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The first mitochondrial 5-methylcytosine map in a non-model teleost (*Oreochromis niloticus*) reveals extensive strand-specific and non-CpG methylation

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ABSTRACT

DNA methylation is one of the main epigenetic mechanisms that regulate gene expression in a manner that depends on the genomic context and varies considerably across taxa. This DNA modification was first found in nuclear genomes of eukaryote several decades ago and it has also been described in mitochondrial DNA. It has recently been shown that mitochondrial DNA is extensively methylated in mammals and other vertebrates. Our current knowledge of mitochondrial DNA methylation in fish is very limited, especially in non-model teleosts. In this study, using whole-genome bisulfite sequencing, we determined methylation patterns within non-CpG (CH) and CpG (CG) contexts in the mitochondrial genome of Nile tilapia, a non-model teleost of high economic importance. Our results demonstrate the presence of mitochondrial DNA methylation in this species predominantly within a non-CpG context, similarly to mammals. We found a strand-specific distribution of methylation, in which highly methylated cytosines were located on the minus strand. The *D-loop* region had the highest mean methylation level among all mitochondrial loci. Our data provide new insights into the potential role of epigenetic mechanisms in regulating metabolic flexibility of mitochondria in fish, with implications in various biological processes, such as growth and development.

1. Introduction

The mitochondrion is a cellular organelle that plays a central role in the regulation of respiration and metabolic processes [1], and contains its own genome, which is also known as mitochondrial DNA (mtDNA) or mitogenome. The mitogenome has conserved characteristics across vertebrates: it has light (minus) and heavy (plus) strands that contain 13 protein-coding genes (subunits of the oxidative phosphorylation system), 22 transfer RNAs (tRNAs), two ribosomal RNA genes (12S rRNA and 16S rRNA), and a non-coding control region comprising the *D-loop* and associated promoters [2]. Mitochondrial epigenetics takes its roots at the beginning of the 1970s [3] but the first studies could not find evidence of mtDNA methylation in humans and other vertebrates [4]. Nevertheless, the identification of DNA methyltransferase 1 in mammalian mitochondria and the description of mtDNA methylation dynamics in relation to mitochondrial transcription factors have stimulated research on the mitoepigenome [5,6]. It has been shown in mammals that genetic and epigenetic alterations in mitogenome could have a high impact on a variety of physiological and pathophysiological processes [7]. The mitochondrion plays an important role in regulating the energetic processes in liver as well as in the complex regulation of fish growth through the hepatosomatic axis [8]. Nevertheless, mechanisms of mitogenome functionality (including mtDNA methylation) are still unexplored in teleosts, the most abundant group containing 96% of all fishes and accounting for half of all extant vertebrates [9].

A more detailed and accurate estimation of various mitochondrial

Abbreviations: mtDNA, mitochondrial DNA or mitogenome;; tRNA, transfer RNA;; rRNA, ribosomal RNA;; WGS, whole-genome sequencing;; WGBS, whole-genome bisulfite sequencing;; bp, base pairs.

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epigenetic modifications gained recognition only recently, with the emergence of single-cell resolution methods [10,11]. In mammals, methylated cytosines in mtDNA are predominantly localized within a non-CpG context in contrast to the nuclear CpG methylation pattern [12]. The functional significance of such non-CpG methylation has been described in skeletal muscle cells, where methylation of the PGC-1α promotor was found to control mitochondrial density [13]. Mitochondrial DNA has a lower methylation level than the nuclear genome [14,15], but it varies with tissue type, developmental stage, age, and other factors [16–18]. This variation in mtDNA methylation has led to a certain level of controversy on how to best determine mtDNA methylation [19]. Nevertheless, an increasing number of reports demonstrate the existence of mtDNA methylation in mammals and its functional significance [14,20,21], as well as its dependance on the inviromental factors [22,23]. Despite many knowledge gaps in the current understanding about the functional role of mtDNA methylation, this mitogenome modification is being considered as a promising biomarker tool in diagnostics of different human and animal diseases [24].

Mitochondrial genomes of thousands of teleosts have been sequenced to date, demonstrating that they usually have a conserved number of mitochondrial genes and the typical gene order, base composition, and codon usage which are common among other vertebrates [2,25]. These mitogenome sequences are widely used in various phylogenetic studies [26], but the pattern, conservation and function of mtDNA methylation in fish have been poorly investigated [27]. To the best of our knowledge, there are only two reports in zebrafish (*Danio rerio*), that described methylation of the mitochondrial genome. These studies showed the almost entirely hypomethylated status of mtDNA in sperm and oocytes of *D. rerio* [28] as well as different mtDNA methylation levels during zebrafish embryogenesis [29].

However, there is hardly any information about the methylation patterns of mtDNA in adult fish tissues, in spite of its importance in regulating important biological processes, such as metabolism and growth [30]. Energy metabolism in hepatic cells integrates metabolism of proteins, lipids and carbohydrates, and differences in mitochondrial efficiency are also known to influence growth [8]. In the present study, we determined the global mtDNA methylation patterns in liver of adult Nile tilapia (*Oreochromis niloticus*), since it is one of the most important fish farmed worldwide, with a production of 4.6 million tons in 2018 [31]. Also, its genome assembly is available and we have recently discovered that differences in methylation of the nuclear genome are associated with growth performance [30]. We now showed, using bisulfite sequencing of mitochondrial DNA, that the highest number of methylated cytosines are located within a non-CpG context (82.8%). Moreover, the mitochondrial non-coding *D-loop* region was the most methylated part of the mitogenome, similarly to mammals [32]. We also showed significant differences in average methylation level between light and heavy strands of the Nile tilapia mtDNA.

2. Materials and methods

2.1. Sampling

This study was approved by Nord University's (Bodø, Norway) ethics board and the Norwegian Animal Research Authority (FOTS ID 1042). All procedures involving animals were conducted according to the EU Directive 2010/63 on the use of animals for scientific purposes.

Nile tilapia females (specimens: C_S3, SL_B1, SL_B4, SL_S2, and SL_S5, see Supplementary Table 1) from the third generation of our in-house domestication program were kept in a freshwater recirculating aquaculture system at Nord University's research station (Bodø, Norway). The rearing conditions were as follows: pH = 7.5, temperature = 28 °C, photoperiod adjusted at 11:13 h dark:light. The fish were fed *ad libitum* with 0.15–0.8 mm Amber Neptun pellets (Skretting, Norway). Prior to sampling, five fish were euthanized with clove oil (Sigma Aldrich, USA) using a 1:10 mix of 15 mL clove oil in 95% ethanol diluted in 10 L of freshwater. Liver samples were collected from the left lobe and around the entry point of the portal vein from 5 one-year-old females, snap-frozen in liquid nitrogen and stored at −80°C until DNA extraction.

2.2. DNA extraction and library preparation

Genomic DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Germany) according to the manufacturer's recommendations. DNA purity was assessed by NanoDrop ND-1000 spectrophotometer (Thermofisher Scientific, USA) and its quantity and quality were determined with Qubit (Thermofisher Scientific) and Tape Station Genomic DNA ScreenTape Assay (Agilent Technologies, USA).

Nuclear (linear) DNA digestion was performed with Plasmid-Safe ATP-Dependent DNase (Lucigen, USA) following the manufacturer's protocol. The incubation time at 37 °C was optimized to 16 h. Then, DNAse was inactivated at 70 °C for 30 min. Mitochondrial DNA was further purified from short remaining fragments of nuclear origin using the Zymoclean™ Large Fragment DNA Recovery Kit (Zymo Research, USA). Linearization of mtDNA was performed by random fragmentation with dsDNA Fragmentase (New England Biolabs, USA) for 5 min at 37 °C. Fragmented DNA was cleaned up using the DNA Clean & Concentrator-5 kit (Zymo Research, USA).

One Nile tilapia genomic DNA library (specimen C_S3; Supplementary Table 1) was constructed and sequenced to be used as reference in our study, since the publicly available mitochondrial genome sequences vary significantly between *O. niloticus* wild populations and strains, possibly due to species identification errors and interspecific hybridization events occurring with closely related *Oreochromis* species [33–36]. The gDNA library was prepared using the Nextera DNA Flex Library Prep Kit (Illumina, USA) and quantified using Agilent High Sensitivity D1000 ScreenTape on the 2100 Tape Station instrument (Agilent Technologies, USA). Illumina Miseq genome analyzer (Illumina) was used with paired-end reads (300 base pairs (bp) length) for whole-genome sequencing (WGS).

Bisulfite mitochondrial DNA libraries from the five Nile tilapia specimens (Supplementary Table 1) were constructed using the Pico Methyl-Seq Library Prep Kit (Zymo Research, USA), following the manufacturer's instructions. In short, normalized DNA samples (2.5 ng, 20 μL) were converted with Lightning Conversion Reagent for 8 min at 98 °C, then 1 h at 37 °C, and then cooled to 4 °C. Immediately after that step, the bisulfite-converted samples were desulphonated, purified, and amplified with PrepAmp Primer. The resulting products were purified with the DNA Clean & Concentrator kit (Zymo Research, USA) and further amplified with LibraryAmp Primers. Amplified WGBS libraries were quantified using Agilent High Sensitivity D1000 ScreenTape on the 2100 Tape Station instrument (Agilent Technologies, USA). Illumina NextSeq 500/550 High Output v2.5 flowcell of Illumina Illumina NextSeq 500 genome analyzer (Illumina, USA) with single-end reads of 75 bp length was used for whole-genome bisulfite sequencing (WGBS).

2.3. Bioinformatics and statistical analyses

Raw WGS and WGBS reads were converted to FASTQ format and demultiplexed using bcl2fastq version 2.16 (Illumina, USA) and their quality was examined with the FastQC tool. Raw sequencing reads were filtered by quality (phred 30) and library adapters were trimmed using cutadapt software (version 2.1) [37].

The NOVOPlasty software (version 3.3) [38] was used for *de novo* assembly of the mitochondrial genome of Nile tilapia. For assembly, we used the previously published *Oreochromis niloticus* mitogenome (GU238433.1) as a reference and a k-mer size of 39. The resulting consensus sequence was annotated using the GeSeq web-interface [39]. The overall base composition of the mitogenome was estimated as the ratio between the total number of each nucleotide and the whole assembly size. The obtained annotation was used to define partitions in the subsequent phylogenetic analysis. The phylogenetic relationships

between our specimen and other *Oreochromis* species based on coding sequences (Supplementary Table 2) were reconstructed using maximum likelihood analysis. Phylogenetic reconstruction was performed using RAxML (version 8.1.20) with GTRGAMMA model and default parameters [40]. Branch supports were computed out of 100 bootstrapped trees. The phylogenetic tree topology was drawn in iTOL (version 4) [41]. The blackchin tilapia (*Sarotherodon melanotheron*) and keppi (*S. lohbergeri*) were used as an outgroup.

The mitochondrial genome assembled on this step of our study was used as a reference in the analysis of Nile tilapia mtDNA methylation from WGBS data. To remove possible nuclear DNA fragments of mitochondrial origin (nuMTs) in the analyzed data set, reads were first aligned to the Nile tilapia reference genome (assembly O_niloticus_UMD_NMBU), excluding the mitogenome, and using the Bismark version 0.19.1 pipeline (parameters: –non_directional -q –score-min L,0,-0.2) [42]. The unmapped reads were aligned to the previously *de novo* assembled mitochondrial genome of Nile tilapia (NCBI accession number: MW149239). Output reads were mapped to the original and *in silico*-modified genomes using Bismark version 0.19.1 (parameters: –non_directional -q –score-min L,0,-0.2). MethylKit was used for normalization of the methylation calling data (CpG, CHH, CHG files) and for further calculation of the coverage and methylation levels of each sample [43]. Analysis of methylation calls was performed on two datasets: 1) one to capture possible individual variations among the 5 samples and 2) the second by pooling the data to identify a common pattern in the liver mitochondrial epigenome. These data were normalized by the median and used for the comparative analysis. For the second part of the analysis, we pooled the sequenced data from five individuals and analyzed the output as one average merged sample. We applied background methylation parameters as it was performed by Dou and colleagues [29], with a $>$ 10% methylation cut-off, *i.e.*, setting $<$10% methylation to 0 as background, while keeping the baseline merged data filtering parameters (each analyzed cytosine has coverage $\geq$265). The average methylation levels per gene were calculated as the ratio between methylated and unmethylated cytosines in each gene. The Circos toolkit [44] was used for the visual representation of methylated sites across the mitochondrial genome.

3. Results and discussion

3.1. The mitochondrial genome of Nile tilapia

68,175,294 Illumina paired-end reads were generated from the *O. niloticus* gDNA library. These reads were used for Nile tilapia mitogenome *de novo* assembly (average coverage 426×). The mitochondrial genome of Nile tilapia (NCBI accession number: MW149239) consisted of 16,626 bp in length and contained a conserved number of mitochondrial genes and the typical gene order, base composition, and codon usage, which are common among other vertebrates (Fig. 1). The overall base composition of the genome in descending order was 30.3% C, 28.1% A, 26.2% T and 15.4% G, without a significant AT bias of 54.3%.

To estimate the phylogenetic position of our specimen among other *Oreochromis* species we conducted a maximum likelihood phylogenetic analysis. The intergenic segments and the control region were discarded from the alignments as they contributed to phylogenetic noise and made the phylogenetic tree unstable. Phylogenetic reconstruction of the Nile

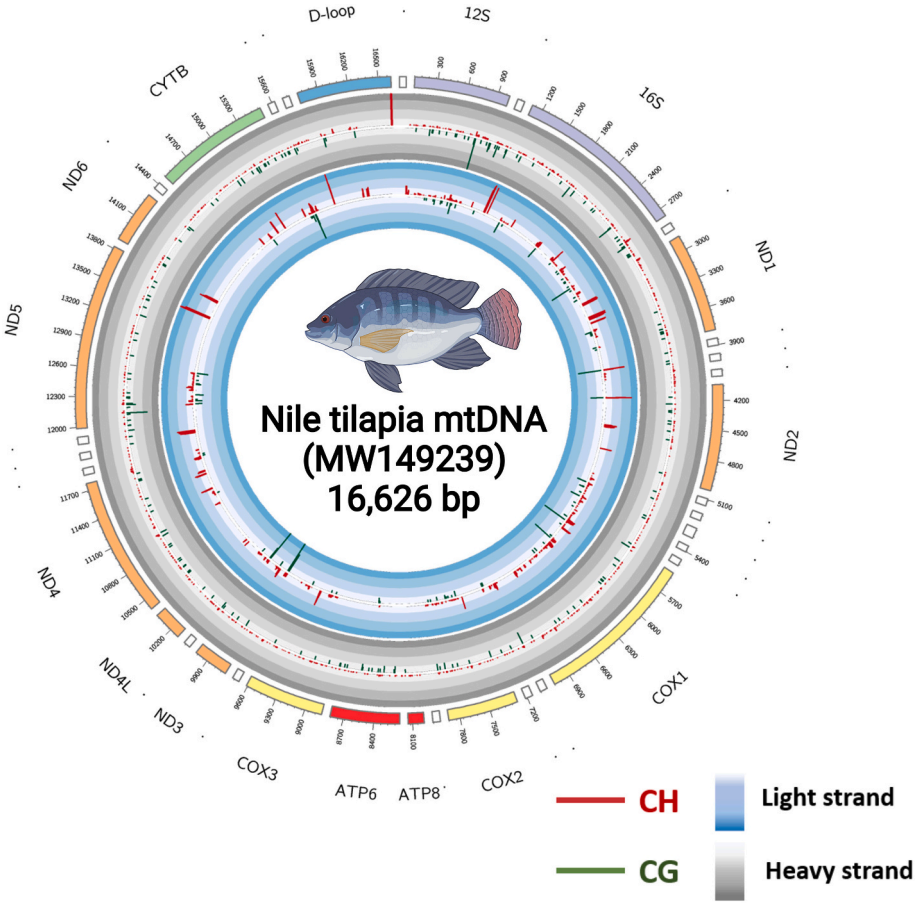

Fig. 1. The mitochondrial genome of Nile tilapia. (A) Graphical map of the complete mitochondrial genome of Nile tilapia with its gene features. Green and red peaks (oriented in and out, respectively) represent the methylation levels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tilapia specimen (MW149239) from Nord University's research station showed that it is very similar to the other Nile tilapia strains previously sequenced (Fig. 2).

3.2. mtDNA methylation in liver of adult Nile tilapia

The total number of reads generated from five specimens ranged from 16,335,889 to 16,960,827 per WGBS-library (NCBI accession numbers are presented in Supplementary Table 2). DNA reads were mapped to the previously assembled mitochondrial genome (NCBI accession number: MW149239) after adapter trimming and quality filtration, and 0.6 to 1.1% reads per WGBS library were mapped to the reference. Mapping and quality trimming statistics are presented in Supplementary Table 3. The sequencing depth of the quality- and adapter-trimmed uniquely mapped mitochondrial reads varied from 167 to 304 times in each of the five libraries (Supplementary Table 3).

As the methylation levels did not differ significantly between individuals (Supplementary Table 4), we used the merged dataset from five WGBS libraries to determine the mitochondrial 5-methylcytosine map for Nile tilapia liver and to describe its general pattern in further analyses. The methylation pattern of the Nile tilapia mitochondrial genome (in five merged liver datasets) covered 21.4% of cytosines (Fig. 3A). The distribution of methylated cytosine varied with the nucleotide context. Out of 3566 methylated cytosines in the Nile tilapia mitochondrial genome, 82.8% were in a non-CpG context and only 17.3% were found as a part of CpG context (Fig. 2B). Methylated cytosines within CpT, CpA, and CpC contexts are defined as a non-CpG cluster. The highest number of methylated cytosines was located within a CpT context (31.9%), followed by CpC (25.7%) and CpA (25.1%), *i.e.* CpN equal 82.7%. The frequencies for CpN dinucleotides and their methylation across the Nile tilapia mitogenome are presented in Supplementary Fig. S1.

A similar proportion of methylated cytosines (CpG to non-CpG context) was observed across all five libraries, as expected. In each individual library, up to 8.4% of methylated cytosines were located within CHH and CHG (where H is any base except G) methylation contexts. Most methylated cytosines were identified in CHH (up to 6.4%) and CHG (up to 2.1%) contexts, and only 2.4% of methylated cytosines were located in a CpG context (Supplementary Fig. S2; Supplementary Table 5).

3.3. Strand-specific distribution of methylated cytosines in mtDNA

We observed an uneven distribution of methylated cytosines with respect to their location on the two light and heavy mtDNA strands. The frequency of methylated cytosines in the cytosine-rich light strand (minus strand) was 37.7%, whereas the heavy strand (plus strand) showed 62.3% (Fig. 1; Fig. 4A). However, this lower number of methylated cytosines located on the light strand had higher average methylation levels than those in the heavy strand (Fig. 1; Fig. 4B). We tested this observation for the light strand using merged data for the analysis. Interestingly, highly methylated cytosines are predominantly located on the light strand of mitochondrial DNA of Nile tilapia (95.4%). Moreover, 97.2% out of them are observed in non-CpG context.

3.4. Methylation of mtDNA genes within CpG and non-CpG contexts

The highest methylation levels among mitochondrial protein-coding genes were observed within *NADH-ubiquinone oxidoreductase chain 5 – nd5* (12.5%), *NADH-ubiquinone oxidoreductase chain 1 – nd1* (11.2%), and *cytochrome b – cytb* (9.9%) genes. At the same time the highest methylation level was found in the non-coding *D-loop* region (18.5%) (Fig. 5). Methylated cytosines were predominantly located within a non-CpG context; for example, within *nd1*, average methylation levels were 8.4% and 2.8% within non-CpG and CpG contexts, respectively. However, a nearly equal proportion of CpG and non-CpG methylated cytosines were found within *NADH-ubiquinone oxidoreductase chain 3 – nd3* (4.4% and 5.5%) and *NADH-ubiquinone oxidoreductase chain 4 L – nd4l* (3.2% and 4.4%), respectively (Fig. 5). A similar distribution was found within the five individual WGBS datasets analyzed. The highest mean methylation levels within CpG context were observed within *nd3* (5.5%), *nd4l* (4.4%), and the *D-loop* (3.1%) (Supplementary Fig. S3; Supplementary Table 5). Interestingly, the same mitochondrial regions and protein-coding genes that have the highest average methylation levels, such as the *D-loop*, and the genes of the membrane arm of respiratory complex I (type I NADH dehydrogenase) *nd1*, *nd2*, and *nd5*, belong to the highly methylated regions/sites. They have the highest methylation levels among all mitochondrial genes. Within a CpG context, the genes of the respiratory complex I *nd3* and *nd4l* have higher average methylation levels than other protein-coding genes.

The average methylation of tRNA genes reached 6.3% (*tRNA-Tyr*). For several tRNA genes (*tRNA-Ile*, *tRNA-Met*, *tRNA-His*, *tRNA-Pro*), methylation was found only within a CpG context. *tRNA-Phe* showed a complete absence of cytosine methylation (Supplementary Fig. S4). The comparative analysis of methylation levels across tRNA and rRNA genes indicated a different pattern compared to mitochondrial protein-coding genes in distribution of methylated cytosines among CpG and non-CpG sites. Here the proportion between CpG and non-CpG methylated nucleotides within a gene tends to have an equal distribution. In comparison to mtDNA encoded tRNAs and rRNAs genes, protein-coding genes are more substantially methylated.

DNA methylation plays a crucial role in the regulation of different processes in eukaryotes [45]. This type of DNA modification is particularly frequent among vertebrates, including teleost fish [46]. In teleosts, DNA methylation participates in different physiological processes, growth and development, adaptation to new environmental conditions, sex determination, and many others [30,47–53]. In contrast to the nuclear genome, methylation in mitochondrial DNA has been given low attention for a long time, and its presence has been disputed [12,54].

The controversial view on methylation of the mitogenome is related

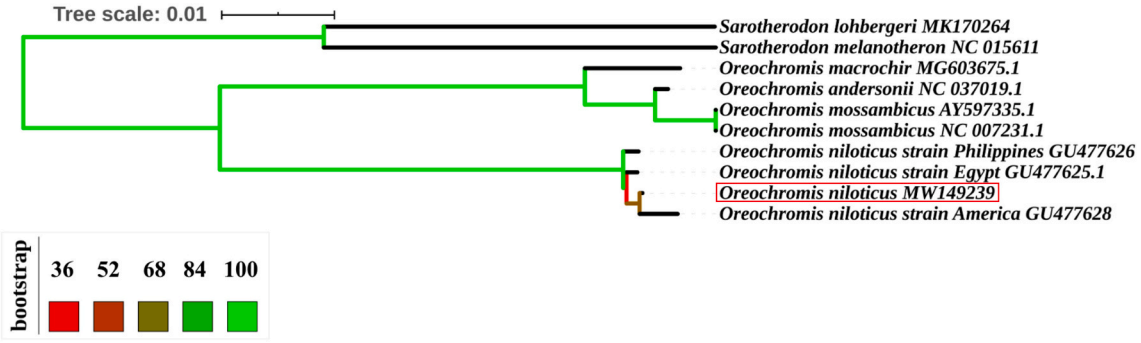

Fig. 2. Maximum likelihood phylogenetic tree reconstruction of several tilapia species based on their coding sequences.

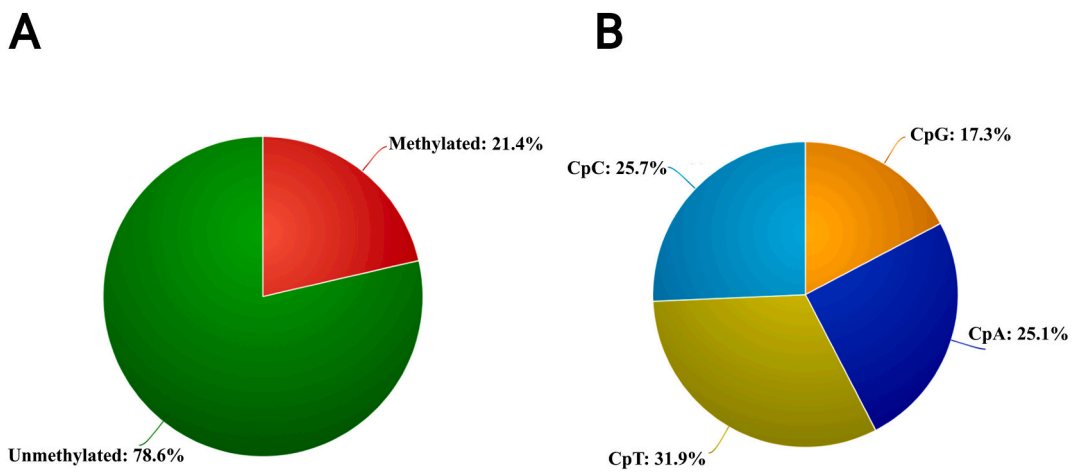

Fig. 3. Mitochondrial DNA methylation in liver of adult Nile tilapia. (A) Presence of methylated cytosines. (B) Nucleotide context of methylated cytosines.

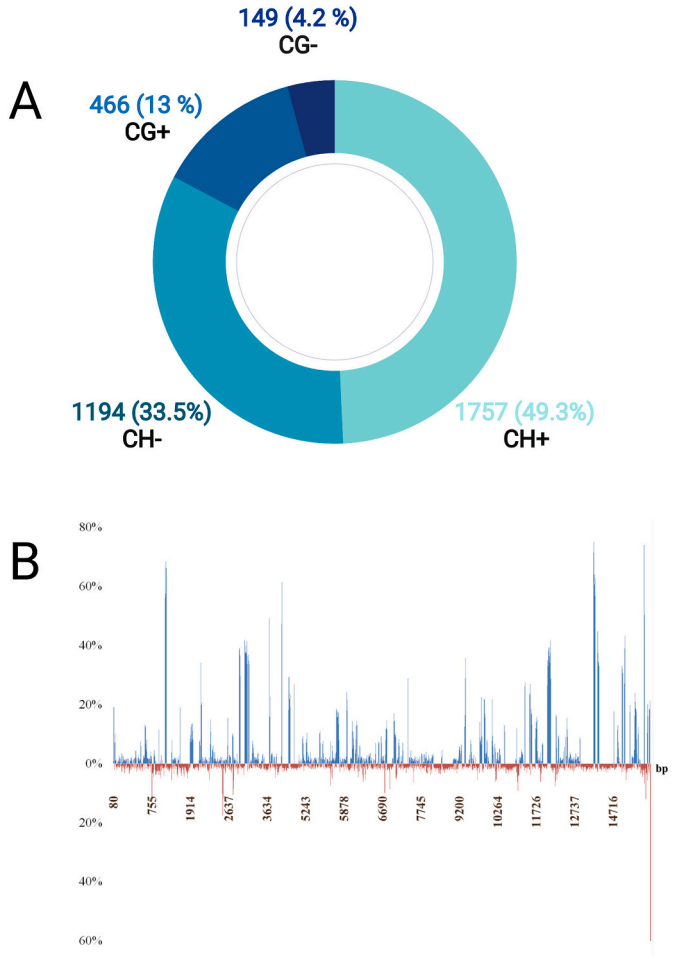

Fig. 4. Strand-specific distribution of methylated cytosines in the mitochondrial genome of Nile tilapia. (A) Strand-specific distribution of methylated cytosines within CpG (CG) and non-CpG (CH) context on light (minus) and heavy (plus) strands of mitochondrial DNA. (B) Strand-specific cytosine methylation levels. The Y-axis shows the percentage of methylated cytosines on light (marked in blue) and heavy (marked in red) strands. The X-axis presents positions on mitogenome. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to its significantly lower level than in the nuclear genome of vertebrates [14,15]. Interestingly mitochondrial DNA methylation predominantly localized within a non-CpG context compared to the nuclear genome [12,15], and mtDNA methylation patterns depend on environmental factors, such as tissue type and developmental stage [16,18,22]. The majority of studies on mtDNA methylation are related to its impact on the origin and development of different human disorders, and only two studies that described the hypomethylation status of mtDNA in zebrafish gametes and changes in mtDNA methylation patterns in zebrafish during embryogenesis have been published [28,29].

In the present study, we obtained the first liver methylome profile in Nile tilapia adult females using modern methods of mitochondrial DNA isolation, whole-genome bisulfite sequencing and advanced bioinformatical tools. In our analysis, we used the high sequencing depth ($>100\times$) as well as high stringency of the cytosine coverage filtering parameters ($\geq 50\times$) to be confident of the presence of methylation marks in the Nile tilapia mitogenome.

In contrast to the nuclear methylation pattern in teleosts, which comprises a clear CpG methylation motif [20] and all other contexts are negligible due to their very low methylation levels, Nile tilapia mtDNA exhibits the opposite, non-CpG methylation profile. We have shown the predominance of non-CpG over the CpG context in relation to the frequency of methylated cytosines. This observation is consistent with earlier reports in mammals [10,25]. Our results are also consistent with the data describing the same parameter (methylation context) in normal human liver cells [32]. Moreover, the Nile tilapia mitogenome had different methylation levels between heavy and light strands. Our data confirmed another interesting feature of mtDNA methylation, where the light strand is highly methylated in the *D-loop* region, as well as *nd5*, *cytb*, and *nd1* genes. However, we revealed that the heavy strand has a higher number (62.4%) of methylated cytosines. Strand-specific analysis has shown the presence of mtDNA methylation peaks within both non-CpG and CpG contexts, on both strands. However, 97.2% out of highly methylated cytosines belonged to a non-CpG context and are located on the light strand, in accordance with previous reports [14,19,29,54–56]. The proportion of methylated cytosines in CpG and non-CpG contexts varied between protein- and non-protein-coding genes. The frequency of methylation within a non-CpG context was higher in mitochondrial protein-coding genes, whereas tRNA and rRNA genes had nearly equal distribution of methylated cytosines within both nucleotide contexts.

4. Conclusions

The mitochondrion as a cell organelle is thought to have originated during the endosymbiotic relationship between prokaryotic and eukaryotic cells during long period of early evolution of life, according

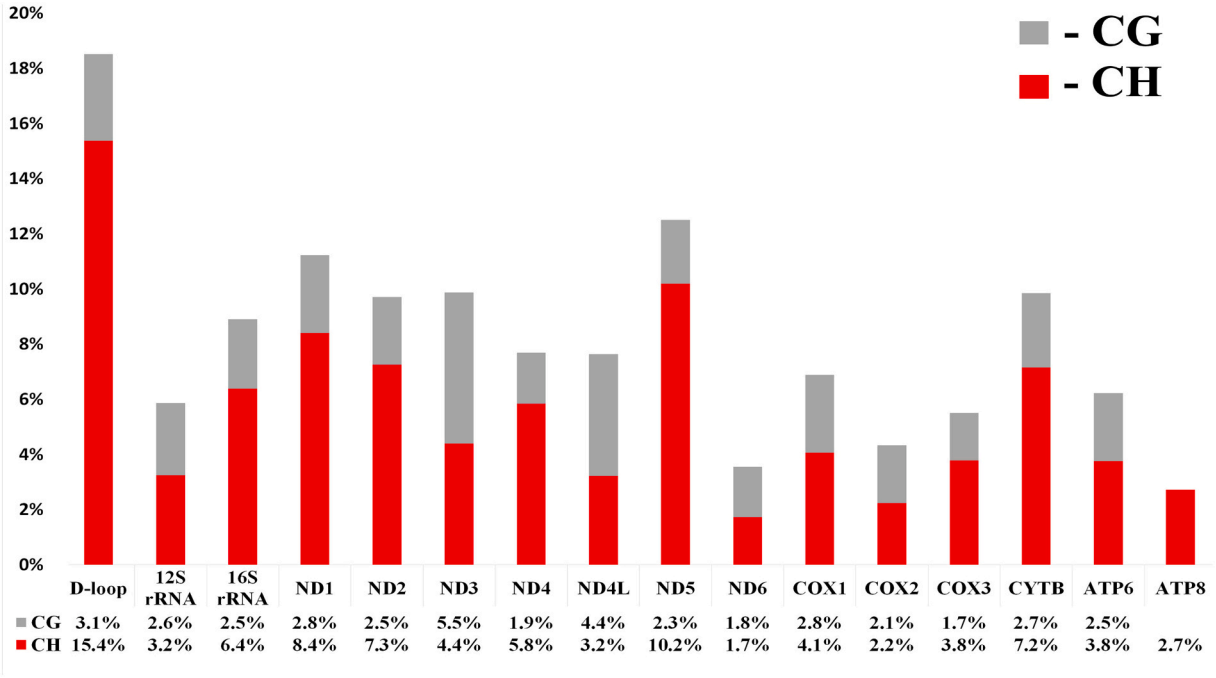

Fig. 5. Average methylation levels of mtDNA protein coding genes and D-loop within CpG and CHH context. The Y-axis shows average methylation percentages.

to the widely accepted endosymbiotic theory. Mitochondria lost most of their functions, becoming the cellular powerhouse in a million years [57]. Mitochondrial genes are involved in essential oxidative phosphorylation processes in animal cells. The high expression of mitochondrial genes is apparently related to the low methylation level previously described in mammal mitogenomes when compared to nuclear genomes [14,15]. In addition, the low 5mC methylation level in mitogenomes could be related to its specific usage and because it is less prevalent than 6mA methylation in prokaryotic genomes [58,59]. Nevertheless, methylation in mtDNA is now known to play an important role in the regulation of mitochondrial gene expression. Particularly, DNA methyltransferase 3B (*DNMT3B*) knockdown not only leads to a global reduction in mtDNA methylation but also increases mitochondrial gene expression [32]. Apart from some scattered information reported in two zebrafish studies, there are no previous reports on mtDNA methylation in teleosts [28,29].

In this paper, we used whole-genome bisulfite sequencing to determine the first mitochondrial methylome map at single-nucleotide resolution in a non-model teleost. In particular, our research provides insights into mitochondrial methylome assessment in adult fish and in an important organ for growth and metabolism regulation, the liver. Hyper- and hypomethylation in nuclear and mitochondrial genomes play important roles in hepatocyte function and are involved in several metabolic conditions in humans [12,60] as well as in growth [8]. Importantly, our data suggest that cytosine methylation is likely ubiquitous throughout the mitochondrial genome in fish. In line with previous reports [32], mitochondrial methylation is not limited to CpG sites [12,32]. In fact, the dinucleotides CpA, CpC, and CpT are predominantly methylated (82.7%), especially in the *D-loop* region within the Nile tilapia mitogenome.

Taken together with previous studies in mammals, our results indicate that mitogenome methylation is likely to play an important role in mitochondrial functionality and long-term metabolic memory in the cell. Common traits in the mitochondrial methylation pattern can be observed throughout the vertebrate evolution from fish to human. This suggests that the presence of methylated cytosines non-CpG context in mtDNA, as well as significant differences in average methylation level between light and heavy mitogenome strands and between different protein-coding genes and other loci, is underpinned by natural selection during hundreds of millions of years of evolution. Similarly to previous studies on higher vertebrates, we found that the light strand is almost twice more methylated than the heavy strand in Nile tilapia, despite containing less cytosines. This is possibly related to the gene distribution in the mitochondrial genome, where most genes are located on the heavy strand.

The potential functional significance of mitochondrial methylation patterns and their association with mitochondrial gene expression and different physiological conditions warrant further investigation. Importantly, this paper sets the foundation for functional studies on the etiology of metabolic disorders in Nile tilapia and paves the way for similar investigations in other fish species.

Data availability

The Nile tilapia mitogenome assembly is publicly available at NCBI under the accession number MW149239. Whole-genome bisulfite sequencing data are available at NCBI with the accession numbers SAMN16393530; SAMN16393534; SAMN16393537; SAMN16393540, and SAMN16393543.

Authors' contributions

MR, KS, FP and JF designed this study, conceptualized the methodology and experiments; MR and AN performed sampling; MR performed library preparation and sequencing; MR, AN and RM analyzed the data; MR and AN wrote the draft manuscript; RM, KS, FP and JF revised the manuscript; JF supervised the study. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2021.07.007.

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