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Comparison of full mitochondrial genomes for the rice weevil, *Sitophilus oryzae* and the maize weevil, *Sitophilus zeamais* (Coleoptera: Curculionidae)

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ABSTRACT

Complete mitochondrial genome sequences were assembled for the rice weevil, *Sitophilus oryzae* (17,602 bp) and the maize weevil, *S. zeamais* (18,105 bp; Coleoptera: Curculionidae: Dryophthorinae), which encode 13 protein coding genes (PCGs), 22 transfer RNAs, and 2 ribosomal RNAs that is typical among Animalia. The A + T-rich control regions of *S. oryzae* (2818 bp) and *S. zeamais* (2832 bp) are the longest described to date among curculionoidea (weevils). Additionally, non-coding intergenic regions have increased in size due to expansion of tandem repeat arrays, but is more pronounced in *S. zeamais* (606 bp) compared to *S. oryzae* (109 bp). A total of 69 and 22 substitution mutations were found among reads from *S. oryzae* and *S. zeamais*, out of which 68 and 2 were predicted in the PCGs respectively, with a majority in NADH Dehydrogenase subunit I. Phylogenetic analyses of coleopteran insects based upon full mitogenomes PCG sequence supported the existence of Curculionoidea and Tenebrionoidea as a monophyletic group, whereas the Cucujoidea and Elateroidea remain paraphyletic. The mitogenomes of these two *Sitophilus* species provide insight into short-term evolutionary relationships among curculionid beetles, and provide resources for the potential deciphering of more broad systematic questions in the Order Coleoptera.

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1. Introduction

The mitochondrion is an important eukaryotic organelle that functions as the respiratory power plant of a cell by generating adenosine triphosphate (ATP) (Cameron, 2014). Insect mitochondrial genomes (mitogenomes) are small in size (14 to 20 kb), compact, circular double stranded DNA molecules that encode 37 genes: 13 protein coding genes (PCGs), 2 ribosomal RNAs (rRNAs; the 12S or small subunit rRNA, rrnS, and the 16S or large rRNA, rrnL), and 22 transfer ribonucleic acids (tRNAs). The products of the 13 PCGs are essential for carrying out oxidative phosphorylation (Attardi and Schatz, 1988; Boore, 1999; Gray et al., 1999; Pirger et al., 2009; Cameron, 2014). A non-coding A + T rich control region contains regulatory elements for DNA replication, transcription and translation that varies in nucleotide length and composition among species (Boore and Brown, 1998). Comparisons of complete mitochondrial genomes have revealed structural and functional variations among taxon (Dowton et al., 2002; Gissi et al., 2008; Cameron, 2014). The relative ease of mitogenome sequence assembly from next generation sequencing (NGS) data has increased accessibility of this type of data for phylogenic reconstructions (Ma et al., 2012; Cameron, 2014; Coates, 2014), mainly by reducing the effects of rare nuclear-integrated copies and other non-target products (Ho and Gilbert, 2010). Molecular phylogenetic analyses have been applied to estimate the evolutionary genomics based on comparisons of complete or near complete mitochondrial genomes (Yu et al., 2007; Cameron et al., 2009; Douglas and Gower, 2010; Coates, 2014), which rely upon an assumed clock-like pace of sequence evolution in the mitochondrial genomes to estimation divergence times (Avise, 1989). Regardless, in instances when high levels of sequence divergence exist between species of Coleoptera, resulting phylogenetic reconstructions have proven difficult to predict ancestral relationships (Sheffield et al., 2009; Song et al., 2010), such that approximations of relationships may be accurate only across relatively short evolutionary time frames (Coates, 2014).

The coleopteran Family Curculionidae (weevils) is numerically the largest group within the Suborder Polyphaga, and consists of about 4600 genera and 51,000 described species of "true weevils" (Oberprieler

Abbreviations: ORF, Open Reading Frame; rRNA, ribosomal ribonucleic acid; tRNA, transfer ribonucleic acid; rrnS, *small rRNA*; rrnL, *large rRNA*; COI, *cytochrome oxidase c subunit 1*; COII, *cytochrome oxidase c subunit 2*; COIII, *cytochrome oxidase c subunit 3*.

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et al., 2007; Bouchard et al., 2011). Many curculionids are significant pests of agriculture, forestry and horticulture across tropical and neotropical environments. Classification into tribes and subfamilies has remained problematic (Crowson, 1955; Alonso-Zarazaga et al., 1999; Oberprieler et al., 2007), mainly due to the sheer number, diversity and abundance of species, as well as varied lifestyles including myrmecophily, predation, coprophagy, endophytic and ectophytic, and high degrees of polyphagy (Marvaldi et al., 2002; Oberprieler, 2004; Oberprieler et al., 2007). Thus, the number of described species accounts for only a small proportion of the actual natural diversity (Erwin, 1982; Oberprieler et al., 2007). Currently, complete or nearly complete mitogenome sequences are reported for only seven curculionid species in 5 subfamilies; *Aegorhinus superciliosus* (subfamily Cyclominae), *Eucryptorrynchus brandti* and *E. chinensis* (subfamily Cryptorhynchinae), *Hylobitelus xiaoi* (subfamily Molytinae), *Naupactus xanthographus* (subfamily Entiminae), and *Rhynchophorus ferrugineus* and *Sphenophorus species* (subfamily Dryophthorinae), and represents only a very small sampling of the total mitogenome diversity across 22 subfamilies (Marvaldi, 1997).

Among the fourteen described species within the genus *Sitophilus*, the maize weevil, *Sitophilus zeamais*, rice weevil, *S. oryzae*, and granary weevil, *S. granarius* (Coleoptera: Curculionidae; Dryophthorinae) cause the greatest levels of damage to stored grain (Longstaff, 1981; Plarre, 2010). The sibling species, *S. zeamais* and *S. oryzae*, infest stored grains as well as other processed and unprocessed stored products (Rees, 2004; Plarre, 2010; Ojo and Omoloye, 2012), but rarely co-occur in the same food materials. In contrast to the restricted range of granary weevils to temperate climates, rice and maize weevils are more widespread and threaten the food security in developing nations through reported damage of 20 to 80% of stored grain products (Giga et al., 1991; Odeyemi and Daramola, 2000; Nukenine et al., 2002; Muzemu et al., 2013). These species are morphologically similar in body structure, with the exception of the granary weevil that has four reddish-yellow spots on the elytra and can fly (Rees, 2004). Genital morphology is frequently used to distinguish *S. oryzae* and *S. zeamais* (Halstead, 1963; Haines, 1991), but difficulties in applying this method has left these species as congeneric and cryptic.

In order to partially address the current lack of comparative mitogenome data among Curculionidae, this study describes an NGS based approach to sequence the complete mitochondrial genomes of *S. oryzae* and *S. zeamais*. Additionally we aimed to compare the mitogenomes of these two weevil species, provide insight into short-term evolutionary trends, and explore the applicability of mitogenome sequences to estimate the phylogenetic relationships among Curculionidae. Furthermore, we hypothesized that substitutions between *Sitiphus* mitogenomics would likely provide for a method for molecular differentiation of these cryptic species. These data are important for understanding near-term phylogenetic relationships among weevils, lay the groundwork for future mitochondrial phylogenomics, and provide a method for differentiation of two cryptic weevil species.

2. Materials and methods

2.1. Ethics statement

There is no need of specific permission for the collection and usage of the insects in this study because these insects are common pests of stored cereals. Neither endangered nor protected species were involved in the study.

2.2. Sampling, DNA extraction and sequencing

Samples of *S. oryzae* and *S. zeamais* were collected on rice and maize grain, respectively, and made during the dry seasons of 2013 and 2014 at eighteen locations in the agricultural zones of Nigeria (Fig. 1). Samples were identified morphologically at the Insect Reference Museum of the Department of Crop Protection and Environmental Biology, University of Ibadan using standard taxonomic keys (Crowson, 1955; Kuschel, 1995; Marvaldi and Lanteri, 2005). The samples were preserved in absolute ethanol and stored at −20 °C until shipment to the Department of Entomology, University of Illinois at Urbana Champaign (UIUC), Champaign, IL, USA.

Samples for each species (>180 individuals each species) were pooled by collection site (Fig. 1) and ground to a powder in liquid nitrogen. Total genomic DNA was extracted from each sample using DNeasy animal tissue kit following the manufacturer's instructions (Qiagen, Hilden, Germany). The resulting extracts from each location was pooled together by species, and quantified by measuring the absorbance at 260 nm using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and 1% gel electrophoresis. Samples were submitted to the W.M. Keck Center for Comparative and Functional Genomics (CCFG), Roy J. Carver Biotechnology Center, UIUC, USA, where Illumina shotgun TruSeq genomic DNA libraries were constructed and sequenced on an Illumina HiSeq 2500 using a TruSeq Rapid SBS sequencing kit version 1.

The raw sequence read data generated from the two insects were preprocessed, assembled and analyzed using the CLC Genomic Workbench 8.5 (CLC Cambridge, MA, USA). Pre-processing of the raw fastq read data from both libraries involved trimming (from low to high quality trimming) of adapter sequences, nucleotides that are <50 base pairs from the ends, and reads with a Phred quality score (q) < 20. A *de novo* assembly was carried out independently on the processed read data from *S. oryzae* and *S. zeamais* using the following parameters: automatic word size option selected with the final k-mer (word) size of 25 as estimated by CLC; contig length ≥200; mismatch cost = 2, insertion and deletion cost = 3; length fraction = 60%, and similarity ≥80%. The best-assembled sequences (contigs) were aligned, manually compared and then compiled into a single contig for each insect species. Processed Illumina reads were mapped back to the assembled mitogenome sequences and single nucleotide polymorphisms (SNPs) predicted by using SNP detection program on CLC Genomic Workbench 8.5 using default parameters: length fraction 0.5; similarity fraction 0.8; deletion cost 3; insertion cost 3 and mismatch cost 2. These nucleotide variants were treated as substitutions within the single copy mitogenome.

Due to the challenges with morphology-based identifications, two sets of species-specific mitochondrial COI primers were used to amplify PCR products from either *S. oryzae* or *S. zeamais* according to Corrêa et al. (2013). Additionally, we designed the primer pair SZ6379F (5′-TTA TTA AAG AAA TAA AAG GAA-3′) and SZ6848R (5′-AAT ATT TGT AAT TTA TCT TTA TG-3′) that is predicted to be *S. zeamais*-specific. Four separate individual weevil of each species were used for molecular species identification.

2.3. Mitogenome annotation, amplification and analyses

BLAST searches at the NCBI with the BLASTn and BLASTx algorithms were used to identify "hits" of *S. oryzae* and *S. zeamais* assemblies to coleopteran mitogenomes available in the Organelle Genome Resources of NCBI (http://www.ncbi.nlm.nih.gov). Protein coding genes (PCGs) were further identified with the use of Open Reading Frame (ORF) Finder at NCBI and CLC Genomic Workbench 8.5 using the invertebrate mitochondrial genetic code. They were further examined and adjusted manually based on the presence of start and stop codon and location of adjacent genes, taking into consideration homologous gene sequences from other known curculionids. Putative cloverleaf secondary structures and anticodons of tRNAs were predicted using tRNAscan-SE v.1.21 (Lowe and Eddy, 1997) and ARWEN v1.2 (Laslett and Canback, 2008) with mito/chloroplast genetic code and the default search options. Alignment with rRNA gene sequences of other curculionids full mitogenome sequences was conducted using the ClustalW algorithm in MEGA 7.0.14 (Kumar et al., 2016) in order to determine gene boundaries. Nucleotide compositions and codon usages were calculated using MEGA 7.0.14, and strand asymmetry (composition skewness)



Fig. 1. Map showing sites from which *Sitophilus species* were collected.

was calculated using the formulae: AT skew = (A − T)/(A + T) and GC skew = (G − C)/(G + C) (Perna and Kocher, 1995). The repeat sequence motifs were identified using Tandem Repeat Finder version 4.09 (Benson, 1999).

The sequence of *S. oryzae* and *S. zeamais* mitogenome control regions were validated by confirmatory sequencing. Four primer pairs for each were designed based on assembled sequence data to flank and amplify the entire control region of our two *Sitophilus* mitogenomes (Supplementary Table 1). Long PCR was performed with GoTaq® Flexi DNA Polymerase (Promega Corporation, USA) and carried out on a BioRad T-100 thermal cycler with initial denaturing at 94 °C for 4 min, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 42–58 °C, and 2–5 min extension at 68 °C. The final extension step was continued for 7 min at 72 °C. All PCR products were detected *via* electrophoresis in 1.0% TAE agarose gel, and purified with DNA Clean and Concentrator −5 Kit (Zymo Research). The purified products were sequenced using the Illumina HiSeq 2500 platform at the CCFG.

2.4. Phylogenetic analysis

The complete mitogenome sequences were downloaded from NCBI for representative species from the sub-Order Polyphaga; across the main super families; Curculionoidea, Tenebrionoidea, Cleroidea, Cucujoidea, Chrysomeloidea, Scarabaeoidea, Staphylinoidea, Scirtoidea, Bostrichoidea, Buprestoidea, Elateroidea, Hydrophiloidea and Dascilloidea. Additionally, representatives of sub-Order Adephaga, Myxophaga and Archostemata were downloaded. Due to the problems faced by phylogenomic efforts for Coleoptera posed by base compositional heterogeneity (Sheffield et al., 2009; Song et al., 2010), an improved phylogenetic resolution was attempted by rooting our trees within the order Coleoptera using Archostemata as outgroup as previously encouraged by Timmermans et al. (2010). The derived amino acid sequences of 13 PCGs were concatenated for each species, and then all aligned using MUSCLE using default parameters. A substitution-based method, Maximum Likelihood (ML) and a distance-based method, Neighbour Joining (NJ) analyses were conducted using MEGA 7.0.14 (Kumar et al., 2016) with node support generated by 1000 bootstrap pseudoreplicates. The Jones-Taylor-Thornton (JTT) model with 5 discrete gamma categories for rates among sites, and complete deletion of the missing data was used during phylogenetic reconstruction.

3. Results

3.1. Sampling, sequencing, and assembly

A total of 378,509,280 and 370,942,848 Illumina reads with an average length of 160 nt were generated for *S. oryzae* and *S. zeamais*, respectively. After quality trimming 322,455,048 paired reads with an average length of 143.3 nt for *S. oryzae*, and 254,486,691 paired reads

with an average length of 143.2 nt were obtained for *S. zeamais*. The *de novo* assembly of Illumina data generated complete mitochondrial genomes for *S. oryzae* (17,602 bp; GenBank accession number: KX373615) and *S. zeamais* (18,105 bp; Accession: KX373614). A total of 1,360,001 Illumina reads were assembled in the *S. oryzae* mitogenome (coverage of 242 to 15,677). In the case of *S. zeamais*, 992,306 reads were assembled (coverage ranging from 168 to 177,637; Supplementary Fig. 1 a & b). Among the mapped reads, a total of 69 and 22 nucleotide substitutions were predicted for each haploid mitogenome.

The diagnostic B primer pair amplified a 450 bp PCR product from *S. zeamais*, whereas no product was generated for *S. oryzae* (Data not shown). Analogously, when using the system designed by Corrêa et al. (2013) an ~950 bp PCR products were generated using COI primer pair SZ1434F with SZ2369R only when using *S. zeamais* template DNA, and *S. oryzae* amplified only when using primer pairs SO1425F with SO2374R (Data not shown).

3.2. Mitogenome annotation, amplification and analyses

Each assembled *Sitophilus* mitogenome encoded the expected ancestral set of 37 genes; 13 PCGs, 22 tRNA genes and 2 rRNA genes (Table 1). Of these, 23 genes are located on the J-strand (9 PCGs and 14 tRNAs), and the remaining 14 on the N-strand (4 PCGs, 8 tRNAs and 2 rRNAs). The 16 overlapping gene sequences in *S. oryzae* and *S. zeamais* mitogenome respectively totaled 133 bp and 245 bp, with the longest overlap of 75 bp between $tRNA^{Met}$ and $tRNA^{Gln}$ with ND2 in *S. zeamais*. The two *Sitophilus* mitogenomes have a non-coding sequence between $tRNA^{Ile}$ and $tRNA^{Gln}$.

The longest spacer region corresponds to the A + T-rich control region. The A + T content of *S. oryzae* mitogenome, 76.3% (39.9% A, 36.4% T, 8.6% G and 15.0% C) and the *S. zeamais* mitogenome, 75.9% (39.9% A, 36.0% T, 8.7% G and 15.4% C), show a strong biased towards A and T nucleotides. The A + T content of PCGs, tRNAs, rRNAs and control regions were ≥70.0% in both mitogenomes (Table 2). Generally, *Sitophilus* mitogenomes are AT- and CG-skewed, with the exception of *S. oryzae* 2nd codon and *S. zeamais* 3rd codon positions that are both TA-skewed. The relative synonymous codon usage (RSCU) showed an overrepresentation of 6 A + T-rich codons (Table 3), while in contrast G + C-rich codons are less frequently used.

All thirteen PCGs encoded by the two *Sitophilus* mitogenomes are initiated with ATN start codon with the exception of ND1 with a TTG codon, and the similar number of complete termination codon (three TAG and nine TAA; with exception for ND5 which uses a TAG stop coding in *S. zeamais* compared to a TAA stop codon in *S. oryzae*, Table 1). The PCGs arrangement is consistent with what is obtained in other coleopteran mitogenomes, nine of them are on J-strand (ND2, COI – III, ATP 6 and 8, ND3, ND6 and Cytb), while the remaining four were found on N-strand (ND1, ND4, ND4L and ND5). The total length of 13 PCGs was 11,123 bp and 11,223 bp, which accounted for 61.44% and 63.76% of the mitogenome sequence from *S. zeamais* and *S. oryzae*, respectively.

The 22 *Sitophilus* tRNAs genes ranged between 63 and 72 bp in length, of which 14 were found on J-strand while 8 were encoded on N-strand (Table 1). Secondary structures were determined for twenty

Table 1
Gene organization of the *Sitophilus zeamais* and *S. oryzae* mitogenomes.

Gene	*Sitophilus zeamais*					*Sitophilus oryzae*		
	Strand	Anti/start/stop codons	Position	Size	IGN	Position	Size	IGN
$tRNA^{Gln(Q)}$	N	ttg	1–69	69		1–67	67	
$tRNA^{Met(M)}$	J	cat	74–141	68	4	74–142	69	6
ND2	J	ata/taa	67–1137	1071	−75	116–1138	1023	−27
$tRNA^{Trp(W)}$	J	tca	1140–1203	64	2	1141–1204	64	2
$tRNA^{Cys(C)}$	N	gca	1203–1265	63	−1	1206–1272	67	1
$tRNA^{Tyr(Y)}$	N	gta	1266–1330	65	0	1272–1336	65	−1
COI	J	ata(att)/taa	1281–2864	1584	−50	1308–2870	1563	−29
$tRNA^{Leu(L1)}$	J	taa	2867–2931	65	2	2873–2937	65	2
COII	J	ata/taa	2908–3615	708	−24	2914–3621	708	−24
$tRNA^{Lys(K)}$	J	ttt	3617–3687	71	1	3623–3693	71	1
$tRNA^{Asp(D)}$	J	gtc	3687–3752	66	−1	3693–3758	66	−1
ATP8	J	att/tag	3753–3908	156	0	3759–3914	156	0
ATP6	J	ata(atg)/taa	3878–4573	696	−31	3908–4579	672	−7
COIII	J	atg/taa	4574–5365	792	0	4580–5371	792	0
$tRNA^{Gly(G)}$	J	tcc	5371–5433	63	5	5381–5443	63	9
ND3	J	ata/tag	5431–5787	357	−3	5441–5797	357	−3
$tRNA^{Ala(A)}$	J	tgc	5786–5851	66	−2	5796–5861	66	−2
$tRNA^{Arg(R)}$	J	tcg	5851–5919	69	−1	5861–5925	65	−1
$tRNA^{Asn(N)}$	J	gtt	5918–5982	65	−2	5924–5988	65	−2
$tRNA^{Ser(S1)}$	J	tct	5982–6050	69	−1	5989–6055	67	0
$tRNA^{Glu(E)}$	J	ttc	6051–6115	65	0	6057–6121	65	1
$tRNA^{Phe(F)}$	N	gaa	6116–6180	65	0	6121–6185	65	−1
ND5	N	att/tag(taa)	6161–7897	1737	−20	6180–7901	1722	−5
$tRNA^{His(H)}$	N	gtg	7898–7962	65	0	7902–7966	65	0
ND4	N	atg/taa	7943–9292	1350	−20	7947–9296	1350	−20
ND4L	N	atg/taa	9286–9567	282	−7	9290–9571	282	−7
$tRNA^{Thr(T)}$	J	tgt	9572–9635	64	4	9576–9639	64	4
$tNA^{Pro(P)}$	N	tgg	9636–9699	64	0	9640–9705	66	0
ND6	J	ata/taa	9702–10,199	498	2	9708–10,205	498	2
Cytb	J	atg/taa	10,199–11,338	1140	−1	10,205–11,344	1140	−1
$tRNA^{Ser2(S2)}$	J	tga	11,333–11,404	72	−6	11,343–11,412	70	−2
ND1	N	ttg/tag	11,426–12,385	960	21	11,436–12,395	960	23
$tRNA^{Leu2(L2)}$	N	tag	12,387–12,451	65	1	12,397–12,460	64	1
rrnL	N		12,455–13,747	1293	3	12,464–13,754	1291	3
$tRNA^{Val(V)}$	N	tac	13,748–13,814	67	0	13,755–13,822	68	0
rrnS	N		13,815–14,600	786	0	13,823–14,608	786	0
Control region			14,601–17,432	2832	0	14,609–17,426	2818	0
tRNA-Ile	J	gat	17,433–17,499	67	0	17,427–17,493	67	0
Repeat region			17,500–18,105	606	0	17,494–17,602	109	0

IGN = Intergenic nucleotides.

Table 2
Nucleotide composition of *Sitophilus* mitogenomes.

Feature	Species	A%	T%	G%	C%	A + T%	AT-skew	GC-skew
Whole genome	*S. zeamais*	39.9	36.0	8.7	15.4	75.9	0.051	−0.278
	S. oryzae	39.9	36.4	8.6	15.0	76.3	0.046	−0.271
1st Codon position	*S. zeamais*	38.6	37.0	8.3	15.7	75.6	0.021	−0.308
	S. oryzae	40.2	36.0	8.5	15.5	76.2	0.055	−0.295
2nd Codon position	*S. zeamais*	41.2	37.0	7.3	14.7	78.2	0.054	−0.336
	S. oryzae	41.1	36.0	8.4	14.9	77.1	0.066	−0.279
3rd Codon position	*S. zeamais*	39.8	34.0	10.5	15.9	73.8	0.079	−0.205
	S. oryzae	38.5	38.0	9.1	14.6	76.5	0.007	−0.232
Protein coding genes	*S. zeamais*	38.9	35.5	9.6	16.0	74.4	0.046	−0.250
	S. oryzae	39.0	35.9	9.5	15.6	74.9	0.042	−0.243
1st Codon position	*S. zeamais*	44.9	38.0	3.1	13.6	82.9	0.083	−0.629
	S. oryzae	42.0	34.0	11.0	13.1	76.0	0.105	−0.087
2nd Codon position	*S. zeamais*	41.9	29.0	14.1	15.5	70.9	0.182	−0.047
	S. oryzae	32.5	36.0	13.3	18.3	68.5	−0.051	−0.161
3rd Codon position	*S. zeamais*	29.9	40.0	11.6	18.9	69.9	−0.144	−0.238
	S. oryzae	42.6	38.0	4.1	15.4	80.6	0.057	−0.579
tRNA genes	*S. zeamais*	40.6	38.0	11.5	10.0	78.6	0.033	0.070
	S. oryzae	40.2	38.0	9.1	12.7	78.2	0.028	−0.165
rRNA genes	*S. zeamais*	41.3	36.6	6.7	15.4	77.9	0.060	−0.394
	S. oryzae	41.6	37.5	6.6	14.3	79.1	0.052	−0.368
Control region	*S. zeamais*	42.5	37.7	5.9	13.9	80.2	0.060	−0.404
	S. oryzae	42.4	36.9	6.8	13.9	79.3	0.069	−0.343

one *S. zeamais* and eighteen *S. oryzae* tRNAs using tRNAscan-SE version 1.21. The remaining tRNA structures were determined using ARWEN version 1.2. All tRNAs were predicted to fold into a clover-leaf secondary structure with appropriate triplets on the anticodon loop, with the exception of $tRNA^{Ser1\ (AGN)}$ in which its dihydrouridine (DHU) arm forms a simple loop. The large rRNA (rrnL) gene was located between $tRNA^{Leu\ (CUN)}$ and $tRNA^{Val}$ while small rRNA (rrnS) was located between $tRNA^{Val}$ and the control region, as obtained in most of the insect mitogenomes. The length of rrnL and rrnS genes was 1291 bp and 786 bp, and 1293 bp and 786 bp in *S. oryzae* and *S. zeamais* respectively. The A + T contents of rRNA genes in *S. oryzae* and *S. zeamais* were 79.1% and 77.9% respectively.

Intergenic spacers, inclusive of the A + T control region ranged from 1 to 2832 bp and 1 to 2818 bp in the *S. zeamais* and *S. oryzae* mitogenomes, respectively. Most intergenic spacers were small (< 6 bp), except for the spacer between $tRNA^{Ser2}$ and ND1 in *S. zeamais* (21 bp) and *S. oryzae* (23 bp) that is common in all other coleopteran mitogenomes. Unique was a long A + T-rich non-coding spacer between $tRNA^{Ile}$ and $tRNA^{Gln}$ in *S. zeamais* (606 bp; 70.1% A + T) and *S. oryzae* (109 bp; 75.2% A + T), with the former having two tandem repeats interspersed with period sizes of 105 (zero mismatches) and 57 (two mismatches) (Supplementary Fig. 2). Moreover, there were 2832 bp and 2818 bp respectively in the *S. zeamais* and *S. oryzae* mitogenome control regions located between rrnS and $tRNA^{Ile}$. The control regions were 80.2 and 79.3% A + T for *S. zeamais* and *S. oryzae*, respectively. Re-sequencing of PCR products from the control region of *S. zeamais* and *S. oryzae* mitogenome confirmed the length and structures predicted above. The minority strand of the *S. oryzae* control regions has 3 predicted tRNA-like structures: one $tRNA^{Tyr(ATA)}$ and two $tRNA^{Leu(TAA)}$–like sequences (Fig. 2).

A total of 68 of 69 and 2 of 22 substitutions were predicted in the PCGs of *S. oryzae* and *S. zeamais* mitogenomes, respectively (Supplementary Tables 2 and 3). Of these substitutions, 41 of 68 (60.3%) and 2 of 22 (9.1%) were non-synonymous in *S. oryzae* and *S. zeamais*, respectively, and most nucleotide changes resulted in amino acids change *via* substitutions.

Table 3
Codon usage and relative synonymous codon usage (RSCU) in the mitogenome of *Sitophilus oryzae*/*S. zeamais*.

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	405/358	1.48/1.55	CCC(P)	67/57	1.08/1.20	GAU(D)	82/76	1.67/1.60
UUC(F)	117/125	0.52/0.45	CCA(P)	74/57	1.19/1.20	GAC(D)	16/19	0.33/0.40
UUA(L)	291/347	2.48/2.47	CCG(P)	12/14	0.19/0.29	GAA(E)	124/97	1.58/1.44
UUG(L)	69/82	0.59/0.59	ACU(T)	125/105	2.02/1.60	GAG(E)	33/38	0.42/0.56
CUU(L)	138/131	0.94/1.17	ACC(T)	48/71	0.78/1.08	UGU(C)	30/33	0.95/1.10
CUC(L)	60/57	0.41/0.51	ACA(T)	63/66	1.02/1.00	UGC(C)	33/27	1.05/0.90
CUA(L)	108/179	1.28/0.92	ACG(T)	11/21	0.18/0.32	UGA(W)	64/59	1.44/1.48
CUG(L)	40/44	0.31/0.34	GCU(A)	31/36	1.82/1.80	UGG(W)	25/21	0.56/0.53
AUU(I)	365/292	1.42/1.62	GCC(A)	14/27	0.82/1.35	CGU(R)	10/12	0.71/1.04
AUC(I)	87/118	0.58/0.38	GCA(A)	20/14	1.18/0.70	CGC(R)	14/12	1.00/1.04
AUA(M)	247/310	1.70/1.63	GCG(A)	3/3	0.18/0.15	CGA(R)	26/15	1.86/1.30
AUG(M)	56/55	0.30/0.37	UAU(Y)	233/273	1.37/1.41	CGG(R)	6/**7**	0.43/0.61
GUU(V)	33/45	1.49/1.28	UAC(Y)	106/115	0.63/0.59	AGU(S)	52/39	0.79/0.58
GUC(V)	17/14	0.46/0.66	CAU(H)	84/82	1.47/1.29	AGC(S)	41/36	0.62/0.53
GUA(V)	48/51	1.69/1.86	CAC(H)	30/45	0.53/0.71	AGA(S)	80/64	1.21/0.95
GUG(V)	5/11	0.36/0.19	CAA(Q)	109/121	1.49/1.40	AGG(S)	41/48	0.62/0.71
UCU(S)	117/114	1.69/1.77	CAG(Q)	37/52	0.51/0.60	GGU(G)	19/16	1.07/0.93
UCC(S)	61/89	1.32/0.92	AAU(N)	405/362	1.50/1.46	GGC(G)	9/12	0.51/0.70
UCA(S)	114/125	1.85/1.73	AAC(N)	134/135	0.50/0.54	GGA(G)	31/32	1.75/1.86
UCG(S)	22/25	0.37/0.33	AAA(K)	530/509	1.75/1.67	GGG(G)	12/9	0.68/0.52
CCU(P)	95/62	1.31/1.53	AAG(K)	76/100	0.25/0.33			



Fig. 2. The tRNA-like structures predicted in the control region of *Sitophilus oryzae*. Analysis was performed using ARWEN v1.2 with the mito/chloroplast genetic code and the default search options.

3.3. Phylogenetic analysis

Maximum Likelihood analyses resulted in clustering of Superfamilies within the Infraorder Cucujiformia, where bootstrap support was shown for the relationships between Curculionoidea, Chrysomeloidea, and Cucujoidea (Log Likelihood −133274.62, gamma shape parameter - 0.4760), but not in support for the position of the Tenebrioidea (Fig. 3). Strong bootstrap support was shown at nodes defining Supfamilies (≥58), with the exception of Chyrsomeloidea, and relationships among Infraorders have node support <50. Specifically, the separation between Infraorders Cucujiformia and Elateriformia still showed strong bootstrap support of 69%, whereas even more basal relationships with Staphyliniformia and Scarabaeiformia remain unresolved. Regardless, clades with representative species from Archostemata, Adephaga and Myxophaga appear supported near the root of the tree. The resulting NJ-based phylogeny (Supplementary Fig. 3) produced a tree topology roughly congruent with that derived from the ML analysis.

4. Discussion

The complete 18,105 *S. zeamais* and 17,602 bp *S. oryzae* mitogenomes were assembled from NGS data generated on an Illumina HiSeq2500. The high nucleotide compositions in both mitogenomes are biased towards A and T and overall AT-skew, which is typical of other insect mitogenomes. This bias has been hypothesized to enhance the optimal replication of mitogenomes, DNA repair, and to facilitate optimal matches with the anticodons of mitochondrial encoded tRNAs (Crozier and Crozier, 1993; Xia, 1996). The gene order of the two *Sitophilus* mitogenomes has the same order and orientation as in the ancestral insect mitochondrial genome (Boore, 1999), and is shared among all of the sequenced mitogenomes from curculionid beetles. The ancestral gene arrangement is not shared among other insect Orders such as Hymenoptera and Lepidoptera, wherein pronounced gene rearrangements are observed (Dowton and Austin, 1999; Coates et al., 2005; Cameron and Whiting, 2008). These results might suggest that gene rearrangements are rare among curculionid beetles.

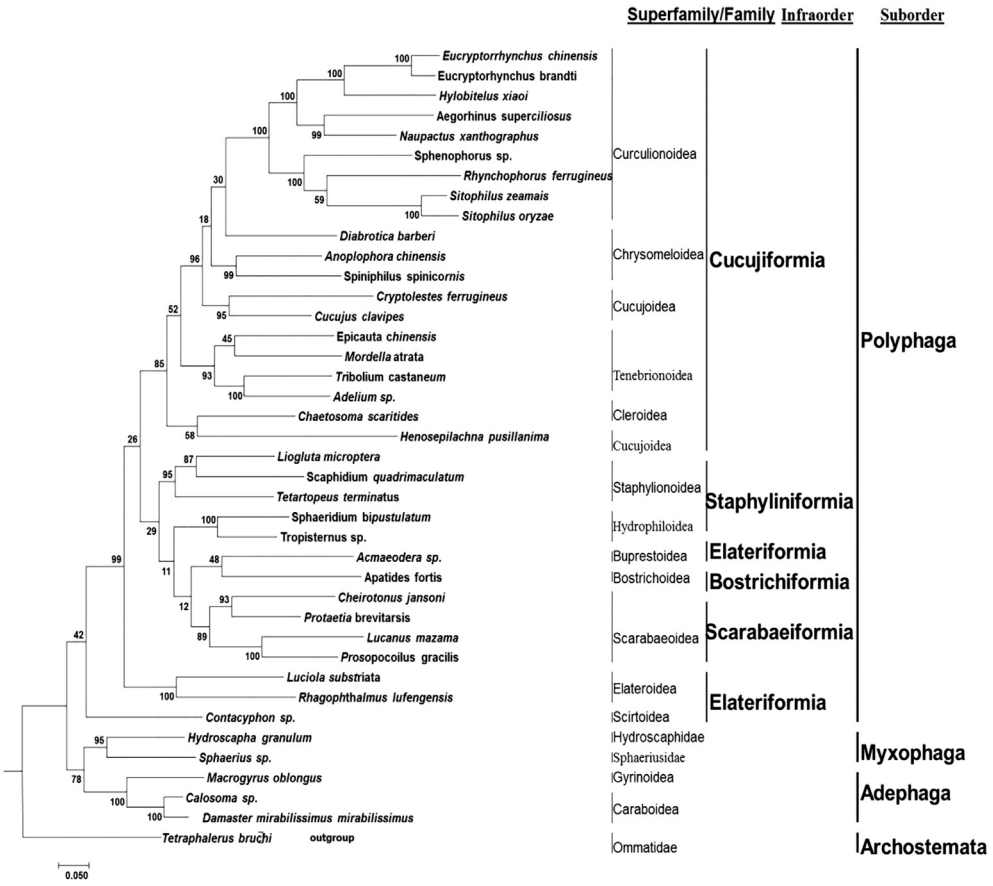


Fig. 3. The Maximum Likelihood estimation of phylogenetic relationships among species from the insect order Coleoptera based on 13 protein coding genes from complete or near-complete mitochondrial genomes. Clustering based upon Superfamily, Infraorder and Suborder is indicated by bars along the right hand side of the phylogenetic tree. *Tetraphalerus bruchi* (Coleoptera: Archostemata: Ommatidae) was used to root the tree as an outgroup.

The 13 PCGs of *Sitophilus* mitogenomes are initiated by ATG, ATA, and ATT codon (methionine and isoleucine), which is in agreement with codons used in other coleopteran mitogenomes (Wang et al., 2013). One exception was found in *S. zeamais* where the ND1 start codon was predicted to be a TTG (leucine) that was also previously reported from the Order Coleoptera; *Trachypachus holmbergi*, *Sphaerius* sp., *Chaetosoma scaritides*, *D.v. virgifera*, *D. barberi* and *Pyrocoelia rufa* (Bae et al., 2004; Coates, 2014; Sheffield et al., 2008). Evidence for use of TTG as the ND1 start codon comes from its conserved location among species which also reduces intergenic spacer size and gene overlap with the adjacent tRNA (Bae et al., 2004; Sheffield et al., 2008). Variation in the ND1 start codon between *S. oryzae* (ATA; methionine) and *S. zeamais* (TTG; leucine) contrasts with the conserved TTG (leucine) ND1 start in *D. v. virgifera* and *D. barberi* mitogenomes (Coates, 2014), which might suggest that start codons may not be conserved in certain coleopteran mitogenomes even over short evolutionary time frames. TAA stop codons are more prevalent compared to TAG in *Sitophilus* mitogenomes, and is consistent with other previously sequenced mitogenomes. In several instances, TAA stop codons were incomplete and could be completed by a posttranscriptional polyadenylation mechanism (Ojala et al., 1981; Cha et al., 2007). Overlapping ORFs for mitochondrial PCGs from our two *Sitophilus* species occurred among ND4 and ND4L, and ND6 and cytb, this was also reported in other insect mitogenomes (Sheffield et al., 2008; Margam et al., 2011, Wang et al., 2013, Coates, 2014).

Sequenced mitogenomes sizes from the Order Coleoptera range from 14,257 bp for *Aspidytes niobe* (Pons et al., 2010) to 20,319 bp for *Protaetia brevitarsis* (Kim et al., 2014), with the number of tandem repeats, intergenic spacers and repetition of other large fragments shown to influence size variation (Rand, 1993; McKnight and Shaffer, 1997). Such expansions in intragenic spaces could be assumed not to affect gene functions and thus be considered selectively neutral. Mitochondrial evolution is believed to favor genome size reduction (Rand, 1993; Boore, 1999) by the selective elimination of random expansions within presumed non-functional intergenic spacers (Burger et al., 2003). The presumption could be supported by relative conservation of the small non-coding intergenic spacers between $tRNA^{Ser2}$ and ND1 in *Sitophilus* sp. that is share among most coleopterans and other insects (Kim et al., 2006; Sheffield et al., 2008, Margam et al., 2011). In contrast, a non-repetitive non-coding intergenic spacers was reported between *Cyphon* sp. ND2 and $tRNA^{Trp}$ (177 bp; Sheffield et al., 2008), and several intergenic spacers between 202 and 322 bp pervade in the *Atta laevigata* mitogenome (Rodovalho et al., 2014). We describe a 109 bp between the $tRNA^{Ile}$ and $tRNA^{Gln}$ in the mitogenome of *S. oryzae* that is absent from other reported mitogenomes in the Superfamily Curculionidae. The same intergenic spacer between $tRNA^{Ile}$ and $tRNA^{Gln}$ in *S. zeamais* in 606 bp and contains a tandem repeat with a perfect 105 bp period size. Given the high sequence and inter-species variability among intragenic spacers, it remains uncertain if these regions could serve as markers for phylogenomic comparisons.

Intergenic spacers from coleopteran mitogenomes range from 216 bp in *Naupactus xanthographus* (Song et al., 2010) to 5654 bp in *P. brevitarsis* (Kim et al., 2014), and are respectively 2831 and 2818 bp in *S. zeamais* and *S. oryzae*. This suggests that control region length variability may play a pronounced role in mitochondrial genome size increases. Changes to the structure of mitogenome control regions may be ambiguous with respect to subsequent effects on replication and initiation of transcription. For instance, conserved structures were observed in the control region of *S. zeamais* and *S. oryzae*, including an ATAGT–like motif, and a poly T-stretch that is believed to be the origin

of minority strand replication, a poly A-stretch was also found downstream $tRNA^{Ile}$ gene in addition to microsatellites $(TA)_{3-6}$. These conserved structures are believed to control replication and transcription in insect mitogenomes (Saito et al., 2005; Oliveira et al., 2007). The three tRNA-like sequences in the *Sitophlus* sp. control region (Fig. 2) were reported previously for other Coleoptera including *Psacothea hilaris* (Kim et al., 2009), *Chrysochroa fulgidissima* (Hong et al., 2009), as well as other insects like *Calameuta idolon* and *C. filiformis* (Korkmaz et al., 2016). These tRNA-like sequences may result from gene duplication that facilitates increased needs for translation (Korkmaz et al., 2016), or may function in the initialization polycistronic transcription. The number of substitution mutations between these tRNA-like regions and corresponding tRNA orthologs may suggest derivation of alternate functions other than translation, but further research is required to test these hypotheses.

The majority of substitution mutations within *S. zeamais* and *S. oryzae* were predicted to result in amino acid changes (non-synonymous) mutations. An analogous scenario was previously reported for *Apis mellifera* (Crozier and Crozier, 1993) and *Diuraphis noxia* (de Jager et al., 2014). NADH Dehydrogenase subunits, ND5, ND2, ND1 and ND4, and ND 5 and ND4, were predicted to contain a majority of intraspecific changes among *S. oryzae* and *S. zeamais* respectively. In *S. oryzae*, ND5 has the highest proportion of non-synonymous substitutions, but the effects on ND5 function and the cause of this predicted directional selection remain unknown. Regardless, these substitutions could serve as markers for the study of population genetic structures and estimation of gene flow within each of these species.

Based on variation in amino acid sequence of all 13 PCGs from 40 coleopteran mitogenomes across 12 Superfamilies from the 5 Infraorders (Cucujiformia, Staphyliniformia, Elateriformia, Scarabaeiformia and Bostrichiformia) of Suborder polyphaga, as well as representatives from Suborders Archostemata, Myxophaga and Adephaga, ML and NJ phlogenetic reconstructions were generally representative of known relationships among Superfamilies (Fig. 3). Topology of resulting phylogenetic trees support Archostemata as a sister group to polyphaga (Coates, 2014). As a whole, the Superfamiles within Infraorder Cucujiformia are supported as distinct groups, whereas relationships among Staphyliniformia, Scarabaeiformia and Bostrichiformia remain more unresolved. Regardless, Elateriformia occupies the basal position in Polyphaga and is in agreement with prediction from prior morphological and molecular genetic studies (Crowson, 1960; Kukalova-Peck and Lawrence, 2004; Coates, 2014). Our phylogenies agreed with earlier morphological taxonomies that placed *Sitophilus* sp., *Rhynchophorus ferrugineus*, and *Sphenophorus* sp. in the Dryophthorinae, wherein *R. ferrugineus* was more closely related to *Sitophilus* sp. compared to *Sphenophorus* sp. Conflict arose in our phylogenetic analysis when Cleroidea, represented by only one family (Cleridae), was grouped together with one of the three Cucujoidea members (Coccinellidae), while the remaining two mitogenomes from Cucujidae form an independent clade, but a similar discrepancy was obtained by Zhang et al. (2016) suggesting that Cucujoidea may be a paraphyletic group. This contrasts with adjacent Curculionoidea and Tenebrionoidea which form a monophyletic group. Analogous reports of paraphyly among Cucujoidea, and monophyly of Curculionoidea and Tenebrionoidea were also made recently (Hunt et al., 2007; Zhang et al., 2016). Although there have been varied reports regarding members that occupy basal lineage in Cucujiformia, with Cleroidea and Tenebrionoidea reported to occupy the more ancestral position (Coates, 2014; Du et al., 2016; Zhang et al., 2016). This and all other phylogenetic reconstructions of Coleoptera are confronted with difficulties in base compositional heterogeneity and high levels of sequence divergence among distant species (Sheffield et al., 2009; Song et al., 2010) that limit the resulting resolution of tree topologies and provide inconclusive evolutionary relationships. Thus, the evolutionary time frame in which coleopteran mitogenome based phylogenies are capable of resolving may likely be limited and is still in the process of being determined, and tree topologies should be interpreted with caution.

Regardless, the phylogenetic analyses of mitogenomes likely are useful for defining short-term evolutionary relationships within the Curculionidae, which may be supported by appropriately grouping two members of the two genus *Eucryptorrhynchus* and *Sitophilus*. This might suggest that mitogenomes data may provide a useful resource for defining phylogenetic relationships, where the accumulation of such data is being facilitated by NGS methods that allow the rapid accumulation of such data (Coates, 2014; Coates and Abel, 2016). Our results and analyses do not provide a comprehensive phylogenetic relationship among major groups of weevils, but provide indication that such analysis will become possible upon the collection of additional novel mitogenomes sequences from species from the Curculionidae.

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