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# Detection of the Centrosomal DNA from the Sperm of Bryophyte *Marchantia polymorpha* L. and *Physcomitrella patens*

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Abstract: The liverwort *Marchantia polymorpha* is dioecious and haploid during most of its life cycle. It is known that fertilization in bryophytes, as in animals, involves sperm and eggs. Recently, we isolated novel DNA from the sperm centrosome of the starfish *Asterina pectinifera*. In the present study, we examined whether the sperm of bryophytes contain this unique DNA. The total DNA in the sperm of *M. polymorpha* was used as a template for polymerase chain reaction (PCR) analysis performed using a centriole-specific DNA primer. Although this primer was designed to specifically amplify the centrosomal DNA of the starfish, it can amplify the centrosomal DNA of many other animals having a similar DNA sequence. The size of the amplified PCR products was approximately 500 bp, which is similar to that of the PCR products amplified from starfish. We determined the nucleotide sequence similarities between the PCR products amplified from the cultured cells of the moss *Physcomitrella patens* and from the sperm of the liverwort *M. polymorpha*. Our results show that DNA similar to that of the starfish centrosomal DNA exists in bryophytes and includes animal centrosomal DNA homologues and candidate centrosomal DNA.

*Keywords:* centrosome, centrosomal DNA, S1 nuclease, bryophyte sperm, liverwort

## Introduction

In most eukaryotic cells, the basal bodies of cilia or flagella and the centrioles in the spindle pole are structurally analogous and interchangeable [1]. Recently, we identified novel DNA from the sperm centrosome of the starfish *Asterina pectinifera* [2]. In starfish, this unique DNA exists in the centrioles of the sperm and the basal bodies of cilia on the blastulae. The localization of the products amplified by polymerase chain reaction (PCR) was investigated by *in situ* PCR with fluorescence-labeled primers. The amplified DNA signal was detected in the sperm centrosome of *A. pectinifera*. Although the centriole-specific DNA (CS) primer was designed to specifically amplify the centrosomal DNA of the starfish, it can amplify the centrosomal DNA of many other animals having a similar DNA sequence (above 95% sequence identity). The size of the PCR products amplified from the DNA of many animals is approximately 500-bp; this is similar to the size of those amplified from the starfish DNA [2,3]. In the present study, we investigated the presence of this unique DNA in the bryophyte sperm. Carothers and Kreitner (1968) have observed centrosomes in the spermatid mother cells of the liverwort *Marchantia polymorpha* [4]. The extraction of sperm from liverworts is easier than that from other plants in which sperm functions as the male gamete. The moss *Physcomitrella patens* has been recognized as a useful model for the study of plant embryogenesis and development for several reasons [5]. Insights into the molecular biology of mosses have been gained come mainly from the studies of this monoecious

moss. Therefore, in this study, the liverwort *M. polymorpha* and the moss *P. patens* were selected to identify the centrosomal DNA in plants. The total DNA of the *M. polymorpha* sperm and the *P. patens* protonemata were used as templates for PCR analysis, which was performed using the CS primer.

## Materials and Methods

### Plant material and culture conditions

The protonemata of *P. patens* ssp. *patens* were cultured on 0.7% agar with 0.01% hyponex (6-10-5). The cultures were grown in 9 cm Petri dishes on medium solidified with 0.7% agar and overlaid with cellophane discs having a diameter of 8 cm (type 325P; AA Packing Ltd, Preston, UK). The plates were grown in a culture room at 25℃ under a regime of 16h light and 8 h darkness. After a 3-week incubation of the *P. patens* plants, the total DNA was extracted according to the specified protocol.

### Sperm isolation

We used the sperm of the bryophyte, *M. polymorpha*, which was collected at Manazuru (Kanagawa) and Chofu (Tokyo). An isolate of the spermatozoon of *M. polymorpha* was taken and a droplet of distilled water was deposited on the antheridium, following which the sperm were released. After 3 repeated centrifugations, each performed at 1,000 x *g* for 1 min, the sperm in the supernatant were sedimented at 1,000 x *g* for 10 min at room temperature. Subsequent steps were carried out at 4℃.

### Nucleic acid isolation

Total DNA was extracted from the protonema or gametpophores using QIAGEN plant tissue Kit according to the manufacturer's protocol.

### PCR analysis

PCR primers measuring 500-bp were designed for the novel DNA from the starfish centrosomal DNA sequence of the partial sequences. The sequences derived from clone K4 were obtained by PCR using the DNA extracted from starfish sperm centrosome as a template, a (CS-primer-F) forward primer (5'-GCAGCTTTCGTCGATTTAAA -3') and a (CS-primer-R) reverse primer (5'-TCA TTTTCTTCACCAGCAAA-3'). This pair of PCR primers is named the *CS-primer*. The PCR was performed using the HotStar Taq polymerase (QIAGEN). The cycling conditions were as follows: first, initial incubation step at 95℃ for 15 min; second, 35 cycles, each performed at 94℃ for 1 min (denaturation), 53℃ for 30 sec (annealing) and 72℃ for 1 min (extension); and third, final extension at 72℃ for 10 min. The following bryophyte RuBisCo primers were used for chloroplast-DNA PCR (positive control): forward, 5'-AGTAACTTTAGGTT TCGTAG-3' and reverse, 5'-TTTCCAAATTTACAA GCAG-3'. Bryophyte (*M. polymorpha*) RuBisCo primers correspond to nucleotides 1020-1039 (forward) and 1370-1389 (reverse). The following bryophyte 18S rRNA primers were used for genomic PCR (positive control): forward, 5'-TACCTGGTTG ATCCTGCC AG-3' and reverse, 5'-AGGTTCACCTA CGGAAACCT -3'. Bryophyte (*M. polymorpha*) 18S rRNA primers correspond to nucleotides 0001 - 0020 (forward) and 1786 - 1805 (reverse). For positive control of PCR was performed under the condition of 35 cycles of 94℃ for 1 min, at 57℃ for 30 sec and at 72℃ for 1 min.

### DNA sequencing analysis

Sequencing was performed using a BigDye Terminator v.3.1 Cycle Sequencing Kit (ABI PRISM 310) as recommended by the manufacturer. Sequences were processed using the program GENETYX-MAC, version 9.0 (Software Development Co., Ltd., Tokyo, Japan).

## Results and Discussion

### Morphological observation of the sperm of liverwort

The sperm of bryophytes (liverworts) can be easily harvested for the purpose of study. Microscopic observation of the liverwort sperm was first carried out by Ikeno in the 1900's [6]. Electron microscopic observation of liverwort sperm was reported by Carothers and Kreitner [4]. With regard to the sperm of the bryophyte *M. polymorpha*, Carothers observed centrosomes in the spermatid mother cells by electron microscopy. The structure of the centriole of the *M. polymorpha* is already confirmed by observations under the electron microscope [7].

Microscopic and electron microscopic analysis of

the liverwort sperm is comparatively easier than that of sperm from other mosses [8] or hornworts [9]. The gametophyte of the liverwort *M. polymorpha* was collected from Manazuru Chou in Kanagawa and Chofu City in Tokyo from March to April. This liverwort is heterothallic.

Figure 1 shows the white solution of the sperm that overflowed on the gametophyte of the male plant of the *M. polymorpha*. The spawning of sperm is indicated in the photograph on the right (Fig. 1). This spawning of sperm was observed on the male-receptacle of the liverwort and appeared as turbid water.

Phase-contrast microscopic photographs of the spermatogenesis of *M. polymorpha* are shown in figure 2. In immature liverworts, the sperm has an outer membrane (Fig. 2, A). During maturation, the outer membrane of the sperm is dissolved and the sperm is then released (Fig. 2, B to T).

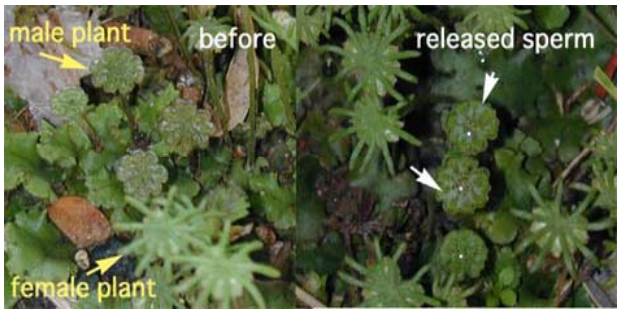


Fig. 1. Gametophytes of the male and female plants of *M. polymorpha* are indicated by yellow arrows (left). White arrows indicate the spawning of the sperm in a water drop on the male-receptacle (right).

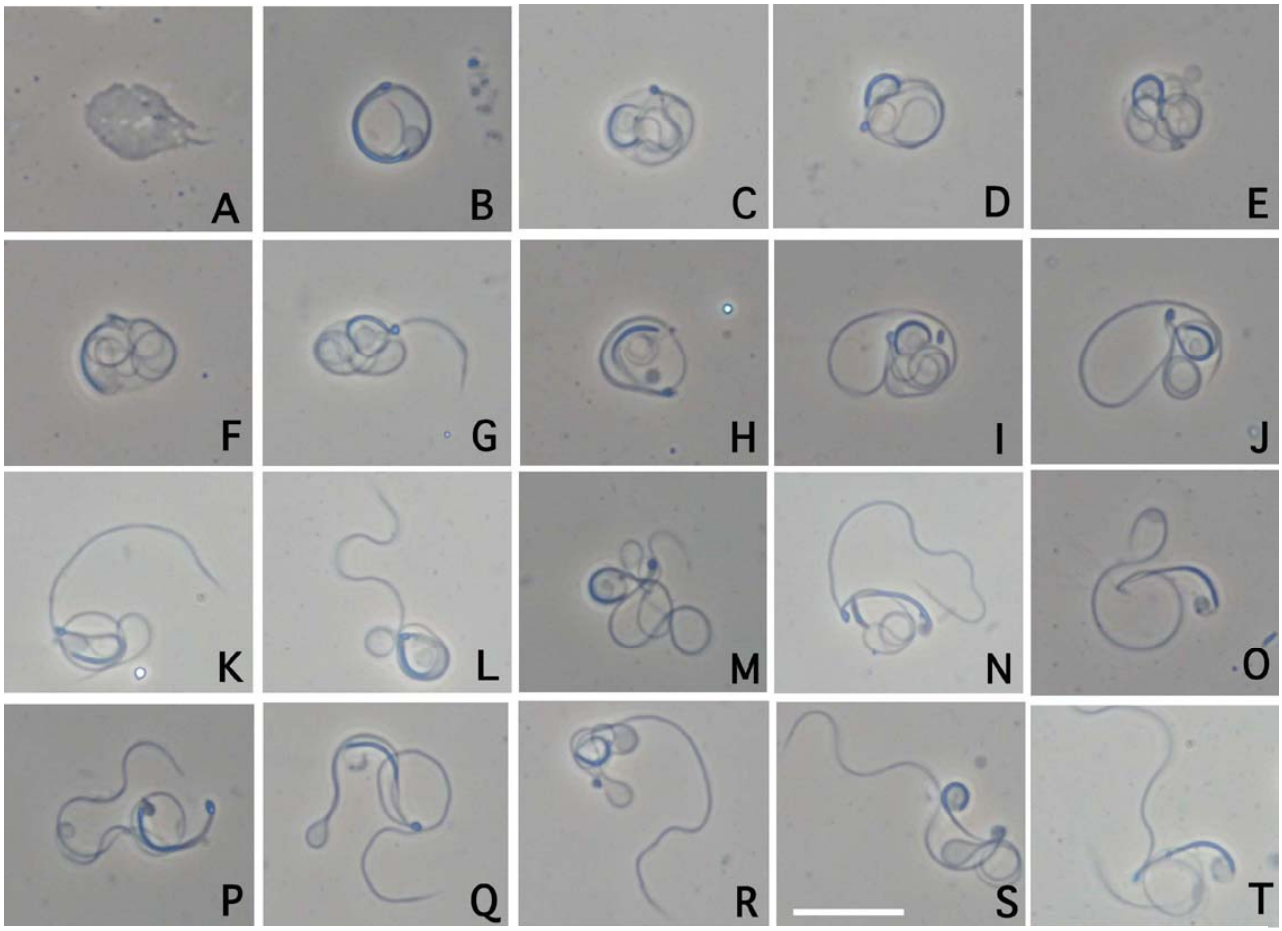


Fig. 2. Phase-contrast microscopic photographs obtained during the sperm maturation of liverwort. The process of spermatogenesis shows in B-T. A shows the outer membrane of the spermatid. Two flagella projected from spherical spermatid cells show in G-N. Two moving flagella are present, and spematids gradually lose the cytoplasm of the spermatid cells (I-S). T shows the mature sperm of the *M. polymorpha*. Scale bar = 10 μm.

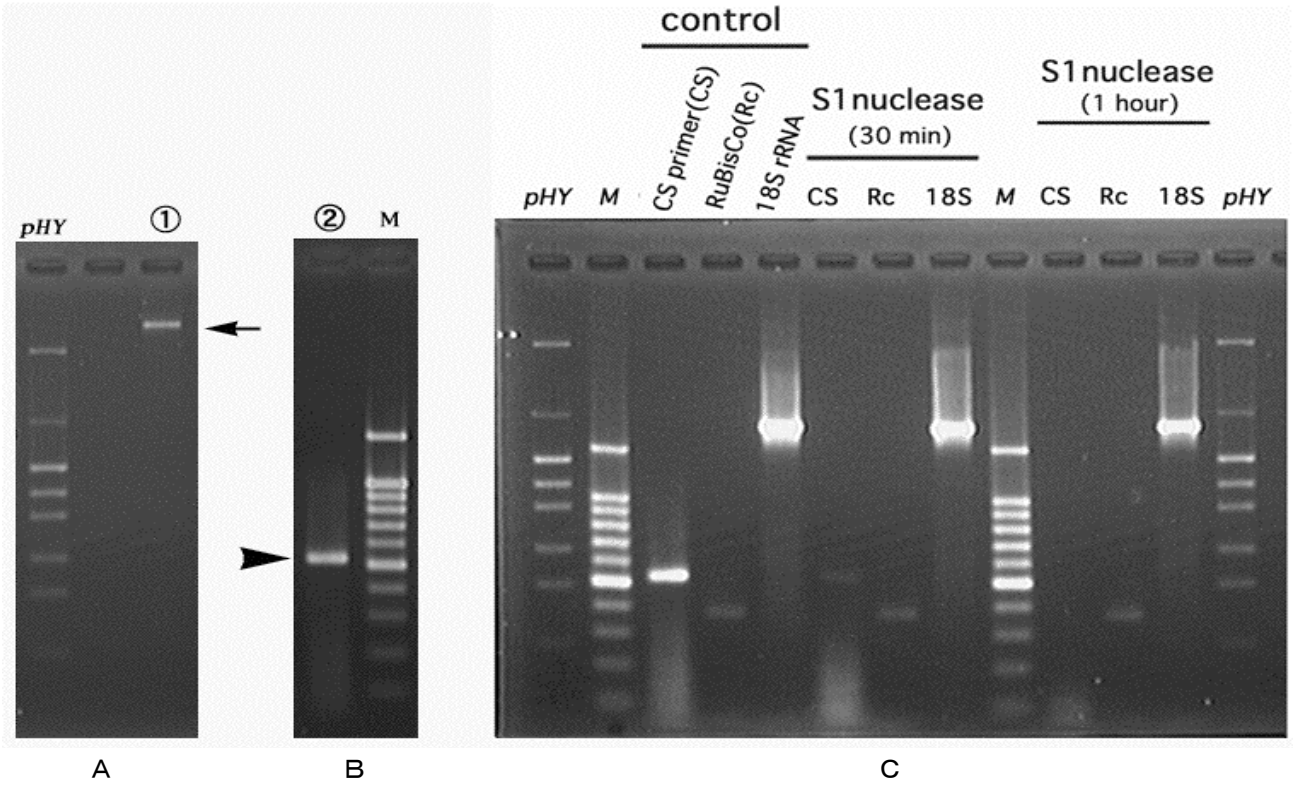


Fig. 3. Total DNA of the *M. polymorpha* shows in A (lane 1, arrow) on the agarose gel (2%) electrophoretic pattern. PCR amplification with CS-primer experiment shows in B. PCR fragments confirmed the 500-bp product (lane 2, arrowhead). In contrast with 18S-rRNA- or RuBisCo-primers, PCR products with the CS-primer were not amplified from the total DNA (template DNA) of the *M. polymorpha*, after treated by S1 nuclease for 1 hour incubation (C). M indicates the 100-bp ladder marker. *pHY* indicates the *pHY* marker of the molecular weight marker.

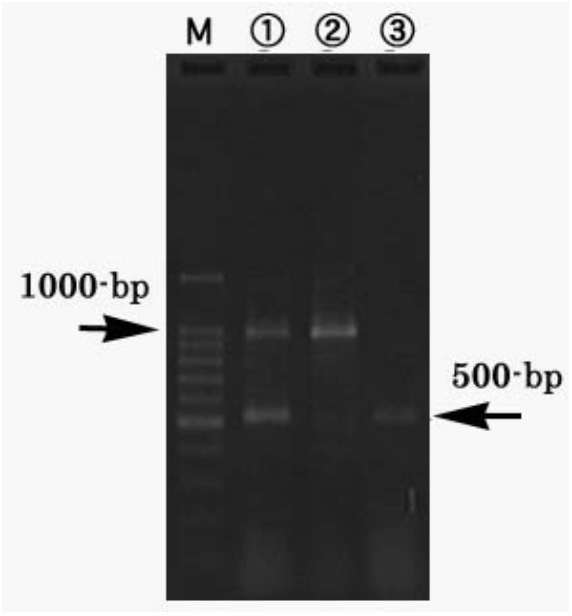


Fig. 4. PCR amplification from the bryophyte *P. patens* is shown in lane 1. Isolated fragments of the 500-bp and 1000-bp products were obtained, (lane 2; 1000-bp, lane 3; 500-bp). M indicates the 100-bp ladder marker.

## PCR amplification and sequence analysis of unique DNA sequences in moss and liverwort

To confirm the presence of unique DNA sequences corresponding to starfish, total DNA was extracted from the male gamete cells (sperm) of the liverwort *M. polymorpha* and from the cultured cells of the moss *P. patens*. To examine whether the unique DNA sequences are present in these plants, PCR amplification was performed with the CS primer in order to amplify the starfish centrosomal DNA homologues. Analysis of the PCR fragments confirmed a 500-bp product (Fig. 3) from the total DNA of *M. polymorpha* sperm. In addition, genomic DNA sequences encoding 18S rRNA and chloroplast DNA encoding the large subunit of RuBisCo (*rbcL*) [10,11] were also amplified as controls for genomic DNA and the organellar DNA, respectively (Fig. 3).

PCR amplification experiments were also performed with the DNA isolated from the

cultured cells of *P. patens* (Fig. 4). An analysis of the amplified fragments confirmed that the protnemema cells of the moss *P. patens* contained the same unique sequences as the plant cells of the liverwort. Further, in the case of *P. patens*, the amplification products of different sizes were obtained. In addition to the 500-bp product, a PCR product of approximately 1000-bp was obtained (Fig. 4).

With respect to animals, the sperm centrosomes of the starfish contain specific novel DNA, and the nucleotide sequence of this DNA has been determined in our recent studies [2,3,12].

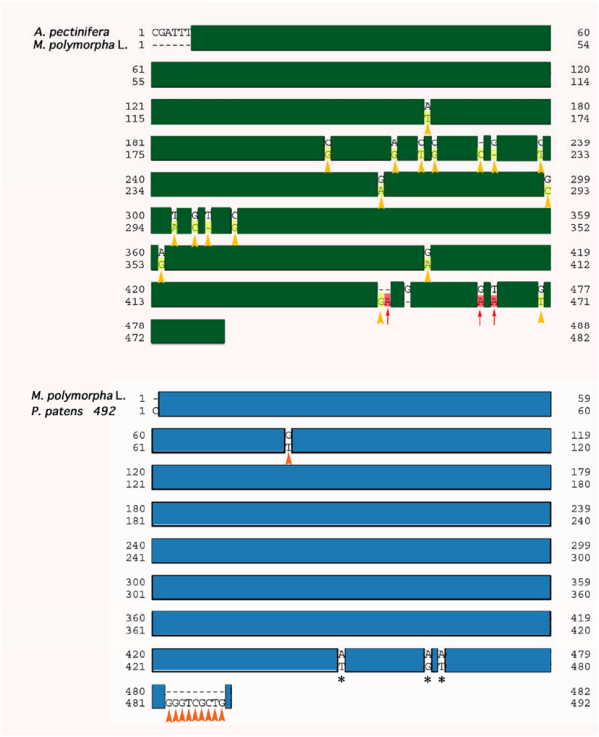


Fig. 5. Comparison between the DNA sequences amplified from the starfish *A. pectinifera* and the liverwort *M. polymorpha*; those sequences were obtained by PCR using the CS primer (top: coincident arrangement is indicated in green). Nucleotide substitution is indicated by yellow arrowhead. The remarkable insertion mutations in the *M. polymorpha* indicated by red arrows, and the remarkable insertion sequences in *P. patens* are indicated by orange triangles (bottom). The asterisk shows nucleotide substitution between *M. polymorpha* and *P. patens*. The numbers on the both sides indicate the nucleic acid residue positions.

A characteristic of this novel DNA is that it is sensitive to single-strand nucleases. For example, this DNA is digested by the S1 nuclease. To determine whether the DNA extracted from our samples also showed this characteristic, it was treated with the S1 nuclease and used for PCR amplification. The untreated total DNA (as a control) extracted from liverwort sperm and the S1 nuclease treated total DNA were subjected to PCR analysis using the CS-primer, RuBisCo primer and 18S rRNA primer, respectively. Using the total DNA as a template for RCR, the amplified PCR fragments were confirmed. However, such PCR amplification was not detected with the CS-primer from S1 nuclease-treated total DNA. The unique DNA in bryophytes was also sensitive to the S1 nuclease since no amplification product was obtained with the CS-primer (Fig. 3C).

The similarities between the nucleotide sequences of the PCR products amplified from the cultured cells of the moss *P. patens* and the sperm of the liverwort *M. polymorpha* were determined and are shown in Figure 5. Figure 5 (bottom) shows the sequences common to *M. polymorpha* and *P. patens* in blue. Figure 5 (top) shows a comparison between the sequences of the starfish and the liverwort; identical sequences are indicated in green. Yellow arrowheads indicate the insertions common to the starfish and the bryophytes. The insertions unique to *M. polymorpha* are denoted by red-arrows. The insertions unique to *P. patens* are indicated by orange arrowhead. The liverwort PCR product sequences were 482 nucleotides long; this liverwort PCR product is 6 nucleotides shorter at the 5' end than the PCR products amplified from the centrosomal DNA of *A. pectinifera*. In contrast, in case of the moss *P. patens*, the PCR product sequences were 492 nucleotides long; this PCR product is 4 nucleotides longer than that of *A. pectinifera*. PCR analysis was carried out with the CS primer to identify similarities between the centrosomal DNA of the starfish and the moss plant. The size of the amplified PCR product was approximately 500-bp, which was similar to that of the PCR product of the starfish. The nucleotide sequences of liverwort showed high homology (95.8% identity) with that from centrosomal DNA of starfish. The nucleotide sequences of *P. patens* also showed high homology (96.3% identity) with that from centrosomal DNA of starfish. There was 99% identity in centrosomal DNA between *M. polymorpha* and *P. patens*. The centrosome exists

not only in most animal cells but also in plant cells [4]. These results of this study suggest that the unique DNA sequences are present in the centrioles of not only the starfish but also the sperm of liverwort *M. polymorpha* and the *P. patens* protonemata.

In bryophytes such as mosses, liverworts, and hornworts, the sperm is the male gamete, as in the case of animals [4,7,8,9]. However, the structure of the sperm of these bryophytes is slightly different from that of animal sperm. The most remarkable difference is that the sperm of these plants have 2 flagella [13]. However, the sperm of all animals have only 1 flagellum. As for the numerical difference of the flagella in which the sperm owns, it is thought with the evidence of the difference that the plants and the animals were able to understand the first divergence that they occurred by a process of the evolution of the eukaryotic cells [14]. The sequence phylogeny and taxonomic distribution of the myosin domain combinations revealed five innovative possibilities that strongly support unikont (animals, fungi, choanozoa, and amoebozoa) monophyly and the primary bikont/unikont (bikont; plants, chromists, and all other protozoa) bifurcation [14]. It is believed that this structural difference in the sperm of plants and animals is an extremely important difference in the divergence of eukaryotes.

In our preliminary PCR experiments performed using the total DNA of the sea urchin *Hemicentrotus pulcherrimus* and the diatom *Chaetoceros gracillis*, we succeeded in amplifying the centrosomal DNA by using the CS primer. The partial DNA sequences of both the sea urchin and diatom were also analyzed, and the homology between both sequences and the centrosomal DNA sequence from the starfish was found to be above 95%. This DNA sequence appears to be highly conserved between species. This novel DNA is expected to be a new tool in the study of the origin of the eukaryotes.

Accession numbers of the genes described here are as follows: AB298452 (*M. polymorpha*) and AB298453 (*P. patens*)

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