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Detecting DNA of multispecies dinoflagellate cysts in the sediment from three estuaries of Makassar strait and fishing port using CO1 primer: Is it CO1 primer suitable for detecting DNA dinoflagellate?

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Abstract:

Most dinoflagellate had a resting cyst in their life cycle. This cyst was developed in unfavorable environmental condition. The conventional method for identifying dinoflagellate cyst in natural sediment requires morphological observation, isolating, germinating and cultivating the cysts. PCR is a highly sensitive method for detecting dinoflagellate cyst in the sediment. The aim of this study is to examine whether CO1 primer could detect DNA of multispecies dinoflagellate cysts in the sediment from our sampling sites. Dinoflagellate cyst DNA was extracted from 16 sediment samples. PCR method using COI primer was running. The sequencing of dinoflagellate cyst DNA was using BLAST. Results showed that there were two clades of dinoflagellate cysts from four locations of study. Clade 1 was dominated by samples from the Jeneberang Estuary (JB), Maros Estuary (M) and Pangkep Estuary(P), while clade 2 was dominated by samples from the Paotere Port (PP). The genetic distance varied between DNA dinoflagellate cyst samples ranging from 0.5 -0.6. The closest genetic distance was between sample of JB1 and sample of JB2, while the farthest genetic distance was sample PP1 and PP2. The primer CO1 was not suitable for dinoflagellate cyst DNA due to only picking one DNA, which was a diatom (*Licmophora* sp).

Keywords: CO1 primer, DNA dinoflagellate cyst, Makassar Strait, Maros-Pangkep and Jeneberang Estuary, PCR method.

Introduction:

Most of the marine dinoflagellate produces cysts which are approximately 200 species of the 2000 marine dinoflagellate [1]. The cyst could create a seed bank for future bloom, consequently, cysts play an important contribution to harmful algal bloom dynamics [2–4]. Dinoflagellate resting cyst could leave in unfavorable environmental condition due to having to resist wall cell, this morphological cell as well maske resting cyst distribute wide geographically [5–7]. Dinoflagellate cysts studied conventionally use a method that required morphological observations and isolating, germinating, or cultivating the cysts [1]. There is a disadvantage in identifying, surveying cysts in the field, which is time-consumption and requiring high skill in identification for low abundance or unknown cyst [1]. The other conventional method for dinoflagellate cysts study was using the most probable number (MPN), however, this method was not appropriate for large-scale sampling due to the time-consuming and required high labor for processing of pre-treatment and observation [8].

The dinoflagellates DNA mass per cell is two to three magnitude greater than other planktonic algae, it also shares the number of unique nuclear characteristics and belongs to the largest genomes known [6]. Dinoflagellates are also very unique due to their nuclear genome structure and regulation [9]. The dinoflagellate genome has several characteristics, such as the genom which e is the largest compared to other eukaryotes groups up to 250 pg , the dinoflagellate genome has a high number of repeated and redundancy DNA[10]. There are several factors

that could cause DNA content changes and variation within a dinoflagellate cell, namely mitosis process (vegetative cell division, zygosis/meiosis process (sexual recombination), and infection by parasites [6]. The most recognized changes of DNA content due to mitosis and sex, those processes result in the large changes of DNA mass, it is quantized 2-fold with each replication, division, or fusion [6].

The molecular approach has been applied for studying the phytoplankton in the water column, benthic substrates, and also in aerosol by qPCR (quantitative polymerase chain reaction) which have high accuracy, rapid quantification, fast and easy to perform also require limited specialized equipment [6,11–14]. The most important and critical thing for using the PCR technique is the ability of the primer to amplify the specific DNA template[15].This method could estimate accurately a targeted DNA from species-rich natural sample with a high sensitivity and specificity[7,16]. However, there was a lack of studies used qPCR to identify and estimate dinoflagellate resting cysts[1,8] due to several reasons namely: low DNA extraction yield, dinoflagellate cyst has an extracellular DNA in bottom sediment, consequently could get overestimation of targeted cyst, [8] and estimation error could be produced due to a different number of rDNA copy between vegetative and resting stages [8]

In eukaryotes, critical housekeeping genes was found invariability with a large number and most abundance of tandem copy [10.] Figueroa et al [10] further explained that rDNA regions consist of two parts which are nucleolar organizer regions that contain the gene of 18S, 5.8S, and 28S rRNAs (transcribed as the 45S ribosomal precursor) and outside the NOR that have a gene of 5S rRNA. Each nucleolar organizing region contains a cluster of tandemly repeated rRNA genes that are separated from each other by non-transcribed spacer DNA. To detect and describe a new species as well support a taxonomy of dinoflagellate, ribosomal gene sequences and internal transcribed spacers (ITS) could be a useful tools[17,18.]

Several previous studies regarding dinoflagellates cysts DNA have been explored and more focus on using single species of dinoflagellate cysts DNA. However, the detection of multispecies of dinoflagellate cysts DNA has been poorly studied in natural surface sediment samples. In this paper, we focused on detecting multispecies of dinoflagellate cyst DNA using CO1 primer. The aim of this study to detect multispecies dinoflagellate cyst from the sediment using CO1 primer.

Materials and Methods:

Research sites

Sediment samples were collected from three estuaries of Makassar Strait (Jeneberang, Maros and Pangkep Estuary) and one fishing port (Paotere Port, Makassar, South Sulawesi Indonesia) (Fig.1). Those areas were selected due to several reasons, such as there were a big river (Jeneberang, Maros and Pangkep River) with a high industrial activity along the river and those estuarine have a high anthropogenic and fishing activity with a high level of organic pollution, consequently those areas have a high potential for Harmful Algal Blooms (HABs) occurrence.

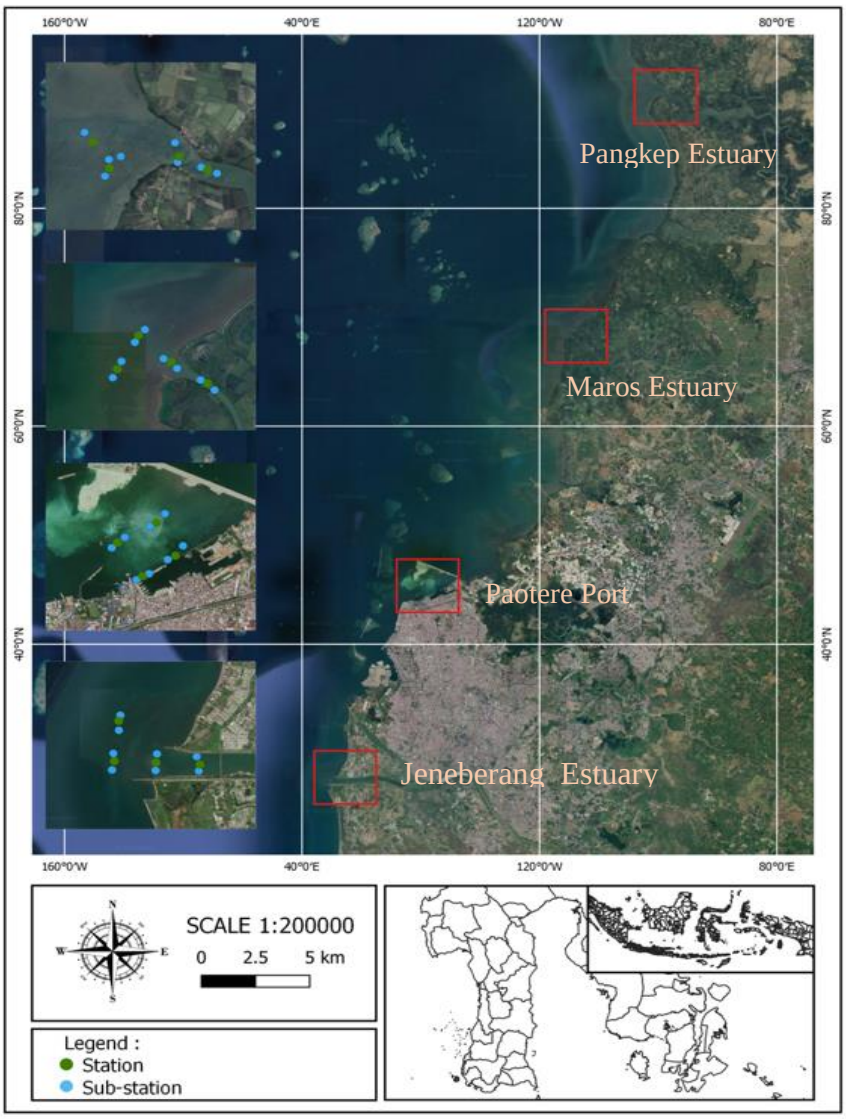

Figure 1. Study site map (this map was adopted from [19]

Sample sediment was taken from 0 – 3 cm from the surface and then placed in a plastic container and put in the refrigerator at 10ºC until samples were analyzed. Four to five grams (wet weight) of each sediment sample was suspended in a separate container with 50 ml of filtered seawater (by GF/C filter, Whatman), sonicated for 15 s (Granbo 008 digital ultrasonic cleaner), and sieved through stain steel sieved with the mesh size of sieved 250 µm, 125 µm and 20 µm to obtain the size fraction measuring less than 20 µm. The cyst fraction was suspended into 200 ml filtered seawater then transferred into 1 L bottle.

DNA extraction and PCR amplification

One liter of water sieved dinoflagellate cyst were filtered using Whatman filter paper of 0.22 µm. Then the filter paper was extracted by following the procedure of the DNA extraction kit miniprep ZymoBiomicsTM. Dinoflagellate cyst genetic diversity analysis was performed using Cytochrome C Oxidase Subunit I (COI) primer according [20]. CO1 gene amplification was conducted using PCR according to Reference [21]. The amplification of the CO1 gene was carried out using PCR with an initial denaturation program at 94°C for 2 minutes after 30 cycles, 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1.5 minutes and final extension 72°C for 7 minutes. PCR reaction consists of Pure Taq Ready To-Go PCR Beads kit, 1 µL for each primer (10 pmol), 3µL template DNA, and sterile water until the final volume of 25 µL. PCR processed in the 2720 Thermal Cycler. The PCR amplification products were then separated using 2% agarose gel electrophoresis. DNA fragments from agarose gels were documented using the Gel Documentation System (Biometra). Finally, the DNA fragment size was measured using the 100bp Plus DNA Ladder.

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Sequencing

CO1 primers was used for amplifying a genomic DNA from dinoflagellate cyst species. The Qiagen Purification Gel Extraction Kit (Qiagen, CA, California was used for purifying The PCR-amplified products. Genus and species-specific PCR-amplified products obtained from sediment samples, such as those from 4 locations and 4 stations for each location were excised from the gel, purified using QIAquick Gel extraction Kit (Qiagen) and directly sequenced. The DNA samples were sent to PT. Genetics Science for the sequencing process. Sequencing was carried out using the automatic sequencer AB-3130 (Applied Biosystem). Sequencing results were viewed manually using the sequence navigator program.

Data analysis

The results of the sequences were cut on the front and back ends using Chromas (http://technelysium.com.au/wp/chromas/) while looking at the quality of the sequences produced by PCR. The forward and reverse sequences are combined to get a longer sequence. In reverse sequence, the sequence is reversed using reverse complement online via https://www.bioinformatics.org/sms/rev_comp.html . After that, the sequences are combined with forward and merged. The sequences results were analyzed using the MEGA 6.0 program [22] to determine the similarity of the sequences. The sequences of each sample were aligned with the existing sequences in the Gene Bank database using the BLAST-N (basic local alignment search tool-nucleotide) program through https://blast.ncbi.nlm.nih.gov/Blast.cgi. Data were presented descriptively

Results:

Electrophoresis results showed that the gene from our sample has the size of 700-800 bp with good quality (Fig. 2), shows that CO1 primer succeeded to generate visible band on the gel.

The results of DNA analysis showed that genetically the dinoflagellate cyst population from 4 locations were DNA closely related. This can be seen from the phylogenetic tree which only consists of 2 clades (Fig. 3). Clade 1 was dominated by dinoflagellate cyst DNA from the Jeneberang Estuary (JB), Maros Estuary (M) and Pangkep Estuary(P), while clade 2 was dominated by dinoflagellate cyst DNA from the Paotere Port (PP). However, one sample from Paotere Port (PP) was included in clade 1.

To measure genetic difference between two population of dinoflagellate cyst, the present study has measured a genetic distance. Based on genetic distance analysis, clades 1 and 2 have genetic distances ranging from 0.5 -0.6. The closest genetic distance was between sample of JB1 and sample of JB2 accounting for 0.083, while the farthest genetic distance was sample of PP1 and PP2 where PP1was in clade 1 and PP2 was in clade 2 with the value of 0.607 (Table. 1). This result indicated that there was high diversity of DNA dinoflagellate cyst between clade 1 and 2.

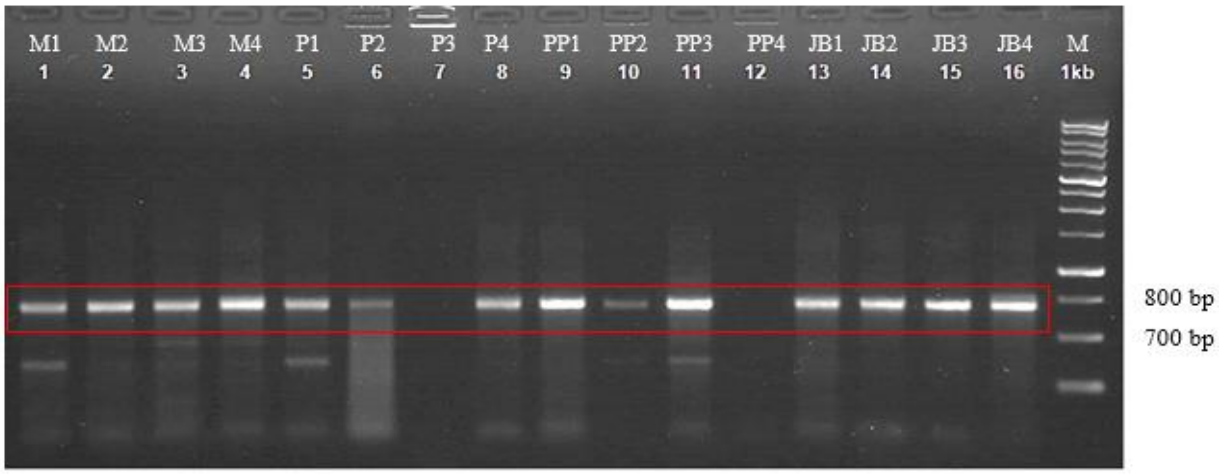

Figure 2. PCR products using CO1 primers and template dinoflagellate cyst DNA from field sediment samples. The expected length of fragment is 700 bp- 800 bp. 100+: molecular marker (1 kb DNA Ladder). M1-M4 : Maros Estuary; P1-P4: Pangkep Estuary; PP1-PP4 : Paotere Port and JB1-JB4 : Jeneberang Estuary was sample code location. 1 – 4 symbols for station; M : marker : molecular weight marker. The red line was the targeted gene. Note: For genom electrophoresis, 1% agarose was used with 75 volt for 90 minutes dan for genom isolation, 2% agarose was used with 75 volt for 90 minutes.

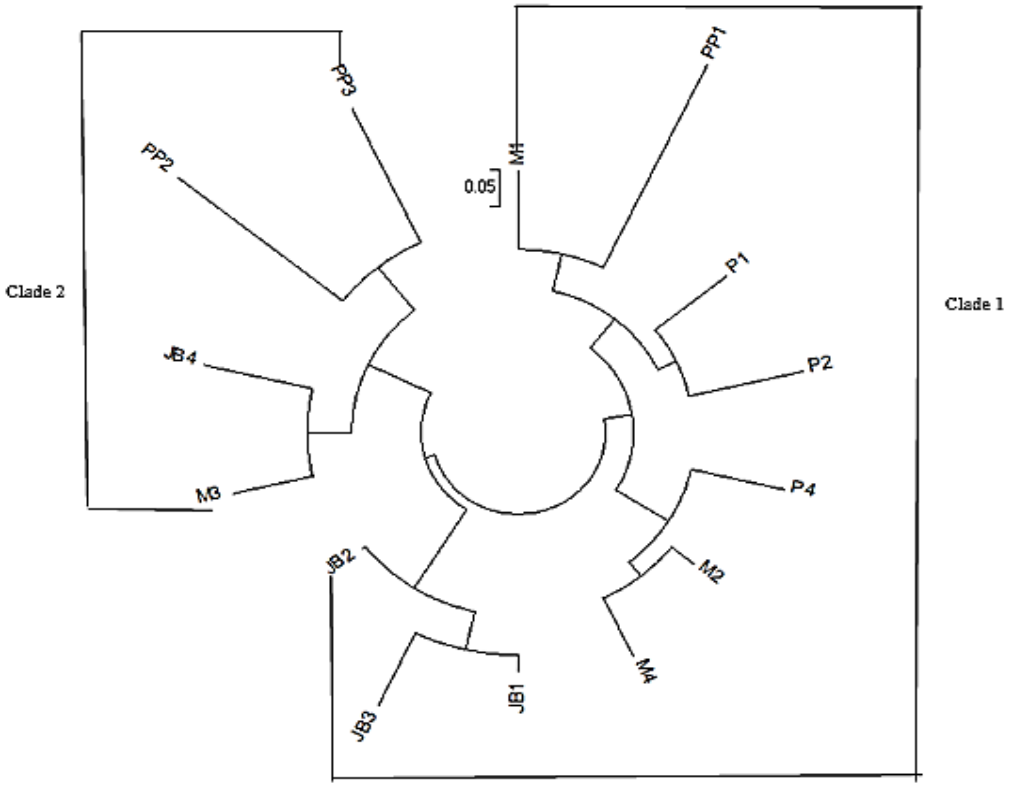

Figure 3. Phylogenic tree dinoflagellate cyst from sampling site. Note: JB is Jeneberang Estuary, M is Maros Estuary, P is Pangkep Estuary, and PP is Paotere Port. 1 - 4 is a symbol for a station.

Table 1. Genetic distance between dinoflagellate cyst samples from 4 locations

Sample Code	Genetic Distance													
	M1	M2	M3	M4	P1	P2	P4	JB1	JB2	JB3	JB4	PP1	PP2	PP3
M1														
M2	0.302													
M3	0.382	0.284												
M4	0.335	0.124	0.304											
P1	0.277	0.261	0.387	0.289										
P2	0.316	0.332	0.355	0.378	0.251									
P4	0.369	0.183	0.410	0.209	0.318	0.349								
JB1	0.368	0.340	0.375	0.345	0.374	0.358	0.396							
JB2	0.311	0.288	0.325	0.291	0.306	0.315	0.334	0.083						
JB3	0.394	0.368	0.399	0.371	0.331	0.347	0.365	0.137	0.171					
JB4	0.448	0.367	0.238	0.366	0.416	0.350	0.451	0.413	0.369	0.391				
PP1	0.427	0.465	0.544	0.484	0.538	0.429	0.474	0.586	0.506	0.582	0.554			
PP2	0.444	0.450	0.488	0.501	0.522	0.519	0.541	0.541	0.486	0.545	0.485	0.607		
PP3	0.554	0.470	0.406	0.475	0.488	0.502	0.574	0.498	0.465	0.520	0.378	0.594	0.480	

Note: JB: Jeneberang Estuary, M: Maros Estuary, P: Pangkep Estuary, PP: Paotere Port. Number 1 – 4 indicated station.

Discussion:

Based on the electrophoresis result (Fig. 2), the DNA bands were well amplified except for line 1 and line 7 which were not successfully amplified, so that only 14 samples could be successfully analyzed. The phylogenetic tree using the Maximum Likehood method produced two clades where clade I contained species from 4 locations representative, while clade 2 was dominated by samples from Paotere Harbor (PP). In addition, some lines also show several bands in one line, but they are not the target gene. Furthermore, gene sequencing of the dinoflagellate cyst samples showed similarities to the diatoms group, namely *Licmophora* sp. (accession number: MT684612.1) and *Licmophora* sp (accession number: MT684615.1) in the percentage of 78.9% blast-n. These results indicated that a genetic combination of the four sites was not too strongly related. It was suspected that the low similarity value is due to the very low data in the gene bank for both diatoms and dinoflagellate cyst species. The sequences in the gene bank specifically for dinoflagellates could not be detected using CO1 primers, while the group of diatoms that could be detected using the same primers was the species group *Licmophora* sp. Specifically for the Dinoflagellate group, each species used different target genes such as *Archaeperidinium saanichi* using 24S rRNA primer[2,] *Cochlodinium polykrikoides* with 28S rRNA primer[7], *Cochlodinium* sp. using 28s rRNA primer [23], *Gymnodinium* sp. with primer of 18S rRNA [24], *Alexandrium fundyense* NA1 ribosomal primer [6,] *Akashiwo sanguinea* with the primer of RNA with Oligo (dT)18 [25], *Alexandrium tamaranses* using Dino5SF1 and Dino5SR1 primer [26] and *Protoperidinium claudican* with the primer of 18S rRNA[27].

Based on the results of the study, there was one individual from the Maros area (M) and one individual from the Jeneberang Estuary (JB) which was included in clade 2 along with the dominant sample from Paotere Harbor (PP). We assumed that there is a possible similarity of species between samples M and JB with PP. The primer CO1 was not suitable for detecting dinoflagellate cyst DNA due to only picking one DNA, which was a diatom (*Licmophora* sp).

Conclusion:

The primer CO1 is not suitable for detecting dinoflagellate cyst DNA due to only picking one DNA, which is a diatom (*Licmophora* sp). For the future study, we recommend other barcoding gen that are more suitable for algae, such as matK, rbcL, psbA-traH, and ITS primer for detecting DNA of dinoflagellate cysts

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Besides, the Figures and images, which are not ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in Universitas Hasanuddin.

Author's Contributions:

NR: Designed a sampling method, conducted field sampling, data analysis and interpretation, writing a draft manuscript, submitting a manuscript.
AAH: Designed primer, and DNA data interpretation
Andi Parenrengi: Running DNA analisis, DNA interpretation, and writing a method of manuscript
SA: Designed primer, and DNA data interpretation, final editing and proofreading of manuscript before submitting.

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الكشف عن الحمض النووي لكيسات دينوفلاجيلات متعددة الأنواع في الرواسب من ثلاثة مصبات أنهار لمضيق ماكاسار وميناء الصيد باستخدام بؤادي جين COI:هل هو مناسب للكشف عن دينوفلاجيلات الحمض النووي؟

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الخلاصة:
تمتلك معظم دينوفلاجيلات كيس راحة في دورة حياتها. ويتطور هذا الكيس في ظروف بيئية غير مواتية. تتطلب الطريقة التقليدية لتحديد كيس الدينوفلاجيلات في الرواسب الطبيعية ملاحظة مورفولوجية وعزل وإنبات وزراعة الأكياس. تفاعل البلمرة المتسلسل يعد طريقة حساسة للغاية للكشف عن أكياس دينوفلاجيلات (dinoflagellate)في الرواسب. إن الهدف من هذه الدراسة هو فحص بواديء جين CO1 وهل يمكن استخدامها للكشف عن الحمض النووي الدنا لأكياس دينوفلاجيلات متعددة الأنواع في الرواسب من مواقع أخذ العينات بالدراسة الحالية. تم استخلاص الحامض النووي DNA لأكياس دينوفلاجيلات من 16 عينة رسوبية. تم استخدام طريقة تفاعل البلمرة المتسلسل باستخدام بؤادي لجين CO1. ودرس تسلسل DNA لكيسات دينوفلاجيلات باستخدام التطابق (BLAST). أظهرت النتائج أن هناك من اثنان من Clades لأكياس الدينوفلاجيلية من أربعة مواقع للدراسة. سيطرت عينات من مصبات (Jeneberang) و (Maros) و (Pangkep) على Clade 1 ، بينما سيطرت عينات من ميناء (Paotere) على Clade 2. اختلفت المسافة الجينية بين عينات DNA لكيسات الدينوفلاجيلات التي تراوحت بين 0.5 - 0.6. كانت أقرب مسافة وراثية بين عينة JB1 وعينة JB2 ، بينما كانت أبعد مسافة وراثية هي عينة PP1 و PP2. لم يكن CO1 مناسبًا للكشف عن الدنا لكيسات الدينوفلاجيلات بسبب تشخيص دنا واحد فقط ، والذي كان عبارة عن طحلب دياتوم (Licmophora sp).

الكلمات المفتاحية: بؤادئ COI , دنا اكياس ثنائية الاسواط, مضيق مكاسار, ماروس بانجكيب ومصب جينبيرانخ, تفاعل البلمرة المتسلسل.