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Caution and Curation for Complete Mitochondrial Genome from Next-Generation Sequencing: A Case Study from *Dermatobranchus otome* (Gastropoda, Nudibranchia)

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ABSTRACT

Mitochondrial genome is an important molecule for systematic and evolutionary studies in metazoans. The development of next-generation sequencing (NGS) technique has rapidly increased the number of mitogenome sequences. The process of generating mitochondrial genome based on NGS includes different steps, from DNA preparation, sequencing, assembly, and annotation. Despite the effort to improve sequencing, assembly, and annotation methods of mitogenome, the low quality and/or quantity sequence in the final map can still be generated through the work. Therefore, it is necessary to check and curate mitochondrial genome sequence after annotation for proofreading and feedback. In this study, we introduce the pipeline for sequencing and curation for mitogenome based on NGS. For this purpose, two mitogenome sequences of *Dermatobranchus otome* were sequenced by Illumina Miseq system with different amount of raw read data. Generated reads were targeted for assembly and annotation with commonly used programs. As abnormal repeat regions present in the mitogenomes after annotation, primers covering these regions were designed and conventional PCR followed by Sanger sequencing were performed to curate the mitogenome sequences. The obtained sequences were used to replace the abnormal region. Following the replacement, each mitochondrial genome was compared with the other as well as the sequences of close species available on the Genbank for confirmation. After curation, two mitogenomes of *D. otome* showed a typically circular molecule with 14,559 bp in size and contained 13 protein-coding genes, 22 tRNA genes, two rRNA genes. The phylogenetic tree revealed a close relationship between *D. otome* and *Tritonia diomea*. The finding of this study indicated the importance of caution and curation for the generation of mitogenome from NGS.

Keywords: *Dermatobranchus otome*, mitochondrial genome, PCR, Sanger sequencing, NGS, analysis pipeline, curation

INTRODUCTION

Mitochondrial genome is the genetic material of mitochondria, a cytoplasmic organelle. Mitochondrial genome of nudibranch is a circular molecule with a common size in the range of 13,880–15,059 bp (Karagozlu et al., 2016; Do et al., 2019). Typically, mitochondrial genome of nudibranch contains 13 protein-coding genes, 22 transfer RNA (tRNA) genes, two rRNA genes. Because of its importance for systematics and phylogenetic reconstruction, the increasing number of mitochondrial genome of nudibranch was sequenced and analyzed over the past decades (https://www.ncbi.nlm.nih.gov/genome/browse#!/organelles/).

Traditionally, mitochondrial genome is sequenced by primer walking method (Boore et al., 2005). The drawback of this method is labor-intensive, time-consuming and less chance to be successful because too many primers and PCR rounds have to use. To reduce this drawback, long-range PCR plus primer walking was introduced. In this method, before primer walking is applied, long-range PCR was employed to sequence long DNA fragments that overlap each other to cover the whole mitochondrial genome. With long-range PCR plus primer walking, sequencing of mitochondrial genome becomes easier, but this method is still slow and costly because a large number of primers have to use as primer walking method (Cameron, 2014). For example, sequencing of an insect mitochondrial genome requires up to 40–50 primers

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(Cameron, 2014). Hence, the introduction of next-generation sequencing (NGS) could be considered as a revolution for mitochondrial genome sequencing.

Compared to Sanger sequencing, NGS is highly accurate and better capable of nucleotide polymorphism detection (Jex et al., 2008). In this method, libraries prepared from PCR products are labeled with coded DNA-reference tags (Timmermans et al., 2010). Subsequently, the reads are generated from DNA fragments of mitochondrial genome by NGS system. Finally, the read data from NGS is targeted for automated assembly and annotation. Together with the introduction of NGS machine, different programs were developed to check and filter raw read data, then assemble and annotate clean data to generate mitochondrial genome sequence. For assembly software, MITObim (Hahn et al., 2013) and NOVOPlasty (Dierckxsens et al., 2017) are frequently used. After assembly, annotation programs as DOGMA (Wyman et al., 2004), MOSAS (Sheffield et al., 2010), MITOS (Bernt et al., 2013) are required for the annotation step. Because of high automation from sequencing to annotation, the advantage of NGS is fast and generation of large raw data. However, due to relying on automation, the quality of mitochondrial genome sequence can be influenced by sequencing, assembly, and annotation methods (Timbó et al., 2017). This suggests the requirement for the curation of mitochondrial genomes generated by NGS.

In practical work, the problem of low quality and/or quantity mitochondrial genome sequence generated from NGS usually challenges us. As an example, this problem occurred as we sequenced the mitochondrial genome of *Dermatobranchus otome*. *D. otome* is a nudibranch in the family Arminidae, order Nudibranchia (Gastropoda). This species was described in Japan and also recorded in Korea (Baba, 1992; Jung et al., 2014). To date, the mitochondrial genome of *D. otome* was not available, and the sequence was generated to investigate mitogenome structure and phylogeny of the species. However, when sequencing and automatic assembly for the mitochondrial genome with NGS, we detected there was an abnormal repeat region in the sequence that could not be fully solved by sequencing another sample with an increase in raw read data for the region coverage. Given the existing problem, primers covering the abnormal region and PCR plus Sanger sequencing were performed to curate the mitochondrial genome. After curation, the revised mitochondrial genome was used for structure and phylogenetic analyses.

MATERIALS AND METHODS

Sample collection and mitochondrial genome sequencing

Steps of mitochondrial genome sequencing based on NGS are briefly described in Fig. 1. At first, *D. otome* samples were collected from Ulleungdo Island, Korea (37°32′20.40″N, 130°50′19.82″E) by scuba diving. The collected samples were preserved in absolute ethanol and deposited in the Department of Biotechnology, Sangmyung University, Korea (SMU0080–SMU0081). Total DNA of *D. otome* was extracted from the specimens using E.Z.N.A.® Mollusc DNA Kit (Omega Bio-tek, Norcross, GA, USA). Before NGS started, partial cytochrome c subunit 1 (COI) gene of specimens was sequenced to confirm the species (data not shown).

Library preparation was conducted with Illumina Truseq Nano DNA sample prep kit (Illumina, San Diego, CA, USA) following the manufacture's instruction. The quality and band size of libraries were estimated by Agilent 2100 bioanalyzer

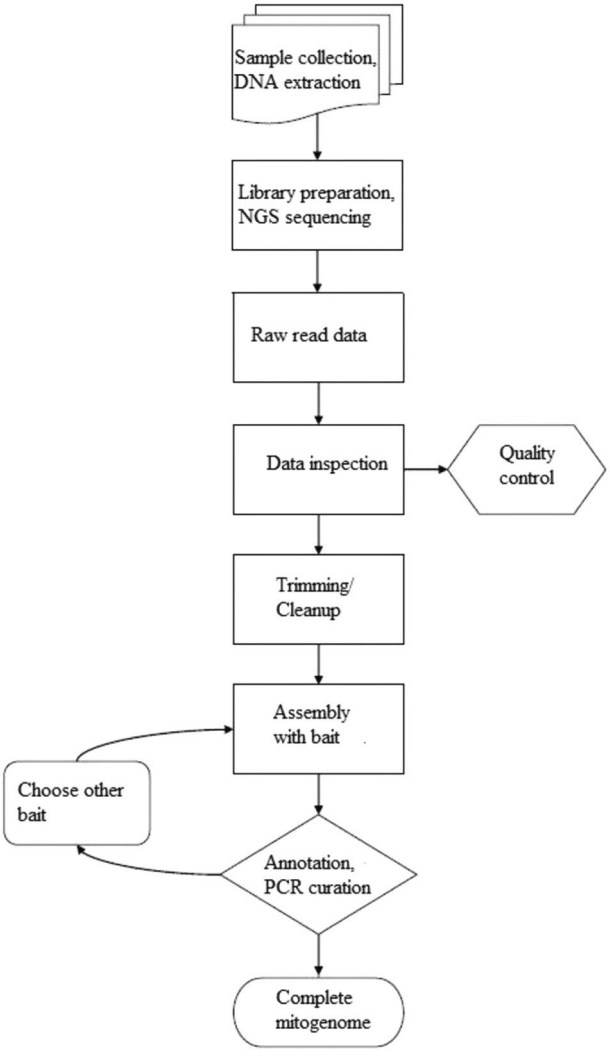

Fig. 1. Flowchart illustrates pipeline for generating mitogenome based on next-generation sequencing (NGS). Polymerase chain reaction (PCR) curation is a necessary step after sequence annotation.

(Agilent, Santa Clara, CA, USA). Paired end reads of mitochondrial genome were generated from prepared libraries by Illumina Miseq system. After checking raw read data and removing adapters, the reads were used for assembly by MITObim (Hahn et al., 2013). COI fragment of *D. otome* was used as bait for assembly. The mitochondrial genome sequence was annotated on MITOS web server with genetic code of invertebrate (Bernt et al., 2013).

The confirmation of 13 protein-coding genes and two ribosomal RNA genes was performed with BLAST searches on the GenBank to align with homologous genes from other nudibranchs. The identification and structure prediction of tRNAs were performed in MITOS web server (Bernt et al., 2013) and ARWEN (Laslett and Canbäck, 2008). The circular map of *D. otome* complete mitochondrial genome was generated and annotated using Geneious 9.1 software (Kearse et al., 2012). The skewness was assessed using the formula: AT skew = [A−T]/[A + T], GC skew = [G−C]/[G + C] (Perna and Kocher, 1995).

Mitochondrial genome curation

After annotation for the first sample of *D. otome*, we detected that there was an abnormal region located between $tRNA^{Cys}$ and $tRNA^{Gln}$. The region was 1,654 bp in size, with four repeats (Appendix 1). The new bait has been used for reassembly, but the problem still existed. For curation of this sequence, two independent methods were approached, which included sequencing another *D. otome* sample with more raw read data and running PCR with a primer set that covers the repeat region. The second sample of *D. otome* was sequenced with approximately 1.5 times more raw data generated from NGS compared to the first sample. Except that, other steps of sequencing, assembly, and annotation were similarly performed as the first sample. In the second method, a set of primers that covers the whole low quality region was designed, and PCR was performed with 2× TOPsimple DyeMIX-Tenuto mastermix (Enzynomics, Daejeon, Korea) and condition in Appendix 2.

Compared to the first sequence, the second sequence was better but there was still an abnormal repeat that was located between *nd2* and *cox1* (Appendix 1). To overcome this problem, a primer set that cover the whole abnormal region was designed for PCR performance (Appendix 2). PCR products from both primer sets were sequenced by ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The generated sequences were used to curate abnormal regions of *D. otome* mitochondrial genomes from NGS.

Phylogenetic analysis

After two mitochondrial genome sequences of *D. otome* were curated, amino acid sequences from 13 protein-coding genes were translated and concatenated for phylogenetic analysis. Other mitochondrial genomes of nudibranchs were obtained from the GenBank. Phylogenetic tree based on concatenated amino acid sequences was reconstructed by Maximum Likelihood and Bayesian methods. The best evolutionary model was searched in MEGA X software (Kumar et al., 2018). Following the finding, Maximum Likelihood method was performed with Le and Gascuel model, and 1,000 bootstrap replicates in MEGA X software (Le and Gascuel, 2008; Kumar et al., 2018). Meanwhile, Bayesian inference was performed for 2,000,000 generations, and sampling was conducted every 1,000 generations by MrBayes v3.2.6 (Ronquist et al., 2012).

RESULTS AND DISCUSSION

Mitochondrial genome curation

Before curation, the sizes of *D. otome* mitochondrial genomes of the first and second samples were 16,054 and 14,789 bp, respectively (Appendix 1). Although both sequences contained an abnormal repeat region, this region in the second sample was short, with only one repeat compared to the long region with four repeats in the first sample (Appendix 1). The abnormal repeat region has not been modified even though several parameters changed in assembly programs. Also, the region has not appeared in the mitochondrial genomes of related species available in the GenBank. Because of these abnormal repeat regions, fragments amplified from PCR that cover while regions were used to replace them in the mitochondrial genomes. Curation resulted in two complete mitochondrial genome sequences of *D. otome* (GenBank accession numbers: MT527185 and MT527186), and both sequences were 14,559 bp in length (Fig. 2). Overall, two mitochondrial genome sequences are almost identical, except for a difference in 19 nucleotides, including 14 nucleotides in coding sequences. However, this difference did not affect amino acid sequences. To summarize, the analysis pipeline for mitochondrial genome sequencing based on NGS with a notation on curation was introduced in Fig. 1. According to the pipeline, curation step should be considered after the annotation of mitochondrial genome.

Complete mitochondrial genome structure

After curation by the experiments, the complete mitochondrial genome of *D. otome* was obtained. The entire mitochondrial genome of *D. otome* consisted of 37 genes, which is characteristic of other nudibranch mitochondrial genomes (Fig. 2). The size of *D. otome* mitochondrial genome is comparable to that of *Tritonia diomedea* (14,540 bp) and *Melibe leonine* (14,513 bp) (Sevigny et al., 2015). The circular mitochondri-

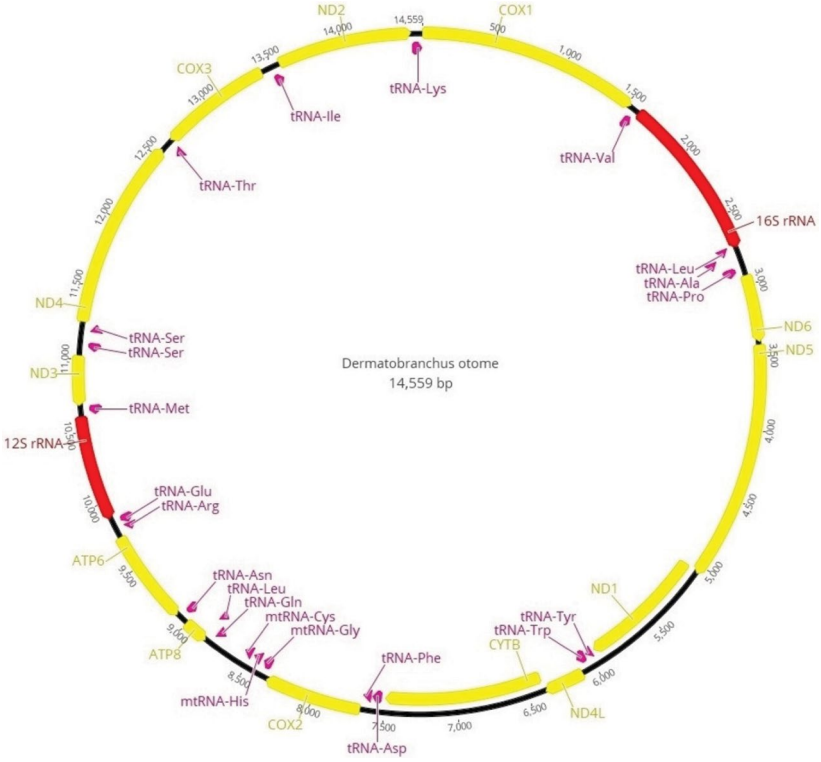

Fig. 2. Complete mitogenome sequence of *Dermatobranchus otome* after curation. Two sequences are almost identical so one sequence is shown here. The sequence was annotated and indicated in Geneious 9.1.

al genome contained 13 protein-coding genes (PCGs) (*atp6*, *atp8*, *cox1-3*, *cytb*, *nd1-6*, *nd4l*), two ribosomal RNA genes (12S rRNA and 16S rRNA) and 22 tRNAs (one gene for each amino acid and two genes for each leucine and serine). Of the 37 genes, 24 genes were encoded on the H-strand, and 13 genes were encoded on the L-strand (Table 1, Fig. 2).

The nucleotide composition of *D. otome* showed A = 26.03%, C = 14.47% G = 20.34%, and T = 39.16% with total G−C of 34.81%. Nine of 13 protein-coding genes had negative AT-skew value, and all 13 protein-coding genes had positive GC-skew value (Table 2). For the whole mitochondrial genome, there were negative AT-skew and positive GC-skew. This suggested the bias for the T and G nucleotides.

Protein-coding genes

Of 13 PCGs, the longest was the *nad5* gene, and the shortest was the *atp8* gene. Nine (*cox1*, *cox2*, *cytb*, *nad1*, *nd2*, *nad4*, *nad4l*, *nd5*, and *nd6*) of the 13 PCGs were encoded on the H-strand while the other four genes (*atp6*, *atp8*, *cox3*, and *nd3*) were encoded on the L-strand.

For initiation, most genes used ATN as a start codon. Among them, eight genes started with ATG, while ATA was observed in *nd6*. Exceptionally, GTG was used for initiation of *cox1* and *nd2* while it was TTG in *nd4l* and *nd1*. TAA is the most common codon used for termination. Eight of 13 PCGs terminated with TAA codon. TAG stop codon was used for four genes (*atp6*, *cox1*, *nd4* and *nd5*) and there was only one gene (*nd3*) using single T as the stop codon.

tRNA and rRNA genes

There were total 22 tRNAs with sizes from 53 to 69 bp (Table 1). Eight of the 22 tRNA genes were encoded on the L-strand and others were encoded on the H-strand. Prediction of secondary structures suggested that the majority of tRNAs of *D. otome* exhibit typical clover-leaf structure (Fig. 2). However, $tRNA^{Gln}$, $tRNA^{Leu}$, and $tRNA^{Tyr}$ remarkably reduced the thymidine-pseudouridine-cytidine (TΨC) loop compared to tRNA with one bp. In addition, the absence of the dihydrouridine (DHU) arm was observed in $tRNA^{Ser}$ (UCU anticodon). This absence can be explained by the deficiency of the dihydrouridine arm, and it is typical characteristics of metazoan mitochondrial genomes (Cameron, 2014).

There were two ribosomal RNA genes detected in the mitochondrial genome of *D. otome*. The large ribosomal RNA (16S rRNA) was 1,151 bp in size and encoded on H-strand, and small ribosomal RNA (12S rRNA) was 734 bp in size and encoded on L-strand.

Table 1. Gene structure of the mitochondrial genome of *Dermatobranchus otome*

Gene	Position		Size (bp)	Codon		Intergenic nucleotide	Strand
	From	To		Start	Stop		
cox1	1	1,530	1,530	GTG	TAG	0	H
$tRNA^{Val}$	1,542	1,608	67			11	H
16S rRNA	1,596	2,746	1,151			−13	H
$tRNA^{Leu}$	2,717	2,783	67			−30	H
$tRNA^{Ala}$	2,780	2,846	67			−4	H
$tRNA^{Pro}$	2,871	2,935	65			24	H
nd6	2,940	3,404	465	ATA	TAA	4	H
nd5	3,421	5,070	1,650	ATG	TAG	16	H
nd1	5,048	5,959	912	TTG	TAA	−23	H
$tRNA^{Tyr}$	5,975	6,027	53			15	H
$tRNA^{Trp}$	6,033	6,098	66			5	H
nd4l	6,099	6,392	294	TTG	TAA	0	H
cytb	6,385	7,512	1,128	ATG	TAA	−8	H
$tRNA^{Asp}$	7,532	7,599	68			19	H
$tRNA^{Phe}$	7,604	7,669	66			4	H
cox2	7,670	8,344	675	ATG	TAA	0	H
$tRNA^{Gly}$	8,346	8,414	69			1	H
$tRNA^{His}$	8,434	8,495	62			19	H
$tRNA^{Cys}$	8,509	8,571	63			13	H
$tRNA^{Gln}$	8,731	8,793	63			159	L
$tRNA^{Leu}$	8,790	8,853	64			−4	L
atp8	8,828	9,007	180	ATG	TAA	−26	L
$tRNA^{Asn}$	9,011	9,076	66			3	L
atp6	9,086	9,751	666	ATG	TAG	9	L
$tRNA^{Arg}$	9,754	9,815	62			2	L
$tRNA^{Glu}$	9,816	9,882	67			0	L
12S rRNA	9,883	10,616	734			0	L
$tRNA^{Met}$	10,617	10,684	68			0	L
nd3	10,684	11,035	352	ATG	T--	−1	L
$tRNA^{Ser}$	11,060	11,128	69			24	L
$tRNA^{Ser}$	11,197	11,251	55			68	H
nd4	11,257	12,570	1,314	ATG	TAG	5	H
$tRNA^{Thr}$	12,596	12,659	64			25	L
cox3	12,655	13,434	780	ATG	TAA	−5	L
$tRNA^{Ile}$	13,488	13,554	67			53	H
nd2	13,555	14,490	936	GTG	TAA	0	H
$tRNA^{Lys}$	14,494	14,559	66			3	H

H or L indicates that the gene is encoded by the heavy or light strand.

Intergenic region

There were 21 intergenic regions in the mitochondrial genome of *D. otome* with a total length of 482 bp. The number of intergenic nucleotides ranged from 1 bp to 159 bp. The longest intergenic space is located between $tRNA^{Cys}$ and $tRNA^{Gln}$ (159 bp), containing 64.15% A−T and 35.85% G−C. There were nine overlapping regions between genes with the range of 1 bp to 30 bp. The longest overlap was located between 16S rRNA and $tRNA^{Leu}$.

Phylogenetic analysis

Two sequences of *D. otome* and nudibranch sequences obtained from the GenBank were used to infer the phylogenetic trees. Bayesian inference and Maximum likelihood trees indicated the consistency of phylogenetic relationships among nudibranchs (Fig. 4). As shown in Fig. 4, two *D. otome* mitochondrial genomes formed a cluster. *D. otome* was sister to other species in the suborder Cladobranchia as *Tritonia diomedea* and *Melibe leonine* with high support.

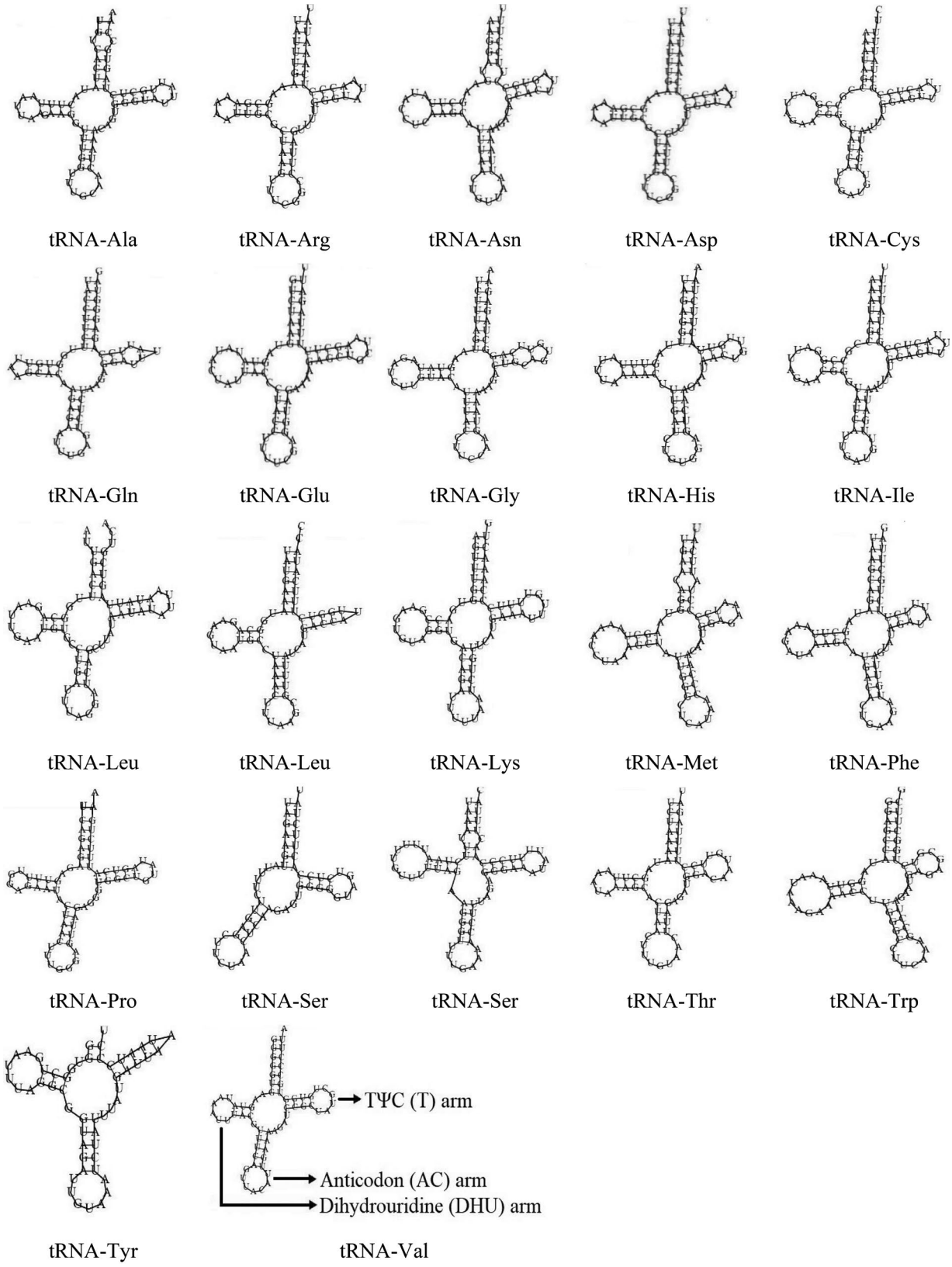

Fig. 3. Secondary structures of 22 transfer tRNA from *Dermatobranchus otome* mitogenome. The label of tRNAs corresponds to their amino acids. Structural elements in tRNAs are illustrated as for tRNA-Val.

Table 2. Base composition and skewness of *Dermatobranchus otome* mitogenome (MT527185)

Region	A%	C%	G%	T%	A-T%	G-C%	AT-skew	GC-skew
Mitogenome	26.03	14.47	20.34	39.16	65.19	34.81	−0.201	0.169
atp6	34.08	17.27	20.12	28.53	62.61	37.39	0.089	0.076
atp8	33.33	14.44	16.11	36.12	69.45	30.55	−0.040	0.055
cox1	21.96	15.42	21.24	41.38	63.34	36.66	−0.307	0.159
cox2	24.3	14.96	22.96	37.78	62.08	37.92	−0.217	0.211
cox3	34.36	18.46	19.23	27.95	62.31	37.69	0.103	0.020
cytb	22.07	16.31	19.86	41.76	63.83	36.17	−0.308	0.098
nd1	22.37	14.36	20.72	42.55	64.92	35.08	−0.311	0.181
nd2	21.05	11.65	22.97	44.33	65.38	34.62	−0.356	0.327
nd3	35.8	17.05	22.73	24.42	60.22	39.78	0.189	0.143
nd4	22.37	11.72	21.39	44.52	66.89	33.11	−0.331	0.292
nd4l	21.77	11.9	21.09	45.24	67.01	32.99	−0.350	0.279
nd5	22.48	13.7	20.48	43.34	65.82	34.18	−0.317	0.198
nd6	21.72	8.39	23.01	46.88	68.6	31.4	−0.367	0.466
12S rRNA	25.48	16.89	18.39	39.24	64.72	35.28	−0.213	0.043
16S rRNA	33.01	12.25	18.77	35.97	68.98	31.02	−0.043	0.210

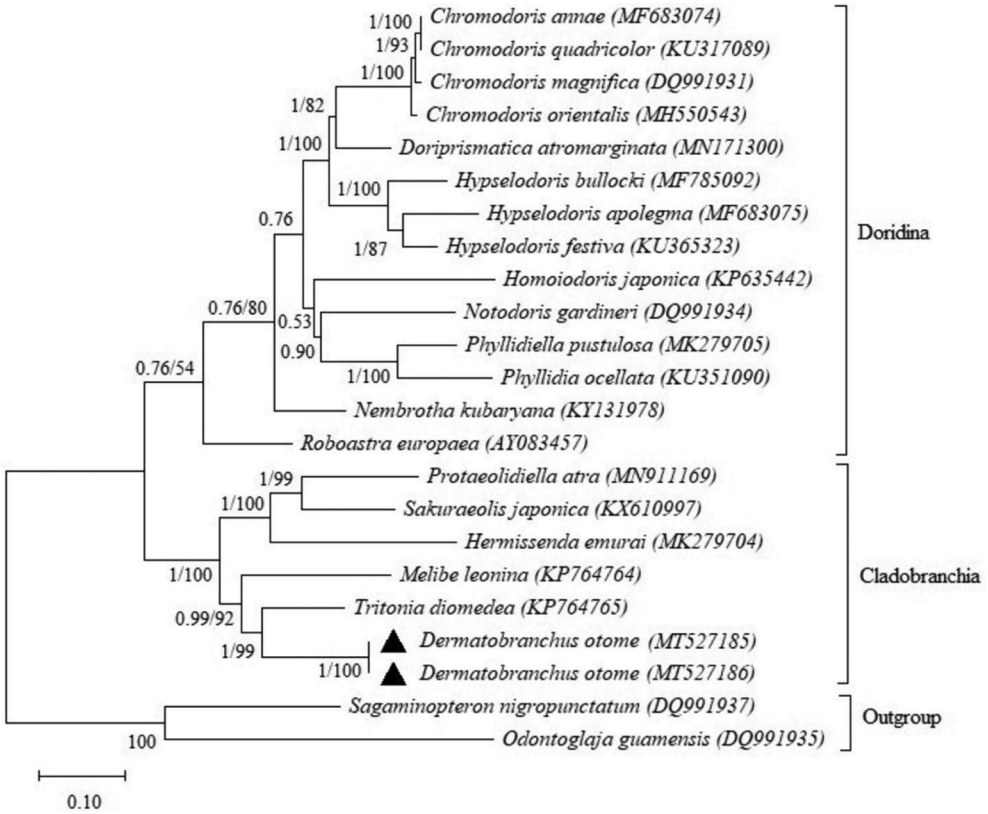

Fig. 4. Phylogenetic tree shows position of *Dermatobranchus otome* in the order Nudibranchia. Genbank accession numbers are placed next to species names. Two sequences of *D. otome* mitogenome are marked with black triangle. The tree was reconstructed based on amino acid sequences of 13 protein-coding genes by Maximum Likelihood and Bayesian methods. *Smaragdinella calyculata* and *Odontoglaja guamensis* (order Cephalaspidea) were used as the outgroup. The node values indicate bootstrap value (right) and posterior probability value (left). Bootstrap values > 50 (maximum likelihood) and posterior probability values > 0.5 (Bayesian inference) are shown at the nodes.

The phylogenetic tree also indicated that there are two suborders of nudibranchs, Cladobranchia and Doridina. Our finding is consistent with previous reports on relationships of nudibranchs based on mitochondrial genome analyses (Sevigny et al., 2015; Do et al., 2019).

Curation of automatic assembly and annotation results

Good quality of mitochondrial genome sequence is a critical step for systematic and phylogenetic studies. Traditionally, mitochondrial genome sequencing is performed by primer walking method or long-range PCR plus primer walking method. However, these approaches are time-consuming and costly. The development of NGS has significantly boosted the effort of mitochondrial genome sequencing (Cameron, 2014). Since the first introduction, NGS showed it as a rapid and productive method for the generation of the mitochondrial genome. However, sequencing, assembly, and annotation process can induce variability in sequence quality (Timbó et al., 2017). The curation of sequence after annotation is important to improve accuracy and quality of the mitochondrial genome. This study showed method for curation of *D. otome* mitochondrial genome by conventional PCR and Sanger sequencing. After curation, two mitochondrial genomes of *D. otome* were qualified for structure and phylogenetic analyses.

Along with NGS, a number of automated programs have been introduced and applied to work with NGS data. It is clear that the development of these programs has facilitated mitochondrial genome sequencing. However, it is also reported that assembly and annotation programs may vary remarkably in their quality, and little has been known about the accuracy of the deposited mitochondrial genome sequences and their annotations (Timbó et al., 2017). As the results of the present study, the low quality region in the mitochondrial genome of *D. otome* was still present even though more raw read data was added to cover the sequence. Therefore, the need for mitochondrial genome curation are emphasized after automation pipelines are employed to assembly and annotate the sequence.

In conclusion, NGS together with automation analyses has proven as a rapid and productive method for generating mitochondrial genome sequences. To fulfil the error from this method, our finding demonstrated that caution and curation for mitochondrial genome sequence with conventional PCR and Sanger sequencing are required. This procedure should be considered for any mitochondrial genome generated from NGS. In addition, more sophisticated assembly and annotation programs are demanded to reduce the error and improve the accuracy of mitogenome sequencing.

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CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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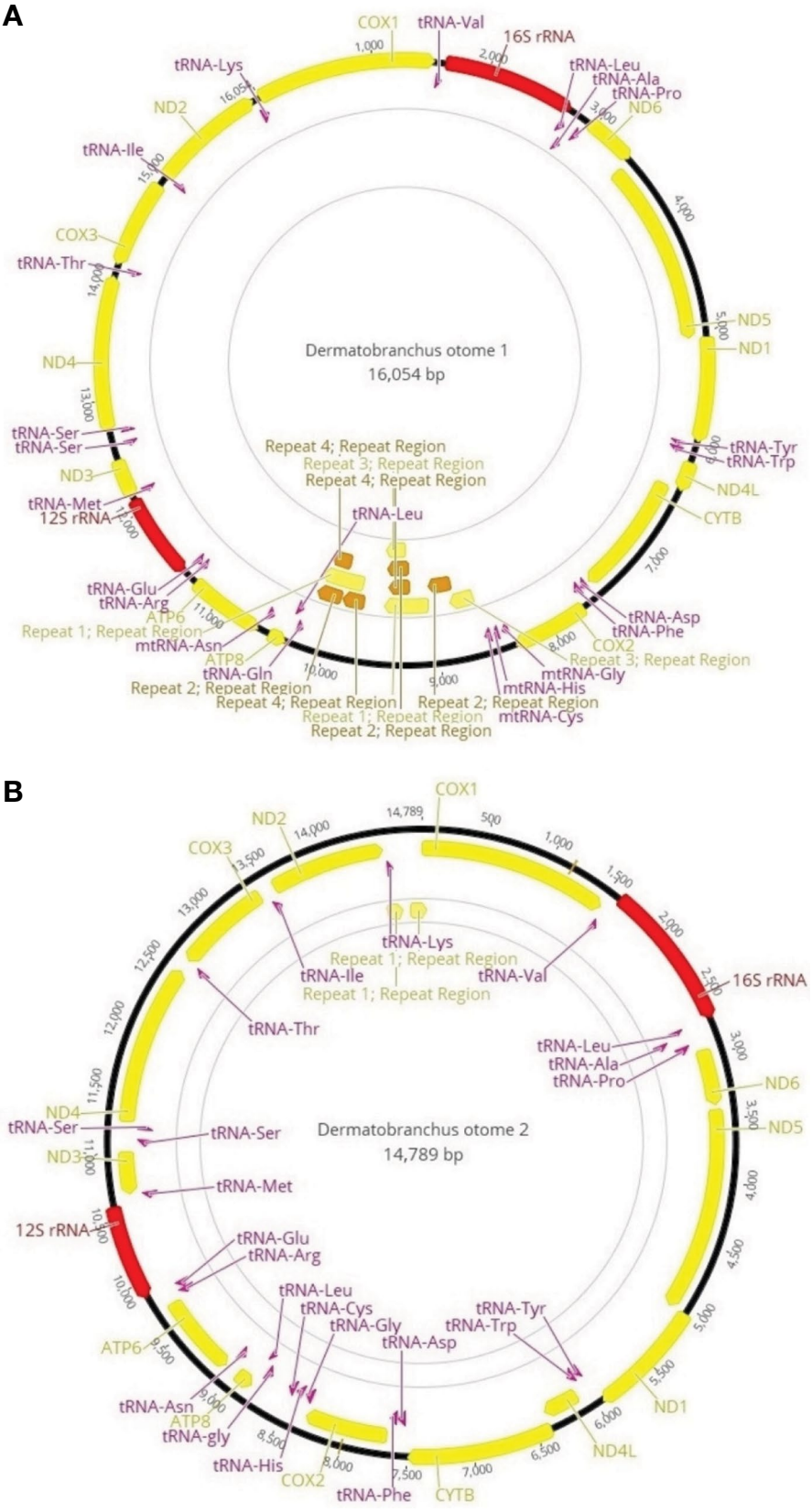

Appendix 1. Mitogenome sequence of *Dermatobranchus otome* before curation with PCR: A, Sample 1; B, Sample 2.

Appendix 2. Primers and PCR condition used for curation of mitogenomes of *Dermatobranchus otome*

Sample	Primer set	Sequence (5′-3′)	PCR condition
1	F1	GAGACTACTGTTATTGCTACATCA	Initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 30 s, extension at 72°C for 45 s; followed by a final extension at 72°C for 5 min.
	R1	GTTTACGAAGATGGGTAGAGCC	
2	F2	CACTTTAATTTTAGCAGGACGT	Initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 30 s, extension at 72°C for 45 s; followed by a final extension at 72°C for 5 min.
	R2	AGTGGCTGCTATAAAAGTATCA	