RF-066 Development of a population genotyping technique in the toxic microalgae and clarification of impacts of human activity on the globalization (Abstract of the Final Report)

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[Abstract]

For population genotyping of six HAB (harmful algal bloom) species, we tried to develop polymorphic microsatellite (MS) markers. We succeeded in developing >10 polymorphic MS markers in two species, 2-3 polymorphic markers in the other two species, and the markers are under development at one species. Results of population genotyping clearly showed significant population differentiation in three of four species, suggesting that the genetic structuring and genetic isolation have occurred among the regional populations in Japan. The population genotyping data in a species suggested a possible gene flow by human assisted dispersal.

In 2006 and 2007, we detected directly the toxic dinoflagellate *Alexandrium tamarense* from oyster's spats, had just transferred from area A to B. We detected 6-120 cells from outside of a young oyster collector and 21-1,488 cells exhausted from the young oysters as the fecal pellets per collector. Other microalgal species were also contained in the fecal pellets and calculated as 1,500-13,600 cells per collector. Considering factors of abundances of *A. tamarense* around the farming ground of oyster spats, filtration rate of oysters and estimation of the total amount of oyster spats carried from the area A to B per year (28 million collectors), it was roughly calculated that oyster spats have carried out ca. several million-liter of seawater per year, implying that the translocation acts as a biological career of toxic microalgae.

Thirty-eight samples were collected from seawater tanks of live fish tracks at a fish market nearby the area B in 2006-2007 and microalgal species were detected from 25 of the 38 samples and some HAB species were also contained. Tracks carry 200 to 10,000 liters of seawaters and 10-20 tracks come to the market per day and it is estimated that ca. 100,000 liter of seawater are carried from geographically distant area per day and discharged into the sea in front of the market, suggesting introduction of HAB species and disturbing the biodiversity in the area B.

1. Introduction

Since cargo vessel ballast water was first suggested as a vector in the dispersal of non-indigenous marine plankton some 90 years ago, compelling evidence from a number of investigators suggests that these organisms have expanded their geographic range by not only natural dispersal but also human-assisted mean[1-3]. Only the ship's ballast water and translocation of shellfish stocks are not vectors for leading accidental transfer of toxic microalgae, but live fish tracking, lumber and sea sand transfer also. Some of them have been overlooked as the vectors so far and these human activities would probably act as carriers of toxic microalgae, resulting in enhancement of globalization of HAB (Harmful Algal Bloom) events.

We believe that an assessment of genetic relationships among HAB populations with highly polymorphic genetic markers provides the most promising approach to elucidate their mixing and dispersal. However, ribosomal DNA data are insufficient to test the dispersal theories, and at the time there was no available genetic marker for any harmful algal bloom species that could unambiguously distinguish between indigenous and introduced flora. Thus, the development of highly polymorphic genetic markers and a genotyping method to briefly distinguish between indigenous and introduced populations was needed to test these theories. Microsatellites are highly polymorphic genetic markers comprising tandemly repeated short nucleotide sequences that are ubiquitous and abundant in eukaryotic genomes[4, 5]. Recently, there has been a rapid increase in studies using microsatellite markers to estimate effective gene flow and clarify dispersal mechanisms in natural eukaryotic populations. Similarly, population genetics methods have been rapidly applied to the ecological research of microalgal species, as evidence by the development of microsatellite markers for *Alexandrium tamarense*[6], *A. minutum*[7], *A. catenella*[8] and *Heterosigma akashiwo*[9].

Development of methods to detect and quantify transferring HAB (Harmful Algal bloom) species via translocation of shellfish stocks and live fish tracking and investigation of current situations of HAB transfer via human activity as are also research topics of urgency for us.

2. Research Objective

In this study, we develop techniques for distinguishing regional populations by genotyping and comparing the genetic structures in six HAB species using highly polymorphic genetic markers and in order to reveal the mechanism for the globalization. We explore the fine-scale diversity, population genetic structure, and biogeography of the target species in Japanese coastal waters and also try to detect the impact of natural and human-assisted dispersals on the genetic structure and gene flow. Additionally we compile a database for the genotyped individuals and populations to assert the origin of a newly appeared population.

Due to increasing the human activities, there can be many potential pass way in initiating non-indigenous toxic microalgae to geographically distant area. International Maritime Organization (IMO) and other international bodies has already progressed by the international

framework “Global Ballast Water Management Programme[10)]” to regulate the transfer of harmful aquatic organisms by ships and these efforts will be expected to reduce a dispersal risk of toxic algal species. As mentioned above, only the ship’s ballast water and translocation of shellfish stocks are not vectors for leading accidental transfer of toxic microalgae, but live fish tracking, lumber and sea sand transfer also. Therefore, development of the methods for detecting and quantifying HAB species contained in the spats or seawater tanks in live fish tracks is an urgent issue for us in order to know the current situation of transportation of HAB species via translocation of shellfish stocks and live fish tracking. Therefore, we verify the current situation of the transportation of HAB species from a coastal area to another area with these human activities and also evaluate the impact of human activities on gene flow of HAB species.

3. Materials and Methods

(1) Development of a population genotyping technique

Water samples containing the target species in this study were taken by collaborators and brought back to the lab and living vegetative cells were isolated by micropipetting and established many clonal strains in every species (Table 1). We incubated the clonal strains in2/f culture medium under appropriate incubation conditions for harvest and stored in a freezer until use. Total genomic DNA was extracted from the harvested cells using DNeasy Plant Mini Kit (QIAGEN) or a modified CTAB method. Microsatellite regions of each species were isolated following a dual-suppression-PCR technique[6, 7, 9)] or compound SSR primer method[11)]. In the species in which microsatellite markers were successfully developed, characterization of each microsatellites and genotyping of every clonal strains were performed, i.e. the PCR success percentage, the number of alleles, gene diversity, Hardy–Weinberg equilibrium (HWE), linkage disequilibrium, the observed and expected heterozygosity were tested using several software for population genetics.

(2) Validation of current situations of harmful and toxic microalgal transportation by human activity

1) Detection and quantification of HAB species contained in oyster spats transferred from production area

In the spring of 2006 and 2007, we obtained Japanese oyster spats, had just transferred from area A of northern Japan to B-area of western Japan and also from area B to area C of western Japan (only in 2006) by tracking. It took already 36 hours during the transfer from area A to area B (distance of 1,280 km) since they had been exposed in air and three hours from area B to area C (distance of 200 km). Approximately, 100-250 individuals of young oysters, whose shell heights are 2-40 mm were adhered to a scallop shell (collector). After brushing the surface of spats we soaked the spats into filtered seawater for six hours to make the young oysters exhaust their fecal pellets and after that removed the oyster spats from the seawater. Two days later, the filtered seawater was concentrated until 5 mL aliquots by nylon mesh (20 μm of opening). We counted the cell numbers of HAB species outside and inside of young oysters by normal light microscope and estimated the abundances per collector as outside and inside of young oysters.

2) Detection and quantification of HAB species from seawater tanks in live fish tracks

We collected seawater samples from 38 live fish tracks in total at a large fish market where is located near B-area for two years of 2006 and 2007. Five liters of seawater was sampled from each seawater tank of the tracks. After when gone back to lab, seawaters were sieved by nylon mesh (15 μm of opening) for concentration of microalgae and the samples were observed by normal light microscopy to identify the species and enumerated the abundances per track in every species.

4. Results and Discussions

(1) Development of a population genotyping technique

We established clonal strains in every species (Table 1) and tried to develop microsatellite markers in three species.

Table 1. View of progress of the population genopying technique

Species	Culture strains for microsatellite isolation*	Strains in total	Sampling area	Development of Microsatellite marker**	Number of polymorphic imarkers	Genotyping technique**
Chattonella ovata	○	93	Hiroshima Bay, Harima-Nada (Hyogo Pref.), Gokasyo Bay (Mie Pref.)	○	12	○
Dinophysis acuminata	○	80	Hiroshima Bay, Inokushi Bay (Ohita Pref.)	△	3	○
Dinophysis fortii	○	>100	Hiroshima Bay, Yatsushiro Bay (Kumamoto Pref.), Tottori coasts (Tottori Pref.)	△	3	△
Gymnodinium catenatum	○	81	Inokushi Bay (Oita Pref.), Senzaki Bay (Yamaguchi Pref.), Miyanokawachi Bay (Kumamoto Pref.)	△	2	○
Heterocapsa circularisquama	○	125	Ago Bay, Mikawa Bay (Aichi Pref.), Kusuura Bay (Nagasaki Pref.)	○	15	○
Pyrodinium bahamense	○	4	Masinloc Bay, Honda Bay (Phillipine)	X	0	X

*, Four strains are available: ○; **, ○: Developed; △: Developping; X: quit developing

In *C. ovata*, we succeeded in developing 12 polymorphic microsatellite markers. Each locus showed either one or two bands for each individual, indicating homozygous or heterozygote state in a diploid. These microsatellite loci are sufficiently variable for the study of genetic structure in *C. ovata.* We analyzed genetic structures of three populations sampled from Hiroshima Bay (n=17), Harima-Nada (Hyogo Pref.) (n=40) and Gokasyo Bay (Mie Pref.) (n=36) using 7 microsatellite markers. The number of alleles per locus ranged from 5 to 11 with an average of 8.0. The observed and expected heterozygosities ranged from 0.38 to 0.98 and from 0.55 to 0.77, respectively. After Bonferroni correction, two populations significantly deviated from Hardy-Weinberg equilibrium HWE (P<0.05). In Fisher's exact test, no significant population differentiation was detected, suggesting no structuring of these three populations, reflecting recent expansion of the populations. Because, the appearance of this species in Japan was reported from 20 years ago, however, the red tide was reported for the first time in Hiroshima Bay in 2004 and

followed by Gokasyo Bay and Harima-Nada in 2007. Recently, appearance of this species has also been confirmed in China and Mexico, therefore, we would like to compare the genetic structure among the world populations using the developed markers and compile a database of the genotypic data in each individual and population.

In *H. circularisquama*, we succeeded in developing 15 polymorphic microsatellite markers (Fig. 1). Each locus showed clear single bands for each individual, indicating homozygous state in a haploid. After Bonferroni correction, no linkage disequilibria were detected between loci ($P > 0.05$). The number of alleles at the 15 loci ranged from 2 to 6 with an average of 3.4, and the estimate of gene diversity (Nei 1987) varied between 0.205 and 0.684. We conducted the genotyping using 45 clonal strains and all the strains were divided into different genotypes (Fig. 2), suggesting that these microsatellite loci are sufficiently variable to allow the population genetic study of *H. circularisquama*. We analyzed genetic structures of three populations sampled from Ago Bay in 2006 (n=45), Hamanako (Shizuoka Pref.) in 2007 (n=40) and Kusuura Bay (Kumamoto Pref.) in 2007 (n=40) using 14 microsatellite markers. The number of alleles per locus ranged from 2 to 6 with an average of 3.9. The gene diversity ranged from 0.28 to 0.75

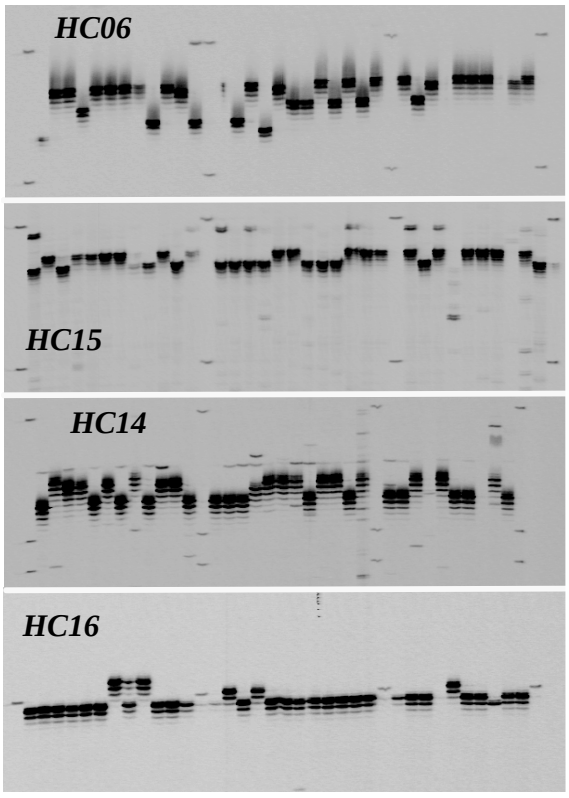

Fig. 1. Gel electrophoresis patterns of PCR products amplified by microsatellite markers in *Heterocapsa circularisquama*. Characters show the locus names.

No.	genotype	No.	genotype
1.	AAAAAAABBABDCBC	26.	BCBAAACBBCADCBB
2.	AEADBAEBA. BACBC	27.	BDBCBAEAACDDCCC
3.	AEACAAEBBCBCCAC	28.	BEACBBCBBCBDCBB
4.	BCBCBAABACBDCBB	29.	BEAABAABBCBDCBB
5.	AEBABAAABCBDCCB	30.	BEBABAAABCBDABB
6.	AEACBACBBCACBBC	31.	BBADBAEBACADCAC
7.	AEBABCAABCBDCAC	32.	ABAAABCABCCDACA
8.	BEADAAAAACBDC. C	33.	ADBCBAABACDDCAC
9.	BBBAACABBCBBCAC	34.	AEBAAAABCABDAAC
10.	BEBCBADABCBDCAB	35.	AC. DBAABAABDACB
11.	ADBCAAEBACBDCBC	36.	BEBDBBCBACCDACB
12.	ABAABACBBCCDABA	37.	ABBABACBBCBDCBA
13.	AEBAAACBBCADCCB	38.	ACBCAABABCBDDBB
14.	ABBAABAAAABDCAA	39.	AEBABACABCADCCC
15.	AEBAAAACACCDCBB	40.	AEAABACBBCDDCBB
16.	BABBABECBCADCBB	41.	AABCABABBACDCBC
17.	ADACBACABCCDABB	42.	ACBCBAEABCBDCBA
18.	ACBCBABBACADCBB	43.	AE. ABAAAACADACB
19.	ACACAACBBCBDCAB	44.	BEACBACAAACDCAA
20.	BEBAAAAABCBDCCB	45.	BEAAAAABBCCDCAB
21.	ACACAAAABABDABB		
22.	AEACBAAAACCDCAB		
23.	ACAABAEABBAACAB		
24.	BEACBAABBCBDCBB		
25.	AEAABAFBACCDCAB		

Fig. 2. Genotyping of each clonal strain of *Heterocapsa circularisquama* by microsatellites (n=45, isolated from Ago Bay). All strains were divided into different genotypes.

with an average of 0.49. Interestingly, significant population differentiations were detected among all possible pairwise populations in Fisher's exact test, suggesting that structuring of these three populations had already progressed since the appetence of this species was firstly confirmed in

Japan (Uranouchi Bay, Kochi Pref.) in 1988. In comparison of the allelic frequency, the number of alleles shared in all three populations, between Ago Bay and Hamanako, Ago Bay and Kusuura Bay and Hamanako and Kusuura Bay were 31, 9, 1 and 1 respectively. Population specific alleles detected only in Ago Bay, Hamanako and Kusuura Bay were 4, 8 and 1, respectively. Thus, Ago Bay and Hamanako populations share many same alleles, but a lack of sharing some alleles were confirmed populations between former two and Kusuura Bay. Accordingly, it is possible that Kusuura Bay's population was brought about from a part of either population. Pearl oysters and Japanese oysters have frequently been transferred by shipping to areas more suitable for their growth in western Japan[12], and this evidence may support a possible introduction of *H. circularisquama* to Kusuura Bay from another area by human assisted dispersal. Further work is necessary to reveal the expansion history of populations in the western part of Japan.

Regarding the cultivation of *Dinophysis* species, no one has succeeded it until very recently, although many researchers of HAB fields had done many trials since the beginning of phytoplankton history over 200 years. Therefore, the large parts of DSP (diarrhetic shellfish poisoning) researches such as their growth and toxin production physiology in *Dinophysis* and also the toxicology of shellfish are still remaining unknown. In 2006, Park et al. (2006) succeeded in cultivating and maintaining *D. acuminata* by feeding *D. acuminata* with a ciliate species *Myrionecta rubra* for the first time in the history[13]. By their great discovery plus collaboration with Dr. T. Kamiyama (National Research Institute of Fisheries and Environment of Inland Sea, Japan), we succeeded in cultivating *D. acuminata* too very recently. Furthermore, fortunately, for the first time in the world at present, we also succeeded in propagating *D. fortii*, which is the most important DSP causing species. At present, we can propagate *D. acuminata* and *D. fortii* at >5,000 cells ml^{-1} and 2,500 cells mL^{-1}, respectively (Fig. 3). Therefore, the microsatellite markers of these species are under development.

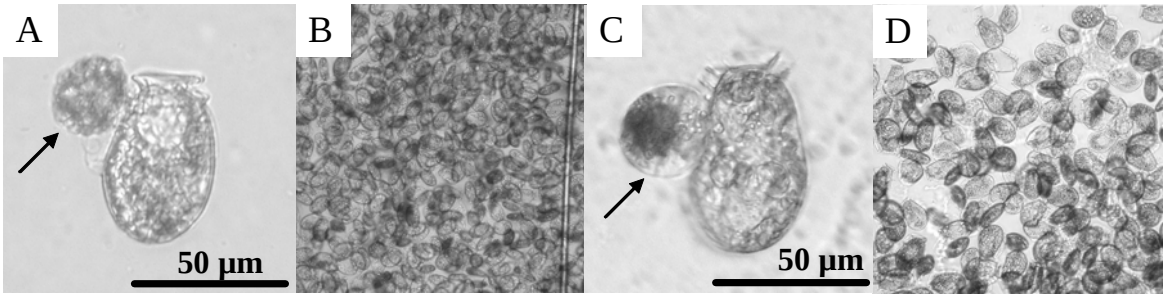

Fig. 3. Cultivation success of *Dinophysis acuminata and D. fortii*
A, *D. acuminata* feeding on a prey; B, harvested cells of *D. acuminata*;
C, *D. fortii* feeding on a prey; D, harvested cells of *D. fortii*;
Arrow shows the prey of *Dinophysis* (*Mesodinium rubra*).

In *D. acuminata*, we succeeded in developing 3 polymorphic microsatellite markers. Each locus showed clear single bands for each individual, indicating homozygous state in a haploid. PCR amplification success in the three markers using 47 clonal strains isolated from Hiroshima Bay was 97.9-100% and the number of alleles per locus ranged from 7 to 14 with an average of 11.0. The

gene diversity ranged from 0.66 to 0.87 with an average of 0.79, suggesting that these loci are sufficiently variable to allow the population genetic study of *D. acuminata*. In a pairwise comparison with Inokushi Bay populations by Fisher's exact test, a significant population differentiation was detected ($P<0.05$), suggesting that structuring and genetic isolation have occurred between two populations. We are going to develop further polymorphic markers in near future.

In *Gymnodinium catenatum*, we isolated the microsatellite regions, determined 413 of the sequences and designed a microsatellite primer in each region of the 42 good candidates chosen from the sequences. We screened the primers showing better PCR amplification using 4 DNA samples and obtained 13 candidates as the result of screening. We then tried to characterize them using 40 DNA samples, unfortunately, only two polymorphic markers were obtained. One marker detected a significant differentiation in pairwise comparison between Inokushi Bay and Senzaki Bay (Yamaguchi Pref.) populations. Therefore, we are willing to continue the development and if you succeed in developing some more useful markers, we believe that these markers are available to discriminate populations.

In *Pyrodinium bahamense*, the microsatellite markers are under development, however, we have only four clonal strains at present and we, therefore, need to collect many more strains. We are planning to do more sampling of *P. bahamense* with an international collaboration with NFRDI in Philippine.

(2) Detection of the transportation of the toxic microalgae by human activity

1) Direct detection and quantification of HAB species from oyster spats during translocation

In March 2006 and April 2007, we detected the vegetative cells of toxic dinoflagellate *Alexandrium tamarense* from both oyster's spats translocated from area A to B. In the samples carried from area A in 2006, we detected 6 cells of *A. tamarense* in the surface of a collector (outside of oysters) and 21 cells exhausted from the young oysters as the fecal pellets per collector. In the sample of 2007, we detected 42 cells of *A. tamarense* in the surface of a collector (outside of oysters) and 720 cells of *A.tamarense* exhausted from the young oysters as the fecal pellets per collector (as inside of oysters). Most of the *A. tamarense* cells, detected as inside of oysters, were the ones exhausted from fecal pellets of oysters and they were still living in the pellets at least for ca. 36 hours (Fig. 4).

In the samples translocated from area B in 2006, we detected 120 cells of *A. tamarense* in the surface of a collector and 1,488 cells from inside of young oysters per collector. Most of the *A. tamarense* cells, detected as inside of oysters, were the ones exhausted from fecal pellets and they were still living in the pellets, as observed the samples from the area A. They swam out as vegetative cells within 1-2 days after release from the pellets. As additional information, a higher amount of other microalgal species were also contained in every sample of fecal pellets and calculated as 1,500-13,600 cells per collector. Considering the factors of the cell density of *A. tamarense* around the farming ground of oyster spats when the spats were translocated and filtration rate of oysters, it was estimated that a single collector carries ca. 0.5-liter of natural

seawater. The history of the translocation of oyster spat is considerably long, >100 years between the area A and B and ca.200 tracks have carried the oyster spats from the area A to area B per year in recent three years (an oyster manager, personal communication) and it is equivalent to 28 million collectors. In conclusions, oyster spats have carried out ca. several million-liter equal of natural seawater per year from the area A to area B in our rough estimation, although it fluctuates drastically from year to year.

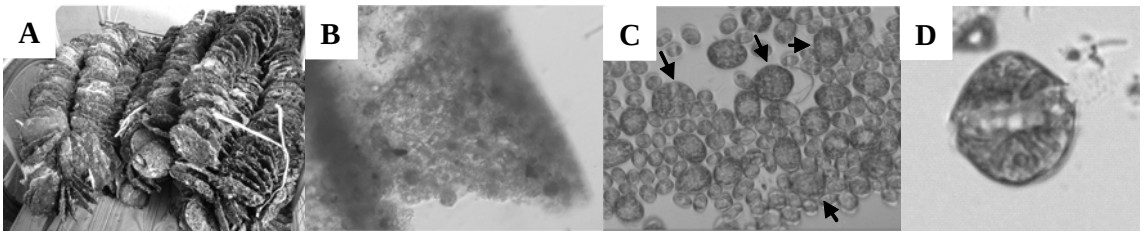

Fig.4. Oyster spats and the feces exhausted from young oysters contained the toxic dinoflagellate *Alexandrium tamarense*. A, oyster spats; B, the feces exhausted from a young oyster; C, the microalgal cells released from the feces; D, a vegetative cell of the toxic dinoflagellate *A. tamarense* swam out from the feces.

2) Detection and quantification of HAB species from live fish tracks

Phytoplankton contained HAB and non-HAB species were detected in 25 of 38 live fish tanks. We detected five toxic microalgae; *Dinophysis caudata*, *D. fortii*, *D. acuminata*, *Lingulodinium polyedrum*, and *Pseudo-nitzschia* spp. and five harmful microalgae; *Chattonella marina*, *C. ovata*, *Cochlodinium polykrikoides*, *Coscinodiscus wailesii*, *Eucampia zodiacus*. In this fish market, a trailer transports ca. 200 to 10,000 liter of natural seawater. Based on the number of tracks coming to the market, it was estimated that ca. 100,000 liters of natural seawater are daily transported from geographically distant area and discharged the seawaters into the sea in front of the market.

We revealed that a large amount of cell numbers of HAB species have been introduced from a area to another area via translocation of shellfish stocks and transfer of living fish by tracking, strongly suggesting the human activity have influenced not only the microalgal community or the population structures but also the biodiversities. The transportation of shellfish will not regulate by low unless some microbe, listed in international treaty, are detected. However, the present study demonstrated that high amounts of toxic microalgae easily travel to geographically distant area via transportation of aquatic organisms. In order to minimize the risk of these artificial transfers in one way or another, legal or administrative countermeasures should be taken. To prevent new bio-invasion and preserve biodiversity in marine ecosystems, researchers also should provide useful tools and protocols for monitoring the accidental transfers of HAB species via aquatic organisms.

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