Recent developments in molecular diagnostic technology for HAB detection

Nagai S,
Fisheries Research Agency of Japan, Hiroshima, Japan, snagai@affrc.go.jp

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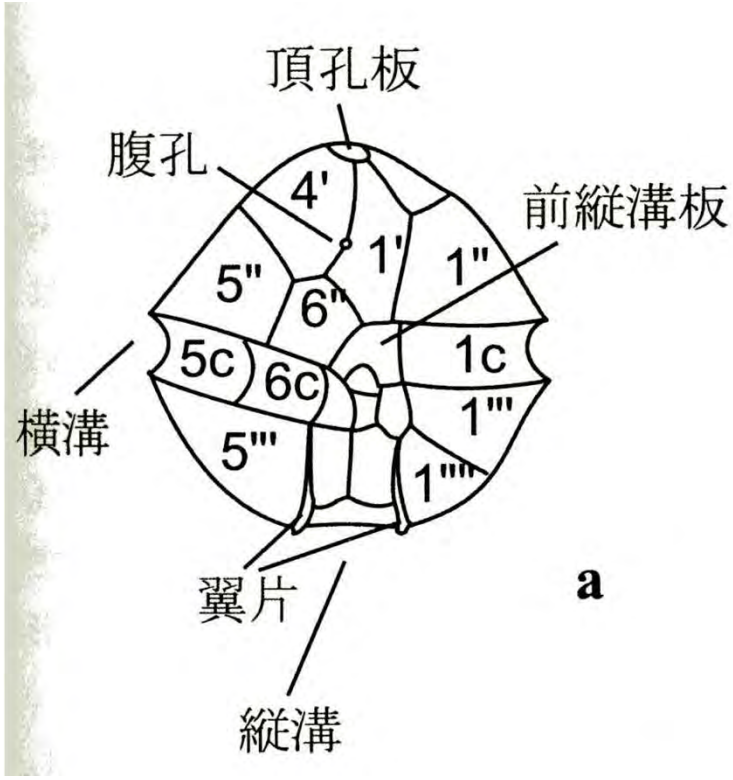
Since 1998
FEIS
Natl. Res. Inst. of Fisheries & Environment of Inland Sea

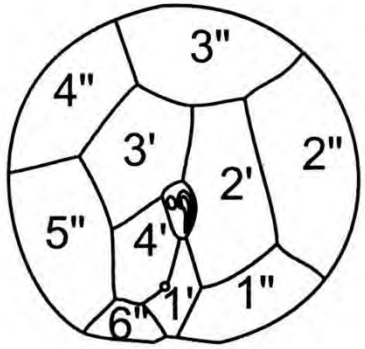
FRA
Fisheries Research Agency

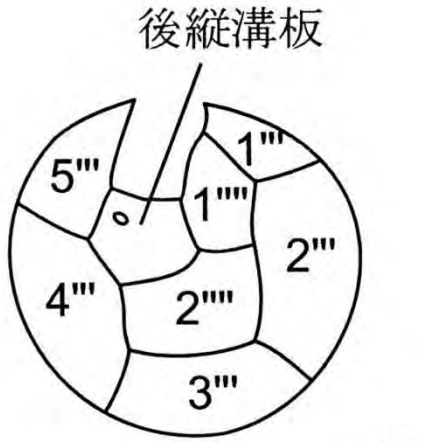
Contents of my talk

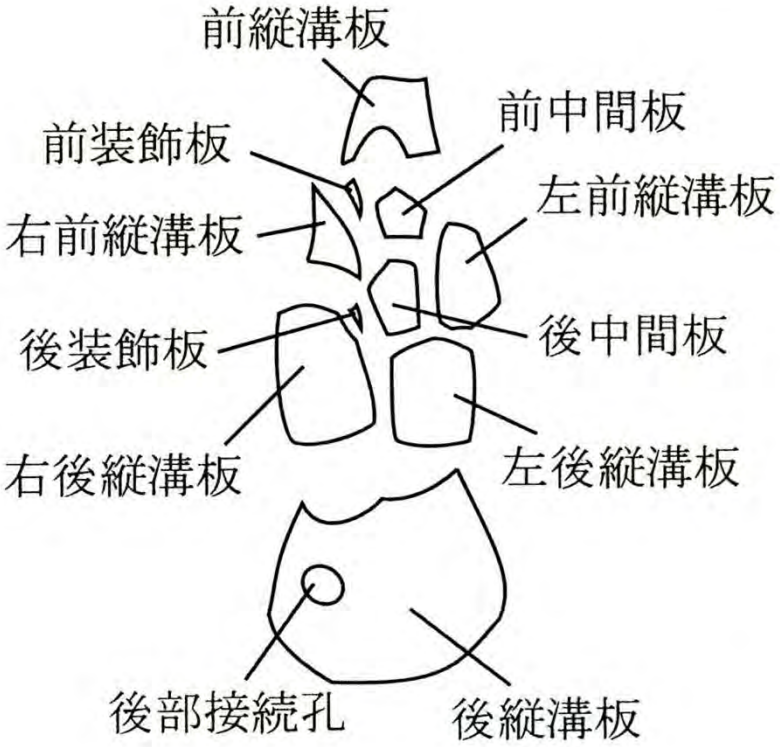
- Present situation of HAB monitoring in Japan

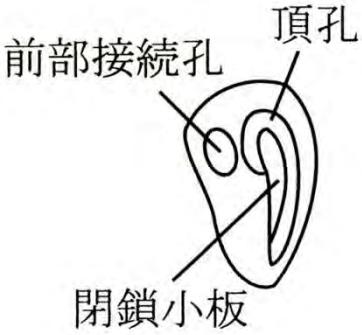
- PCR-RFLP
- FISH (Fluorescence In Situ Hybridization)
- Real time q-PCR
- LAMP (Loopmediated isothermal Amplification)
- Multiplex-PCR
- Metagenome by 454 sequencing





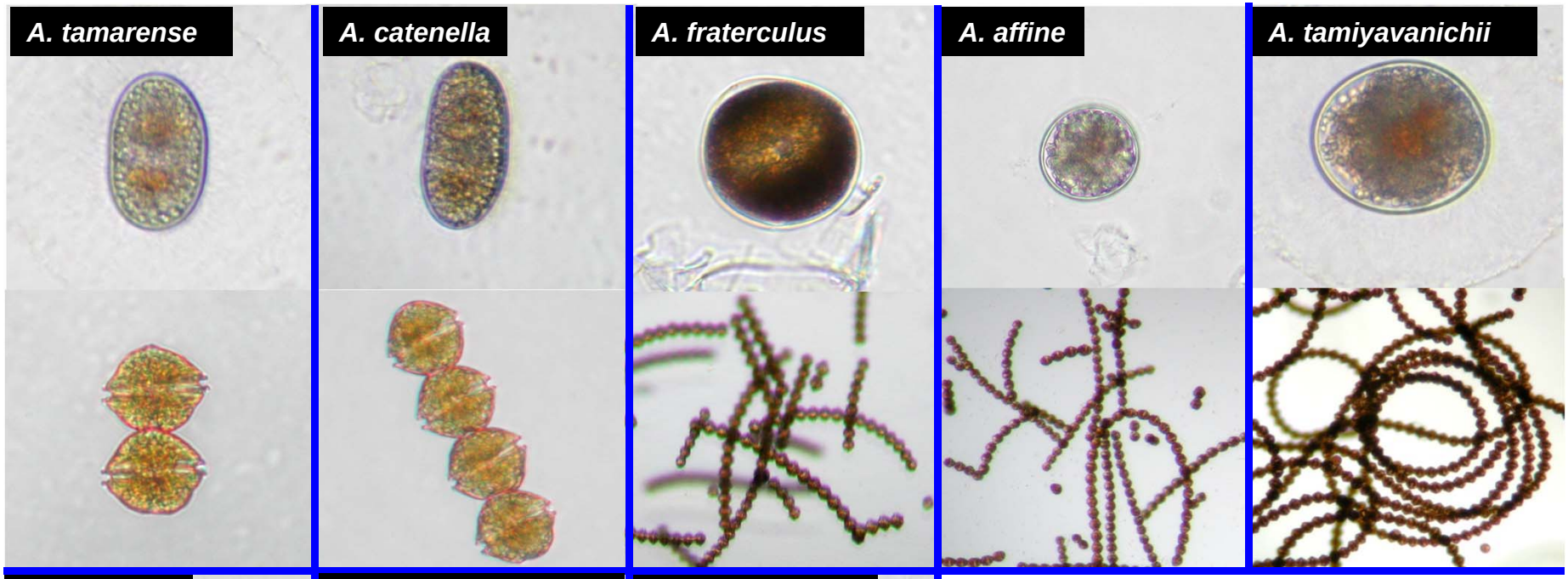


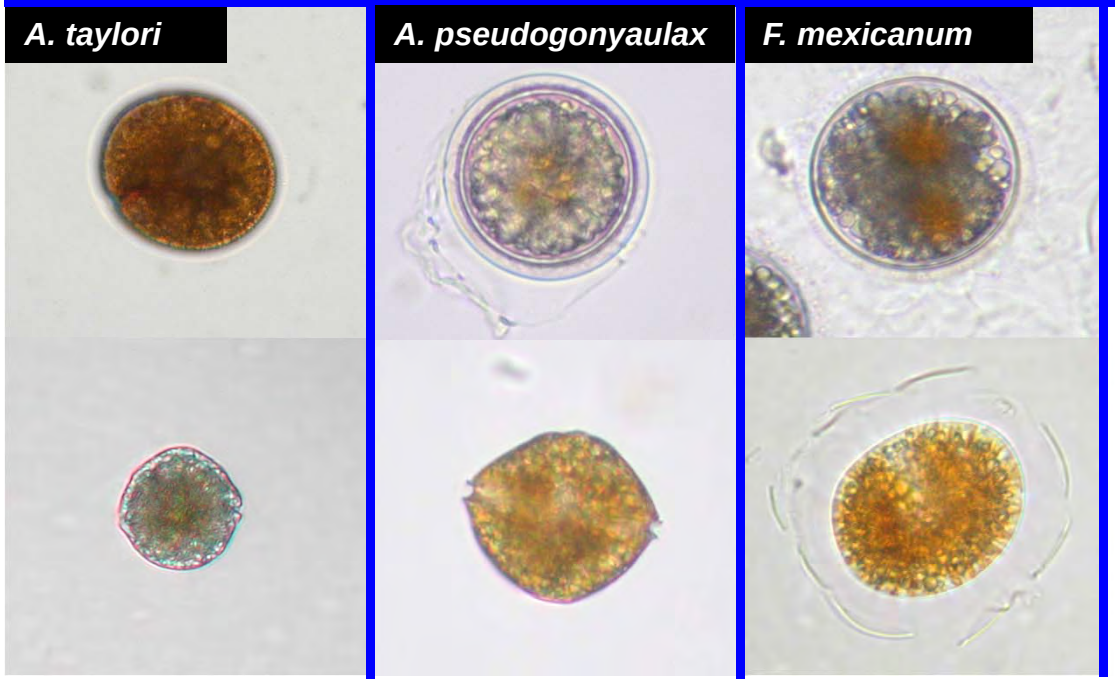




the criteria of classification of species

***Alexandrium*の鎧版配列模式図（研究代表者　福代康夫氏科研費報告書より引用）**



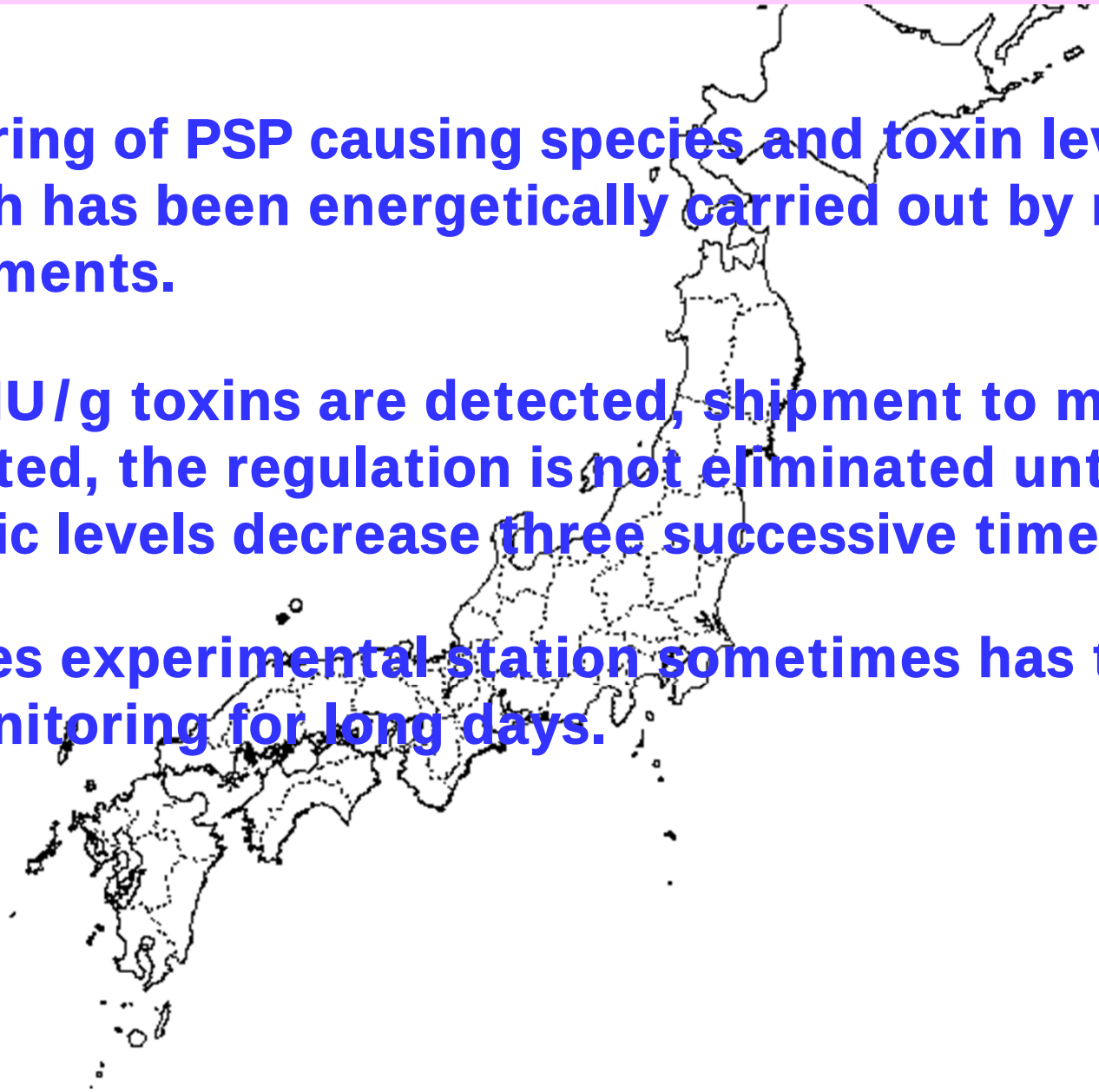


The genus is classified into 28 species.

10 species is toxic.

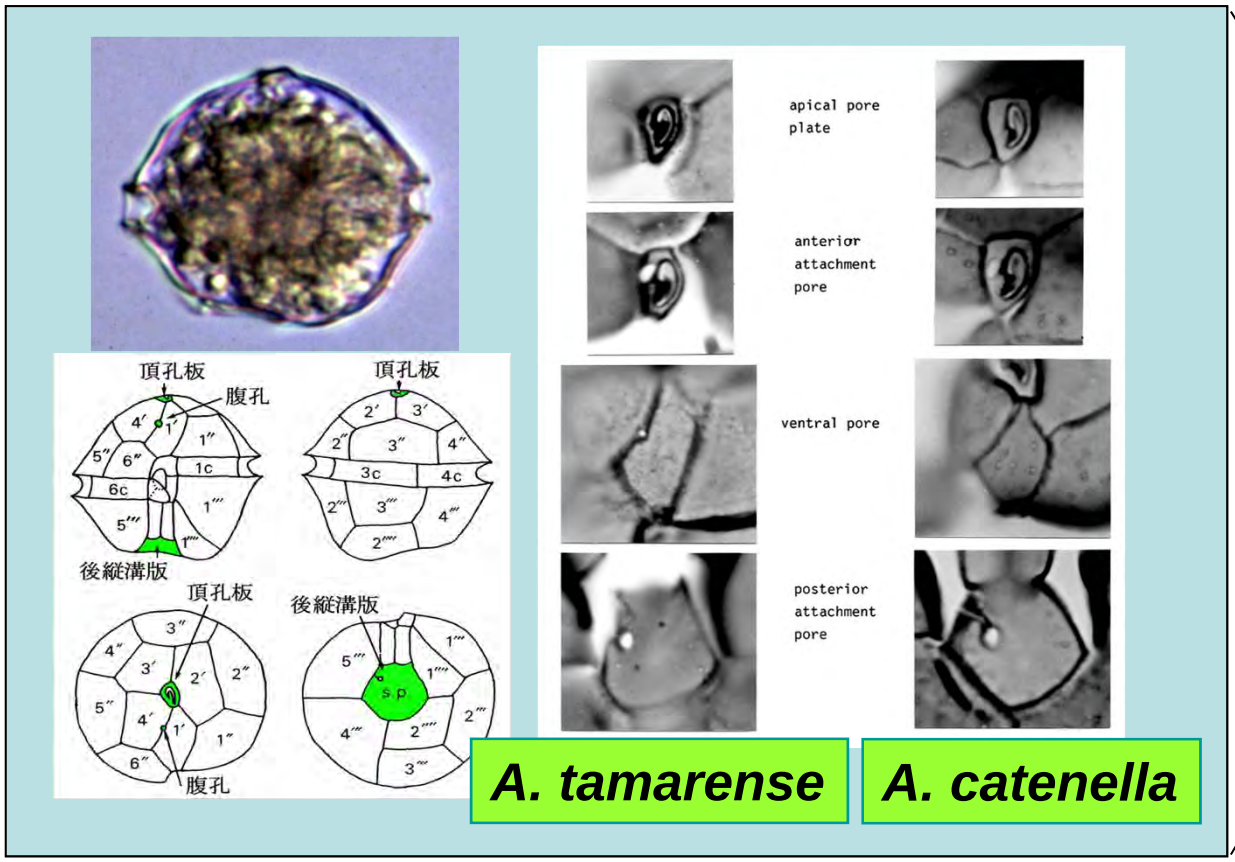
At least 10 species appear in Japanese coasts,
4 species are toxic.

Monitoring system of PSP in Japan



- Monitoring of PSP causing species and toxin levels in shellfish has been energetically carried out by regional governments.
- If >4 MU/g toxins are detected, shipment to market is prohibited, the regulation is not eliminated until when the toxic levels decrease three successive times.
- Fisheries experimental station sometimes has to continue the monitoring for long days.

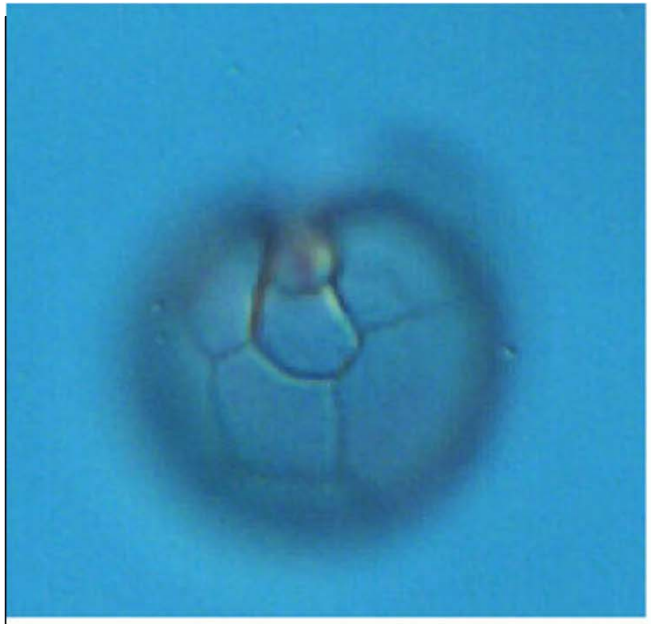
Monitoring of toxic phytoplankton

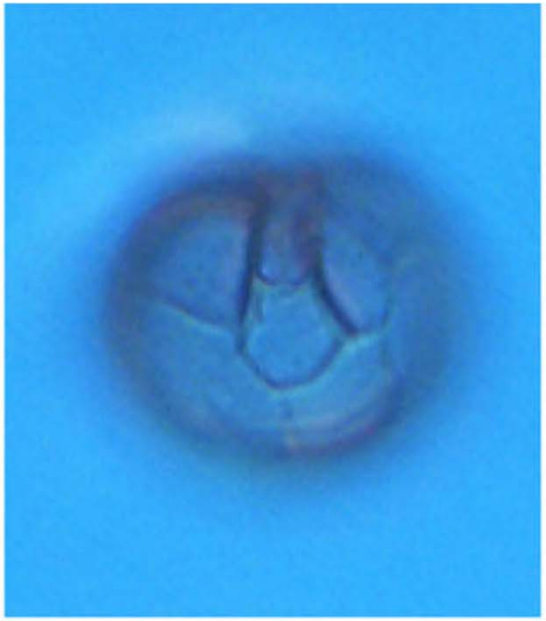


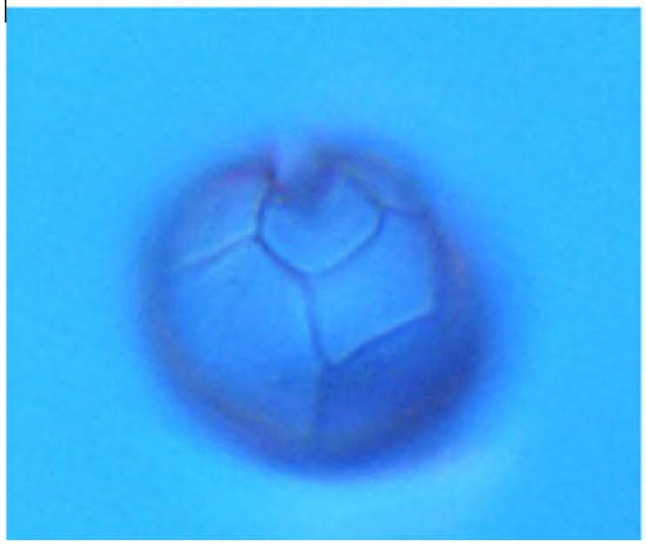
It is not so easy to distinguish the plate morphology.

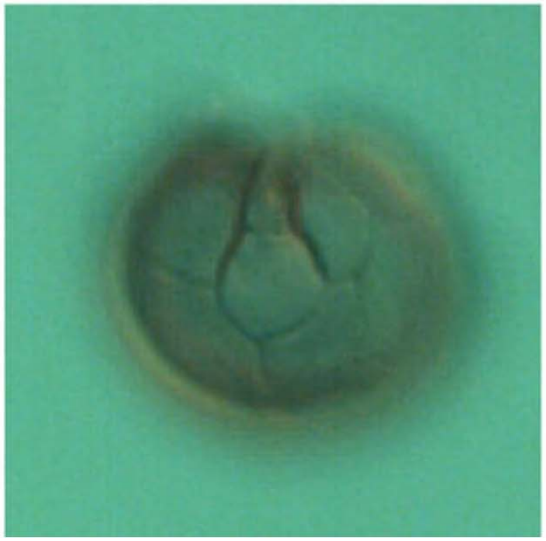
It is required knowledge, experiences and skills.

Very complicated specimens were sampled in Tottori Pref. in May 2006



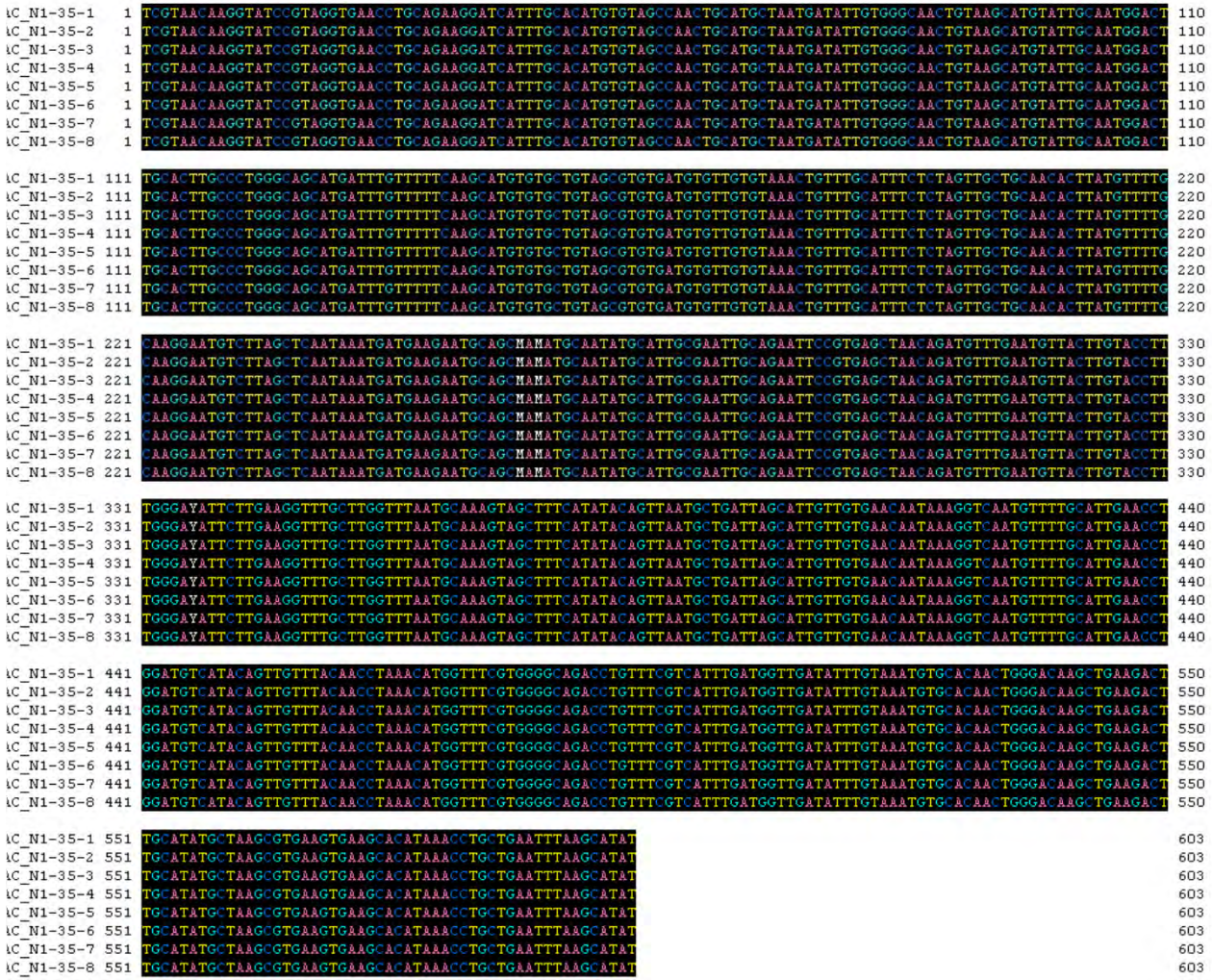






Posterior sulcal plate is similar to *A. tamarense.*

Alignment of *A. catenella* (ITS)



- **Kagawa Pref.**
- **Mie Pref.**
- **Hiroshima Pref.**
- **Tottori Pref.**

Sequences are all identical to typical Japanese *catenella*.

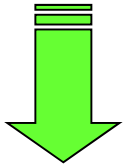
Color of culture

Growth at high temp.

Finally this specimen was jugged as *A. catenella*.

We have an annual training course on identification of toxic phytoplankton (sponsored by Fisheries Agency of Japan)

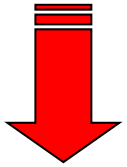
By frequent personnel reshuffling of regional governmental researchers



every 2-3 years

Lack of experience and skills. In addition…
Lack of infrastructure by dwindling budgets.

→This is the real situation of HAB monitoring in Japan



Development of easy, accurate, inexpensive molecular identification tools has been required.

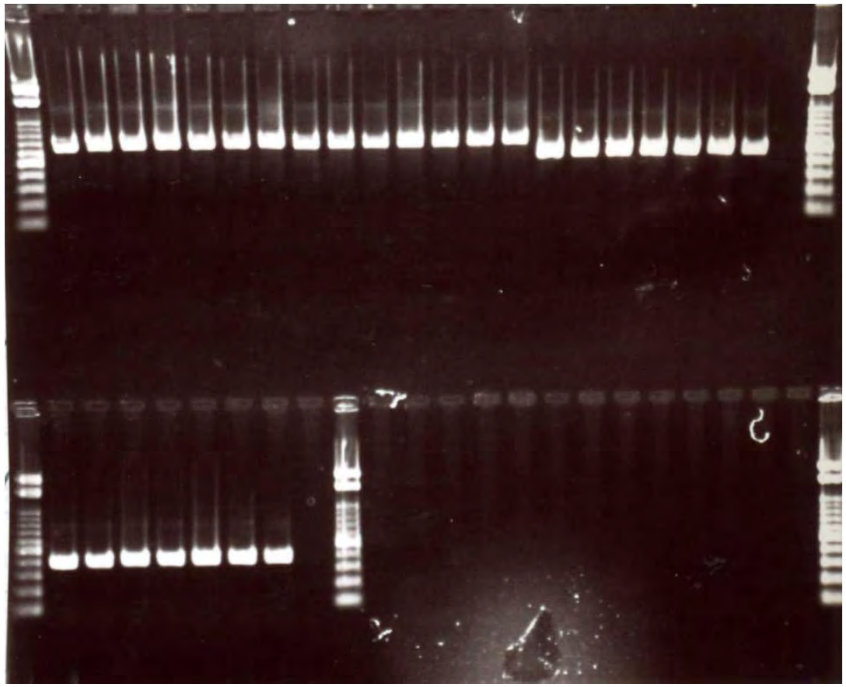
Contents of my talk

Species identification of HAB species by use of molecular technique

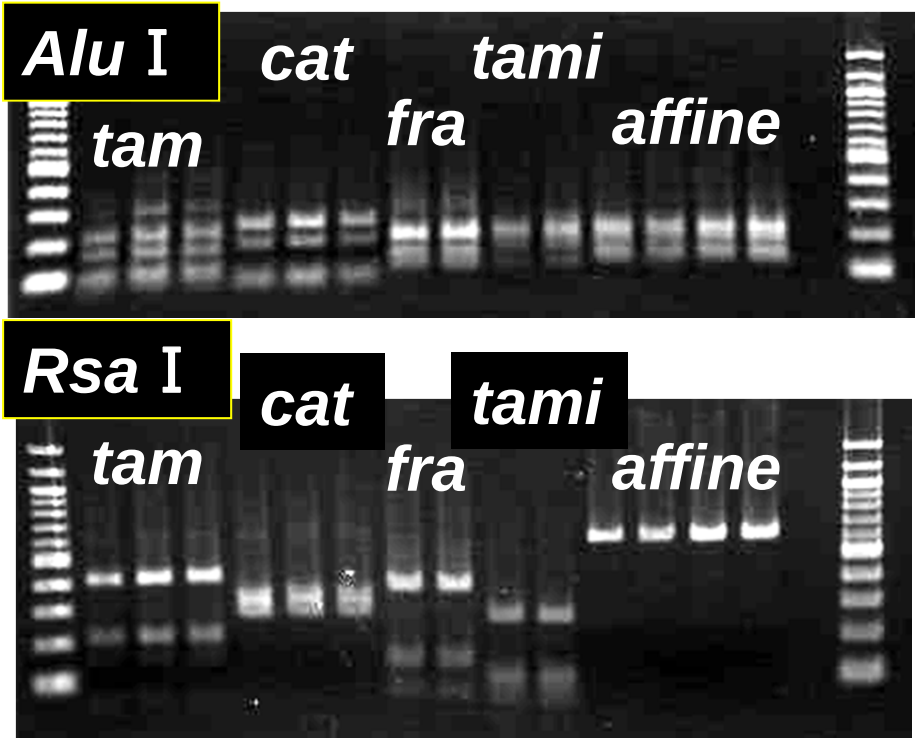
- **PCR-RFLP**
- **FISH (Fluorescence In Situ Hybridization)**
- **Real time q-PCR**
- **LAMP (Loop Mediated Amplification)**
- **Multiplex-PCR**
- **Metagenome by 454 sequencing**

PCR－RFLP (Restriction Fragment Length Polymorphism)

PCR amplification → Digestion by enzymes → Restriction site depends



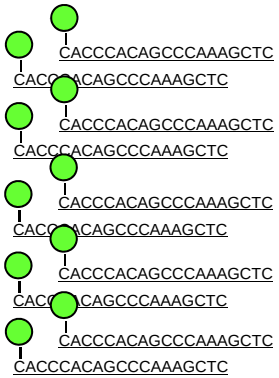
Nuclear SSU, LSU or ITS



By Scholin et al. (1995), Adachi et al. (1996)

Fluorescence *In Situ* Hybridization (FISH)

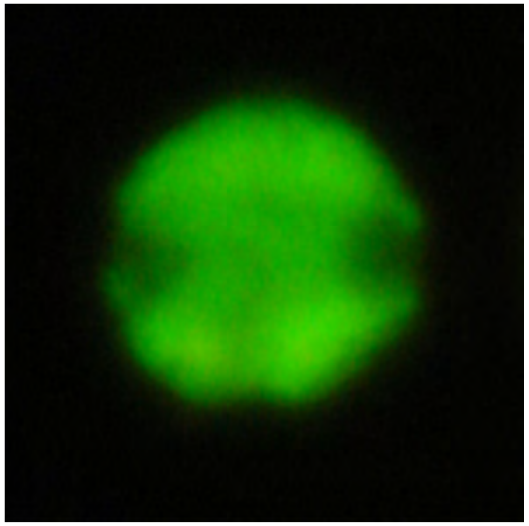
Target : ribosomal DNA or RNA



FITC labeled rRNA targeted probe

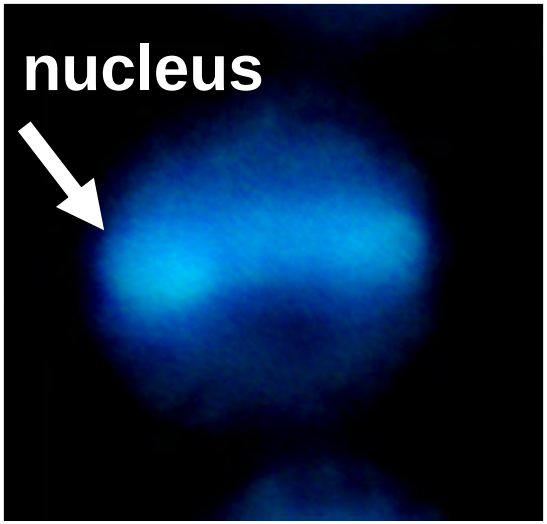
hybridization

FISH



by FITC-labeled probe

DAPI staining



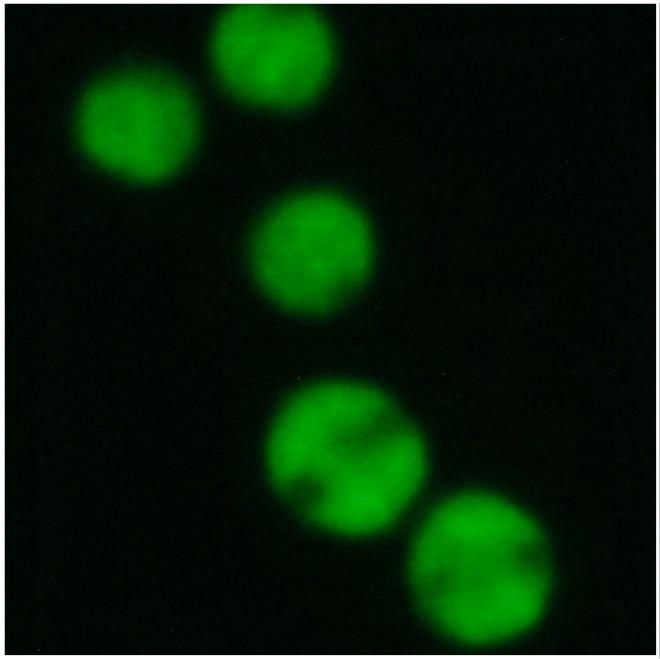
Horseshoe-like shape

In rRNA FISH,

Ribosomal RNA genes are expressed in the ribosome.
RNA-target probe works only in cytoplasm.
There are so many copies in cytoplasm.

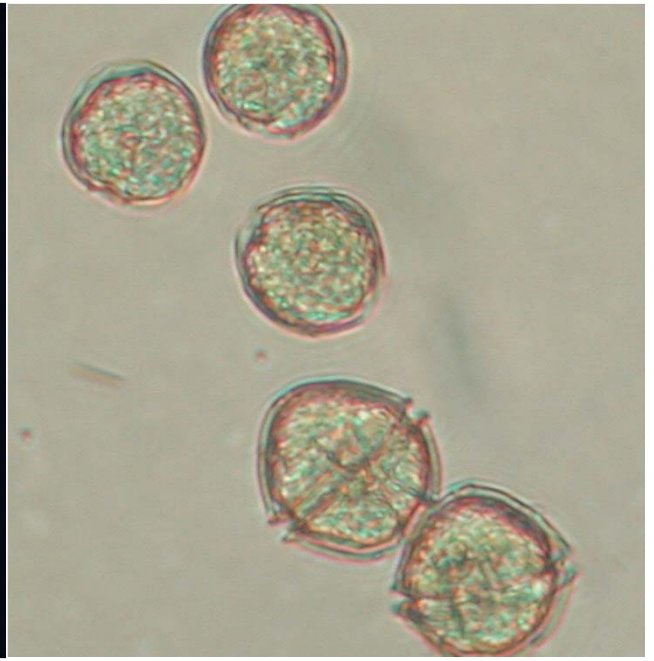
→High florescent intensity is obtained.

Double staining of *Alexandrium* cells by FITC-FISH and Calcofluor-White MR2



FITC-labeled probe

Calcofluor-white

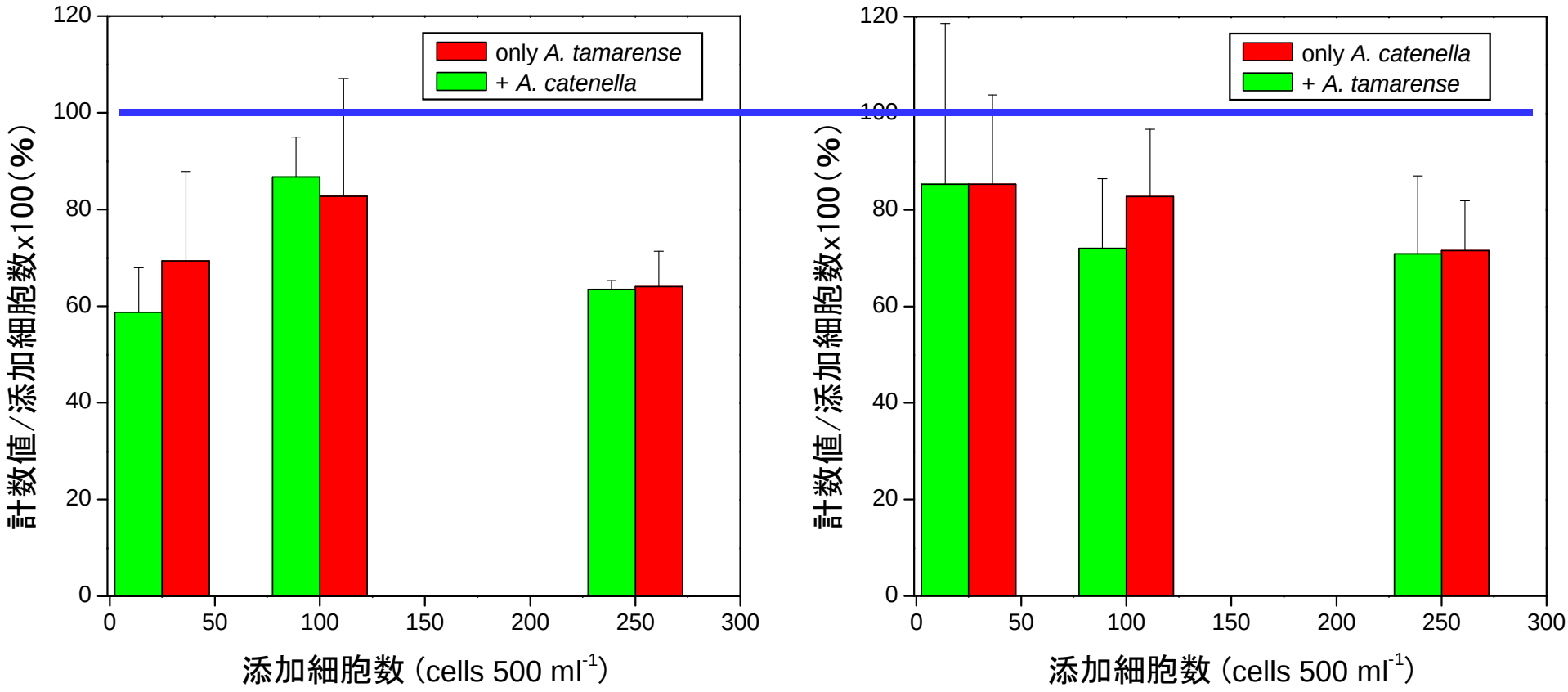


Normal Light

No need to extract RNA and we can observe the cell morphology.
→Good point: FISH is possible to quantify.

However, the amount of RNA varies depending conditions.
→weak point: RNA levels depend on individuals in natural sample.

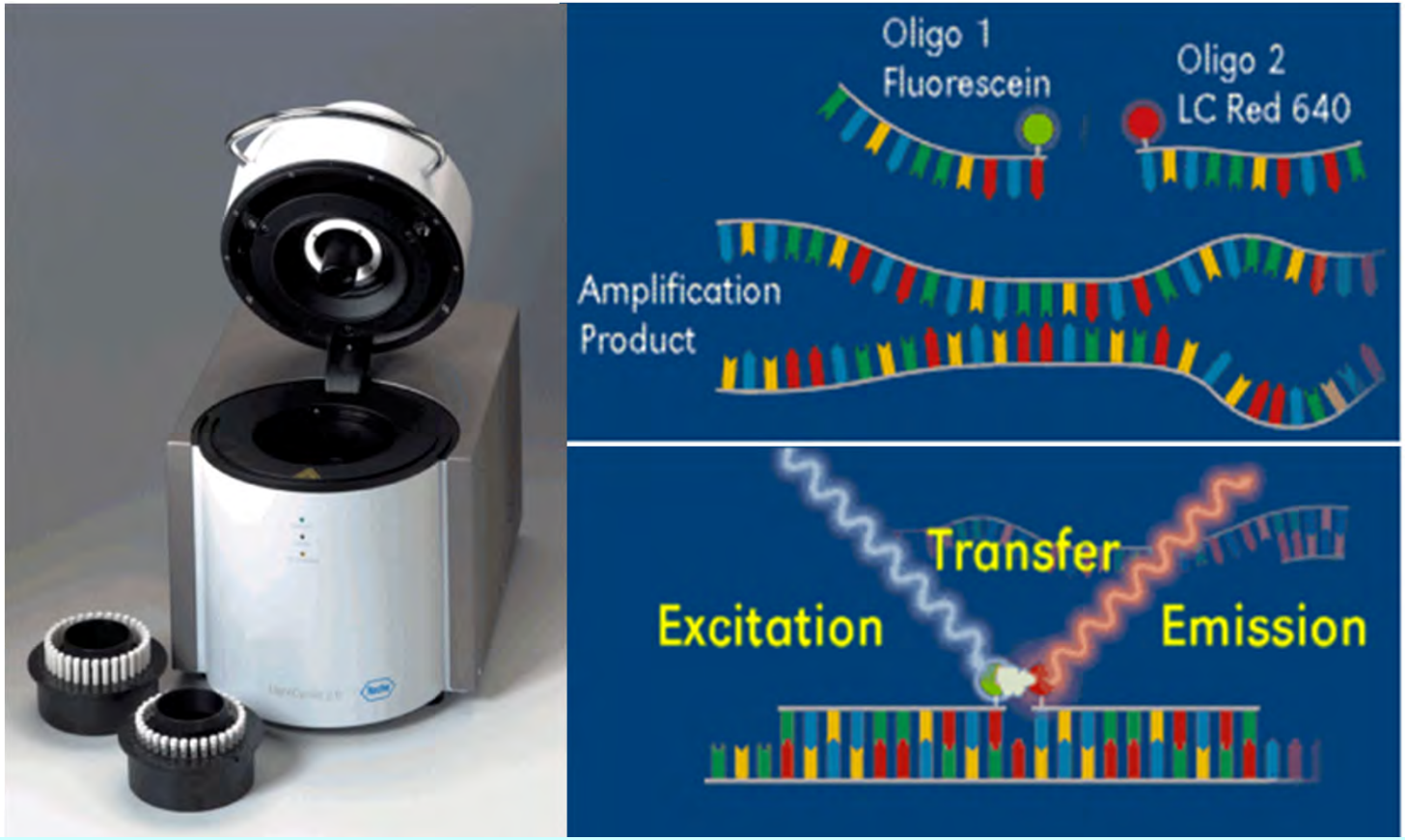
Enumeration of *Alexandrium* cultured cells mixed with natural plankton samples



A 500 mL of natural sample was concentrated to 500 µL and *Alexandrium* cultured cells were added and enumerated the cell density.

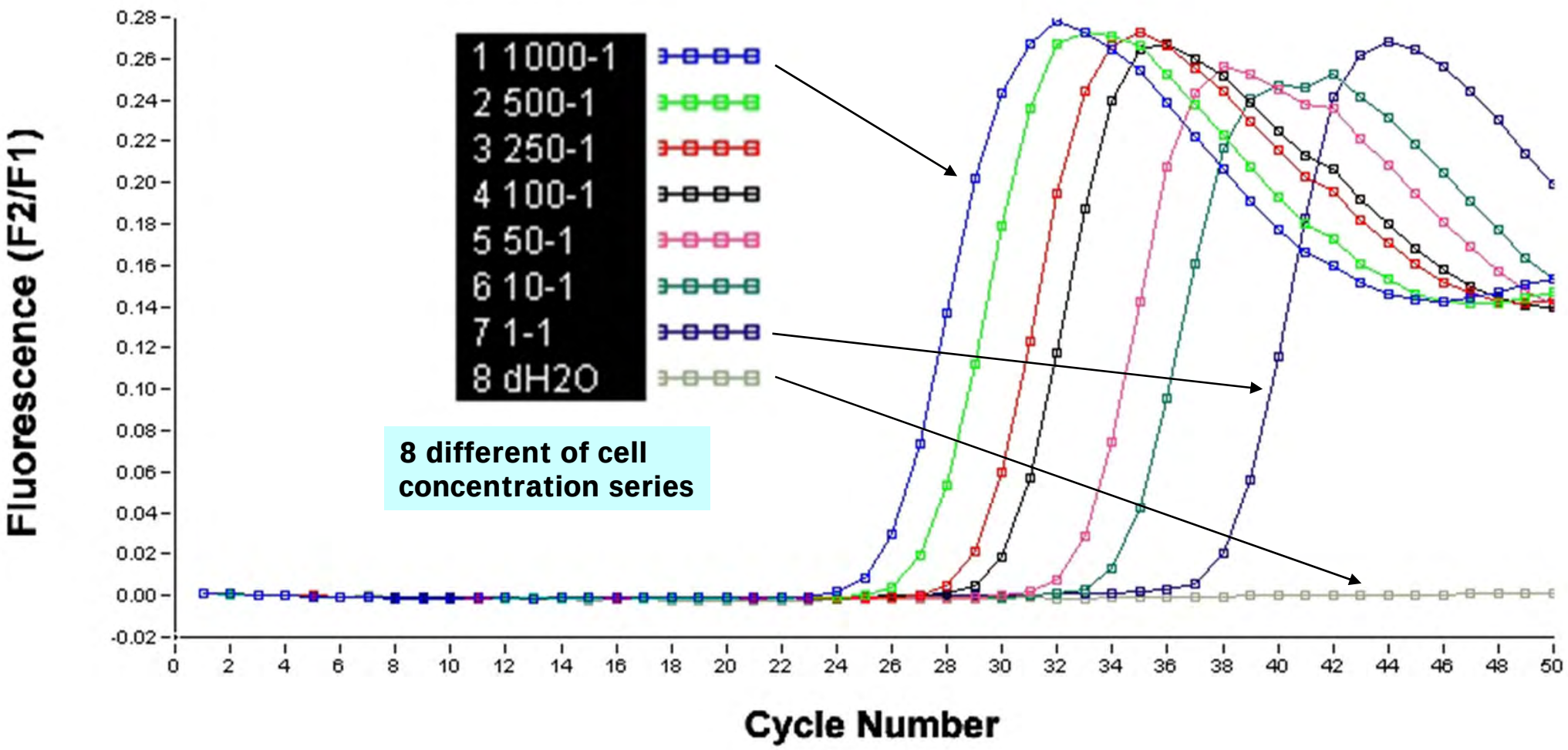
Recovery rate: 60-85% in both species

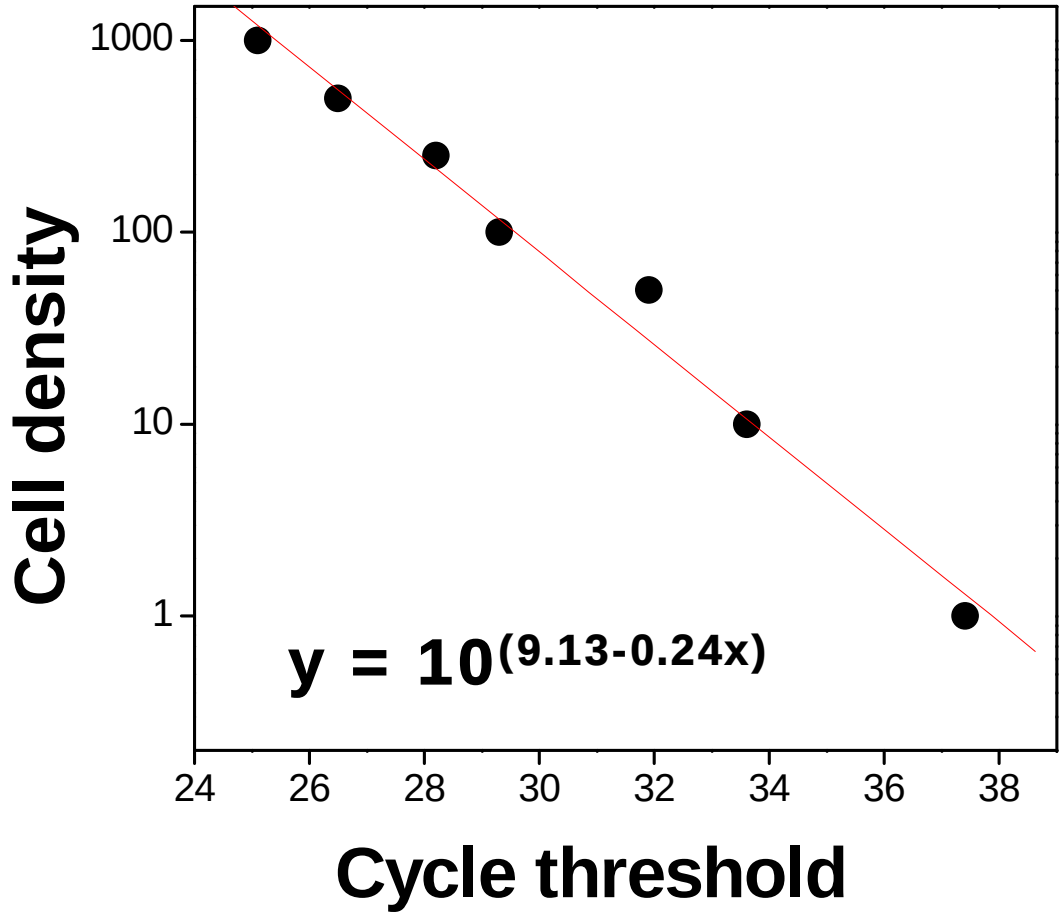
Detection and quantification of HAB species by real time q-PCR



As PCR-amplification increases, probes hybridize to the target gene and fluorescence intensity increases, therefore, it is possible to quantify the amount of DNA containing samples.

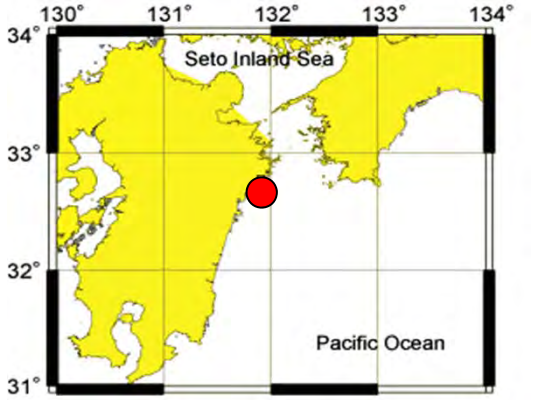
Real time q-PCR to draw the calibration curve in vegetative cells of *G. catenatum*.





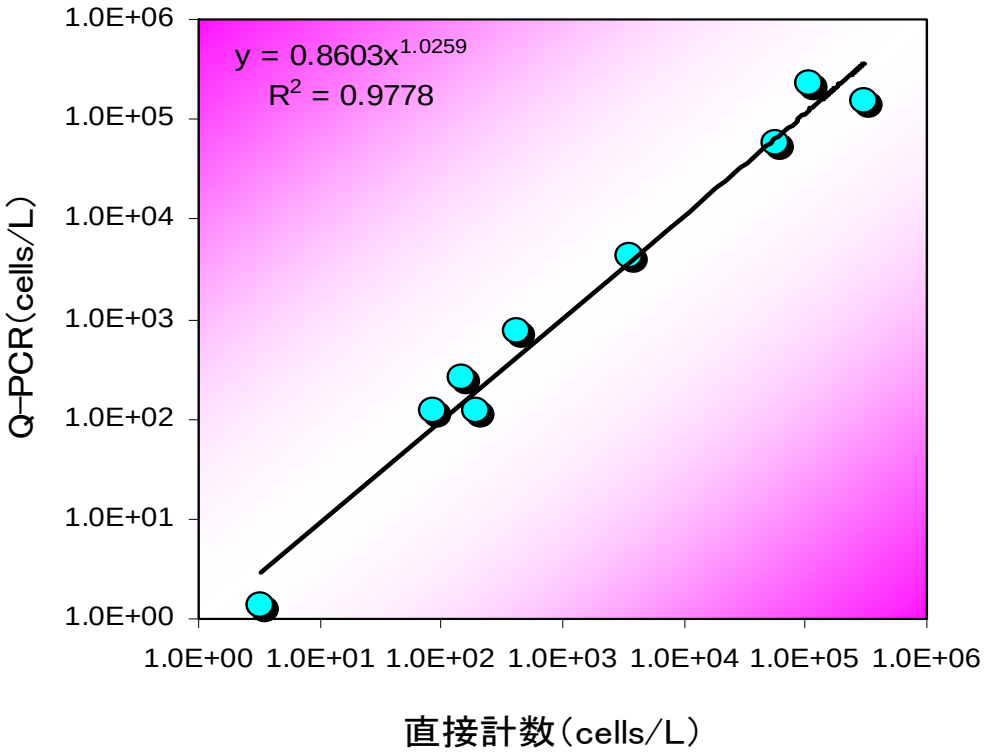
Estimation of cell abundance from the cycle threshold in *G. catenatum*.

Abundance of vegetative cells in *G. catenatum* by real time q-PCR (Jan-May in 2006)



	direct count		Q-PCR	
	Mean (n=3)	SD	Mean (n=3)	SD
23-Jan	3	3	1	2
13-Feb	414	137	751	236
20-Feb	192	5	120	37
6-Mar	85	31	119	12
14-Mar	147	49	265	56
20-Mar	3,500	1,645	4,177	1,128
17-Apr	57,000	45,902	56,544	16,860
26-Apr	110,000	10,000	226,011	65,933
1-May	303,333	92,916	152,222	31,183

Unit: cells/L

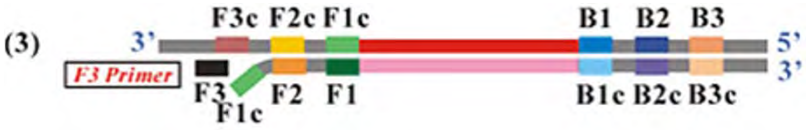


Correlation between direct count and quantification by Q-PCR

LAMP (Loopmediated isothermal Amplification)

◇No need for a step to denature double stranded DNA into a single stranded form.

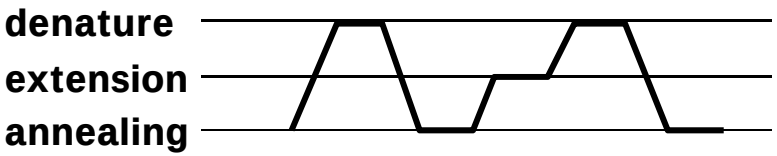
→*BST* polymerase synthesizes complement DNAs with dissociating the double strand.



◇The whole amplification reaction takes place continuously under isothermal conditions（60 to 65℃）

→Easy to operate with simple equipments (blockheater or dry oven)

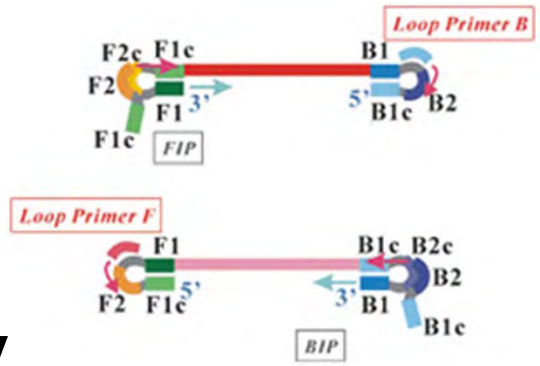
Normal PCR



LAMP

Characteristics of LAMP

◇Amplification starts from several points by use of 6 primers to recognize 8 distinct regions.
→It occasionally causes none-specific amplification.

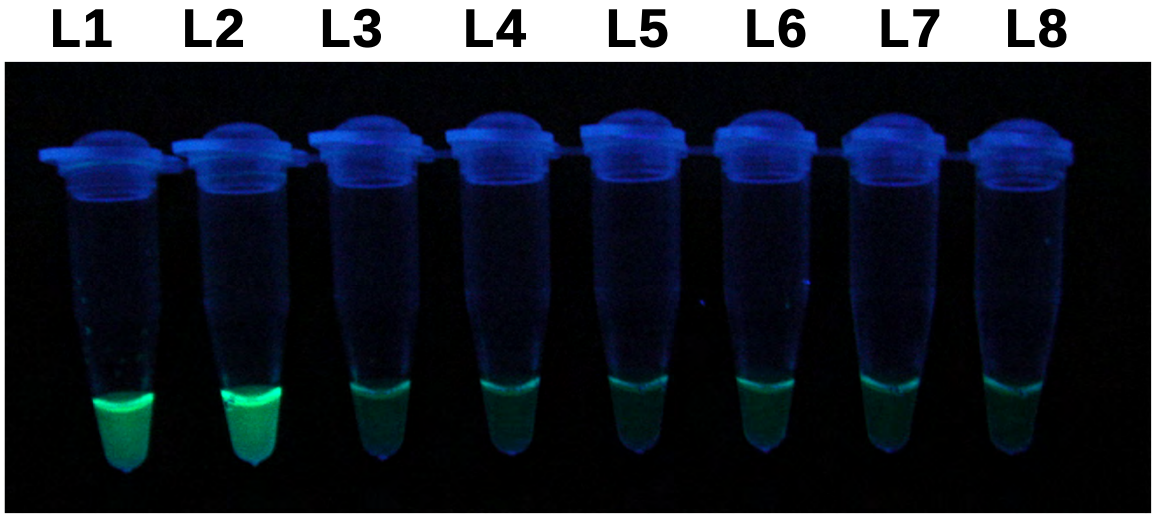


◇Extremely High amplification efficiency
→Isothermal amplification occurs from several regions.
→DNA will be amplified 10^9-10^{10} times in 15-60 minutes

◇Magnesium pyrophosphate, a by-product of the amplification reaction, is produced in proportion to the amount of amplified products.
→White turbidity can be visually observed.

◇Total cost can be reduced, as LAMP dose not require special reagents or sophisticated equipments.

Easy detection by LAMP



Lane1, *A. tamarense* (5ng)
L2, *A. tamarense* (single cell)

L3, *A. catenella* (>10ng)
L4, *A. tamiyavanichii* (>10ng)
L5, *A. fraterculus* (>10ng)
L6, *A. affine* (>10ng)
L7, *A. minutum* (>10ng)
L8, *A. ostenfeldii* (>10ng)
（Lane3-8 emit no fluorescence)

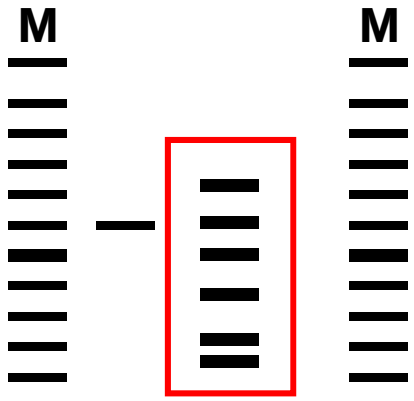
LAMP primers for *A. tamarense* is able to specifically amplify *A. tamarense* DNA!

Development of a multiplex PCR assay for simultaneous detection of 6 *Alexandrium* species

Multiplex PCR:

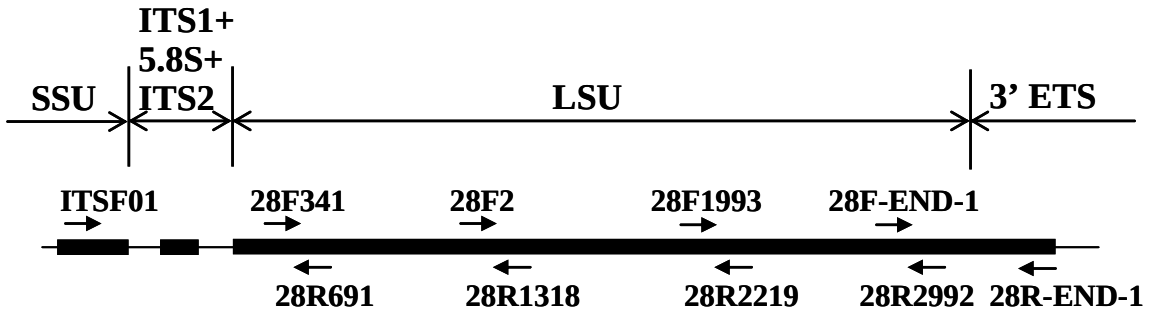
amplifying several target regions at one time by mix of different primer pairs.

The length of PCR product was changed in every species. Therefore, six species are detectable at one time.



Target region:

Nuclear LSU, D1/D2 region or nuclear 5.8S-rDNA+ITS regions

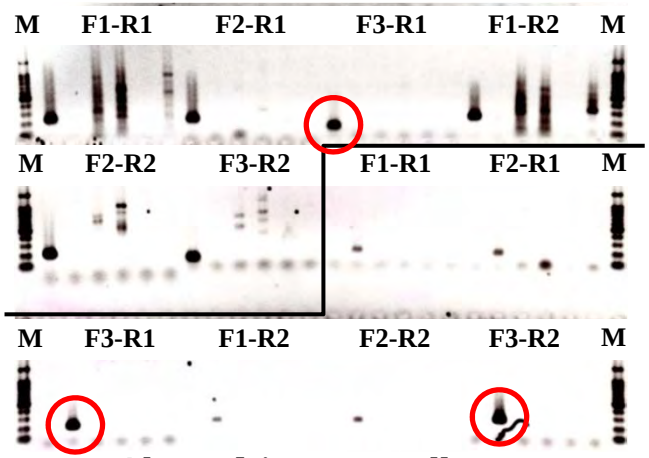


Primer design and screening

Species Strain name (Accession number)	Primer name	Sequence	Region	Posion*	selected primers
Alexandrium tamarense	Atama-F1	GATTTGCTTGGTGGGAGTGTTGC	D1D2	408-430	
AT0104H15 (AB565483)	Atama-F2	CTTGCTTGACAAGAGCTTTGG	D1D2	432-452	
	Atama-F3	ACCTTTGCACATGAATGATAAGTC	D1D2	508-531	○
	Atama-R1	CATCCCCAAGCACAGGAAC	D1D2	622-640	○
	Atama-R2	AAGCATCCCCAAGCACAGG	D1D2	625-643	
Alexandrium catenella	Acat-F1	TAAACCAACTGGGATCTCTTC	D1D2	39-59	
AC0206MIE20 (AB565484)	Acat-F2	CTTCAGTAATTGCGCATGAACC	D1D2	56-77	
	Acat-F3	CAAAGTAAACAGACTTGATTTCCTC	D1D2	416-440	○
	Acat-R1	CCAGACACATTTAACAAACATGC	D1D2	593-615	
	Acat-R2	GAAAGCAACCTCAAGGACAAG	D1D2	630-650	○
Alexandrium tamiyavanichii	Atami-F1	AAGCTTGCTGTGGGTACAGA	ITS	90-109	○
AT0112T06 (AB436948)	Atami-F2	GGGTACAGATTGCATGCGTTG	ITS	101-121	
	Atami-R1	TACAGCTCACAGCAATGCAG	ITS	415-434	○
	Atami-R2	GTTAACAAGCAACACACACCAATG	ITS	537-560	
Alexnadrium fraterculus	Afra-F1	GCTTTGAATTGTGTTTGTGAAC	D1D2	111-132	○
AF0703MIE01 (AB436941)	Afra-F2	AAGAGAGTTAAATGAGTTTGCAC	D1D2	360-382	
	Afra-R1	GCTAGTTATTTGTGCATGTGCTG	D1D2	496-518	
	Afra-R2	TCACCAAACACATGCCTGAG	D1D2	592-611	
	Afra-R3	GTCAGTGTTAAAGCTTGTGGG	D1D2	668-688	○
Alexandrium affine	Affin-F1	CTTGCTTCAAGCTGGTATGTC	ITS	98-118	○
KAGAWA-37 (AB565485)	Affin-F2	CATGGCTTGCAATCGCAACC	ITS	133-152	
	Affin-R1	GCACAGATACACTTGGAAGAG	ITS	397-417	
	Affin-R2	GTCAATGTTCACCATTTCACCA	ITS	564-585	○
Alexandrium pseudogoniaulax	Apseu-F1	ACCAGCGGAGGTACAGTTGC	D1D2	141-160	
KAGAWA-39 (AB565486)	Apseu-F2	GGGTGGTAAATTTCACGCAAG	D1D2	274-294	○
	Apseu-R1	ACACAGTAAACCCATGCGCAG	D1D2	450-470	
	Apseu-R2	TGGCAACAGCTGACAATCGCA	D1D2	650-670	○

*, They are shown the positions from the 5' termini of the PCR amplicons. The circles show the primers selected as the best combination to amplify species-specifically.

Alexandrium tamarense



Alexandrium catenella

PCR cocktail and conditions

Cocktail of multiplex-PCR

No.	reagents	(X 1)
1	microstellite type-it (Qiagen)	5.0
2	Atama-F3 (10 μM)	0.2
3	Atama-R1	0.2
4	Acat-F3	0.2
5	Acat-R2	0.2
6	Atami-F1	0.2
7	Atami-R1	0.2
8	Afra-F1	0.2
9	Afra-R3	0.2
10	Affin-F1	0.2
11	Affin-R2	0.2
12	Apseu-F2	0.2
13	Apseu-R2	0.2
14	dH2O	1.6
15	template DNA	1.0
	Total (μL)	10.0

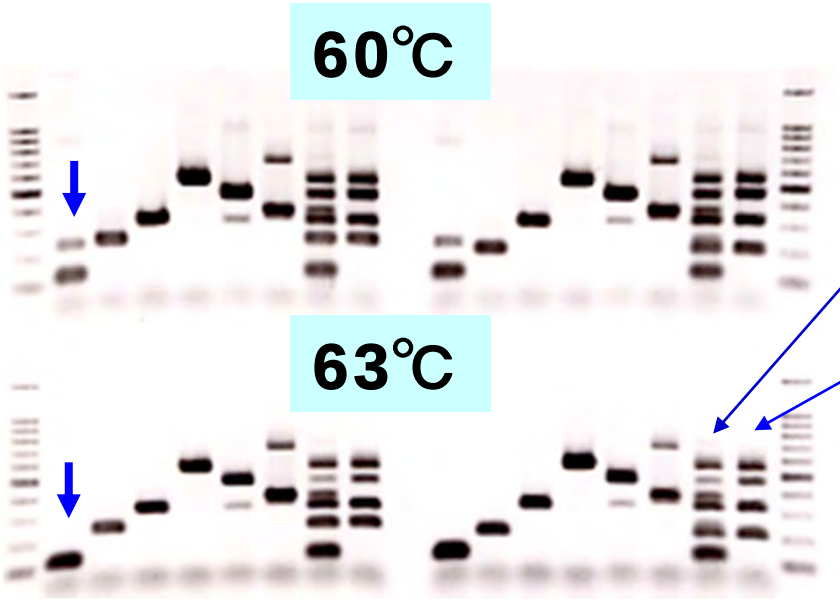
PCR condition

step	temp.	time	cycle
1	95	5 min	1
2	95	30 sec	30
	61	90 sec	
	72	30 sec	
3	60	30 min	1
	4	∞	

Characteristics:

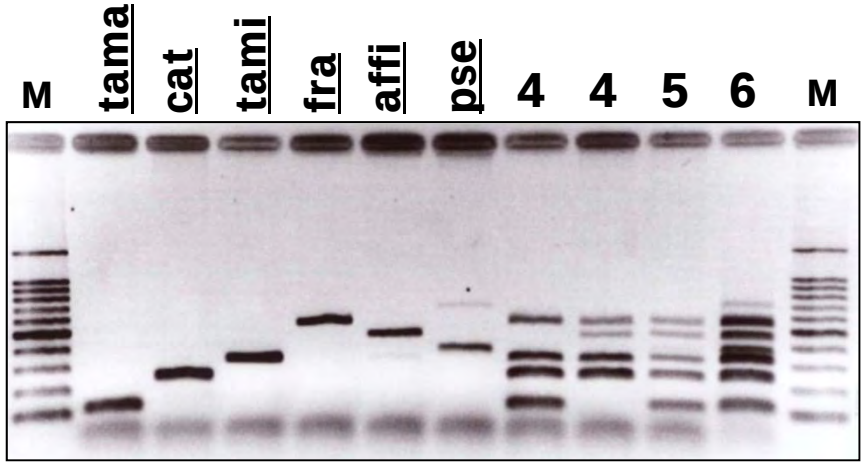
12 primers are mixed.
PCR kit for multiplex is used.
Annealing is longer than usual.

Optimization of PCR conditions



All 6 species DNAs were mixed.

Following 4 species DNAs were mixed.
A. affine, *catenella*, *fraterculus*, *tamiyavanichii*



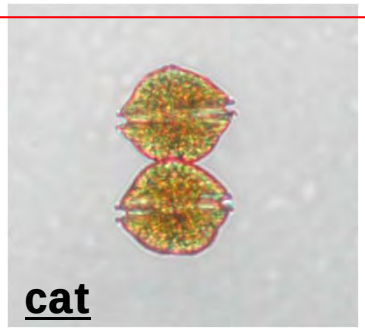
Single cells PCR

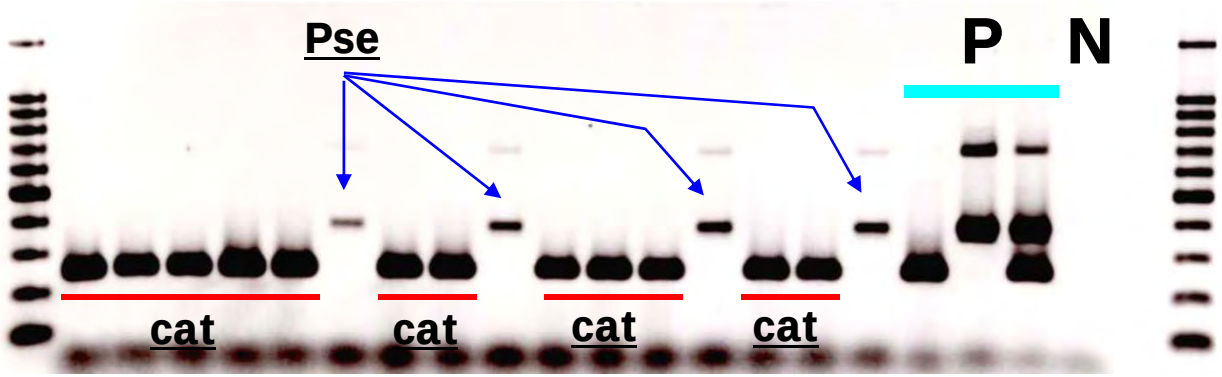
Succeed brilliantly
***Congratulation*!**

Application to natural samples

To establish *A. catenella* clonal strains from a sediment form New Zealand, I was doing sediment incubation, but many small *A. pseudogoniaulax* cells appeared in that sample. It was very difficult to exactly distinguish, therefore, I checked them by Multiplex-PCR.

Single cell-Multiplex PCR was carried out.





Succeed brilliantly
***Congratulation*!**

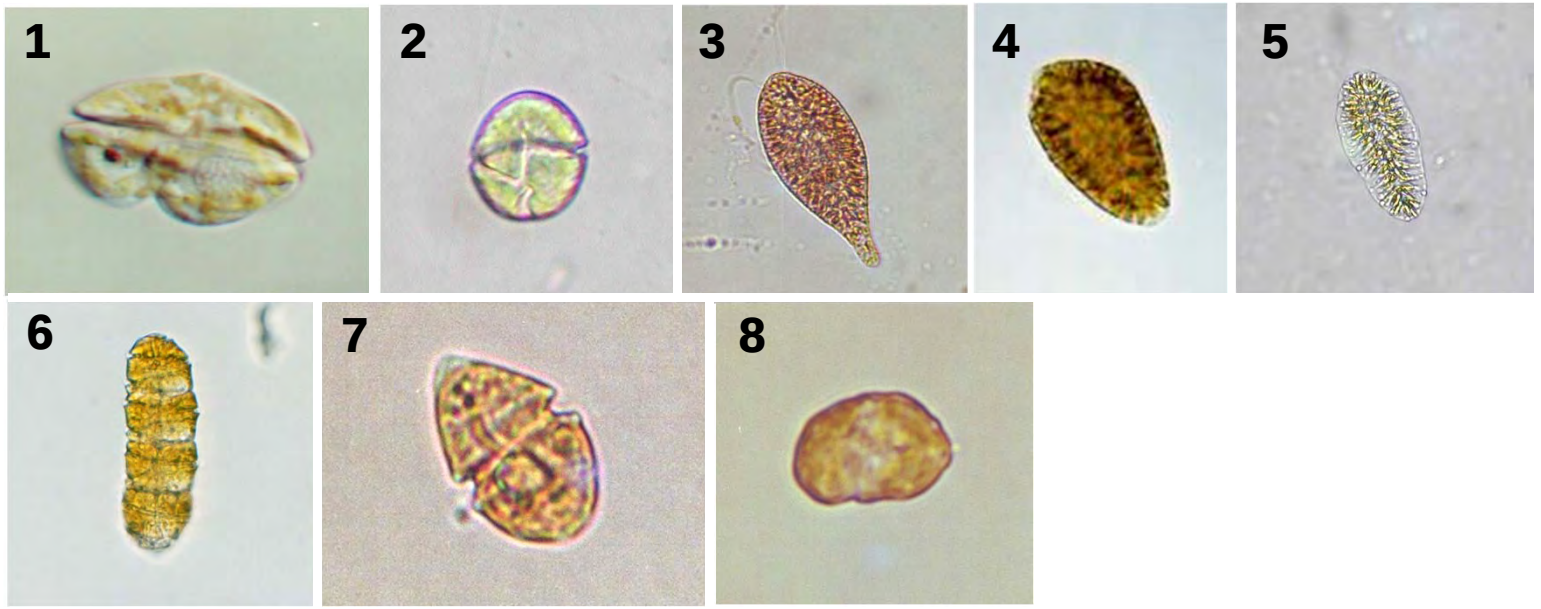
Species-specific amplification by the multiplex-PCR technique developed in this study.

Species	Location	Reference*	Strain number	field samples	Positive	Negative	mis identification
Alexandrium affine (Harima Nada, Kagawa Pref., Japan)	34° 17' N, 134° 28' E	this study	2		2	0	0
A. catenella (Kitanada, Tokushima Pref., Japan)	34° 12' N, 134° 27' E	Nagai et al. 2006a	6		6	0	0
A. catenella (Ago Bay, Mie Pref., Japan)	34° 17' N, 136° 49' E	Nagai et al. 2006a	6		6	0	0
A. catenella (Inokushi Bay, Oita Pref., Japan)	32° 47' N, 131° 53' E	Nishitani et al. 2007b	12		12	0	0
A. catenella (Kakuriki-Nada, Nagasaki Pref. Japan)**	32° 48' N, 129° 46' E	this study		12	12	0	0
A. fraterculus (Ago Bay, Mie Pref., Japan)	34° 17' N, 136° 49' E	Nagai et al. 2009b	3		3	0	0
A. pseudonogiaulax (Harima Nada, Kagawa Pref., Japan)	34° 16' N, 134° 28' E	this study	2		2	0	0
A. pseudonogiaulax (HiroshimaBay, Hiroshima Pref., Japan)**	34° 16' N, 132° 16' E	this study		12	12	0	0
A. tamarense (Okhotsk Sea, Hokkaido, Japan)	45° 10' N, 143° 42' E	Nagai et al. 2007a	12		12	0	0
A. tamarense (Jinhae Bay, Korea)	35° 03' N, 128° 43' E	Nagai et al. 2007a	12		12	0	0
A. tamarense (Hiroshima Bay, Hiroshima Pref., Japan)**	34° 16' N, 132° 16' E	this study		12	12	0	0
A. tamiyavanichii (Fukuyama Bay, Hiroshima Pref., Japan)	34° 26' N, 133° 26' E	Nagai et al. 2003	10		10	0	0
A. ostenfeldii (Funka Bay, Hokkaido, Japan)	42° 16' N, 140° 33' E	Nagai et al. in press	5		0	5	0
A. taylori (Shioya Bay, Okinawa Pref., Japan)	26° 40' N, 128° 06' E	this study	5		0	5	0
Flagilidium mexicanum (Hiroshima Bay, Hiroshima Pref., Japan)	34° 16' N, 132° 16' E	this study	1		0	1	0
Cochlodinium polykrikoides (Harima-Nada, Hyogo Pref., Japan)	34° 41' N, 134° 52' E	Nagai et al. 2009a	2		0	2	0
Chattonella ovata (Fukuyama Bay, Hiroshima Pref., Japan)	34° 26' N, 133° 26' E	Nishitani et al. 2007a	2		0	2	0
Gymnodinium catenatum (Inokushi Bay, Oita Pref., Japan)	32° 47' N, 131° 53' E	this study	2		0	2	0
Heterocapsa circularisquama (Ago Bay, Mie Pref., Japan)	34° 17' N, 136° 49' E	Nagai et al. 2007b	2		0	2	0
Heterosigma akashiwo (Hiroshima Bay, Hiroshima Pref., Japan)	34° 16' N, 132° 16' E	Nagai et al. 2006b	2		0	2	0
Karenia brevis (Florida, USA)	27° 70' N, 82° 80' W	CCMP718	1		0	1	0
Karenia brevis (Florida, USA)	27° 37' N, 82° 58' W	CCMP2228	1		0	1	0
Karenia degitata (Fukuyama Bay, Hiroshima Pref., Japan)	34° 26' N, 133° 26' E	this study	2		0	2	0
Karenia mikimotoi (Inokushi Bay, Oita Pref., Japan)	32° 47' N, 131° 53' E	Nishitani et al. 2009	2		0	2	0
Total			92		101	27	0

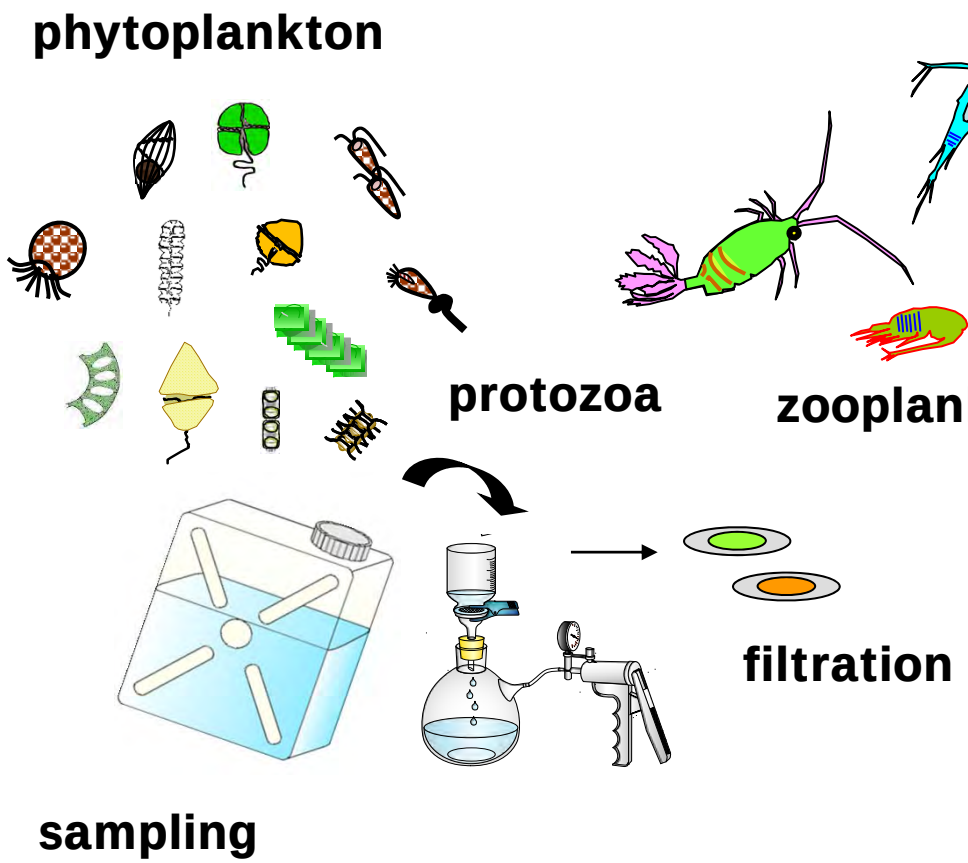
*, **, *Alexandrium catenella, A. tamarense and A. pseudogoniaulax* cells were obtained from seawaters in April, 2010 and single cells were used for the species identification.

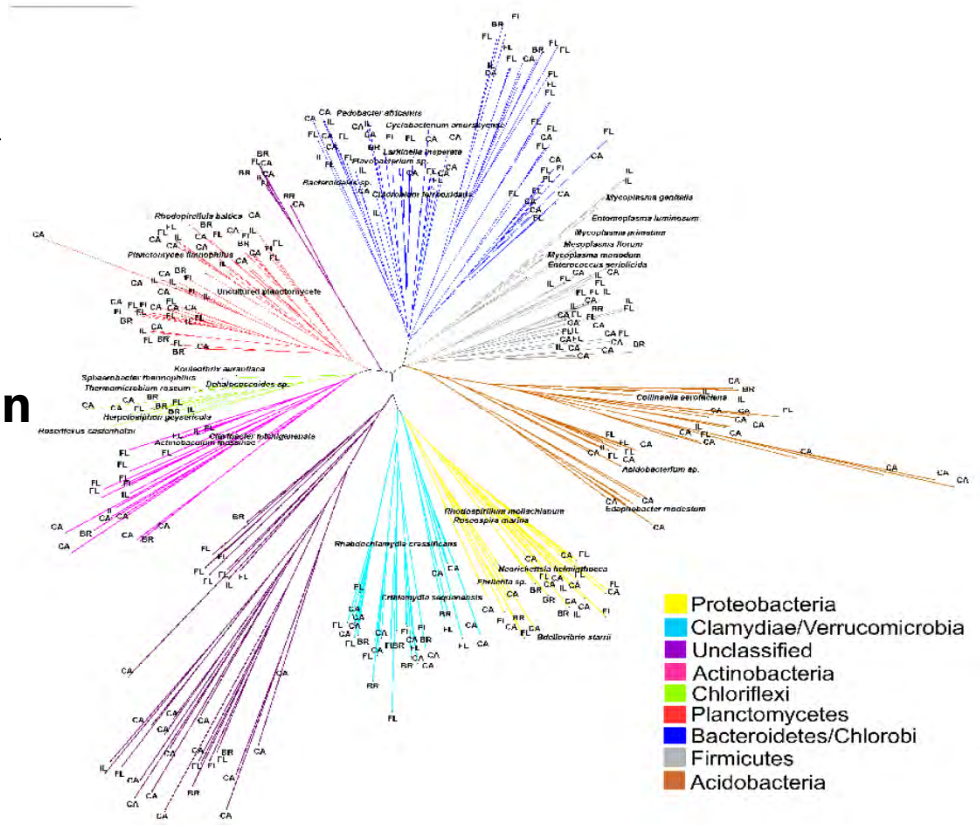
I am developing the multiplex PCR for these species at present.

Karenia brevis*, *K. degitata*, *Chattonella antiqua*, *C. marina*, *C. ovata*, *Cochlodinium polykrikoides*, *Heterocapsa circularisquama*, *Heterosigma akashiwo



Metagenome analysis of marine plankton using next generation sequencer





Background of research

- **Cell delicateness in most species**

  Cells do not fossilize in most of the species.

- **Hardness to know the species had appeared in the past**

  Preservation of appearance records is important.

- **Difficulty of identification of species <10μm in size**

  Registration of sequence data with morphological information should be enhanced.

Development of next generation sequencer

- **Mechanization from 2008**

  Companies are developing one machine after another.

- **Incredible performances**

  Coming of sequence revolution

- **Transition from metagenome to petagenome**

  We have to struggle with huge amounts of genetic data. Not only hard- but also software should be developed.

- **Changes of monitoring style, from morphology based to genetic**

  We can apply metagenome analysis to monitoring of coastal environments by 454 sequencing.

What is metagenome analysis?

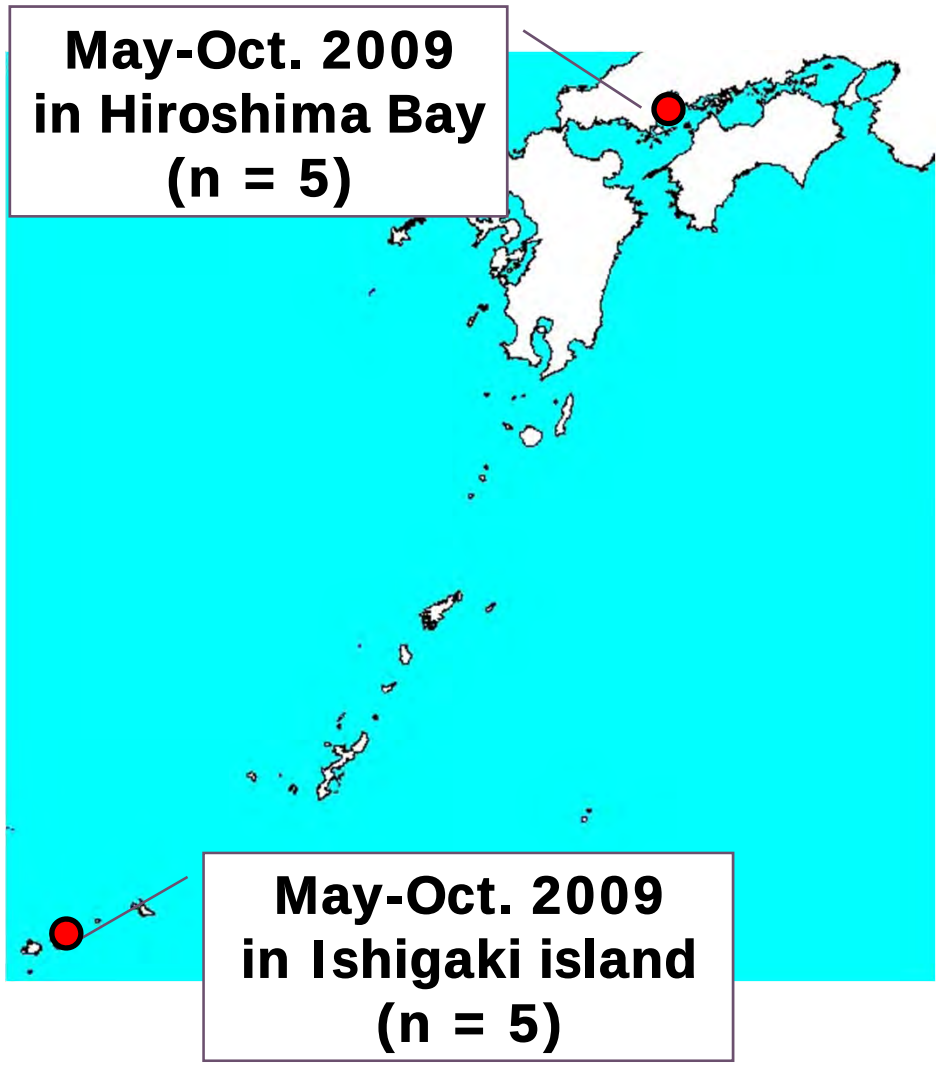
PCR amplification using universal primers and 454 sequencing

Records of all species in marine ecosystems and database construction

Comparison of marine biodiversity / investigation of its distribution / detection of new invasive species

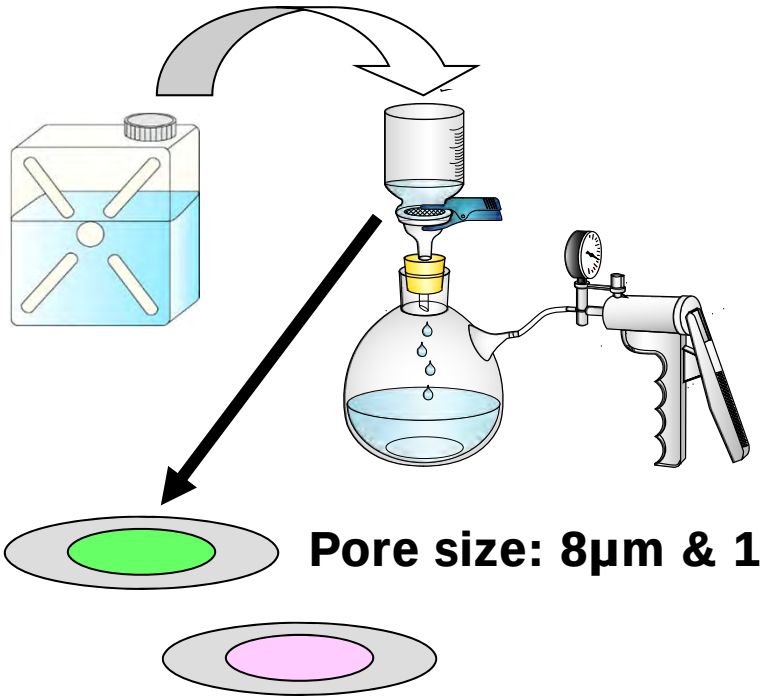
Materials & Methods

- **plankton sampling**



- **harvest**

Filtration of 500 mL seawater by nylon mesh (Φ75μm)



trap plankton on filters and DNA extraction from filters

454 sequencing by universal-PCR

PCR

from 20 filters of 10 samples

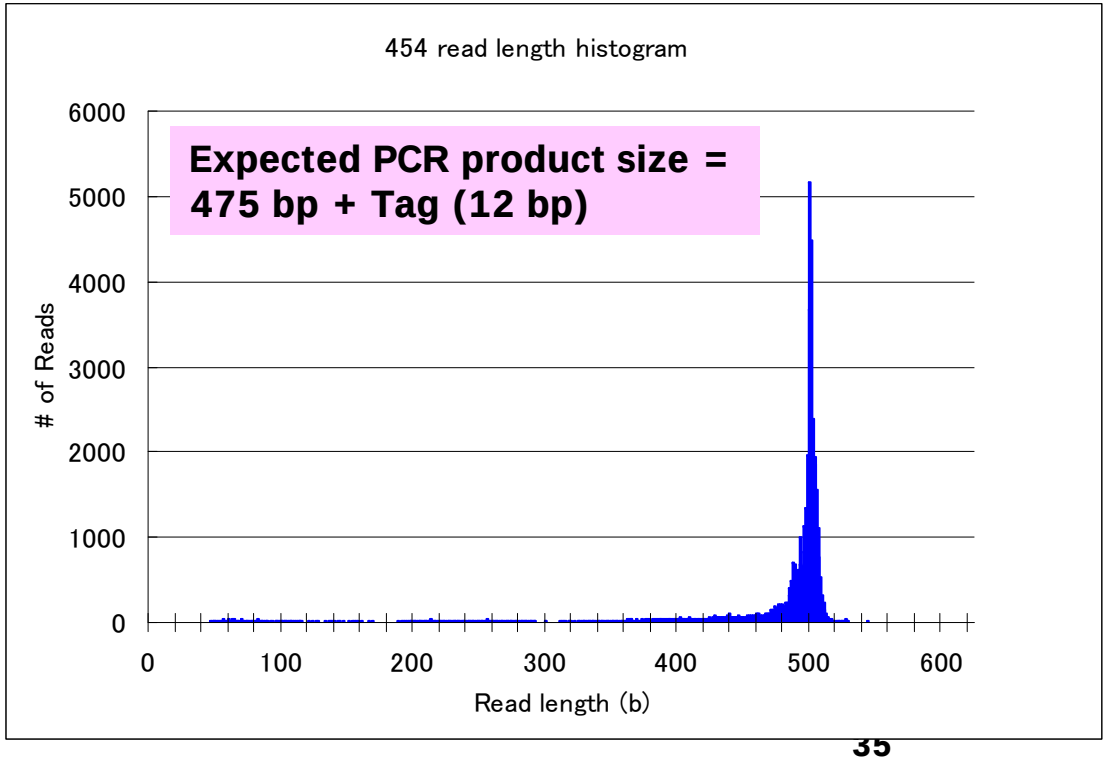
Purification by a column

10 samples were mixed together.

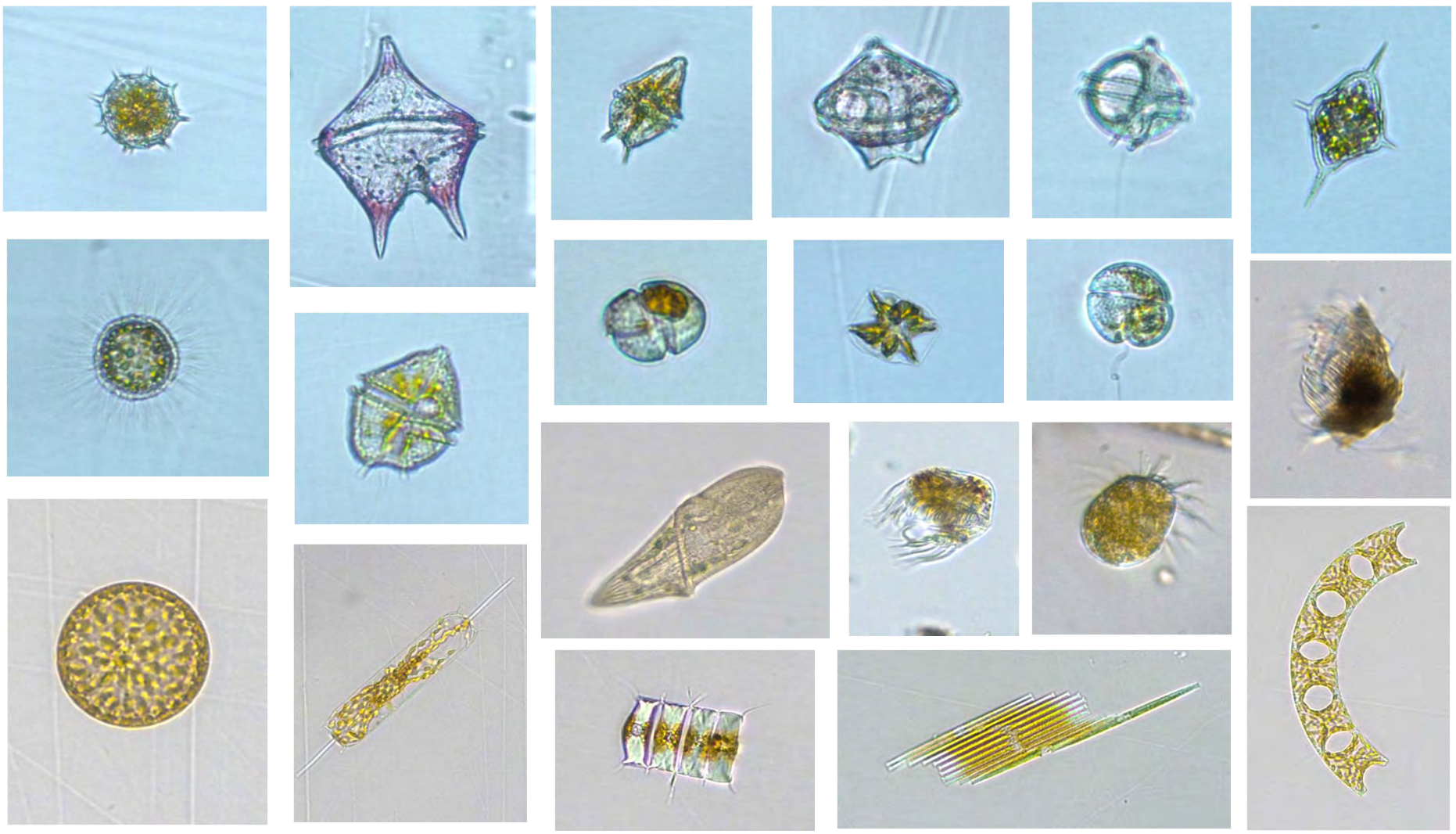
454 sequencing

Blast search

Contents	Results
Total amount of sequences	50,521
Total amount of bases (bp)	23,270,509
Average read length	461
No hit numbers of sequences in Blast	10
Numbers of GC (bp)	11211023
GC contents (%)	48



Plankton species in Hiroshima Bay



Result of Blast search for 50,521 seq.

- Only 3 or 4 sequences hit to bacterial genomes.
- Only 10 sequences did not hit to anything.
- <30 sequences hit to terrestrial plants or insects.

Samples	sequences
Hiroshima Bay (n=5)	33,545
Ishigaki islands (n=5)	15,889
unknown	1,087

Summary of algal data from Hiroshima Bay

Ranking	Phylum	Class	OTUs identified	total OTUs	unidentified OTUs	Identification success %	Total sequence
1	Heterocontophyte	Dinophyceae	154	200	46	77.0	6,070
2	Heterocontophyte	Bacillariophyceae	110	114	4	96.5	2,440
3	Haptophyta	Haptophyceae	44	57	13	77.2	570
4	Cryptophyta	Cryptophyceae	31	31	0	100.0	972
5	Chlorophyta	Prasinophyceae	23	32	9	71.9	1,072
6	Rhodophyta	Florideophyceae	16	16	0	100.0	147
7	Chlorophyta	Ulvophyceae	15	15	0	100.0	46
8	Heterocontophyte	Chrysophyceae	14	19	5	73.7	274
9	Chlorophyta	Chlorophyceae	12	12	0	100.0	316
10	Heterocontophyte	Pheophyceae	12	12	0	100.0	32
11	Euglenopyceae	Euglenopyceae	11	11	0	100.0	52
12	Heterocontophyte	Oomycetes	10	10	0	100.0	75
13	Chlorophyta	Trebouxiophyceae	5	5	0	100.0	87
14	Heterocontophyte	Xanthophycea	5	5	0	100.0	17
15	Heterocontophyte	Synuraphyceae	5	5	0	100.0	14
16	others		25	34	9	73.5	854
17	unknown picoplankton		0	28	28	0.0	712
	Total		492	606	114	81.2	13,750

Identification success: 73.7-100.0%

Genetic information is well supported in several classes.

Results of Ciliophora from Hiroshima Bay

	Phylum	Class	OTUs identified	total OTUs	unidentified OTUs	Identification success %	Total sequence	Similarity % Min	Max	Mean	SD
1	Litostomatida	Pleurostomatida	1	1	0	100.0	4			99.3	
2	〃	Haptorida	2	2	0	100.0	56	98.7	98.8	98.8	
3	〃	Vestibuliferida	1	1	0	100.0	1			100.0	
4	Spirotrichea	Choreotrichida, Tintinida	14	14	0	100.0	1125	95.4	99.4	97.0	1.3
5	〃	Stichotrichida	7	7	0	100.0	83	90.6	100.0	95.8	2.5
6	〃	Oligotrichida	6	6	0	100.0	107	92.4	99.6	96.4	2.0
7	〃	Choreotrichida	5	5	0	100.0	27	94.6	100.0	97.9	2.2
8	〃	unidentified	0	5	5	0.0	8	91.7	100.0	96.8	3.7
9	Prostomatea	Prorodontida	0	1	1	0.0	2			98.2	
10	Oligohymenophorea	Scuticociliatida	1	1	0	100.0	1			93.1	
11	Oligohymenophorea	Peritrichia	1	1	0	100.0	7			98.9	
12	unidentified		0	7	7	0.0	18	91.4	100.0	96.5	2.9
	Total		38	51	13	74.5	1,439				

Identification success: 38/51(74.5%), 1,439 sequences in total

Protozoa from Hiroshima Bay

Phylum	Class	OTUs identified	total OTUs	unidentified OTUs	Total sequence	Similarity % Min	Max	Mean	SD
Rizaria	Cercozoa, Foraminifera, Radiolaria	53	66	13	1132	87.0	100.0	95.2	3.3
Apicomplexa	Conoidasida, Sporozoea	8	12	4	41	86.2	100.0	94.4	3.3
〃	unknown	1	19	18	574	87.6	100.0	96.8	2.7
Stramenopiles	Bicosoecidea	6	6	0	25	88.4	100.0	94.5	4.1
〃	Oomycetes	10	10	0	22	88.6	100.0	94.3	4.0
〃	Labyrinthulea	16	16	0	40	85.0	100.0	96.0	3.6
〃	Pirsonia	3	3	0	14			94.2	4.3
〃	unknown	0	16	16	227			96.8	1.7
Amoebozoa	Flabellinea	2	2	0	4	98.6	99.0	98.8	
Fungi		45	62	17	117	84.9	100.0	97.8	1.9
	合計	144	212	68	2196				

Identification success, 144/212 (67.9%), 2,196 seq. in total

Summary in Hiroshima Bay samples

	Phylum	Class	Numbers of species	Numbers of sequences	unidentified	Identification success %	Total
Algae	9	28	492	606	113	81.2	13,750
Ciliophora	1	5	38	51	13	74.5	1,439
Animaria	16	31	167	167	0	100.0	2,014
Protozoa	4	?	99	150	51	66.0	2,079
Fungi	?	?	45	62	17	72.6	117
unknown Eukaryote	—	—	0	522	522	0.0	13,902
Total			841	1558	716	54.0	33,301

More registration of sequence to database is needed.

Hiroshima Bay = 1,558 OTUs

Ishigaki island = 1,361 OTUs

	Number of species	Number of Accession
Shared	**862**	**581**
Only Ishigaki	**489**	**770**

What can we do?

- **Record of all components in marine ecosystems**

  It is possible to cover all components by reading 200,000 seq.

- **Good bio-indicators of water quality and useful detection tools for new invasion and comparison of marine biodiversity**

  This is an effective tool for conservation study of marine environments.

- **Quantification of abundances**

  It will be a more efficient technique for monitoring of planktons.
  It will be one of the most powerful monitoring tools.

- **Full automatic analyzer in future**

  Full automatic robot analyzer, from sampling to analysis, is desirable.

Summary of molecular techniques

	accuracy	operability	experience	cost	equipment	quantification
PCR-RFLP	◎	◎	◎	◎	◎	X
FISH	○	○	△	◎	△	○△
SH	◎	○	○	◎	X	○
Q-PCR	○	○	△	○	△	○
LAMP	◎	◎	◎	◎	◎	X
Multiplex-PCR	◎	◎	◎	◎	◎	X
DNA microarray	○	○	○	△X	X	X
metagenome	○	X	X	X	XX	X
	◎, very good	◎, very good	◎, no need	◎, inexprensive	◎, inexprensive	◎, possible

Which is the best technique?

It depends on the purpose and we have to choose it according to our purposes.