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Effective DNA extraction methods for mitochondrial phylogenomics of the sea urchins

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Abstract

In the present study, we evaluated the efficiency of a minimally destructive and a destructive mitochondrial DNA extraction method. The first method extracted total DNA from epidermis and muscles of the spines and yielded varying PCR efficiency values that are dependent on the template concentration, which could be attributed to contamination by pigment and/or genomic DNA in the tissue. The second method extracted mitochondrial DNA from fractionated mitochondria obtained from tissues and coelomic fluid inside the test. Using this method, PCR products and purified mitochondrial DNA were used for sequencing on a next generation sequencer (Ion PGM). Direct sequencing of the purified mitochondrial DNA led to complete recovery of the mitochondrial genomes; however, contaminants were present. PCR failure was commonly encountered using the first method but was absent using the second method. The two extraction methods were compared to the commonly used destructive method that extracted total DNA from gonad or coelomic fluid inside the test. PCR results indicated that the destructive method exhibited similar performance as the first method. The present study generated 11 complete and one partial mitochondrial genome sequences for 12 species. The complete mitochondrial sequences from the order Cidaroida were sequenced for the first time. Phylogenetic trees constructed based on individual genes showed various topologies. Phylogenetic relationships estimated from all protein-coding regions of the mitochondrial genome were consistent with the order-level relationships estimated from molecular and morphological data in previous studies.

Introduction

Molecular phylogenetic studies on sea urchins have been primarily conducted to examine or verify the phylogeny based on morphological and fossil evidence. However, the scope of phylogenetic studies remain limited (e.g. Littlewood and Smith, 1995; Stockley *et al*., 2005). Smith *et al*. (2006) constructed a comprehensive phylogeny of echinoids. The study compiled morphological information and gene sequence data for three loci (18Sr, 28Sr, and 16Sr) from 46 genera with representatives from 28 families and 13 of the 14 orders of living echinoids; molecular and fossil estimates of divergence times were compared and examined. The resulting phylogenetic tree showed high congruence with that constructed based on morphological characters (compare discussion in Kroh & Smith 2010). Some regions of the mitochondrial genome were successfully obtained to determine phylogenetic relationships within a family or a genus, which often encouraged evolutional discussion in developmental biology (e.g. Matsuoka & Hatanaka 1991; Jeffery *et al*. 2003; Lee 2003; Kinjo *et al*. 2008; Hart *et al*. 2011). However, analysis of phylogenetic relationships within orders or among families is not straightforward. Phylogenetic trees constructed from different loci

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produced various topologies, indicating that the evolutionary rates of certain loci can differ between clades, as Smith *et al*. (1992) showed for rRNA. The locus or combination of loci is an important consideration in phylogenetic analysis (Jeffery *et al*. 2003). In addition, mitochondrial and nuclear markers can show conflicting results, as determined by previous results on commercially exploited sea urchin *Tripneustes gratilla* (Bronstein *et al*. 2016). Therefore, analysis of multiple markers is highly recommended.

We are currently compiling the West Pacific sea urchins to analyze their classification and ecology (see Kanazawa *et al*. 2019). It is desirable to adopt a minimally destructive DNA extraction method to preserve the specimens for morphological and other subsequent analyses (e.g. Mason *et al*. 2011). DNA extraction from gonads is a highly reliable method for sea urchins; however, extraction requires extensive destruction of the specimens. Herein, we attempted to perform phylogenetic analysis using whole mitochondrial genomes. The mitochondrial sequences of 30 echinoid species were registered in the GenBank database in January 2019. Considering the diversity of sea urchins, this is very small number, and data from the representatives from 13 orders are lacking. Our project aimed to fill many of these gaps. Long-PCR is an important method used efficient generation of sequences. Therefore, a minimally destructive DNA extraction method and long-PCR method was required to effectively investigate sea urchin phylogeny. Here, we present preliminary results of our project on DNA extraction, PCR, and phylogenetic analysis methods that are suitable for sea urchins.

Materials and Methods

A total of 12 sea urchin species representing 12 genera and four orders were collected throughout Japan (Table 1, detailed localities are shown in Saitoh *et al*. 2019). The analyzed specimens are deposited in Kanagawa University under the Kanazawa Collection.

TABLE 1. Specimens examined in this study. Voucher code in the Kanazawa Collection of Kanagawa University is shown. Detailed localities are shown in Saitoh *et al*. (2019). See Figure 1 for Extraction method 1–3.

Species	Voucher code	Locality	Extraction method	Used tissues	yielded DNA amount	Accession No.
Order Camarodonta						
Family Echinometridae						
Colobocentrotus mertensii	OKN_19	Okinawa	2	inner test tissue	0.7 µg	MK609487
Family Toxopneustidae						
Pseudoboletia maculata	KUC_33	Kouchi	3	inner test tissue	4.7 µg	MK609486
Toxonopneustes pileolus	KUC_13	Kouchi	3	inner test tissue	1.8 µg	
Tripneustes gratilla	KUC_16	Kouchi	3	inner test tissue	1.2 µg	MK609485
Order Cidaroida						
Family Cidaridae						
Prionocidaris baculosa	KMR05	Kouchi	1	gonad	224.6 µg	MK609482
Stylocidaris reini	KMR12	Kouchi	1	coelomic fluid	15.2 µg	MK609483

...Continued on next page

TABLE 1. (Continued)

Species	Voucher code	Locality	Extraction method	Used tissues	yielded DNA amount	Accession No.
Order Diadematoida						
Family Diadematidae						
Echinothrix calamaris	KUC_07	Kouchi	2	inner test tissue	1.4 µg	MK609484
Order Spatangoida						
Family Eurypatagidae						
Eurypatagus ovalis	-	Mie	1	spines	unmeasurable	-
Linopneustes murrayi	-	Mie	1	spines	unmeasurable	-
Family Loveniidae						
Lovenia elongata	-	Shimane	1	spines	unmeasurable	-
Family Maretiidae						
Nacospatangus alta	-	Kanagawa	1	spines	unmeasurable	-
Family Spatangidae						
Spatangus luetkeni	-	Kanagawa	1	epidermis	8.3 µg	-

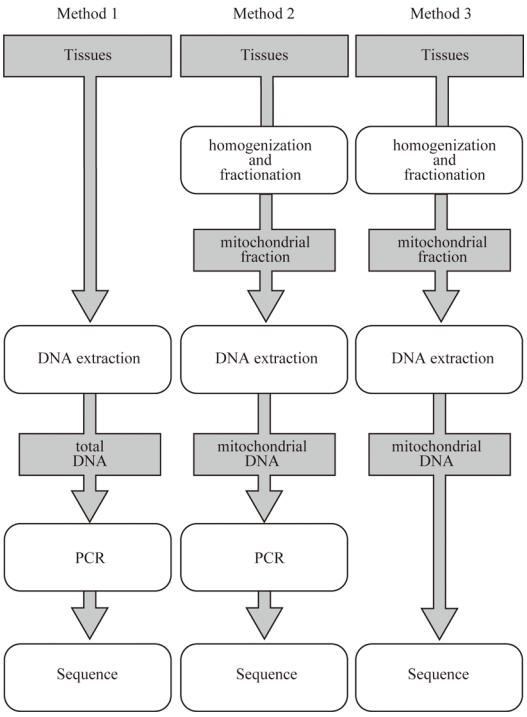

FIGURE 1. Schematic diagram of three methods to get sequence.

To maintain the integrity of the specimens, two minimally destructive DNA extraction procedures were evaluated, namely, extractions from spines (a well-known minimally destructive method; e.g. Bronstein *et al*. 2016) and extraction from coelomic fluid and tissues inside the test. Several spines with basal muscles removed from the test of each specimen were used. For comparison, a commonly used destructive method that extracts DNA from tissues inside tests (gonad or coelomic fluid) was additionally employed; tissues inside tests and coelomic fluid were extracted after removing the lantern through the peristome. Figure 1 shows the three extraction methods.

Coelomic fluid fractionation.

The method described by Cantatore *et al*. (1974) was modified and used for coelomic fluid fractionation. Coelomic fluid with inner test tissues (presumed gonad or ampulla) was extracted through the peristome using a syringe with an 18G needle. The fluid was centrifuged at 1200 ×g for 10 min, after which the precipitate was extracted and added with sucrose-TEK buffer (0.25 M sucrose, 100 mM Tris, 1 mM EDTA, 240 mM KCl, pH 7.6). The precipitate with the buffer was homogenized with a dounce homogenizer on ice. The homogenate was centrifuged at 1200 ×g for 10 min; the precipitate contains fragmented cells and nuclei. Genomic DNA was extracted from the precipitate (not used in this study). Afterwards, the supernatant was centrifuged at 3000 ×g for 10 min to precipitate the pigments. Next, the supernatant was centrifuged at 15,000 ×g for 13 min, and the precipitate was used for mitochondrial DNA extraction.

DNA extraction and purification.

The method described below was used for DNA extraction from the spines and the fractionated precipitates containing nuclei or mitochondria from the inner tissues. Samples were digested overnight at 40 ºC in 300 µL of TNES-Urea buffer (Asahida *et al*., 1996: 10 mM Tris, 125 mM NaCl, 10 mM EDTA2Na, 1 % SDS, 6 M Urea, pH 7.5) containing 20 µL of 20 mg/mL Proteinase K. Impurities were removed with PCI-CIA (phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol), and DNA was purified by ethanol precipitation and resuspended in nuclease-free water. The DNA yields for all species are shown in Table 1.

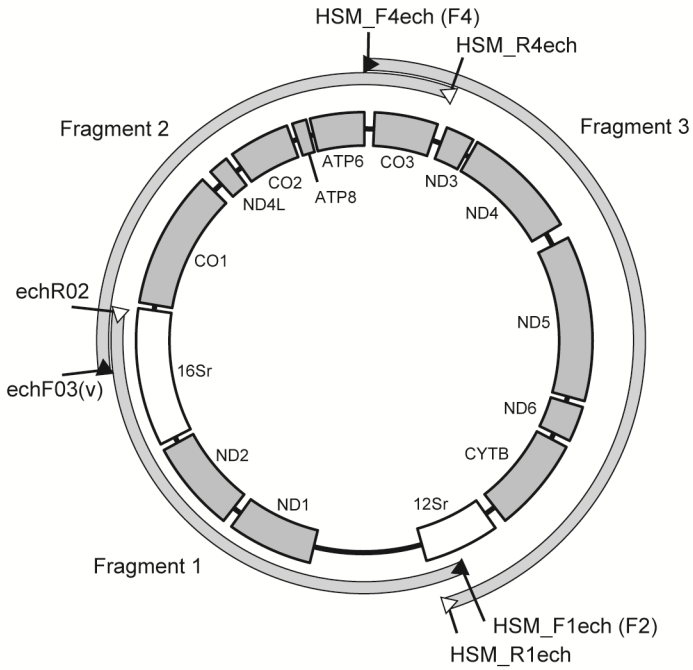

FIGURE 2. Location of major genes on a sea urchin mitochondrion and division into PCR fragments. Primers were designed using well-preserved regions, and the whole mitochondrial genome was divided into three to obtain overwrapped PCR products.

PCR.

The primers used in Wakayama *et al*. (2016) and improved primers (Table 2) were used for PCR amplification. Whole mitochondrial genomes were amplified as a series of two or three mutually overlapping

fragments (Fig. 2). The fragments were approximately 5,000 to 13,000 bp in length (estimated lengths: fragments 1 and 2, 5,000 bp; fragment 3, 7,000 bp; fragment 1+2 10,000 bp; fragment 3+1, 13,000 bp), and the overlaps ranged from 170 to 840 bp. PrimeSTAR GXL DNA Polymerase or Tsk Gflex DNA Polymerase (Takara) was used to amplify the fragments. Betaine solution (5 M) was added as PCR enhancer at 1/40 to 1/10 volume of the PCR mixture using PrimeSTAR GXL Polymerase. Template DNA concentration was measured using a spectrophotometer and adjusted to 10 to 20 ng/10 µL PCR mixture. The annealing temperature was set to 50, 55, or 60 ºC.

TABLE 2. Primers used in this study. HSM_F1ech and HSM_F2, echF03 and echF03v, and HSM_F4 and HSM_F4ech anneal almost the same position respectively. HSM_F2 and HSM_F4 were used for spatangoids. The primer echF03v is almost the same as echF03 but is adapted to a variety of sea urchins.

Fragment 1	
HSM F1ech (forward)	5'-AAGTTTAAAACTCAAAGGACTTGGCGG-3'
HSM_F2 (forward)	5'-CGTCAGATCAAGGTGCAGC-3'
echR02 (reverse)	5'-GTAGATAGAAACTGACCTGGCTCTCG-3'
Fragment 2	
echF03 (forward)	5'-CCTGTTTACCAAAAACATCGCTCC-3'
echF03v (forward, improved)	5'-TCGCCTGTTTACCAAAAACATCGC-3'
HSM R4ech (reverse)	5'-GCAGATAGGAATGCATGCGGCTTGAAAC-3'
Fragment 3	
HSM F4ech (forward)	5'-GAACATTTAAATTATGGCTCATCAAC-3'
HSM F4 (forward)	5'-GGCTCATCAACACCCWT-3'
HSM R1ech (reverse)	5'-GAGTGACGGGCGATGTGTACGCATTCCAG-3'

DNA sequencing and data processing.
Shotgun sequencing was performed on an Ion PGM instrument (Thermo Fisher scientific). PCR fragments from each species were mixed at an equal molar ratio, digested with enzymes (The Ion Xpress Plus Fragment Library Kit, ThermoFisher scientific) to the target fragment size of 400 bp, and barcoded to generate the libraries using Ion Xpress Barcode Adapters (Thermo Fisher scientific). In some cases, the DNA from fractionated mitochondria were directly fragmented without PCR amplification. Multiple samples were pooled and sequenced concurrently in one run. After sequencing, contigs were assembled using the sequence assembly software SeqMan NGen (DNASTAR), generating a contig longer than the whole mitochondrial genome. Both termini of the contig were combined to obtain a circular mitochondrial genome.

Phylogenetic analysis.
A total of 12 whole mitochondrial genome sequences acquired in the present study and 25 additional sequences (24 sea urchins and 1 sea cucumber as an outgroup) from the GenBank database were used for phylogenetic analysis. Individual protein-coding regions and combined data from all the target regions were used for the analyses. Each protein-coding region was removed from the whole mitochondrial sequences, adjusted, and aligned using MAFFT (Katoh & Standley, 2013). Gapped bases were excluded from the analysis. Partitionfinder2 (Lanfear *et al*., 2016) was used for data partitioning and substitution model selection. The first, second, and third codon positions of individual protein-coding regions were identified and used as input to the software as the initial partitions. Combined data and selected models suitable to each partition, which were recovered by Partitionfinder2, were used for Bayesian analysis (seven subsets: SYM+I+G for the first codon of ATP6, ND1, ND3, ND4L, and ND6; GTR+I+G for the second codon of ATP6, COII, COIII, CYTB, ND1, ND4L, and ND6; GTR+I+G for the first codon of ATP8, ND2, ND4, and ND5; GTR+I+G for the second codon of ATP8, ND2, ND3, ND4, and ND5; SYM+I+G for the first codon of

COII, COIII, and CYTB; SYM+I+G for the first codon of COI; and F81+I for the second codon of COI). Mrbayes (Huelsenbeck and Ronquist, 2001) was used for Bayesian analysis. For maximum likelihood analysis, concatenated results from Partitionfinder2 (Subset construction is same as Bayes analysis) were analyzed with the GTR gamma mode using RAxML (Stamatakis, 2014).

Results

Sequencing of PCR products.

Total DNA extracted from spines and inner tissues were contaminated by pigments; on the other hand, no contamination was detected from DNA extracted from the fractionated mitochondria. For the total DNA extracted from spines and inner tissues, PCR efficiency was dependent on template DNA concentration (Fig. 3). Amplification was not successful when very low template concentrations were used. PCR efficiency was higher at the optimal template concentration and dramatically reduced when the DNA concentration was increased beyond the optimal template concentration. The optimal concentration of the template DNA concentration varied depending on the species. PCR products were more easily amplified using DNA extracted from the fractionated mitochondria than DNA extracted using other two methods tested. Sequencing of the PCR products returned satisfactory results. Whole mitochondrial genomes of nine species were successfully sequenced based on the above procedure.

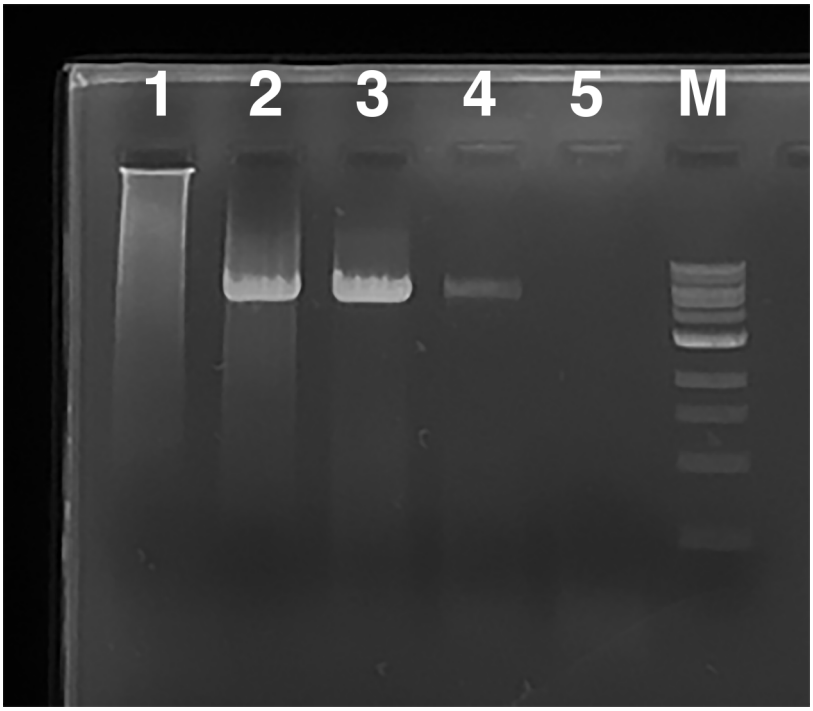

FIGURE 3. A typical electrophoresis image of PCR product using total DNA extracted from spines. Fragment 3 (approximately 7200 bp) was PCR amplified in different concentrations of template DNA. Lane 1: 5 ng template. Lane 2: 10 ng template. Lane 3: 20 ng template. Lane 4: 30 ng template. Lane 5: 40 ng template. They were reacted in 10 μL volume PCR mixture. Lane M: 1000 bp DNA Ladder. Lowest and highest template DNA concentrations caused no amplification. Different concentrations of template DNA caused different amplification rates.

Sequencing of fractionated mitochondria.

The amount of DNA obtained from the mitochondrial fraction varied depending on the size and shape of the sea urchins, and the final DNA yields were usually low (Table 1). Sequencing libraries that were directly generated from the DNA extracted from fractionated mitochondria were found to contain higher amounts of contaminants; for instance, the chromosomes of *Vibrio* and/or *Campylobacter* were additionally sequenced. In addition, in some cases, the use of non-PCR DNA as template resulted in insufficient coverage and incomplete assemblies. Two whole and one partial (*Toxopneustes pileolus*) mitochondrial genomes were acquired based on this procedure.

Phylogenetic analysis.

Analyses based on independent protein-coding regions produced various tree topologies. The combined data, which contains all the protein-coding regions, also produced different topologies. The two clades were interchanged between Bayesian and maximum parsimony analyses. For the combined data, excluding the third codon produced topologies that were consistent between the two analyses (Fig. 4).

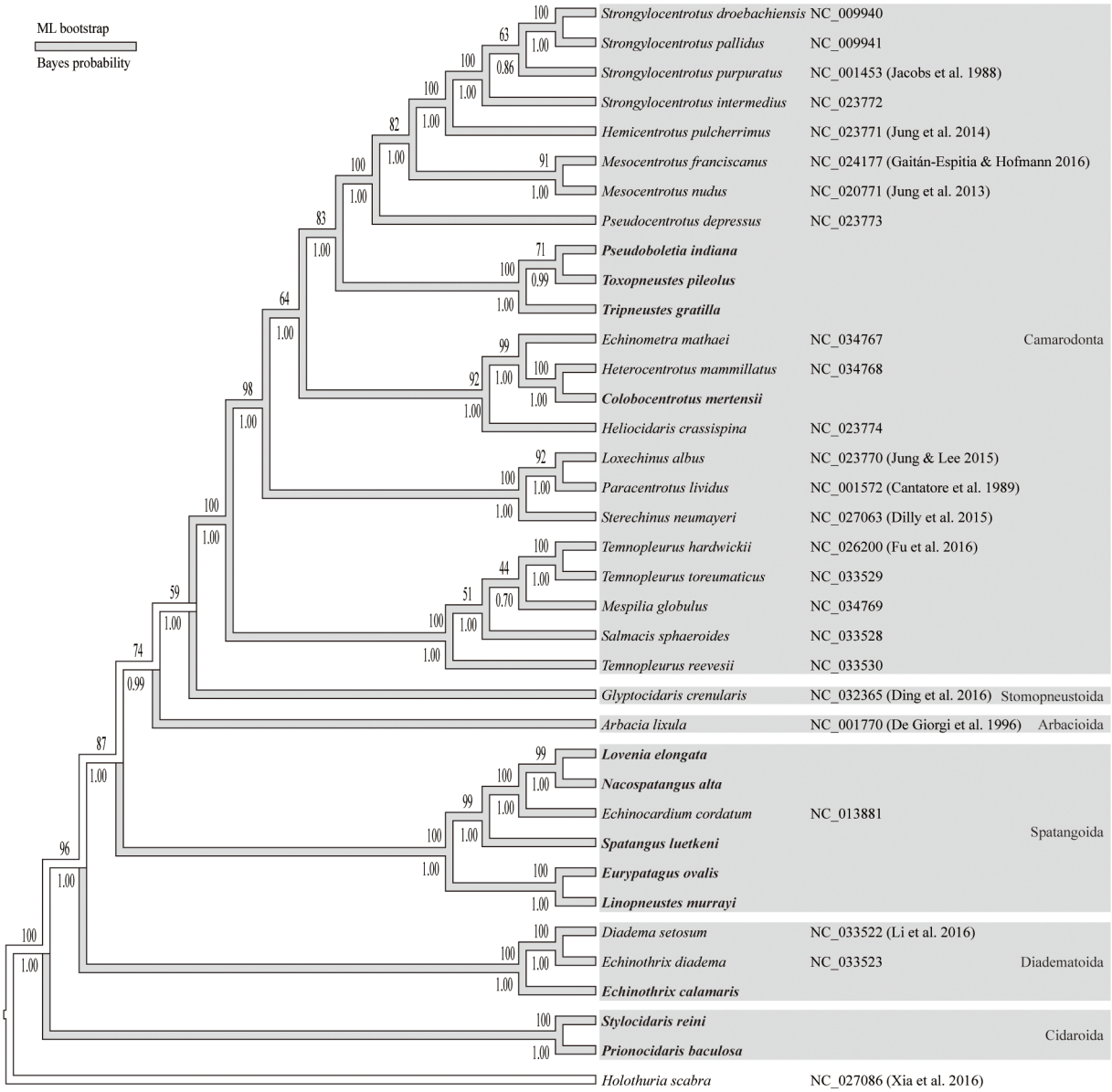

FIGURE 4. A phylogenetic tree based on all protein-coding regions excluding third codon. Bootstrap values are shown above branches, and bayesian posterior probabilities are shown below branches. Sequences obtained in this study are shown in bold.

Discussion

DNA yield obtained by different extraction methods

The DNA yield varied depending on the species and extraction method (Table 1). The DNA yield from the purified mitochondria was low, whereas the yield from gonad tissues extracted using the destructive method was considerably higher. However, in the present study, the amount of starting material available for DNA extraction was not uniform. Moreover, the total DNA contained not only mitochondrial DNA but also nucleic DNA. Therefore, the differences in efficiency between the three methods were not discussed in the present study. However, a simple destructive method is likely to produce superior DNA yield compared to minimally destructive methods.

PCR and sequencing

DNA extraction from the epidermis and basal muscle of spines has been previously described (e.g. Coupé *et al*. 2011; Stockley *et al*. 2005). This method has advantage of redundancy; in particular, the removal of a few spines leads to minimal damage on the test for morphological observation and subsequent analysis. However, as pointed out in Wakayama *et al*. (2016), certain difficulties can be encountered during PCR; using the total DNA extracted from spines as template DNA, we observed that a small difference in the concentration of template DNA resulted in a remarkable difference in PCR efficiency (Fig. 3). To obtain PCR products with high efficiency, it is necessary to significantly reduce the template concentration. Increasing the concentration of the DNA template was found to reduce the PCR efficiency, indicating the presence of an inhibitor. The DNA extracted from spines or inner tissues were usually stained with pigment; the DNA extracts of sea urchins with deep red or purple color were strongly pigmented, whereas the DNA extracts of sea urchins with lighter colors showed lighter color. Contaminants, such as pigments, are likely to inhibit the PCR reaction. Or genomic DNA included in total DNA is likely to attract the primers.

The mitochondrial DNA purified from fractionated mitochondria can be directly used for sequencing; however, optimal results were not consistently obtained. Results revealed the abundance of bacterial contaminants, including *Vibrio* and *Campylobacter*, which reduced the covelage of the target sequences. These contaminants can result in insufficient sequence coverage. Increasing the coverage will require increasing the number of total reads to be sequenced; however, the total DNA yield is insufficient for repetitive sequencing. Purified mitochondria are more likely to obtain good PCR amplification results. The PCR of the purified mitochondria have the advantage of low contamination with genomic DNA or pigment.

Our current findings indicated that sufficient mitochondrial genome sequence data can be obtained using DNA extracted from the spines, tissues inside the test, and coelomic fluid as PCR template. DNA extraction using destructive methods are not necessary, and using minimally destructive methods can maintain the integrity of the tests for subsequent morphological or anatomical examination.

Taken together, we sequenced 11 complete and one partial mitochondrial genome of 12 sea urchin species; of these, the sequences of ten species were generated for the first time. The mitochondrial sequence of *Tripneustes gratilla* was found to be 99.0% and 99.6% identical to those of KY268294 and NC034770 respectively. The sequence of *Nacospatangus alta* was found to be 99.3 % identical to NC_023255.1. The above results demonstrated that the reliability of the method proposed in this study is comparable to those of other studies. In addition, the complete mitochondrial sequence for the order Cidaroida was determined for the first time. The mitochondrial genomes of all sea urchins analyzed showed the same gene arrangement and orientation (Fig. 2).

Phylogeny of the sea urchins

Phylogenetic analyses based on individual regions did not produce a stable topology; different topologies were produced from analyses of different regions. These results are consistent with those reported previous studies (e.g. Jeffery *et al*., 2003; Brosseau *et al*., 2012), suggesting that mitochondrial DNA independent regions that comprise several hundred to a thousand bases contain insufficient information to examine phylogenetic relationships among higher-level clades, such as orders. In a phylogenetic tree constructed using all the protein-coding regions, the two clades were swapped with each other between the two analysis methods. However, removal of the third codon produced consistent results, thereby indicating that the third codon introduced noise in the current analysis.

The combined data, which contain all the protein-coding regions of mitochondrial genome without third codon, produced a similar tree topology to the tree generated by Smith *et al*. (2006), who used three loci, namely, nuclear 18S, 28S, and mitochondrial 16S rRNA. Excluding multiple branches in their phylogenetic tree, the phylogenetic relationships among family-level clades were found to be consistent with our current findings. Analysis based on the combined data returned similar results to those generated using the protein-coding regions of the mitochondrial genomes; both datasets probably retain information suitable for family-to order-level phylogenetic estimation.

The results of phylogenetic analysis in the present study indicated discrepancies in the genus or family relationships. For example, we observed discrepancies for the genus *Temnopleurus* in Camarodonta and family Loveniidae in Spatangoida based on the views given by traditional classification based on morphological data (Fig. 4).

In our phylogenetic tree, the species belonging to the genus *Temnopleurus* were distributed in two

separate branches but comprised a clade together with species belonging to the genera *Mespilia* and *Salmacis*. The monophyly of these three genera were also described by Jeffery *et al*. (2003). They conducted molecular analyses based on the mitochondrial 16S rRNA, COI and nuclear 18S rRNA. Their results showed that *Temnopleurus* species are distributed in two separate branches and form a clade with members of the genera *Mespilia* and *Salmacis*. And they concluded that two subgenera of *Temnopleurus* should each be given full generic status. The result in present study supported the view of Jeffery *et al*. (2003) by more abundant genetic data.

For the family Loveniidae, Kroh and Smith (2010) concluded that the genera *Lovenia* and *Echinocardium* comprise a sister group and belong to the family Loveniidae. However, based on our results, the genus *Nacospatangus*, which belongs to other family Maretiidae based on the results of Kroh and Smith (2010), comprises a sister group along with the genus *Lovenia* and belongs to the clade with the genus *Echinocardium*. The monophyly of the clade is supported by the high bootstrap values and high posterior probabilities. The families Loveniidae and Maretiidae form a sister group in Kroh and Smith (2010). A re-assessment of these two families using a larger taxon sampling is necessary.

Conclusions

1. The destructive DNA extraction procedure resulted in higher DNA yields than the minimally destructive DNA extraction method.

2. The DNA extracted from fractionated mitochondria returned low yields, but no difficulties in PCR amplification were encountered.

3. Even for DNA extracted from fractionated mitochondria, PCR amplification before sequencing is recommended to avoid the sequencing of unwanted bacterial contaminants.

4. Protein coding regions excluding the third codon is effective in family-to order-level phylogenetic estimation for sea urchins.

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