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Comparison of complete mitochondrial DNA sequences between old and new world strains of the cowpea aphid, *Aphis craccivora* (Hemiptera: Aphididae)

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ABSTRACT

Mitochondrial DNA provides useful tools for inferring population genetic structure within a species and phylogenetic relationships between species. The complete mitogenome sequences were assembled from strains of the cowpea aphids, *Aphis craccivora*, from the old (15,308 bp) and new world (15,305 bp) using shotgun Illumina HiSeq 2500 sequence reads. The *A. craccivora* mitogenomes have 13 protein coding genes (PCGs), 22 transfer RNAs and 2 ribosomal RNAs in the same order and orientation found among Insecta. The two *A. craccivora* mitogenomes do not contain long intergenic repeats as found in other members of the family Aphididae. Comparisons between strains indicated a 99.7% sequence identity, wherein 57 and 11 substitutions were respectively predicted among reads from new and old world strains. Among these substitutions, 31 and 2 were in PCGs of new world and old world aphids, respectively, and out of which 28 and 2 were nonsynonymous. A majority of the substitutions were in NADH Dehydrogenase subunit 1. Phylogenetic analysis of full mitogenomes sequences predicted that Aphididae as monophyletic and clustered with Pterocommatinae and Aphidinae at subfamily level.

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1. Introduction

Aphididae, an important Family within the sub-Order Sternorrhyncha of Hemiptera that contains approximately 5000 described species of aphids, of which around 250 species are pests of agriculture, forestry and horticulture (Blackman and Eastop, 1984; Grimaldi and Engel, 2005). Aphids can cause damage to plants by direct feeding, and also by vectoring several viral pathogens. The cowpea aphid, *Aphis craccivora* (Hemiptera: Aphididae), principally feeds on the phloem of leguminosae plant species throughout the world. The cowpea aphid causes significant damage to cultivated cowpea, *Vigna unguiculata*, even when at low population densities due to vectoring of the potyviruses cowpea aphid-borne mosaic virus (Thottappilly and Rossel, 1985; Atiri et al., 1986; Coceano et al., 1998) and blackeye cowpea mosaic virus (Dijkstra et al., 1987). The viruses cause cowpea mottling, chlorosis, and seed shriveling (Brunt et al., 1990), which directly contribute to severely reduced yields (Bock and Conti, 1974; Singh and Emden, 1979). These two viruses can propagate via vertical transmission from parent to progeny seeds (Bennett, 1969; Gillaspie et al., 1993; Bashir and Hampton, 1996) which makes their control difficult, and highlights the need to develop effective measures to control initial viral infection by the principal insect vector, *A. craccivora*.

Similar to other aphids in the Family Aphididae, *A. craccivora* has a complex life cycle with distinct morphologies, diphenism, social (swarming) behavior, ability to form galls in plants, and has tight obligate associations with bacterial endosymbionts; all of which make this group of insects intriguing for evolutionary and ecological study (Blackman and Eastop, 1984; Minks and Harrewijn, 1987; Huang and Qiao, 2014). The intra-ordinal relationships and identification of strains among aphid species is incomplete due to the dearth of useful morphological characters that are taxonomically informative (Foottit, 1997). On the other hand, molecular sequence data have provided information that has resolved ecological and taxonomic relationships among insect species. The maternally inherited mitochondrial genome (mitogenome) of insects is short, ~13 to 18 kb, and has been widely used in

Abbreviations: NWAC, new world *Aphis craccivora*; OWAC, old world *Aphis craccivora*; ORF, open reading frame; rRNA, ribosomal ribonucleic acid; tRNA, transfer ribonucleic acid; 12S RNA, small rRNA; 16S RNA, large rRNA; COXI, cytochrome oxidase c subunit 1; COXII, cytochrome oxidase c subunit 2; COXIII, cytochrome oxidase c subunit 3; ND, NADH dehydrogenase subunit; ATP, ATP synthase subunit; CYTB, cytochrome *b*.

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phylogenomic, molecular systematic, population genetic, and evolutionary analyses (Coates et al., 2005; Cameron, 2014; Coates, 2014). These applications of mitogenome sequence data have broadened our knowledge about molecules needed for phylogenetic analysis and for defining intra-ordinal relationships (Timmermans and Vogler, 2012; Cameron, 2014).

The total number of complete insect mitochondrial genomes in the NCBI GenBank (ncbi.nlm.nih.gov/genome/browse) is 967 at the time of this publication, but only nine complete genomes are from the Superfamily Aphidoidea. This collection includes one complete and one partial mitogenome from the genus *Aphis* (ncbi.nlm.nih.gov/genome/browse), where the partial sequence from *A. craccivora* was reconstructed from transcript data and lacks sequence from two PCGs and two rRNAs (ND2, ND5, 16S rRNA and 12S rRNA; Song et al., 2016). Furthermore, the order and orientation of genes could not be determined from this prior transcript-based assembly. Herein full mitogenome sequence data were generated for *A. craccivora* using Illumina sequencing methods, and applied to define haplotype variation among strains collected from California, USA (new world) and Ghana, Africa (old world). These data are important contributions to the collection of mitogenomes for the Aphidoidea which are useful for evolutionary and phylogenetic studies. Furthermore, this study provides information directed to continuing efforts to understand the *A. craccivora* population dynamics and substructure that may contribute to the development of novel management strategies for this species.

2. Material 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 crops. Neither endangered nor protected species were involved in the study.

2.2. Sequencing and assembly

A. craccivora samples from Africa (old world) were collected from the cowpea fields at the Savanna Agricultural Research Institute (Tamale, Ghana) during the main crop season in 2014; Aphid samples from USA (new world) were collected from a 'hotspot' location for this pest at the University of California Kearney Agricultural Research and Extension Center (Parlier, California) during two main crop seasons in 2014 and 2015. All samples were stored in plastic bags with MiniPax® sorbent (Multisorb Technologies; Buffalo, New York) pads prior to DNA extraction. DNA from each individual aphid was extracted by using ArchivePure DNA Tissue kit (5 Prime Gmbh; Hilden Germany). >200 individual aphid DNA samples were pooled into individual old world *Aphis craccivora* (OWAC) and new world *Aphis craccivora* (NWAC) bulk samples. The OWAC and NWAC samples were processed individually at 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 paired end sequencing was performed on an Illumina HiSeq 2500 using a TruSeq Rapid SBS sequencing kit version 1.

All portions of the reads processing and assembly were performed by using the CLC Genomics Workbench v 8.5 (Qiagen Valencia, CA). Fastq formatted Illumina sequence reads data from OWAC and NWAC libraries were trimmed to remove adapter sequences and low quality and shorter reads with Phred quality scores <20 ($q < 20$) and read lengths < 64 nt. The *de novo* assembly of a reference *A. craccivora* mitogenome was generated from trimmed reads data from both OWAC and NWAC libraries using the fast assembly option with the word size (*k*-mer) automatically optimized by the CLC Genomics Workbench algorithms.

Oligonucleotide primers were designed to span four mitochondria genome assembly gaps and scaffolds using the IDT DNA online program with the draft sequence as template. Polymerase chain reaction (PCR) was conducted by using *GoTaq* from Promega (Wisconsin) following the manufacturer's suggested conditions using individual primer pairs (Supplementary Table 1) and bulk OWAC and bulk NWAC samples as templates. The elongation steps for PCR reactions were conducted at 68 °C instead of the usual 72 °C. The resulting PCR products were sequenced at the Roy J. Carver Biotechnology Center, UIUC, after cleaning with Qiagen PCR cleaning kit (Valencia, CA).

2.3. Mitogenome annotation and sequence analyses

Resulting contigs were used to query against *Aphis gossypii* (NC024581) and *Aphis glycines* (KT889380) mitochondria sequences with the blast algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1997) in the CLC Genomics Workbench. Contigs putatively containing mitogenome sequence (*E*-value less than e-100) were annotated further. Open reading frame for each gene on each mitochondrial genome was predicted by the ORF tool (http://web.expasy.org/translate/) and confirmed with the protein BLAST tool on NCBI. All tRNAs were identified by using ARWEN 1.2 (Laslett and Canback, 2008) and tRNAscan-SE 1.21 (Schattner et al., 2005) online tools. Gene boundaries of large and small rRNAs were determined by aligning them with rRNA gene sequences of other Aphidoidea full mitogenome sequences using the ClustalW algorithm in MEGA 6 (Tamura et al., 2013). Nucleotide compositions and codon usage were thereafter calculated using MEGA 7.0.14, and strand asymmetry was determined using the formulae: AT skew = (A − T)/(A + T) and GC skew = (G − C) / (G + C) (Perna and Kocher, 1995).

2.4. Haplotype variation and differentiation between strains

Inter-strain haplotype variation was estimated by alignment of *A. craccivora* OWAC and NWAC mitogenome sequences using MEGA 6.0, and subsequent estimation of the number of variant sites conducted using DiffSeq from the EMBOSS package (Rice et al., 2000). For the estimation of intra-strain haplotype variation, all trimmed reads OWAC and NWAC libraries were mapped to their respective reference mitogenome sequence using default parameters on the CLC Genomics Workbench v 8.5. The position and frequency of substitutions (haplotype variants) were made with at least 15% of reads to call a substitution "real".

2.5. Phylogenetic analysis

Ten complete and two partial mitochondrial genome sequences from aphid species were downloaded from NCBI GenBank, along with *Drosophila melanogaster* as the outgroup. These downloaded sequences and the two *A. craccivora* mitogenomes assembled in this study (OWAC and NWAC) were used in all subsequent phylogenetic analyses. The nucleic acid from the 13 PCGs were concatenated for all mitogenomes, and amino acid translations made using the invertebrate mitochondrial code were subsequently aligned using Clustal W with the default settings in MEGA 6 (Tamura et al., 2013). The Maximum Likelihood analyses were conducted using CLC genomics workbench with node support generated by 1000 bootstrap pseudo-replicates, using the Kimura 2-parameter model with 5 discrete gamma categories for rates among sites and complete deletion of sites with any missing data.

3. Results

3.1. Sequencing and assembly

A total of 159,458,074 paired end Illumina reads (mean length of 170.9 nt) and 223,396,402 paired reads (mean 170.6 nt) were respectively generated from the OWAC and NWAC libraries (NCBI accession number SUB2404790). Raw reads were quality (Phred score 20, one ambiguity allowed) and length (longer than 64 bp) trimmed after

Illumina adapters trimming, resulting 126,082,594 paired reads (mean 156.1 nt) for OWAC, and 183,087,386 paired reads (mean 157.7 nt) for NWAC. BLAST searches against *Aphis gossypii* (NC024581) mitochondrial sequence identified two large overlapping contigs from the combined *A. craccivora* assembly data (E-values = 0). The two large contigs were 13 and 9 kbp, and both containing scaffolds. Aligning these two large contigs from *A. craccivora* against other aphid mitochondrial genome sequences resulted in a single large contig of >15 kb that covered the whole *A. craccivora* mitogenome. PCR with four sets of primers (Supplementary Table 1) successfully amplified across four regions with ambiguous bases (Ns) that resulted from the assembly of paired end Illumina data, and subsequent Sanger sequencing and alignment with the draft mitogenomes resulted in the complete filling of these gaps. After gap filling, complete mitogenomes were submitted to GenBank for the 15,308 bp *A. craccivora* old world (OWAC; accession number: KX447141) and the 15,305 bp new world (NWAC; accession number: KX447142) strains.

3.2. Mitogenome annotation and sequence analyses

The complete annotated *A. craccivora* mitochondrial genomes for OWAC and NWAC encode the expected ancestral set of 37 genes comprised of 13 PCGs, 22 tRNAs and 2 rRNA genes (Table 1). The 13 PCGs span a total of 11,591 bp for both OWAC and NWAC, and account for 75.72 and 75.73% of total *A. craccivora* mitochondrial sequences, respectively. There was no CDS length variation between strains. Nine of the PCGs were on the forward strand (COXI, COXII, ATP8, ATP6, COXIII, ND3, ND6, CYTB and ND2) while the remaining four were on the reverse strand (ND5, ND4, ND3L and ND1; Table 1). The arrangement of the PCGs was consistent with those of other Insecta (Table 1 and Fig. 1). The putative start codons of PCGs used by OWAC are identical to NWAC, all starting with ATN. The termination codon TAA was used in all PCGs in both OWAC and NWAC.

There were 22 tRNAs predicted by the ARWEN 1.2 and tRNAscan-SE for both OWAC and NWAC, with 15 and 7 respectively on the forward and reverse strands (Table 1). Typical clover-leaf like structures were predicted for all *A. craccivora* tRNAs except $tRNA^{ser(gct)}$ (Supplementary Fig. 2A and B). The length of the tRNAs ranged from 62 bp to 75 bp. The boundaries of rRNA genes were verified by sequence alignment with other aphid mitogenomes. There were the two expected $tRNA^{leu}$ genes in *A. craccivora* with TAA and TAG anticodons in respectively OWAC and NWAC. The 1263 bp 16S rRNA (A + T = 85.5%) and 769 bp 12S rRNA (A + T = 84.8%) genes are located on the negative strand and respectively located between $tRNA^{Leu}$ and $tRNA^{Val}$, and between $tRNA^{Val}$ and the control region. No sequence variation was observed between rRNA genes in the two *A. craccivora* strains.

The nucleotide compositions across the two aphids mitogenomes were highly A + T biased (83.7%). DNA AT and GC skew describe the abundance of adenine over thymine and guanine over cytosine on the leading strain, respectively (Lobry, 1996). AT skew is calculated by A = (A − T) / (A + T) and GC skew is defined by C = (C − G) / (C + G). The two aphids mitogenomes were strongly A-skewed (0.073) and C-skewed (−0.259; Supplementary Table 2A and B). The A + T content of PCGs, tRNAs, rRNAs and control regions was >80% with the

Table 1
Annotation and gene organization of the old world *A. craccivora* (OWAC; Ghana, Africa) and new world *A. craccivora* (NWAC; California, USA) mitogenomes.

Gene	Strand	Anti/start/stop codons	OWAC (KX447141)		NWAC (KX447142)	
			Position	Size	Position	Size
COXI	+	ATT/TAA	1–1561	1561	1–1561	1561
tRNA(Leu)	+	taa	1561–1630	70	1561–1630	70
COXII	+	ATA/TAA	1633–2304	672	1633–2304	672
tRNA(Lys)	+	ctt	2306–2380	75	2306–2380	75
tRNA(Asp)	+	gtc	2380–2441	62	2380–2441	62
ATP8	+	ATA/TAA	2451–2600	150	2451–2600	150
ATP6	+	ATT/TAA	2581–3234	654	2581–3234	654
COXIII	+	ATG/TAA	3234–4019	786	3234–4019	786
tRNA(Gly)	+	tcc	4019–4081	63	4019–4081	63
ND3	+	ATT/TAA	4082–4435	354	4082–4435	354
tRNA(Ala)	+	tgc	4436–4499	64	4436–4499	64
tRNA(Arg)	+	tcg	4499–4563	65	4499–4563	65
tRNA(Asn)	+	gtt	4562–4630	69	4562–4630	69
tRNA(Ser)	+	gct	4629–4691	64	4629–4691	63
tRNA(Glu)	+	ttc	4694–4757	64	4694–4757	64
Gap			4758–4936	159	4758–4935	158
tRNA(Phe)	−	gaa	4937–5002	66	4936–5001	66
ND5	−	ATT/TAA	5003–6673	1671	5002–6672	1671
tRNA(His)	−	gtg	6723–6791	71	6723–6791	69
ND4	−	ATG/AAA	6794–8051	1257	6791–8048	1257
ND4L	−	ATG/TAA	8107–8400	294	8104–8397	294
tRNA(Thr)	+	tgt	8399–8460	62	8396–8457	62
tRNA(Pro)	−	tgg	8462–8531	70	8459–8528	70
ND6	+	ATA/TAA	8523–9023	501	8520–9020	501
CYTB	+	ATG/TAA	9027–10,142	1116	9024–10,139	1116
tRNA(Ser)	+	tga	10,149–10,214	66	10,146–10,211	66
ND1	−	ATT/TAA	10,225–11,160	936	10,222–11,157	936
tRNA(Leu)	−	tag	11,161–11,225	65	11,158–11,222	65
16S rRNA	−		11,226–12,488	1263	11,223–12,485	1263
tRNA(Val)	−	tac	12,489–12,550	62	12,486–12,547	62
12S rRNA	−		12,562–13,330	769	12,559–13,327	769
Control region			13,425–13,981	557	13,422–13,978	557
tRNA(Tyr)	+	ata	13,632–13,697	66	13,629–13,694	66
tRNA(Ile)	+	gat	13,976–14,039	64	13,973–14,036	64
tRNA(Gln)	−	ttg	14,036–14,103	68	14,033–14,100	68
tRNA(Met)	+	cat	14,110–14,172	63	14,107–14,169	63
ND2	+	ATT/TAA	14,173–15,150	978	14,170–15,147	978
tRNA(Trp)	+	tca	15,146–15,211	66	15,147–15,212	66
tRNA(Cys)	−	gca	15,202–15,270	69	15,202–15,270	69

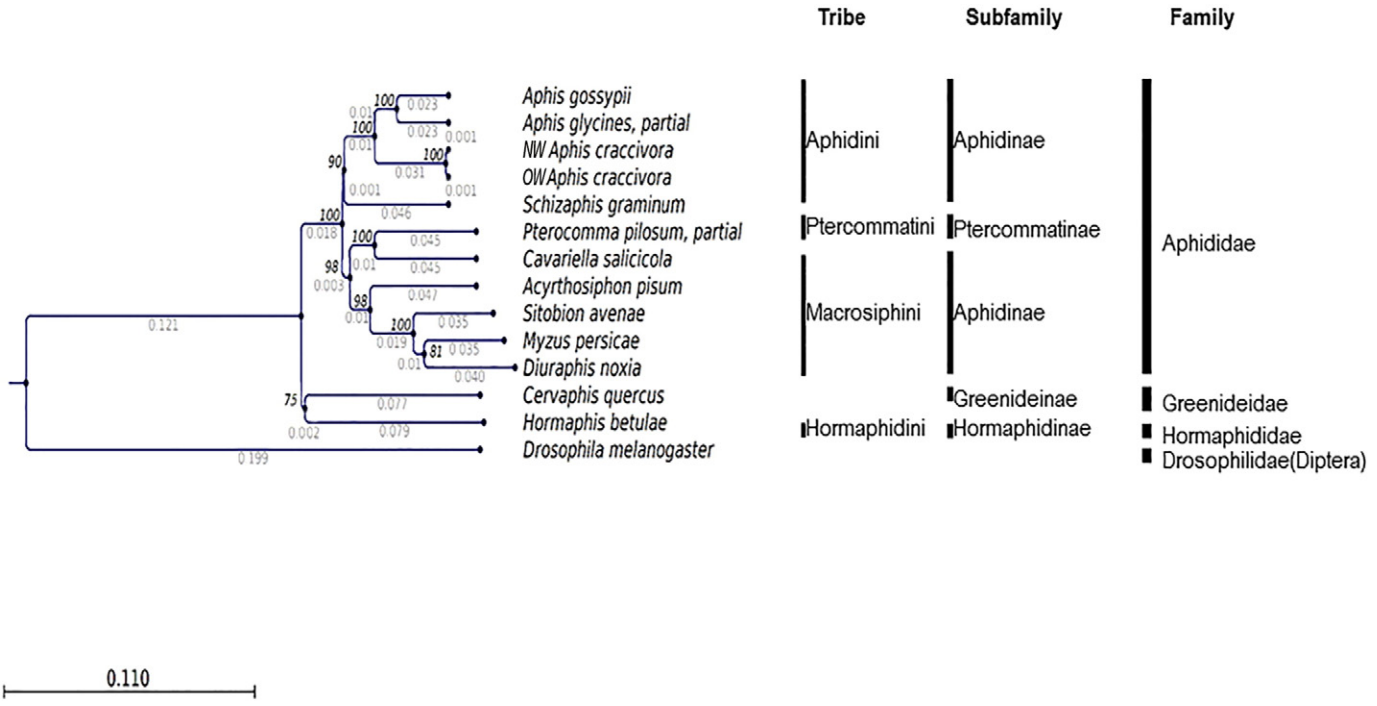


Fig. 1. The maximum likelihood (ML) estimation of phylogenetic relationships among species in the Hemipteran Family Aphididae based on complete or near complete mitochondrial genome sequences. Clustering based upon Tribe, Subfamily and Family are indicated by bars along the right hand side of the phylogenetic tree. *Drosophila melanogaster* was used to root the tree. *Aphis gossypii* (KJ669654); *Aphis glycines* (KC840675); *Schizaphis graminum* (AY531391); *Pterocomma pilosum* (KC840676); *Cavariella salicicola* (KC332935); *Acyrthosiphon pisum* (FJ411411); *Sitobion avenae* (KJ742384); *Myzus persicae* (KU236024); *Diuraphis noxia* (KF636758); *Cervaphis quercus* (KF254841); *Hormaphis betulae* (KT875793); *Drosophila melanogaster* (U37541).

exception of the 2nd codon positions of the PCGs (75%). A significant over representation of nine A + T-rich codons and less frequently used G + C-rich codons was observed in the relative synonymous codon usage (Supplementary Table 3).

3.3. Haplotype variation and differentiation between strains

A total of 33 nucleotide substitutions were predicted between the 15,308 consensus alignment between *A. craccivora* OWAC and NWAC mitogenomes (0.216%). Additional inter-strain variation was observed at three nucleotide deletions found in NWAC at positions 4675 (−/T) and 6741 (−−/AT) relative to OWAC assembly. All of these deletions occurred within the intergenic region.

Variation within *A. craccivora* strains was estimated by mapping trimmed Illumina reads back to their respective mitogenome assembly. Specifically, among the total processed reads from the OWAC library, 696,653 (0.55%) mapped to the assembled OWAC mitogenome sequence assembly, with a mean coverage/sequence depth of 5356 (range from 716 to 74,948; Supplementary Fig. 1A). Analogously, a total of 1,393,064 Illumina reads from the NWAC library (0.76%) were mapped to the NWAC mitochondrial genome assembly (mean coverage/sequence depth was 11,351; range from 1970 to 144,518; Supplementary Fig. 1B). From among the mapped reads a total of 57 and 11 haplotype variants were respectively predicted among NWAC and OWAC reads; 31 and 2 were predicted in PCGs, and 1 and 2 in intergenic spacers of NWAC and OWAC, respectively (Supplementary Table 3). The frequency of haplotype variants at polymorphic sites ranged from 15.3 to 49.3% and 16.0 to 43.5% for NWAC and OWAC, respectively (Supplementary Table 2A and B). Substitutions occurred within COXI, ND5, ND4, 16S and 12S rRNAs with the greatest proportion within ND5. Out of these 31 and 2 substitutions predicted in PCGs, 28 and 2 were nonsynonymous in NWAC and OWAC aphids, respectively.

Table 2A
Predicted haplotype variants among reads for the old world *A. craccivora* mitochondrial DNA.

Reference position	Type	Reference	Allele	Zygosity	Count	Coverage	Frequency	Forward/reverse balance	Average quality	Location	Type of SNPs
6670	SNV	T	A	Heterozygous	1691	4959	34.10	0.29	34.69	ND5	Nonsynonymous
6673	SNV	T	A	Heterozygous	1711	4880	35.06	0.31	34.75	ND5	Nonsynonymous
6677	SNV	T	A	Heterozygous	1939	4963	39.06	0.32	34.77	Intergenic spacer	Nonsynonymous
6687	SNV	T	A	Heterozygous	1915	4406	43.46	0.32	34.40	Intergenic spacer	Nonsynonymous
13,406	SNV	C	A	Heterozygous	3169	10,244	30.93	0.34	37.29		
13,549	SNV	G	T	Heterozygous	1552	9144	16.97	0.47	37.38		
13,553	SNV	A	T	Heterozygous	1526	9037	16.88	0.46	37.22		
13,558	Insertion	–	C	Heterozygous	1363	8507	16.02	0.45	36.58		
13,560	SNV	T	A	Heterozygous	1483	8410	17.63	0.45	36.82		
13,700	Deletion	C	–	Heterozygous	767	2102	36.48	0.44	36.45		
13,703	Insertion	–	T	Heterozygous	367	2222	16.51	0.34	37.93		

3.4. Phylogenetic analysis

The ML-based phylogenetic tree had a log likelihood of −100,148.35 and an estimated gamma shape parameter of 0.56. Furthermore, the tree defined the relationship among the mitogenomes from nine species in the Aphidoidea superfamily of insects, including our two strains of *A. craccivora* (Fig. 1). This analysis placed the two *A. craccivora* strains within the Aphididae, and clustered them in a clade adjacent to *A. glycines*. The ML tree defined Aphidini as a monophyletic group with *Schizaphis graminum* at the base and the most ancestral in this clade comprised of the Aphidinae. Interestingly, *Pterocomma pilosum* was clustered together with *Cavariella salicola* despite being in different Subfamilies. Topology of the remaining portions of the phylogenetic tree supported Greenideidae and Hormaphididae as most basal of the Aphididae. Within the Aphididae the Subfamily Pterocommatinae is most closely related to the Aphidinae, while tribes Aphidini, Pterocommatini, Macrosiphini and Hormaphidini were all monophyletic.

4. Discussion

The assembly of Illumina sequence reads from genomic shotgun libraries has emerged as a rapid method for obtaining completed mitochondrial genome sequences (Knaus et al., 2011; Ma et al., 2011). The technique has been applied to species in the insect Orders Coleoptera (Coates, 2014; Ojo et al., 2016) and Lepidoptera (Coates and Abel, 2016). In the current study we analogously assembled entire mitochondrial genome sequences for two strains of *A. craccivora*, the 15,308 bp OWAC and 15,305 bp NWAC, from NGS data. This size of these *A. craccivora* mitogenomes is congruent with previously sequenced aphid mitogenomes ranging from the lowest in *Sitobion avenae* (15,108 bp; Zhang et al., 2016), *Schizaphis graminum* (15,721; Wang et al., 2014), and *Diuraphis noxia* (15,784 bp; Zhang et al., 2014) to the largest in *Acyrthosiphon pisum* (16,971 bp; Wang et al., 2013). Variation in the total length of insect mitogenomes mostly results from insertion/deletion mutations within intergenic spacers, and various repeat regions including the AT-rich control regions (Arunkumar and Nagaraju, 2006), and includes examples among species of aphids (Wang et al., 2013). Varying degrees of variation in lengths of the control region occur within and between species (Mancini et al., 2008), but remain static between stains of *A. craccivora*. A region of tandem repeats was previously reported between $tRNA^{Glu}$ and $tRNA^{Phe}$ among the aphid species *C. salicicola*, *S. graminum* and *A. pisum* with 2 to 7 complete divergent repeat units depending on species (Wang et al., 2013). This repeat region was also reported in *Aphis gossypii* (KJ669654 from 4747 to 5530). A 178 bp gap was present in the orthologous region of the mitogenome assemblies from both *A. craccivora* strains, but no discernable repeat was identified using Tandem Repeat finder. However, three interspersed $CA(A/T/G)(A)_{2-5}(T)_{3-5}AC[C]T$ sequences were present in this *A. craccivora* mitogenome region, suggesting that a highly cryptic, degraded, or pro-repeat may be present. Further mitogenomes sequences from phylogenetically related aphid species will be necessary in order to discern the evolutionary trajectory of this mitogenome region.

Despite the relatively high average depth of coverage (≥5356-fold), small gaps of ambiguous bases were obtained in the initial *A. craccivora* mitogenome assemblies from paired end Illumina HiSeq data. Subsequent gap filling by PCR amplification and Sanger sequencing of fragments revealed that these gaps were comprised of stretches of 23 to 25 A nts. Such homopolymer stretches are known to be problematic among reads from the Roche 454 systems (Luo et al., 2012) but are not reported for Illumina read data, however the latter technology is prone to errors within inverted repeat and high GC regions (Nakamura et al., 2011). The basis for these gaps at homopolymers remains unknown, but might be related to the highly biased A + T content or the potential ambiguous placement of reads during assembly. This was analogously observed during the assembly of the AT-rich control region of the beetle species, *Diabrotica virgifera virgifera* and *D. barberi* using Illumina HiSeq read data.

The A + T content in *A. craccivora* is high (83.6%) and shows a strong A-skew (0.073), which is typical of insect mitochondrial genomes and might be a result of selection for optimal translation and DNA repairs (Crozier and Crozier, 1993; Xia, 1996). The nine most frequent codons in *A. craccivora* (phenylalanine, leucine, isoleucine, methionine, serine, tyrosine, glutamine, asparagine and lysine) are A + T-rich and also show a preferential presence of A or T nucleotides at the wobble position (Supplementary Table 3, Supplementary Fig. 2A and B). The gene order of PCGs in the two *A. craccivora* mitogenomes was arranged in the same order and orientation as in the ancestral mitogenome (Boore, 1999), that is also observed among other Aphididae (Wang et al., 2013). The initiation codons of the 13 PCGs of *A. craccivora* are mainly ATT, ATA and ATG, which are similarly used in other insect mitogenomes (Margam et al., 2011; Wang et al., 2013). The TAA stop codon is used exclusively in both *A. craccivora* strains, and is also commonly used among other reported aphid mitochondrial genes (Wang et al., 2014).

In contrast to the standard order of PCGs in the *A. craccivora* mitogenomes, the translocation of tRNA genes appears to have occurred through evolutionary time. Specifically, $tRNA^{Tyr}$ has moved from the conserved position between $tRNA^{Cys}$ and COXI in the Drosophilid, *D. yakuba*, and the aphids *C. salicicola*, *S. graminum*, and *A. pisum*, to the AT-rich control region between 12S rRNA and $tRNA^{Ile}$ in *A. craccivora* (Table 1). The same translocation has not been observed in any other aphid mitogenomes (Wang et al., 2013). In contrast, a series of tRNA deletions and novel insertions have occurred throughout aphid mitogenomes evolution. For example, the $tRNA^{Ser}$ between $tRNA^{Asn}$ and $tRNA^{Glu}$ in the ancestral gene order is retained in *C. salicola* and *A. craccivora*, but is deleted in *S. graminum* and *A. pisum*. In contrast, *S. graminum* and *A. pisum* respectively encode duplicate copies of $tRNA^{Gln}$ and $tRNA^{Met}$. Although no additional tRNA copies were observed in *A. craccivora*, the movement, deletion, and translocation of tRNA genes appear relatively prevalent among the Aphididae, but the consequence of these changes on function of the mitochondrion remains unknown.

The monophyly of the tribe Aphidini and family Aphididae predicted in our ML-based analysis is congruent with the results of previous studies into phylogenetics of the Aphididae (Wang et al., 2014; Song et al., 2016). In contrast to these prior studies, our resulting tree showed that the Subfamily Aphidinae was not monophylytic due to the inclusion of Pterocommatinae with high node support, where this was due to the nesting of *Pterocomma pilosum* within the same clade as *Cavariella solicicola* (Fig. 1). An analogous discrepancy showing *Pterocomma* as a sister group of *Cavariella* was obtained previously that based conclusions drawn from reconstructions based on COXII gene sequence data (von Dohlen et al., 2006).

Alignment of *A. craccivora* OWAC and NWAC sequences resulted in a 15,308 bp consensus sequence between which 33 nucleotide substitutions were predicted (0.216% divergence). Based upon a simple assumption of a standard molecular clock of 2% and 1.15% change per million years respectively for *Drosophila* and Lepidoptera (Powell et al., 1986; Brower, 1994), divergence of *A. craccivora* strains may have occurred ~0.108 to 0.188 mya (108,000–188,000 ya). Moreover, among the 31 and 2 substitutions predicted within PCGs between NWAC and OWAC, only 2 in NWAC were non-synonymous (amino acid changes). This observation of nucleotide mutation biased as silent evolutionary neutral sites and subsequent low rate of protein sequence evolution might suggest that strong negative purifying selection may act to cull less fit functional variants. Analogous effects of purifying selection have been shown between the mitogenomes of other closely related insect species (Coates, 2014). The high number of substitutions between *A. craccivora* strains occurred within the ND5 gene, which was also reported in the mitogenome of *Diuraphis noxia* (de Jager et al., 2014) and suggests that there may be an unequal rate of molecular

evolution across the mitochondrial genome sequence within this taxa. Alternatively, genes from the mitogenomes have been transposed (copied) to aphid nuclear genomes (Sunnucks and Hales, 1996), wherein many have been degraded into pseudogenes (International Aphid Genomics C., 2010). This might suggest the sequencing of total genomic shotgun libraries may contain reads derived from transposed and diverged mitochondrial gene sequences, and thus confound results obtained from alignments aimed to estimate intraspecific variation. Evidence for this potential alignment of reads from heterogeneous sources (e.g. mitogenome and nuclear copies) may reside in the relatively high reads coverage regions of the NWAC ND5 gene (Supplementary Table 2B; Supplementary Fig. 1B). Therefore, methods that enrich for the mitochondrion may be best suited for assessing population variation in this mitochondrial genome, and interpretation of the high degree of ND5 sequence variation within *A. craccivora* strains should be exercised with caution in light of the potential alignment of heterogeneous reads.

Agunbiade et al. (2013) and Song et al. (2016) have built an initial transcriptome for *A. craccivora*, in which only a few partial mitochondrial transcripts were identified. The *A. craccivora* mitochondrial sequence reported by (Song et al., 2016; KT889380) contains 14,425 bp, which contains ten strings of Ns ranging from 2 bp to 339 bp. Multiple alignment of three *A. craccivora* mitochondrial genome sequences (Supplementary Fig. 3) showed four gaps ranging from 3 bp to 744 bp in the reported Asian *A. craccivora* mitochondrial sequence. In the coding region, ND2, ND5, 16S rRNA and 12S rRNA were partially reported. The present study provides the first complete mitogenome sequences for *A. craccivora*, and comparisons that estimate variation between the old world (Ghana, Africa) and the new world (California, USA) strains. The predicted haplotypes variations could be used to differentiate female lineages within *A. craccivora* populations, and potentially be applied to predict any temporal or spatial haplotype structuring that could aid in devising methods to control this destructive agricultural pest. For example, we have observed differences between old world and new world *A. craccivora* populations in their ability to infest cowpea genotypes carrying specific resistance genes (Huynh et al., 2015), and associating molecular profiles with important phenotype differences could be informative for guiding cowpea resistance breeding and for field-level pest management decisions.

Table 2B
Predicted haplotype variants among reads for the new world *A. craccivora* mitochondrial DNA.

Reference position	Type	Reference	Allele	Zygosity	Count	Coverage	Frequency	Forward/reverse balance	Average quality	Location	Type
186	SNV	C	T	Heterozygous	1183	6427	18.41	0.41	37.28	Cox1	Synonymous
5607	SNV	T	A	Heterozygous	2224	13,671	16.27	0.17	37.76	ND5	Nonsynonymous
6564	SNV	T	A	Heterozygous	2771	16,131	17.18	0.48	36.86	ND5	Nonsynonymous
6573	SNV	C	A	Heterozygous	3175	19,572	16.22	0.47	37.56	ND5	Nonsynonymous
6575	SNV	T	A	Heterozygous	4108	20,527	20.01	0.47	37.47	ND5	Nonsynonymous
6587	SNV	C	A	Heterozygous	6067	22,776	26.64	0.47	37.62	ND5	Nonsynonymous
6590	SNV	G	A	Heterozygous	4135	24,164	17.11	0.47	37.74	ND5	Nonsynonymous
6590	SNV	G	T	Heterozygous	7654	24,164	31.68	0.44	35.85	ND5	Synonymous
6594	SNV	T	A	Heterozygous	5861	36,019	16.27	0.48	37.30	ND5	Nonsynonymous
6604	SNV	A	T	Heterozygous	7185	42,559	16.88	0.46	35.83	ND5	Nonsynonymous
6607	SNV	C	A	Homozygous	13,218	40,295	32.80	0.48	37.09	ND5	Nonsynonymous
6611	Deletion	T	–	Heterozygous	7173	40,823	17.571	0.48	36.02	ND5	Nonsynonymous
6613	SNV	T	A	Heterozygous	8675	43,118	20.12	0.47	36.94	ND5	Nonsynonymous
6614	MNV	TC	AA	Heterozygous	11,788	43,118	27.34	0.49	37.15	ND5	Nonsynonymous
6615	SNV	C	A	Heterozygous	9337	43,118	21.65	0.47	37.28	ND5	Nonsynonymous
6615	Deletion	C	–	Heterozygous	6609	43,118	15.33	0.48	33.72	ND5	Nonsynonymous
6625	Deletion	T	–	Heterozygous	19,794	61,175	32.36	0.49	36.81	ND5	Nonsynonymous
6628	SNV	T	A	Heterozygous	23,004	61,554	37.37	0.47	36.87	ND5	Nonsynonymous
6630	SNV	T	A	Heterozygous	23,505	61,395	38.28	0.48	36.80	ND5	Nonsynonymous
6633	Deletion	C	–	Homozygous	25,635	64,979	39.45	0.46	33.40	ND5	Nonsynonymous
6642	SNV	T	A	Heterozygous	25,200	81,500	30.92	0.45	36.93	ND5	Nonsynonymous
6646	Deletion	T	–	Heterozygous	20,688	77,899	26.56	0.44	36.82	ND5	Nonsynonymous
6653	Deletion	CC	–	Heterozygous	15,908	84,169	18.90	0.44	33.64	ND5	Nonsynonymous
6653	Replacement	CC	A	Heterozygous	28,668	84,169	34.06	0.46	36.32	ND5	Nonsynonymous
6653	MNV	CC	AA	Heterozygous	17,616	84,169	20.93	0.44	36.77	ND5	Nonsynonymous
6676	SNV	T	A	Heterozygous	20,690	109,315	18.93	0.43	36.27	ND5	Nonsynonymous
6708	SNV	A	T	Heterozygous	15,306	92,223	16. 60	0.43	35.72	ND5	Nonsynonymous
6717	SNV	C	A	Homozygous	7230	33,542	21.56	0.34	37.34	Intergenic spacer	Nonsynonymous
6726	SNV	T	A	Heterozygous	5149	24,628	20.91	0.31	37.70	ND4	Nonsynonymous
11,964	SNV	T	A	Heterozygous	3541	19,530	18.13	0.48	37.19	16S RNA	Nonsynonymous
12,002	SNV	C	A	Heterozygous	4558	17,379	26.23	0.48	37.48	16S RNA	Nonsynonymous
13,607	SNV	G	T	Heterozygous	705	4473	15.76	0.44	36.59	12S RNA	Synonymous
13,639	SNV	A	T	Heterozygous	4530	13,752	32.94	0.47	37.23		
13,642	SNV	G	A	Heterozygous	3536	13,490	26.21	0.48	37.09		
13,642	Deletion	G	–	Heterozygous	2340	13,490	17.35	0.44	34.98		
13,647	SNV	G	T	Heterozygous	5297	13,884	38.15	0.45	37.20		
13,659	SNV	C	A	Heterozygous	2561	16,472	15.55	0.40	36. 90		
13,659	SNV	C	T	Heterozygous	3384	16,472	20.54	0.37	37.24		
13,659	Deletion	C	–	Heterozygous	4476	16,472	27.17	0.41	36.16		
13,697	Deletion	C	–	Heterozygous	18,605	37,747	49.29	0.41	36.51		
13,700	MNV	AT	TA	Homozygous	7833	38,167	20.52	0.45	37.76		
13,717	SNV	G	T	Heterozygous	9462	19,898	47.55	0.40	37.53		
13,720	SNV	G	A	Heterozygous	7736	19,233	40.22	0.40	37.38		
13,726	SNV	T	A	Heterozygous	5748	18,250	31. 50	0.42	37.40		
13,751	SNV	C	T	Heterozygous	1473	7140	20.63	0.49	36.88		
13,761	SNV	C	A	Heterozygous	608	3936	15.45	0.46	36.80		

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