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# Sequence Analysis of 5.8S rDNA and the Internal Transcribed Spacer Region in Dinoflagellate *Heterocapsa* Species (Dinophyceae) and Development of Selective PCR Primers for the Bivalve Killer *Heterocapsa circularisquama*

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Algal exposure assays revealed that all tested strains of *H. circularisquama* and *H. illdefina* were markedly toxic to juvenile pearl oysters. *H. triquetra*, and *Heterocapsa* sp. 3 and sp. 5 caused low levels of mortality in juveniles (1.7–7.5%), whereas *H. lanceolata, H. horiguchii* and *Heterocapsa* sp. 4 caused no mortality. Three strains of *H. circularisquama* alone exhibited strong toxicity against bivalves among *Heterocapsa* species isolated in Japan.

The sequencing of internal transcribed spacer (ITS) regions including 5.8S rDNA and phylogenetic analysis were performed for isolates of *Heterocapsa* species. Alignment of the sequences demonstrated that ITS regions were highly conserved among *H. circularisquama* strains, while the distance between *H. circularisquama* and other species ranged from 0.165 to 0.254. Except for *H. horiguchii* and *Heterocapsa* sp. 4, the phylogenetic tree obtained was congruent with morphological identification. *Heterocapsa* species were subjected to PCR amplification with a primer set designed based on a specific signature sequence for *H. circularisquama* in the ITS 2 region. A specific band was detected for all strains of *H. circularisquama* but not other *Heterocapsa* species, indicating that the ITS region is suitable as a species-specific marker for *H. circularisquama*.

**Key words:** dinoflagellate, *Heterocapsa circularisquama*, internal transcribed spacer, ITS, red tide

A marine dinoflagellate, *Heterocapsa circularisquama* Horiguchi, causes red tide blooms which kill bivalves such as pearl oysters, short-necked clams and oysters, presenting a serious problem for shellfish aquaculture in the western area of Japan[10–12]. The *H. circularisquama* bloom specifically affects bivalves, not fish or other marine vertebrates, and is thus a novel red tide[12].

The genus *Heterocapsa* is characterized by the thecal plate arrangement Po, cp, 5', 3a, 7'', 6c, 5s, 5''', 2'''', and the presence of body scales on the cell surface[4]. At the time *H. circularisquama* was described as a new species, ten *Heterocapsa* species had been identified[4]. Among these ten, *H. illdefina* (Herman et Sweeney) Loeblich III *et al.* is the closest relative of *H. circularisquama*[4]. Despite differences in morphology in the fine structure of the body scales and the ultrastructure of the pyrenoid matrix, these two species are almost indistinguishable at the light microscopic level[4]. Thus the fine structure of body scales, which can be observed by transmission electron microscopy (TEM), is an important taxonomic marker for species identification[4–6]. Based on this marker, several new *Heterocapsa* species have been identified in Japanese coastal waters during the monitoring of *H. circularisquama* and assigned the names

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*Heterocapsa* sp. 1 to sp. 6[5]. Consequently, *Heterocapsa* sp. 1 and sp. 2 are newly described as *H. lanceolata* Iwataki and Fukuyo and *H. horiguchii* Iwataki, Takayama and Matsuoka, respectively[6]. Presently, the following eleven species are described as valid members of the genus *Heterocapsa*[6]; *H. arctica* Horiguchi, *H. circularisquama*, *H. horiguchii, H. illdefina, H. lanceolata, H. minima* Pomroy, *H. niei* (Loeblich III) Morrill and Loeblich III, *H. pacifica* Kofoid, *H. pygmaea* Loeblich III *et al.*, *H. rotundata* (Lohmann) Hansen and *H. triquetra* (Ehrenberg) Stein. Under the light microscope, only the cell shape, cell size and positions of organelles can be observed. Using these characteristics, identification of *H. triquetra, H. arctica*, *H. rotundata*, *H. niei*, *H. pygmaea*, *H. lanceolata* and *Heterocapsa* sp. 6 was reported to be relatively easy[5]. In contrast, *H. circularisquaman*, *H. illdefina*, *H. horiguchii*, *Heterocapsa* sp. 4, *Heterocapsa* sp. 3 and *Heterocapsa* sp. 5 have a hemispherical epitheca and hypotheca of almost the same size[5,6]. It is practically impossible to differentiate *H. circularisquaman* from *H. illdefina*, *H. horiguchii* from *Heterocapsa* sp. 4 and *Heterocapsa* sp. 3 from *Heterocapsa* sp. 5 at the microscopic level and the observation of body scales is required for the identification of these species[4–6]. To cope with *H. circularisquama* blooms quickly and to lessen the damage to shellfish aquaculture, a rapid and easy monitoring system for this species is needed. However, as the morphological identification of this species is difficult, an alternative species marker is needed for *H. circularisquama*.

The 5.8S ribosomal RNA gene (rDNA) and flanking internal transcribed spacer (ITS1 and ITS2) regions, which are thought to evolve faster than the 18S and 28S rDNA region, have been used for phylogenetic analysis to resolve taxonomic ambiguities in some dinoflagellates[1,2]. Adachi *et al.* reported homogeneity of this region within each ITS type in *Alexandrium* species and high diversity in this region between the ITS types, indicating that the ITS regions are a useful species-specific genetic marker for dinoflagellates[1,2]. In this study, the sequencing of ITS regions and a phylogenetic analysis were performed for *H. circularisquama*, *H. arctica*, *H. illdefina*, *H. pygmaea*, *H. triquetra*, *H. lanceolata*, *H. horiguchii* and three undescribed *Heterocapsa* species to clarify the phylogenic relationships of *Heterocapsa* species, some of which can be differentiated by the fine structure of body scales. In addition, the specificity of primers designed for the *H. circularisquama*-specific sequences in this region was investigated by use of PCR as a preliminary step in establishing a monitoring system for species associated with the mass mortality of bivalves.

The lethal effect of *H. circularisquama* on pearl oysters has been demonstrated using both cultured and natural cells[14,15]. Matsuyama *et al.* reported that *H. circularisquama* had an inhibitory effect on the clearance in blue mussel whereas *H. triquetra* showed no such effect[13]. However, the toxicity of other *Heterocapsa* species has not been determined. Moreover, *H. lanceolata* and *H. horiguchii* have been detected during blooms of *H. circularisquama*[6] To construct a system for monitoring species associated with the mass mortality of bivalves, it first must be clarified whether other species are toxic or not. Therefore, we also analyzed the toxic effects of isolates of *Heterocapsa* species on juvenile pearl oysters.

## Materials and Methods

### *Cultures*

The clonal isolates of the *Heterocapsa* species used in this study are listed in Table 1. *H. circularisquama* OA1, OK1 and OK3 were isolated from Obama Bay, Fukui prefecture, Japan in August 1998 while *H. lanceolata* TK6-D57 and *H. horiguchii* FK6-D47 were isolated from Tokyo Bay, Tokyo and Fukuyama Bay, Hiroshima Prefecture, Japan, respectively. Other strains were obtained from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton (CCMP), USA and the National Institute for Environmental Studies (NIES), Ministry of Environment, Japan. *H. circularisquama* OA1, OK1 and OK3 were morphologically identified by epifluorescent microscopy (BX-60; Olympus, Tokyo, Japan) and transmission electron microscopy (TEM) (JEM-1010; JEOL, Tokyo, Japan) according to the criteria of Horiguchi[4]. For the observation of body scales by TEM, cells were fixed with osmium vapor for 30 sec, rinsed three times with distilled water and then stained with 2% aqueous uranyl acetate for 15 min. Based on the body scales observed by TEM, *H. pygmaea* NIES472, *H. niei* NIES614 and *H. pygmaea* NIES473 have been assigned as *Heterocapsa* sp. 3, sp. 4 and sp. 5, respectively[5]. Cultures were maintained in modified SWM3 medium[7] (ESM medium[17] for *Heterocapsa* sp. 5 NIES472) at 20°C (5°C for *H. arctica* CCMP445) with a 12L:12D cycle at 50 $\mu E/m^2/s$ with cool white fluorescent bulbs.

### *Analysis of the toxicity of Heterocapsa species*

The toxicity of *Hetetocapsa* species was assessed with an algal exposure assay using juvenile pearl oysters, *Pinctada fucata*, as described previously[14]. Juvenile pearl oysters cultured for 1–2 months after hatching, were obtained from the Tazaki Marine Biological Research Center, Tazaki Pearl

Table 1. *Heterocapsa* species used in this study and their toxicity to juvenile pearl oyster.

| Species | Strains | Origin | Mortality (%)[a] |
|---|---|---|---|
| *H. circularisquama* | OA1 | Obama Bay, Fukui, Japan | 52.5±23.3 |
| | OK1 | Obama Bay, Fukui, Japan | 86.3±8.5 |
| | OK3 | Obama Bay, Fukui, Japan | 96.3±4.8 |
| *H. arctica* | CCMP445 | Baffin Bay, The Arctic | N.D. |
| *H. illdefina* | CCMP446 | The North Pacific, CA, USA | 47.5±9.6 |
| *H. pygmaea* | CCMP1322 | The Gulf of Mexico, TX, USA | 6.3±7.6 |
| | CCMP1490 | The Mediterranean Sea, Italy | 7.5±2.9 |
| *H. triquetra* | NIES7 | Osaka Bay, Osaka, Japan | 1.3±2.5 |
| | CCMP448 | Nantucket Sound, MA, USA | 3.8±2.9 |
| *H. lanceolata* | TK6-D57 | Tokyo Bay, Tokyo, Japan | 0 |
| *H. hoiguchii* | FK6-D47 | Fukuyama, Hiroshima, Japan | 0 |
| *Heterocapsa* sp. 3 | NIES472 | Kashiwazaki, Niigata, Japan | 3.8±2.9 |
| *Heterocapsa* sp. 4 | NIES614 | Iriomote, Okinawa, Japan | 0 |
| *Heterocapsa* sp. 5 | NIES473 | Izuhara, Nagasaki, Japan | 1.7±2.9 |

[a] Percentage of dead juvenile pearl oysters after 24h exposure to the cells of *Heterocapsa* ($10^5$ cells/ml)
N.D., not determined

Co. (Mie, Japan) and Miyuki Fisherman's Productive Association (Wakayama, Japan). They were maintained on *Cheatoceros dydimus* as food at 20°C in the dark. Prior to experiments, the juvenile pearl oysters were transferred to 6-well plates (Becton Deckinson, CA) at 20 individuals/well and starved in sterilized sea water for 24 h. Then, 10 mL of algal culture at 100,000 cells/mL cultivated in the late exponential phase was added to each well and incubated for 24 h at 20°C in the dark. After incubation, the dead juvenile pearl oysters were enumerated under a stereoscopic microscope. Death was judged from the absence of a heart beat[14]. *Heterosigma akashiwo* HaOB0001 isolated from Obama bay in 1998 was used as a negative control[14].

### *Analysis of ITS regions*

Cells in late–exponential phase (150 mL) were harvested by centrifugation at 300×*g* for 10 min and then, total DNA was extracted from the cell pellet as described previously[1]. ITS regions containing 5.8S rDNA were amplified by polymerase chain reaction (PCR) from total DNA with ITSA and ITSB primers complemental to the 3' end of the 16S-like rDNA and the 5'-end of the 28S rDNA[1]. PCR was performed for 30 cycles of 1 min at 94°C, 2 min at 55°C and 3 min at 72°C with a final elongation step of 7 min at 72°C using a DNA Thermal Cycler PJ2000 (Takara, Kyoto, Japan).

The 680 bp products whose sizes correspond to the ITS regions were obtained from all strains. PCR fragments were cloned into a pCR™ II vector according to the manufacturer's instructions (Invitrogen, USA). Two to 4 clones from each strain were selected and sequenced individually with a DNA sequencer 373A (Applied Biosystem Inc., USA) using a Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystem Inc.). The end position of 16S-like rDNA and the starting position of 28S rDNA were determined by comparing with published rDNA sequences from other dinoflagellates[1–3]. The sequences of ITS regions were aligned using the multiple sequence alignment program CLUSTAL W version 1.7[23] and then converted to a distance matrix based on the Jukes-Cantor method[8]. Gap positions were excluded. Phylogenetic trees were constructed from the distance matrix using the neighbor-joining algorithm[19] of CLUSTAL W version 1.7.

The sequence data on ITS regions determined in this study have been deposited in the DDBJ/EMBL/GenBank DNA data bases under accession numbers AB084089 to AB084101. The sequence data on *H. circularisquama* HA92-1 was derived from DDBJ/EMBL/GenBank DNA data bases (accession number; AB049711).

## Results and Discussion

### *Toxicity of Hetrocapsa species*

Nagai *et al.* reported that the mortality of juvenile pearl oysters increased depending on the cell density of *H. circularisquama* up to 50,000 cells/mL and the $LD_{50}$ was approximately 20,000 cells/mL in a 24 h exposure experiment[14]. Considering these findings, we examined the toxicity of

*Heterocapsa* species at an initial density of 100,000 cells/mL. At this cell density, 86.3% and 96.3% of juvenile pearl oysters were dead after 24 h exposure to *H. circularisquama* OK1 and OK3, respectively (Table 1). When *H. circularisquama* OA1 was tested, the mortality was found to be 52.5% which is at least five-fold less than that at the $LD_{50}$ of *H. circularisquama* described previously[14]. At 10,000 cells/mL, exposure to *H. circularisquama* OK3 resulted in approximately 50% mortality, while that to *H. circularisquama* OA1 and OK1 caused 20% mortality (data not shown). These results suggest that toxicity differs among strains. Forty seven percent of juveniles died in an exposure experiment using *H. illdefina* at a cell density of 100,000 cells/mL (Table 1). At 10,000 cells/mL, *H. illdefina* caused 2% mortality among juveniles. When *H. lanceolata*, *H. horiguchii* and *Heterocapsa* sp. 4 were subjected to this assay, the juveniles did not die. *H. pygmaea*, *H. triquetra*, and *Heterocapsa* sp. 3 and sp. 5 showed low levels of mortality among juveniles (Table 1). However, surviving juveniles actively moved their gills similar to the juveniles which were exposed to *H. akashiwo* for 24 h as a negative control and did not die at a cell density of 100,000 cells/mL (data not shown). Matsuyama *et al.* reported that *H. triquetra* had no significant effect on bivalves[13]. Recently, hemolytic activity, which is possibly the factor responsible for mortality in bivalves, has been found in *H. circularisquama*, but *H. triquetra* has been reported to show no hemolytic activity at 50,000 cells/mL[16]. Considering these observations, the toxicity of these species including *H. lanceolata* and *H. horiguchii*, which sometimes occur at the same time as *H. circularisquama*[6], was very low compared to that of *H. circularisquama*. *H. illdefina* has not been found in Japan and no red tides or mass mortality of bivalves caused by this species has been reported here to date, however, our data show that *H. illdefina* as well as *H. circularisquama* should be taken into account for early predictions of the mass mortality of bivalves.

### *ITS analysis*

The sequence analysis showed that the nucleotide lengths of 5.8S rDNA, ITS1 and ITS2 in *Hetrocapsa* species were 161, 229 to 234 and 197 to 205 bp, respectively (Table 2). The length of 5.8S rDNA in *Hetrocapsa* species was conserved and almost identical to that of other dinoflagellates, *Alexandrium catenella* (160 bp)[2], *A. tamarense* (160 bp)[2], *Gymnodinium catenatum* (160 bp)[2] and *Prorocentrum micans* (159 bp)[3], whereas the lengths of ITS1 and ITS 2 varied among dinoflagellates (Table 2). In *A. catenella, A. tamarense* and *G. catenatum*, the length of ITS 2 (192, 197 and 223 bp, respectively) was greater than that of ITS 1 (166, 165 and 192 bp, respectively), whereas in *Heterocapsa* species as well as *Prorocentrum micans*, ITS1 was larger than ITS2. Notably, the ITS1 of *Heterocapsa* species at 229 to 233 bp in length was much larger than that of other dinoflagellates[1–3]. The length of the ITS region containing the 5.8S rDNA region in *H. circularisquama* (596 bp) was homogenous and distinguishable from that in other *Heterocapsa* species. The length of the ITS region from five species of the genus *Alexandrium* was reported to be highly conserved at the intra-species level and variable at the inter-species level. These results suggest that the length of ITS regions is a species-specific character of dinoflagellates[1].

Table 2. Nucleotide lengths of the 5.8S rDNA and ITS regions from *Heterocapsa* species and other dinoflagellates.

| Species | ITS1 | 5.8S rDNA | ITS2 | Total length (bp) |
|---|---|---|---|---|
| *H. circularisquama* | 230 | 161 | 205 | 596 |
| *H. arctica* | 233 | 161 | 203 | 597 |
| *H. illdefina* | 230 | 161 | 203 | 594 |
| *H. pygmaea* | 229 | 161 | 202 | 592 |
| *H. triquetra* | 233 | 161 | 201 | 595 |
| *H. lanceolata* | 230 | 161 | 197 | 588 |
| *H. horiguchii* | 230 | 161 | 203 | 594 |
| *Heterocapsa* sp. 3 | 234 | 161 | 203 | 598 |
| *Heterocapsa* sp. 4 | 230 | 161 | 203 | 594 |
| *Heterocapsa* sp. 5 | 230 | 161 | 197 | 588 |
| *Alexandrium catenella*[1] | 166 | 160 | 192 | 518 |
| *A. tamarense*[1] | 165 | 160 | 197 | 522 |
| *Gymnodinium catenatum*[2] | 192 | 160 | 223 | 575 |
| *Prorocentrum micans*[3] | 212 | 159 | 195 | 566 |

The alignment of the sequences demonstrated that 5.8S rDNA sequences were highly conserved within *Heterocapsa* species and the number of base substitutions between *H. circularisquama* and other *Heterocapsa* species ranged from 0 to 6. Notably, 5.8S rDNA sequences of *H. illdefina*, *H. horiguchii, Heterocapsa* sp. 3, *Heterocapsa* sp. 4 and *Heterocapsa* sp. 5 were identical. In contrast, sequences of both the ITS1 and ITS2 regions were highly divergent between *Heterocapsa* species. ITS regions in *H. circularisquama* OA1, OK1 and OK3 were identical and only three base substitutions were observed between *H. circularisquama* HA92-1 and the three other strains of *H. circularisquama*. The number of different nucleotides in ITS1 and ITS2 between *H. circularisquama* and other *Heterocapsa* species ranged from 43 to 90 and 59 to 73, respectively. From these results, a phylogenetic tree of *Hetrocapsa* species was con-

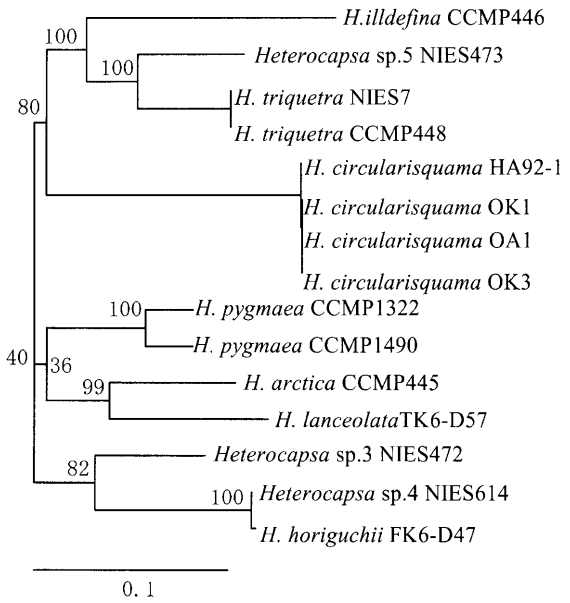


Fig. 1. A molecular phylogenetic tree inferred from nucleotide sequences of 5.8S rDNA, ITS1 and ITS2 in *Heterocapsa* species using the neighbor-joining method. The numbers indicate the percentage of 1000 bootstrap resamplings.

structed using the distance values of the ITS region including 5.8S rDNA (Fig. 1). Except for *H. horiguchii* and *Heterocapsa* sp. 4, the ITS types of *Heterocapsa* species were divergent corresponding to morphological species. The distance value between *H. circularisquama* HA92-1 and the other three strains of *H. circularisquama* was very low (0.002) while that between *H. circularisquama* and other species ranged from 0.165 to 0.254. The distance value was largest between *H. circularisquama* HA92-1 and *H. illdefina* CCMP446. Four strains of *H. circularisquama* were divergent from *H. illdefina* (Fig. 1). *H. lanceolata* and *H. arctica*, the epitheca of which is almost twice the length of the hypotheca[6], were closely related to each other phylogenetically (Fig. 1). The body scales of *H. lanceolata* have a hexagonal basal plate with a central hole, while those of *H. arctica* comprise a triangular basal plate without a hole[6]. *Heterocapsa* sp. 3 and *Heterocapsa* sp. 5 were almost indistinguishable at the light microscopic level[5], but ITS analysis showed that these species were distantly related and *Hetocapsa* sp. 5 was closely related to *H. triquetra* (Fig. 1). The cells of *Hetocapsa* sp. 5 and *H. triquetra* are hemispherical and rhombic, respectively, whereas, in their body scale structure, these two species are almost identical[5]. Only one base substitution was observed between *H. horiguchii* and *Heterocapsa* sp. 4 and the distance value between these species was very low (0.002). *H. horiguchii* and *Heterocapsa* sp. 4 were almost identical in not only cell shape and size but also the structure of the body scales, and were distinguishable only by the basal plate texture of the body scale[5]. The structure of body scales is still an important taxonomic molecular marker for *Heterocapsa* species, however, ITS analysis indicates that the difference in body scale structure does not always correspond to genetic diversity, suggesting that sequence data as well as morphological data are required for the identification of *Heterocapsa* species. Adachi *et al.* reported a low distance value (less than 0.01) for sexually compatible isolates of *Alexandrium catenella* or *A. tamarense*, suggesting that sequence analysis of the ITS region is interpreted as supporting the biological species concept[1]. Although sexual reproduction is not known in *H. circularisquama*, these observations indicate that the ITS region is a useful marker for the identification of *H. circularisquama* and suggest that *Heterocapsa* sp. 3 and sp. 5 are independent species and *H. horiguchii* and *Heterocapsa* sp. 4 should be combined.

The phylogenetic relationships between *H. circularisuama* and 6 species, *H. pygmaea, H. arctica, H. lanceolata, H. horiuchii, Heterocapsa* sp. 3 and *Heterocapsa* sp. 4, were not conclusive because of a low efficiency of reconstruction using a bootstrap analysis (Fig. 1). To decide on a more conclusive phylogenetic relationship between *Heterocapsa* species, more sequence data such as 18S rDNA[3,20,21] or 23S rDNA[20,22] should be analyzed.

### *H. circularisquama-specific PCR primer*

We found a specific signature sequence for *H. circularis-*

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ITSCir primer          3'-CACACGAGACCGTGGAAACT-5'

                          413                 435
H. circularisquama OK1    GTGTG-CTCTG-GCACCTTTGA
H. circularisquama OK3    ····················
H. circularisquama OA1    ····················
H. triquetra NIES7        ·C···-GGT·TCC····CC···
H. triquetra CCMP448      ·C···-GGT·TCC····CC···
H. pygmaea CCMP1322       ·C·CAA··TGT--TG··CC···
H. pygmaea CCMP1490       ·C·CA-G--T·TCTG··CC···
H. arctica CCMP445        ··GCA--C···T·TG··CC···
H. illdefina CCMP446      ·C···TT·T·TAA·····CC··
H. lanceolata TK6-D57     ·····-··T·····-··C····
H. horiguchii FK6-D47     ·C·C···AACTG·TG··CC···
Heterocapsa sp.3 NIES472  ·C·····AACTC·TG··CC···
Heterocapsa sp.4 NIES614  ·C·C···AACTG·TG··CC···
Heterocapsa sp.5 NIES473  ·····-··G·TC·····CC···
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Fig. 2. Signature sequences of *Heterocapsa* species in ITS 2. Numerals represent positions relative to the initial nucleotide of ITS1. A PCR primer ITSCir (5'-TCAAAGGTGCCAGAGCACAC-3') was designed according to the signature sequence of *H. circularisquama*

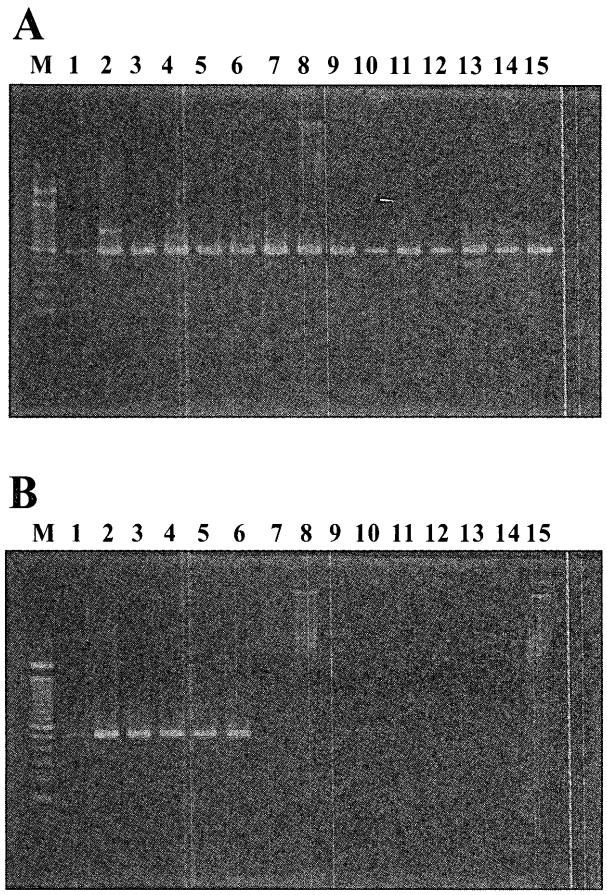


Fig. 3. Ethidium bromide-stained agarose gel showing PCR products of ITS regions from cells of *Heterocapsa* species. The amplification was performed with ITSF-ITSR (A) and ITSF-ITSCir (B). 1, *H. circularisquama* OA1; 2, *H. circularisquama* OK1; 3 and 4, isolates of *H. circularisquama* from Gokasho bay, Mie, Japan; 5 and 6, isolates of *H. circularisquama* from Shido bay, Kagawa, Japan; 7, *H. triquetra* NIES7; 8, *H. pygmaea* CCMP 1490; 9, *H. arctica* CCMP445; 10, *H. illdefina* CCMP446; 11, *H. lanceolata* TK6-D57; 12, *H. horiguchii* FK6-D47; 13, *Heterocapsa* sp. 3 NIES472; 14, *Heterocapsa* sp. 4 NIES614; 15; *Heterocapsa* sp. 5 NIES473; 16, no template; M, 100 bp ladder marker

*quama* in the ITS 2 region and designed a PCR primer, ITSCir(5'-TCAAAGGTGCCAGAGCACAC-3'), for it (Fig. 2). *Heterocapsa* species were subjected to PCR amplification with a ITSA-ITSCir primer set. A specific band was detected from all strains of *H. circularisquama* but not other *Heterocapsa* species (Fig. 3). Four strains of *H. circularisquama* were isolated from Gokasho bay, Mie, Japan on June 9, 2001 (designated HCGK0106 and HCGK0107) and from Shido bay, Kagawa, Japan on September 13, 2001 (designated HCGK0106 and HCGK0107), and were subjected to PCR with the *H. circularisquama*-specific primer set. A specific band was detected (Fig. 3) from these isolates, indicating that the ITSA-ITSCir primer set was a useful tool for selective detection of *H. circularisquama*. Recently, a PCR immunoassay targeting ITS regions was introduced for detection and semiquantification of toxic *Alexandrium* sp[18]. However, there are some problems with the PCR procedures used in the detection and quantification of a target in natural environments, such as an incomplete correlation of the PCR product with the initial amount of target gene[9], and low efficiency of extraction of DNA from phytoplanktonic cells due to contamination by polysaccharides[24]. To solve these problems and quantify the cells of *H. circularisquama* in natural environments, we are now trying to improve the DNA extraction method and develop a quantitative competitive PCR method[9] using the ITSA-ITSCir primer set.

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