Mitochondrial genomes of two eucotylids as the first representatives from the superfamily Microphalloidea (Trematoda) and phylogenetic implications

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Abstract

**Background:** The Eucotylidae Cohn, 1904 (Superfamily: Microphalloidea), is a family of digeneans parasitic in kidneys of birds as adults. The group is characterized by the high level of morphological similarities among genera and unclear systematic value of morphological characters traditionally used for their differentiation. In the present study, we sequenced the complete or nearly complete mitogenomes (mt genome) of two eucotylids representing the genera *Tamerlania* (*T. zarudnyi*) and *Tanaisia* (*Tanaisia* sp.). They represent the first sequenced mt genomes of any member of the superfamily Microphalloidea.

**Methods:** A comparative mitogenomic analysis of the two newly sequenced eucotylids was conducted for the investigation of mitochondrial gene arrangement, contents and genetic distance. Phylogenetic position of the family Eucotylidae within the order Plagiorchiida was examined using nucleotide sequences of mitochondrial protein-coding genes (PCGs) plus RNAs using maximum likelihood (ML) and Bayesian inference (BI) methods. BI phylogeny based on concatenated amino acids sequences of PCGs was also conducted to determine possible effects of silent mutations.

**Results:** The complete mt genome of *T. zarudnyi* was 16,188 bp and the nearly complete mt genome of *Tanaisia* sp. was 13,953 bp in length. A long string of additional amino acids (about 123 aa) at the 5′ end of the cox1 gene in both studied eucotylid mt genomes has resulted in the cox1 gene of eucotylids being longer than in all previously sequenced digeneans. The rrl gene was also longer than previously reported in any digenean mitogenome sequenced so far. The TΨC and DHU loops of the tRNAs varied greatly between the two eucotylids while the anticodon loop was highly conserved. Phylogenetic analyses based on mtDNA nucleotide and amino acids sequences (as a
Background
The Eucotylidae Cohn, 1904, is a group of digenetic renal flukes of birds reported from a diversity of birds worldwide [1, 2]. Eucotylids have a truncated heteroxenous life cycle with a single terrestrial pulmonate snail intermediate host; cercariae encyst within the sporocyst to form metacercariae [3, 4]. Birds are infected by ingesting molluscs containing metacercariae [3].

The system of the Eucotylidae has been unstable with several revisions utilizing somewhat different morphological characters as main diagnostic criteria [1, 5, 6]. Currently, the family contains two subfamilies, the Eucotylinae Skrjabin, 1924, and the Tanaisiinae Freitas, 1951 [1]. Species of the subfamily Tanaisiinae are characterized by the absence of the cervical thickening and cirrus sac and the presence of the seminal receptacle, intercaecal testes and caeca forming a cyclocoel [1, 6]. However, the key diagnostic characters of this subfamily were most recently amended by Lunaschi et al. [2] who described a well-developed cirrus sac with a small cirrus in a species of the Tanaisiinae. The three genera within the Tanaisiinae (Tanaisia Skrjabin 1924, Tamerlania Skrjabin 1924 and Paratanaisia Freitas, 1951) are differentiated by the extent of vitelline fields, the position of testes and the shape of tegumental scales or spines [1, 2, 5, 6]. However, the relative taxonomic value of these characters remains unclear and requires an independent set of characters. DNA sequences provide such an alternative source of data.

The taxonomic status of the genera Tanaisia and Tamerlania remained controversial for decades. Different authors either considered Tamerlania a separate genus or a synonym of Tanaisia, or its subgenus [5–7], mostly based on the similarities in the extent of the vitelline follicle fields. Although Tamerlania is currently recognized as a valid genus, some of its diagnostic characters are shared with both Tanaisia and Paratanaisia [1, 2]. Beside the problems at the genus level, the species boundaries within this group of renal flukes also remain uncertain. Therefore, DNA sequences, especially from fast-mutating genes, are critically important for addressing existing problems of eucotylid evolutionary interrelationships and taxonomy.

Previous molecular phylogenetic studies based on nuclear ribosomal DNA (rDNA) included very few representatives of the Eucotylidae in order to position the family within higher taxa [8–12]. They invariably placed the Eucotylidae within the suborder Xiphidiata as a long-branched clade. However, as mentioned above, more variable molecular markers are needed to re-evaluate the relative weight of morphological criteria traditionally used in eucotylid systematics to solve questions of differentiation among genera and species within this group, while more genes in general need to be used to examine its relationships with other digeneans.

Mitochondrial (mt) genomes represent a rich source of genetic markers with greater variability and have been widely used in parasitic flatworm population genetics and systematics [13–18]. The growing amount of mitogenome data in GenBank improves its utility in molecular phylogenetics of trematodes. However, mitogenome data remain very scarce or completely lacking for many groups of trematodes including the Eucotylidae and the whole superfamly Microphalloidea. To at least partially fill this gap, we have sequenced and annotated complete mitogenome of Tamerlania zarudnyi Skrjabin, 1924 (type species of the genus Tamerlania), and nearly complete mitogenome of Tanaisia sp. collected from birds in Pakistan. The aims of this study were to: (i) characterize and compare the previously unstudied mitogenomes of eucotylids; (ii) explore the phylogenetic relationships among currently sequenced eucotylid genera; (iii) test the monophyly of the suborder Xiphidiata Olson, Cribb, Tkach, Bray & Littlewood, 2003, using available mitogenome data.

Methods
Specimen collection, morphological examination and genomic DNA isolation
Twenty adult specimens of T. zarudnyi Skrjabin, 1924, were collected from kidneys of the house crow
(Corvus splendens) and 15 specimens of Tanaisia sp. were collected from the kidney of the little ringed plover (Charadrius dubius) (Charadriiformes: Charadriidae) in the Swabi district, Khyber Pakhtunkhwa province, Pakistan. Trematode specimens were preserved in 80% ethanol and stored in a freezer [19]. Two digenean specimens from each host were processed for morphological studies according to the protocol recommended by Lutz et al. [19] and identified to species or merely to genus level using published descriptions and keys [1, 2, 5, 6, 20, 21]. For molecular studies, total genomic DNA (gDNA) of three specimens from each species was extracted from ethanol-preserved specimens using Wizard® SV Genomic DNA Purification System (Promega, Madison, WI, USA) according to the protocol described by Gasser et al. [22] following the manufacturer's instructions.

Amplification and analysis of nuclear ribosomal DNA

For the taxonomic identification, the nuclear rDNA ITS region was amplified with the primers BD1 and BD2 [23], and to examine the phylogenetic interrelationships among eucotylid genera, D1–D3 region of the large subunit (LSU) of rDNA (28S rDNA) was amplified utilizing the primers LSU5 (forward) and 1500R (reverse) [9, 24] as described in our previous studies [25, 26]. To determine intraspecific differences, DNA of three specimens from each species was used separately for the amplification of each marker. All resultant positive PCR amplicons were purified using EZNA Gel Extraction Kit (OMEGA Bio-tek Inc., Doraville, GA, USA) and sent to Genewiz Company (Beijing, China) for sequencing. The obtained nucleotide sequences for each marker were assembled using DNAStar v7.1 [27] and Clustal X 1.83 [28] software. Sequence identity (%) across the ITS rDNA region among newly obtained sequences and another eucotyrid, T. valida, the only presently available ITS rDNA of eucotyrid in NCBI GenBank, was calculated using BioEdit 7.0.9.0 [29]. Similarly, to phylogenetic analysis, sequence identity across the D1–D3 region of LSU among newly sequenced and four other eucotyrids, presently available in GenBank, was also determined.

To assess the phylogenetic interrelationships of our specimens within the family Eucotiidae, the newly obtained 28S rDNA sequences were aligned with available sequences of other eucotyrid species, using MEGA X [30]. Renicola sp. was used as the outgroup based on the results of previous studies suggesting the close relationships of Eucotiidae and Renicolidae [8, 9]. The resulting alignment, trimmed to the length of the shortest sequence, was 910 bp long, including a few small gaps due to indels.

Phylogenetic analyses were conducted using Bayesian inference (BI) as implemented in MrBayes version 3.2.6 software [31, 32]. The GTR+G+F model was identified as the best fitting nucleotide substitution model using jmodeltest 2 software [33]. BI analysis was performed using MrBayes software as follows: two parallel Markov chain Monte Carlo (MCMC) chains were run for 10,000,000 generations. The initial 25% of sampled data generated was treated as “burn-in”, and the final 75% of trees was used for calculating Bayesian posterior probabilities (BPP). The phylograms were visualized in FigTree version 1.4 software [34] and annotated in Adobe Illustrator®.

Long PCR-based sequencing of eucotyrid mt genomes

Sequences of short mitochondrial genome fragments (cox3-cytb, rnrL-rnrS) and partial genes (nad4, nad1, cox1, nad5) were obtained using platyhelminth universal primers [35]. The obtained sequences were further used to design six or five pairs of species specific primers (Additional file 1: Table S1) for the amplification of complete or nearly complete mt genomes of our eucotyrids in medium to long overlapping fragments. Long mt genome fragments (2.5–3.5 kb) were amplified by long-PCR reactions using PrimeStar Max DNA polymerase premix (Takara, Dalian, China) following the procedure described in our previous studies [25, 26] and sequenced directly by Genewiz sequencing company (Beijing, China) using the primer-walking strategy.

mtDNA genomes assembly, annotation and analyses

The obtained sequences were carefully examined by checking chromatograms for quality and double peaks following BLASTn analysis to make sure that all amplimers are the desired target sequences. DNA extracted from a single individual of each species was used to infer and annotate its mitogenome, thereby avoiding any intraspecific variation. Eucotyrid mtDNA sequences were assembled using DNAStar v7.1 software [27] and aligned against selected digenean mitogenomes and then against each other using MAFFT 7.149 [36] to determine the relative positions of genes. Boundaries of protein-coding genes (PCGs) were found by searching for open reading frame (ORF; NCBI) and checking alignment against the selected digenean mitogenomes. tRNA sequences and their secondary structure were identified using the MITOS [37] and ARWEN [38] web servers. The two ribosomal RNAs, rrl and rrsS, were determined via a comparison with the mt genome sequences of selected trematode species and their boundaries were assumed to extend to their adjacent genes. The nucleotide identity (%), nucleotide and amino acid composition, A+T/ G+C skewness, codon usage and relative synonymous codon usage (RSCU) for PCGs were determined in PhylloSuite v1.2.1 [39]. The stacked bar chart of amino acids
used for the construction of mt PCGs was drawn using ggplot2 [40]. DnaSP v.6 [41] was used to conduct the sliding window analysis: window size of 200 bp and a step size of 20 bp were implemented to estimate the nucleotide divergence (Pi) among PCGs, rRNAs and tRNAs of the three eucotyloid mitogenomes. Kimura-2-parameter (K2P) genetic distances of the mt PCGs (substitution included = transitions + transversions) were also calculated using MEGA X [30]. To detect tandem repeats of nucleotides in the two non-coding regions of the complete mt genome of T. zarudnyi, we used Tandem Repeat Finder [42] and mreps [43] software. The circular diagram of the mt genomes was drawn with MTVIZ, an online tool of mitochondrial visualization (available at: [http://pacos.informatik.uni-leipzig.de/mtviz/](http://pacos.informatik.uni-leipzig.de/mtviz/)).

**Phylogenetic analyses based on mt genomes**

Phylogenetic analyses were conducted using the two newly sequenced eucotyloid mitogenomes and 28 other selected trematodes mitogenomes of the order Plagiorchiida La Rue, 1957. Schistosoma japonicum belonging to the order Diplostomida Olson, Cribb, Tkach, Bray, and Littlewood, 2003, was used as the outgroup. Two datasets were processed for phylogenetic analyses: dataset 1 containing nucleotide alignment of 11 PCGs, 2 rRNAs and 20 tRNAs and dataset 2 containing amino acid alignment of 11 PCGs. The trnG, trnE and cox3 were excluded from analyses because we were unable to obtain complete sequences of these genes for Tanaisia sp. The PhyloSuite program [39] was used to generate GenBank files of the studied eucotyliids. Fasta files with nucleotide sequences of PCGs, rRNAs and tRNAs were extracted from the GenBank files. The nucleotide sequences of PCGs were translated to their corresponding amino acids using PhyloSuite. Alignments of both datasets were performed separately using the MAFFT program [36] integrated in PhyloSuite, wherein for dataset 1, codon alignment mode was used for PCGs, and normal alignment mode was used for the RNA nucleotide sequences. The alignments were then concatenated (each as a separate set) in PhyloSuite after the removal of ambiguously aligned regions using Gblocks 0.91b [44]. The resulting files were then subjected to ModelFinder [45] to find the most appropriate evolutionary models for both datasets to conduct maximum likelihood (ML) and Bayesian inference (BI) methods of phylogenetic analyses.

For dataset 1, maximum likelihood phylogeny was inferred using IQ-TREE [46] with the GTR+I+F model as the best fit model of nucleotide substitution by performing ultrafast bootstraps [47] with 100,000 replicates. BI phylogeny was inferred using MrBayes 3.2.6 [31, 32] (with default settings) under the GTR+I+G+F model using two MCMC chains for 10,000,000 generations and 1000 sample frequency, in which the initial 25% of trees was discarded as ‘burn-in’ and the rest was used to calculate the BPP.

To remove effects of possible mutation saturation due to silent mutations, dataset 2 (alignment of translated sequences of 11 PCGs) was analyzed in MrBayes with the Jones+I+G+F model as the best fitting model of amino acid evolution. The analysis was conducted using the same parameters as described above for the nucleotide-based BI phylogeny. The phylograms were visualized and annotated in iTOL [48] and Adobe Illustrator®.

**Results and discussion**

**Comparison of nuclear rDNA markers and phylogenetic relationships within Eucotyliidae**

No intraspecific variation was observed in the nucleotide sequences of both markers sequenced from three specimens of each species in this study. The examined eucotyloid collected from C. splendens agreed with the key features of the genus Tamerlania and all qualitative and morphometric characteristics of T. zarudnyi [20] (Table 1: Fig. 1a). Moreover, the 28S rDNA sequences (1255 bp; GenBank accession no. MW131090) showed 99.52% identity with corresponding sequences presently available in GenBank for this species. The minor 28S sequence variability can be explained by the substantial geographic distance between the collection localities of the two sequenced samples. It is worth noting that we collected our material in the region that is relatively close to the area where the species was originally described [49]. The ITS rDNA region of T. zarudnyi was 946 bp in length (GenBank accession no. MW159308); to date, no other Tamerlania sequences are available in GenBank.

The ITS rDNA region of our Tanaisia sp. (935 bp; GenBank accession no. MW159299) showed 96.36–97.64% identity with sequences of T. valida (KX913703–X913711) deposited in the GenBank by authors from Brazil (unpublished). The same authors also deposited 28S rDNA sequences of their two samples of T. valida, Tv6 (1352 bp; KX913713) and Tv12 (1245 bp; KX913714). These samples demonstrated only 95.02% identity with each other, which indicates that the sequences submitted to GenBank as two isolates of T. valida represent two different species. At the same time, the differences between the two Brazilian samples were observed only at the 5’ and 3’ ends of their partial 28S sequences which suggests a problematic quality of sequences at both ends of either both samples or one of them. Upon removal of the potentially problematic 280 bp from the 5’ end and 63 bp from the 3’ end, the remaining 902 bp was 100% identical. Therefore, we used only this 902 bp of 28S rDNA of T. valida in our phylogenetic analysis of the Eucotyliidae and trimmed the remaining sequences to the same length.
A preliminary analysis of the position of the Eucotylidae (including our new sequences) among plagiocotylian digeneans (not shown) did not show any differences compared to previously published phylogenies [8, 9, 12]. Our BI analysis of the interrelationships within the Eucotylidae based on partial 28S rDNA sequences placed the two sequences (one previously available and our new sequence) of *T. zarudnyi* together in a clade with 100% nodal support (Fig. 2). Our specimens identified as *Tanaisia* sp. clustered together with *T. fedtschenkoi* with high nodal support (BPP = 0.95), which was expected because the sequenced sample of *T. fedtschenkoi* originated from Europe and *T. valida* was collected in South America. 28S rDNA sequences of our *Tanaisia* sp. (GenBank accession no. MW139645) showed 98.49% identity with corresponding sequences of *T. fedtschenkoi* (1255 bp; AY116870). Although the key morphological features and dimensions of *Tanaisia* sp. corresponded to those of *T. fedtschenkoi* [20] (Table 1; Fig. 1b), considering the level of divergence in 28S sequences and the lack of ITS rDNA or mitochondrial sequences of *T. fedtschenkoi* in the GenBank, we opted to identify our *Tanaisia* sp. to the genus level only.

Despite the limited amount of currently available sequence data, all three recognized genera of the Tanaisiinae are represented in the phylogeny. Importantly, the type species of all three genera have been sequenced. This provides an opportunity to make some preliminary conclusions regarding the relative value of morphological criteria used in the systematics of the Tanaisiinae. The phylogenetic analysis clearly indicates that *Tanaisia* is phylogenetically closer to *Paratanaisia* than to *Tamerlania* (Fig. 2). The most obvious morphological character shared between the two genera is the relative position of the testes, which are tandem or nearly tandem in *Tanaisia* and *Paratanaisia*, but symmetrical in *Tamerlania*. At the same time, several previous authors considered *Tamerlania* a synonym or a sub-genus of *Tanaisia* [5–7], mostly based on the similarity in the extent of the fields of vitelline follicles, which do not reach posterior margin on both genera. In *Paratanaisia* the vitelline fields extend much further anteriorly and reach the level of intestinal bifurcation [1]. Thus, molecular phylogeny provides evidence that the relative position of testes is more indicative of close evolutionary relationships among taxa of Tanaisiinae than the position of vitelline fields. Although both characters seem to be useful for the practical purpose of differentiation among genera, it is clear that more sequence data with denser taxonomic and geographic sampling are necessary for more definitive conclusions.

### Table 1
Comparative measurements of *Tamerlania zarudnyi* (present study) and *Tanaisia* sp. with *Tamerlania zarudnyi* (previously published) and *Tanaisia fedtschenkoi*, respectively

| Characters                          | *Tamerlania zarudnyi* (present study), Pakistan | *Tamerlania zarudnyi* (Byrd and Denton, 1950), USA | *Tanaisia* sp. (Present study), Pakistan | *Tanaisia fedtschenkoi* (Byrd and Denton, 1950), USA |
|------------------------------------|-------------------------------------------------|-------------------------------------------------|------------------------------------------|-------------------------------------------------|
| Total body length                  | 3.0–3.7                                         | 1.94–3.23                                       | 2.41–3.35                                | 1.62–3.46                                       |
| Body maximum width                 | 0.77–0.86                                       | 0.40–0.66                                       | 0.49–0.74                                | 0.41–0.71                                       |
| Oral sucker length                | 0.19–0.21                                       | 0.13–0.29                                       | 0.14–0.21                                | 0.13–0.22                                       |
| Oral sucker width                  | 0.20–0.27                                       | 0.16–0.32                                       | 0.2–0.29                                 | 0.17–0.29                                       |
| Pharynx length                     | 0.09–0.10                                       | 0.06–0.12                                       | 0.08–0.09                                | 0.05–0.09                                       |
| Pharynx width                      | 0.11–0.13                                       | 0.07–0.14                                       | 0.10–0.12                                | 0.08–0.14                                       |
| Oesophagus length (μm)             | 24–40                                           | 42                                              | 90–109                                   | 34–93                                           |
| Cirrus sac length (μm)             | 80–90                                           | 60–85                                           | 82–110                                   | 78–135                                          |
| Ovary length                       | 0.17–0.18                                       | 0.08–0.21                                       | 0.17–0.21                                | 0.15–0.2                                        |
| Ovary width                        | 0.21–0.26                                       | 0.13–0.23                                       | 0.19–0.21                                | 0.14–0.2                                        |
| Anterior (right) testis length*    | 0.19–0.25                                       | 0.07–0.20                                       | 0.21–0.22                                | 0.15–0.2                                        |
| Anterior (right) testis width*     | 0.21–0.23                                       | 0.07–0.22                                       | 0.20–0.22                                | 0.10–0.2                                        |
| Posterior (left) testis length*    | 0.20–0.24                                       | 0.08–0.20                                       | 0.23–0.35                                | 0.13–0.3                                        |
| Posterior (left) testis width*     | 0.18–0.23                                       | 0.08–0.20                                       | 0.18–0.21                                | 0.10–0.2                                        |
| Unity of caeca from posterior end  | 0.25–0.47                                       | –                                               | 0.29–0.33                                | –                                               |
| Ending of vitellaria from posterior end | 0.42–0.98                      | 0.43–1.06                                       | 0.90–0.97                                | –                                               |
| Eggs length (μm)                   | 32–35                                           | 32–50                                           | 32–34                                    | 33–38                                           |
| Eggs width (μm)                    | 16–21                                           | 25–32                                           | 11–17                                    | 10–19                                           |

*Anterior/posterior in case of *Tanaisia* sp. and right/left in case of *Tamerlania zarudnyi*
The complete mitogenome of *T. zarudnyi* (GenBank accession no. MW334947) was 16,188 bp long and the partial mitogenome of *Tanaisia* sp. (GenBank accession no. MW334948) was 13953 bp in length. The mitogenome of *T. zarudnyi* was one of the largest mt genomes among trematodes sequenced so far, next to *Echinostoma paraensei* (20,298 bp; KT008005; direct GenBank submission), *E. revolutum* (17,030 bp; MN496162; [50]) and *Schistosoma spindale* (16,901 bp; DQ157223; [51]).

We were unable to amplify the fragment containing non-coding regions (partial *trnE-LNCR-trnP-SNCR-partial cox3*) of the mt genomes of *Tanaisia* sp. This was likely due to the presence of repetitive AT-rich sequences resulting in difficulties in PCR amplification. Similar region(s) in other studies of mitochondrial genomes of flatworms have also been shown as problematic [51–54]. This is a more general issue with the Sanger sequencing, which is often unable to sequence complete non-coding region(s) of mt genomes having several tandem repeats [54]. Recently, the PacBio single-molecule real-time sequencing method was used to characterize long and complicated repetitive regions of flatworm mitogenomes [54, 55]. Nonetheless, the nearly complete mt genome of *Tanaisia* sp. sequenced in the present study proved to be sufficient for phylogenetic reconstructions and a variety of comparative analyses.

The mt genomes of both sequenced eucotylids lacked the *atp8* gene and all the genes were encoded on the same strand (H strand). Genes were either separated by non-coding intergenic sequences (1–320 bp), located immediately one after another or even overlapped by 1–40 bp. The general architecture and comparison of orthologous sequences for the two studied eucotylid mitogenomes are summarized in Table 2. The complete circular mt genome of *T. zarudnyi* (Fig. 3a) contained the typical flatworm 36 mitochondrial genes with overall A–T content
of 60.5%. The A–T content of the partial mt genome of *Tanaisia* sp. was 56.7%. The A–T content in both eucotylid mitogenomes was within the range observed in other trematode mitogenomes including xiphidiates, e.g. 51.7% in *P. westermani* (AF219379; direct GenBank submission) and 65.24% in *Plagiorchiis maculosus* (MK641809; [25]). The nucleotide composition and skewness in overall mitogenomes, individual genes and each codon position (1st, 2nd, 3rd) of the studied eucotylids are listed in the table (Additional file 2: Table S2).

**Protein-coding genes and codon usage**

The shortest protein-coding gene in the mt genomes of the studied eucotylids was *nad4L* (270–273 bp), whereas the longest was *cox1* (2055–2061 bp). Notably, the size of the *cox1* gene in eucotylid mt genomes was the largest among all currently sequenced digeneans. This is due to the presence of a long additional amino acid tract (about 123 aa) at the 5’ end of the *cox1* gene in both studied eucotylids. The size of the *cox1* gene reported from the majority of plagiordiiid trematodes varies from 1533 bp in *Fasciola hepatica* [56, 57] to 1567 bp in *Plagiorchiis maculosus* [25]. However, in the mt genomes of diplodostomidan species, the size of the *cox1* gene is significantly longer and ranges from 1611 bp in *Cotylurus marcogliesei* [18] to 1830 bp in *Schistosoma mansoni* [58], which has 60 additional amino acids at the start of the *cox1* gene.

All PCGs of the studied eucotylid mt genomes used ATG or GTG as start codons, whereas the most frequent stop codon was TAG (for 11/12 PCGs in each species) followed by TAA used for *nad4L* in *T. zarudnyi* and for *nad2* in *Tanaisia* sp. (Table 2). The abbreviated or incomplete stop codons T or TA were not used in these mitogenomes. The most frequently used codons in mitochondrial PCGs of both eucotylids were UUU, UUG and GUU, while the least used codons were ACA, CAA and AAC. Codon CAA was entirely missing in PCGs of *Tanaisia* sp. Thus, codons ending in U or G were predominant (≥ 83%), resulting in high AT skewness in the third codon position (−0.682 in *T. zarudnyi* and −0.768 in *Tanaisia* sp.) (Additional file 2: Table S2). Similarly, leucine (L1 + L2), valine, phenylalanine and glycine were the most frequent amino acids in the PCGs of the eucotylid mitogenomes, which were also observed in other xiphidiates [25, 26, 59–61]. Codon usage and relative synonymous codon usage (RSCU) of both eucotylid mitogenomes are presented here (Additional file 3: Figure S1).

**Transfer and ribosomal RNA genes**

All 22 tRNA genes found in the complete mtDNA of *T. zarudnyi* ranged in length from 61 bp (*trnS1*) to 73 bp (*trnK*) (Table 2). Sequences of two tRNA genes, *trnE* and *trnG*, were not obtained for the mt genomes of *Tanaisia* sp. whereas the remaining 20 tRNAs ranged from 62 bp (*trnS1*) to 70 bp (*trnH, trnM, trnA and trnT*) in *Tanaisia* sp. All tRNA sequences could be folded into the typical cloverleaf structure, except *trnS1*, which lacked the dihydrouridine (DHU) arm in both eucotylid mitogenomes (Additional files 4 and 5: Figure S2 and S3). Similarly, standard anticodons were found in all tRNAs of these eucotylid mitogenomes. The nucleotide identity and substitution model among the tRNAs of the two eucotylid mitogenomes are also presented (Additional file 4 and 5: Figure S2 and S3). The ΨYC and DHU loops were highly variable while the anticodon loop was highly conserved with only one (*trnH, trnV, trnL*) or two (*trnK*) nucleotide substitutions. We also found the non-canonical or non-Watson-Crick pairs in different stems (acceptor, anticodon, ΨYC and DHU), as commonly found in other trematode mitogenomes [16, 55, 62]. G–T was the most common non-canonical base pair in the DHU stem of tRNAs in both eucotylids. The nucleotide substitutions among different stems were mostly compensatory or hemi-compensatory base changes (G–T ↔ A–T and C–A to T–A) where there was a single nucleotide mutation in a base pair maintaining their bond in the mitochondrial tRNA of other species.

The large subunit of mitochondrial rRNA (*rrnL*) was 1244 bp long in *T. zarudnyi* and 1231 bp long in *Tanaisia* sp., which is longer than previously reported in any other trematode mitogenomes. The size of the small rRNA subunit (*rrnS*) was within the range reported for other trematode mitogenomes (Table 2). *rrnL* and *rrnS* were separated by *trnC*. Both eucotylid mitogenomes had shared gene boundaries in *trnT–rrnL–trnC–rrnS*, which has been observed in nearly all flatworms characterized so far.

**Non-coding regions and intergenic sequences**

Apart from short intergenic sequences (1–23 nt), one long stretch of intergenic sequences of 162 nt between *nad4* and *trnQ* in the mt genomes of *T. zarudnyi* and 320 nt between *trnY* and *trnL1* in the mt genome of *Tanaisia* sp. were also found. The complete mitogenome of *T. zarudnyi* contains two non-coding regions (NCRs): a large non-coding region (LNCR; 875 bp) and a short non-coding region (SNCR; 835 bp). Both NCRs are located at the usual positions reported in other digeneans (between *trnG* and *cox3*) and separated by *trnE*. The LNCR contains two sets of identical sequences (367 bp each), separated from each other by a stretch of 111 nucleotides. Each set of sequences as well as the gap between them is capable of forming putative secondary structures containing several stem loops (Fig. 3c). Microsatellite-like
simple sequence repeats (SSRs) of TA52 were also found in the SNCR of *T. zarudnyi*. These microsatellite-like sequences can be folded in three stem-loop structures where the A–T bond is replaced by A–A or G–A at three positions (a single instance in each stem).

**Table 2** Comparison of the annotated mitochondrial genomes of *Tamerlania zarudnyi* and *Tanaisia* sp.

| Gene      | Position From:To | Size (bp) | IGN | Codon Start/Stop | Identity (%) |
|-----------|------------------|-----------|-----|------------------|--------------|
| *Tamerlania zarudnyi*/Tanaisia sp. | | | | | |
| cox3      | 1/1 651/120      | 651/120   |    | ATG/CGG TAG/TAG  | 13.12        |
| trnH      | 667/123 734/192  | 68/70     | 15/2| GGT/GTG TAG/TAG  | 71.61        |
| cytB      | 738/198 1850/3130| 1113/1113 | 3/5 | GGT/GTG TAG/TAG  | 71.61        |
| trnF      | 1853/1313        | 270/273   | 2/2 | GGT/ATG TAA/TAG  | 63           |
| nad4L     | 2083/1546 3366/2850| 1284/1305 | -40/-40 | ATG/ATG TAA/TAG | 65.44        |
| trnQ      | 3529/2857 3593/2922| 65/66     | 162/6| GGT/ATG TAA/TAG  | 66.67        |
| trnF      | 3604/2930 3669/2994| 66/65     | 10/7| GGT/ATG TAA/TAG  | 78.79        |
| trnM      | 3692/3014 3762/3083| 71/70     | 22/19| GGT/ATG TAA/TAG  | 73.61        |
| atp6      | 3765/3089 4301/3622| 537/534   | 2/5 | GGT/ATG TAA/TAG  | 46.95        |
| nad2      | 4301/3626 5170/4492| 870/867   | -1/3| GGT/ATG TAA/TAG  | 69.31        |
| trnV      | 5176/4502 5241/4567| 66/66     | 5/9 | GGT/ATG TAA/TAG  | 68.18        |
| trnA      | 5249/4577 5318/4646| 70/70     | 7/9 | GGT/ATG TAA/TAG  | 84.29        |
| trnD      | 5325/4653 5392/4716| 68/64     | 6/6 | GGT/ATG TAA/TAG  | 61.76        |
| nad1      | 5396/4722 6292/5639| 897/918   | 3/5 | GGT/ATG TAA/TAG  | 75.05        |
| trnN      | 6295/5642 6364/5707| 70/66     | 2/2 | GGT/ATG TAA/TAG  | 64.29        |
| trnP      | 6375/5711 6440/5778| 66/68     | 10/3| GGT/ATG TAA/TAG  | 75           |
| trnl      | 6452/5786 6521/5852| 70/67     | 11/7| GGT/ATG TAA/TAG  | 78.57        |
| trnK      | 6541/5846 6613/5929| 73/67     | 19/10| GGT/ATG TAA/TAG  | 63.01        |
| nad3      | 6616/5930 6972/6280| 357/351   | 2/– | GGT/ATG TAA/TAG  | 68.91        |
| trnS1     | 6987/6283 7047/6344| 61/62     | 14/2| GGT/ATG TAA/TAG  | 54.84        |
| trnW      | 7062/6348 7130/6414| 69/67     | 14/3| GGT/ATG TAA/TAG  | 69.57        |
| cox1      | 7134/6418 9188/8478| 2055/2061| 3/3 | GGT/ATG TAA/TAG  | 74.29        |
| trnT      | 9193/8502 9257/8571| 65/70     | 4/23| GGT/ATG TAA/TAG  | 74.29        |
| rrnL      | 9258/8572 10501/9802| 1244/1231| 73     | GGT/ATG TAA/TAG  | 73.3         |
| trnC      | 10502/9803 10570/9868| 69/66     | 74.29|
| rrnS      | 10571/9869 11328/10645| 758/777     | 80.76|
| cox2      | 11329/10646 11925/11245| 597/600     | 74.5 |
| nad6      | 11926/11251 12375/11697| 450/447     | 5/5   | GGT/ATG TAA/TAG  | 59.56        |
| trnY      | 12382/11701 12446/11764| 65/64     | 6/3 | GGT/ATG TAA/TAG  | 73.13        |
| trnL1     | 12457/12085 12521/12150| 65/66     | 10/320 | GGT/ATG TAA/TAG | 79.1         |
| trnS2     | 12532/12160 12604/12228| 73/69     | 10/9 | GGT/ATG TAA/TAG  | 61.64        |
| trnL2     | 12611/12229 12679/12297| 69/69     | 6/–  | GGT/ATG TAA/TAG  | 78.57        |
| trnR      | 12679/12302 12746/12364| 68/63     | 1/4  | GGT/ATG TAA/TAG  | 64.71        |
| nad5      | 12749/12367 13444/13953| 1596/1587 | 2/2 | GGT/ATG TAA/TAG  | 60.53        |
| trnE      | 14344/– 14411/–| 68/–     | –1/– | GGT/ATG TAA/TAG  | 74.29        |
| LNCn      | 14412/– 15286/–| 875/–    | GGT/ATG TAA/TAG | 74.29        |
| trnG      | 15287/– 15353/–| 67/–     | GGT/ATG TAA/TAG | 74.29        |
| LNCn      | 15353/– 16188/–| 835/–    | GGT/ATG TAA/TAG | 74.29        |

bp: base pairs; IGN: intergenic nucleotide; LNCR: large non-coding region; SNCR: short non-coding region

Sliding window analysis and nucleotide diversity

The sliding window analysis (window size = 200, step size = 20) of the aligned mitogenomic sequences of the two studied eucotylids showed relatively low nucleotide diversity (Pi values) of *rrnS* (0.143), *rrnL* (0.209), *nad1* (0.227), *cox1* (0.229), *cox2* (0.251) and *cytb* (0.272) while
genes with relatively high nucleotide diversity included \textit{atp6} (0.386), \textit{nad5} (0.363), \textit{nad6} (0.357) and \textit{nad4L} and \textit{nad4} (0.329 each) (Fig. 4a). A similar result was obtained from the Kimura-2-parameter (K2P) genetic distance analysis of 11 PCGs (substitutions included = transitions + transversions) where \textit{nad1}, \textit{cox1}, \textit{cox2} and \textit{cytb} had the lowest K2P genetic distances, while \textit{atp6}, \textit{nad5}, \textit{nad4L} and \textit{nad6} had comparatively high K2P genetic distances (Fig. 4b). These analyses and average sequence identity (Table 2) consistently indicated that \textit{atp6}, \textit{nad5}, \textit{nad4L} and \textit{nad6} are fast-evolving genes in the two eucotylid mitogenomes.

Similarly high levels of nucleotide variation in \textit{atp6}, \textit{nad5} and \textit{nad6} genes have been reported in mt PCGs of a variety of flatworms [63–65]. Since hypervariable genes are more suitable to resolve recently diverged lineages

\textbf{Fig. 3} Organization of the mitochondrial genome of the two eucotylids (a, b) with secondary structures of one set of repeated sequences and gap between them present in the large non-coding regions (LNCR) and short microsatellite in the short non-coding region (SNCR) of \textit{Tamerlania zarudnyi} (c). Grey colour indicates the intergenic sequences and NCRs. Arrow in (b) denotes the partial sequences of \textit{cox3} gene of the mitogenome of \textit{Tanaisia} sp.
[66], we consider the fast-evolving \( \text{atp6}, \text{nad5} \) and \( \text{nad6} \) to be suitable molecular markers for identification, systematics and population level studies in the family Eucotylidae.

**Phylogeny of xiphidiatan trematodes and other selected trematodes based on mt genomes**

Both ML and BI analyses (based on dataset 1) with the respective models produced phylogenetic trees with identical branch topologies and only minor differences in nodal support (Fig. 5). The BI phylogeny based on concatenated amino acid sequences of 11 PCGs (dataset 2) was consistent with that resulting from ML and BI phylogenies based on nucleotide sequences (Fig. 6). The only notable difference was the position of the clade containing members of the families of the suborder Prococephalata Olson, Cribb, Tkach, Bray & Littlewood, 2003. In the nucleotide-based tree, it clustered together with the clade of the suborder Echinostomata (Fig. 5), while in the amino acid-based tree it formed a clade with the Opisthorchiata La Rue, 1957, and one of the clades representing paraphyletic xiphidiates (Fig. 6). This difference between phylogenies based on mitochondrial nucleotides and amino acids sequences is consistent with our previous study [26] and several other studies, including those published very recently [50].

Regardless of the dataset and model used, all phylogenies supported the paraphyletic nature of the superfamily Gorgoderoidea sensu Curran, Tkach & Overstreet, 2006, and suborder Xiphidiata. Importantly, eucotylids...

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**Fig. 4** Comparative analyses of two eucotylid mitogenomes. **a** Sliding window analysis of the alignment of 11 protein-coding genes (PCGs), 2 rRNAs and 18 coalescent tRNAs (\( \text{trn}E \) and \( \text{trn}G \) are omitted as we were unable to obtain their sequences for one of the species). The black line represents nucleotide variation in a window of 200 bp (step size = 20 bp, with the values inserted at its mid-point). Gene boundaries are indicated by colour with mean variation ratio per gene shown above each gene. The region indicated by the circle in \( \text{cox}1 \) gene shows nucleotide variation in the additional sequences at the 5' end of this gene in both studied eucotylid mt genomes. **b** The Kimura-2-parameter distance (K2P) among 11 PCGs of eucotylid mitogenomes.
did not show close affinity to any other members of the Plagiorchiida included in the analysis and appeared on the trees as either a sister clade to the remaining ingroup taxa or as a member of a polytomy. Olson et al. [9] classified Paragonimidae and Dicrocoeliidae within the superfamily Gorgoderoidea based on the phylogenetic analysis using nuclear rDNA genes. However, recently published data based on 28S rDNA did not support the close relationship between the Paragonimidae and other families included in the superfamily Gorgoderoidea [12]. Our analyses suggest that the Paragonimidae might be closer to the Brachycladiidae than to the Dicrocoeliidae. The position of the Dicrocoeliidae and the Eucotylidae (Microphalloidea) outside the clade uniting other xiphidiatan trematodes (Brachycladiidae and Paragonimidae) further strengthens the suggestion expressed by Suleman et al. [26] that the content of the suborder Xiphidiata may need to be reconsidered with more sequence data.

Conclusions
In this study, we sequenced the ITS rDNA region and 28S rDNA gene as well as complete or nearly complete mitochondrial genomes of two eucotylids, *T. zarudnyi* and *Tanaisia* sp. The complete mitochondrial genome of *T. zarudnyi* was the fourth largest mt genome of all available trematode mt genomes. The presence of a long additional string of amino acids (about 123 aa) at the 5’ end of the *cox1* gene in mt genomes of both studied eucotylids increases the size of the *cox1* gene, which is longer than in any of the previously sequenced trematodes and probably any flatworm. Similarly, the *rrnL* gene was longest among those reported so far from digeneans. The TΨC and DHU loops of the tRNAs varied greatly between the two eucotylids while the anticodon loop was highly conserved. Our analyses of the average sequence identity combining nucleotide diversity and Kimura-2-parameter distances between the two eucotyloid mitogenomes suggested that *atp6, nad5, nad4L* and *nad6* genes are better molecular markers for the species differentiation and population-level studies of eucotylids. Phylogenetic analyses based on mitochondrial nucleotide sequences (PCGs+RNAs) and concatenated amino acid sequences (11 PCGs) showed a lack of close relationship of the Eucotylidae with any major clade within the Plagiorchiida. Furthermore, our analyses did not support the classification of Paragonimidae and Dicrocoeliidae within the superfamily Gorgoderoidea. Similarly, the position of the Dicrocoeliidae and Eucotylidae (Microphalloidea)
outside the clade uniting other xiphidiatan trematodes strengthened the proposal that the content of the sub-order Xiphidiata and the interrelationships between its constituent families may need to be reconsidered.

**Supplementary information**

The online version contains supplementary material available at https://doi.org/10.1186/s13071-020-04547-8.

Additional file 1: Table S1. Sequences of primers used to amplify and sequence the mitochondrial genomes of *Tamerlania zarudnyi* and *Tanaisia* sp.

Additional file 2: Table S2. Nucleotide composition and AT/GC skewness of the mitochondrial genome of *Tamerlania zarudnyi* and *Tanaisia* sp.

Additional file 3: Figure S1. Relative synonymous codon usage (RSCU) for the protein-coding genes of two eucotylid mitogenomes. Codon families are labeled on the x-axis. Values on the top of the bars indicate percentages of each amino acid used for the construction of protein-coding genes.

Additional file 4: Figure S2. Secondary structures of tRNAs (trnH-trnK) in eucotylid mitogenomes with nucleotide substitutions highlighted.

Additional file 5: Figure S3. Secondary structures of tRNAs (trnS1-trnG) in eucotylid mitogenomes with nucleotide substitutions highlighted, except trnE and trnG.

**Abbreviations**

BI: Bayesian inference; ML: Maximum likelihood; MCMC: Metropolis-coupled Markov chain Monte Carlo; PCGs: Protein-coding genes; tRNAs: Transfer RNAs; rRNAs: Ribosomal RNAs; RSCU: Relative synonymous codon usage; dN/dS: Non-synonymous/synonymous; TRs: Tandem repeats; NCRs: Non-coding regions; SNCR: Short non-coding region; LNCR: Long non-coding region; K2P: Kimura-2-parameter.

**Acknowledgements**

The authors thank Ms Rong Li for technical assistance.

**Authors' contributions**

Suleman performed the experiments, analyzed data and drafted the manuscript. MSK, HU and NM participated in specimen collections. XQZ directed and supervised this study. JM, ME and VVT contributed to data analysis, the writing and revision of the manuscript. All authors read and approved the final manuscript.

**Funding**

This study was supported by the National Natural Science Foundation of China (grant no. 31702225), the International Science and Technology Cooperation Project of Gansu Provincial Key Research and Development Program (grant no. 17J7WA031) and the Agricultural Science and Technology Innovation Program (ASTIP) (grant no. CAAS-ASTIP-2016-LVRI-03).

**Availability of data and materials**

The datasets supporting the findings of this article are included within the article and its additional files. The nuclear ITS and 28S rDNA nucleotide sequences generated in this study for the two eucotylids were deposited in the GenBank database under the accession numbers MW159308 and MW131090 for *Tamerlania zarudnyi* and MW159299 and MW139645 for *Tanaisia* sp. The mitogenomic sequences of *Tamerlania zarudnyi* and *Tanaisia* sp. are available in GenBank under the accession numbers MW334947 and MW334948, respectively.

**Fig. 6** Phylogeny of the order Plagiorchiida based on Bayesian inference (BI) using concatenated amino acid sequences of mitochondrial protein-coding genes. Rectangles indicate BPP = 100; other values are given above the nodes. *Schistosoma japonicum* is the outgroup.
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