef flats and these two (they are called Type A and Type B) are distinguishable by color pattern of spines, ecological distribution pattern and habitat preference: Type A sea-urchins which have white-tipped or entirely white spines inhabit the moat and rock pools and frequently aggregated, while Type B with entirely brown spines are mainly found in burrows probably excavated by themselves on the reef edge of wave-exposed environments and generally avoided contact with other individuals. They also reported that the two types are different in the agonistic behavior: Type B is more aggressive than Type A, and Type B living in burrows exhibits remarkable agonistic behavior against the intruders by driving them away from their own burrows [3].

On the other hand, Uehara and Shingaki [4, 5] have reported that *E. mathaei* from the Okinawan coast can be divided into four different types (Types A, B, C and D) which are distinguishable by color pattern of spines and gamete incompatibility. The spines of Types A, B, C and D are white-tipped or entirely white, entirely brown, dark-brown or green and uniform black, respectively. Uehara and Shingaki [4, 5] have extensively examined the external features of eggs and sperm,

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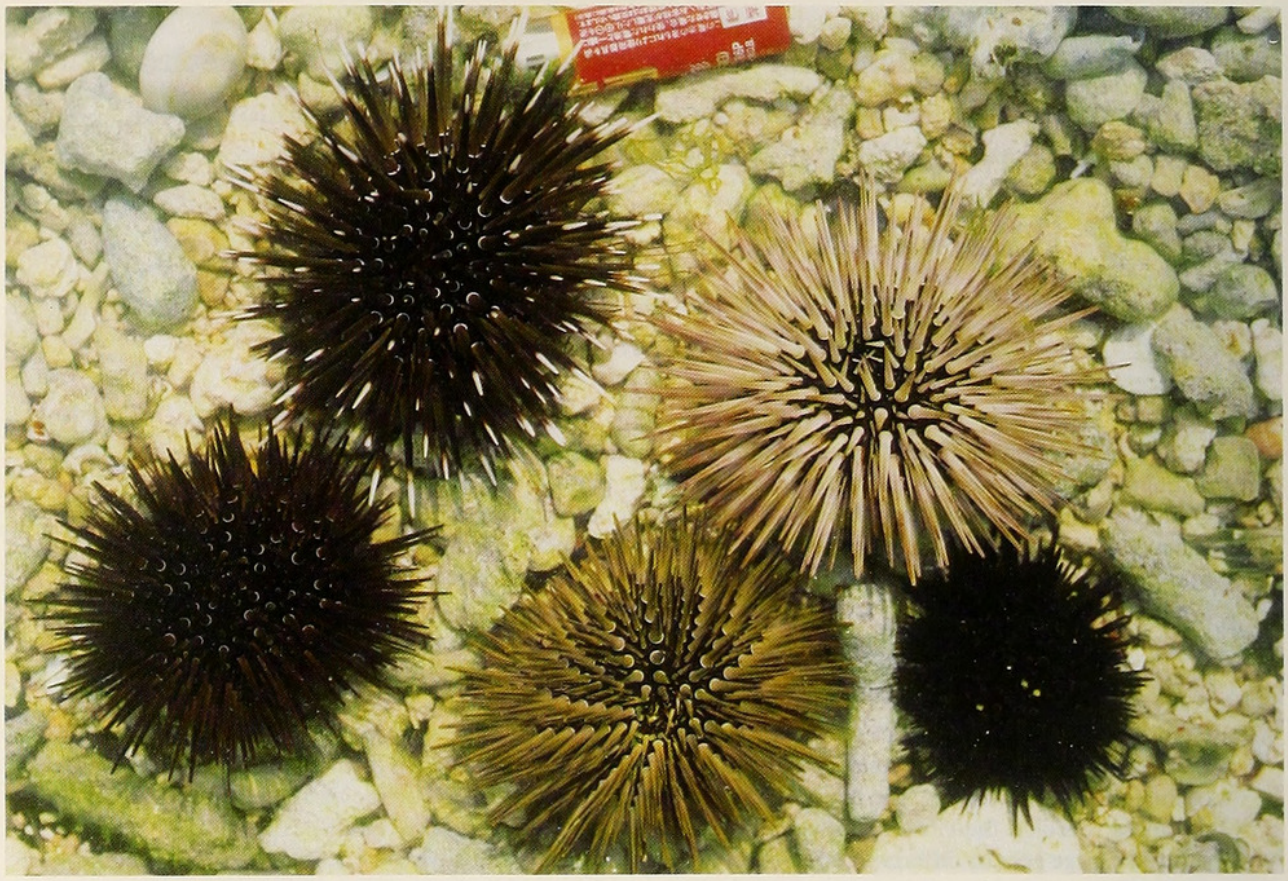

FIG. 1. Four types of the sea-urchin, *E. mathaei* (Blainville) from Okinawa Island, Japan. The left in the upper row is Type A with white-tipped spines. The right in the upper row is Type B with entirely brown spines. The left two sea-urchins in the lower row are Type C; the left of those is Type C with dark-brown spines and the right of those is Type C with green spines. The right in the lower row is Type D with uniform black spines. This picture was offered by the courtesy of Dr. T. Uehara, The University of the Ryukyus.

cross fertilization between types, larval and adult morphology, karyotypes, spawning and distribution patterns, and suggested that the four types might be four different species. Figure 1 shows four types of *E. mathaei* from Okinawa Island. Thus, the taxonomic problem as to whether these four types are indeed different species merits biochemical and genetic scrutiny. The molecular approach would provide useful information on the taxonomic relationships and also for the phylogenetic relationships which can not be obtained by morphological approaches. The elucidation of such problems is very attractive and valuable for understanding the speciation and evolution of the sea-urchin, *E. mathaei*.

Among many biochemical methods used for taxonomic studies, enzyme electrophoresis has been most widely used as one of the powerful techniques to distinguish the morphologically very similar species and to investigate the genetic and/or evolutionary relationships among taxa [6]. One of the present authors (N.M.) has been studying the echinoid phylogeny and taxonomy by using the molecular techniques such as enzyme electrophoresis and immunological method, and found that enzyme electrophoresis is a reliable method in the field of echinoid phylogeny and taxonomy [7–14].

In this paper, we report on the results of an electrophoretic investigation designed to clarify the taxonomic situation, the genetic and evolutionary relationships of the four types of the sea-urchin, *E. mathaei* (Blainville). In addition to these taxonomic problems, we also discuss on enzyme variation within populations of the sea-urchin estimated electrophoretically from population genetic standpoint.

MATERIALS AND METHODS

Sea-urchins

The four types (Types A, B, C and D) of the sea-urchin, *Echinometra mathaei* (Blainville), used in this study were collected from the coast near the Sesoko Marine Science Center, The University of the Ryukyus, Sesoko Island, Okinawa Prefecture, in February 1986. The Type A sea-urchins were collected from the moat and rock pools by snorkeling, and the others from the reef edge at low tide. For comparison, the Type A sea-urchins were also collected in August 1988 from the coast near the Sabiura Marine Park Research Station, Kushimoto, Wakayama Prefecture, which is located in the middle of the main island of Japan (Fig. 2). The number of individuals collected was 100 in total: 20 each of Type A from Kushimoto and Okinawa, 17 of Type B, 23 of Type C and 20 of Type D. The Okinawan Type C sea-urchins were divided into two populations on the basis of the color pattern of spines: one is characterized by dark-brown spines (13 individuals) and the other by green spines (10 individuals).

Immediately after collection, the guts and gonads were cut out from live specimens, and throughly washed in filtered sea water. They were then frozen in dry ice and transported to the

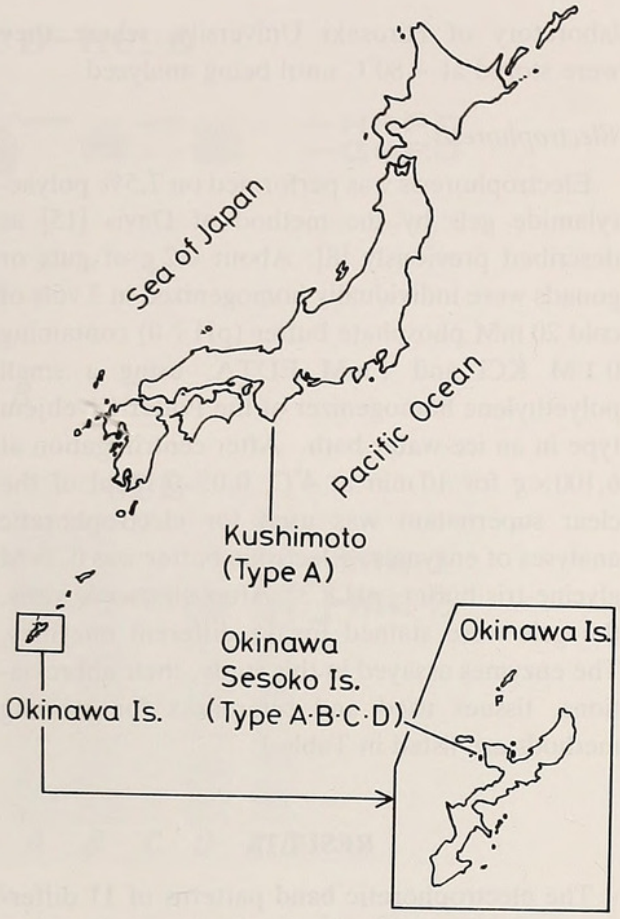

FIG. 2. Map showing the collecting localities of the four types (Types A, B, C and D) of *E. mathaei*, from Japanese waters used in this study.

TABLE 1. Enzymes and tissues assayed in the present electrophoretic study

Enzyme	Abbreviation	Tissue	Stain reference
Alcohol dehydrogenase	ADH	Gut	28
Glucose-6-phosphate dehydrogenase	G6PD	Gonad	29
Hexose-6-phosphate dehydrogenase	H6PD	Gonad	12
Malate dehydrogenase	MDH	Gut	30
Malic enzyme	ME	Gut	28
Octanol dehydrogenase	ODH	Gut	28
Sorbitol dehydrogenase	SDH	Gut	30
Xanthine dehydrogenase	XDH	Gut	30
Superoxide dismutase	SOD	Gut	28
Hexokinase	HK	Gonad	30
Alkaline phosphatase	ALK	Gonad	28
Esterase	EST	Gut	30
Peroxidase	PO	Gut	30
Amylase	AMY	Gut	31
Leucine amino peptidase	LAP	Gut	28

laboratory of Hirosaki University, where they were stored at −80°C until being analyzed.

Electrophoresis

Electrophoresis was performed on 7.5% polyacrylamide gels by the method of Davis [15] as described previously [8]: About 0.2 g of guts or gonads were individually homogenized in 3 vols of cold 20 mM phosphate buffer (pH 7.0) containing 0.1 M KCl and 1 mM EDTA, using a small polyethylene homogenizer of the Potter-Elvehjem type in an ice-water bath. After centrifugation at 6,100×*g* for 10 min at 4°C, 0.05–0.10 ml of the clear supernatant was used for electrophoretic analyses of enzymes. Electrode buffer was 0.38 M glycine-tris buffer, pH 8.3. After electrophoresis, the gels were stained for 15 different enzymes. The enzymes assayed in this study, their abbreviations, tissues used and references for staining methods are listed in Table 1.

RESULTS

The electrophoretic band patterns of 11 different enzymes observed in the four types (Types A, B, C and D) of *E. mathaei* are shown in Figure 3. From these band patterns, 28 genetic loci were identified. The major features of variation in 15 enzymes are summarized as follows:

Six enzymes (ADH, G6PD, H6PD, SDH, XDH and HK) from the four types all exhibited a single monomorphic active band of the same electrophoretic mobility. LAP also exhibited a single band of activity, but the band of Type D moved faster than those of the other three types.

PO exhibited two active bands in each type (PO-1 and PO-2), and the respective bands showed the same electrophoretic mobility among the four types. The fast band (PO-2) always showed a higher activity than the slow band (PO-1).

ME consistently appeared as two active bands (ME-1 and ME-2), of which the fast band showed higher activity. ME-2 showed single- and triple-banded phenotypes in Types A and D. This was interpreted as a diallelic system at a single locus coding for a dimeric protein, with single-banded pattern corresponding to the homozygous state and triple-banded pattern to the heterozygous state. The electrophoretic mobility of ME-1 and ME-2 did not differ in the four types.

ODH in Types A and C showed single- and triple-banded phenotypes as ME-2 in Types A and D. These band patterns were also interpreted as representing homozygosity and heterozygosity at a single locus coding for a dimeric protein, respectively. A similar variation has also been observed with ODHs of other sea-urchin species belonging to the order Echinoida, such as *Strongylocentrotus intermedius, Echinostrephus aciculatus* and *Heterocentrotus mammillatus* [9, 14]. The fast bands of homozygous state showed the faster mobility in Types A and B than in Types C and D.

ALK consistently appeared as two bands (ALK-1 and ALK-2) of similar activity. Although this enzyme was monomorphic in the four types, the active bands varied in mobility between the types.

MDH showed two active zones in each type (MDH-1 and MDH-2). Of these the faster zone (MDH-2) exhibited single- and triple-banded phenotypes in all types as in the case of ME-2 and ODH, thus suggesting the diallelic system at a single locus coding for a dimeric protein. A similar polymorphism has also been observed in *Anthocidaris crassispina* belonging to the same family Echinometridae [13]. The respective band of MDH-2 showed the same electrophoretic mobility among the four types, while the mobility of the slower bands (MDH-1) varied between the types; MDH-1 showed a single- and double-banded phenotypes in Type A. This suggests the presence of a diallelic system at a single locus coding for a monomeric protein: the single-banded pattern corresponds to the homozygous state, and the double-banded pattern to the heterozygous state. The MDH-1 bands of Types B, C and D were monomorphic and showed the same electrophoretic mobility which was slower than the MDH-1 of Type A.

EST activity was detected as several bands which were grouped into five zones (EST-1 to EST-5). EST-1 was double-banded in Type D, but single-banded in the other types. This may probably be due to the presence of two different alleles at a single locus. The mobilities of EST-2, EST-3 and EST-4 were the same in all types. The faster

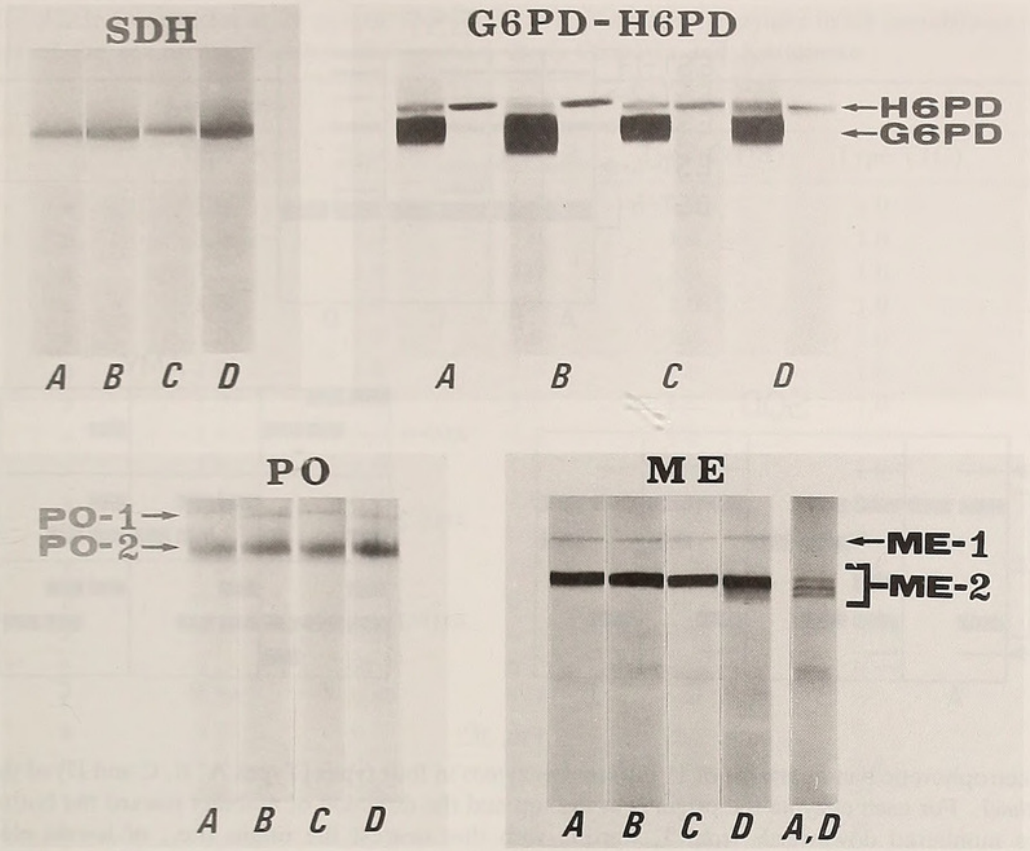
SDH
G6PD-H6PD
←H6PD
←G6PD
A B C D
A B C D
PO
ME
PO-1→
PO-2→
←ME-1
]←ME-2
A B C D
A B C D A,D

FIG. 3A.

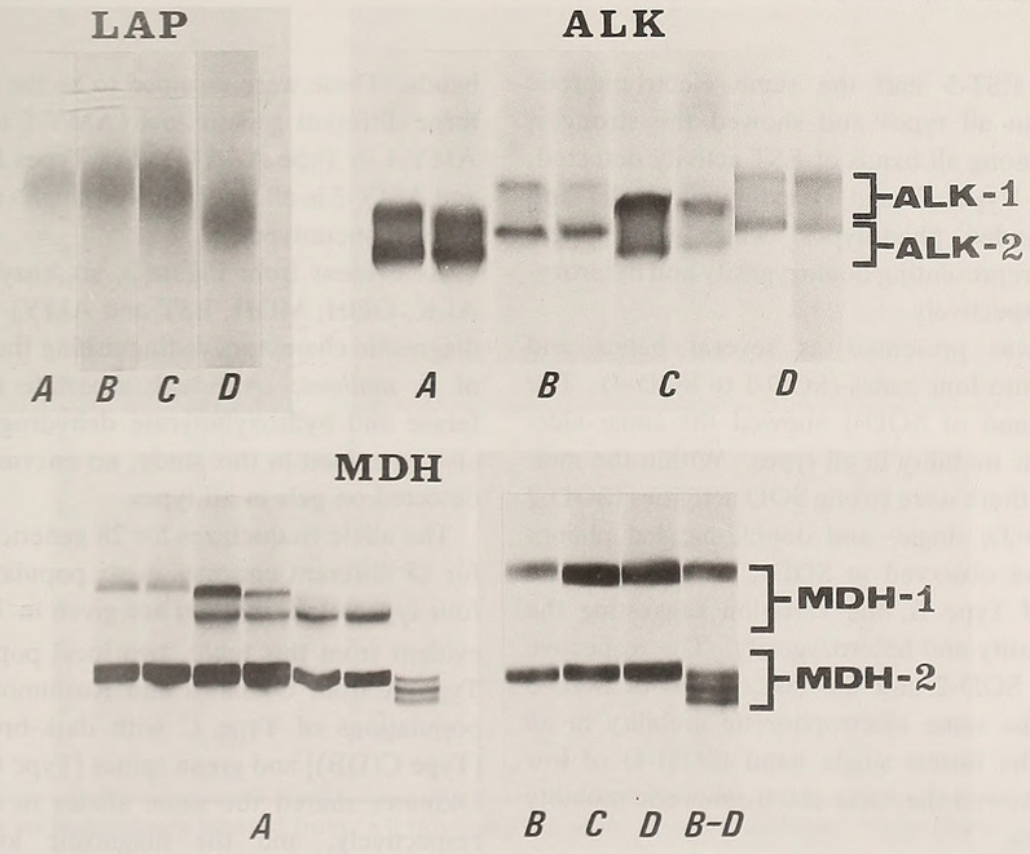
LAP
ALK
]←ALK-1
]←ALK-2
A B C D
A B C D
MDH
]←MDH-1
]←MDH-2
A
B C D B-D

FIG. 3B.

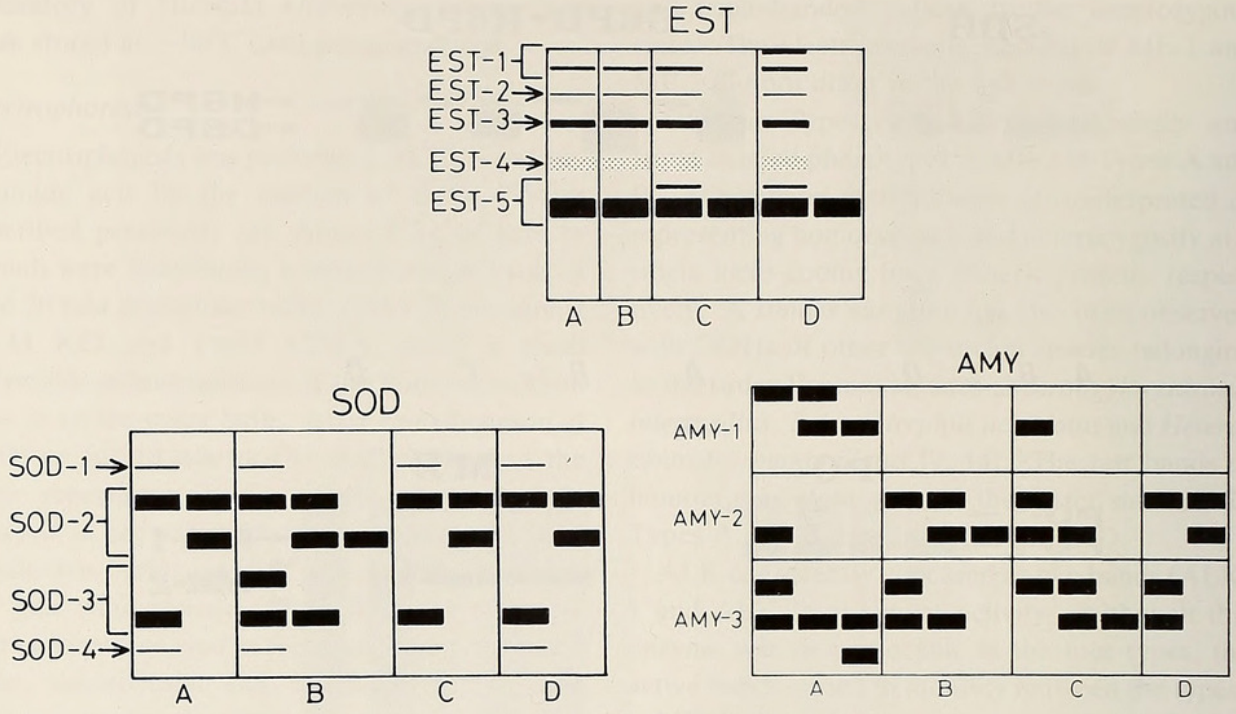

FIG. 3C.

FIG. 3. Electrophoretic band patterns of 11 different enzymes in four types (Types A, B, C and D) of the sea-urchin *E. mathaei*. For each enzyme the origin is at the top and the direction of mobility toward the bottom. Genetic loci are numbered downwards from 1, starting with that nearest the origin (i.e., of lowest electrophoretic mobility). The letters of A, B, C and D marked under the respective band pattern show Types A, B, C and D of *E. mathaei*, respectively.

band of EST-5 had the same electrophoretic mobility in all types and showed the strongest activity among all bands of EST activity detected. EST-5 in Types C and D exhibited single- and double-banded phenotypes, which were interpreted as representing homozygosity and heterozygosity, respectively.

SOD was presented as several bands and grouped into four zones (SOD-1 to SOD-4). The slowest band of SOD-1 showed the same electrophoretic mobility in all types. Within the middle zones there were strong SOD activities (SOD-2 and SOD-3), single- and double-banded phenotypes were observed in SOD-2 of all types and SOD-3 of Type B, the variation suggesting the homozygosity and heterozygosity. The respective bands of SOD-2 and the faster band of SOD-3 showed the same electrophoretic mobility in all types. The fastest single band (SOD-4) of low activity showed the same electrophoretic mobility in all types.

AMY activity was also detected as several bands. These were assumed to be the products of three different genetic loci (AMY-1 to AMY-3). AMY-1 in Type A, AMY-2 in Types B, C and D and AMY-3 in all types showed single- and double-banded phenotypes.

As evident from Figure 3, six enzymes (LAP, ALK, ODH, MDH, EST and AMY) were useful diagnostic characters distinguishing the four types of *E. mathaei*. Although aspartate aminotransferase and hydroxybutyrate dehydrogenase were also examined in this study, no enzyme band was detected on gels in all types.

The allele frequencies for 28 genetic loci coding for 15 different enzymes in six populations of the four types of *E. mathaei* are given in Table 2. As evident from this table, two local populations of Type A from Okinawa and Kushimoto and two populations of Type C with dark-brown spines [Type C(DB)] and green spines [Type C(G)] from Okinawa shared the same alleles in each locus, respectively, and the diagnostic locus distinguishing those populations was not found. On the

TABLE 2. Allele frequencies at 28 genetic loci coding for 15 different enzymes in six populations of four types of the sea-urchin, *Echinometra mathaei*, from Okinawa and Kushimoto

Locus	Allele	Kushimoto	Okinawa				
		Type A	Type A	Type B	Type C(DB)	Type C(G)	Type D
ADH	a	1.0	1.0	1.0	1.0	1.0	1.0
G6PD	a	1.0	1.0	1.0	1.0	1.0	1.0
H6PD	a	1.0	1.0	1.0	1.0	1.0	1.0
SDH	a	1.0	1.0	1.0	1.0	1.0	1.0
XDH	a	1.0	1.0	1.0	1.0	1.0	1.0
HK	a	1.0	1.0	1.0	1.0	1.0	1.0
PO-1	a	1.0	1.0	1.0	1.0	1.0	1.0
PO-2	a	1.0	1.0	1.0	1.0	1.0	1.0
ME-1	a	1.0	1.0	1.0	1.0	1.0	1.0
ME-2	a	1.0	0.97	1.0	1.0	1.0	0.91
	b	0	0.03	0	0	0	0.09
LAP	a	1.0	1.0	1.0	1.0	1.0	0
	b	0	0	0	0	0	1.0
ODH	a	0.21	0.11	0	0.64	0.56	0
	b	0	0	0	0.36	0.44	1.0
	c	0.79	0.89	1.0	0	0	0
ALK-1	a	0	0	1.0	0	0	1.0
	b	0	0	0	1.0	1.0	0
	c	1.0	1.0	0	0	0	0
ALK-2	a	0	0	1.0	0	0	1.0
	b	1.0	1.0	0	1.0	1.0	0
MDH-1	a	0	0	1.0	1.0	1.0	1.0
	b	0.55	0.29	0	0	0	0
	c	0.45	0.71	0	0	0	0
MDH-2	a	1.0	0.97	0.94	1.0	0.95	0.93
	b	0	0.03	0.06	0	0.05	0.07
EST-1	a	0	0	0	0	0	1.0
	b	1.0	1.0	1.0	1.0	1.0	0
EST-2	a	1.0	1.0	1.0	1.0	1.0	1.0
EST-3	a	1.0	1.0	1.0	1.0	1.0	1.0
EST-4	a	1.0	1.0	1.0	1.0	1.0	1.0
EST-5	a	0	0	0	0	0.35	0.15
	b	1.0	1.0	1.0	1.0	0.65	0.85
SOD-1	a	1.0	1.0	1.0	1.0	1.0	1.0
SOD-2	a	0.63	1.0	0.62	0.60	0.50	0.63
	b	0.37	0	0.38	0.40	0.50	0.37
SOD-3	a	0	0	0.27	0	0	0
	b	1.0	1.0	0.73	1.0	1.0	1.0
SOD-4	a	1.0	1.0	1.0	1.0	1.0	1.0
AMY-1	a	0.44	0.59	0	0	0	0
	b	0.56	0.41	0	1.0	1.0	0
AMY-2	a	0	0	0.37	0.22	0.06	0.72
	b	1.0	1.0	0.63	0.78	0.94	0.28
AMY-3	a	0	0.21	0.16	0.44	0.25	0.50
	b	0.95	0.79	0.84	0.56	0.75	0.50
	c	0.05	0	0	0	0	0

Alleles are correspondingly lettered from "a", this being the allele of lowest mobility. Type C(DB) and Type C(G) represent Type C sea-urchins with dark-brown spines and those with green spines, respectively.

TABLE 3. Genetic variation in six populations of four types of the sea-urchin, *Echinometra mathaei*, from Okinawa and Kushimoto

Parameter	Kushimoto	Okinawa				
	Type A	Type A	Type B	Type C(DB)	Type C(G)	Type D
No. of alleles per locus	1.18	1.21	1.19	1.14	1.21	1.22
Proportion of polymorphic loci (%)	17.9	21.4	18.5	14.3	21.4	22.2
Expected average heterozygosity per locus (%)	6.7	5.5	6.3	6.3	7.3	7.1

TABLE 4. Genetic identities (above diagonal) and genetic distances (below diagonal) between six populations of four types of the sea-urchin, *Echinometra mathaei*, from Okinawa and Kushimoto

Type	1	2	3	4	5	6
1. Type A (Kushimoto)	—	0.990	0.856	0.899	0.900	0.727
2. Type A (Okinawa)	0.010	—	0.851	0.886	0.881	0.725
3. Type B (Okinawa)	0.155	0.161	—	0.850	0.844	0.865
4. Type C(DB) (Okinawa)	0.106	0.121	0.163	—	0.992	0.782
5. Type C(G) (Okinawa)	0.105	0.127	0.170	0.008	—	0.774
6. Type D (Okinawa)	0.319	0.322	0.145	0.246	0.256	—

Type C(DB) and Type C(G) represent Type C sea-urchins with dark-brown spines and those with green spines, respectively. Genetic identities and genetic distances were calculated by the method of Nei [16].

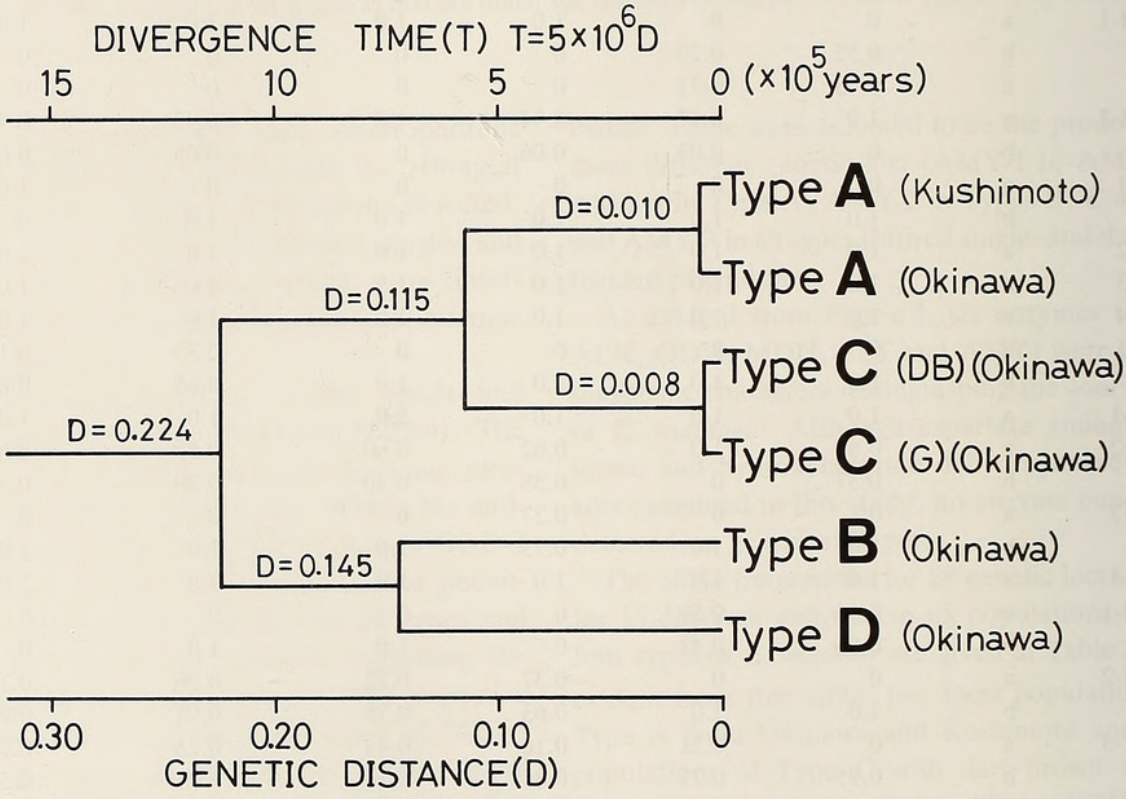

FIG. 4. A molecular phylogenetic tree showing the genetic relationships among six populations of four types of *E. mathaei* from Okinawa and Kushimoto. It was constructed from Nei's genetic distances by using the UPGMA clustering method of Sneath and Sokal [17]. The divergence time estimated from the Nei's equation [18] using the genetic distance is given in the phylogenetic tree.

other hand, the four types showed the different allelic compositions in several genetic loci.

With respect to the degree of enzyme variation within populations, Table 2 shows that enzymes (*e.g.*, G6PD or HK) involved in glucose metabolism (catalysing steps in, or adjacent to, the glycolytic pathway and tricarboxylic acid cycle) were on average less variable than those (*e.g.*, SOD or AMY) involved in other reactions, which contain many that are relatively non-specific with respect to substrate. Table 3 summarizes the extent of genetic variation in six populations. The number of alleles per locus was in the range of 1.14–1.22, with a mean of 1.19, the proportion of polymorphic loci (P), in the range of 14.3–22.2%, with a mean of 19.3%, and the expected average heterozygosity per locus (H), in the range of 5.5–7.3%, with a mean of 6.5%.

In order to quantify the degree of genetic differentiation among six populations of the four types, the genetic identity (I) and genetic distance (D) between each population were calculated by the method of Nei [16] from the allele frequency data in Table 2. Table 4 shows the matrices of I and D values between all pairs of the six populations. The high I values were found between two populations of Type C (DB and G) and between two local populations of Type A (I=0.992 and I=0.990). When the I values between the four different types were compared with each other, the I values between Types A and C were higher (I=0.881–0.900), while those between Types D and A or C were lower (I=0.725–0.782). Figure 4 shows the molecular phylogenetic tree for six populations of the four types which was constructed from the Nei's genetic distance matrix by using the unweighted pair-group arithmetic average (UPGMA) clustering method of Sneath and Sokal [17]. The molecular phylogenetic tree revealed the following: The four type are divided into two large clusters. One consists of Types A and C, and the other of Types B and D. The mean genetic distance between these two clusters is 0.224. Namely, Type A is more closely related to Type C than to the other types (D=0.115), and Type B is more closely related to Type D than to the other types (D=0.145). Further, the affinity between Types A and C is higher than that between Types B and D. The divergence time (T) of the four types estimated from the genetic distance (D) by the Nei's equation [18] is also given in the phylogenetic tree. The molecular phylogenetic tree with the divergence time provides much valuable information with respect to the evolutionary divergence or the speciation process of the four types of *E. mathaei.*

DISCUSSION

Enzyme variation within populations

Soon after protein electrophoresis became widely used as a method for screening genetic variation, it became clear that certain enzymes were on average more variable than others. On the basis of their works with flies of the genus *Drosophila,* Gillespie and Kojima [19] and Kojima *et al.* [20] proposed that enzyme heterogeneity was related to enzyme function; i.e., those enzymes involved in glucose metabolism are less variable than those involved in other reactions. This holds true for the sea-urchin enzymes here studied; the non-glucose metabolizing enzymes (the mean H=7.9%) were substantially more variable than the glucose enzymes (the mean H=3.2%). Similar results have also been obtained in many other sea-urchin species reported previously [8, 9, 13, 14]. Kojima also stated that substrate heterogeneity was reflected in enzyme heterogeneity [20]. However, the enzyme heterogeneity may be explained by a different way; In general, glucose metabolizing enzymes are of functional importance, and therefore functional constraint of the enzyme molecules is stronger than that of other non-glucose metabolizing enzymes. The more strict constraint would decrease the neutral regions of the molecules and the probability of a mutational change (amino acid replacement) being not harmful (*i.e.*, selective neutral) is smaller for the glucose metabolizing enzymes than for other non-glucose metabolizing enzymes. Thus, the Kojima's findings can be explained easily by the neutral theory of molecular evolution.

We have previously reported on the amount of genetic variation within populations of various echinoderm species [13]. According to it, the average heterozygosity per locus (H=6.5%) in six

populations of the four types of *E. mathaei* was comparable to H values of many other echinoderm species living in shallow water, but considerably lower than H values of echinoderms in deep-sea. On the basis of electrophoretic studies on genetic variation in marine invertebrates, Ayala and Valentine [21] suggested that marine invertebrates from trophically stable environment, such as deep-sea generally show higher genetic variation than those from trophically unstable environment, such as shallow water in temperate latitudes. Kimura [22] described in his neutral theory that most mutations at molecular level are selectively neutral and most of the remainings mildly deleterious. Therefore, the latter mildly deleterious genes would be selected in unstable environment such as shallow water. On the other hand, in more stable environment such as deep-sea, some of such mildly deleterious genes can function and may be maintained in populations. As a result, the degree of genetic variation in marine invertebrates from unstable environment such as shallow water would become lower than that from stable environment such as deep-sea. The prediction of Ayala and Valentine [21] does not seem to be contradictory to the neutral theory. Further, the difference in the degree of genetic variation between invertebrates from shallow water and deep-sea may be closely related to the population size. Namely, it is expected that the population size of invertebrates from deep-sea is much larger than that of invertebrates from shallow water. Accordingly, deep-sea invertebrates of large population size would maintain higher genetic variability within the populations as compared with shallow water invertebrates of the small population size.

Taxonomic situation of the four types of E. mathaei

As evident in Figure 3 and Table 2, the four types (Types A, B, C and D) of the sea-urchin, *E. mathaei,* from the Okinawan coast do not share gene pools in spite of their sympatric distribution. Namely, they are fixed for different alleles at 7 genetic loci, LAP, ODH, ALK-1, ALK-2, MDH-1, EST-1 and AMY-1. The six enzymes including the above seven genetic loci are diagnostic enzymes that are very useful molecular characters for distinguishing the four types of very similar morphology. According to a number of biochemical taxonomic studies on a variety of animal taxa, distinct species which are reproductively isolated are typically fixed for different alleles at the same locus. On the other hand, conspecific populations generally differ from one another in frequencies of the same alleles. The present electrophoretic data clearly show that there is no gene flow between the four types in spite of their sympatric distributions. This is a strong evidence for that they are genetically distinct and separate species. In contrast, the two local populations of Type A from Okinawa and Kushimoto and the two populations of Type C with dark-brown and with green spines showed identical electrophoretic patterns in all enzymes assayed and almost the same allele frequencies at the 28 genetic loci scored. This clearly shows that the two populations of Type C belong to one and the same species and the difference in spine color is simply of individual variation within the species.

As evident from the phylogenetic tree shown in Figure 4, the D values between the four types were significantly higher than those obtained by interpopulational comparison: those between two local populations of Type A from Kushimoto and Okinawa and between the two populations of Type C [Type C(DB) and Type C(G)]. The D values between Type A and Type C and between Type B and Type D are 12 to 18 times as large as those between populations of the same type. Further, the D value between the cluster of Type A and Type C and that of Type B and Type D is 22 to 28 times as large as the interpopulational values of the same types. When compared with many other electrophoretic data on various animal groups hitherto reported (see the review of Ayala [23]), the D values between two local populations of Type A and between two populations of Type C are equivalent to those reported between conspecific populations, while the D values between the four types are comparable to those between incipient species or closely related species. More recently, one of the present authors (N. M.) has electrophoretically examined the degree of genetic differentiation among six local Japanese populations of the sea-urchin, *Anthocidaris crassispina,* which belongs to the same family as *E. mathaei.* As a result, D-values between six conspecific local

populations of the sea-urchin were in the range of 0.008–0.069 [13]. The D-values between the four types examined in this study are considerably higher than those between conspecific six local populations of *A. crassispina*. Judging from the genetic distances between the four types in addition to their different allelic compositions, they should be considered as four distinct, but closely related species of the genus *Echinometra* of the family Echinometridae.

Evolutionary relationships among the four types

The phylogenetic tree among the four types of *E. mathaei* (Fig. 4) revealed that Type A is more closely related to Type C than to other types and that Type B is more closely related to Type D than to other types. It also shows that Type A is considerably different genetically from Type B. The large genetic differentiation between Type A and Type B has also been suggested by non-molecular studies; Uehara and Shingaki [4, 5] did not succeed in reciprocal cross fertilization between Type A and Type B. They also reported that their karyotypes differ from each other, though their diploid chromosome numbers are equal to each other (2n=42). Further, the two types are also different in the number of tubercles on the madreporite and in the skeletal structure of larva. The present electrophoretic results well accord with these non-molecular evidence. However, it is difficult to estimate the genetic relationships of the four types quantitatively by non-molecular data. On the other hand, the molecular approach can provide valuable information to the estimation of their phylogenetic relationships.

Speciation process of the four types

According to the morphological studies by Uehara and Shingaki [5], Type C is similar to Type D in several morphological characters, though they are largely differentiated from each other at molecular level. In a previous biochemical systematic study on the genetic relationships among six members of the family Echinometridae from Japanese waters, Matsuoka and Suzuki [14] reported that *E. mathaei* (Type A was used as the representative sea-urchin of the four types) is closely related to the endemic Japanese sea-urchin, *A. crassispina*. In appearance, Type D sea-urchins resemble *A. crassispina*. Further, Uehara and Shingaki [5] reported that Type C and Type D have the same shaped trifurcated spicules in their tube feet. Similar trifurcated spicules are also observed in the tube feet of *A. crassispina*. Further, Types C and D have intermediate characters between Types A and B in the skeletal structure of larva and the number of tubercles on madreporite. These morphological evidence suggest that Type C·D-like sea-urchin might be the ancestral form of the four types of *E. mathaei*. The phylogenetic tree (Fig. 4) shows not only their genetic relationships, but also the sequence of their evolutionary divergence. According to Nei [18], genetic distance (D) corresponds well with the divergence time (T) from the common ancestor, and T of two taxa can be estimated by $T=5\times 10^6 D$ (years). Application of this equation to the molecular dendrogram constructed from the D values (Fig. 4) leads to a speculation that the ancestral form of the four types that might be Type C·D-like sea-urchin diverged into two lineages (one is Type C-like lineage and the other Type D-like lineage) 1.1 million years ago (MY), and that thereafter, Types A and B derived from Type C-like and Type D-like sea-urchins 0.6–0.7 MY ago, respectively. Namely, Types A and B sea-urchins may be more recent species than Types C and D, and the former two seem to be more predominant species than the latter two. In fact, the distributional ranges of Types A and B are wider than those of Types C and D. In paticular, Type A distributes widely by Sagami Bay in the central region of the main island of Japan. Further, in Okinawan reef flats, Types A and B are more frequently found than Types C and D. Namely, the population size of Types A and B appears to be larger than that of Types C and D.

Nisiyama [24] described in his monograph that the genus *Echinometra* is one of the oldest genera of the family Echinometridae and its evolutionary origin dates back at least to the Miocene. However, the previous biochemical systematic study on the family Echinometridae clearly demonstrated that the evolutionary origin of the genus *Echinometra* is more recent geological age of the late

Pliocene (about 2 MY ago) [14]. Further, the present molecular evidence strongly suggests that the four types of *E. mathaei* had speciated in more recent geological age of the middle Pleistocene.

In recent years, many authors have used mitochondrial DNA to study evolutionary relationships of organisms. However, Nei [25] suggested that the resolving power of mitochondrial DNA is not necessarily higher than that of protein electrophoresis. This is particularly so when the restriction enzyme technique is used. According to the estimation of Nei [25], electrophoresis is expected to survey about 100 nucleotides per locus. If we examine 60 loci by electrophoresis, it is equivalent to studying 6,000 nucleotides. This is much larger than the number of nucleotides (895) sequenced by Brown *et al.* [26] for human and ape mitochondrial DNAs. In their study of the evolution of human and ape mitochondrial DNAs, Ferris *et al.* [27] used eighteen 6-base enzymes and one 4-base enzyme. The average number of restriction sites per sequence for all 6-base enzyme was 42, whereas the number for the 4-base enzyme was 7. Therefore, the total number of nucleotides assayed is $42\times6+7\times4=280$. This number is even smaller than the number of nucleotides sequenced by Brown *et al.* [26]. Protein electrophoresis may be one of the powerful techniques of measuring genetic divergence in the evolutionary studies.

In conclusion, the four types of *E. mathaei* from Japanese waters should be classified as four separate and distinct species of the genus, *Echinometra,* on account of their genetic distinction verified by the present biochemical genetic study. In the near future, the taxonomic description and the appropriate species names should be given to these four sibling species of the genus, *Echinometra.*

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