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An integrated pipeline for NGS and annotation of the complete mitochondrial genome of the giant intestinal fluke, *Fasciolopsis buski* (lankester, 1857) Looss, 1899 (Digenea: Fasciolidae)

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Abstract

Helminths include both parasitic nematodes (roundworms) and platyhelminths (trematode and cestode flatworms) that are abundant, and are of clinical importance. The genetic characterization of parasitic flatworms using advanced molecular tools is central to the diagnosis and control of infections. Although the nuclear genome houses suitable genetic markers (e.g., in ribosomal (r) DNA) for species identification and molecular characterization, the mitochondrial (mt) genome consistently provides a rich source of novel markers for informative systematics and epidemiological studies. In the last decade, there have been some important advances in mtDNA genomics of helminths, especially lung flukes, liver flukes and intestinal flukes. *Fasciolopsis buski*, often called the giant intestinal fluke, is one of the largest digenean trematodes infecting humans and found primarily in Asia, in particular the Indian subcontinent. Next-generation sequencing (NGS) technologies now provide opportunities for high throughput sequencing, assembly and annotation within a short span of time. Herein, we describe a high-throughput sequencing and bioinformatics pipeline for mt genomics for *F. buski* that emphasizes the utility of short read NGS platforms such as Ion Torrent and Illumina in successfully sequencing and assembling the mt genome using innovative approaches for PCR primer design as well as assembly. We took advantage of our NGS whole genome sequence data (unpublished so far) for *F. buski* and its comparison with available data for the *Fasciola hepatica* mtDNA as the reference genome for design of precise and specific primers for amplification of mt genome sequences from *F. buski*. A long-range PCR was carried out to create a NGS library enriched in mt DNA sequences. Two different NGS platforms were employed for complete sequencing, assembly and annotation of the *F. buski* mt genome. The complete mt genome sequences of the intestinal fluke comprise 14,118 bp and is thus the shortest trematode mitochondrial genome sequenced to

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date. The noncoding control regions are separated into two parts by the tRNA-Gly gene and donot contain either tandem repeats or secondary structures, which are typical for trematode control regions. The gene content and arrangement are identical to that of *F. hepatica*. The *F. buski* mtDNA genome has a close resemblance with *F. hepatica* and has a similar gene order tallying with that of other trematodes. The mtDNA for the intestinal fluke is reported herein for the first time by our group that would help invesigate Fasciolidae taxonomy and systematics with the aid of mtDNA NGS data. More so, it would serve as a resource for comparative mitochondrial genomics and systematic studies of trematode parasites.

Keywords

Fasciolopsis buski, Mitochondria, Next generation Sequencing, Contigs

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Introduction

Fasciolopsis buski, often called the giant intestinal fluke, is one of the largest digenean trematode flatworms infecting humans and found primarily in Asia and the Indian subcontinent, also occurring in Taiwan, Thailand, Laos, Bangladesh, India, and Vietnam. The trematode predominates in areas where pigs are raised, they being the most important reservoirs for the organism and where underwater vegetables viz. water chestnut, lotus, caltrop and bamboo are consumed. It is an etiological agent of fasciolopsiasis, a disease that causes ulceration, haemorrhage and abscess of the intestinal wall, diarrhoea, and even death if not treated properly. Interestingly, most infections are asymptomatic with high rates of infection (up to 60%) in India and the mainland China (Le *et al.,* 2004). Among animals, pigs are the main reservoir of *F. buski* infection. In India, the parasite has been reported from different regions including the Northeast and variations in the morphology of the fluke have been observed from different geographical regions (Roy & Tandon, 1993). *F. buski* occurs in places with warm, moist weather and is the only single species in the genus found in aquatic environments. The complex life cycle combined together with the specific immune evasion traits of parasites make research and drug or vaccine programs for intestinal flukes very difficult; consequently, new methods to control this parasite are required. Being one of the most important intestinal flukes from epidemiological point of view, *F. buski* seeks considerable attention from the scientific community and the available gene sequences for the organism on the public domain remain scarce thereby restricting research avenues. Therefore, fasciopsiasis has become a public health issue and is of major socioeconomic significance in endemic areas.

Metazoan mitochondrial (mt) genomes, ranging in size from 14 to 18 kb, are typically circular and usually encode 36–37 genes including 12–13 protein-coding genes, without introns and with short intergenic regions (Wolstenholme, 1992). Due to their maternal

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inheritance, faster evolutionary rate change, lack of recombination, and comparatively conserved genome structures mitochondrial DNA (mtDNA) sequences have been extensively used as molecular markers for studying the taxonomy, systematics, and population genetics of animals (Li *et al.,* 2008*;* Catanese, Manchado & Infante*,* 2010). At the time of writing this manuscript, quite a number of complete metazoan mt genomes are already deposited in GenBank (Benson *et al*., 2005) and other public domain databases viz. Mitozoa (D'Onorio de Meo *et al.,* 2011), mainly for Arthropoda, Mollusca, Platyhelminthes, Nematoda, and Chordata (Chen *et al.,* 2009). Presently, the class Trematoda comprises about 18,000 nominal species, and the majority of them can parasitize mammals including humans as their definitive host (Olson *et al*., 2003). Despite their medical and economical significance, most of them still remain poorly understood at the molecular level. In particular, the complete mt genomes of the species belonging to the family Fasciolidae are not at all available in the public domain. Complete or near-complete mt genomes are now available for 15 odd species or strains of parasitic flatworms belonging to the classes Trematoda and Cestoda. To date, a PCR-based molecular characterization using ITS1&2 molecular markers for *F. buski* have been carried out (Prasad *et al.,* 2007). However, further datasets generated by high-throughput sequencing and comparative transcriptome analysis could bring a more comprehensive understanding of the parasite biology for studying parasite-host interactions and disease as well as parasite development and reproduction, with a view towards establishing new methods of prevention, treatment or control.

Until quite recently, sequencing of mt genomes was somewhat challenging and a daunting task. It has been approached using the conventional strategy of combining long-range PCR with subsequent primer walking. The paradigm shift caused by the third generation sequencing technologies have paved the way for Next-Generation Sequencing (NGS) technologies, which encourages proposals for more straightforward integrated

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pipelines for sequencing complete mt genomes (Jex, Littlewood & Gasser, 2010) that are more cost effective and less time consuming.

Here in, we present a straightforward approach for reconstructing novel mt genomes directly from NGS data generated from total genomic DNA extracts. We took advantage of the whole genome sequence data for *F. buski* (our unpublished results), generated by NGS and its comparison with the existing data for the *F. hepatica* mt genome sequence to design precise and specific primers for amplification of mt genome sequences of *F. buski*. We then carried out long-range PCR to create a NGS library enriched in mt DNA sequences. We utilized two different next generation sequencing platforms to completely sequence the mitochondrial genome, and applied innovative approaches to assemble the mitochondrial genome *in silico* and annotate it. When verifying one region of the assembly by Sanger sequencing it was found to match our assembly results. The purpose of the present study was to sequence the mt genome of *F. buski* for the first time with a novel strategy, compare its sequences and gene organization, identify any adaptive mutations in the 12 protein-coding genes of the intestinal parasite species, and to reconstruct the phylogenetic relationships of several species of Trematoda and Cestoda in the Phylum Platyhelminths, using mtDNA sequences available in GenBank.

Material & Methods

Parasite material and DNA Extraction

Live adult *F. buski* were obtained from the intestine of freshly slaughtered pig, *Sus scrofa* domestica at local abattoirs meant for normal meat consumption and not specifically for this design of study. The worms recovered from these hosts represented the geographical isolates from Shillong (co-ordinates 25.57°N 91.88°E) area in the state of Meghalaya, Northeast India. Eggs were obtained from mature adult flukes by squeezing between two glass slides. For the purpose of DNA extraction, adult flukes collected from different host animals were

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processed singly; eggs recovered from each of these specimens were also processed separately. The adult flukes were first immersed in digestion extraction buffer [containing 1% sodium dodecyl sulfate (SDS), 25 mg Proteinase K] at 37°C for overnight. DNA was then extracted from lysed individual worms by standard ethanol precipitation technique (Sambrook, Fitsch & Maniatis, 1989) and also extracted from the eggs on FTA cards using Whatman's FTA Purification Reagent. DNA was subjected to a series of enzymatic reactions that repair frayed ends, phosphorylate the fragments, and add a single nucleotide 'A' overhang and ligate adaptors (Illumina's TruSeq DNA sample preparation kit). Sample cleanup was done using Ampure XP SPRI beads. After ligation, ~300-350 bp fragment for short insert libraries and ~500 – 550 bp fragment for long insert libraries were size selected by gel electrophoresis, gel extracted and purified using Minelute columns (QIAGEN). The libraries were amplified using 10 cycles of PCR for enrichment of adapter-ligated fragments. The prepared libraries were quantified using Nanodrop and validated for quality by running an aliquot on High Sensitivity Bioanalyzer Chip (Agilent). 2X KapaHiFiHotstart PCR ready mix (KapaBiosystemsInc, Woburn, US) reagent was used for PCR. the Ion torrent library was made using Ion Plus Fragment library preparation kit (Life Technologies, Carlsbard, US) and the Illumina library was constructed using TruSeqTM DNA Sample Preparation Kit (Illumina, Inc, US) reagents for library prep and TruSeq PE Cluster kit v2 along withTruSeq SBS kit v5 36cycle sequencing kit (Illumina, Inc, US) for sequencing.

Primer design strategy and Polymerase Chain Reaction (PCR)

~16 million 100 base-paired end reads were available for *F. buski* as a part of an independent attempt towards whole genome sequencing of *F. buski*. In order to recover mtDNA coding sequences from this data, *Fasciola hepatica* mt genome with accession AF216697.1 was retrieved from GenBank as a reference mt Genome and alignment using Bowtie (v2-2.0.0-beta6/bowtie2 --end-to-end --very-sensitive --no-mixed --phred64) (Langmead *et al.,* 2009).

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In all, 1625 paired end reads were obtained, which were aligned to different intervals in the *F. hepatica* mt genome, covering ~ 3 kb of the 14 kb *F. hepatica* mt genome. Accordingly, primers were designed at these regions, using sequence information from *F. buski* to ensure optimum primer designing as shown in Table 1. Long-range PCR was carried out using 10 ng of genomic DNA from *F. buski* and the following PCR conditions: 10 ng of FD-2 DNA with 10 uM Primer mix in 10 ul reaction PCR cycling conditions – 98° C for 3min, 35 cycles of 98° C for 30 sec, 60 for 30 sec, 72 for 2 min 30sec, final extension 72° C for 3 min and 4° C hold. The bands were gel-eluted corresponding to different products and pooled for NGS library construction (Fig. 1).

NGS Library construction, sequencing and assembly

The pooled PCR products were sheared to smaller sizes using Bioruptor. One each of Ion Torrent and Illumina library was constructed as per manufacturers' protocols. Briefly, PCR products were sonicated, adapter ligated and amplified for x cycles to generate a library. The libraries were sequenced to generate 14k reads of an average of 150 nt SE reads on Ion Torrent, and 1.3 million reads of 72 nt SE reads on Illumina GAIIx. High quality and vector filtered reads from Ion Torrent and Illumina sequencing were assembled (hybrid-assembly) using Mira-3.9.15 (http://sourceforge.net/apps/mediawiki/mira-assembler). The hybrid assembly generated 776 contigs. All 776 contigs were then used as input for CAP3 assembler which generated 38 contigs. The contigs were further filtered to remove short and duplicate contigs. Finally, only 14 contigs were retained and ORF prediction was carried out using ORF Finder (Open Reading Frame Finder) (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The schematic outline of the assembly is depicted in Fig. 2.

A manual examination of the 14 contigs revealed overlaps amongst all of them (except C30) (Fig. 2) and in collinear arrangement when compared with the *F. hepatica* mitochondrial

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sequence. The 14 contigs were manually joined wherever overlaps (minimum overlap > 5) were found and that resulted in two individual contigs, which, in turn, were assembled into one single contig with the addition of a couple of 'N's'. To resolve the remaining gaps between the two contigs as well as to confirm the assembly both the regions were amplified and Sanger sequenced. The Sanger sequencing was carried out by designing two primers for both the contigs flanking the 'Ns' to resolve this gap and to verify the assembly as well as closure of the gap that was remaining after joining the contigs manually. The Sanger data in two regions was used to replace the NGS assembly-derived data to refine the assembly and obtain one single contig with no gaps. Region 1 was a ~500 nt overlapping region between C2 and C16. Region 2 was sequenced using one primer in C24 and the second primer in C26. Considering the finished mitochondrial genome, i.e., from position 1 to 14118, two primer pairs were designed as detailed below:

Set 1: fw primer position # 7395-7414(Length=20)

FORWARD PRIMER: TGGTTATTCTGGTTGGGGAG

rev primer position # 8137-8159(Length=23)

REVERSE PRIMER: AACCCTCCTATAAGAACCCAAAG (RC=)

CTTTGGGTTCTTATAGGAGGGTT

The Sanger sequence data and NGS assembly aligned to each other with 94% identity. Twenty-nine out of 494 positions showed discordance between the Sanger sequencing and NGS-derived sequencing for this region (Fig. 3). These discordances consist of 19 gaps and 10 mismatches that can be introduced by either the sequencing chemistry (for e.g homopolymeric stretches in Ion Torrent) or an assembly artifact (eg. Ns). Overall, the Sanger sequencing confirmed the assembly pipeline and also corrected errors that are commonly observed in NGS pipelines.

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Set 2: fw primer position # 4634-4655(Length=22),

FORWARD PRIMER: TAGGGTTATTGGTGTTAACCGG

reverse primer position #4961-4937(Length=25)

REVERSE PRIMER: CAACAAACCAACAACTATACATCCC

REV PRIMER RC:- GGGATGTATAGTTGTTGGTTTGTTG

The region between contigs C24 and C26 did not show any overlap. The forward primer was 94 bp inward from the junction on C24 and the reverse primer was 112 bp outward from the junction on C26. The expected region based on assembly for contigs 24 and 26 and the Sanger results are shown in Fig. 4. The bases in brown colour within brackets are the bases that fill the gap between C24 and C26. Sanger sequencing of the region between C24 and C26 enabled gap-filling of a region that was not sequenced/assembled by the NGS approach and enabled assembly of the mitochondrial genome into one single draft genome.

To confirm our findings reported herein, whole genomic DNA from an independent *F. buski* sample replicate (Sample FD3) was used and Sanger sequencing was performed on two separate regions (Sample FD3-Region C24-C26 and Sample FD3-Region C2-C16) as described above. The regions from two independent biological sample replicates (FD2 and FD3) by Sanger sequencing exhibited 98-99% identity and thus validated our results (Fig. 5).

The data pertaining to this study is available in the National Centre for Biotechnology Information (NCBI) Bioproject database with Accession: PRJNA210017 and ID: 210017.

The contig assembly files are deposited in NCBI Sequence Read Archive (SRA) with Accession: SRR924085.

In silico analysis for nucleotide sequence statistics, protein coding genes (PCGs) prediction, Annotation and tRNA prediction

Sequences were assembled and edited both manually and using CLC Genome Workbench V.6.02 with comparison to published flatworm genomes. The platyhelminth genetic code

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(Telford *et al*., 2000) was used for translation of reading frames. Protein-coding genes were identified by similarity of inferred amino acid sequences to those of other platyhelminth mtDNAs available in GenBank. Boundaries of rRNA genes both large (rrnL) and small (rrnS) were determined by comparing alignments and secondary structures with other known flatworm sequences. The program ARWEN (Laslett & Canbäck, 2008) was used to identify the tRNA genes (trns). To find all tRNAs, searches were modified to find secondary structures occasionally with very low Cove scores (<0.5) and, where necessary, also by restricting searches to find tRNAs lacking DHU arms (using the nematode tRNA option). Nucleotide codon usage for each protein-encoding gene was determined using the program Codon Usage) at http://www.bioinformatics.org/sms2/codon_usage.html. The ORFs and codon usage profiles of PCGs were analyzed. Gene annotation, genome organization, translation initiation, translation termination codons, and the boundaries between protein-coding genes of mt genomes of the two fasciolid flukes were identified based on comparison with mt genomes of other trematodes reported previously (Le *et al.,* 2002). The mtDNA genome of *F. buski* was annotated taking *F. hepatica* as a reference genome using several open source tools viz. Dual Organellar Genome Annotator (DOGMA) (Wyman, Jansen & Boore, 2004), Organellar Genome Retrieval System (OGRe) (Jameson *et al.,* 2004) and Mitozoa database (D'Onorio de Meo *et al.,* 2011). The newly sequenced and assembled *F. buski* mtDNA was sketched with GenomeVX at http://wolfe.ucd.ie/GenomeVx/ with annotation files from DOGMA (Wyman, Jansen & Boore, 2004).

Phylogenetic Analysis

The 12 PCGs were concatenated and a super matrix was created in Mesquite (Maddison & Maddison, 2001) and run in MrBayes (Ronquist & Huelsenbeck, 2003). Phylogenetic analyses of concatenated nucleotide sequence datasets for all 12 PCGs were performed using Bayesian inference [BI]). MrBayes was executed using four MCMC chains and 106

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generations, sampled every 1,000 generations. Each of the 12 genes was treated as a separate unlinked data partition. Bayesian posterior probability (BPP) values were determined after discarding the initial 200 trees (the first 2×105 generations) as burn-in. Using the phylogeny estimated from the nuclear ribosomal DNA data set, pictograms of full mitochondrial genes indicating the gene order were aligned next to the individual 'leaves' of the tree (Fig. 6).

Results and discussion

Gene contents and organization

The intestinal fluke *F. buski* has a mt genome typical of those of most platyhelminths (Fig. 7A). The circular genome consists of 14118 nt bp and is almost similar to that of *Fasciola hepatica* (Fig. 7B). The 12 protein-coding genes fall into the following categories: nicotinamide dehydrogenase complex (nad1–nad6 and nad4L subunits); cytochrome c oxidase complex (cox1–cox3 subunits); cytochrome b (cob) and adenosine triphosphatase subunit 6 (atp6). Two genes encoding ribosomal RNA subunits are present: the large subunit (rrnL or 16S) and small subunit (rrnS or 12S), which are separated by trnC, encoding the transfer RNA (tRNA) for cysteine. As in other mt genomes, there are 22 tRNA genes, denoted in the figure by the one-letter code for the amino acid they encode. Leu and Ser are each specified by two different tRNAs, reflecting the number and base composition of the relevant codons. As in other flatworms, all genes are transcribed in the same direction (Fig. 7). Genes lack introns and are usually adjacent to one another or separated by only a few nucleotides. However, some genes overlap, most notably nad4, nad4L and with regions of the long non coding region, which is almost 500nt length.

Nucleotide composition and codon usage

Invertebrate mt genomes tend to be AT-rich (*Malakhov, 1994*), which is a notable feature in PCGs of several parasitic flatworms. However, nucleotide composition is not uniform among

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307 the species (Table 2). Values for >70% AT are seen in all *Schistosoma* spp. except for *S.*
308 *mansoni* (68.7%), whereas *F. buski* and *Fasciola hepatica* are 60% AT rich and *Paragonimus*
309 *westermani,* only 50 % AT rich. Cytosine is poorly represented in *F. Buski*. The annotation
310 and nucleotide sequence statistics are enumerated in Tables 2- 5. The gene content and
311 arrangement are identical to those of *F. hepatica*. ATG and GTG are used as the start-codons
312 and TAG and TAA, the stop-codons.

313 Among species considerable differences in base composition in PCGs are reflected in
314 differences in the protein sequences. However, the redundancy in the genetic code provides a
315 means by which a mt genome could theoretically compensate for base-composition bias.
316 Increased use of abundant bases in the (largely redundant) third codon position accounts for
317 the fact that base composition bias would be less marked in the first and second codon
318 positions. A phylogenetic tree was computed concatenating all the annotated 12 PCGs that
319 completely accounted for the platyhelminth phylogeny with the represenative species (Fig.
320 8). *F. buski* came in the same clade with *F. hepatica* while *Ascaris* species formed the
321 outgroup. The outgroup *Ascaris lumbricoides* displayed a different gene order that was
322 aligned adjacent to the phylogenetic leaf nodes (Fig. 8).

323 **Transfer and ribosomal RNA genes**
324 A total of 22 tRNAs were inferred along with structures (Fig. 9). The complete annotation
325 along with their GC percentage is shown in Table 6. tRNA-Leu had the highest GC
326 composition and the length varied between 60-70 nt bases. The tRNA genes generally
327 resemble those of other invertebrates. A standard cloverleaf structure was inferred for most of
328 the tRNAs. Exceptions include tRNA(S) in which the paired dihydrouridine (DHU) arm is
329 missing as usual in all parasitic flatworm species (also seen in some other metazoans) and
330 also tRNA(A) in which the paired DHU-arm is missing in cestodes but not in trematodes (and
331 not usually in other metazoans) and hence, was also seen in *F. buski*. Structures for tRNA(C)

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vary somewhat among the parasitic flatworms. A paired DHU-arm is present in *F. buski*, which is not seen in *Schistosoma mekongi* and cestodes. A comparative synteny for all the 12 protein coding genes and 22/23 tRNAs for the representative platyhelminth parasites can be seen across all the species under study (Fig. 8).

Conclusions

Although mt genomes of only a few parasitic flatworms have been sequenced, some general points can be made. The mtDNA of *F. buski* didnot exhibit any surprising gene order composition or their organization relative to other invertebrates. As usual atp8 was absent, which is not without a precedent among invertebrates. Some typical secondary structures were inferred for some tRNA genes. Again, however, mt tRNA genes are less conserved in metazoans as compared to their nuclear counterparts. Gene order is similar or identical among most of the flatworms investigated, which might be expected for a taxon at this level of taxonomic heirarchy. In conclusion, the complete mtDNA sequences of *F. buski* will add to the knowledge of the trematode mitochondrial genomics and will aid in phylogenetic studies of the family Fasciolidae.

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412 **Figure Legends**

413 **Figure 1 - Gel images of the long range PCR products**

414 Long-range PCR carried out using 10 ng of genomic DNA from F. buski (FD2 and FD3
415 samples). Gel-eluted bands corresponding to different products that were pooled for NGS
416 library construction are shown.

417 **Figure 2 - Strategy for MIRA and CAP3 Assembly for mtDNA NGSdata**

418 Iontorrent and Illumina High quality and vector filtered Reads assembled (hybrid assembly)
419 using mira-3.9.15. 776 contigs were generated from the hybrid assembly. All the 776 contigs
420 were fed in CAP3 assembler. Post filtering 14 contigs were retained. From 14 contigs
421 overlapping contigs were joined and 2 contigs were formed, which were finally joined as one
422 with the addition of couple of N's. Predicted ORFs were compared against F. hepatica coding
423 regions. Region 1 is a ~500 nt overlapping region between C2 and C16. Region 2 was
424 sequenced using one primer in C24 and the second primer in C26.

425 **Figure 3 - Assembly confirmation of the ~500 nucleotide region between C2-C16.**

426 Primers spanning the 500bp overlap junction between contig 2 and contig 16 are marked in
427 green font. Sanger sequenced region (query) and NGS assembly (subject) were aligned with
428 94% identity with strong supportive E-values (0.0). Twentynine out of 494 positions showed
429 discordance between the Sanger sequencing and NGS- derived sequencing consisting of 19
430 gaps and 10 mismatches that may be introduced by either sequencing chemistry (eg.
431 homopolymeric stretches in Ion Torrent) or an assembly artifact (eg. Ns).

432 **Figure 4 - Assembly confirmation for the C24-C26 region**

433 Region between contigs C24 and C26 showing no overlap regions. Forward primer is 94 bp
434 inward from the junction on C24 and the reverse primer 112 bp outward from thejunction on
435 C26. The bases in brown colour in brackets are those that fill the gap between C24 and C26.

436

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Figure 5 - Sanger sequencing confirmatory results for FD2 and FD3 replicate samples.
Two separate regions from two independent biological samples sequenced by Sanger methods showing 98-99% identity between samples FD2 (subject) and FD3 (query) in the regions C2-C16 and C24-C26.

Figure 6 - Phylogenetic analysis of the concatenated 12 protein coding genes from the platyhelminth mtDNA.
Differences in the gene order in the mitochondrial genomes of parasitic flatworms from the Trematoda and Cestoda and taking Nematoda (Ascaridida) as an outgroup. Phylogenetic analyses of concatenated nucleotide sequence datasets for all 12 PCGs were performed using Bayesian Inference using four MCMC chains and 106 generations, sampled every 1,000 generations. Bayesian posterior probability (BPP) values were determined after discarding the initial 200 trees (the first 2◊105 generations) as burn-in. Using the phylogeny estimated from the nuclear ribosomal DNA data set, pictograms of full mitochondrial genes are indicated next to the individual ëleavesí of the tree.

Figure 7 - Circular genome map of Fasciola hepatica and Fasciolopsis buski.
The manual and in silico annotations with appropriate regions for F. buski (7A) and annotated GenBank flat file for F. hepatica (7B) were drawn into a circular graph in GenomeVX depicting the 12 PWGs and 22tRNAs.

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462 **Figure 8 - Synteny map of the representative species for the platyhelminth mtDNA.**

463 A comparative synteny for all the 12 protein coding genes and 22/23 tRNAs for the

464 representative platyhelminth parasites (Schistosoma spp, F. buski, Fasciola hepatica,

465 Paragonimus westermani). X-axis represents substitution rates per unit.

466

467 **Figure 9 - 22 tRNA secondary structures predicted using ARWEN.**

468

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469 **Table 1.** Primer sequences used in the study

Primer name	Primer Sequence	Product	Expected Length	Observed Length (bp)
F1	TACATGCGGATCCTATGG	P1	1525	500, 700, 1000
F2	AAAGACATACAAACAACAAC			
F3	TCTTTAGTGTATTCTTTGGGTCATG	P2	2660	3000
F4	AACAACCCCAACCTACCCT			
F5	GTTTGTTGAGGGTAGGTTGGGG	P3	1623	1600
F6	CAAATCATTAATGCGAGG			
F7	CTTTTTGATGCCTGTGTTCATAG	P4	2010	2000
F8	ACCTTTCAAACAATCCCCCA			
F9	CGGATTTATAGATGGTAGTGCCTG	P5	1037	1000
F10	CCGGATATACACTAACAAACATAATTAAG			
F11	GTTTGTTAGTGTATATCCGGTTGAAG	P6	2361	2200
F12	GGCAGCAACCAAAGTAGAAGA			
F13	TATTTCTTGGTTGTTGGAGGCTAT	P7	3783	4000, 8000
F14	TCTATAGAACGCAACATAGCATAAAAG			

470

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Table 2. Mitochondrial DNA Nucleotide sequence statistics information of Platyhelminths

Sequence type	DNA	DNA	DNA	DNA	DNA	DNA	DNA	DNA	DNA	DNA	DNA	DNA	DNA
Length	14,118, bp circular	14,462bp circular	14,965bp circular	14,277bp circular	13,510 bp	13,875bp circular	15,003bp circular	14,415bp circular	14,085bp circular	13,670bp circular	13,709bp circular	14,281 bp circular	14,284 bp circular
Organism Name	***Fasciolopsis buski***	***Fasciola hepatica***	***Paragonimus westermani***	***Opisthorchis felineus***	***Opisthorchis viverrini***	***Clonorchis sinensis***	***Schistosoma haematobium***	***Schistosoma mansoni***	***Schistosoma japonicum***	***Taenia saginata***	***Taenia solium***	***Ascaris lumbricoides***	***Ascaris suum***
Accession	Submitted to GenBank	NC_002546	NC_002354	EU921260	JF739555	FJ381664	NC_008074	NC_002545	NC_002544	NC_009938	NC_004022	JN801161	NC_001327
Modification Date	submitted	01-FEB-2010	01-FEB-2010	18-AUG-2010	05-APR-2012	01-JUL-2010	14-APR-2009	14-APR-2009	01-FEB-2010	14-APR-2009	01-FEB-2010	01-DEC-2011	11-MAR-2010
Weight (single-stranded)	4396.507	4,499.496 kDa	,652.101 kDa	4,437.683 kDa	4,197.397 kDa	4,311.834 kDa	4,658.966 kDa	4,482.165 kDa	4,371.002 kDa	4,242.425 kDa	4,251.992 kDa	4,428.619 kDa	4,429.981 kDa
Weight (double-stranded)	8721.667	8,934.244 kDa	9,246.535 kDa	8,820.283 kDa	8,346.532 kDa	8,571.888 kDa	9,266.949 kDa	8,904.302 kDa	8,700.11 kDa	8,443.711 kDa	8,467.723 kDa	8,443.711 kDa	8,822.899 kDa
Annotation table													
Featutre type	**Count**	**Count**	**Count**	**Count**	**Count**	**Count**	**Count**	**Count**	**Count**	**Count**		**Count**	**Count**
CDS	12	12	12	12	12	12	12	12	12	12	12	12	12
Gene	12	12	12	12	12	12	12	12	12	12	12	12	12
Misc. feature	1	1	-	-	-	-		1	1	-	-	1	2
rRNA	2	2	1	2	2	2	2	2	2	2	2	2	2
tRNA	22	22	23	22	20	22	22	23	23	22	22	22	22

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Table 3. Atomic composition and Nucleotide distribution Table of *Fasciolopsis buski* mtDNA

Ambiguous residues are omitted in atom counts.					
As single stranded			**Nucleotide**	**Count**	**Frequency**
Atom	Count	Frequency	Adenine (A)	2509	0.178
hydrogen (H)	174924	0.376	Cytosine (C)	1281	0.091
carbon (C)	139209	0.299	Guanine (G)	3925	0.278
nitrogen (N)	48681	0.105	Thymine (T)	6334	0.449
oxygen (O)	88120	0.19	Purine (R)	0	0
phosphorus (P)	14049	0.03	Pyrimidine (Y)	0	0
As double stranded			Adenine or cytosine (M)	0	0
hydrogen (H)	346023	0.375	Guanine or thymine (K)	0	0
carbon (C)	275774	0.299	Cytosine or guanine (S)	0	0
nitrogen (N)	103549	0.112	Adenine or thymine (W)	0	0
oxygen (O)	168590	0.183	Not adenine (B)	0	0
phosphorus (P)	28098	0.03	Not cytosine (D)	0	0
			Not guanine (H)	0	0
			Not thymine (V)	0	0
			Any nucleotide (N)	69	0.005
			C + G	5206	0.369
			A + T	8843	0.626

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Table 4. Codon usage for *F. buski* mtDNA genome

AmAcid	Codon	Number	/1000	Fraction
Ala	GCG	22.00	4.74	0.20
Ala	GCA	26.00	5.60	0.24
Ala	GCT	46.00	9.91	0.43
Ala	GCC	14.00	3.02	0.13
Cys	TGT	239.00	51.49	0.80
Cys	TGC	58.00	12.49	0.20
Asp	GAT	90.00	19.39	0.85
Asp	GAC	16.00	3.45	0.15
Glu	GAG	63.00	13.57	0.68
Glu	GAA	30.00	6.46	0.32
Phe	TTT	442.00	95.22	0.85
Phe	TTC	79.00	17.02	0.15
Gly	GGG	119.00	25.64	0.28
Gly	GGA	67.00	14.43	0.16
Gly	GGT	201.00	43.30	0.48
Gly	GGC	31.00	6.68	0.07
His	CAT	19.00	4.09	0.73
His	CAC	7.00	1.51	0.27
Ile	ATT	168.00	36.19	0.86
Ile	ATC	28.00	6.03	0.14
Lys	AAG	42.00	9.05	1.00
Leu	TTG	263.00	56.66	0.36
Leu	TTA	193.00	41.58	0.27
Leu	CTG	70.00	15.08	0.10
Leu	CTA	53.00	11.42	0.07
Leu	CTT	117.00	25.20	0.16
Leu	CTC	26.00	5.60	0.04
Met	ATG	101.00	21.76	0.58
Met	ATA	72.00	15.51	0.42
Asn	AAA	39.00	8.40	0.34
Asn	AAT	64.00	13.79	0.56
Asn	AAC	11.00	2.37	0.10
Pro	CCG	16.00	3.45	0.27
Pro	CCA	10.00	2.15	0.17
Pro	CCT	27.00	5.82	0.45
Pro	CCC	7.00	1.51	0.12
Gln	CAG	19.00	4.09	0.63
Gln	CAA	11.00	2.37	0.37
Arg	CGG	34.00	7.32	0.33
Arg	CGA	15.00	3.23	0.14
Arg	CGT	44.00	9.48	0.42

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Arg	CGC	11.00	2.37	0.11
Ser	AGG	100.00	21.54	0.25
Ser	AGA	49.00	10.56	0.12
Ser	AGT	88.00	18.96	0.22
Ser	AGC	17.00	3.66	0.04
Ser	TCG	30.00	6.46	0.07
Ser	TCA	25.00	5.39	0.06
Ser	TCT	69.00	14.86	0.17
Ser	TCC	28.00	6.03	0.07
Thr	ACG	7.00	1.51	0.14
Thr	ACA	14.00	3.02	0.27
Thr	ACT	19.00	4.09	0.37
Thr	ACC	11.00	2.37	0.22
Val	GTG	114.00	24.56	0.22
Val	GTA	95.00	20.47	0.19
Val	GTT	270.00	58.16	0.53
Val	GTC	34.00	7.32	0.07
Trp	TGG	174.00	37.48	0.60
Trp	TGA	115.00	24.77	0.40
Tyr	TAT	160.00	34.47	0.83
Tyr	TAC	32.00	6.89	0.17
End	TAG	118.00	25.42	0.65
End	TAA	63.00	13.57	0.35

Counts of di-nucleotides in *F. buski* mtDNA

1.pos\2.pos	A	C	G	T
A	467	191	766	1072
C	217	204	262	593
G	656	347	1263	1645
T	1158	530	1619	2997

Frequency of di-nucleotides in *F. buski* mtDNA

1.pos\2.pos	A	C	G	T
A	0.033	0.014	0.055	0.077
C	0.016	0.015	0.019	0.042
G	0.047	0.025	0.09	0.118
T	0.083	0.038	0.116	0.214

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Table 5. mtDNA annotation of *F. buski* and comparison with *Fasciola* hepatica

	Gene	Length in *F. hepatica*	Gene Prediction Length in *F. buski*	% of *F. hepatica* CDS covered in *F. buski*
1	nad3	118	97	82.20
2	nad2	288	257	89.24
3	cox1	510	470	92.16
4	nad1	300	278	92.67
5	cox2	200	194	97.00
6	cox3	213	210	98.59
7	nad5	522	515	98.66
8	cob	370	366	98.92
9	nad6	150	149	99.33
10	nad4L	90	90	100.00
11	nad4	423	423	100.00
12	atp6	172	172	100.00

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Table 6. transfer RNA (tRNA) annotations of the *Fasciolopsis buski* mtDNA

Sl. No.	Contig Start	Contig End	tRNA ID	Single letter symbol	Codon	Length	GC %
1	657	722	mtRNA-His	H	GUG	66	30.3
2	3358	3424	mtRNA-Gln	Q	UUG	67	35.8
3	3438	3501	mtRNA-Phe	F	GAA	64	39.1
4	3511	3576	mtRNA-Met	M	CAU	66	40.9
5	4966	5030	mtRNA-Val	V	UAC	65	32.3
6	5048	5113	mtRNA-Ala	A	UGC	66	40.9
7	5113	5180	mtRNA-Asp	D	GUC	68	38.2
8	6093	6157	mtRNA-Asn	N	GUU	65	41.5
9	6162	6219	TV-loop mtRNA-Pro	P	UGG	58	31
10	6236	6298	mtRNA-Ile	I	GAU	63	47.6
11	6301	6367	mtRNA-Lys	K	CUU	67	38.8
12	6739	6799	D-loop mtRNA-Ser	S1	GCU	61	41
13	6812	6874	mtRNA-Trp	W	UCA	63	34.9
14	8458	8522	mtRNA-Thr	T	UGU	65	30.8
15	9523	9588	mtRNA-Cys	C	GCA	66	50
16	11462	11527	mtRNA-Tyr	Y	GUA	66	39.4
17	11526	11590	mtRNA-Leu	L1	UAG	65	49.2
18	11589	11653	D-loop mtRNA-Ser	S2	UGA	65	36.9
19	11659	11722	mtRNA-Leu	L2	UAA	64	37.5
20	11720	11791	mtRNA-Arg	R	UCG	72	37.5
21	13364	13432	mtRNA-Gly	G	UCC	69	30.4
22	13446	13509	mtRNA-Glu	Q	UUC	64	43.8

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Figure 1.

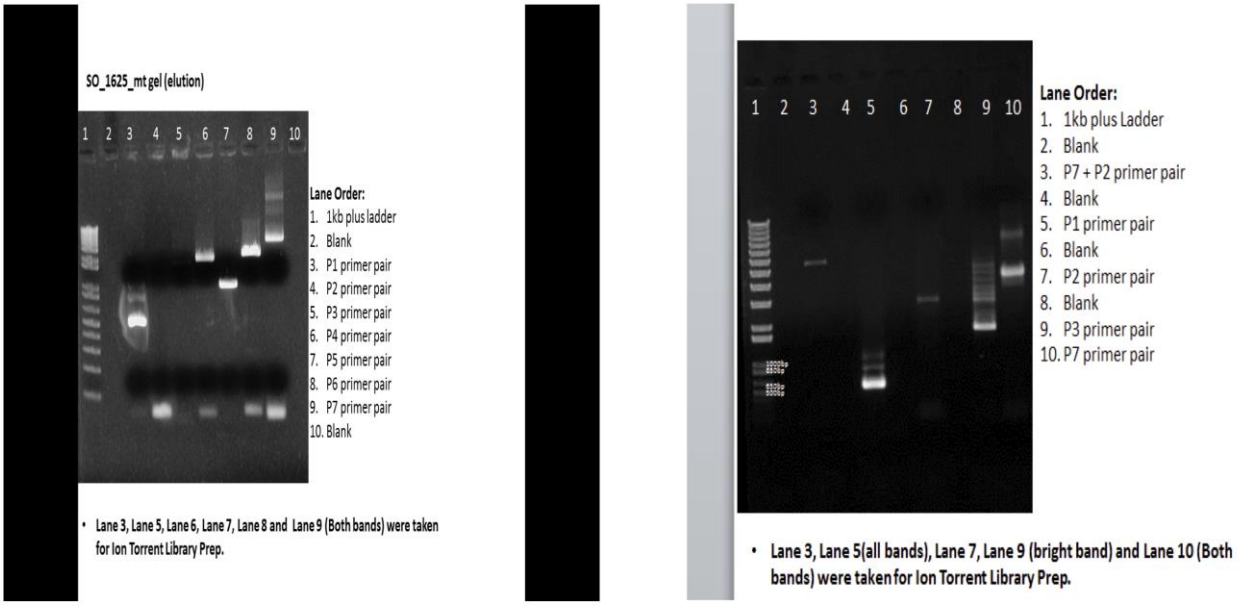

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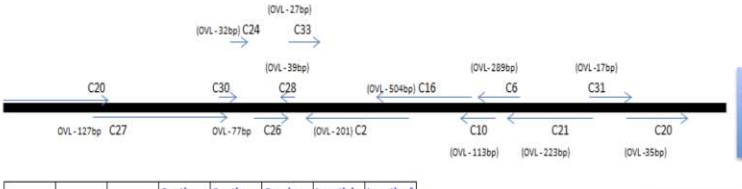
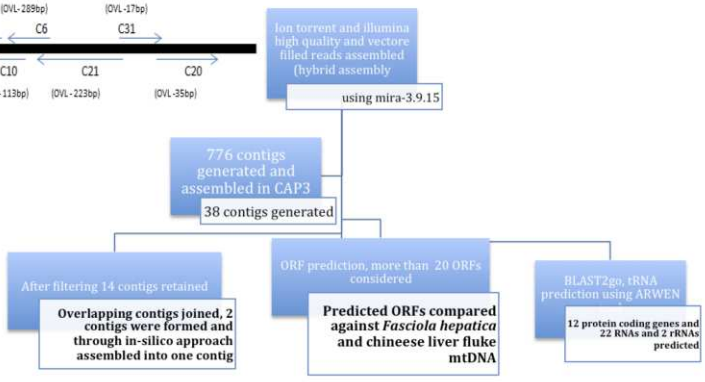
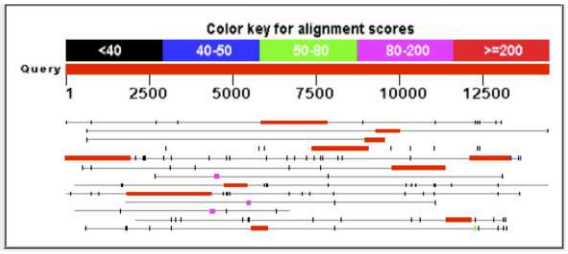
Figure 2.



Contig	FH Start	FH End	Contig start	Contig End	Overlap bp	Length in FH	Length of Contig
C20	1	1974	1970	3945	127	1974	1976
C27	1848	4410	1	2577	77	2563	2577
C30	4334	4505	12	183	32	172	172
C24	4474	4619	4	149	-129	146	146
C26	4749	5469	12	747	39	721	736
C28	5431	5569	141	1	27	139	-139
C33	5543	6069	47	565	201	527	519
C2	5869	7869	2012	1	504	2001	-2010
C16	7366	9073	1745	10	113	1708	-1734
C10	8961	9588	668	37	289	628	-630
C6	9300	9997	711	2	223	698	-708
C21	9775	11407	1644	10	17	1633	-1633
C31	11391	12134	33	771	35	744	739
C20	12100	13306	3	1213		1207	1211

A





B

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Figure 3.

TGGTTATTCTGGTTGGGGAGTTGATTTTTTGATGTTTTCTTTGCATTTGGCGGGGGTTTCTAGTATTTTTAGGTCTATTAAATTTATTTGTACTATAGTGGAAGTGATGTTTGAGGAAGGAACAGGACGTTTAA
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>NGS_ASSEMBLY (SUBJECT)

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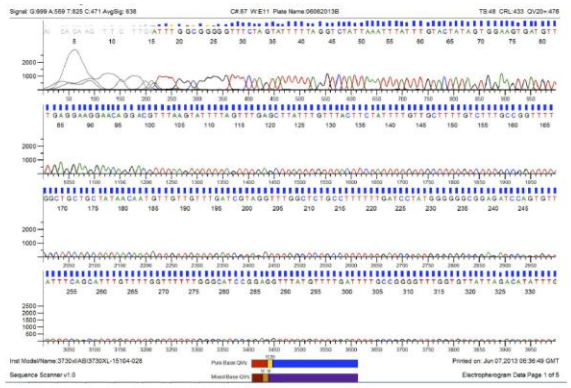

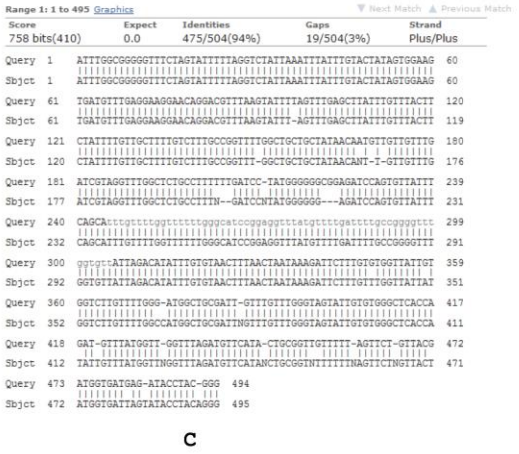

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Figure 4.

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Figure 5.

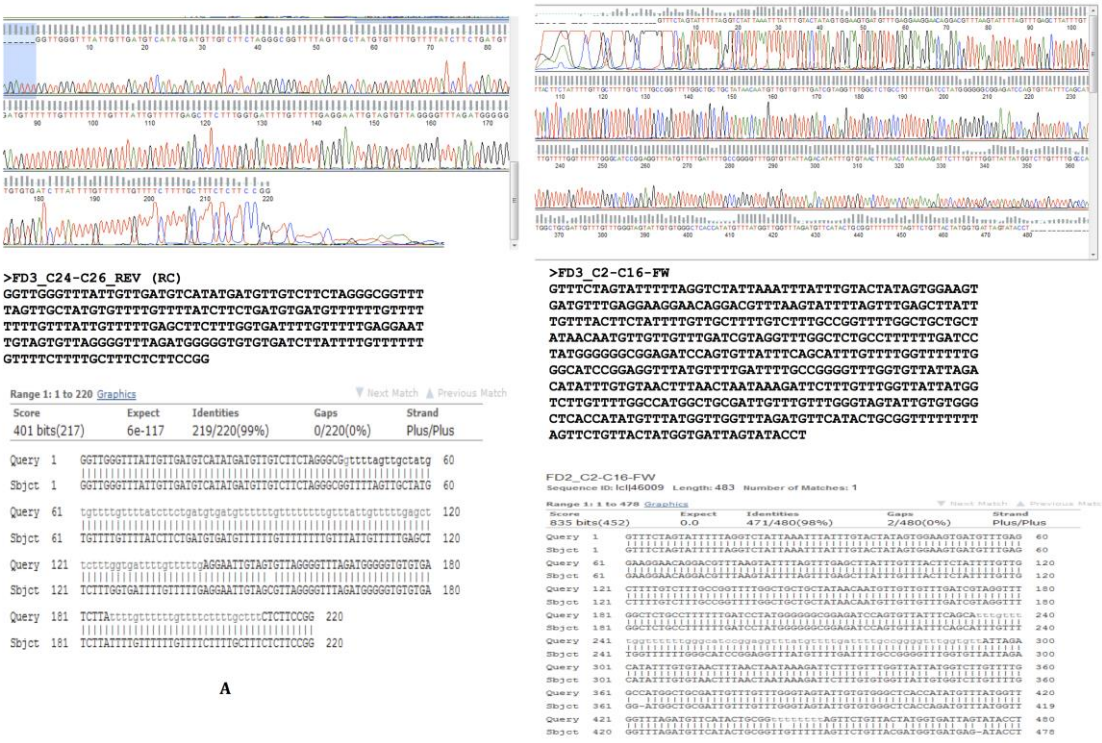

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Figure 6.

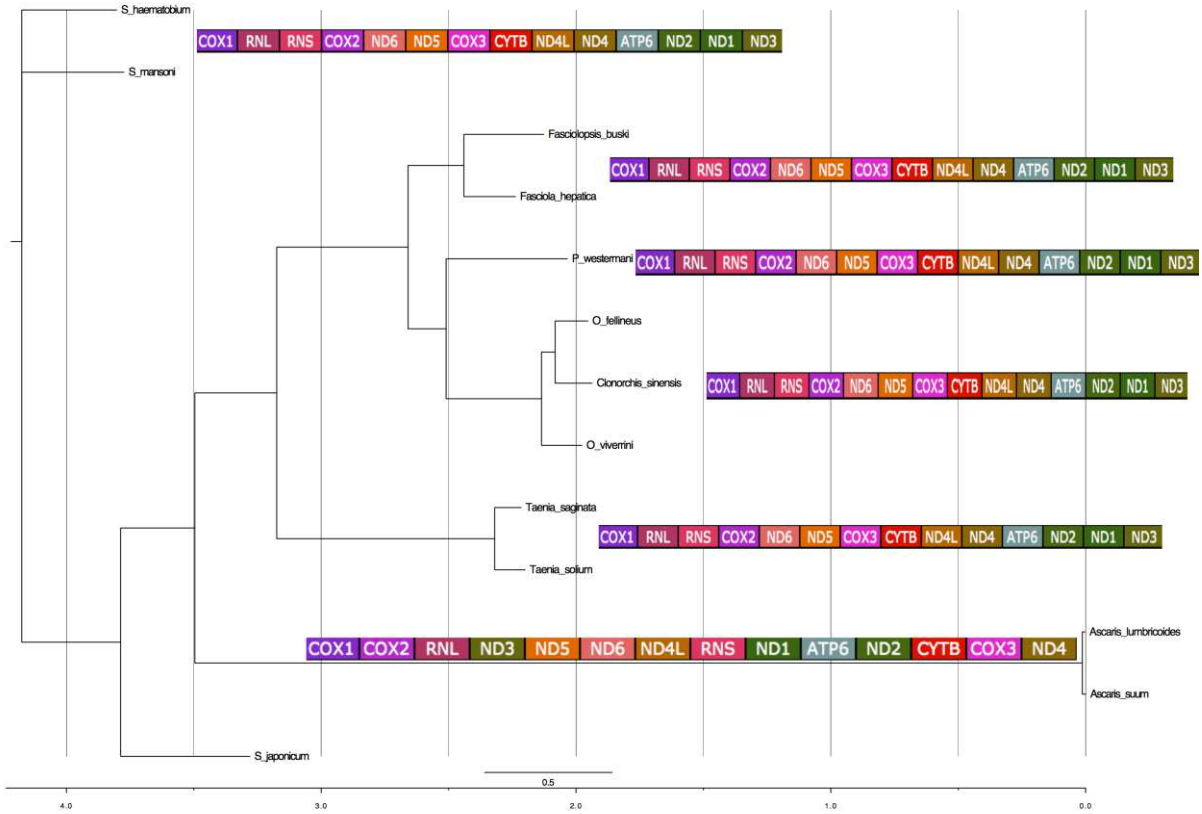

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Figure 7.

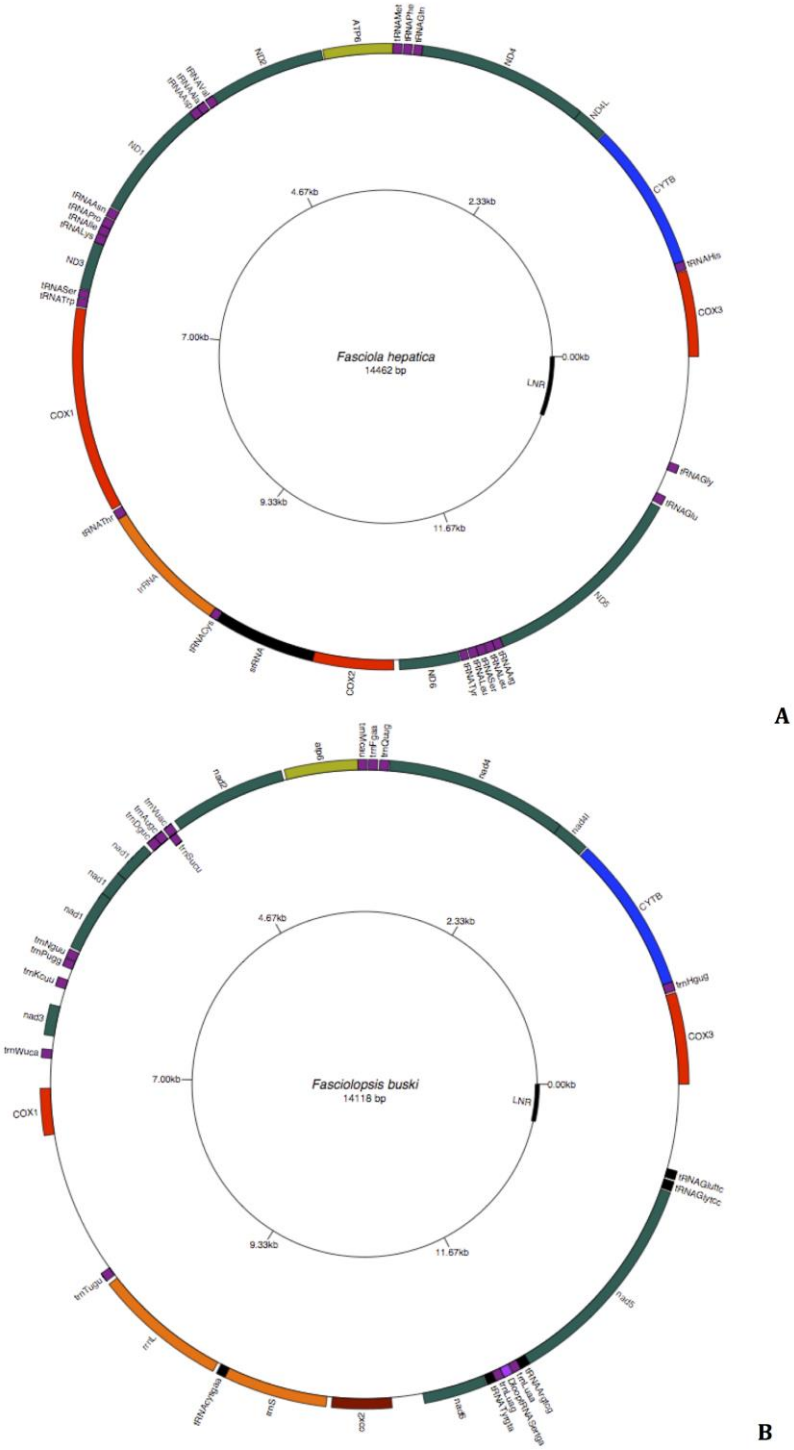

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Figure 8.

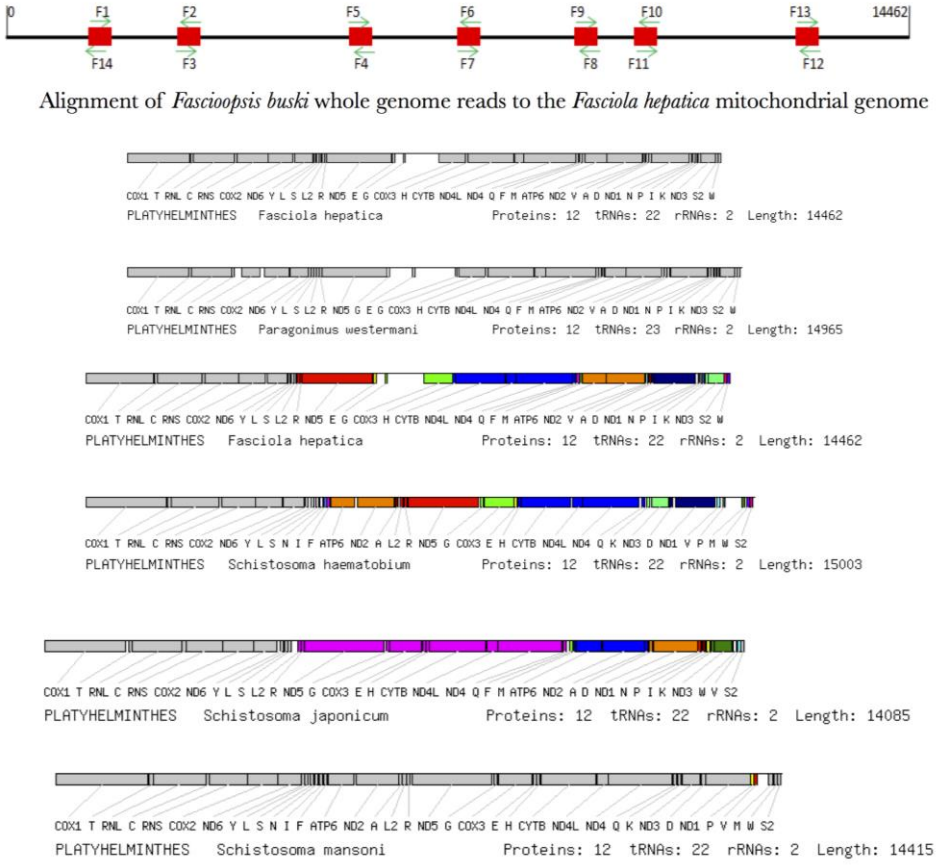

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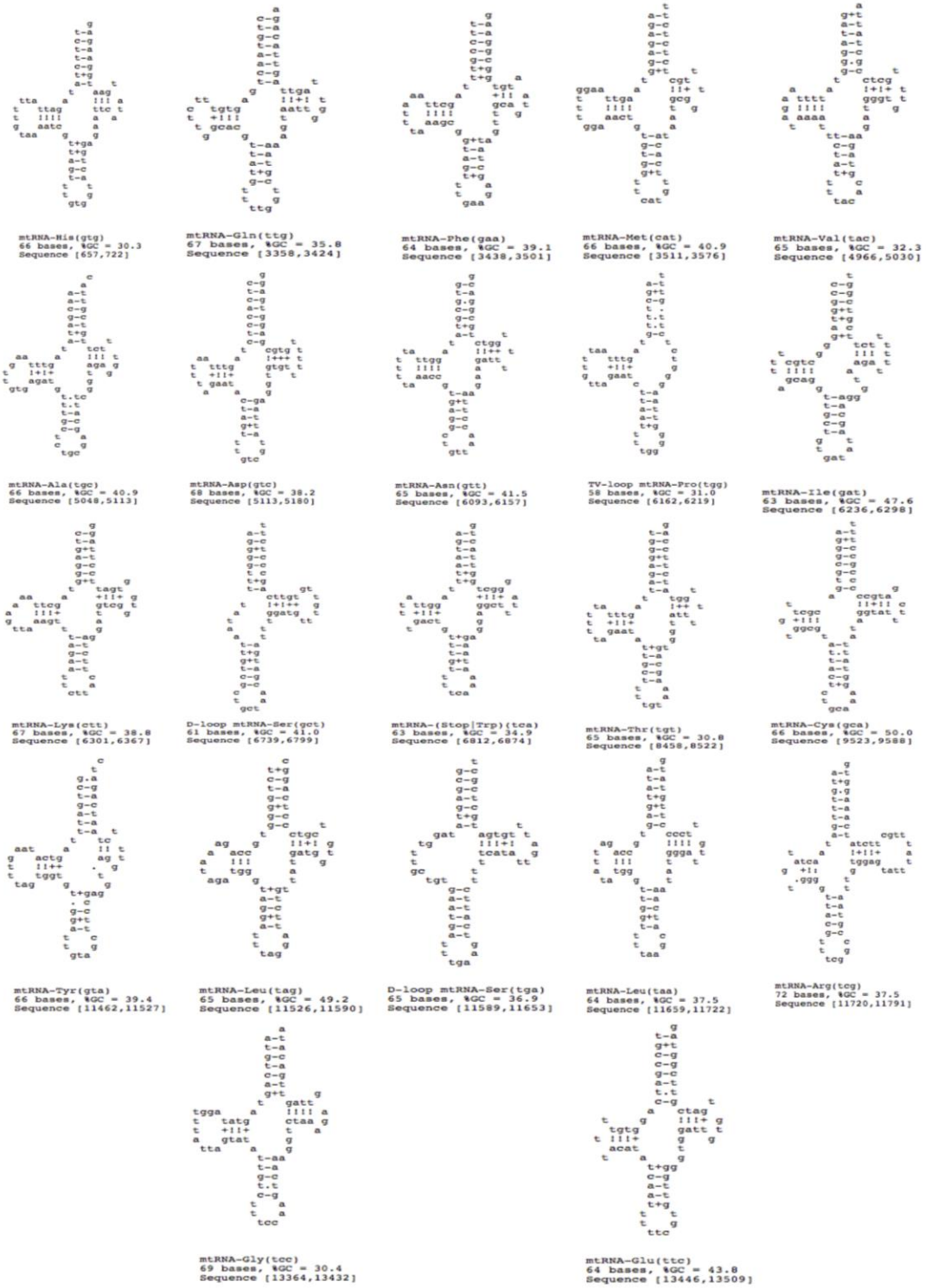
Figure 9.



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REVIEWER'S COMMENTS

Jun 22

InCoB2013 <incob2013@easychair.org>

to me

Dear Devendra Biswal

We have evaluated all the comments received and concluded that your paper

Submission: 81

Title: An integrated pipeline for next-generation sequencing and annotation of the complete mitochondrial genome of the giant intestinal fluke, Fasciolopsis buski (Lankester, 1857) Looss, 1899 (Digenea: Fasciolidae).

requires substantial revisions (for details see reviewers' comments) with regards your methodology that will most likely require additional experiments before it might be suitable for publication in BMC Genomics InCoB2013 Supplement Issue. If you are willing to carry out these revisions you are requested to submit a revised version by July 13. The revised version will undergo a second review before a 'accept' or 'reject' decision is reached.

1) Adhere to BMC authors' guidelines to ensure that the manuscript is accurate, complete, and optimally formatted. Upload the manuscript as one (1) PDF file containing text plus tables and figures. Changes in text should be visible in red color (use in Word under "Review" functions the "compare two versions of document" option)

The attachment (1 zip-compressed folder) should contain:

Word file of revised manuscript

Figures as separate files (format see below)

Response letter (PDF)

Supplementary files, if applicable

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2) Figures

Each figure should include a single illustration and should fit on a single page in portrait format. If a figure consists of separate parts, it is important that a single composite illustration file be submitted which contains all parts of the figure.

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Please read our figure preparation guidelines for detailed instructions on maximising the quality of your figures. (http://www.biomedcentral.com/ifora/figures)

Formats

The following file formats can be accepted:

PDF (preferred format for diagrams)

DOCX/DOC (single page only)

PPTX/PPT (single slide only)

EPS

PNG (preferred format for photos or images)

TIFF

JPEG

BMP

Figure legends

The legends should be included in the main manuscript text file at the end of the document, rather than being a part of the figure file. For each figure, the following information should be provided: Figure number (in sequence, using Arabic numerals - i.e. Figure 1, 2, 3 etc); short title of figure (maximum 15 words); detailed legend, up to 300 words.

Regards,

InCoB2013 Publication Co-chairs

Christian Schoenbach, Shoba Ranganathan and Bairong Shen

---------- REVIEW 1 ----------

PAPER: 81

TITLE: An integrated pipeline for next-generation sequencing and annotation of the complete mitochondrial genome of the giant intestinal fluke, Fasciolopsis buski (Lankester, 1857) Looss, 1899 (Digenea: Fasciolidae).

AUTHORS: Devendra Biswal, Sudip Ghatani, Jollene Shylla, Ranjana Sahu, Nandita Mullapudi, Alok Bhattacharya and Veena Tandon

OVERALL EVALUATION: 2 (accept)

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REVIEWER'S CONFIDENCE: 3 (medium)

---------- REVIEW ----------

The article by Biswal et al details the process used to sequence the mt genome of F. buski and the associated findings. This article is appropriate for BMC Genomics.

I have several comments on the article:

1. It would benefit from being corrected for English and typos.

2. Figure and Table legends are minimal, and do not provide all the details required to understand them. e.g Figure 4 - what does the horizontal axis represent? i.e scale. Plus details of the coloured boxes is not given in the legend, only in the text.

3. I don't think the table numbering in the text matches the actual tables, e.g. nucleotide composition across species doesn't appear in Table 1 (see page 11 in text). The authors should consider the use of supplementary tables for some of their results tables.

4. Reference 12 appears to be incomplete.

5. Given the primer design and sequencing strategy, is it really surprising that the mt genome of F. buski is "almost" similar to that of F. hepatica? How can we be sure that this isn't an artefact of design but really is the biological truth?

---------- REVIEW 2 ----------

PAPER: 81

TITLE: An integrated pipeline for next-generation sequencing and annotation of the complete mitochondrial genome of the giant intestinal fluke, Fasciolopsis buski (Lankester, 1857) Looss, 1899 (Digenea: Fasciolidae).

AUTHORS: Devendra Biswal, Sudip Ghatani, Jollene Shylla, Ranjana Sahu, Nandita Mullapudi, Alok Bhattacharya and Veena Tandon

OVERALL EVALUATION: -2 (reject)

REVIEWER'S CONFIDENCE: 3 (medium)

---------- REVIEW ----------

The authors make use of data that are not yet publicly available (F buski WGS not published so far). If

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submission #81 is published, interested parties would not be able to reproduce the authors' work. In addition, the authors should make ALL data publicly availabe, deposit it an an approptiate repository and obtain accession numbers and/or provide data sets as additional files.

Primer design/PCR: based on the authors's writing this reviewer is not convinced of the results. It seems the entire results are based on one (1) DNA sample (FD-2) without appropriate replicates.

Sanger-sequncing cofirmed region: specify in the manuscript text which region was confirmed. Why only one region and not two regions from replicate samples.

- novelty/originality: yes

- importance to field: limited

- appropriatness for this journal: yes

- sound methodology: partially

- quality of data or experimental results: partially

- support of discussion/conclusions by results: partially

- references to prior work: yes

- length, organization and clarity (language): no; the manucript requires major editing

- quality of display items: partially acceptable; manuscript appeared to be prepared in a hurry; the majority of tables would suit additional data (supplement) but do not fit as display items in the manuscript. It would help if the authors glean from similar published papers what has been shown and how it was displayed.

- compliance with standards (e.g. MIAME) etc.(if applicable): partially

- accessibility of data/software/websites: partially

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Response to reviewers' comments on Paper 81 submitted to BMC Genomics through INCOB 2013 easychair

Dear Sir

Re: Submission Paper 81

Title: **An integrated pipeline for next-generation sequencing and annotation of the complete mitochondrial genome of the giant intestinal fluke, Fasciolopsis buski (Lankester, 1857) Looss, 1899 (Digenea: Fasciolidae)**

Please find attached a revised version of our manuscript "An integrated pipeline for next-generation sequencing and annotation of the complete mitochondrial genome of the giant intestinal fluke, Fasciolopsis buski (Lankester, 1857) Looss, 1899 (Digenea: Fasciolidae).

The attachment includes Word file and also a pdf file of revised manuscript with tables and figures

- Figures as separate files
- Response letter (PDF)

We would like to thank the reviewers for their time and their valuable comments.

The reviewers' comments were highly insightful and enabled us to greatly improve the quality of our manuscript. In the following lines are our point-by-point responses to each of the comments of the reviewers.

Response to comments of reviewers

Reviewer 1

1. It would benefit from being corrected for English and typos.

Response: The manuscript is revised with the help of a language expert addressing typo and grammatical errors.

2. Figure and Table legends are minimal, and do not provide all the details required to understand them. e.g Figure 4 - what does the horizontal axis represent? i.e scale. Plus details of the coloured boxes is not given in the legend, only in the text.

Response: Figure legends have been enhanced with appropriate modifications.

3. I don't think the table numbering in the text matches the actual tables, e.g. nucleotide composition across species doesn't appear in Table 1 (see page 11 in text). The authors should consider the use of supplementary tables for some of their results tables.

Response: Tables have been arranged properly with correct numbering throughout the text.

4. Reference 12 appears to be incomplete.

Response: reference 12 is corrected as detailed below:

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Jex AR, Littlewood DTJ, Gasser RB: Toward next-generation sequencing of mitochondrial genomes—focus on parasitic worms of animals and biotechnological implications. Biotechnol. Adv 2010, 28:151-159.

5. Given the primer design and sequencing strategy, is it really surprising that the mt genome of F. buski is "almost" similar to that of F. hepatica? How can we be sure that this isn't an artefact of design but really is the biological truth?

The results indeed are really surprising as Fasciola hepatica is a common liver fluke, while F. buski is an intestinal fluke. However, the outcome is not an artifact of design as we went for Sanger validation of another biological replicate for the two regions, which we had validated for the original sample; this has been elaborated in the revised manuscript. Besides, F. buski and Fasciola hepatica belong to the same family (Fascioloidae) and hence, a striking similarity may not be ruled out.

Reviewer 2:

Query: The authors make use of data that are not yet publicly available (F buski WGS not published so far). If submission #81 is published, interested parties would not be able to reproduce the authors' work. In addition, the authors should make ALL data publicly availabe, deposit it an an approptiate repository and obtain accession numbers and/or provide data sets as additional files.

Response: As suggested, raw data were uploaded for the mtDNA seq part (Illumina FastQ files for now) to SRA. The data pertaining to this study is available in the National Centre for Biotechnology Information (NCBI) Bioproject database with Accession: ***PRJNA210017 and ID: 210017****. The contig assembly files are deposited in NCBI Sequence Read Archive (SRA) with* ***Accession: SRR924085****.*

Query: Primer design/PCR: based on the authors' writing this reviewer is not convinced of the results. It seems the entire results are based on one (1) DNA sample (FD-2) without appropriate replicates.

Response: Typically NGS experiments being cost-prohibitive are conducted on single specimens. Validation (of a subset) is done on replicates. But as per the reviewer's suggestions we are happy to inform you that we confirmed the findings by carrying out experiments on another reported whole genomic DNA from an independent F. buski sample (Sample FD3). Sanger sequencing was performed on two separate regions SAMPLE FD3-

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Region C24-C26 and SAMPLE FD3-Region C2-C16 as described in the manuscript. Two separate regions from two independent biological samples showed 98-99% identity.

Query: Sanger-sequencing confirmed region: specify in the manuscript text which region was confirmed. Why only one region and not two regions from replicate samples.

Response: To confirm our findings reported whole genomic DNA from an independent F. buski sample replicate (Sample FD3) was used and Sanger sequencing was performed on two separate regions (Sample FD3-Region C24-C26 and Sample FD3-Region C2-C16) as described above.

Query: manuscript appeared to be prepared in a hurry; the majority of tables would suit additional data (supplement) but do not fit as display items in the manuscript. It would help if the authors glean from similar published papers what has been shown and how it was displayed.

Response: The manuscript is greatly enhanced with error corrections and proper display of figures and tables throughout the manuscript taking cue from other publications on similar notes.

We hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript suitable for publication in BMC Genomics.

We shall look forward to hearing from you in a positive note at your earliest convenience.

Sincerely,

Veena Tandon

and Alok Bhattacharya

(Corresponding authors)

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