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# Comparative analysis of the complete mitochondrial genomes of three *Zeugodacus* species (Insecta: Tephritidae: Dacinae) and their phylogenetic relationships with other congeners

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**Received** 16 April 2023
**Accepted** 24 July 2023
**Published** 8 September 2023

**Academic Editors** Marianna Simões, Monika Eberhard

**Citation:** Yong H-S, Song S-L, Chua K-O, Liew YJM, Suana IW, Lim P-E, Chan K-G, Eamsobhana P (2023) Comparative analysis of the complete mitochondrial genomes of three *Zeugodacus* species (Insecta: Tephritidae: Dacinae) and their phylogenetic relationships with other congeners. Arthropod Systematics & Phylogeny 81: 747–759. https://doi.org/10.3897/asp.81.e105025

## Abstract

The complete mitogenomes of fruit flies *Zeugodacus* (*Javadacus*) *calumniatus*, *Z.* (*Javadacus*) *heinrichi* and *Z.* (*Sinodacus*) *hochii* have similar gene order and contain 37 genes and a non-coding region. They share an identical start codon for the respective protein-coding genes (PCGs), an identical TAA stop codon for 11 PCGs, TAG for *cob*, and an incomplete T stop codon for *nad5*. The cloverleaf structure of most of the tRNAs is similar in the three *Zeugodacus* species. Phylogenetic analyses reveal *Z.* (*Parasinodacus*) *cilifer* to be external to two main clades: (A) monophyletic subgenus *Zeugodacus*; and (B) subgenera *Javadacus* and *Sinodacus*. The present results indicate that the taxonomic status of some taxa needs clarification. *Z. calumniatus* is genetically very similar to *Z. tau* and is not congruent with its current placement in the *munda* complex. *Z. mukiae* NC_067083 is genetically very similar to *Z. scutellaris*, but differs significantly from *Z. mukiae* MG683384 of the *arisanicus* (*arisanica*) complex. On the other hand, *Z. propredíaphorus* is genetically distinct from and not a synonym of *Z. diaphorus*. *Z. caudatus* sensu stricto from Indonesia forms a sister lineage with *Z. diversus*, instead of with the Malaysian and Chinese taxa of *Z. caudatus* sensu lato. A notable incongruence is the sister lineage of *Z.* (*Sinodacus*) *hochii* and *Z.* (*Javadacus*) *heinrichi* among other taxa of subgenus *Javadacus*. A more extensive taxon sampling, particularly the subgenus *Sinodacus* (and other subgenera), is needed to clarify/resolve their subgenus status.

## Keywords

Fruit fly, mitogenomics, phylogeny, systematics, *Zeugodacus* subgenera

# 1. Introduction

The fruit fly genus *Zeugodacus* Hendel, 1927 (considered previously, and still by some researchers, as a subgenus of genus *Bactrocera* Macquart, 1835) consists of 13 subgenera with some 200 species worldwide (Hancock and Drew 2018a, 2018b). The larvae of many *Zeugodacus* species have cucurbits as host plants. Some 17 species have been listed as pest of cucurbits: 11 species of fruit pest and six species of flower pest (Doorenweerd et al. 2018). *Zeugodacus cucurbitae* (Coquillett, 1899), a fruit pest, is the economically most important species. Based on a global checklist, 195 species are found in the Asia-Pacific and one species in Africa (Doorenweerd et al. 2018). New species are, however, continuously being discovered (Yong et al. 2015a; Kunprom and Pramual 2019; Prabhakar et al. 2019; Leblanc et al. 2019; Drew and Romig 2022).

To date, the subgenus names of genus *Zeugodacus* have not been applied consistently, for example, *Z. cucurbitae* has been treated as a member of subgenus *Javadacus* (Hancock and Drew, 2018b; Leblanc, 2022; Starkie et al., 2022) and subgenus *Zeugodacus* (San Jose et al. 2018; Zhang et al. 2023), *Z. tau* as subgenus *Javadacus* (Hancock and Drew 2018b; Starkie et al. 2022) and subgenus *Zeugodacus* (San Jose et al. 2018; Zhang et al. 2023), and *Z. triangularis* as subgenus *Sinodacus* (Starkie et al. 2022; Zhang et al. 2023) and subgenus *Zeugodacus* (Hancock and Drew 2018b).

Based on molecular phylogenetic analysis, some of the subgenera (as applied by the researchers) within the genus *Zeugodacus* are recovered as polyphyletic or paraphyletic (San Jose et al. 2018; Starkie et al. 2022; Zhang et al. 2023). However, the assignments of some *Zeugodacus* species to subgenera are subjected to emendation (Hancock and Drew 2018a, 2018b). Hancock and Drew (2018b) opined that analysing small groups of subgenera separately would enable fine-tuning of the subgeneric limits established so far.

Mitochondrial genomes (mitogenomes) of insects have been extensively studied and applied particularly to studies regarding phylogeny and evolution (Cameron 2014). They have been shown to be suitable for resolving higher-level phylogeny of Paraneopteran insects (Li et al. 2015). Compared to partial sequences of single or multiple mitochondrial and nuclear genes (Yong et al. 2015a; San Jose et al. 2018; Prabhakar et al. 2019; Starkie et al. 2022), there are relatively few studies of the genus *Zeugodacus* based on complete mitogenomes. To date, the mitogenomes of some 14 species of the genus *Zeugodacus* (not including the unnamed cryptic species such as in *Z. caudatus* species complex) are available in the NCBI GenBank. Of these, four species are fruit pests, five species are flower pests, and five species are non-pest. Furthermore, fewer *Zeugodacus* mitogenomes have been reported compared to genus *Bactrocera* (Yong et al. 2021; Zhang et al. 2023).

In view of the lack of mitogenomic studies in the genus *Zeugodacus* and the unresolved systematic status of some taxa, we sequenced and annotated the complete mitogenomes of *Z.* (*Javadacus*) *calumniatus* (Hardy 1970), *Z.* (*Javadacus*) *heinrichi* (Hering, 1941) and *Z.* (*Sinodacus*) *hochii* (Zia, 1936) to determine their genomic features, and phylogenetic relationships with other congeners. *Z. calumniatus* and *Z. heinrichi* are non pest, while *Z. hochii* is a Cucurbitaceae fruit pest (Doorenweerd et al. 2018). At the time of this study (sequencing performed in September 2018), there were no reports on the mitogenomes of these three species. The present study is still the first report on the mitogenomes of *Z. calumniatus* and *Z. heinrichi*. These whole mitogenomes will serve as a useful dataset for studying the genetics, systematics and phylogeny of the *Zeugodacus* genus and subgenera in particular, and tephritid fruit flies in general.

# 2. Materials and methods

## 2.1. Sample collection and mitochondrial DNA extraction

The male fruit flies of *Z. calumniatus* and *Z. heinrichi* were collected by H-S Yong and IW Suana on the way to Rinjani, Lombok, Indonesia (8°33′54.00″S, 116°21′3.60″E) on 6 November 2015; *Z. hochii* was collected by H-S Yong in the garden of the Institute of Biological Sciences, Universiti Malaya, Kuala Lumpur, Malaysia (3°07′9.00″N, 101°39′13.79″E) on 29 October 2011. They were collected by means of cue-lure, preserved in absolute ethanol and stored in a –20 °C freezer until used for DNA extraction. The specimens were identified according to existing literature (Drew and Romig 2013, 2016), and verified with published *cox1* sequences in GenBank. The isolation of mitochondria and the extraction of mitochondrial DNA (mtDNA) were carried out according to the method of Yong et al. (2015b, 2016a). No permits are needed to study these fruit flies; they are not endangered or protected by law.

## 2.2. Library preparation and genome sequencing

The methods described by Yong et al. (2016b) and Song et al. (2018) were used for sample and library preparation (using Nextera DNA Sample Preparation Kit), and genome sequencing using the Illumina MiSeq Desktop Sequencer (2 × 150 bp pair-end reads) (Illumina, USA). The mitogenome sequences have been deposited in the GenBank under the accession numbers: *Z. calumniatus* OQ730413; *Z. heinrichi* OQ730414; and *Z. hochii* OQ730415.

## 2.3. Mitogenome analysis

Analysis of mitogenome, gene annotation, visualization and comparative analysis are detailed in Yong et al.

(2021). Gene annotation of the assembled mitogenome was first carried out at MITOS web-server (http://mitos.bioinf.uni-leipzig.de/index.py) (Bernt et al. 2013). The nucleotide composition, amino acid frequency and relative synonymous codon usage (RSCU) were determined using MEGA X (Kumar et al. 2018). DnaSP 6 (Rozas et al. 2017) was used to estimate the ratios of non-synonymous substitutions (Ka) and synonymous substitutions (Ks) for the PCGs. The AT and GC skewness were determined according to Perna and Kocher (1995). Palindromes (inverted repeats) in the control region were checked with Tandem Repeats Finder (Benson 1999). Blast ring image generator (BRIG) (Alikhan et al. 2011) was used to create the circular map of the mitogenomes. Transfer RNA (tRNA) genes were identified by MITOS web-server (Bernt et al. 2013).

### 2.4. Mitogenomes from GenBank and phylogenetic analysis

The mitogenomes of *Zeugodacus* taxa available from GenBank (Table S1: subgenera based on Hancock and Drew 2018a, 2018b) were used for phylogenetic comparison. Two species of genus *Ceratitis* (*C. fasciventris* NC_035497 and *C. rosa* NC_053847) were used as outgroup taxa.

Alignment of nucleotide sequences and reconstruction of phylograms followed those described in Yong et al. (2015a, 2015b, 2016a, 2016b) and Song et al. (2018). Briefly, the gene sequences were aligned by MAFFT version 7 (Katoh and Standley 2013) and subsequently edited and trimmed using BioEdit v.7.0.5.3 (Hall 1999). Kakusan v.3 (Tanabe 2007) was used to determine the best-fit nucleotide substitution models for maximum likelihood (ML) analysis selected using the corrected Akaike Information Criterion (Akaike 1973).

Phylograms of 13 concatenated PCGs, and 15 mt-genes (13 PCGs and 2 rRNA genes) were reconstructed using TreeFinder (Jobb et al. 2004). Bootstrap values (BP) were generated via 1000 ML bootstrap replicates. Bayesian analyses were conducted using the Markov chain Monte Carlo (MCMC) method via Mr. Bayes v.3.1.2 (Huelsenbeck and Ronquist 2001), with two independent runs of $2\times10^6$ generations with four chains, and with trees sampled every 200$^{th}$ generation. Likelihood values for all post-analysis trees and parameters were evaluated for convergence and burn-in using the "sump" command in MrBayes and the computer program Tracer v.1.5 (http://tree.bio.ed.ac.uk/software/tracer/). The first 200 trees from each run were discarded as burn-in (where the likelihood values were stabilized prior to the burn-in), and the remaining trees were used for the construction of a 50% majority-rule consensus tree. Phylogenetic trees were viewed and edited by FigTree v.1.4 (Rambaut 2012). Uncorrected pairwise (*p*) genetic distances were estimated using PAUPb10 software (Swofford 2002).

A ML/BI phylogenetic tree based on the partial *cox1* sequences of selected closely related *Zeugodacus* taxa, with *Dacus* species as outgroup taxa, was reconstructed to elucidate their phylogenetic relationship.

## 3. Results

### 3.1. Mitogenome features

The mitogenomes of *Z. calumniatus*, *Z. heinrichi* and *Z. hochii* had similar gene order and contained 37 genes (13 protein-coding genes – PCGs, 2 rRNA genes, and 22 tRNA genes) and a non-coding region (A + T-rich control region) (Table 1; Fig. 1). The three whole mitogenomes were AT-rich, ranging from 71.1% (*Z. hochii*) to 73.5% (*Z. calumniatus*), with positive AT and negative GC skewness values (Table S2).

All three *Zeugodacus* species had 15 intergenic regions and overlaps in 12 regions (Table 1). The longest spacing sequence (34 bp in *Z. calumniatus*, 38 bp in *Z. heinrichi*, 35 bp in *Z. hochii*) was between *trnR* and *trnN* genes. This sequence had clear stem-loop structures (Fig. S1). The longest overlap in all three species was 65 bp between the *trnS2* and *nad1* genes.

**Table 1.** Gene order and organization of the mitochondrial genome of *Zeugodacus calumniatus* (Zca), *Z. heinrichi* (Zhe) and *Z. hochii* (Zho). *Minus (–) sign indicates overlap.

| Gene | Strand | Size (bp) Zca/Zhe/Zho | Intergenic sequence* Zca/Zhe/Zho | Start codon Zca/Zhe/Zho | Stop codon Zca/Zhe/Zho |
|---|---|---|---|---|---|
| *trnI*(atc) | J | 66/66/66 | –3/–3/–3 | | |
| *trn*Q(caa) | N | 69/69/69 | 8/10/8 | | |
| *trn*M(atg) | J | 69/69/69 | 0/0/0 | | |
| *nad2* | J | 1023/1023/1023 | 9/9/10 | ATA/ATT/ATT | TAA/TAA/TAA |
| *trnW*(tga) | J | 68/68/68 | –8/–8/–8 | | |
| *trnC*(tgc) | N | 63/63/63 | 1/0/1 | | |
| *trnY*(tac) | N | 67/67/67 | –2/–2/–2 | | |
| *cox1* | J | 1539/1539/1539 | –5/–5/–5 | TCG/TCG/TCG | TAA/TAA/TAA |
| *trnL2*(tta) | J | 66/66/66 | 4/4/4 | | |
| *cox2* | J | 690/690/690 | 5/5/5 | ATG/ATG/ATG | TAA/TAA/TAA |
| *trnK*(aag) | J | 71/71/71 | 0/1/0 | | |
| *trnD*(gac) | J | 67/67/68 | 0/0/0 | | |

| Gene | Strand | Size (bp) Zca/Zhe/Zho | Intergenic sequence* Zca/Zhe/Zho | Start codon Zca/Zhe/Zho | Stop codon Zca/Zhe/Zho |
|---|---|---|---|---|---|
| *atp8* | J | 162/162/162 | –7/–7/–7 | ATT/ATT/ATT | TAA/TAA/TAA |
| *atp6* | J | 678/678/678 | –1/–1/4 | ATG/ATG/ATG | TAA/TAA/TAA |
| *cox3* | J | 789/789/789 | 6/6/6 | ATG/ATG/ATG | TAA/TAA/TAA |
| *trnG*(gga) | J | 65/65/65 | –3/–3/–3 | | |
| *nad3* | J | 357/357/357 | 4/4/3 | ATA/ATA/ATA | TAA/TAA/TAA |
| *trnA*(gca) | J | 66/66/66 | 4/4/4 | | |
| *trnR*(cga) | J | 64/64/67 | 34/38/35 | | |
| *trnN*(aac) | J | 65/65/65 | 0/0/0 | | |
| *trnS1*(agc) | J | 68/68/68 | 0/0/0 | | |
| *trnE*(gaa) | J | 68/68/68 | 18/18/18 | | |
| *trnF*(ttc) | N | 66/66/66 | 0/0/0 | | |
| *nad5* | N | 1720/1720/1720 | 15/15/15 | ATT/ATT/ATT | T--/T--/T-- |
| *trnH*(cac) | N | 65/66/65 | 3/3/3 | | |
| *nad4* | N | 1341/1341/1341 | –7/–7/–7 | ATG/ATG/ATG | TAA/TAA/TAA |
| *nad4L* | N | 297/297/297 | 2/2/2 | ATG/ATG/ATG | TAA/TAA/TAA |
| *trnT*(aca) | J | 65/65/65 | 0/0/0 | | |
| *trnP*(cca) | N | 66/66/66 | 2/2/2 | | |
| *nad6* | J | 525/525/525 | –1/–1/–1 | ATT/ATT/ATT | TAA/TAA/TAA |
| *cob* | J | 1137/1137/1137 | –2/–2/–2 | ATG/ATG/ATG | TAG/TAG/TAG |
| *trnS2*(tca) | J | 67/67/67 | –65/–65/–65 | | |
| *nad1* | N | 1020/1020/1020 | 10/10/10 | ATA/ATA/ATA | TAA/TAA/TAA |
| *trnL1*(cta) | N | 65/65/65 | 0/0/–1 | | |
| *rrnL* | N | 1327/1328/1329 | 0/0/0 | | |
| *trnV*(gta) | N | 72/72/72 | –1/–1/–1 | | |
| *rrnS* | N | 793/793/793 | 0/0/0 | | |
| Control region | J | 946/943/945 | | | |

## 3.2. Protein coding genes and codon usage

The A + T content for the 13 PCGs of the three *Zeugodacus* mitogenomes ranged from 68.6% (*Z. hochii*) to 71.5% (*Z. calumniatus*), with negative AT and GC skewness values (Table S2). The 1st codon position had positive GC skewness values, while the 2nd and 3rd codon positions had negative GC skewness values.

For the individual PCGs, the A+T content ranged from 65.0% for *cox3* to 80.4% for *nad4L* in *Z. calumniatus*, 65.3% for *cox3* to 77.8% for *nad4L* and *nad6* in *Z. heinrichi*, and 63.1% for *cox3* to 77.4% for *nad4L* in *Z. hochii* (Table S3). All the PCGs had negative AT skewness values (Table S3); *nad1*, *nad4*, *nad4L* and *nad5* had negative GC skewness values, the other nine PCGs had positive GC skewness values.

*Zeugodacus calumniatus*, *Z. heinrichi* and *Z. hochii* shared an identical start codon for the respective PCGs (Table 1). The most common start codon was ATG (in 6 PCGs – *cox2*, *atp6*, *cox3*, *nad4*, *nad4L*, *cob*), followed by three ATA (*nad2*, *nad3*, *nad1*), three ATT (*atp8*, *nad5*, *nad6*), and one TCG (*cox1*). The three species had an identical TAA stop codon for 11 PCGs (*nad2*, *cox1*, *cox2*, *atp8*, *atp6*, *cox3*, *nad3*, *nad4*, *nad4L*, *nad6*, *nad1*), one PCG had TAG (*cob*), and one PCG (*nad5*) had an incomplete T stop codon (Table 1).

The frequency of individual amino acids varied among the congeners of *Zeugodacus* (Fig. 2). However, the most frequently utilized codons were highly similar in these mitogenomes. The frequency of individual amino acids was very similar in the three congeners. The predominant amino acids (with frequency above 200) in all the three mitogenomes were glycine, isoleucine, leucine2, phenylalanine, serine2, and valine (Table S4). Cysteine had the lowest frequency of 44 in *Z. calumniatus* and 45 in *Z. heinrichi* and *Z. hochii*.

Analysis of the relative synonymous codon usage (RSCU) revealed that there was no biased usage of A/T than G/C at the third codon position (Table S5; Fig. 2). The frequency of each codon was similar across the three *Zeugodacus* mitogenomes. The most commonly used codon was UUA encoding for leucine2, and the least commonly used codon was AGG encoding for serine1 (Table S5; Fig. 2).

The Ka/Ks ratio (an indicator of selective pressure on a PCG) was less than 1 for all the 13 PCGs in the three *Zeugodacus* mitogenomes, indicating purifying selection (Table S6; Fig. 3). The *cox1* gene had the lowest ratio (Ka/Ks = 0.006) for *Z. calumniatus* and *Z. heinrichi*, and the third lowest for *Z. calumniatus* and *Z. hochii* (Ka/Ks = 0.013) as well as *Z. heinrichi* and *Z. hochii* (Ka/Ks = 0.017).

## 3.3. Ribosomal RNA genes and transfer RNA genes

Of the two rRNA genes in the three *Zeugodacus* mitogenomes, *rrnS* (793 bp in all three mitogenomes) was much shorter than *rrnL* (1327 to 1329 bp) (Table 1). They were AT-rich, ranging from 76.6% (*Z. hochii*) to 77.8% (*Z. calumniatus*), with positive AT skewness and negative GC skewness values (Table S2).

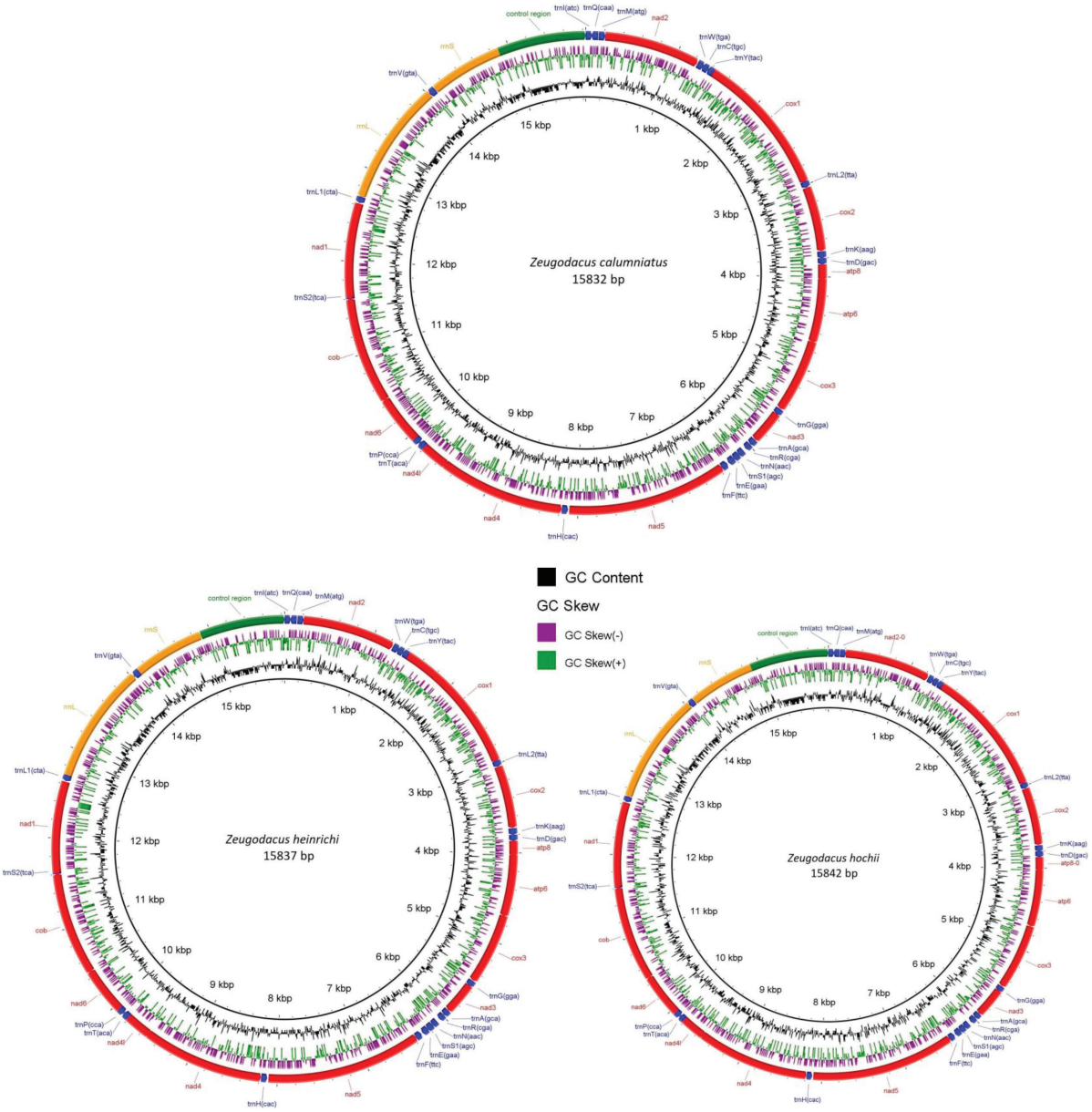


**Figure 1.** Complete mitogenomes of *Zeugodacus calumniatus*, *Z. heinrichi* and *Z. hochii*, with BRIG visualization showing the protein-coding genes, rRNA genes and tRNA genes. GC skew is shown on the outer surface of the ring whereas GC content is shown on the inner surface. The anticodon of each tRNA gene is shown in parentheses.

The tRNA genes were AT-rich, ranging from 74.3% (*Z. hochii*) to 74.8% (*Z. calumniatus*), with negative AT skewness and positive GC skewness values (Table S2). The cloverleaf structure of most of the tRNAs was similar in the three *Zeugodacus* species (Fig. 4). They lacked the DHU loop for serine S1 (*trnS1*), and had short DHU stem (3 bp) for asparagine, isoleucine, leucine L1, leucine L2, lysine, and tyrosine. Phenylalanine (*trnF*) in *Z. calumniatus* and *Z. heinrichi* lacked the TΨC loop. The discriminator base in lysine was A for *Z. hochii*, but G for *Z. calumniatus* and *Z. heinrichi*.

## 3.4. Control region

The control region of the three mitogenomes was AT-rich, ranging from 83.0% (*Z. hochii*) to 85.3% (*Z. calumniatus*), with positive AT skewness and negative GC skewness values (Table S2). It was flanked by *rrnS* and *trnI* genes respectively, with 946 bp in *Z. calumniatus*, 943 bp in *Z. heinrichi* and 945 bp in *Z. hochii*. A long poly-A stretch was present in the same posterior region of the three mitogenomes – 21 bp in *Z. calumniatus* and *Z. hochii*, and 23 bp in *Z. heinrichi*. There was a long poly-T stretch in the same middle region – 18 bp in *Z. caluminatus*, and 19 bp in *Z. heinrichi* and *Z. hochii*.

The simple tandem repeats in the control region common to the three mitogenomes were: $(ATT)_2$, $(TAA)_2$, $(TAT)_2$, $(TTAAA)_2$, $(TTAA)_3$, $(TA)_3$, and $(TA)_6$. In addition, there were repeats present only in a single mitogenome as well as in two of the three mitogenomes. Some nucleotide motifs in one or more mitogenomes were simple tandem repeats as well as palindromes – ATAATA,

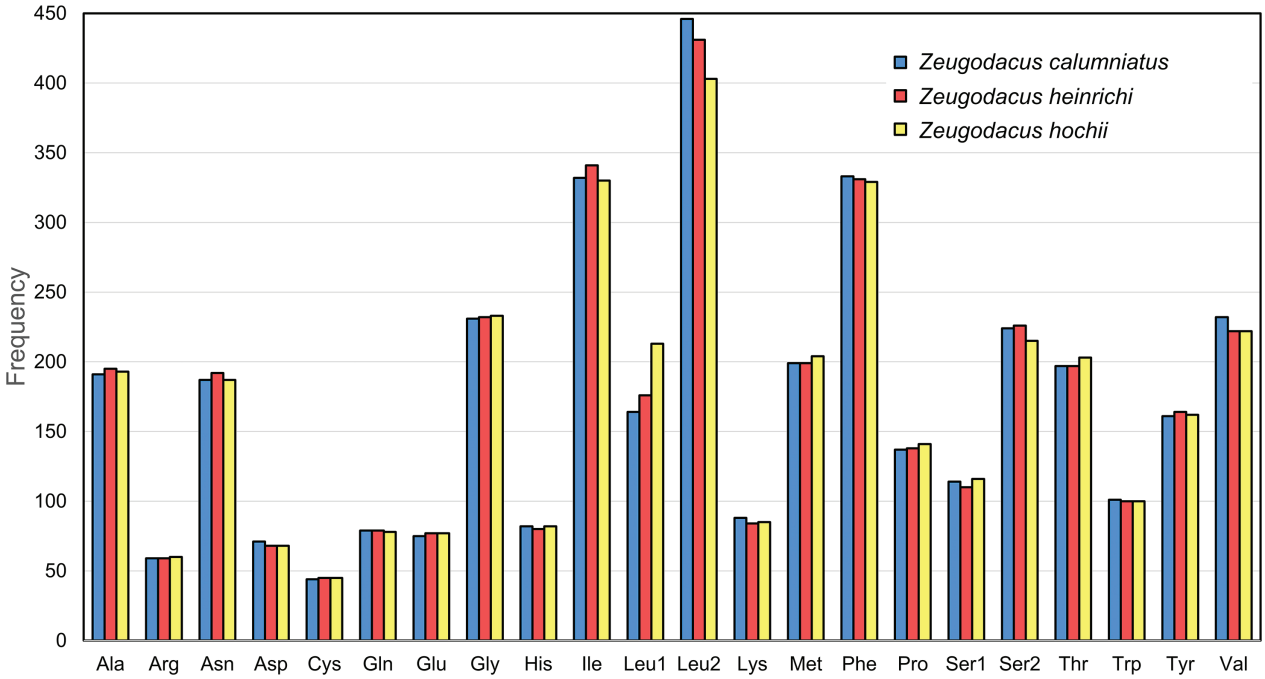


**Figure 2.** Amino acid frequency (Top) and relative synonymous codon usage (Bottom) of PCGs in the *Zeugodacus* mitogenomes generated using MEGA X (https://www.megasofware.net/). Zca, *Zeugodacus calumniatus*; Zhe, *Zeugodacus heinrichi*; Zho, *Zeugodacus hochii*.

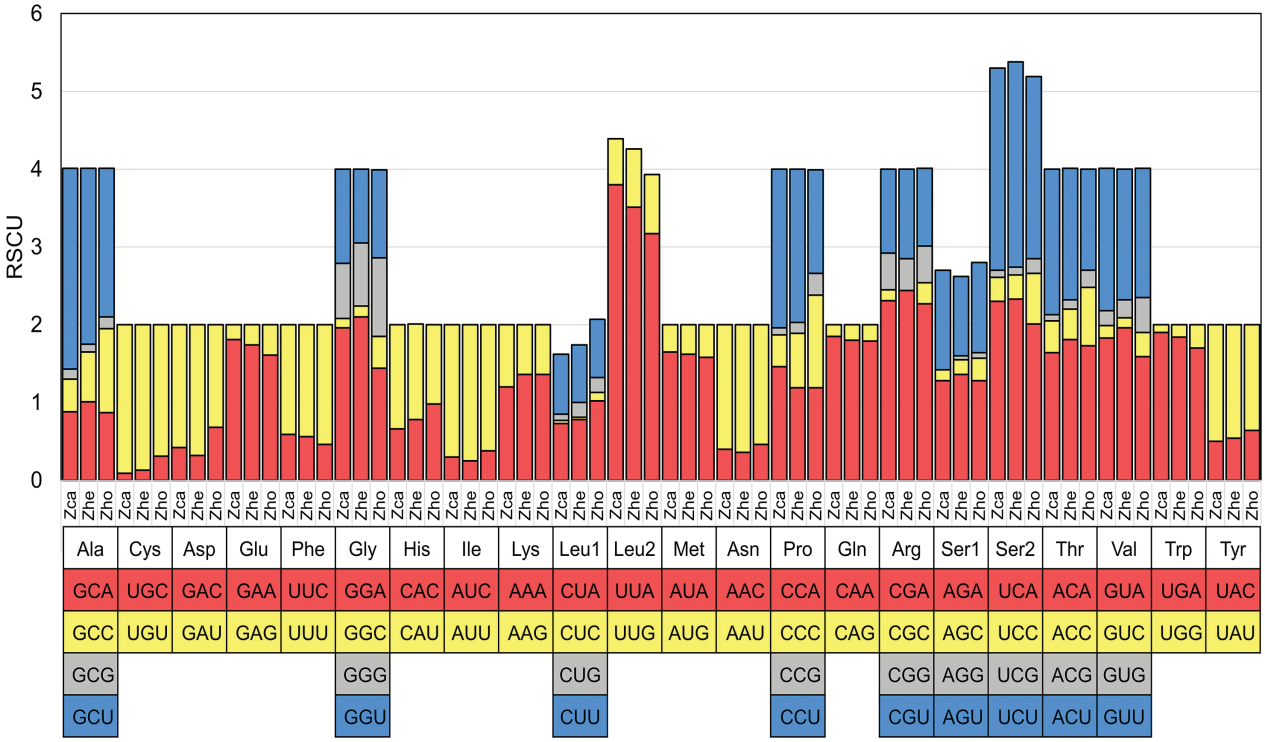


**Figure 3.** Box plot for pairwise divergence of Ka/Ks ratio (mean ± SD, and range) for 13 protein-coding genes (PCGs) of three *Zeugodacus* mitogenomes (*Z. calumniatus*, *Z. heinrichi*, *Z. hochii*) generated using DnaSP6.0. (http://www.ub.edu/dnasp).

TATTAT, ATTAATTA, AATAAAATAA, TAATTAAT, and AATAAAATAAAATAA.

Two palindromes in the control region were common to the three mitogenomes – TAAAAT (n = 5 in *Z. calumniatus* and *Z. heinrichi*, n = 6 in *Z. hochii*); and TTAATT (n = 4 in *Z. calumniatus*, n = 1 in *Z. heinrichi*, n = 3 in *Z. hochii*). Three palindromes (AATTAA, ATTTTA, GATTAG) were common to *Z. calumniatus* and *Z. heinrichi*, while two (AATTTTAA, ATTAATTA) were common to *Z. heinrichi* and *Z. hochii*. The palindromes present only in one mitogenome were: TTAAAATT and AAAATTTTAAAA in *Z. calumniatus*, TTTAATTT in *Z. heinrichi*, and AATTAA, ATAAAATA and CGGGGC in *Z. hochii*.

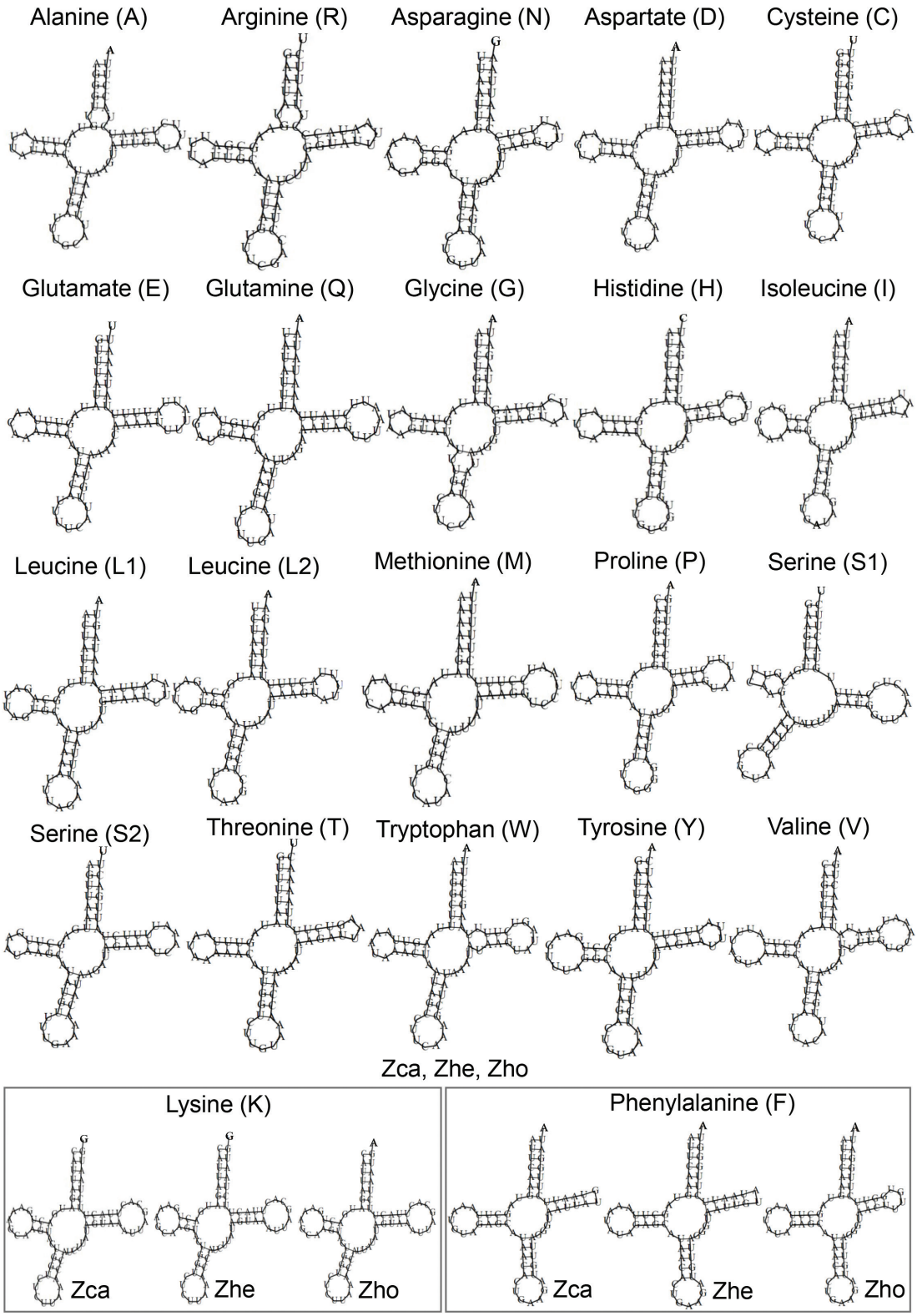


**Figure 4.** Cloverleaf structure of the 22 inferred tRNAs in the mitogenomes of *Zeugodacus calumniatus* (Zca), *Z. heinrichi* (Zhe) and *Z. hochii* (Zho).

## 3.5. Phylogenetic analysis/ relationship

The phylogenetic trees based on 13 PCGs and 15 mt-genes (13 PCGs and 2 rRNA genes) revealed identical topology with very good nodal support based on ML and BI methods (Fig. 5). *Z.* (*Parasinodacus*) *cilifer* was external to two main clades: (1) Clade A comprising taxa of subgenus *Zeugodacus* (as defined by Hancock and Drew 2018a, 2018b) – *Z. diaphorus*, *Z. propredipho-rus*, *Z. scutellaris*, *Z. mukiae*, *Z. scutellatus*, *Z. cauda-tus* species complex, *Z. diversus*, *Z. triangularis*, and *Z. strigifinis* [*Z. mukiae* NC_067083 might be a misidentified taxon – see Discussion]; and (2) Clade B containing other

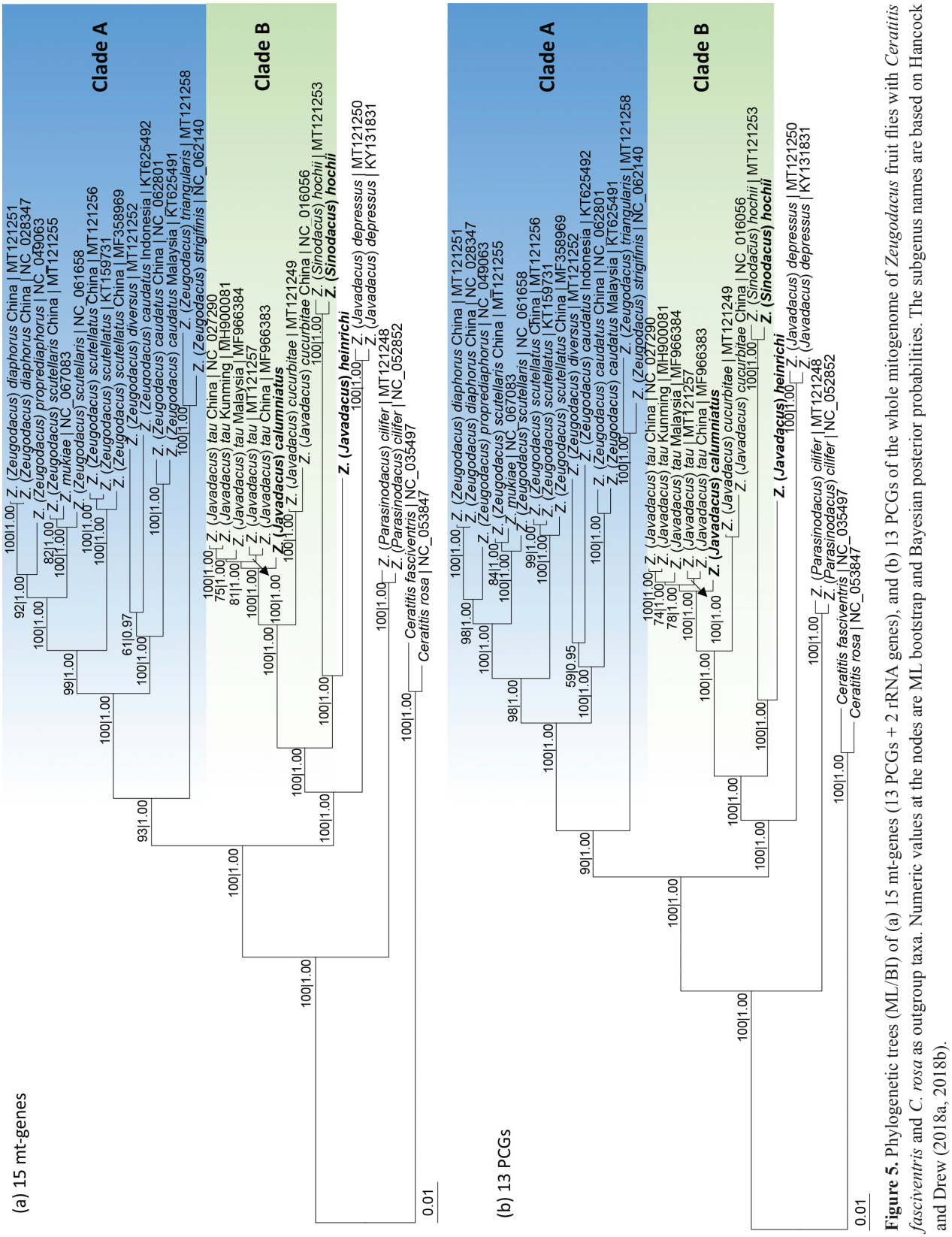


**Figure 5.** Phylogenetic trees (ML/BI) of (a) 15 mt-genes (13 PCGs + 2 rRNA genes), and (b) 13 PCGs of the whole mitogenome of *Zeugodacus* fruit flies with *Ceratitis fasciventris* and *C. rosa* as outgroup taxa. Numeric values at the nodes are ML bootstrap and Bayesian posterior probabilities. The subgenus names are based on Hancock and Drew (2018a, 2018b).

subgenera (*Javadacus* and *Sinodacus*) – *Z. calumniatus*, *Z. tau*, *Z. cucurbitae*, *Z. heinrichi*, *Z. hochii*, and *Z. depressus*.

The sister lineage of *Z. triangularis* and *Z. strigifinis* was external to the other taxa of subgenus *Zeugodacus* in Clade A. Of the other taxa of subgenus *Zeugodacus*, *Z. mukiae* NC_067083 formed a sister lineage with one of the two *Z. scutellaris* taxa (Fig. 5). The genetic distance (based on 15 mt-genes) between *Z. mukiae* and *Z. scutellaris* was $p$ = 0.4% and 0.9%, and the distance between the two *Z. scutellaris* taxa was $p$ = 0.7% (Table S7). In addition, *Z. diversus* formed a sister lineage with *Z. caudatus* Indonesia in a subclade containing *Z. caudatus* Malaysia and *Z. caudatus* China.

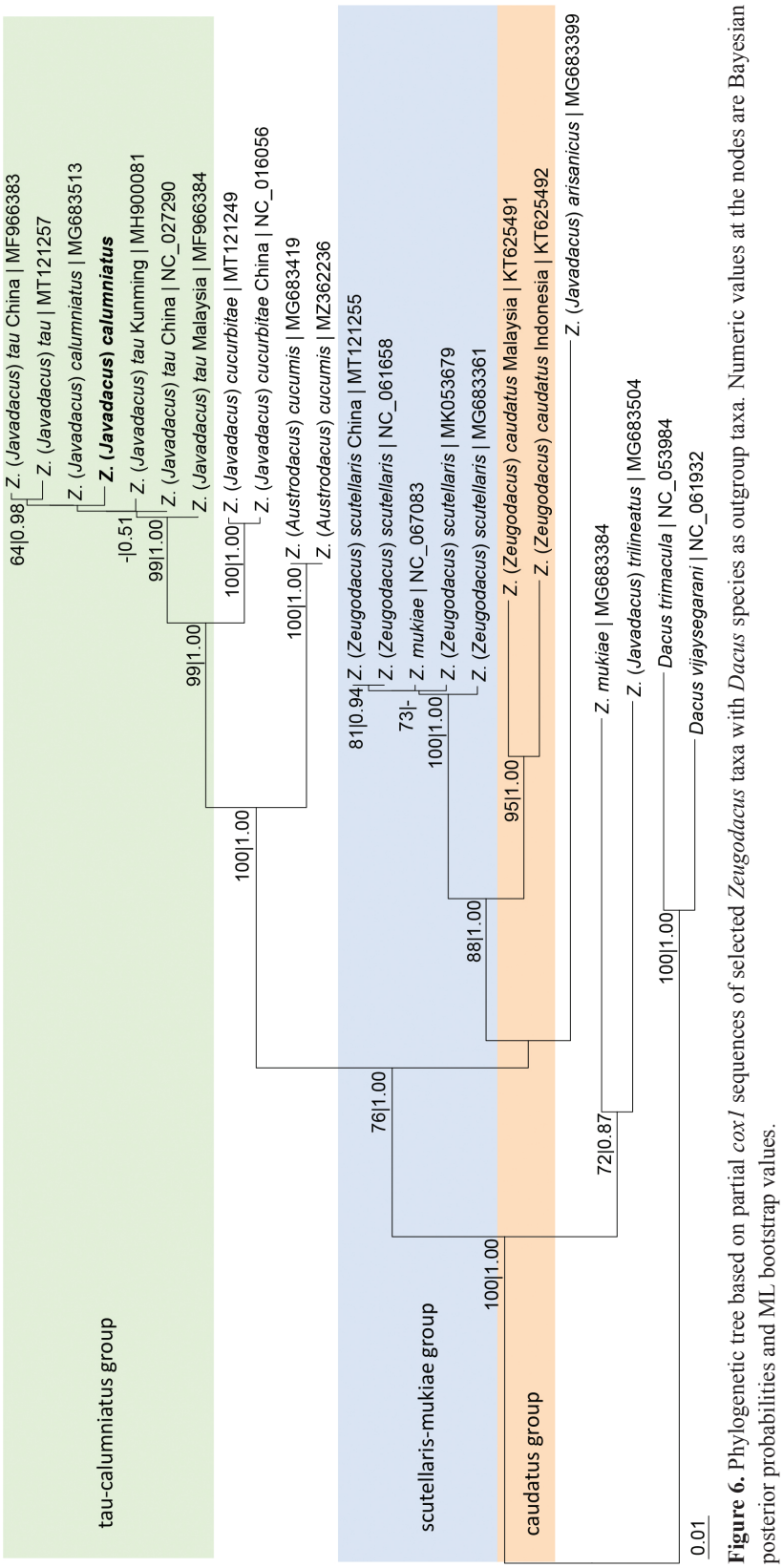


**Figure 6.** Phylogenetic tree based on partial *cox1* sequences of selected *Zeugodacus* taxa with *Dacus* species as outgroup taxa. Numeric values at the nodes are Bayesian posterior probabilities and ML bootstrap values.

In Clade B, *Z. calumniatus* formed a sister lineage with *Z. tau* in a subclade containing also *Z. cucurbitae*, while *Z. heinrichi* and *Z. hochii* formed a sister lineage in another subclade; *Z. depressus* was sister/external to the remaining Clade B taxa. A notable incongruence was the sister lineage of *Z.* (*Sinodacus*) *hochii* with *Z.* (*Javadacus*) *heinrichi* among other taxa of subgenus *Javadacus* (Fig. 5).

Figure 6 depicts the molecular phylogeny of selected *Zeugodacus* taxa with *Dacus* species as outgroup taxa based on partial *cox1* gene. Most of the nodes were fully supported.

## 4. Discussion

Like other tephritid fruit flies, as well as other insects, the mitogenomes of *Z. calumniatus*, *Z. heinrichi* and *Z. hochii* have the three main clusters of characteristic tRNA genes (Fig. 1): (1) I-Q-M (isoleucine, glutamate and methionine); (2) W-C-Y (tryptophan, cysteine and tyrosine); and (3) A-R-N-S1-E-F (alanine, arginine, asparagine, serine S1, glutamate and phenylalanine) (Cameron 2014). They also have the atypical cloverleaf structure of serine S1 (*trnS1*), which is common in all Metazoa (Jühling et al. 2012).

The A-T rich control region of the three *Zeugodacus* mitogenomes possesses both similar and dissimilar features, such as a long poly-A stretch, a long poly-T stretch, tandem repeats and palindromes. Due to its high variability, lack of purifying selection and higher substitution rate, this non-coding control region has been explored for its phylogenetic utility. For example, it has been reported to be of possible phylogenetic utility in some groups of Hemiptera (Li and Liang 2018), a powerful marker for phylogenetic inference in echinoids (Bronstein et al. 2018), and successful for differentiating the BPH (Brown Plant Hopper, *Nilaparvata lugens*) populations (Anand et al. 2022).

The *cox1* gene, with very low Ka/Ks ratio (0.006 to 0.017) in the three *Zeugodacus* mitogenomes of the present study, representing fewer changes in amino acids, supports its use as a molecular marker for species differentiation and DNA barcoding (Doorenweerd et al. 2020; Lopez-Vaamonde et al. 2021). Genes with very low Ka/Ks ratio, such as *cox1*, *atp6* and *cox3* (Fig. 3), reflect the purifying selection that acts on most protein-coding genes. This suggests that any mutations that reduce their function would be quickly eliminated from the population due to their deleterious effects on fitness. In this study, the *atp8* gene has a comparatively higher Ka/Ks ratio (Fig. 3). There are similar results in other insect groups, such as the true bugs in which *atp8* shows sign of positive selection (Gonçalves et al. 2022).

In the present study, *Z. mukiae* NC_067083 is genetically very similar to *Z.* (*Zeugodacus*) *scutellaris*, with $p = 0.4$–$0.9\%$ based on 15 mt-genes (Table S7), and $p = 0.3$–$0.4\%$ based on partial *cox1* sequences (Table S8), indicating that it may be a misidentification, as it differs from the taxon *Z. mukiae* MG683384 with $p = 12.6\%$ based on partial *cox1* sequences (Table S8; Fig. 6). A similar incorrect taxonomic identification has also been inferred for *Z. calumniatus*, with maximum intraspecific genetic distance of $p = 8.8\%$ based on partial *cox1* sequences (Kunprom and Pramual 2019).

Previous work has shown that the 'canonical' *Z. mukiae* is a member of the *arisanicus* (*arisanica*) complex and not the *scutellaris* complex (Hancock and Drew 2018b; San Jose et al. 2018). Additionally, it was shown that *Z. mukiae* MG683384 forms a lineage with *Z. trilineatus* and *Z. arisanicus* (San Jose et al. 2018). However, based on partial *cox1* sequences of selected taxa (this study), *Z. arisanicus* is sister to subgenus *Zeugodacus* and does not form a sister lineage with *Z. mukiae* and *Z. trilineatus* (Fig. 6). *Z. arisanicus* was previously assigned to the subgenus *Parazeugodacus* (San Jose et al. 2018).

*Zeugodacus calumniatus* is genetically very similar to *Z. tau* with $p = 0.8\%$ based on 15 mt-genes (Table S7; Fig. 5); the intraspecific genetic distance of *Z. tau* is $p = 0.2$–$0.7\%$. Based on partial *cox1* sequences, the genetic distance between *Z. calumniatus* and *Z. tau* is $p = 0.6$–$1.0\%$ (Table S8; Fig. 6); the intraspecific genetic distance of *Z. calumniatus* is $p = 0.4\%$. In an earlier study based on partial *cox1* sequences from bp 50–700, the intraspecific uncorrected genetic distance of the *Z. tau* taxa from China, Bangladesh, India (Meghalaya, north of Bangladesh) and Malaysia ranges from $p = 0$ to $p = 0.72\%$ (Yong et al. 2017). In the finding of Kunprom and Pramual (2019), the closest genetic distance between *Z. calumniatus* from Indonesia and *Z. tau* is $p = 0.2\%$ based on partial *cox1* sequences. *Z. calumniatus* is placed in the *munda* complex by Hancock and Drew (2018b); it is grouped with the lineage (*Z. cucurbitae* – *Z. tau*) based on molecular phylogeny (San Jose et al. 2018).

The taxonomic status of *Z. calumniatus* needs clarification as it is morphologically very similar to *Z. tau* (Drew and Romig 2013, 2016); it "is similar in most respects to tau (Walker) and is differentiated by the presence of the prominent brown mark extending over the m crossvein" (Hardy 1974). Nonetheless, in the present study, *Z. calumniatus* is distinctly separated from the *Z. tau* taxa in the *calumniatus-tau* sister lineage of subgenus *Javadacus* (Fig. 5). There are similar examples of closely related tephritid fruit flies with small genetic distance, for example, *Bactrocera carambolae* and *B. dorsalis* (currently accepted as good species) with $p = 1.2\%$ based on 15 mt-genes (Yong et al. 2016a).

In the current taxonomic treatment, *Zeugodacus propredipharus* (previously *Bactrocera propredipharus* Wang et al., 2008) is synonymised with *Zeugodacus diaphorus* (previously *Bactrocera diaphora*) (Drew and Romig 2013). The present phylogenetic analysis reveals that *Z. propredipharus* is genetically distinct from *Z. diaphorus*, with a genetic distance of $p = 2.9$–$3.0\%$ based on 15 mt-genes; the intraspecific genetic distance of *Z. diaphorus* is $p = 0.1\%$ (Table S7). This is concordant with the phylogenetic analysis by Wang et al. (2020) which indicates the two taxa to be closely related. An integrative study based on multiple individuals and comprehensive sampling is needed to elucidate the species status of *Z. propredipharus*.

It is noteworthy that *Z. diversus* forms a sister lineage with *Z. caudatus* Indonesia in a subclade containing *Z. caudatus* Malaysia and *Z. caudatus* China (Fig. 5). Earlier molecular phylogeny has shown the Malaysian and Chinese taxa of *Z. caudatus* to be genetically very different from and hence not conspecific with *Z. caudatus* sensu stricto from Indonesia (Yong et al. 2015a, 2016b). Further taxonomic work is needed to formally erect the Malaysian population as a new species.

A notable incongruence in the present study is the grouping of *Z.* (*Sinodacus*) *hochii* with *Z.* (*Javadacus*) *heinrichi* among other taxa of subgenus *Javadacus*

(Fig. 5). An earlier study on molecular phylogeny also indicates the grouping of *Z.* (*Sinodacus*) *hochii* with *Z.* (*Javadacus*) *heinrichi* in the same clade (San Jose et al. 2018). This raises the question whether *Z. heinrichi* is a member of subgenus *Sinodacus*, or *Z. hochii* a member of subgenus *Javadacus*. Assuming that the subgenus *Sinodacus* forms a lineage in the same clade as the subgenus *Javadacus*, the subgenus status of *Z.* (*Javadacus*) *depressus* (the external taxon to the other taxa in Clade B) also needs clarification as it has been assigned to subgenus *Paradacus* Perkins, 1938 in some studies (see Jeong et al. 2017). The recent study of Zhang et al. (2023) on the mitogenomes of tephritid fruit flies has recovered paraphyletic/polyphyletic subgenera within the genus *Zeugodacus*. Our present results add to these inconsistencies. A more extensive taxon sampling, particularly the subgenus *Sinodacus* (and other subgenera), is needed to clarify/resolve their subgenus status. Independent sources of information from across the genome (e.g. independent nuclear genes) are also important to confirm/establish their taxonomic relationships.

In summary, we have successfully sequenced and annotated the whole mitochondrial genomes of *Z.* (*Javadacus*) *calumniatus*, *Z.* (*Javadacus*) *heinrichi* and *Z.* (*Sinodacus*) *hochii*. The genome features are similar in the three species. Phylogenetic analysis based on the mt-genes reveals two major clades of the *Zeugodacus* taxa: (A) monophyletic subgenus *Zeugodacus*, and (B) subgenera *Javadacus* and *Sinodacus*; *Z.* (*Parasinodacus*) *cilifer* is external to the two main clades. It reveals the incongruence of *Z.* (*Sinodacus*) *hochii* forming a sister lineage with *Z.* (*Javadacus*) *heinrichi*. It also indicates the need to clarify the taxonomic status of *Z. mukiae* NC_067083 and *Z. calumniatus*. On the other hand, the results indicate the possible valid species status of *Z. propredіaphorus* (genetically distinct from and likely not a synonym of *Z. diaphorus*). A broad taxon sampling of subgenus *Sinodacus* and other subgenera will help to clarify their taxonomic status and phylogeny.

## 5. Acknowledgements

We thank our respective institutions for their support of our research on tephritid fruit flies. H-S Yong is supported by MoHE-HIR Grant (H-50001-00-A000025) and Universiti Malaya (H-5620009). Our thanks also go to Dr. Leonardo Gonçalves and another reviewer for their valuable suggestions in improving the manuscript.

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# Supplementary Material 1

## Tables S1–S8

**Authors:** Yong H-S, Song S-L, Chua K-O, Liew YJM, Suana IW, Lim P-E, Chan K-G, Eamsobhana P (2023)
**Data type:** .docx
**Explanation note: Table S1.** List of *Zeugodacus* mitogenomes from GenBank, and subgenera based on Hancock and Drew (2018a, 2018b). — **Table S2.** A + T content (%), AT and GC skewness of three *Zeugodacus* mitogenomes. Zca, *Zeugodacus calumniatus*; Zhe, *Zeugodacus heinrichi*; Zho, *Zeugodacus hochii*. — **Table S3.** Base composition, A + T content (%), AT and GC skewness of the 13 protein coding genes in three *Zeugodacus* mitogenomes. — **Table S4.** Amino acid frequency for the 13 protein coding genes of three *Zeugodacus* mitogenomes. — **Table S5.** Relative synonymous codon usage for the 13 protein coding genes of *Zeugodacus* mitogenomes. — **Table S6.** Ka/Ks ratio for the 13 protein coding genes of the *Zeugodacus* mitogenomes. Zca, *Zeugodacus calumniatus*; Zhe, *Zeugodacus heinrichi*; Zho, *Zeugodacus hochii*. — **Table S7.** Pair-wise genetic distance (%) of *Zeugodacus* taxa based on 15 mt-genes (13 protein-coding genes and 2 rRNA genes). — **Table S7.** Pair-wise genetic distance (%) of *Zeugodacus* taxa based on 15 mt-genes (13 protein-coding genes and 2 rRNA genes). (Cont.) — **Table S8.** Pair-wise genetic distance (%) of *Zeugodacus* taxa based on partial *cox1* sequence.

**Link:** https://doi.org/10.3897/asp.81.e105025.suppl1

# Supplementary Material 2

## Figure S1

**Authors:** Yong H-S, Song S-L, Chua K-O, Liew YJM, Suana IW, Lim P-E, Chan K-G, Eamsobhana P (2023)
**Data type:** .docx
**Explanation note:** Stem-loop structure of spacing sequence between *trnR* and *trnN* genes in three *Zeugodacus* mitogenomes. Left, *Z. calumniatus*; centre, *Z. heinrichi*; right, *Z. hochii*.

**Link:** https://doi.org/10.3897/asp.81.e105025.suppl2