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Molecular Discrimination of Dinoflagellates *Cochlodinium Polykrikoides* Margalef, *Gyrodinium Impudicum* Fraga et Bravo and *Gymnodinium Catenatum* Graham using RAPD-PCR Method

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Abstract

Randomly amplified polymorphic DNA (RAPD) analysis was used to study genetic relationships among *C. polykrikoides*, *G. impudicum* and *G. catenatum*, which possess similar morphological features. Four of 12 primers were selected and 59 amplification products ranged from 0.2 kb to 3.0 kb. The number of polymorphic products in *C. polykrikoides*, *G. impudicum* and *G. catenatum* was 16 (27.1%), 8 (13.5%), and 16 (27.1%), respectively, while 17 were monomorphic. Number of species-specific bounds was 26 (44.0%), 34 (57.6%), 26 (44.0%) in *C. polykrikoides*, *G. impudicum* and *G. catenatum*, respectively. The genetic similarity between *C. polykrikoides* and *G. impudicum*/*G. catenatum* was 0.83, whereas *G. impudicum* and *G. catenatum* was 0.78. Our results suggest that *C. polykrikoides*, *G. impudicum* and *G. catenatum* are extremely different on the basis of RAPD analysis, despite similarity based on their morphology. The RAPD technique appears to be efficient in detecting genetic variation in these dinoflagellates.

Key words – *Cochlodinium polykrikoides*, DNA polymorphism, *Gymnodinium catenatum*, *Gyrodinium impudicum*, PCR, RAPD

Introduction

The chain-forming dinoflagellates *Cochlodinium polykrikoides* Margalef, *Gyrodinium impudicum* Fraga et Bravo and *Gymnodinium catenatum* Graham are difficult to discriminate under the light microscope fixed Lugols solution because of their similar morphology[6,7]. *Cochlodinium polykrikoides* occurred for the first time in 1982 in Korean waters and blooms have presented continuously since after then, linked with massive mortality of cultured fish and the species is regarded as a fish killer[15]. While *G. impudicum* was known to be non-toxic in Spain [12], there was no report on *G. impducum* red tide in Korean coastal waters and damage to fish or shellfish [15]. *Gymnodinium catenatum* produces toxins, such as gonyautoxins and saxitoxin, and this species is responsible for outbreaks of paralytic shellfish poisoning (PSP) in Korean coastal waters[15]. When the three species concur in summer, differentiation of toxic and non-toxic phytoplankton is essential for biotoxin risk assessment. In an attempt to resolve these problems, we previously applied fluorescent lectin probes to differentiate them and proposed the utility of the probes as a tool for the discrimination both in culture and in nature[5,6].

However, randomly amplified polymorphic DNA (RAPD) analysis is known to provide a rapid way to detect DNA polymorphism among individuals or populations in a species[21,22]. Also, this method is less expensive than the sequence-based methods[10]. Recently,

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RAPD has been applied to know the intraspecific and interspecific variations and genetic relationships in *G. catenatum*[1,4]. Although fungi, green algae and red algae were subjected to RAPD analysis previously[2,3,18,20], a little was investigated to identify harmful dinoflagellates using RAPD. In this study, we performed RAPD analysis to understand the intraspecific and interspecific variations and genetic relationship among strains of *C. polykrikoides*, *G. impudicum* and *G. catenatum* collected from Korea and from Japan and Spain.

Materials and Methods

Cultures

Isolates of *C. polykrikoides* CP-1, CP-2, CP-3, CP-4 was obtained from the Korean coasts of Tongyong, Gohung, Kunsan, the Japanese coast of Harimanada, respectively. Isolates of *G. impudicum* GI-1, GI-2, GI-3, GI-4 were collected from the coast of Tongyong. *Gymnodinium catenatum* GC-1 (Spanish strain collected from Vigo) was provided by Dr. Donald Anderson, Woods Hole Institution, USA, and the clones GC-2, GC-3, GC-4 were isolated from Chinhae Bay, Korea. All isolates were grown in f/2-Si medium[13] containing an anitbiotic mixture[14] at 20℃ under a photon flux from cool white fluorescent tubes of 50 mol m-2 s-1 in a 14: 10 h L:D cycle.

Isolation of DNA

Cultures were harvested during the exponential phase by centrifugation. They were immediately kept at -20℃ until molecular analysis. Cells were thawed in 500 L of extraction buffer (100 mM Tris-HCl, pH 8.0; 40 mM EDTA) and 150 L of 10% sodium dodecyl sulphate (SDS), and incubated at 55℃ for 30 min. The supernatant was extracted twice with phenol:chloroform: isoamylalchol (25:24:1, v/v/v) prior to ethanol precipitation.

RAPD analysis

Prior to polymerase chain reaction (PCR), DNA concentrations were adjusted to 50 ng L-1. PCR reactions were performed in a final volume of a 20 L mixture: 1.25 unit *Taq* DNA polymerase (Ex *Taq* TM, Bioneer Co.); 10 Ex *Taq*TM buffer (Bioneer Co.); 2.5 mM dNTPS (Bioneer Co.); 50 ng template DNA; and 100 pmol of RAPD primer. Amplifications were performed with the Perkin-Elmer thermocycler 2400. The thermocycling profile included an initial denaturation step of 94℃ for 4 min, followed by 35 cycles of 1 min at 94℃, primer annealing for 1 min at 55℃, and extenstion for 2 min at 72℃. Amplification products (20 L) were separated for 40 min at 100V on 1.8% agarose TBE gels. These were stained with 10 L ethidium bromide added to the gel and 0.25 L ethidium bromide mL-1 TBE buffer to the TBE running buffer. Gels were illuminated with UV light and photographed with Polaroid 667 positive/negative instant film. Twelve random primers (Seoulin UniPrimerTM Kit I) were screened (Seoulin Scientific Co., Ltd., http://www.seoulin.co.kr), but URP-1, URP-2, URP-3 and URP-9 were eventually selected for analysis. RAPD bands were scored as present/absent (p/a = 1/0) and only well-resolved bands were considered.

Data analysis

Individual data set from each primer were combined to make data matrix, and phylogenetic relationship was constructed using NTSYS-PC program version 2.02[19].

Results

Amplified PCR products using URP-1, URP-2, URP-3 and URP-9 in this study showed various polymorphic fragments, ranging in size from 0.2 kb to 3.0 kb (Fig. 1). A total of 59 DNA fragments was obtained from the isolates of the three species using the four primers (Table 1). The number of amplified products by four primers ranged from 13-17, with average 14.7 bands per

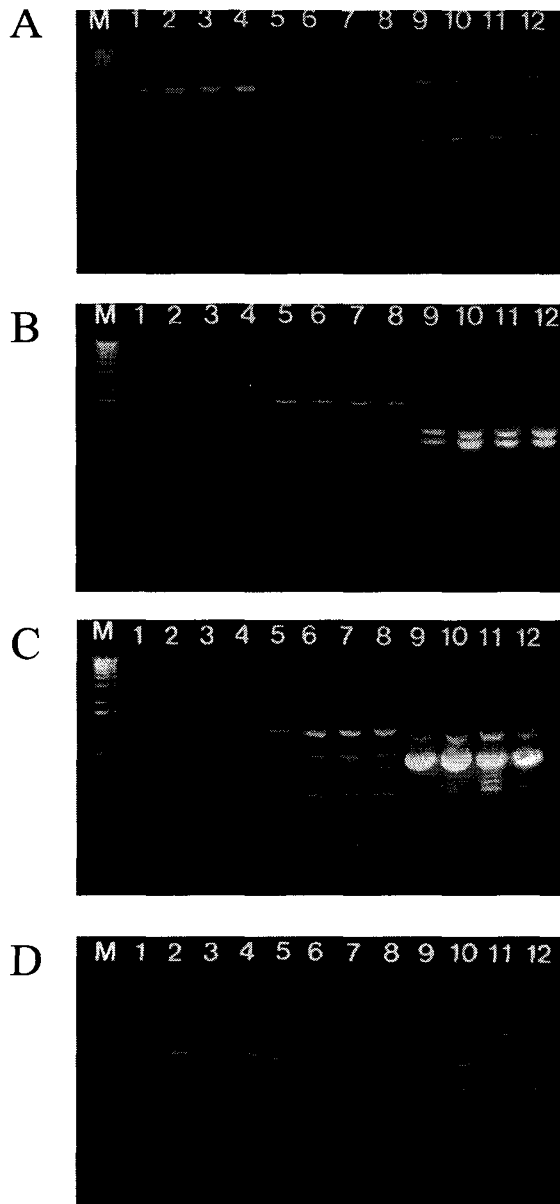

Fig. 1. Agarose gel electrophoresis pattern of randomly amplified dinoflagellates template DNA.
The template DNA was amplified using the primer URP-1 (A), URP-2 (B), URP-3 (C) and URP-9 (D) and separated on a 1.8% agarose gel. M, 1 kb Plus DNA Ladder marker; 1-4, three isolates of *Cochlodnium polykrikoides* (CP-1, CP-2, CP-3) were collected from the Korean coast of Tongyong, Gohung, Kunsan, respectively, the rest of one isolate (CP-4) was provided from Japan (the coast of Harimanada); 5-8, four isolates of *Gyrodinium impudicum* (GI-1, GI-2, GI-3, GI-4) were collected from the coast of Tongyong; 9-12, three isolates of *Gymnodinium catenatum* (GC-2, GC-3, GC-4) were collected from Chinhae Bay, Korea and GC-1 was Spanish strain isolated from Vigo.

primer. Among 59 bands, the number of polymorphic products in *C. polykrikoides, G. impudicum, G. catenatum* was 16 (27.1%), 8 (13.5%), 16 (27.1%), respectively, while 17 DNA fragments (28.8%) were common. Species-specific bands of *C. polykriioides, G. impudicum, G. catenatum* amounted to 26 (44.0%), 34 (57.6%), 26 (44.0%), respectively. In *C. polykriioides*, polymorphism varied from 14.2% to 35.2% depending on primers, from 0~21.4% in *G. impudicum* and from 17.6 42.8% in *G. catenatum*. Profiles of PCR products obtained from three dinoflagellates using the four primers were shown in Fig. 1. Repeated amplification under the same PCR conditions resulted in stable banding pattern. Amplification patterns of *C. polykriioides* (lane 1-4), *G. impudicum* (lane 5-8) and *G. catenatum* (lane 9-12) were very different depending on primers. However, similar banding patterns were observed in the isolates of each *C. polykrikioides, G. impudicum* and *G. catenatum*. PCR products in *G. catenatum* between 300 bp and 400 bp were observed in a specific bands. The phylogenetic tree based on genetic distance calculated from RAPD data was shown in Fig. 2. *Cochlodinium polykrikoides, G. impudicum* and *G. catenatum* had significantly lower degrees of similarity coefficients of 0.83, with between *C. polykrikoides* and *G. impudicum/G. catenatum*. However, the relationship between *G. impudicum* and *G. catenatum* showed 0.78 of similarity distance, indicating that two species was closer than that of *C. polykriioides*.

Discussion

Adachi *et al.*[1] and Bolch *et al.*[4] reported that RAPD-PCR method was useful to discriminate isolates of each *G. catenatum*, while our results showed very low resolution among isolates of *C. polykrikoides, G. impudicum* and *G. catenatum*, respectively. However, the similarity coefficient among species was relatively low (0.78~0.83; 0~1 scale), confirming that *C. polykrikoides*,

Table 1. Random primers and the number of observed bands in four isolates of *Coclodinium polykrikoides* (CP-1, CP-2, CP-3, CP-4), *Gyrodinium impudicum* (GI-1, GI-2, GI-3, GI-4) and *Gymnodinium catenatum* (GC-1, GC-2, GC-3, GC-4)

Primer	No. of PCR products	No. of polymorphic products (%)			No. of monomorphic products			No. of specific products (%)		
		CP	GI	GC	CP	GI	GC	CP	GI	GC
URP-1	14	2 (14.2)	3 (21.4)	6 (42.8)	4	4	4	8 (57.1)	7 (50.0)	4 (28.5)
URP-2	17	6 (35.2)	3 (17.6)	3 (17.6)	5	5	5	6 (35.2)	9 (52.9)	9 (52.9)
URP-3	15	5 (33.3)	2 (13.3)	3 (20.0)	5	5	5	5 (33.3)	8 (53.3)	7 (46.6)
URP-9	13	3 (23.0)	0 (0)	4 (30.7)	3	3	3	7 (53.8)	10 (76.9)	6 (46.1)
Total	59	16 (27.1)	8 (13.5)	16 (27.1)	17	17	17	26 (44.0)	34 (57.6)	26 (44.0)

Note: Band frequencies among the isolates in *C. polykrikoides, G. impudicum* and *G. catenatum* were calculated identically.

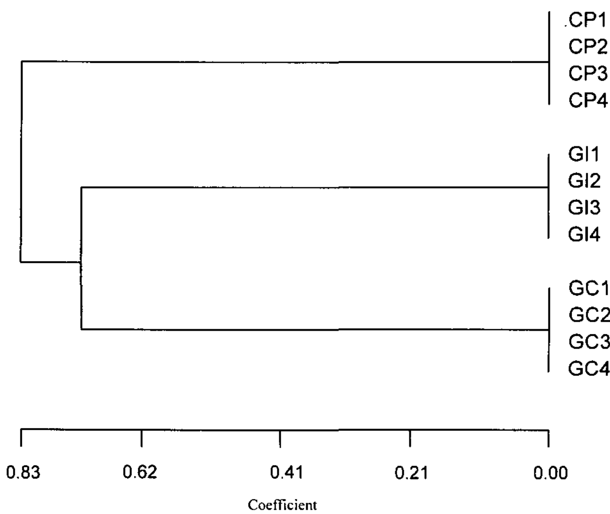

Fig. 2. Dendrogram consisting of dinoflagellates based on the NTSYS program version 2.02. Twelve isolates were shown in Fig. 1.

G. impudicum and *G. catenatum* are genetically different dinoflagellates. A previous study of sequence analysis of several isolates of *C. polykrikoides* from the East Sea, the South Sea and the West Sea, revealed the same nucleotide sites in the internal transcribed spacer (ITS) regions[9]. Our present data also show the same genetic similarity on the basis of RAPD patterns among three isolates CP-1, CP-2 and CP-3 from Tongyong, Gohung, Kunsan, respectively, and genetic identity even between Korean isolates and Japanese isolate collected from Harimanda. Park and Park[17] showed that the analyses of large sub-unit (LSU) rDNA sequences of *C. polykrikoides* and *G. impudicum* showed only 74.9% similarity and also sequence analyses of ITS had quite different nuceotide alignments with 55% similarity[7]. RAPD analysis in our present study was observed in very different DNA fragments between *C. polykrikoides* and *G. impudicum*, indicating an extensive divergence of two species. Costas *et al.*[11] and Zardoya *et al.*[23] suggested that sequence analysis targeted LSU rDNA of *G. impudicum* and *G. catenatum* showed a high genetic divergence and therefore a different phylogenetic relationship. As can be seen in Fig. 2, the similarity coefficient of *G. impudicum* and *G. catenatum* based on RAPD bands had significantly lower. Since the number of specific products of three dinoflagellates also persisted approximately half bands in a total of PCR fragments (Table 1), they were significantly different species at even the genomic DNA level. Our current result agrees well with Costas *et al.*[11], Zardoya *et al.*[23], Park and Park[17] and Cho *et al.*[7] suggestions. An establishment of RAPD-PCR is expected to be useful for the genetic characterization of morphologically closely related dinoflagellates. Meanwhile, Kim *et al.*[15] and Cho *et al.*[8] suggested that *G. impudicum* was more closely related to *G. catenatum* rather than *C. polykrikoides* by sequence analysis of ITS regions. Likewise, phylogenetic relationship based on RAPD markers also showed that the

placement of *G. impudicum* was closer to *G. catenatum* than *C. polykrikoides*. Thus, *C. polykrikoides*, *G. impudicum* and *G. catenatum* are clearly different species and *G. impudicum* may belong to the genus of *Gymnodinium* on the basis of phylogenetic analysis and genetic comparison (sequence analysis of ITS region, RAPD patterns).

Adachi *et al.*[1] suggested that Japanese isolate of *G. catenatum* might belong to the same population of European strains. However, Bolch *et al.*[4] have reported that Australian isolates of *G. catenatum* observed higher level of RAPD diversity within even the same regions. However, the three Korean isolates of *G. catenatum* from the same sampling site found almost a identical RAPD patterns (Fig. 1), suggesting that the divergence of Korean strains of *G. catenatum* is very low, compared to Australian *G. catenatum*. Following Adachi *et al.*[1] suggestion, possibly Korean *G. catenatum* is attributed to the same population of Japanese and European strains because of the same DNA polymorphisms with Korean and Spanish *G. catenatum* (Fig. 1). Furthermore, in the case of *G. impudicum*, isolated from the same seawater sample, examined the same RAPD patterns, also *C. polykrikoides* collected from different regions observed the same RAPD patterns (Fig. 1). Thus, Korean isolates of *C. polykrikoides*, *G. impudicum* and *G. catenatum* are associated with lower genetic divergence within the population of Korean waters. Bolch *et al.*[4] suggested that the reason why there was a higher divergence of *G. catenatum* was because the mating system was a complex, multigroup and outbreeding system. It is known that *G. catenatum* is expected to be easily the induction of sexual reproduction by unfavorable environmental conditions and is possible to be a high substantial genetic differentiation among the waters. In contrast sexual reproduction way provide, the possibilities of genetic divergence in *C. polykrikoides* and *G. impudicum* are lower than that of *G. catenatum* due to the limitation of their sexual recombination within the population. From the previous studies[1,3], a spatially homogenizing effect of gene flow within the population of *G. catenatum* would be minimized compared to *C. polykrikoides* and *G. impudicum*. As more data on geographically separated regions in *C. polykrikoides* and *G. impudicum* become available, a more decisive conclusion about the genetic aspect might be possible.

In conclusion, although it is difficult to discriminate *C. polykrikoides* from *G. impudicum* and *G. catenatum* fixed with Lugol soultion because of similar features under the light microscope, similarity coefficient based on RAPD-PCR patterns has significantly lower degree. That confirms that *C. polykrikoides*, *G. impudicum* and *G. catenatum* are extremely different species with genomic DNA levels. However, Korean isolates of *C. polykrikoides*, *G. impudicum* and *G. catenatum* may occupy homogenous populations and any of genetic differentiation against all localities in waters, with even heterogeneous environments. Possibly, dinoflagellates habituded at Korean, Japanese and European waters are limited to genetic divergence within populations, although they are geographically separated.

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초록 : RAPD-PCR 방법을 이용한 *Cochlodinium polykrikoides*, *Gyrodinium impudicum*, *Gymnodinium catenatum*의 분자생물학적 진단

조은섭
(남해수산연구소)

형태적으로 매우 유사한 적조생물 *C. polykrikoides*, *G. impudicum*, *G. catenatum*을 RAPD 방법을 이용하여 유전적 유연관계를 조사했다. 12개의 primer 중 4종류만 선택되었고 증폭된 밴드수는 59개이며 그 크기는 0.2에서 3.0 kb까지였다. *C. polykrikoides*, *G. impudicum*, *G. catenatum*의 다형화된 밴드수는 16개, 8개, 16개로 각각 나타났다. 반면에, 17개의 밴드만 동일하였다. *C. polykrikoides*, *G. impudicum*, *G. catenatum*의 종 특이적인 밴드수는 26개, 34개, 26개로 각각 보였다. *C. polykrikoides*와 *G. impudicum*/*G. catenatum*의 유전적 유사성은 0.83이며, *G. impudicum*과 *G. catenatum*은 0.78로 나타났다. 이러한 결과로 볼 때 형태적으로는 유사하게 보이지만, RAPD 분석에 의하면 *C. polykrikoides*, *G. impudicum*, *G. catenatum*은 현저하게 상이한 적조생물이다. 앞으로 RAPD 기법을 이용하면 이러한 와편모조류의 유전적 변이를 탐색하는데 유용할 것으로 보인다.