The Complete Mitochondrial Genome of the Caecal Fluke of Poultry, *Postharmostomum commutatum*, as the First Representative from the Superfamily Brachylaimoidea

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**INTRODUCTION**

*Postharmostomum commutatum* (Platyhelminthes: Brachylaimoidea), a parasite of the caeca of poultry, has been frequently reported from many countries and regions, including China. However, the molecular epidemiology, population genetics and phylogenetics of this parasite are poorly understood. In the present study, we determined and characterized the complete mitochondrial (mt) genome of *P. commutatum*, as the first representative from the superfamily Brachylaimoidea. The mt genome of *P. commutatum* presents a clear bias in nucleotide composition with a negative AT-skew on average (−0.306) and a positive GC-skew on average (0.466). Phylogenetic analyses showed that *P. commutatum* (superfamily Brachylaimoidea) and other ten members of the order Diplostomida were recovered as sister groups of the order Plagiorchida, indicating that the order Diplostomida is paraphyletic. This is the first mt genome of any member of the superfamily Brachylaimoidea and should represent a rich source of genetic markers for molecular epidemiological, population genetic and phylogenetic studies of parasitic flukes of socio-economic importance in poultry.
Chromadorea nematodes, which lack *atp8* gene (Boore, 1999). Due to its maternal inheritance, high genome copy numbers, fast evolutionary rate, simple genetic structure and lack of recombination, mt genome sequences have been widely used in molecular epidemiological, population genetic and phylogenetic studies at various taxonomic levels of different parasitic worms (Zhang et al., 2017; Wu et al., 2018; Jin et al., 2019).

The digeneans (subclass Digenea) are distributed worldwide and comprise ~18,000 described species (Kostadinova and Pérez-Del-Olmo, 2019). The phylogeny and classification of digeneans have been substantially revised with analyses of the two nuclear ribosomal RNA genes (Olson et al., 2003; Littlewood et al., 2015; Pérez-Ponce de León and Hernández-Mena, 2019). Recently, mt genomic datasets have also been used to understand the phylogenetic relationships of digeneans (Webster and Littlewood, 2012; Brabec et al., 2015; Briscoe et al., 2016; Chen et al., 2016; Locke et al., 2018). A major difference between the most accepted classification based on nuclear rRNA genes and mt genome phylogenies is the order Diplostomida. All previous phylogenetic analyses based on nuclear rRNA genes have supported the monophyly of this order within the subclass Digenea (Olson et al., 2003; Littlewood et al., 2015; Pérez-Ponce de León and Hernández-Mena, 2019). However, mt genome phylogeny rejected the monophyly of this order because *Clinostomum complanatum* (Schistosomatoidae), *Alaria americana*, *Hysteromorpha triloba*, *Tylodelphys immer*, *Cardiocephaloides medioconiger*, *Cotylurus marcogliesei*, *Posthodiplostomum centrichtid*, *Cyathocotyle prussica* (Diplostomoidea) and two *Diplostomum* species were recovered as sister groups of order Plagiorchiida, not the order Diplostomida (Brabec et al., 2015; Chen et al., 2016; Locke et al., 2018). The order Diplostomida currently consists of three superfamilies (Brachylaimoidea, Diplostomoidea and Schistosomatoidea). The superfamily Brachylaimoidea has potential veterinary importance and complex taxonomic history. Currently, the superfamily Brachylaimoidea contains at least 20 valid species that consist of parasites of mammals and birds (Heneberg et al., 2016). Despite their importance, no mt genome had been sequenced and characterized for any members of the superfamily Brachylaimoidea.

The objectives of the present study were to determine and analyze the complete mt genome of *P. commutatum*, as the first representative from the superfamily Brachylaimoidea, and to assess the systematic and phylogenetic position of this fluke within the subclass Digenea using concatenated protein sequences derived from all coding genes.

**MATERIALS AND METHODS**

**Parasites and Total Genomic DNA Isolation**

Adult specimens of *P. commutatum* were collected from a naturally infected chicken in Hunan province of China. Adult worm specimens were washed separately in physiological saline, identified preliminarily to species based on morphological features described previously (Pojmaniska, 2002), fixed in 70% (v/v) ethanol and stored at −20°C until further use. DNA extraction was performed from individual flukes using a commercially available kit (Wizard™ SV Genomic DNA Purification System, Promega) according to the manufacturer’s instructions. The molecular identity of each specimen was further verified by PCR using an established method and then sequenced (Bowles and McManus, 1994). The mt cox1 sequences of *P. commutatum* samples showed 99% similarity with that of *P. commutatum* from *Gallus gallus* in Brazil (GenBank accession no. MFH19409) (Valadão et al., 2018). Our phylogenetic analyses based on mt cox1 sequences of *P. commutatum* and relatives showed that two *P. commutatum* isolates grouped together, suggesting that the *Postharmostomum* isolate from present study represented *P. commutatum* (Figure S1).

**Sequencing and Assembling**

High molecular weight genomic DNA was extracted from an adult fluke and agarose-gel electrophoresis (1%) was used to verify DNA integrity. After fragmentation (400–500 bp) of this DNA by shearing using G-tubes (Coavris M220), a paired-end genomic library (about 320 bp inserts) was constructed using TruSeq™ DNA Sample Prep Kit (Illumina). All sequencing was carried out on Illumina Hiseq 4000 platform and data recorded in FASTQ format. The clean reads were obtained from raw reads by removing adaptor sequences, highly redundant sequences, reads that contained more than 10% ambiguous positions (N) and low-quality reads. Clean reads were assembled into contigs with Geneious 11.1.5 (Kearse et al., 2012) based on mt cox1 conserved sequence motifs. The assembly parameters were minimum overlap identity 99.5%, minimum overlap 150 bp and maximum gap size 5 bp. The assembly generated a large contig ending with overlapping fragments. As this structure allowed a single circular organization of the mt genome, we assumed that the complete mt genome had been assembled. The completeness of the mt genome assembly was further verified by long PCR experiment using five pairs of primers (Table S1) which were designed in the conserved regions.

**Annotations**

The assembled mt genome was annotated with the MITOS webserver (Bernt et al., 2013). The boundaries of protein-coding genes and rRNA genes were determined by alignment with the homologous genes of *C. complanatum* using the computer program MAFFT 7.122 with the option (L-INS-I) (Katoh and Standley, 2013). Amino acid sequences of 12 protein-coding genes were inferred using MEGA 6.0 (Tamura et al., 2013). Translation start and stop codons were identified based on comparison with those of *C. complanatum* reported previously (Chen et al., 2016). The identification, boundary delimitation and secondary structure folding of 22 tRNA genes were identified using ARWEN (Laslett and Canbäck, 2008) and the program tRNAscan-SE (Lowe and Chan, 2016) under the default search model, with the “other mitochondrial” sequence source and the “invertebrate mitochondrial” genetic code, and manual adjustment. The Ka/Ks ratio was calculated for nucleotide sequences of all 12 mt protein-coding genes of *P. commutatum* and other digeneans using DnaSP v5 (Librado and Rozas, 2009).
Phylogenetic Analysis
All mt genome sequences of subclass Digenea (Table 1), along with an outgroup of the subclass Monogenea (Gyrodactylus derjavinoides; GenBank accession number EU293891) (Huyse et al., 2008), were obtained from GenBank and combined for phylogenetic analysis. The deduced amino acid sequences of 12 protein-coding genes were aligned individually using MAFFT 7.122. The well-aligned conserved blocks were identified using Gblocks 0.91b with default parameters using the option for a less stringent selection (Talavera and Castresana, 2007). The individual amino acid or concatenated amino acid alignments and newick trees have been stored in a publicly available data repository (Accession ID: 25084; Study Accession URL: http://purl.org/phylo/treebase/phylows/study/TB2:S25084).

TABLE 1 | Mitochondrial genome sequences of digeneans sequenced completely prior to the present study and used for phylogenetic analysis.

| Order             | Family               | Species                        | Size (bp) | GenBank accession number |
|-------------------|----------------------|--------------------------------|-----------|--------------------------|
| Diplostomida      | Cyathocotylidae      | Cyathocotyle prussica          | 13,665    | NC_039780                |
|                   | Clinostomidae        | Clinostomum complanatum       | 13,796    | NC_027082                |
|                   | Diplostomidae        | Alaria Americana              | 13,836    | MH536507                 |
|                   |                      | Diplostomum pseudospathaceum  | 14,099    | KR289764                 |
|                   |                      | Diplostomum spathaceum        | 14,784    | KR289763                 |
|                   |                      | Hysterocephrus triloba        | 13,655    | MH536511                 |
|                   |                      | Posthodiplostomum centrarchid | 14,561    | MH536512                 |
|                   |                      | Tylolephytus immer            | 14,193    | MH536513                 |
| Schistosomatidae  |                      | Schistosoma spindale          | 16,901    | DQ157223                 |
|                   |                      | Schistosoma haematobium       | 15,003    | DQ157222                 |
|                   |                      | Schistosoma japonicum         | 14,087    | JQ781215                 |
|                   |                      | Schistosoma mansoni           | 14,415    | NC_002545                |
|                   |                      | Schistosoma mekongi           | 14,072    | AF217449                 |
|                   |                      | Schistosoma marginosum        | 15,167    | AP017709                 |
|                   |                      | Trichobilharzia szidat        | 14,293    | NC_086411                |
| Strigeidae        |                      | Trichobilharzia regent        | 14,838    | DQ859919                 |
|                   |                      | Cardiocephaloides medioconger | 15,107    | MH536506                 |
|                   |                      | Cotylurus marcogliei          | 13,815    | MH536509                 |
| Plagiorchiida     | Brachycladiidae      | Brachycladium goliath          | 15,229    | NC_029757                |
|                   | Dicrocoeliidae       | Dicrocoelium dentriticum      | 14,884    | NC_025280                |
|                   |                      | Dicrocoelium chinensis        | 14,917    | NC_025279                |
|                   |                      | Eurytrema pancreaticum        | 15,031    | KP241855                 |
| Echinocasmodiae   |                      | Echinocasmodius japonicus     | 15,865    | NC_030518                |
| Echinostomatidae  |                      | Artyechinostomum sultryfex    | 14,567    | NC_037150                |
|                   |                      | Echinostoma caproni           | 14,150    | AP017706                 |
|                   |                      | Isthmiophora hortense         | 14,994    | KR082182                 |
|                   |                      | Echinostoma miyagawai         | 14,416    | NC_039632                |
|                   |                      | Hypoderaea sp.                | 13,180    | KM111525                 |
|                   |                      | Echinostoma paraensei         | 20,298    | KTO08005                 |
| Fasciolidae       |                      | Fasciola gigantica            | 14,478    | KF543342                 |
|                   |                      | Fasciola hepatica             | 14,462    | AF216697                 |
|                   |                      | Fasciola jacksoni             | 14,952    | KX787886                 |
|                   |                      | Fascioloides magna            | 14,047    | NC_029481                |
|                   |                      | Fasciolopsis buski            | 14,833    | NC_030528                |
| Gastrodiscidae    |                      | Homalopogaster paloniae       | 14,490    | KT266674                 |
| Gastrothylacidae  |                      | Gastrothylax crumarifer       | 14,801    | NC_028733                |
|                   |                      | Fischoechidus colboidi        | 14,256    | KX169164                 |
|                   |                      | Fischeoeridus elongatus       | 14,120    | NC_028001                |
| Heterophyidae     |                      | Haplorchis taichi             | 15,130    | NC_022433                |
|                   |                      | Metagonimus yokogawai         | 15,258    | NC_023249                |
| Himasthiidae      |                      | Acanthoparyphium sp.          | 14,191    | MG792058                 |
| Notocotylidae     |                      | Ogmocotyle sikae              | 14,307    | NC_027112                |
| Opisthorchiidae   |                      | Metorchis orientalis          | 13,834    | NC_028008                |
|                   |                      | Opisthorchis felineu          | 14,277    | EU921260                 |
|                   |                      | Opisthorchis sinensis         | 13,875    | FJ381684                 |
|                   |                      | Opisthorchis viveni           | 13,510    | JF39555                  |
| Paramphistomidae  |                      | Paramphistomum cervi          | 14,023    | KT199897                 |
|                   |                      | Calicophoros microbathrioides | 14,028    | NC_027271                |
|                   |                      | Explanatum explanatum         | 13,968    | NC_027968                |
|                   |                      | Orthocoeleus streptocoelium  | 13,800    | KM659177                 |
| Troglorematidae   |                      | Paragonimus heterocercus      | 13,927    | NC_034430                |
|                   |                      | Paragonimus ohirai            | 14,818    | NC_032032                |
|                   |                      | Paragonimus westermanni       | 14,103    | NC_027673                |
Phylogenetic analyses were conducted using Maximum likelihood (ML) and Bayesian inference (BI). ML analysis was computed using PhyML 3.0 (Guindon et al., 2010). For ML analysis, it was partitioned by gene, and bootstrapping frequencies (BS) was performed using the rapid bootstrapping option with 100 iterations, the JTT (genes 1–6; cytb, cox3, nad2, nad4L, nad5 and nad6), LG (genes 7–8; cox2 and nad4), MtArt (genes 9–10; cox1 and nad1) and MtREV (genes 11–12; atp6 and nad3) models were used as selected by ProtTest 2.4 (Abascal et al., 2005) based on the Akaikie information criterion (AIC). BI analysis was performed using MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003), two independent runs with four incrementally heated Metropolis-coupled Markov chains Monte Carlo were run for two million generations, with tree sampling conducted at every 200 generations. The first 25% of the sampled trees were discarded as burn-in, and the remaining trees were used to calculate Bayesian posterior probabilities (Bpp). The potential scale reduction factor approached 1 and the average split frequency of less than 0.01 were used to represent the convergence of the two simultaneous runs. For BI analysis, the dataset was partitioned by gene, and the amino acid model for each gene was estimated from above models with model-given frequencies and gamma distributed rates. PhyloBayes 3.3b (Lartillot and Philippe, 2004) was run using the site-heterogeneous mixture CAT model, and the analysis was stopped when the conditions considered to indicate a good run were reached (maxdiff <0.1 and minimum effective size >300). The phylogenetic trees were visualized using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS AND DISCUSSION

Genome Organization and Composition

We sequenced the P. commutatum genome and produced over 3 Gb of Illumina short-read sequence datasets. A total of 14,070,228 × 2 raw reads with the size of 250 bp were generated and 13,411,012 × 2 clean reads were obtained for assembly of the mt genome. The entire mt genome sequence of P. commutatum (GenBank accession no. MN200359) was 13,799 bp in size (Figure 1). We further confirmed the completeness of mt genome assembly using five pairs of primers covering the whole 13,800 bp-long assembled sequence to amplify the entire mt genome of P. commutatum. All five fragments (~2–4 kb each) were successfully confirmed by long PCR amplification (Figure S2). This complete mt genome was slightly shorter than some other digenetic parasites (such as Echinostoma caproni, Fischioederius elongatus and Schistosoma japonicum) but was slightly longer than some digenetic parasites (such as C. compostatum, C. prussica and Opisthorchis viverini) (Table 1). This difference is mainly due to the total fraction of non-coding sequences. This circular mt genome contains 12 protein-coding genes (cox1-3, nad1-6, nad4L, atp6 and cytb), 22 tRNA genes, two rRNA genes (rrnL and rrnS) and a non-coding (control or AT-rich) region (Table 2 and Figure 1). The gene orders are the same as those of flukes of the order plagiorchiida, such as O. viverinii, C. compostatum and Fasciola gigantica (Cai et al., 2012; Liu et al., 2014; Chen et al., 2016), but distinct from those of blood flukes, such as S. japonicum (Zhao et al., 2009) and S. turkestanicum (Wang et al., 2011). Additionally, the mt genes of P. commutatum overlap by 47 bp in five locations (1 to 40 bp per location) (Table 2). The mt genome of P. commutatum has 15 intergenic regions, which range from 1 to 24 bp in size. The longest region is between nad4 and tRNA-Gln (Table 2).

The nucleotide composition of the complete mt genome of P. commutatum is biased toward A+T (64.8%), in accordance with mt genomes of other digenetic parasites (Zhao et al., 2009; Wang et al., 2011; Suleman et al., 2019b). AT- and GC- skews are a measure of compositional asymmetry. In P. commutatum mt genome, AT-skews were always negative, while the values of GC-skew were positive (Table 3). The AT-skew value observed was −0.306 on average, ranging from −0.448 (nad6) to −0.140 (rrnS). The average GC-skew value observed is 0.466, ranging from 0.339 (22 tRNA) to 0.737 (nad3) (Table 3). In all mt genome sequences of flatworms (including tapeworms, nematodes and trematodes) reported to date (Liu et al., 2013; Briscoe et al., 2016; Zhao et al., 2016), the GC skew is positive due to the very low C content in mt genomes.

Protein-Coding Genes

A total of 3,405 amino acids are encoded by the P. commutatum mt genome. The enriched A+T content was reflected in the codon usage. In P. commutatum mt genome, Leu and Phe are the most frequently encoded amino acids, and Gln is the least frequent (Table 4). Individually, the most frequently used
### TABLE 2 | The organization of the mt genome of *Postharmostomum commutatum*.

| Gene/Region | Positions | Size (bp) | Number of aa* | Ini/Ter codons | Anticodons | ln |
|-------------|-----------|-----------|---------------|----------------|------------|----|
| cox3        | 1–654     | 654       | 217           | ATG/TAA        |            | +8 |
| tRNA-His (H)| 663–729   | 67        |               |                | GTG        | +3 |
| cytb        | 733–1,850 | 1,115     | 372           | ATG/TA         |            | 0  |
| nad4        | 1,851–2,114| 264       | 87            | GTG/TA         |            | -40|
| nad4        | 2,075–3,370| 1,296     | 431           | ATG/TAA        |            | +24|
| tRNA-Gln (Q)| 3,395–3,455| 61        |               | ATG/TAA        |            | 0  |
| tRNA-Phe (F)| 3,454–3,517| 64        |               | GAA            |            | +1 |
| tRNA-Met (M)| 3,519–3,586| 68        |               | CAT            |            | 0  |
| atp6        | 3,587–4,093| 507       | 168           | GTG/TA         |            | 0  |
| nad2        | 4,094–5,037| 944       | 314           | ATG/TA         |            | 0  |
| tRNA-Val (V)| 5,038–5,099| 62        |               | TAC            |            | +2 |
| tRNA-Ala (A)| 5,102–5,164| 63        |               | TGC            |            | 0  |
| tRNA-Asp (D)| 5,165–5,229| 65        |               | GTC            |            | 0  |
| nad1        | 5,230–6,144| 915       | 304           | ATG/TA         |            | +14|
| tRNA-Asn (N)| 6,159–6,222| 84        |               | GTT            |            | +1 |
| tRNA-Pro (P)| 6,224–6,290| 67        |               | TGG            |            | 0  |
| tRNA-Ile (I)| 6,291–6,355| 65        |               | QAT            |            | -1 |
| tRNA-Lys (K)| 6,355–6,420| 66        |               | CCT            |            | 0  |
| nad3        | 6,421–6,777| 357       | 118           | ATG/TA         |            | +3 |
| tRNA-Ser(AGN)(S1)| 6,781–6,839| 59      |               | GCT            |            | +9 |
| tRNA-Trp (W)| 6,849–6,911| 63        |               | TCA            |            | +12|
| cox1        | 6,924–8,498| 1,575    | 524           | ATG/TA         |            | +20|
| tRNA-Thr(T)| 8,519–8,581| 63        |               | TGT            |            | +1 |
| rrnL        | 8,583–9,553| 971      |               |                |            | 0  |
| tRNA-Cys (C)| 9,554–9,617| 64        |               | GCA            |            | 0  |
| rrnS        | 9,618–10,349| 732     |               |                |            | +4 |
| cox2        | 10,354–10,955| 602     | 200           | ATG/TA         |            | 0  |
| nad6        | 10,956–11,403| 448    | 149           | ATG/T          |            | 0  |
| tRNA-Tyr (Y)| 11,404–11,468| 65      |               | GUA            |            | 0  |
| tRNA-LeuCUN(L1)| 11,469–11,535| 67      |               | TAG            |            | -3 |
| tRNA-LeuCUN(S2)| 11,533–11,601| 69      |               | TGA            |            | +17|
| tRNA-LeuCUN(L2)| 11,619–11,682| 64      |               | TAA            |            | -1 |
| tRNA-Arg (R)| 11,682–11,747| 66      |               | TGG            |            | +2 |
| nad5        | 11,750–13,313| 1,564   | 521           | ATG/T          |            | 0  |
| tRNA-Glu (E)| 13,314–13,375| 62      |               | TTC            |            | 0  |
| Non-coding region (NC)| 13,376–13,733| 358 |               |                |            | 0  |
| tRNA-Gly (G)| 13,734–13,799| 66      |               | TCC            |            | 0  |

*The inferred length of amino acid (aa) sequence of 12 protein-coding genes; Ini/Ter codons: initiation and termination codons; ln, Intergenic nucleotides (between the current gene and the next gene).

### TABLE 3 | Nucleotide composition and skews of *Postharmostomum commutatum* mitochondrial genome.

| Gene | Nucleotide frequency | A+T (%) | AT-skew | GC-skew |
|------|----------------------|---------|---------|---------|
|      | A (%)                | G (%)   | T (%)   | C (%)   |
| atp6 | 20.9                 | 25.8    | 43.6    | 9.7     | 64.5  | -0.352 | 0.454 |
| cox1 | 20.4                 | 25.3    | 44.2    | 10.1    | 64.6  | -0.368 | 0.433 |
| cox2 | 23.1                 | 25.9    | 40.4    | 9.6     | 63.5  | -0.272 | 0.419 |
| cox3 | 20.5                 | 24.3    | 43.3    | 9.9     | 65.8  | -0.377 | 0.421 |
| cytb | 22.8                 | 23.5    | 43.3    | 10.4    | 66.1  |  -0.310 | 0.386  |
| nad1 | 19.7                 | 29.8    | 43.4    | 7.1     | 63.1  | -0.376 | 0.615 |
| nad2 | 21.8                 | 25.4    | 43.1    | 9.7     | 64.9  | -0.328 | 0.446 |
| nad3 | 18.2                 | 29.7    | 47.6    | 4.5     | 65.8  | -0.447 | 0.737 |
| nad4 | 20.4                 | 27.0    | 44.7    | 9.7     | 65.1  | -0.373 | 0.564 |
| nad4L| 23.9                 | 25.8    | 43.2    | 7.1     | 67.1  | -0.288 | 0.564 |
| nad5 | 19.4                 | 28.2    | 44.8    | 7.6     | 64.2  | -0.396 | 0.575 |
| nad6 | 18.3                 | 28.3    | 48.0    | 5.4     | 66.3  | -0.448 | 0.680 |
| rrmS | 26.5                 | 26.0    | 35.1    | 12.4    | 61.6  | -0.140 | 0.354 |
| rrmL | 27.4                 | 24.7    | 37.2    | 10.7    | 64.6  | -0.152 | 0.395 |
| 22 tRNA| 27.0                 | 24.7    | 36.1    | 12.2    | 63.1  | -0.144 | 0.339 |
| Total| 22.5                 | 25.8    | 42.3    | 9.4     | 64.8  | -0.306 | 0.466 |
amino acid was TTT (Phe; 8.93%), followed by TTG (Leu; 7.76%), TTA (Leu; 6.91%) and GTT (Val; 5.15%) (Table 4). All of the 12 identified protein-coding genes begin with ATG (cox1, cox2, cox3, cytb, nad1, nad2, nad3, nad4, nad5 and nad6) or GTG (atp6 and nad4L) as their start codons. Seven of the 12 genes appear to use TAA (cox3, nad4 and nad3) or TAG (nad4L, atp6, nad1 and cox1) as the stop codon, while the other genes end with incomplete codon TA (cytb, nad2 and cox2) or T (nad5 and nad6). This is very common in worm mt genomes, such as tapeworms (Nakao et al., 2007), nematodes (Hu et al., 2002; Hu et al., 2003; Kim et al., 2006) and trematodes (Chang et al., 2016). It is hypothesized that the mRNAs ending in T or TA are converted to TAA by post-transcriptional polyadenylation (Ojala et al., 1981).

### Transfer RNA Genes and Ribosomal RNA Genes

The sizes of 22 tRNA genes identified in mt genome of *P. commutatum*, ranging from 59 to 69 bp in length. A standard four-arm cloverleaf structure was inferred for most of the tRNA genes. However, the tRNA-Ser<sup>UCN</sup> (S1) gene shows an unorthodox structure, with the paired dihydrouridine (DHU) arm missing, as usual in all parasitic trematodes (also seen in some cestodes and nematodes) (Nakao et al., 2002; Hu et al., 2003). Structures for tRNA-Cys (C) and tRNA-Ser<sup>UCN</sup> (S2) often vary somewhat among the parasitic trematodes. A paired DHU-arm of these tRNA genes is not seen in *Haplorchis taichui*, *S. mansoni* (Blair et al., 1999; Lee et al., 2013), but it is present in *P. commutatum*. The rrnL gene of *P. commutatum* is located between tRNA-Thr and tRNA-Cys genes, and rrnS gene is located between tRNA-Glu and cox2 genes. The sizes of the rrnL and rrnS genes for *P. commutatum* were 971 bp and 732 bp, respectively (Table 2). The A+T contents of the rrnL rrnS genes for *P. commutatum* are 64.6% and 61.6%, respectively. The sizes and A+T contents of the two rrn genes for *P. commutatum* are almost similar to those of other digeneans sequenced to date, such as that of *Clonorchis sinensis*, *Paragonimus ohirai* and *Uvitellina sp.* (Cai et al., 2012; Le et al., 2019; Suleman et al., 2019a).

### Non-Coding Region

The mt genome sequences of flukes contain usually two non-coding regions (NC) of significant size difference (Yan et al., 2013; Le et al., 2019; Suleman et al., 2019b). However, in *P. commutatum* mt genome, there is only one NC (Table 2 and Figure 1). The NC is located between the tRNA-Glu and tRNA-Gly, and lacks any

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**Table 4 | Codon usage of Postharmostomum commutatum mitochondrial protein-coding genes.**

| Amino acid | Codon | Number | Frequency (%) | Amino acid | Codon | Number | Frequency (%) |
|------------|-------|--------|---------------|------------|-------|--------|---------------|
| Phe        | TTT   | 305    | 8.93          | Met        | ATA   | 83     | 2.43          |
| Phe        | TTC   | 5      | 0.15          | Met        | ATG   | 102    | 2.99          |
| Leu        | TTA   | 236    | 6.91          | Thr        | ACT   | 43     | 1.26          |
| Leu        | TTG   | 265    | 7.76          | Thr        | ACC   | 5      | 0.15          |
| Ser        | TCT   | 89     | 2.61          | Thr        | ACA   | 25     | 0.73          |
| Ser        | TCA   | 24     | 0.7           | Asn        | AAT   | 51     | 1.5           |
| Ser        | TCG   | 31     | 0.91          | Asn        | AAC   | 2      | 0.06          |
| Tyr        | TAT   | 42     | 1.29          | Lys        | AAA   | 34     | 1.0           |
| Tyr        | TAC   | 37     | 1.08          | Lys        | AAG   | 65     | 1.9           |
| Stop       | TAA   | 3      | 0.88          | Ser        | AGT   | 95     | 2.78          |
| Stop       | TAG   | 4      | 1.12          | Ser        | AGC   | 8      | 0.23          |
| Cys        | TGT   | 96     | 2.81          | Ser        | AGA   | 63     | 1.85          |
| Cys        | TGC   | 3      | 0.09          | Ser        | AGG   | 44     | 1.29          |
| Trp        | TGA   | 58     | 1.64          | Val        | GTC   | 12     | 0.35          |
| Trp        | TGG   | 64     | 1.87          | Val        | GTA   | 91     | 2.67          |
| Leu        | CTT   | 45     | 1.32          | Val        | GTG   | 135    | 3.96          |
| Leu        | CTC   | 2      | 0.06          | Ala        | GCT   | 74     | 2.17          |
| Leu        | CTA   | 13     | 0.38          | Ala        | GCA   | 30     | 0.88          |
| Leu        | CGT   | 7      | 0.21          | Ala        | GCC   | 4      | 0.12          |
| Pro        | CCT   | 48     | 1.41          | Ala        | GCA   | 30     | 0.88          |
| Pro        | CCC   | 3      | 0.09          | Ala        | GCG   | 33     | 0.97          |
| Pro        | CCA   | 17     | 0.5           | Asp        | GAT   | 63     | 1.84          |
| Pro        | CCG   | 14     | 0.41          | Asp        | GAC   | 5      | 0.15          |
| His        | CAT   | 46     | 1.35          | Glu        | GAA   | 26     | 0.76          |
| His        | CAC   | 7      | 0.21          | Glu        | GAG   | 51     | 1.49          |
| Gln        | CAA   | 14     | 0.41          | Gly        | GGT   | 138    | 4.04          |
| Gln        | CAG   | 18     | 0.53          | Gly        | GCC   | 13     | 0.38          |
| Arg        | CGT   | 39     | 1.14          | Gly        | GGA   | 63     | 1.85          |
| Arg        | CGC   | 2      | 0.06          | Gly        | GGG   | 75     | 2.2           |
| Arg        | CCG   | 12     | 0.35          | Ile        | ATT   | 107    | 3.13          |

*Excluding abbreviated stop codons (TA and T). Stop = Stop codon.*
tandem repeats. Its size is 358 bp and A+T contents is 76.5%. One NC was also identified in *H. taichu* and *Fasciolopsis buski* mt genomes (Lee et al., 2013; Ma et al., 2017). Although the function of non-coding regions is currently unknown, the high A+T content predicts an involvement in the initiation of replication (Keddie et al., 1998).

### Non-Synonymous/Synonymous Substitution Ratio of Protein-Coding Genes

The non-synonymous (Ka)/synonymous (Ks) substitutions ratio is particularly useful for characterizing evolutionary relationships between mt protein-coding genes in closely-related species (Fay and Wu, 2003). The Ka/Ks ratio was calculated for nucleotide sequences of all 12 mt protein-coding genes of *P. commutatum* and other digeneans (Table 1). The Ka/Ks ratio is a measure of selective pressures acting on genes, which indicates either negative (Ka/Ks <1) or positive (Ka/Ks >1) or that positive and negative selection counter-balance each other (Ka/Ks = 1) (Meganathan et al., 2011; Li et al., 2012). In the *P. commutatum* mt genome, *atp6* appeared to have the highest Ka/Ks ratio (2.230), while *cox1* is the lowest Ka/Ks ratio (0.154) (Figure 2). Herein, the Ka/Ks ratio of nine protein-coding genes (*cox1, cox2, cox3, nad2, nad3, nad4, nad4L, nad6* and *cyt b*) was <1 (range: 0.154 to 0.989), suggesting that these mt protein-coding genes of digeneans are under purifying selection. The Ka/Ks ratio of three protein-coding genes (*nad1, nad5* and *atp6*) was >1 (range: 1.169 to 2.230), indicating that these mt protein-coding genes of digeneans have evolved under positive or Darwinian selection. A similar pattern is also observed for nematode mt genomes (Liu et al., 2013).

### Comparison With Other Selected Digeneans Mt Genomes

The amino acid sequences of *P. commutatum* were compared with other digeneans (Table 5). In addition, the amino acid sequence similarities between *P. commutatum* and three species from the order Plagiorchiida ranged from 22.6–72.1% (Meganathan et al., 2011; Li et al., 2012). In the *P. commutatum* mt genome, *atp6* appeared to have the highest Ka/Ks ratio (2.230), while *cox1* is the lowest Ka/Ks ratio (0.154) (Figure 2). Herein, the Ka/Ks ratio of nine protein-coding genes (*cox1, cox2, cox3, nad2, nad3, nad4, nad4L, nad6* and *cyt b*) was <1 (range: 0.154 to 0.989), suggesting that these mt protein-coding genes of digeneans are under purifying selection. The Ka/Ks ratio of three protein-coding genes (*nad1, nad5* and *atp6*) was >1 (range: 1.169 to 2.230), indicating that these mt protein-coding genes of digeneans have evolved under positive or Darwinian selection. A similar pattern is also observed for nematode mt genomes (Liu et al., 2013).

### TABLE 5 | Pairwise identities (%) in mitochondrial amino acid sequences between *Postharmostomum commutatum* and other representative digeneans.

| Gene/Genome | Diplostomida | Plagiorchiida | Diplostomida |
|-------------|--------------|---------------|--------------|
|             | CC | CP | EP | EM | OV | SH | SJ | TR |
| atp6        | 32.1 | 30.4 | 22.6 | 31.5 | 31.0 | 20.2 | 28.0 | 27.4 |
| cox1        | 71.8 | 78.2 | 65.2 | 72.1 | 70.4 | 69.3 | 71.0 | 73.4 |
| cox2        | 53.0 | 59.4 | 52.3 | 42.5 | 48.0 | 52.0 | 47.0 | 48.7 |
| cox3        | 33.6 | 29.0 | 25.8 | 31.8 | 29.0 | 22.6 | 20.4 | 24.5 |
| cyt b       | 63.2 | 65.0 | 60.2 | 66.7 | 61.8 | 46.0 | 50.1 | 49.2 |
| nad1        | 54.7 | 50.8 | 47.3 | 49.0 | 48.0 | 39.4 | 40.9 | 44.0 |
| nad2        | 31.8 | 29.5 | 23.1 | 27.7 | 30.1 | 26.2 | 29.2 | 23.0 |
| nad3        | 54.2 | 52.2 | 43.5 | 53.4 | 46.6 | 44.9 | 40.7 | 50.0 |
| nad4        | 49.3 | 49.3 | 45.9 | 47.3 | 45.2 | 31.4 | 31.8 | 33.3 |
| nad4L       | 58.6 | 59.8 | 46.0 | 62.1 | 55.2 | 30.2 | 33.3 | 31.4 |
| nad5        | 46.3 | 47.0 | 35.7 | 52.0 | 36.7 | 32.4 | 30.9 | 31.9 |
| nad6        | 39.2 | 38.9 | 33.6 | 39.6 | 40.3 | 24.8 | 30.9 | 32.9 |
| EmtG        | 37.9 | 43.8 | 37.3 | 37.8 | 35.6 | 29.8 | 35.2 | 29.6 |

†Digeneans: CC, Clinostomum complanatum; CP, Cyathocotyle prussica; EP, Eurytrema pancreaticum; EM, Echinostoma miyagawai; OV, Opisthorchis viverrini; SH, Schistosoma haematobium; SJ, Schistosoma japonicum; TR, Trichobilharzia regenti; EmtG, entire mitochondrial genome.
The amino acid sequence similarity between *P. commutatum* and the selected three species from the order Diplostomida ranged from 20.2–71.0% (Table 5). These results show that the superfamily Brachylaimoidea (represented by *P. commutatum*) was more closely related to the members of order Plagiorchiida than it was to the members of order Diplostomida. Based on identity, COX1 was the most conserved protein, whereas ATP6 was the least conserved (Table 5).

**Phylogenetic Analyses**

The present study included three superamilies (Diplostomoidea, Schistosomatoidea and Brachylaimoidea) from the order Diplostomida and phylogenetic analysis showed that the order Diplostomida was paraphyletic with strong support in BI (Bpp = 1.0, Figure 3) and ML (BS = 100, Figure 4), but was weakly supported in PhyloBayes (Bpp = 0.5, Figure 5) analyses. The monophyly of the superfamily Diplostomoidea was weakly supported with BI (Bpp = 0.87, Figure 3), and was strongly supported in ML (BS = 100, Figure 4) and PhyloBayes (Bpp = 1.0, Figure 5) analyses. The superfamily Schistosomatoidea, however, was not monophyletic in all of the three phylogenetic analyses in this study. One species (C. complanatum) from the superfamily Schistosomatoidea was more closely related to *C. prussica* (Diplostomoidea) (Figure 3) or *P. commutatum* (Brachylaimoidea) (Figures 4 and 5) than it was to the other 8 species from the superfamily Schistosomatoidea.

Eleven species representing three superfamilies (Diplostomoidea, Schistosomatoidea and Brachylaimoidea) from the order Diplostomida were more closely related to the members of order Plagiorchiida than they were to the other eight species from the order Diplostomida. Our results were consistent with those of previous studies from mt genome datasets. For example, Brabec et al. (2015) sequenced the mt genomes of two species of diplostomids, and their phylogenetic analyses recovered the family Diplostomidae as the sister group of the order Plagiorchiida, although those relationships were supported by a low nodal support value.

![Figure 3](image-url)
Chen et al. (2016) generated the complete mt genome of *C. complanatum* and performed a phylogenetic analysis with mt genomes, indicating that *C. complanatum* is the sister group of the order Plagiorchiida with strong support in ML analyses. Most recently, Locke et al. (2018) determined the complete mt genome of seven diplostomoids representing three families (Diplostomidae, Strigeidae and Cyathocotylidae) and their mt genome phylogenetic tree yielded the order Diplostomida as paraphyletic because strigeids, diplostomids and clinostomids were recovered as sister groups of the order Plagiorchiida, not the order Diplostomida. No species from this superfamily (Brachylaimoidea) of the order Diplostomida were included in previous analyses of mt genome datasets (Brabec et al., 2015; Briscoe et al., 2016; Chen et al., 2016; Locke et al., 2018). In the present study, the determination of the mt genome of *P. commutatum* allows a reassessment of the phylogenetic relationships of digeneans. Our results confirm and expand on recent analyses showing a paraphyletic pattern of mt genome evolution in the order Diplostomida (Brabec et al., 2015; Briscoe et al., 2016; Chen et al., 2016; Locke et al., 2018).

The work of Olson et al. (2003) created robustness and stability in higher systematics within the subclass Digenea based on nuclear rRNA genes. Monophyly of the order Diplostomida has also been established previously in nuclear rRNA genes (Olson et al., 2003; Littlewood et al., 2015; Locke et al., 2018; Pérez-Ponce de León and Hernández-Mena, 2019). The mt genomic phylogenetic relationships of the order Diplostomida revealed a conflict with the rDNA phylogeny. Locke et al. 
(2018) discussed the possible causes of the mt genome topology and noted that the discrepancy occurs along short internal branches at the base of longer terminal branches, which could be related to a rapid radiation and incomplete lineage sorting. Along these short internal branches, mt genomes of digeneans may have a lower phylogenetic signal than nuclear genomes, exacerbating effects of incomplete taxon sampling (Hedtke et al., 2006; Philippe et al., 2011). In addition, both mt and nuclear genome data for representatives of the Diplostomida and of the early divergent lineages of the Plagiorchiida are needed to address the relationships of the two major lineages of the Digenea (Locke et al., 2018). Although the number of digenean mt genome sequences is increasing, to date, mt genomes of many lineages of digeneans are underrepresented or not represented. Insufficient taxon sampling for digenean mt genomes might be the cause of the discordance between the mt and nuclear datasets. Therefore, more mt genomes of digenean species representing families that have not yet been sequenced should be included in future analysis to resolve the taxonomic problems of digeneans because mt genome sequences have been shown to resolve deep-level relationships in many metazoan groups (Littlewood, 2008) and the use of mtDNA sequences has been considered promising (Philippe et al., 2011).

CONCLUSION

The present study determined the complete mt genome sequence of Postharmostomum commutatum, which shares some similarity with, and interesting differences to, other digeneans. Phylogenetic analyses showed that P. commutatum was recovered as
sister group of the order Plagiorchiida, supporting that the order Diplostomida is paraphyletic. The availability of the *P. commutatum* mt genome should represent a rich source of genetic markers for molecular epidemiological, population genetic and phylogenetic studies of parasitic flukes of socio-economic importance in poultry.

**DATA AVAILABILITY STATEMENT**

Publicly available datasets were analyzed in this study. This data can be found here: GenBank accession no. MN200359.

**ETHICS STATEMENT**

All procedures involving animals in the present study were approved and this study was approved by the Animal Ethics Committee of Hunan Agricultural University (No. 43321503).

**AUTHOR CONTRIBUTIONS**

G-HL and Y-TF conceived and designed the study, and critically revised the manuscript. Y-TF and Y-CJ performed the experiments. Y-TF and Y-CJ analyzed the data. G-HL and Y-TF drafted the manuscript. Y-TF and Y-CJ helped in study design, study implementation, and manuscript preparation. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.01037/full#supplementary-material

**TABLE S1** | Primers used for assembly validation.

**FIGURE S1** | Inferred phylogenetic relationships among *P. commutatum* and other relatives based on mitochondrial cox1 sequences utilizing maximum likelihood (ML) using Omithobiliarca canaliculata as an outgroup.

**FIGURE S2** | PCR amplicons from the mitochondrial genome of *Postharmostomum commutatum*. Amplicons generated with the *P. commutatum* primers. M: DL5000 DNA marker, 1: Validation_01, 2: Validation_02, 3: Validation_03, 4: Validation_04, 5: Validation_05, 6: Negative control.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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