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Intraspecific grouping of a harmful bloom-forming raphidophyte *Heterosigma akashiwo* based on its chloroplastic genetic signatures

SHIN-ICHIRO AKASE[1], TAKESHI YOSHIKAWA[2]*, NORIKO HAYAKAWA[1], HIROTO MAEDA[2], KEIZO NAGASAKI[3], YUJI TOMARU[3] & TAIZO SAKATA[2]

[1] *The United Graduate School of Agricultural Sciences, Kagoshima University, 1–21–24 Korimoto, Kagoshima 890–0065, Japan*
[2] *Laboratory of Microbiology, Faculty of Fisheries, Kagoshima University, 4–50–20 Shimoarata, Kagoshima 890–0056, Japan*
[3] *Harmful Algal Bloom Division, National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, 2–17–5 Maruishi, Ohno, Saeki, Hiroshima 739–0452, Japan*

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Abstract: Previously, five intraspecific genetic signatures were established at the flanking and intergenic spacer (IGS) regions on the chloroplast genome of the typical harmful bloom-forming raphidophyte *Heterosigma akashiwo*. In order to assess its diversity, grouping of 30 *H. akashiwo* strains isolated from coastal waters of western Japan was conducted by detecting the genetic signatures using the polymerase chain reaction (PCR) and cleaved amplified polymorphic sequence (CAPS) techniques. Consequently, they were divided into five categories: G1–G5. The present categorization results based on the chloroplastic genetic signatures did not show any apparent correlation with locality of the tested strains, but corresponded comparatively well to the categorization results based on sensitivity patterns to a double-stranded DNA virus HaV (*H. akashiwo* virus). These results may account for the reliability of the grouping technique based on chloroplastic genetic signatures and its applicability to the analysis of the propagation processes and population dynamics of *H. akashiwo* blooms.

Key words: *Heterosigma akashiwo*, intraspecific grouping, chloroplast genome, intergenic spacer region, genetic signatures

Introduction

Heterosigma akashiwo (Hada) Hada (Raphidophyceae) is a typical harmful algal bloom (HAB)-causing microalga occurring in coastal waters of subarctic and temperate areas of both the northern and southern hemispheres. As it often causes mortality of cultured fish such as salmon, yellowtail, red sea bream and greater amberjack (Yamochi 1989; Imai & Itakura 1991; Honjo 1993; Smayda 1998), practical techniques to predict its occurrence and to control its blooms are urgently required. For the last few decades, methods for forecasting red tide outbreaks have been studied by monitoring environmental factors such as water temperature, salinity, and inorganic nutrients (Honjo et al. 1991; Matsuyama et al. 1995). On the other hand, a number of bacteria and viruses killing HAB-causing microalgae were isolated and investigated to assess the possibility of their practical use as biological agents to control HABs (eg. Lovejoy et al. 1998; Yoshinaga et al. 1999; Nagasaki et al. 1999; Furusawa et al. 2003; Mayali & Azam 2004).

Recent studies have revealed that the clonal composition of microalgal blooms is highly diverse and the clonal dominancy fluctuates dynamically throughout the blooming period. Shankle et al. (2004) reported intraspecies succession of *Prorocentrum micans* (Dinophyceae) during its blooms by using randomly amplified polymorphic DNA (RAPD) analysis. Tarutani et al. (2000) found that *H. akashiwo* populations were composed of distinct types of clones that have different viral sensitivity spectra, and pointed out the possibility that both abundance and clonal composition of *H. akashiwo* populations were controlled by viral infection; later, from the viewpoint of viral sensitivity spectra, Tomaru et al. (2004) indicated possible intraspecies succes-

*Corresponding author: Takeshi Yoshikawa; e-mail, yoshi@fish.kagoshima-u.ac.jp

sion within *H. akashiwo* blooms.

In order to conduct a precise investigation on algal bloom dynamics, molecular genetic markers with resolution at an intraspecific level are expected to work as powerful tools for monitoring clonal composition changes in natural blooms. Molecular techniques such as RAPD, restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE), single-strand conformational polymorphism (SSCP) and microsatellites have been applied for the purposes mentioned above (de Bruin et al. 2003; Nagai et al. 2004). However, low reproducibility involved in RAPD analysis often leads to misinterpretation (MacPherson et al. 1993; Halldén et al. 1996; Rabouam et al. 1999); as for the other techniques, nucleotide sequence information at a polymorphic genomic locus is a prerequisite. In addition, due to the separation capacity of polyacrylamide gels, DNA fragment size targeted by DGGE or SSCP is restricted to about 500 bp. False bands produced by heteroduplex formation during DGGE analysis should be taken into account (Curtis & Craine 1998), especially when heterogeneous DNA isolated directly from seawater samples *in situ* is used. Cloning of microsatellites is highly laborious and costly.

Akase et al. (2004) established intraspecific molecular markers for *H. akashiwo* by comparing the nucleotide sequences of *rbcL*, *rbcS*, *psbA*, IGS and flanking regions on the chloroplast genome of four *H. akashiwo* strains. Consequently, five genetic signatures were recognized in the *trnL* upstream region and the *trnL-rbcL* and *rbcS-cfxQ* IGS regions, and the four strains tested in the previous study were grouped into two groups by PCR and cleaved amplified polymorphic sequence (CAPS) techniques (Akase et al. 2004). However, due to the insufficient sample size, the usefulness of these genetic signatures for identification of *H. akashiwo* clones has not been confirmed. The objectives of the present study are to attempt grouping of an additional 26 *H. akashiwo* strains from different localities, to elucidate the relationship between the genetic variation and geographical distribution of the *H. akashiwo* strains, and to evaluate the usefulness of the five genetic signatures for identifying *H. akashiwo* strains.

Materials and Methods

Microalgal strains and extraction of their DNA

Heterosigma akashiwo strains used in this study are listed in Table 1. Selection of *H. akashiwo* clones was conducted according to the results of their viral sensitivity spectra given by Tomaru et al. (2004) who divided them into six host groups. Eleven clones from the host groups A (1, 46, 72), B (77, 55), C (58, 59), E (16, 81), and F (37, 39) (Tomaru et al. 2004) were tested in the present experiments. The strains NIES-5 and NIES-6 (Watanabe & Hiroki 1997) were obtained from the National Institute for Environmental Studies (NIES, Japan). All the microalgal cells were cultivated in Provasoli's enrichment seawater medium (PES medium) at 23°C under a 12 hr : 12 hr (light : dark) cycle with an irradiance of $5.0\times10^{2}\ \mu E\ m^{-2}\ s^{-1}$.

After cultivation for 2 weeks under the conditions described above, the microalgal cells were collected by centrifugation at $1{,}000\times g$ for 10 min. The total DNA was purified using the nucleic acid purification kit SepaGene (Sanko Junyaku, Tokyo, Japan) according to the manufacturer's instructions.

Discrimination of the *H. akashiwo* strains by PCR and CAPS

The nucleotide substitution sites A, B and D, the indel site C, and the inversion site E were PCR-amplified; then, the amplicons were digested with restriction enzymes except for sites A and C (Figs. 1, 2; for details see Akase et al. (2004)). The PCR amplification profiles consisted of 35 cycles of 1 min at 95°C, 2 min at 67°C (for signature A), 55°C (for signature B), 45°C (for signature C), or 50°C (for signatures D and E), and 3 min at 72°C followed by 5 min at 72°C. All the amplicons and their restricted fragments were electrophoresed on 2% agarose gels or 5% polyacrylamide gels.

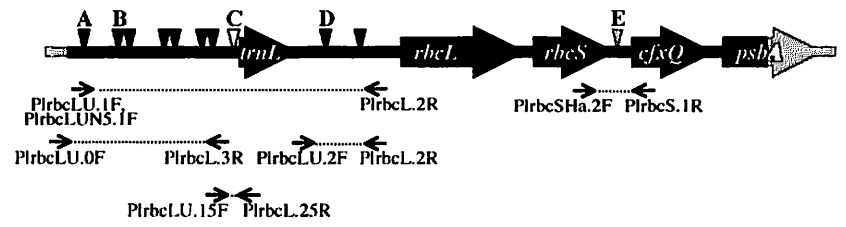

Fig. 1. Gene structure of *trnL*, *rbcL*, *rbcS*, *cfxQ* and *psbA* encoded on the *Heterosigma akashiwo* chloroplast genome. The gene-coding regions and their encoding directions are shown as arrows with thick lines. The regions where nucleotide sequences have been determined (Akase et al. 2004) are shown as black lines. Nucleotide substitution, deletion and inversion sites found among the *H. akashiwo* strains are denoted as closed, open and shaded triangles, respectively, and those that were used as genetic signatures for strain discrimination are labeled as A–E. The loci on which the oligonucleotide primers for the PCR and CAPS assays were designed and the regions that are amplified by these primers are shown below as thin arrows and dotted lines, respectively.

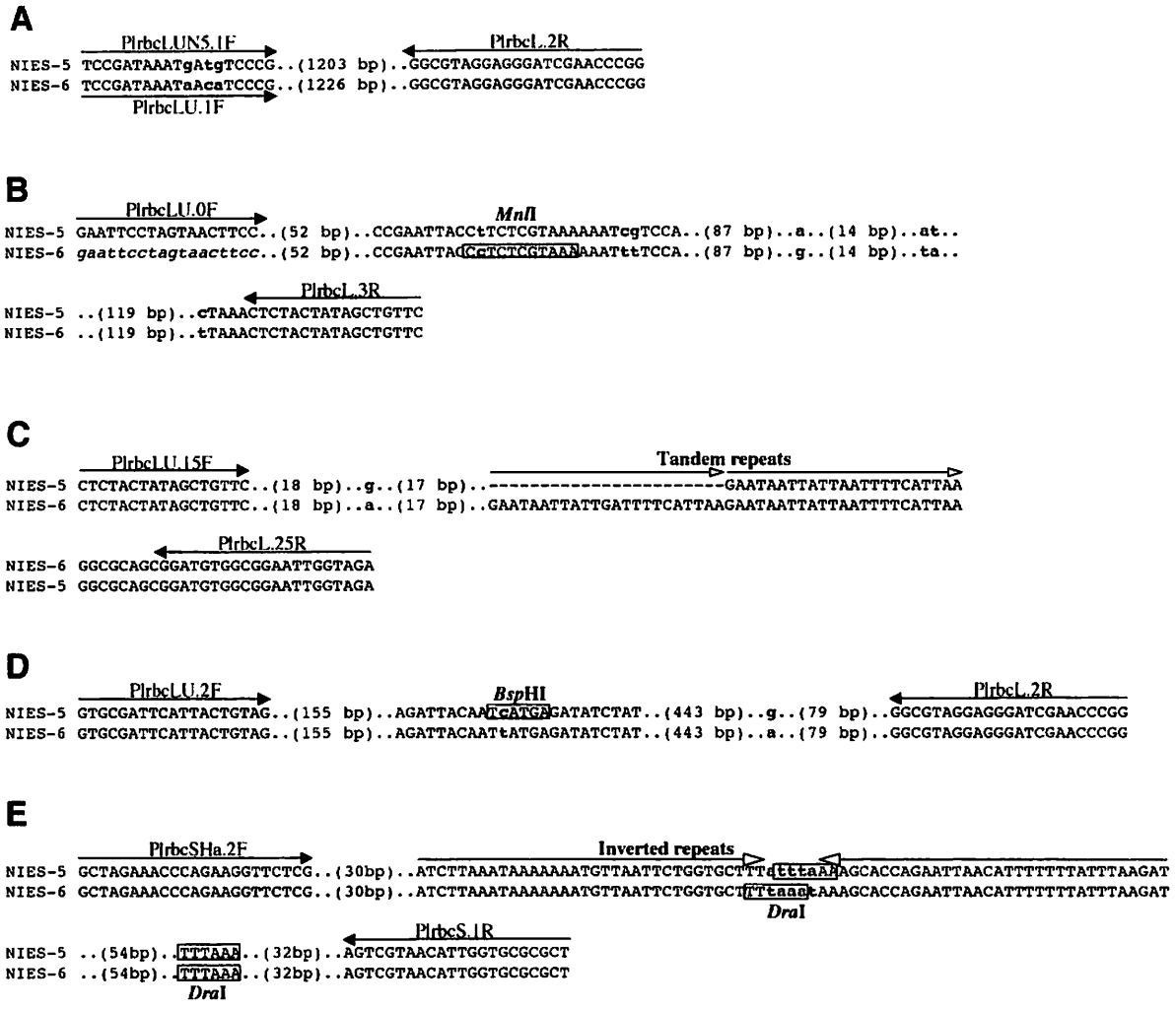

Fig. 2. Nucleotide sequence comparisons at the chloroplastic genetic signature sites between the N5 and N6 group strains of *Heterosigma akashiwo*. The nucleotide substitutions and indels are shown as bold small letters and dashes, respectively. The tandem and inverted repeat sequences are shown as arrows with open arrowheads. The loci of the oligonucleotide primers and the target sites of restriction enzymes used for the PCR and CAPS assays are shown as closed arrows and boxes, respectively. These genetic signatures are mapped on Fig. 1 (closed, open, and shaded triangles, A–E). Nucleotide sequences shown in italic small letters signify that these sequences originated from the primer PlrbcLU.0F, not from the chloroplast genome.

Results

As shown in Figs. 3A, B and D, the PCR amplification of signature A with the group-specific primers PlrbcLUN5.1F and PlrbcLU.1F, and the CAPS analyses of signatures B and D grouped the *Heterosigma akashiwo* strains UR94, KG95, TBm, USm, TK1030-1 and H00621OB01 into the N5 group, represented by the band profiles of strain NIES-5, while the other strains grouped into the N6 group, represented by the band profiles of strain NIES-6. On the other hand, the strains AI97521 and KB98621-1 had no tandem repeats at the indel site C, as they produced shorter amplicons 105 bp in length as in the case of the N5 group strains (Fig. 3C). In addition, the CAPS analysis of signature E grouped the strains KG95, TBm, USm, TK1030-1 and H00621OB01 into the N6 group, while the strains GS95, MZm, 1, 77, 46, 55 and 72 grouped into the N5 group (Fig. 3E).

Table 1 summarizes the grouping of the 30 *H. akashiwo* strains tested in the present experiments based on the combination of the genetic signatures A–E; consequently, the strains were categorized into five groups: G1–G5 (Table 1). All the strains isolated from the Seto Inland Sea belonged to G3, G4, and G5, except for TK1030-1, and shared four or five N6 type signatures out of five. On the other hand, the strains isolated from the other coastal environments facing the Pacific Ocean, the Sea of Japan, and the East China Sea were distributed throughout all five categories (G1–G5).

Discussion

In general, the molecular evolutionary rates of chloroplast genomes maintained by terrestrial plants are relatively slow (Wolfe et al. 1987). However, due to their low func-

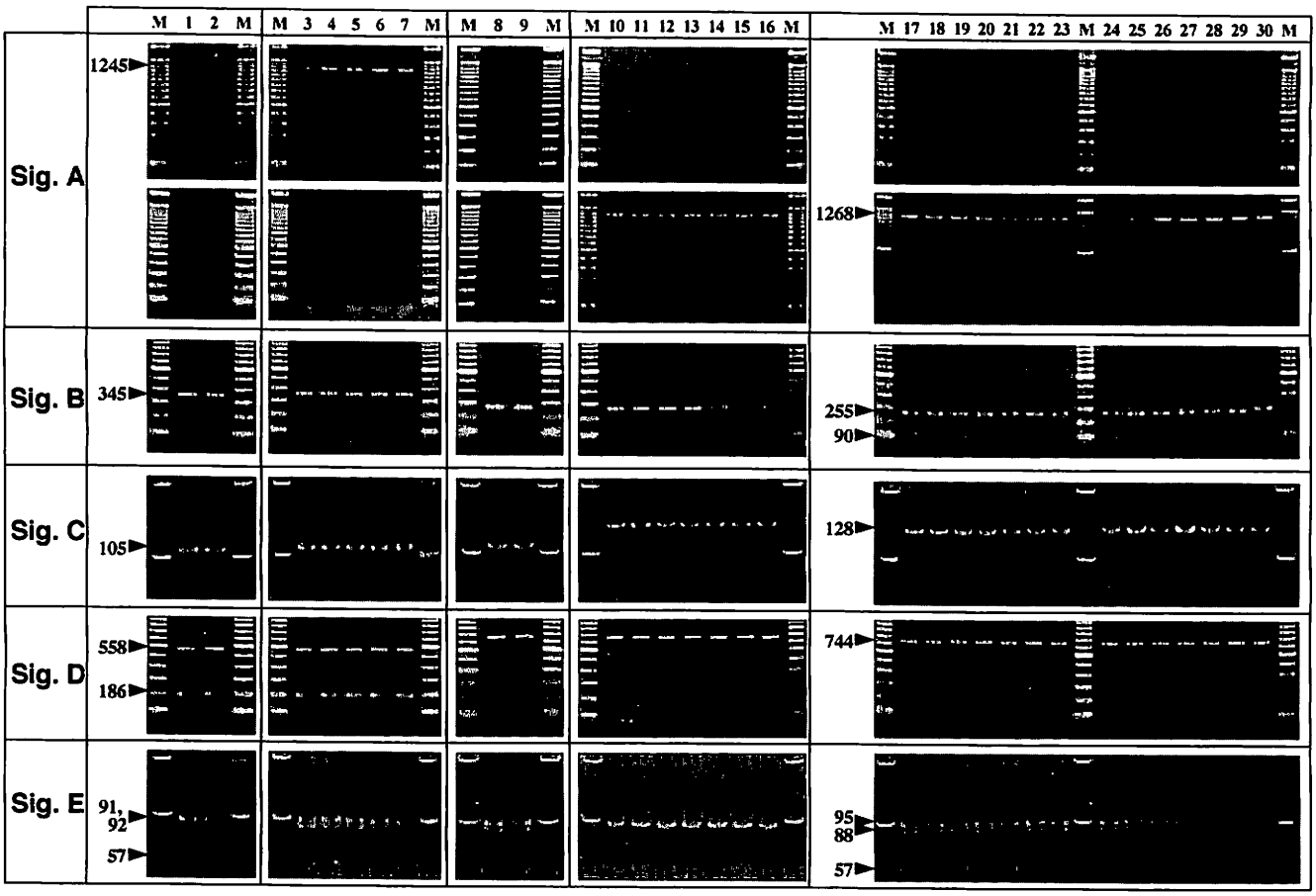

Fig. 3. Differentiation of the 30 *Heterosigma akashiwo* strains by the PCR and CAPS assays shown in Fig. 2. Panels Sig. A–E indicate the electrophoresis results of the PCR or CAPS shown in Fig. 2A–2E, respectively. In the panel Sig. A, primer PlrbcLUN5.1F specific to the N5 group strains (upper photographs) and primer PlrbcLU.1F specific to the N6 group strains (lower photographs) were used. The DNA fragment sizes are indicated as base pairs with arrowheads. The lane numbers correspond to the strain numbers listed in Table 1. Lane M: 100 Base-Pair Ladder (Amersham Biosciences, NJ, USA). Sig. A, B and D, electrophoresed on 2% agarose gel; Sig. C and E, electrophoresed on 5% native polyacrylamide gel.

tional constraints, IGS regions exhibit moderate evolutionary rates and are considered useful for measuring diversity at the intraspecies level. As for some marine algae and terrestrial plants, the IGS regions between large and small subunit genes of RuBisCO (*rbcL* and *rbcS*), and between the transfer RNA genes *trnL* and *trnF* have been successfully applied to enable intraspecific discrimination (Maggs et al. 1992; Zuccarello & West 1997; Kamiya et al. 1998; Ingvarsson et al. 2003; Dobes et al. 2004). In the same way, based on the analysis of five genetic signatures (Akase et al. 2004), we divided the 30 *H. akashiwo* strains into five categories: G1–G5 (Table 1). Figure 4 represents the number of the strains isolated from each sampling point and the categories they belong to. Although a large proportion of strains belonging to categories G3–G5, which have four or five N6 type signatures, occurred mainly in the Seto Inland Sea, there was no apparent correlation between their areas of occurrence and the categories they belonged to. These results suggest that clonal composition of *H. akashiwo* populations in individual marine environments is highly diverse, and the chloroplastic genetic signatures do not necessarily reflect the geographical distributions. Furthermore, the categorization of the *H. akashiwo* clones did not show any apparent seasonal change, although the influence of only analyzing a limited number of clones and sampling bias should not be ignored.

Tomaru et al. (2004) monitored the dynamics of *H. akashiwo* clones and their viruses in Hiroshima Bay in 2000 and divided 90 *H. akashiwo* clones into 6 groups (Host Group A–F) based on their virus sensitivity spectra. Comparison between the categories based on the chloroplastic genetic signatures (this study) and those based on the virus sensitivity spectra (Tomaru et al. 2004) showed a considerable correspondence; i.e., Host Groups A and B fell into category G4, and Host Groups C, E, and F fell into category G5. The genotypic categorization and the phenotypic grouping results suggest the possibility that genetic signatures may be useful in analysis of *H. akashiwo* population dynamics. It is of interest that the polymorphism found at the chloroplastic genetic signature loci supported the di-

Table 1. Classification of the *H. akashiwo* strains according to the chloroplastic genetic signature typing.

Strain numbers	Strains[a]	Sampling points	Isolation years	Signatures[b]					Categories
				A	B	C	D	E	
1	NIES-5[c]	Gokasho Bay	1966	N5	N5	N5	N5	N5	G1
2	UR94	Uranouchi Bay	May 1994	N5	N5	N5	N5	N5	
3	KG95	Kagoshima Bay	April 1995	N5	N5	N5	N5	N6	G2
4	TBm	Tachibana Bay	November 1996	N5	N5	N5	N5	N6	
5	USm	Usuki Bay	November 1996	N5	N5	N5	N5	N6	
6	TK1030-1	Tokuyama Bay	30 October 1998	N5	N5	N5	N5	N6	
7	H00621OB01	Obama Bay	21 June 2000	N5	N5	N5	N5	N6	
8	AI97521	off-Aioi	21 May 1997	N6	N6	N5	N6	N6	G3
9	KB98621-1	Kobe Port	21 June 1998	N6	N6	N5	N6	N6	
10	GS95	Gokasho Bay	May 1995	N6	N6	N6	N6	N5	G4
11	MZm	Kagoshima Bay	November 1996	N6	N6	N6	N6	N5	
12	H00607-1 (1, A)	Hiroshima Bay	7 June 2000	N6	N6	N6	N6	N5	
13	H00616-MB01 (77, B)	Hiroshima Bay	16 June 2000	N6	N6	N6	N6	N5	
14	H00619-10 (46, A)	Hiroshima Bay	19 June 2000	N6	N6	N6	N6	N5	
15	H00623-06 (55, B)	Hiroshima Bay	23 June 2000	N6	N6	N6	N6	N5	
16	H00721-1 (72, A)	Hiroshima Bay	21 July 2000	N6	N6	N6	N6	N5	
17	NIES-6[d]	Osaka Bay	1979	N6	N6	N6	N6	N6	G5
18	K95-Ho[d]	Kagoshima Bay	1995	N6	N6	N6	N6	N6	
19	K95-Hy[d]	Kagoshima Bay	1995	N6	N6	N6	N6	N6	
20	KM95	Yatsushiro-Kai	April 1995	N6	N6	N6	N6	N6	
21	NM97414	Nomi Bay	14 April 1997	N6	N6	N6	N6	N6	
22	HFK-1	Tajiri Port	15 July 1998	N6	N6	N6	N6	N6	
23	H00612-3 (16, E)	Hiroshima Bay	12 June 2000	N6	N6	N6	N6	N6	
24	H00616-07 (37, F)	Hiroshima Bay	16 June 2000	N6	N6	N6	N6	N6	
25	H00619-1 (39, F)	Hiroshima Bay	19 June 2000	N6	N6	N6	N6	N6	
26	H00619-MB03 (81, E)	Hiroshima Bay	19 June 2000	N6	N6	N6	N6	N6	
27	H00623-09 (58, C)	Hiroshima Bay	23 June 2000	N6	N6	N6	N6	N6	
28	H00623-10 (59, C)	Hiroshima Bay	23 June 2000	N6	N6	N6	N6	N6	
29	K01-Hs	Kagoshima Bay	April 2001	N6	N6	N6	N6	N6	
30	K01-Hu	Kagoshima Bay	April 2001	N6	N6	N6	N6	N6	

[a] The clone numbers and the host groups to which Tomaru et al. (2004) referred are shown in parentheses (clone number, host group).
[b] The signatures that showed the same band profiles as the N5 and N6 groups in Fig. 3 are described as N5 and N6, respectively.
[c] Strains that were classified into the N5 group in a previous report (Akase et al. 2004).
[d] Strains that were classified into the N6 group in a previous report (Akase et al. 2004).

versity results concerning *H. akashiwo* clones indicated by viral infectivity spectra (Tomaru et al. 2004), even though these grouping were made from quite different viewpoints. Thus, chloroplastic signatures may be useful for monitoring changes in microalgal clonal composition, which fluctuates dynamically during red tide blooms and year by year in terms of viral sensitivity (Tarutani et al. 2000; Tomaru et al. 2004). Use of chloroplastic signatures also has the advantage of being applicable to *H. akashiwo* cells that have become unculturable due to the initiation of encystment or viral infection (Nagasaki et al. 1996; Tomaru et al. 2004), since PCR or CAPS analyses at the chloroplastic signature loci are able to detect polymorphism of microalgal DNA isolated directly from seawater samples *in situ* without necessitating the isolation and cultivation of *H. akashiwo* clones. Further investigations of the *H. akashiwo* chloroplast genome sequences will probably unveil potentially useful genetic signatures. By analysing these signatures, clonal composition dynamics in red tide populations is expected to be observable in greater detail.

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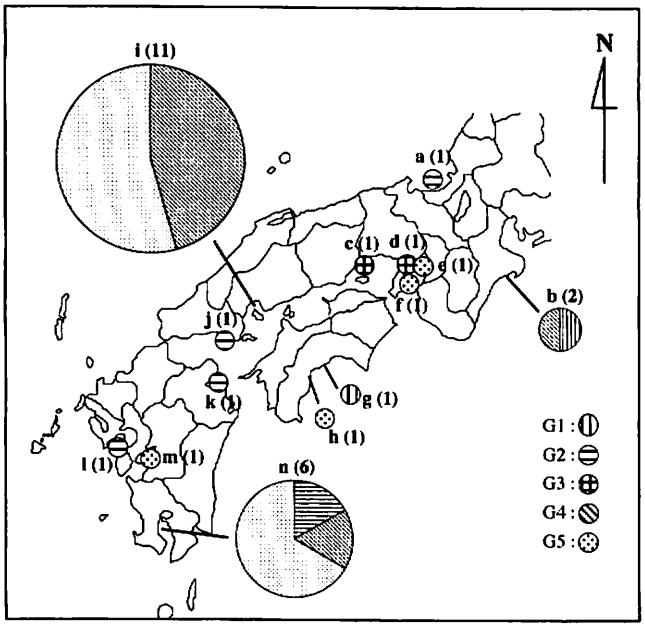

Fig. 4. Numbers and categories of *Heterosigma akashiwo* strains isolated from western Japan. Sizes of the pie charts indicate the number of strains, which are also shown in parentheses. Proportion of the strains belonging to each category is indicated as a sector. The sampling points are as follows: a, Obama Bay (Fukui Prefecture); b, Gokasho Bay (Mie); c, off-Aioi (Hyogo); d, Kobe Port (Hyogo); e, Osaka Bay (Osaka); f, Tajiri Port (Osaka); g, Uranouchi Bay (Kochi); h, Nomi Bay (Kochi); i, Hiroshima Bay (Hiroshima); j, Tokuyama Bay (Yamaguchi); k, Usuki Bay (Oita); l, Tachibana Bay (Nagasaki); m, Yatsushiro-Kai (Kumamoto); n, Kagoshima Bay (Kagoshima).

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