Comparison of the mitochondrial genomes of the Old and New World strains of the legume pod borer, *Maruca vitrata* (Lepidoptera: Crambidae)

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Abstract. *Maruca vitrata* (Fabricius, 1787) is a cryptic pantropical species of Lepidoptera that are comprised of two unique strains that inhabit the American continents (New World strain) and regions spanning from Africa through to Southeast Asia and Northern Australia (Old World strain). In this study, we de novo assembled the complete mitochondrial genome sequence of the New World legume pod borer, *M. vitrata*, from shotgun sequence data generated on an Illumina HiSeq 2000. Phylogenomic comparisons were made with other previously published mitochondrial genome sequences from crambid moths, including the Old World strain of *M. vitrata*. The 15,385 bp *M. vitrata* (New World) sequence has an 80.7% A+T content and encodes the 13 protein-coding, 2 ribosomal RNA and 22 transfer RNA genes in the typical orientation and arrangement of lepidopteran mitochondrial DNAs. Mitochondrial genome-wide comparison between the New and Old World strains of *M. vitrata* detected 476 polymorphic sites (4.23% nucleotide divergence) with an excess of synonymous substitution as a result of purifying selection. Furthermore, this level of sequence variation suggests that these strains diverged from ∼1.83 to 2.12 million years ago, assuming a linear rate of short-term substitution. The de novo assemblies of mitochondrial genomes from next-generation sequencing (NGS) reads provide readily available data for similar comparative studies.

Key words: Lepidoptera, *Maruca vitrata*, mitogenome, New World

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Introduction

The mitochondrial genome (mitogenome) encodes proteins that are involved in electron transport chain and oxidative phosphorylation that are essential for the energy production of the cell (Wolstenholme, 1992; Cameron, 2014). Insect mitogenomes are circular double-stranded DNA (dsDNA) molecules that typically range in size from 14 to 20 kb (Boyce et al., 1989; Wolstenholme, 1992) and contain a conserved set of 13 protein coding genes (PCGs), 22 transfer RNAs (tRNAs), and 2 ribosomal RNA genes (rRNAs) that are required for the translation of mitogenome-encoded proteins (Boore, 1999). Insect mitogenomes usually contain a large A+T-rich, non-coding control region that functions in the initiation of transcription and the replication of the mtDNA (Wolstenholme, 1992; Cameron, 2014), where the length is highly variable among different lineages, due to high rates of nucleotide substitution, insertions/deletions, and variable numbers of tandem repeats (Fauron and Wolstenholme, 1980; Inohira et al., 1997). Animal mitogenomes have a highly conserved gene content, maternal inheritance, and lack recombination (Avise, 2000), which have facilitated their use in the studies of phylogenetic relationships, population genetic structuring, as well as comparative evolution (Zhang et al., 1995; Nardi et al., 2003; Arunkumar et al., 2006). Comparisons between various insect mitogenomes have enabled the estimation of short-term evolutionary patterns within and between closely related species (Ballard, 2000; Coates et al., 2005). Although the gene content of the metazoan mitogenomes is highly conserved, variable lengths of A+T-rich coding regions, gene order, and orientation of tRNAs and PCGs are observed among insects (Hong et al., 2009).

To date, complete or near complete mitogenomes have been sequenced from more than 60 arthropod species (Cameron, 2014). Although the insect order Lepidoptera contains approximately 200,000 species, 72 complete or near complete mitogenomes representing 17 families in the lepidopteran lineage Ditrysia are available (Cameron, 2014). Among the family Crambidae, mitogenomes have been obtained for eight species: Ostrinia nubilalis and O. furnacalis (Coates et al., 2005), Diatraea saccharalis (Li et al., 2011), M. vitrata (Old World) (Margam et al., 2011b), Cnaphalocrocis medinalis (Chai et al., 2012), Chilo suppressalis (Chai et al., 2012), Paracycnotis prodigalis (Ye et al., 2013), and Spoladea recurvalis (He et al., 2015). Complete mitogenome sequences for Lepidoptera have been used to study the divergences between sibling and congeneric species (Yukuhiro et al., 2002; Coates et al., 2005) and to facilitate population-level studies (Kim et al., 2006). Furthermore, full mitogenomes for Lepidoptera are important for the determination of phylogenetic relationships and the identification of cryptic species (Lee et al., 2006; Chai et al., 2012) at various taxonomic levels.

Obtaining full mitogenome sequences has been a challenging and resource demanding task when sequenced by long-range PCR and subsequent primer walking (Huys et al., 2007) or overlapping short polymerase chain reaction (PCR) products (Coates et al., 2005). Recently, next-generation sequencing (NGS) technologies have led to more straightforward pipelines for the assembly of complete mitogenome sequences (Jex et al., 2010a; Knaus et al., 2011). Mitogenomes have been sequenced from NGS libraries prepared from long-PCR amplified sequences (Maricic et al., 2010; Morin et al., 2010; Horn et al., 2011) or from sequence captured DNA molecules (Vasta et al., 2009). Long-range PCR strategies may remain useful for NGS-based sequencing, especially when limited genetic material can be recovered from small invertebrates (Jex et al., 2010b). Ultra-deep sequencing on NGS platforms can acquire data that are sufficient for the subsequent assembly of full mitogenomes at high read depth (Knaus et al., 2011). Also, due to the high copy number of mitochondria in most eukaryotic cells, NGS can reduce the impact of rare nuclear-integrated copies of mitochondria in most eukaryotic cells, NGS can reduce the impact of rare nuclear-integrated copies of mitochondrial DNAs or other non-target products (Gilbert et al., 2007; Ho and Gilbert, 2010).

Members from the genus Maruca (Lepidoptera: Crambidae) are serious insect pests that feed on cowpea crops throughout the tropical and sub-tropical regions, from northern Australia and East Asia through sub-Saharan Africa to the Caribbean, Central America and Hawaii (Sharma, 1998). Feeding by M. vitrata causes considerable crop loss and reduced yields to cultivated cowpea in many African countries, and is a target for improved and sustainable control methods (Agunbiade et al., 2012). The comparison of M. vitrata mitochondrial cytochrome c oxidase subunit I (cox1) sequence data obtained from samples collected in Africa and Puerto Rico revealed unexpectedly high levels of sequence variation, which suggested the existence of cryptic species or undefined strains (Margam et al., 2011a). A recent study also confirmed the existence of different subspecies of M. vitrata in Asia and Africa and Maruca spp. in Oceania and Latin America based on cox1 data (Malini et al., 2015). An analogous relationship among New and Old World species has also been predicted between Helicoverpa zea and H. armigera, the corn earworm, and cotton bollworm (Behere et al., 2007), but accurate estimates of time since divergence between these species are lacking. As a result of the lack of morphological characters for subspecies sorting between the Old and New World strains of M. vitrata, Illumina HiSeq
2000 sequencing reads were obtained from M. vitrata (New World) and used to successfully assemble the full mitogenome sequence. These data were applied to refine previous haplotype divergence estimates and provide an estimated time of divergence between the New and Old World strains, in ongoing efforts to describe the evolutionary divergence in M. vitrata.

Materials and methods

Sample collection and DNA extraction

Insect samples of M. vitrata adults were collected from white bean (Phaseolus vulgaris) near Lares, Puerto Rico. All specimens were preserved in 100% ethanol and stored at 4 °C. Total genomic DNA was extracted from the adult specimens using the DNeasy animal tissue kit following the manufacturer’s instructions (QIAGEN, Valencia, California). The quality of the DNA was assessed through electrophoresis on a 1% agarose gel before submission for library construction and sequencing.

Illumina HiSeq 2000™ library construction and sequencing

A shotgun genomic DNA library was constructed using the TruSeq DNA sample prep kit (Illumina, San Diego, California). Briefly, 1 µg of genomic DNA was sonicated on a Covaris M220 (Covaris, Woburn, Massachusetts) using the default methods for sonication to a size of 500 bp library, as described by the manufacturer. Sonicated DNA was blunt-ended, 3′-end A-tailed and ligated to an indexed adaptor. The adaptor-ligated gDNA was amplified by PCR to selectively enrich for those fragments that have adapters on both ends. Amplification was carried out for eight cycles with the Kapa HiFi polymerase (Kapa Biosystems, Woburn, Massachusetts), to reduce the likelihood of multiple identical reads, due to preferential clonal amplification. The library was size selected on a 2% agarose gel for fragments 600 bp to 800 bp in length. The size-selected library was run on Agilent bioanalyzer DNA7500 LabChips (Agilent, Santa Clara, California), to determine the average fragment size and to confirm the presence of DNA of the expected size range, and quantitated by qPCR on an ABI 7900. The indexed library was loaded onto a single lane of an eight-lane flowcell for cluster formation and paired-end sequenced at 100-bp lengths on an Illumina HiSeq 2000™ using the TruSeq SBS sequencing kits version 3. The raw .bcl files were converted into FASTQ files using the software Casava 1.8.2 (Illumina). All shotgun genomic DNA library construction and Illumina HiSeq 2000™ sequencing were carried out at the W. M. Keck Center for Comparative and Functional Genomics, Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign.

Mitogenome assembly

FASTQ output was trimmed of read data with PHRED quality scores < 20 using the script TrimmingReads.pl (Patel and Jain, 2012; NGSToolKit). A total of 30 million reads from the trimmed FASTQ sequence were loaded into the Velvet Assembler program v.1.2.10 (Zerbino and Birney, 2008) and assembled using the de Bruijn graph method with a hash size (k-mer) = 21, no coverage cutoff, and a minimum contig length (−min_contig_lgth) = 10,000. Querying the National Center for Biotechnology Information (NCBI) non-redundant (nr) nucleotide database with the blastn algorithm (Altschul et al., 1997; http://blast.ncbi.nlm.nih.gov/Blast.cgi) identified resulting contigs containing mitogenome sequence. The contigs that produced database ‘hits’ were filtered for those with E-values ≥ 10^-40 and percent similarities ≥ 75 to identify contigs with encoded mitochondrial gene sequences.

Sequence annotation

Coding sequences for each M. vitrata (New World) mitochondrial gene were aligned to mitochondrial gene sequences from crambid mitogenomes (GenBank Accessions: JF339041, JN246082, FJ240227, HM751150, AF467260, NC_020094, and KJ739310) (Supplementary Table 1) using the CLC Genomics Workbench v6.5.2. The 5′ end of the mitochondrial genes was inferred to be the first legitimate start codon in the open reading frame, and manual adjustments were made when needed. The 3′ end was inferred to be at the first in-frame stop codon encountered. When the stop codon was located within the sequence of a downstream gene encoded on the same strand, a truncated stop codon (T) adjacent to the beginning of the downstream gene was designated as the termination codon. The positions and secondary structures for the tRNA genes were confirmed using ARWEN v1. 2 (Laslett and Canbäck, 2008; http://mbio-serv2.mbioekol.lu.se/ARWEN/index.html). The graphical circular structure of the mitogenome was drawn using GenomeVx, a suite of tools for generating physical maps of plastid and mitogenomes (Conant and Wolfe, 2008; http://wolfe.ucd.ie/GenomeVx/). All annotations were transferred to Sequin 13.05 (http://www.ncbi.nlm.nih.gov/Sequin/) and submitted to the GenBank database under the accession number KJ466365.

Genome sequence analysis

The nucleotide sequences, codon usage, and relative synonymous codon usage (RSCU) in the M. vitrata
vitrata (New World) mitogenome were calculated with MEGA 5.2.2 (Tamura et al., 2004). Nucleotide composition skew analysis was carried out by the formula: AT skew = [A−T]/[A+T] and GC skew = [G−C]/[G+C], respectively (Perna and Kocher, 1995).

The DNA and protein sequence identities were calculated across the Crambidae using the FASTA program, a tool for biological sequence comparison (Pearson and Lipman, 1988; http://fasta.bioch.virginia.edu/fasta_www2/). The online program, microsatellite repeats finder (http://insilico.ehu.es/mini_tools/microsatellites/) was used to analyse the A+T-rich control region for tandem repeats (repeat units ≥ 2 nucleotides and arrays of 4 ≥ units).

Estimation of divergence times

The nucleotide sequence of M. vitrata (Old World; GenBank accession number HM751150) was downloaded in the FASTA format and aligned with that of the M. vitrata New World sample (GenBank accession number KF466365) using MEGA 5.2.2 (Tamura et al., 2004). Gaps in the resulting alignment due to ambiguous bases (Ns) in HM751150 overhangs were deleted. The number of nucleotide differences between the two aligned sequences were calculated using DiffSeq from the EMBOS Package (Rice et al., 2000). A clock-like rate of 2% mitochondrial DNA sequence change per million years has been estimated among Drosophila species (Powell et al., 1986) and a 1.115% per million year estimate was proposed among Lepidoptera (Brower, 1994), which to date is the divergence time based on the substitution rate across a 11,250 bp of the consensus alignment between M. vitrata strains (inter-species substitution rate/clock rate).

Results and Discussion

Mitogenome assembly and annotation

Paired-end Illumina sequencing generated a total of 336,472,660 reads, of which 30 million were subsampled and used to generate a 15,369 bp de novo assembly of the previously uncharacterized M. vitrata (New World) mitogenome (Table 1 and Fig. 1). The mitogenome of M. vitrata (New World) was similar to the size previously reported from other Lepidoptera, that range from 15,314 bp in Coreana raphaelis (Lepidoptera: Lycaenidae) (Kim et al., 2006) to 15,928 bp in Bombyx mandarina (Lepidoptera: Bombycidae) (Yukuhiro et al., 2002). The M. vitrata (New World) mitogenome contained the 13 PCGs, 22 tRNAs, and 2 rRNA genes that are typical for bilaterian metazoans (Wolstenholme, 1992; Table 1 and Fig. 1). As in all other lepidopteran mitogenomes, of the total 37 genes, 23 were encoded on the major strand, whereas the 14 others were encoded on the minor strand (Table 1 and Fig. 1). The 13 PCGs spanned 11,369 bp and accounted for 73.90% of the entire sequence. The M. vitrata (New World) mitogenome had the typical Ditrysian gene order trnM-trnL-trnQ-nad2 (Yukuhiro et al., 2002; Coates et al., 2005; Kim et al., 2006; Lee et al., 2006; Cameron and Whiting, 2008; Hong et al., 2008), which differed from the ancestral gene order trnL-trnE-trnM-nad2 found in other insects (Cameron, 2014). This was first reported by Taylor et al. (1993) in lycaenids and noctuids, but has now been found in five lepidopteran superfamilies (Papilionoidea, Noctuoidea, Pyraloidea, Tortricoida, and Bombycoidea) and a synapomorphy of Ditrysia (Salvato et al., 2008).

The M. vitrata (New World) mitogenome showed a high A+T content 80.70% (A = 40.30%, T = 40.4%, G = 7.99%, and C = 11.31%) (Table 2), and this is consistent with the high A+T content of insect (Clary and Wolstenholme, 1985; Crozier and Crozier, 1993) and lepidopteran mitogenomes (Liu et al., 2008). The nucleotide composition of the PCGs and the AT- and GC-skew calculated for all available crambid mtDNA genomes are presented in Table 2. The average AT-skew of the crambid mitogenomes was 0.006, ranging from −0.024 to 0.03, whereas the average GC-skew was −0.195, ranging from −0.26 to −0.17. The AT-skew of M. vitrata (New World) was −0.002. A similar negative AT-skew was also previously reported for C. medinalis (Chai et al., 2012) and Spolādea recurvalis (He et al., 2015) (Table 2). This indicates a bias for T over A nucleotides. Consistent with other lepidopteran mitogenomes that exhibit negative GC skewness, the GC-skew of M. vitrata (New World) was −0.17, indicating a bias for C over G nucleotides.

Protein coding genes

The putative start codons of PCGs used by M. vitrata (New World) (ATN; Table 1) were identical to those previously known in animal mitogenomes (Wolstenholme, 1992). CGA was used as the start codon for cox1 as previously reported for lepidopterans such as Bombyx mori (Yukuhiro et al., 2002), O. nubilalis and O. fuscacalis (Coates et al., 2005), S. recurvalis (He et al., 2015), Adoxophyes honmai (Lee et al., 2006), C. raphaelis (Kim et al., 2006), Antheraea pernyi (Liu et al., 2008), B. mandarina (Pan et al., 2008), Ochrogaster lunifer (Salvato et al., 2008), Artogeia melete (Hong et al., 2009), Eriogyna pyretoorm (jiang et al., 2009), Hypantria cunea (Liao et al., 2010) and M. vitrata (Old World) (Margam et al., 2011b). The termination codon TAA was used in all PCGs with the exception of nad3, which had TAG. Also, consistent with some other insect mitogenomes,
Table 1. Annotation of the full mitogenome of *Maruca vitrata* (New World)

| Gene  | Strand | CDS positions | Size (bp) | Start codon | Stop codon | Anticodon |
|-------|--------|---------------|----------|-------------|------------|-----------|
| trnM  | J      | 1..68         | 68       | CAT         |            |           |
| trnI  | J      | 69..133       | 65       | GAT         |            |           |
| trnQ  | N      | 130..200      | 71       | TTA         |            |           |
| nad2  | J      | 269..1255     | 987      | ATA         | TAA        |           |
| trnW  | J      | 1257..1324    | 68       | TCA         |            |           |
| trnC  | N      | 1316..1382    | 67       | GCA         |            |           |
| trnT  | N      | 1383..1451    | 69       | GTC         |            |           |
| cox1  | J      | 1458..2988    | 1531     | CGA         | T–         |           |
| trnL1 | J      | 2988..3056    | 69       | TAA         |            |           |
| cox2  | J      | 3056..3737    | 682      | ATG         | T–         |           |
| trnK  | J      | 3737..3809    | 73       | CTT         |            |           |
| trnD  | J      | 3811..3878    | 68       | GTC         |            |           |
| atp8  | J      | 3879..4040    | 162      | ATC         | TAA        |           |
| atp6  | J      | 4034..4708    | 675      | ATG         | TAA        |           |
| cox3  | J      | 4715..5503    | 789      | ATG         | TAA        |           |
| trnG  | J      | 5505..5573    | 69       | TCC         |            |           |
| nad3  | J      | 5570..5926    | 357      | ATA         | TAG        |           |
| trnA  | J      | 5924..5992    | 69       | TGC         |            |           |
| trnR  | J      | 5990..6054    | 65       | TCG         |            |           |
| trnN  | J      | 6054..6119    | 66       | GTT         |            |           |
| trnS1 | J      | 6122..6189    | 68       | GCT         |            |           |
| trnQ  | J      | 6189..6256    | 68       | TTC         |            |           |
| trnF  | N      | 6255..6324    | 70       | GAA         |            |           |
| nad5  | N      | 6307..8058    | 1752     | ATT         | TAA        |           |
| trnH  | N      | 8058..8126    | 69       | GTG         |            |           |
| nad4  | N      | 8125..9465    | 1341     | ATG         | TAA        |           |
| nad4L | N      | 9465..9746    | 282      | ATA         | TAA        |           |
| trnT  | J      | 9758..9823    | 66       | TGT         |            |           |
| trnP  | N      | 9823..9890    | 68       | TGG         |            |           |
| nad6  | J      | 9892..10422   | 531      | ATT         | TAA        |           |
| cytB  | J      | 10426..11574  | 1149     | ATG         | TAA        |           |
| trnS2 | J      | 11573..11638  | 66       | TGA         |            |           |
| nad1  | N      | 11660..12790  | 1131     | ATG         | TAA        |           |
| trnL2 | N      | 12791..12859  | 69       | TAG         |            |           |
| rrsL  | N      | 12894..14197  | 1304     | TAC         |            |           |
| trnV  | N      | 14210..14276  | 67       | TAC         |            |           |
| rrsS  | N      | 14280..15044  | 765      |            |            |           |

A+T-rich region: 15045..15385 341

J, major strand; N, minor strand.

Table 2. Nucleotide composition in the whole mitogenomes of *Maruca vitrata* (New World) and other available mitogenomes from six additional species from the lepidopteran Family Crambidae

| Species                      | A    | T    | A+T  | AT-Skew | G    | C    | G+C  | GC-Skew |
|------------------------------|------|------|------|---------|------|------|------|---------|
| *Cnaphalocrocis medinalis*   | 40.36| 41.58| 81.94| -0.01   | 7.45 | 10.61| 18.06| -0.17   |
| *Chilo suppressalis*         | 40.64| 40.03| 80.67| 0.01    | 7.39 | 11.94| 19.33| -0.24   |
| *Diatraea saccharalis*       | 40.87| 39.15| 80.02| 0.02    | 7.42 | 12.56| 19.98| -0.26   |
| *Ostrinia furnacalis*        | 41.46| 38.92| 80.38| 0.03    | 7.91 | 11.71| 19.62| -0.19   |
| *Ostrinia nubilalis*         | 41.36| 38.81| 80.17| 0.03    | 8.02 | 11.82| 19.83| -0.19   |
| *Paracyclosperma prodigalis* | 40.84| 40.71| 81.55| 0.002   | 7.54 | 10.91| 18.45| -0.18   |
| *Spolasma recurvata*         | 39.48| 41.43| 80.91| -0.024  | 7.79 | 11.3 | 19.09| -0.18   |
| *Maruca vitrata* (Old World) | 40.14| 39.89| 80.03| 0.003   | 8.21 | 11.76| 19.97| -0.18   |
| *Maruca vitrata* (New World)| 40.27| 40.44| 80.7 | -0.002  | 7.99 | 11.31| 19.3 | -0.17   |

1Partial mitogenome sequences that lacked portions of the AT-rich control region sequence.
2Partial mitogenome sequences for which the *cox1* gene sequence is publicly unavailable.
cox1 and cox2 both had incomplete (T–) stop codon (Coates et al., 2005; Salvato et al., 2008) (Table 1). The presence of incomplete stop codons is a molecular feature shared with all lepidopteran mitogenomes sequenced to date (Brower, 1994; Yukuhiro et al., 2002; Coates et al., 2005; Kim et al., 2006; Lee et al., 2006; Cameron and Whiting, 2008; Hong et al., 2008) and with many arthropod mitogenomes (Boore, 1999). The motif TTAG has been observed immediately upstream of the putative cox1 CGA start codon in some lepidopteran mitogenomes and has been proposed to serve in non-standard initiation (Yukuhiro et al., 2002). We observed the motif ATAG immediately upstream of the CGA start codon of the M. vitrata (New World) cox1 gene. This motif is conserved in some lepidopteran mitogenomes and lacking in others (Margam et al., 2011b). Among the available crambid mitogenomes, O. furnacalis and O. nubilalis both have the motif TTAG upstream of the start codon of the cox1 gene, while M. vitrata (Old World) has ATAG. Reduction in mitogenome size has led to minimal intergenic space as well as overlap between adjacent genes in M. vitrata (New World). Atp8 and atp6 PCGs had a seven-nucleotide overlap (Table 1). This feature is common to all lepidopteran mitogenomes known (Brower, 1994; Yukuhiro et al., 2002; Coates et al., 2005; Kim et al., 2006; Lee et al., 2006; Cameron
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| Amino acid | Codon | Count | RSCU | Amino acid | Codon | Count | RSCU |
|------------|-------|-------|------|------------|-------|-------|------|
| Ala (A)    | GCU   | 78    | 2.22 | Pro (P)    | CCU   | 75    | 2.21 |
|            | GCC   | 7     | 0.21 |            | CCC   | 9     | 0.29 |
|            | GCA   | 40    | 1.21 |            | CCA   | 43    | 1.14 |
|            | GCG   | 1     | 0.05 |            | CCG   | 2     | 0.06 |
| Cys (C)    | UGU   | 26    | 1.31 | Gln (Q)    | CAA   | 63    | 2    |
|            | UGC   | 4     | 0.23 |            | CAG   | 0     | 0    |
| Asp (D)    | GAU   | 65    | 1.32 | Arg (R)    | CGU   | 13    | 0.9  |
|            | GAC   | 3     | 0.22 |            | CGC   | 0     | 0    |
| Glu (E)    | GAA   | 72    | 1.72 |            | CGA   | 41    | 2.71 |
|            | GAG   | 6     | 0.13 |            | CGG   | 1     | 0.08 |
| Phe (F)    | UUU   | 344   | 1.81 | Ser1 (S)   | AGU   | 32    | 0.75 |
|            | UUC   | 32    | 0.19 |            | AGC   | 3     | 0.05 |
| Gly (G)    | GGU   | 67    | 1.23 |            | AGA   | 81    | 1.89 |
|            | GGC   | 4     | 0.09 |            | ACG   | 0     | 0    |
|            | GGA   | 122   | 2.15 | Ser2 (S)   | UCU   | 109   | 2.77 |
|            | GGG   | 15    | 0.22 |            | UCC   | 4     | 0.06 |
| His (H)    | CAU   | 57    | 1.34 |            | UCA   | 87    | 2.39 |
|            | CAC   | 12    | 0.35 |            | UCG   | 4     | 0.09 |
| Ile (I)    | AUU   | 443   | 1.88 | Thr (T)    | ACU   | 89    | 2.23 |
|            | AUC   | 26    | 0.12 |            | ACC   | 9     | 0.31 |
| Lys (K)    | AAA   | 93    | 1.77 |            | ACA   | 58    | 1.12 |
|            | AAG   | 14    | 0.23 |            | ACG   | 2     | 0.04 |
| Leu1 (L)   | CUU   | 27    | 0.31 | Val (V)    | GUU   | 86    | 2.22 |
|            | CUC   | 3     | 0.04 |            | GUC   | 2     | 0.03 |
|            | CUA   | 23    | 0.68 |            | GUA   | 59    | 1.71 |
|            | CUG   | 0     | 0     |            | GUG   | 3     | 0.04 |
| Leu2 (L)   | UUA   | 481   | 4.86 | Trp (W)    | UGA   | 94    | 2    |
|            | UUG   | 11    | 0.12 |            | UGG   | 0     | 0    |
| Met (M)    | AUA   | 267   | 1.83 | Try (Y)    | UAU   | 183   | 1.84 |
|            | AUG   | 23    | 0.17 |            | UAC   | 13    | 0.16 |
| Asn (N)    | AAU   | 238   | 1.94 |            |        |       |      |
|            | AAC   | 8     | 0.06 |            |        |       |      |

Transfer and ribosomal RNA genes

The published mitogenome of *M. vitrata* (Old World) is incomplete and thereby has 19 tRNAs and a part of the rRNA reported. However, the *M. vitrata* (New World) mitogenome encoded the 22 tRNAs typically found in animal mitogenomes (Boore, 1999) and all formed the canonical cloverleaf-like tRNA secondary structure, with the exception of *trnS1*, in which its dihydrouridine (DHU) arm formed a simple loop. The lack of DHU arm in *trnS1* is a common condition in metazoan mitogenomes (Taanman, 1999) but is not found in all lepidopterans. For example, *A. honmai* has all the tRNAs with a complete cloverleaf structure (Lee et al., 2006). Of the 22 tRNA genes, 14 genes were encoded on the majority strand, while eight were encoded on the minority strand. The length of tRNAs ranged from 63 bp to 71 bp. Consistent with what is observed in all other insect mitogenomes, the mitogenome of *M.*...
Table 4. Comparison of the percent nucleotide and amino acid identity in the 13 protein coding gene sequences of *Maruca vitrata* (New World) with orthologs in 6 other crambid lepidopteran mitogenomes [Mv – *M. vitrata* (Old World), Cs (C. *suppressalis*), Cm (*C. medinalis*), Ds (*D. saccharalis*), Of (*O. furnacalis*), On (*O. nubilalis*), Pp (*Paracyphoriza prodigalis*), Sr (*Spoladea recurvalis*); coxI gene sequence not available]

| Genes | DNA (%) | Protein (%) | DNA (%) | Protein (%) | DNA (%) | Protein (%) | DNA (%) | Protein (%) |
|-------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|
| Mv    | cs      | 95.7        | 97.7    | 84.6        | 80.8    | 86.5        | 83.8    | 78.9        | 75.0        | 82.2        | 80.8        |
| cs    | 94.3    | 99.4        |         |             |         | 89.5        | 94.1    | 85.6        | 93.9        |             |             |
| cm    | 94.9    | 97.8        |         |             |         | 90.0        | 93.8    | 85.9        | 92.5        |             |             |
| cm    | 94.6    | 98.5        |         |             |         | 89.0        | 94.7    | 86.2        | 91.6        |             |             |
| d2    | 93.8    | 90.6        |         |             |         | 81.2        | 64.8    | 78.6        | 61.8        |             |             |
| d1    | 93.9    | 98.7        |         |             |         | 83.7        | 85.4    | 90.4        | 93.3        |             |             |
| d3    | 94.3    | 99.1        |         |             |         | 83.8        | 83.8    | 88.4        | 90.6        |             |             |
| d4    | 95.7    | 97.5        |         |             |         | 87.6        | 84.3    | 91.6        | 90.1        |             |             |
| d4l   | 96.1    | 90.5        |         |             |         | 89.4        | 87.1    | 91.8        | 88.2        |             |             |
| d5    | 96.0    | 98.3        |         |             |         | 85.7        | 83.6    | 88.5        | 87.0        |             |             |
| d6    | 96.8    | 94.3        |         |             |         | 82.1        | 73.4    | 85.6        | 80.2        |             |             |
| cytb  | 97.5    | 98.4        |         |             |         | 86.4        | 91.1    | 89.3        | 91.6        |             |             |

| Genes | DNA (%) | Protein (%) | DNA (%) | Protein (%) | DNA (%) | Protein (%) | DNA (%) | Protein (%) |
|-------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|
| Of    | cs      | 83.9        | 80.5    | 83.9        | 80.8    | 83.1        | 78.4    | 87.3        | 87.2        |             |             |
| cs    | 87.4    | 90.1        |         |             |         | 87.2        | 89.7    | 85.8        | 84.4        |             |             |
| cm    | 88.6    | 95.7        |         |             |         | 87.9        | 94.3    | 88.3        | 93.7        |             |             |
| cm    | 88.1    | 93.8        |         |             |         | 87.8        | 93.0    | 87.1        | 91.6        |             |             |
| cm    | 86.4    | 90.9        |         |             |         | 86.2        | 90.9    | 86.9        | 89.7        |             |             |
| atp8  | 85.8    | 73.6        |         |             |         | 85.2        | 71.7    | 82.1        | 66.0        |             |             |
| atp6  | 85.6    | 89.7        |         |             |         | 85.2        | 89.7    | 83.7        | 86.2        |             |             |
| nad3  | 84.4    | 86.1        |         |             |         | 84.2        | 86.0    | 83.5        | 80.9        |             |             |
| nad4  | 87.9    | 82.4        |         |             |         | 87.7        | 82.6    | 86.7        | 81.4        |             |             |
| nad4l | 89.7    | 82.8        |         |             |         | 89.0        | 83.9    | 88.3        | 81.7        |             |             |
| nad5  | 87.6    | 84.3        |         |             |         | 87.2        | 83.9    | 86.4        | 84.1        |             |             |
| nad6  | 83.9    | 75.1        |         |             |         | 83.3        | 74.6    | 83.7        | 72.9        |             |             |
| cytb  | 87.2    | 90.3        |         |             |         | 87.5        | 90.8    | 86.1        | 85.6        |             |             |

*Maruca vitrata* (New World) contained two rRNAs with a total length of 2,069 bp. The large rRNA (*rrnL*) had a length of 1,304 bp, while the small rRNA (*rrnS*) had a length of 765 bp (Table 1).

**Non-coding regions**

The *M. vitrata* (New World) mitogenome contained characteristics typical of other lepidopteran mitogenomes. The 7 bp intergenic spacer located between *trnS2* and *nad1* contained the ATACTAA motif, which is conserved across the order Lepidoptera (Cameron and Whiting, 2008), including *M. vitrata* (Old World). This motif is possibly fundamental to site recognition by the transcription termination peptide (mtTERM protein) (Taanman, 1999) and is present in most insect mitogenomes (Cameron and Whiting, 2008). Insect mitogenomes often contain a non-coding A+T-rich region that varies considerably in length among insect species, or even within the same species (Zhang and Hewitt, 1997). Consistent with findings from other insects, the largest non-coding region was the A+T-rich control region, although it varies in size in crambids (Hua et al., 2008; Oliveira et al., 2008; Ma et al., 2009). Tandem repeat sequences are common in the control region of insects and can vary in length and copy number (Dotson and Beard, 2001). Repeats in Lepidoptera share greater degrees of conservation than in other insect groups (Cameron and Whiting, 2008). The A+T rich control region in *M. vitrata* (New World) was estimated at 341 bp and contained (AT) tandem repeats. The A+T-rich region contained a conserved ATAG motif followed by an 18 bp poly-T stretch. This feature has been found in *C. medinalis*, *C. suppressalis* and *D. saccharalis*, but the length of the poly-T stretch varies between species. This structure is suggested to function as a signal for mitochondrial DNA replication initiation (Hu et al., 2010; Yin et al., 2010; Kim et al., 2010). A poly-A stretch commonly observed in other lepidopteran mitogenomes was also found immediately upstream of *trnM* in *M.
vitrata (New World). The (AT)$_4$, (AATT)$_4$ and (AT)$_5$ repeats in the M. vitrata control region were predicted (Supplementary Table 2) of which (AT)$_5$ was similar to an (AT)$_8$ in Hyphantria cunea (Liao et al., 2010) and (AT)$_7$ in Ochrogaster lunifer (Salvato et al., 2008).

Comparative genome analysis and estimation of divergence times

Protein coding sequence was shown to be highly similar between M. vitrata (New World) and M. vitrata (Old World) mitogenomes (see above; Table 4). Comparison between M. vitrata (New World) and M. vitrata (Old World) coding sequences found 476 nt substitutions in the 11,250 nt consensus alignment (4.23% divergence), of which 74 (15.5%) were non-synonymous mutations. This suggests that purifying selection may be curbing the rate of amino acid change in Maruca. Novel non-synonymous mutations were directly shown to be under negative selection in haplotypes derived from mutator strains of mice (Stewart et al., 2008) such that the accumulation of mildly deleterious mutations might lead to reduced fitness and result in removal from the population. The alignment of entire mitogenome sequences from M. vitrata (Old World) and M. vitrata (New World) resulted in a 14,039 consensus (gaps excluded) and showed a total of 526 nucleotide differences (3.75%) (Supplementary Table 2) of which (AT)$_9$ repeats in the control region were (AT)$_8$ in O. cunea (Liao et al., 2010) and (AT)$_7$ in O. lunifer (Salvato et al., 2008). This short-term change identified here provides valuable information regarding variation between genetically distinct strains of a species that is analogous to that shown by comparisons between other closely related insect species (Coates et al., 2005; Coates, 2014). Specifically, these whole mitochondrial genome analyses provide estimates of divergence time and short-term patterns of sequence evolution that are more accurate compared to the use of single mitochondrial gene sequence data. Although not explored in the current study, the rapid de novo assembly of mitogenomes from NGS sequence data presents future opportunities for phylogenomic studies within and among species of Lepidoptera.

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Supplementary material

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