Zoological Systematics, 43(4): 356–386 (October 2018), DOI: 10.11865/zs.201833

ORIGINAL ARTICLE

Biased heteroplasmy within the mitogenomic sequences of *Gigantometra gigas* revealed by sanger and high-throughput methods

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Abstract Few studies have explored the differences between Sanger and HTS methods in the results of mitogenome sequencing. We used a single individual of insect to study the differences between the sequences given by Sanger and PCR-free HTS methods. Here we provided evidence for biased results of sequencing due to different methods in the mitochondrial genes of *atp6*, *atp8*, *cox1*, *cox2*, *cox3*, *Cytb*, *nad2*, *nad3*, *nad4*, *nad5*, *rrnS*, *rrnL*, *trnH*, *trnI*, and control region at various degrees. Especially, in *cox1*, the differently sequenced nucleotides account for 2.6% of the complete length. Furthermore, the highest value of the intraspecific genetic distance based on K2P accounts for 2.5% using a barcode fragment size of *cox1* (651 bp, Sanger), while the maximum distance of the corresponding *cox1* fragment obtained by the two sequencing methods was 5.0%. We revealed that the methods of Sanger and HTS may give different sequencing results of mitochondrial genes, which may reflect the heteroplasmy of mitogenomes within an insect individual. Therefore, researchers should be very cautious in using the mixed data of a gene given by different methods of sequencing.

Key words Sanger and high-throughput sequencing, mitogenome, *cox1*, COI, heteroplasmy, giant water strider.

1 Introduction

For the past three decades, the analysis of mitochondrial DNA has been proven as an exceptionally useful tool for DNA barcoding, phylogeography, and phylogenetics (Avise *et al*., 1987; Avise, 2004; Simon *et al*., 2006; Kayal *et al*., 2013; Crampton-Platt *et al*., 2016), mainly because of its abundance in tissues, simple structure, and rapid rate of evolution (Brown *et al*., 1982). Among the mitochondrial genes, *cox1* is one of the most pervasive molecular markers in identifying species, inferring genetic structures of population, and reconstructing phylogeny, and mitochondrial genes are also applied at various degrees. As for the studies of molecular systematics based on mitogenomes, all of the protein-coding genes (PCGs) are

Received 8 January 2018, accepted 30 June 2018
Executive editor: Fuqiang Chen

regularly included as molecular markers.

There has been a rapid accumulation of the sequences of mitogenomes, which are widely used in extensive studies (Crampton-Platt *et al*., 2016). In the past few years, DNA metabarcoding has emerged as a fast and effective approach to characterizing environment samples with complete mitogenomes in contrast to short DNA markers used before (Brandon-Mong *et al*., 2015; Kartzinel *et al*., 2015; Leray *et al*., 2015; Evans *et al*., 2016). Comprehensive mitogenomic analysis has also been applied in the studies of population genetics and phylogeographic history of various animals (Morin *et al*., 2010; Ma *et al*., 2012; Keis *et al*., 2013; Chang *et al*., 2017). As for phylogenetic analyses, the use of mitogenomes has intensified for deep analysis and testing different approaches of phylogenetic reconstruction (Boore, 2006; Cameron, 2014a, b; Andújar *et al*., 2015).

Since the rapid increasing demand in the amount of available mitogenomes in the past decade, HTS technologies have made a significant breakthrough in the advances of data generating methods. Such improvement in the methods of HTS significantly reduce analytical cost in obtaining the sequences of complete mitogenomes in large scale. Based on the HTS technologies, a simple and widely used approach allowing multiplex sequencing and assembling was developed, which can be used to simultaneously acquire a bulk of full mitogenomes of different species from pooled animals without DNA enrichment or amplification in advance (Tang *et al*., 2014, 2015). The rapid developments of HTS technologies make it possible to utilize mitogenomic information quickly and cost-efficiently in various domains of ecological and evolutionary studies. In the meantime, Sanger method is still widely used in obtaining the sequences of mitochondrial genes in lab. However, it has not received enough attention how different the sequences of mitogenomes obtained by Sanger and HTS methods can be in the water strider.

As insects have advantage in immense biodiversity and mitogenome has an outsized impact on entomological genetics, we selected a special species in true bugs (Hemiptera: Heteroptera), *Gigantometra gigas*, which is the largest water strider with narrow distribution in Hainan Island of China and northern Vietnam (Fig. 1C) and has ever been suggested as extinct by some entomologists (personal communication), to serve as the material for studying the potentially different mitogenomic sequences obtained by different methods of sequencing. The large body size (length 30–40 mm; Fig. 1) can provide abundant genomic DNA from a single individual for the contrast analysis between Sanger and HTS methods with repetitions for each of them. In this study, the complete mitogenomic sequences of the giant water strider obtained by Sanger and PCR-free HTS methods was assembled and annotated respectively, which provides an important genomic resource of the mysterious water strider for conservation biologists. We found significant systematic differences in the sequences of mitogenome obtained by Sanger and HTS methods. Besides, the sequences of ITS-1 and ITS-2 in the nuclear rDNA (nrDNA) cluster also showed differences between Sanger and HTS methods, just at a low degree. This study may call attention to the systematic differences between Sanger and PCR-free HTS methods. It should be very cautious to use the mixed data of a gene given by different methods of sequencing in ecological and evolutionary studies.

2 Materials and methods

2.1 Sampling and DNA extraction

The specimen of *Gigantometra gigas* (1♂), which was used for comparing the results of the two different sequencing methods, was collected by Yanhui Wang, Qiang Xie, and Hesheng Wang from Yinggeling Nature Reserve, Hainan, China, on July 7th, 2014. Moreover, we collected another 23 specimens of the species, which were used for measuring the intraspecific genetic distances, from Yinggeling Nature Reserve, Hainan (HNYG), Diaoluoshan Nature Reserve, Hainan (HNDL), and northern Vietnam (VIET). These specimens collected for experiments had been preserved in 95% ethanol and stored at −20°C until DNA extraction. Total genomic DNA was extracted from the muscle tissue of thorax by the CTAB-based method (Reineke *et al*., 1998). These voucher specimens were deposited at Institute of Entomology, College of Life Sciences, Nankai University.

2.2 PCR amplification and sequencing

For the single individual of *G. gigas* investigated for the impact of different sequencing methods, total genomic DNA extracts were divided into six parts. Three of them were sequenced with the HTS platform by China National GeneBank (BGI-Shenzhen, China), and the other three were used for regular PCR and then sequenced by Sanger method. For each copy of the genomic DNA extracts latterly used for Sanger sequencing, the overlapped short fragments were separately

amplified, sequenced, and manually assembled to provide three independent results of sequencing the complete mitogenome and nrDNA cluster (Sanger *et al.*, 1977). The clone with pEASY-T3 (a TA cloning vector) was used in the sequencing of complete sequences of *cox1*, and 25 different clones were selected randomly and sequenced, respectively. These fragments were amplified using perfectly matched primers (Table S1). The PCR amplification was carried out in a 50-μL reaction system containing 6 μL 10× LA PCR Buffer II (Mg^{2+} Plus), 6 μL of dNTP mixture (2.5 mM), 0.5 μL LA Taq (TaKaRa Biotechnology, Dalian, China), 2 μL of each primer (10 μM), 2 μL DNA template and 31.5 μL distilled water. The thermal cycling program of the PCR consisted of an initial denaturation at 94°C for 2 min, 35 thermal cycles (94°C for 30 s, 43–52°C for 30 s, 72°C for 1 min) and a final extension at 72°C for 8 min. Colony PCR was carried out in a 25-μL reaction volume followed by 30 amplification cycles.

For the HTS method, three independent DNA libraries based on the same DNA extracts were separately constructed with an insert size of 250 bp following manufacturer's instruction, and then sequenced with 150 bp paired-end (PE) on an Illumina HiSeq 4000 platform. Raw reads were filtered by removing reads containing adaptor contamination, poly-Ns (>5bp Ns) and PE reads with >10 bases of low quality scores (<20) (Zhou *et al*., 2013; Tang *et al*., 2014, 2015). *De novo* assembly for each repeat was performed by *SOAPdenovo-Trans*-bin-v1.03 under different K-mer settings (Xie *et al*., 2014). Then the assemblies were blasted against a mitogenome database containing full mitogenomes from 24 heteropteran species (Table

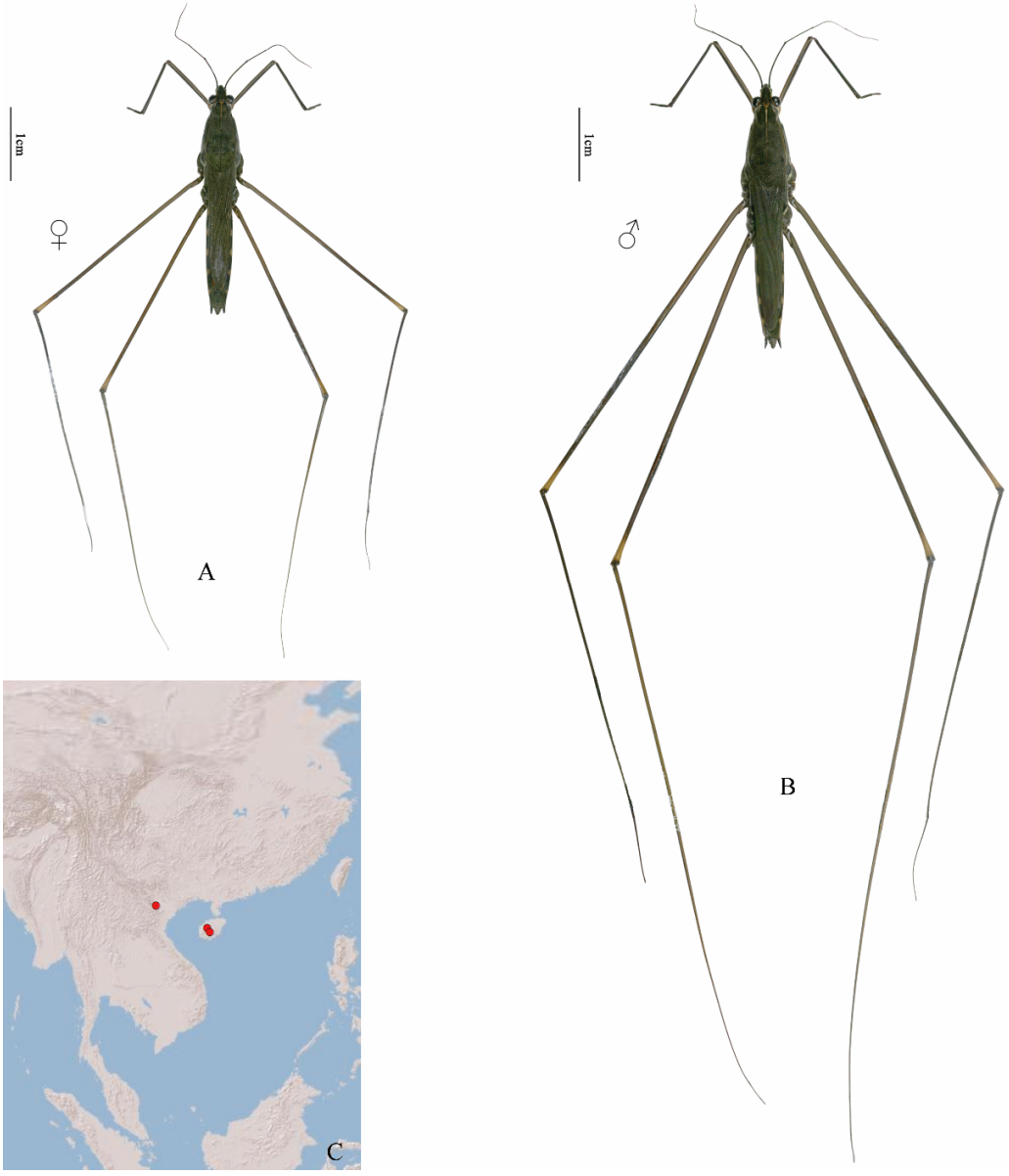


Figure 1. *Gigantometra gigas*. A. Female, dorsal view. B. Male, dorsal view. C. The narrow distribution of *G. gigas*.

S2), which were downloaded from GenBank, using the program BLAST+ (Camacho *et al*., 2009). The sequences of nrDNA cluster were searched against 18S and 28S rDNA databases containing 30 heteropteran species, respectively (Table S2). The statistical results show that the assembly results are the best under K-mer=71 (HTS1 and HTS3) and K-mer=61 (HTS2). Then the scaffolds of mitochondrial origin were annotated using the Mitos web server (Bernt *et al*., 2013). The protein coding regions were verified using ORF Finder provided by the NCBI website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

While for the 23 individuals investigated for the genetic distance of *G. gigas*, the gene *cox1* in barcode fragment size were sequenced by Sanger method.

2.3 Analyses and comparison of the sequences

For mitogenomes, the sequence files obtained by Sanger method were proof read and assembled into a complete sequence in BioEdit 7.2.6 (Hall, 1999). The protein coding regions were identified using ORF Finder implemented by the NCBI website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The rRNA genes were identified by sequence homology with the published mitochondrial sequences of insect from GenBank and the corresponding secondary structure models were constructed referring to those of the other true bugs (Hemiptera-Heteroptera), *i.e*., *Agriosphodrus dohrni* (Li *et al*., 2011), *Alloeorhynchus bakeri* (Li *et al*., 2012a), *Stenopirates* sp. (Li *et al*., 2012b), and *Aquarius paludum* (Cui *et al*., 2012). The tRNA genes were found by tRNAscan-SE search Server v.1.21 with invertebrate mitochondrial codon predictors and a cove score cut off of 5 (http://lowelab.ucsc.edu/tRNAscan-SE/). Only a few of tRNA genes that could not be detected by tRNAscan-SE were identified by comparing to those of the other heteropterans (Lowe & Eddy, 1997). Strand asymmetry was calculated using the formulas: AT skew = (A−T) / (A+T) and GC skew = (G−C) / (G+C), which were displayed using CGView Server (Grant & Stothard, 2008). For the nrDNA cluster, we ensured the accurate boundaries of different genes by aligning to the published nrDNA cluster of heteropterans with MEGA7 (Kumar *et al*., 2016). Moreover, we also mapped the individual HTS sequencing reads back onto the HTS scaffolds to show the basis of heteroplasmy sites between the two sequencing methods and showed the coverage of short sequence fragments at each position using Geneious 10 (Kearse *et al*., 2012). The R package Seqcombo was used to show the different nucleotides in the sequences of 13 PCGs obtained by Sanger and HTS methods.

2.4 Analysis of intraspecific genetic distances using *cox1*

The analysis of intraspecific nucleotide variability in the most regular barcode fragment of *cox1* (651 bp, Sanger) was calculated with the model of Kimura 2-parameter (K2P) using MEGA7 (Kimura, 1980; Kumar *et al*., 2016). We separately analyzed genetic distances of the individuals within and between the three collecting sites (HNYG, HYDL and VIET). Moreover, the genetic distance of the corresponding different sequences of the *cox1* obtained by the two sequencing methods were measured as well.

3 Results

3.1 Mitogenome organization and structure

The complete mitochondrial genome sequence of *G. gigas* was a double-stranded circular DNA molecule, which was 15,348 bp in size with Sanger sequencing (Fig. S1). This mitogenome totally contained the typical 37 genes (two rRNAs, 13 PCGs and 22 tRNAs) and a non-coding region (control region), with the same gene order as *Drosophila yakuba* (Clary & Wolstenholme, 1985), which were shown in Table S3. Gene overlaps were observed at 15 gene junctions and involved a total of 61 bp, of which the longest overlap (20 bp) existed between *cox2* and *trnK*. In addition to the control region, 63 nucleotides were dispersed in seven intergenic spacers, ranging in size from 1 to 25 bp. The longest spacer sequence was located between *trnY* and *cox1*.

3.2 Sequences obtained by Sanger and HTS methods

The three mitogenomic sequences of *G. gigas* independently obtained by Sanger method were identical, of which the complete length was 15,348 bp (Fig. S1), and has been deposited in the GenBank (accession number: MF177288). While, the three independent results of mitogenomic sequences obtained by HTS method were different and thus the three repetitions of HTS sequencing were separately called as HTS1, HTS2, and HTS3. In the result of HTS1, a complete sequence

of mitogenome was obtained (GenBank accession number: MF177289). While for the latter two repetitions, an incomplete mitogenomic sequence was feedback for each, in which the sequences of *rrnS* and *rrnL* were obtained partially and the sequence of *trnV* was absent in the result of HTS2 (GenBank accession number: MF177290), and the sequences of *rrnS* and the 5'-half of control region were absent in that of HTS3 (GenBank accession number: MF177291). The detailed information of each sequenced mitochondrial gene were listed in Table S4.

On the whole, there were 148 different nucleotide sites in the sequences obtained by Sanger and HTS methods. Among them, certain inconsistent nucleotide sites exhibited systematic differences between the two sequencing methods. For the three repetitions of HTS sequencing, the consistency ratio of the results reached by HTS sequencing accounts for 46% of the 148 different nucleotide sites.

The differently sequenced nucleotides distribute in 10 PCGs, two rDNAs (*rrnS* and *rrnL*), two tDNAs (*trnI* and *trnH*), and control region. The different nucleotides in the sequences of 10 PCGs, i.e., *atp6, atp8, cox1, cox2, cox3, Cytb, nad2, nad3, nad4*, and *nad5*, obtained by Sanger and HTS methods were shown in Figure 2. Among all the sequences of 13 PCGs obtained by Sanger and HTS methods, the sequences of *cox1* had the biggest differences. Taking the results of all three repetitions of HTS into account, there are totally 40 different nucleotide sites between the *cox1* sequences obtained by Sanger and HTS methods (Table 1).

Besides, the sequences of rDNA, tDNA, and control region showed differences as well in the results obtained by Sanger and HTS methods at various degrees. For the sequences of *rrnS* and *rrnL* in the results of HTS1 compared with Sanger, there were eight different nucleotides and one deletion in *rrnS* (Fig. S2) and 30 different nucleotides coupling with one insertion and one deletion in *rrnL* (Fig. S3). For the sequences of tDNA, there were one different nucleotide in *trnI* in the results of HTS3 compared with Sanger, HTS1, and HTS2, and two different nucleotides in *trnH* in the results of Sanger compared with HTS methods. Additionally, there were 10 different nucleotides in the sequences of control region obtained by the results of HTS2 compared with Sanger and HTS1, and 19 different nucleotides in those of HTS3 compared with Sanger, HTS1, and HTS2. Except for the differences mentioned above, the nucleotide sequences of the remaining genes were the same in the results of Sanger and HTS methods. All the different nucleotides of mitogenomic sequences in the results of Sanger and HTS methods were shown in Table S5, and the base states of different sites were separately calculated in the three assembly results of HTS method (Table S6).

The three independent sequences of nrDNA cluster obtained by Sanger method were the same with a complete length of 6,769 bp (GenBank accession number: MF177292). While for the completeness of the nrDNA cluster, the feedbacks of the three repeats based on the same HTS platform were different. In the assembly of HTS1, a complete sequence of nrDNA cluster was obtained (GenBank accession number: MF177293). While for the latter two repetitions, the nrDNA clusters were incomplete. In the assembly of HTS2 and HTS3, the sequences of 28S rDNA, ITS-1, and ITS-2 were partial and the sequences of 5.8S rDNA were absent (GenBank accession number: MF177294–MF177297). We compared the results of Sanger with those of HTS and found one nucleotide difference in the ITS-1 and one in the ITS-2 regions, respectively (Fig. 3).

3.3 Comparison of intraspecific genetic distances

The genetic distances among individuals of each place and between them using the sequences obtained by Sanger method and that between the two sequencing methods were separately calculated based on the 651 bp *cox1* and shown in Figure 4. The maximum pairwise K2P distance of individuals in HNYG, HNNK, and VIET was 2.1%, 1.8%, and 1.4%, respectively, and they altogether make a total maximum pairwise K2P distance of 2.5% (Fig. 4, Table S7). Surprisingly, the maximum distance of the polymorphic *cox1* sequences from a single individual obtained by different sequencing methods accounted for 5.0%. Besides, the median value of genetic distance within an individual due to the two different sequencing methods was also higher than that between different individuals or even different geographic populations based on Sanger sequencing.

3.4 Verification of the polymorphic sites in *cox1* by cloning

The 25 different single-clones of complete *cox1* sequenced with Sanger method verified the polymorphism of *cox1* within a single individual of *G. gigas*. The total number of polymorphism sites was 101 in the 25 different single-clone sequences (Fig. 5). Among them, there exist 33 sites being in high accordance with the 38 sites which exhibit obvious second-peak in the results of direct Sanger sequencing without cloning (Fig. 6) and the 40 sites which are different between the results of Sanger and HTS (Table 1).

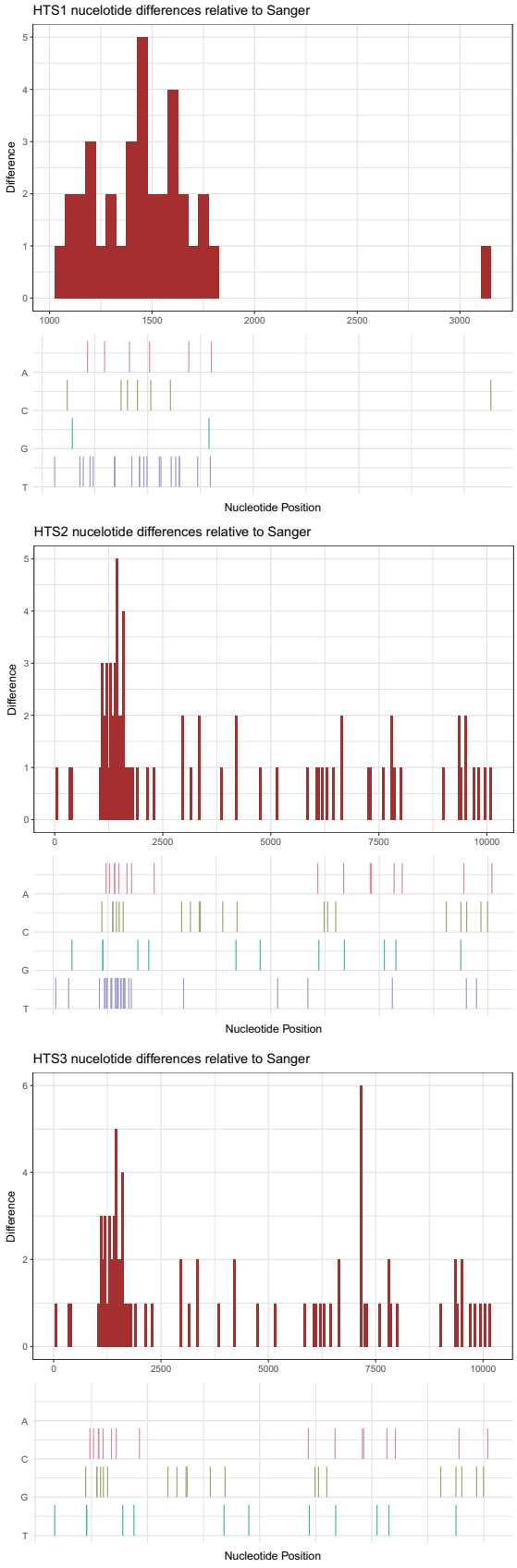


Figure 2. The different nucleotides of all 13 PCGs in mitogenomes obtained by Sanger and HTS sequencing. The different sequences of HTS sequencing are separately compared with the consequence of Sanger method. The horizontal axis stands for the nucleotide position, of which the sequences of 13 PCGs are ordered according to the circular mitochondrial DNA from *nad2* to *nad1* in the clockwise direction. The vertical axis stands for the number of the different sites in the sequences of 13 PCGs, and the different nucleotide in each site of all three HTS sequences compared with Sanger is shown in the corresponding panel.

Figure 3. The different nucleotides in the ITS-1 and ITS-2 regions are shown. The result shows the different nucleotides at nucleotide position np 1897 (G nucleotide and T nucleotide) and np 2790 (C nucleotide and T nucleotide) obtained by Sanger and HTS methods.

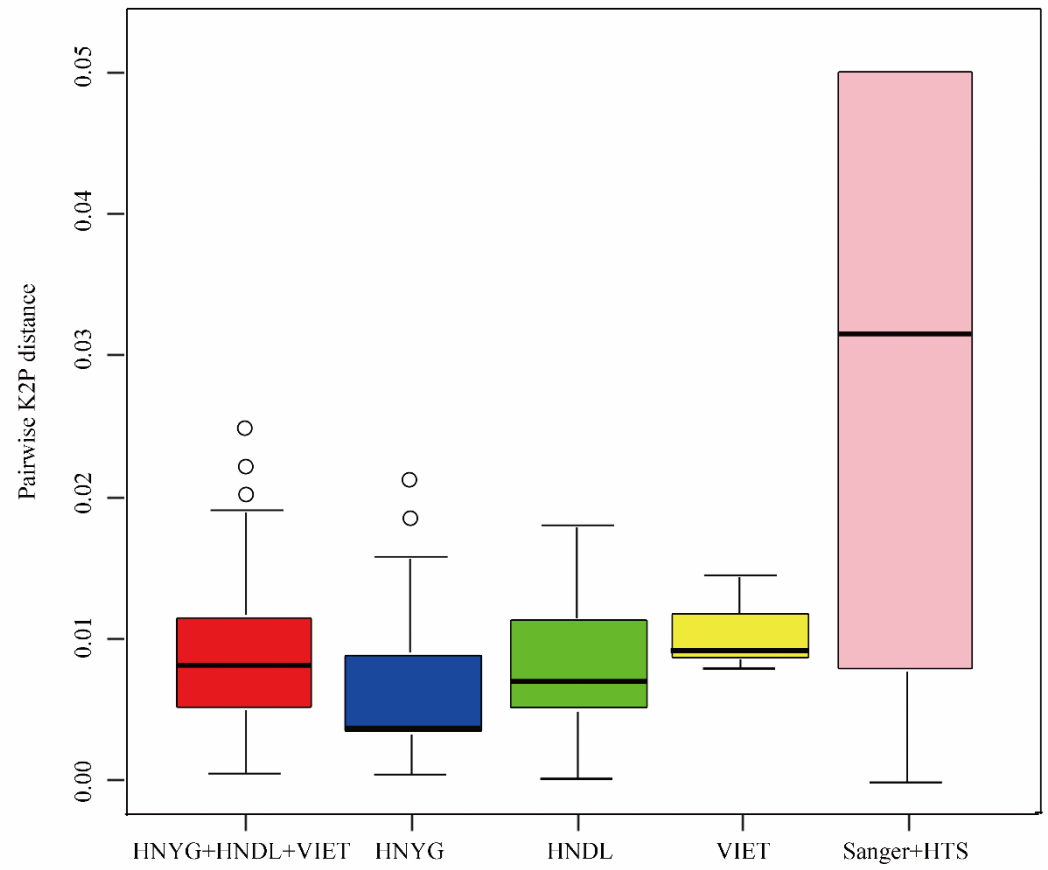


Figure 4. Intraspecific pairwise K2P distance of *G. gigas* based on barcode fragment size of *cox1* (Sanger). The red boxplot shows the genetic distances of individuals in all three collecting sites, and the boxplots (blue, green, and yellow) separately show the distances of individuals within each place (HNYG, HNDL, and VIET). The pink boxplot shows the distances of the corresponding *cox1* sequences obtained by the two sequencing methods. Abbreviation: HNYG—Yinggeling Nature Reserve, Hainan; HNDL—Diaoluoshan Nature Reserve, Hainan; VIET—northern Vietnam.

4 Discussion

4.1 Differently sequenced mitogenomes between Sanger and HTS methods

In recent years, the platform of HTS has made rapid development in virtue of reducing cost for obtaining complete mitogenomes in the fields of DNA barcoding, phylogeography, and phylogenetics. Although Sanger sequencing has been regularly used in lab so far, little attention has been paid to the existence and scale of differences between Sanger and HTS

methods. In this study, we highlight that there exist systematic differences in the sequences of mitochondrial genes obtained by Sanger and HTS methods in a single individual of *G. gigas*. Especially for the complete length of the *cox1*, which is still one of the mostly used markers in population genetics studies (Arriaga-Jiménez & Roy, 2015; Borsa *et al*., 2016; Tonione *et al*., 2016), the discrepant nucleotides between different sequencing methods account for 2.6%, and all the three results of HTS sequencing made remarkable differences compared with Sanger sequences at various degrees. Such results call attention to the possibility and influence of remarkable differences between the results of different sequencing methods.

The systematic differences in mitogenome between Sanger and HTS methods may make influence in delimiting species and reconstructing the tree of life. Nowadays, DNA barcoding is regularly employed as an important part of integrative taxonomy in species description and delimitation (Stoev *et al*., 2010, Hendrich & Balke, 2011; Riedel *et al*., 2013). As for the species with similar morphological characteristics, DNA barcoding is an efficient approach to identify species in the field of biodiversity studies based on the genetic distance of mitochondrial sequences, especially those of *cox1* (Hebert *et al*., 2003a, b; Ward *et al*., 2005; Butcher *et al*., 2012), which relies on the conception that each species has unique DNA barcode and intraspecific variation is typically lower than that of interspecific. However, an increasing number of researchers found that the interspecific variations may be lower than the intraspecific ones and the plausible thresholds may not bode well for delineating closely related species especially for those poorly understanding groups (Ratnasingham & Hebert, 2013). For the true bugs which have been investigated so far, previous studies recommended some thresholds of interspecific distances for delimiting closely species ranging from 2.2% (Raupach *et al*., 2014) to 3.5% (Jung *et al*., 2011). In contrast, the notable systematic differences of complete sequences of the *cox1* within a single insect individual reached 2.6% in our work. Moreover, we found a genetic distance between the geographic populations of HNYG and VIET with a high pairwise K2P distance of 2.5% using a barcode fragment size of *cox1* (Sanger), which were even lower than the maximum distance (5.0%) of corresponding *cox1* sequences owing to the two sequencing methods (Fig. 4). If the systematic differences caused by the

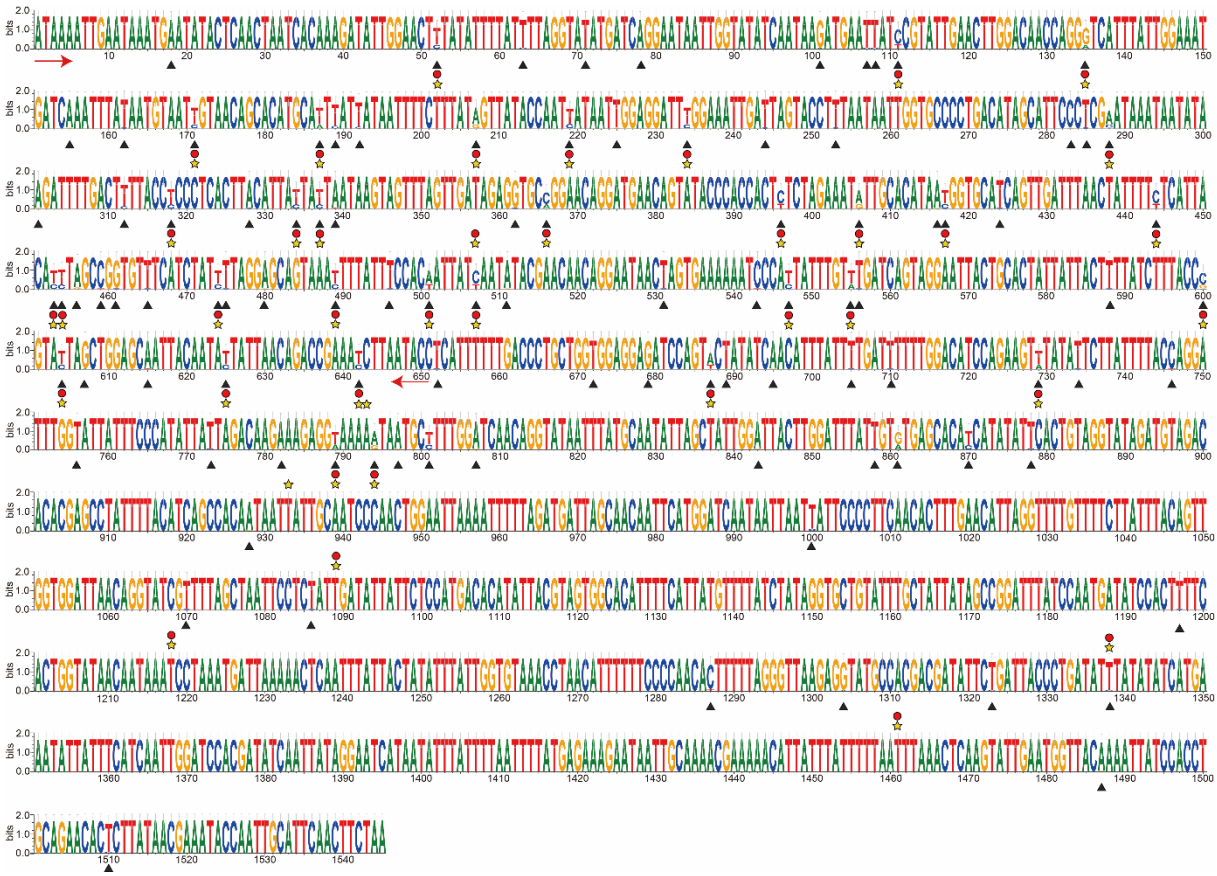
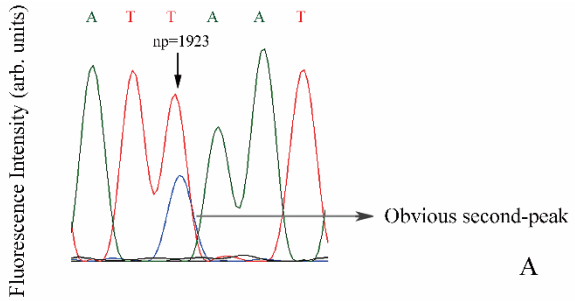


Figure 5. The polymorphism sites among the 25 different cloning sequences of *cox1*. Weblogo 3.0 was used to show the nucleotide content of 25 cloning sequences of *cox1* (Crooks *et al*., 2004). The abscissa stands for the number of the bases, while the ordinate stands for the proportion of nucleotide content provided by the 25 different cloning sequences in the same position. The sequence length between the two arrows stands for the barcode fragment size of *cox1*. The black triangles show the polymorphism positions in the 25 different cloning sequences, the red circles show the positions exhibited obvious second-peak in the results of direct Sanger sequencing without cloning, and the yellow stars show the different sites between the results of Sanger and HTS.

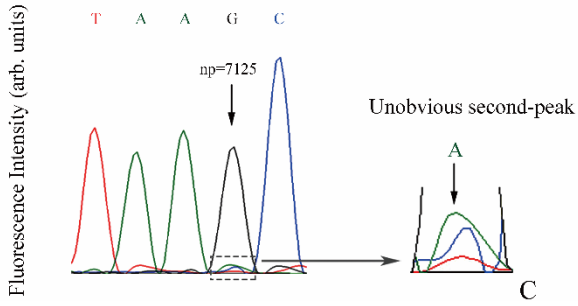
different sequencing methods were taken into account, especially the notable differences observed in the sequences of *cox1*, the species delimitation using DNA barcoding should be carried out with more caution.

Interestingly and not surprisingly, the different nucleotides obtained by the results of different sequencing methods have lower impact on the function or structure of the coding genes embracing such different sites. For the 79 different nucleotides in the sequences of 13 PCGs, the corresponding substitutions in amino acids are mainly synonymous, and only 7 changes are nonsynonymous (Table S5). For the secondary structure of rRNA, 2 of the 8 differently sequenced nucleotides in the sequences of *rrnS* situated at a base pair, which is C-G in Sanger while U-A in HTS1 (Fig. S2). For the secondary structure of *trnH*, there was a similar case in which a base pair was suggested as A-U by Sanger and as G-C by HTS (Fig. S4). These substitutions in the sequences of *rrnS* and *trnH* are compensatory.



	Sanger	HTS1	HTS2	HTS3
np=1923	T	C	C	C
Amino acid	I	I	I	I

B



	Sanger	HTS1	HTS2	HTS3
np=7125	G	G	A	A
Amino acid	L	L	L	L

D

Figure 6. Two examples of the heteroplasmic sites in Sanger sequencing which correspond to the differently sequenced sites. Panels A and B indicate the sites at which the second-peak is obviously higher than the third-peak and fourth-peak, and the base state of the second-peak can be obtained by at least one result of HTS. The different fluorescence densities of base situated at np 1923 in the *cox1* are shown in the panel A, and the panel B shows the nucleotides with amino acids at np 1923 in the results of Sanger and HTS methods. The nucleotides are C in the results of HTS sequencing, while the corresponding nucleotides are T in the results of Sanger method in both positions, and the different nucleotides lead not to the amino acids changed. Panels C and D indicate the site at the unobvious second-peak, which is slightly higher than the third-peak and fourth-peak, and the base state of the second-peak can also be obtained by at least one result of HTS. Panel C shows the unobvious second-peak at np 7125, and the nucleotide and amino acid of the site in the results of Sanger and HTS methods are shown in panel D. The amino acids are listed using single-letter amino acid abbreviations.

4.2 Heteroplasmy as the primary source for the differently sequenced mitogenomes

Previously, a series of studies have demonstrated the presence of heteroplasmy in mitochondrial DNA at various levels, which include the heteroplasmic sites between different mitogenomes within a single mitochondrion of a lysed human HL-60 cell (Reiner *et al.*, 2010), the heteroplasmic sites between different organs within an individual of zebrafish (Magalhães *et al.*, 2016), and the polymorphic organization of mitogenome between individuals of hymenopterans due to horizontal gene transfer or recombination (Sun *et al.*, 2011; Mao *et al.*, 2013). Moreover, specific primers were designed to amplify and obtained low frequency of haplotype in heteroplasmic sequences with a single mitogenome (Kastally & Mardulyn, 2017). Our study further demonstrated the heteroplasmy presenting in the genetic substances of a single water strider individual using its muscle tissue of thorax. Considering that most insects are very small and/or slender, sometimes a number of individuals from the same species would be used to ensure the quantity and the quality of the extracted genomic DNAs. In such cases, the heteroplasmy within each individual and the polymorphism between individuals of the same species are aggregated in the sample of genomic DNA and thus make the variation of sequences even more complicated.

Table 1. Detailed information for different nucleotides, together with amino acids in the *cox1* obtained by Sanger and HTS methods.

Site	Nucleotide				Amino acid			
	HTS1	HTS2	HTS3	Sanger	HTS1	HTS2	HTS3	Sanger
1468	T	T	T	C	L	L	L	L
1527	C	C	C	T	I	I	I	I
1545	A	G	G	A	Q	Q	Q	Q
1551	G	G	G	A	G	G	G	G
1587	T	T	T	C	I	I	I	I
1603*	T	T	T	A	F	F	F	I
1623	A	A	A	G	M	M	M	M
1635	T	T	T	C	I	I	I	I
1650	T	T	T	C	F	F	F	F
1704	A	A	A	C	R	R	R	R
1734	C	T	T	C	P	P	P	P
1750	T	T	T	C	L	L	L	L
1753	T	T	T	C	L	L	L	L
1773	T	C	C	T	D	D	D	D
1782	C	C	C	A	A	A	A	A
1812	C	A	A	T	L	L	L	L
1822*	A	A	A	G	I	I	I	V
1833	T	T	T	C	N	N	N	N
1860	C	C	C	T	F	F	F	F
1869	T	T	T	C	H	H	H	H
1870	T	T	T	C	L	L	L	L
1890	T	T	T	C	I	I	I	I
1905	T	T	T	C	N	N	N	N
1917	A	A	A	T	T	T	T	T
1923	C	C	C	T	I	I	I	I
1963	T	T	T	C	L	L	L	L
1971	T	T	T	A	V	V	V	V
2016	C	C	C	G	P	P	P	P
2020	T	T	T	C	L	L	L	L
2041	T	T	T	C	L	L	L	L
2058	T	T	T	C	N	N	N	N
2059*	T	C	C	C	F	L	L	L
2103	A	A	A	T	V	V	V	V
2145	T	T	T	A	V	V	V	V
2199	G	A	A	A	E	E	E	E
2205	T	T	T	A	G	G	G	G
2210	A	A	A	G	S	S	S	S
2355	A	G	G	A	A	A	A	A
2604	A	G	G	A	W	W	W	W
2727	G	A	A	G	P	P	P	P

*The heteroplasmic sites which may also lead to heteroplasmic amino acids revealed by Sanger and HTS methods. The amino acids are listed using single-letter amino acid abbreviations.

The presence of heteroplasmy in mitochondrial DNA was reflected in two aspects. On one hand, after iterative check of the chromatograms of all different nucleotides in mitochondrial genes, we found that 74 inconsistent nucleotides are apparently in heteroplasmic sites, of which the second-peak is obvious to eye (Figs 6A–B) and all can be recovered by at

least one of the corresponding results of HTS method. Meanwhile, there are other 43 heteroplasmic sites of which the second-peak is not obvious but can also be recovered by at least one of the corresponding results of HTS method (Figs 6C–D). Moreover, the ratio of such two types of heteroplasmic sites accounts for 94% of all different nucleotides in the coding genes of mitogenome between the two sequencing methods. On the other hand, according to the differently sequenced sites revealed by Sanger and the three assembly sequences of HTS, 90% of them are suggested to be heteroplasmy ones at which one of the suboptimal bases is the result reached by Sanger method (Table S6). However, as for the 29 different nucleotides situated in the control region, we cannot detect the discernible second-peak at all. Due to the control region is noncoding gene of mitogenome, we could not rule out the probability of pseudogenes.

For the complete sequences of *cox1*, which have the most different nucleotides between the two sequencing methods, 95% of the heteroplasmic sites exhibit obvious second-peak in the chromatogram of *cox1*. And according to such sites, their heteroplasmy can also be witnessed by the assembly results of HTS method but the dominated nucleotide state is different at least in one of the three repeats (Table S6). Furthermore, the 25 different cloning sequences of *cox1* verified mitogenomic heteroplasmy within an insect individual, and the polymorphism sites among cloning sequences were high in accordance with the different sites exhibited obvious second-peak in direct Sanger sequencing without cloning (Fig. 5). It suggested that the different nucleotides between the two sequencing methods were resulted from the mitogenomic heteroplasmy. Surprisingly, the polymorphism sites among cloning sequences concentrated in the 5'-end fragment of *cox1* gene, i.e., the regular barcode fragment, which warn researchers to be cautious of using HTS results in the study of ecology and evolution (Fig. 5). Moreover, the coverage of the nucleotides at each position in the three repetitions of HTS were shown in Figure S5, and the average coverage of the notably heteroplasmic nucleotides in the sequences of *cox1* among three HTS results with 310 reads (>10-fold redundancy) (Timbó *et al*., 2017). Therefore, the primary source for the differently sequenced mitogenomes obtained by Sanger and HTS methods is mainly due to the objective existence of heteroplasmy.

5 Conclusion

In summary, it is worthy to notice that there may exist significant systematic differences in mitogenomic sequence of insects between Sanger and PCR-free HTS methods, especially in the *cox1*, which is one of the common used markers in all kinds of ecological and evolutionary research. In the background that HTS is becoming more and more popular while Sanger method is still widely used, researchers should be very cautious in using the mixed data of a gene given by different sequencing methods, which could be hard to identify and eliminate the potential impact of different sequencing methods.

Funding This project was supported by the National Natural Science Foundation of China (31222051).

Acknowledgements We sincerely thank Mr. Hongxu Mi and Ms. Shuai Jiang (Yinggeling National Nature Reserve, China) for their help in collecting *Gigantometra gigas*. We also thank Prof. Dawei Huang (Nankai University, China) and Mr. Fei Ye (Sun Yat-sen University, China) for helpful discussion. All authors contributed critically to the drafts and gave final approval for publication.

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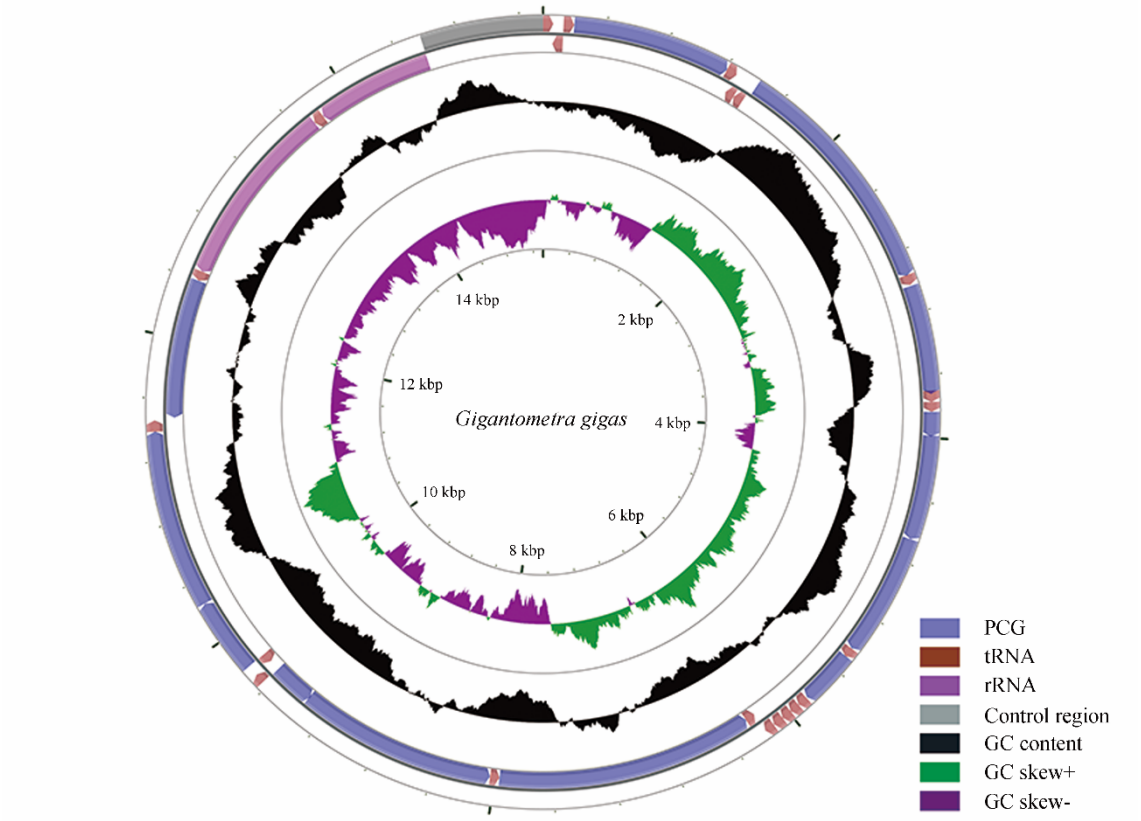

Figure S1. Map of the *Gigantometra gigas* mitogenome using Sanger method (GenBank accession number: MF177288). Genes in the outer circle indicate the direction of transcription of the majority strand (J-strand), and those in the inner circle indicate that of the minority strand (N-strand). The GC content, GC skew+, and GC skew- are separately shown in the circle.

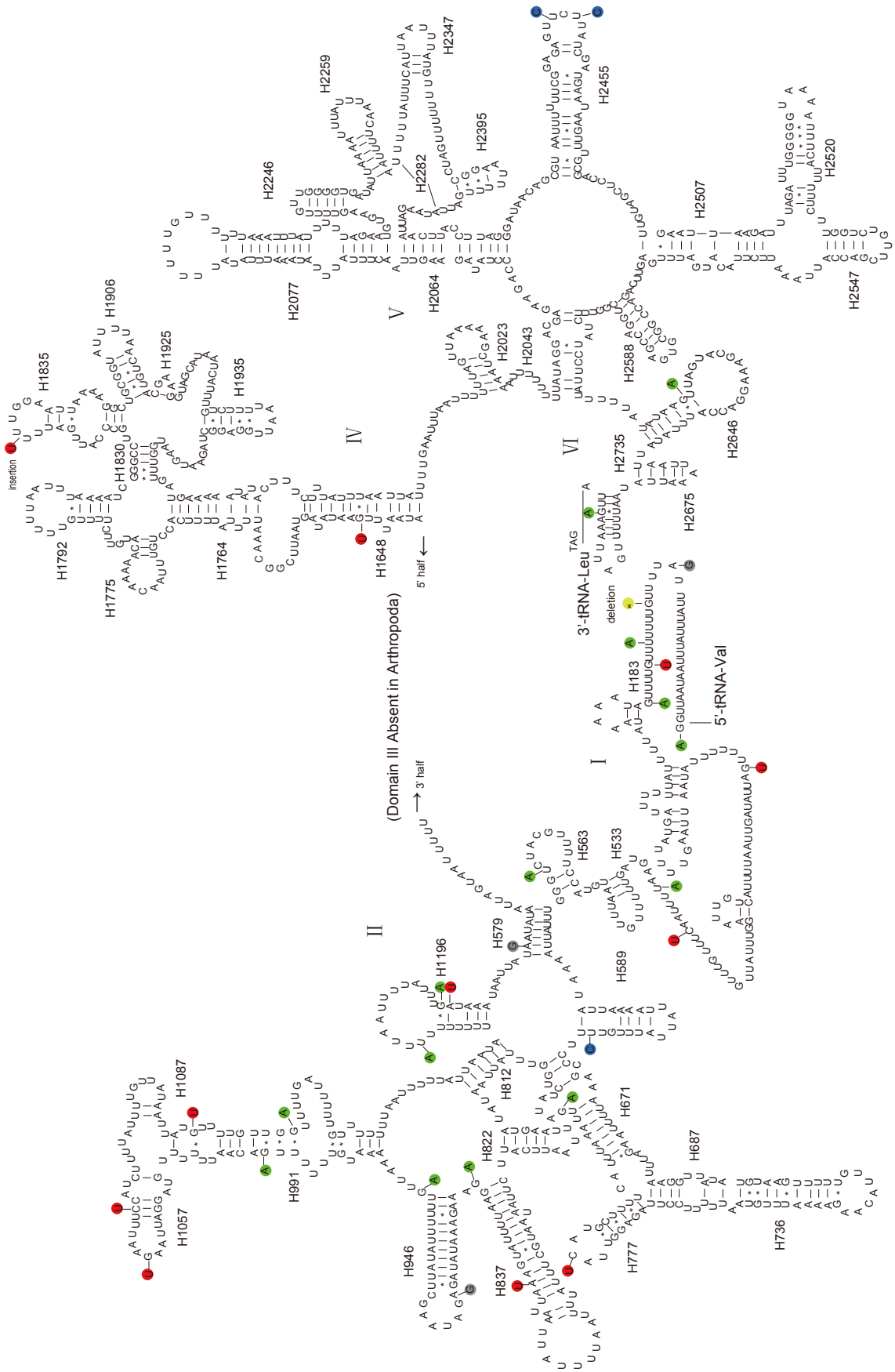

Figure S2. Inferred secondary structure of *rrnS* in *G. gigas* obtained by the results of Sanger and HTS1. The nucleotides in color represent the differences in the results of HTS1 compared wih Sanger. Base pairing is indicated as follows: standard canonical pairs by lines (C - G, G - C, A - U, U - A); wobble GU pairs by asterisks (e.g. G * U); other non-canonical pairs by open circles (e.g. A ○ G, A ○ C, U ○ U).

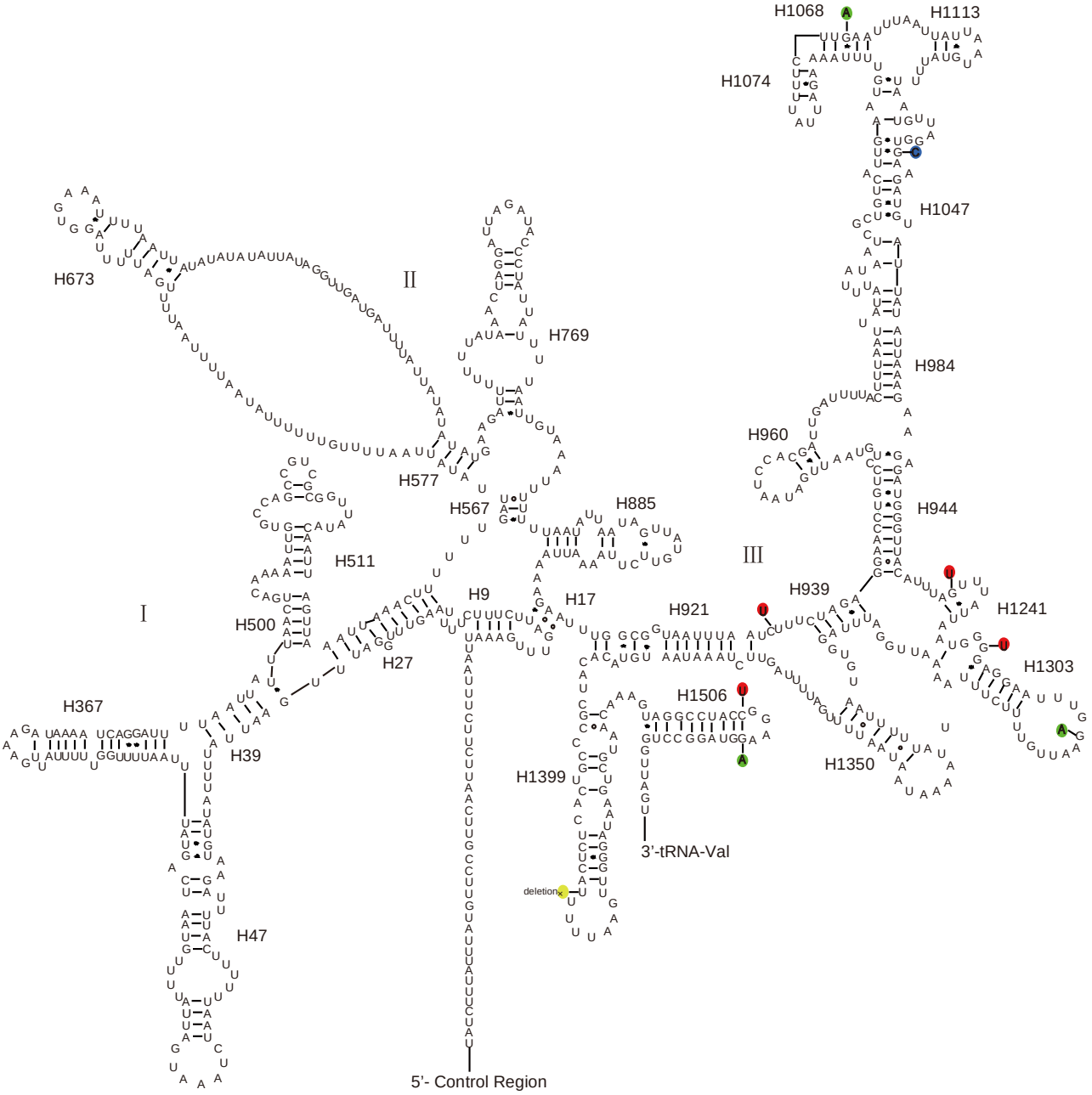

Figure S3. Inferred secondary structure of *rrnL* in *G. gigas* obtained by the results of Sanger and HTS1. The nucleotides in color represent the differences in the results of HTS1 compared with Sanger. Base pairing is indicated as follows: standard canonical pairs by lines (C - G, G - C, A - U, U - A); wobble GU pairs by asterisks (e.g. G * U); other non-canonical pairs by open circles (e.g. A ○ G, A ○ C, U ○ U).

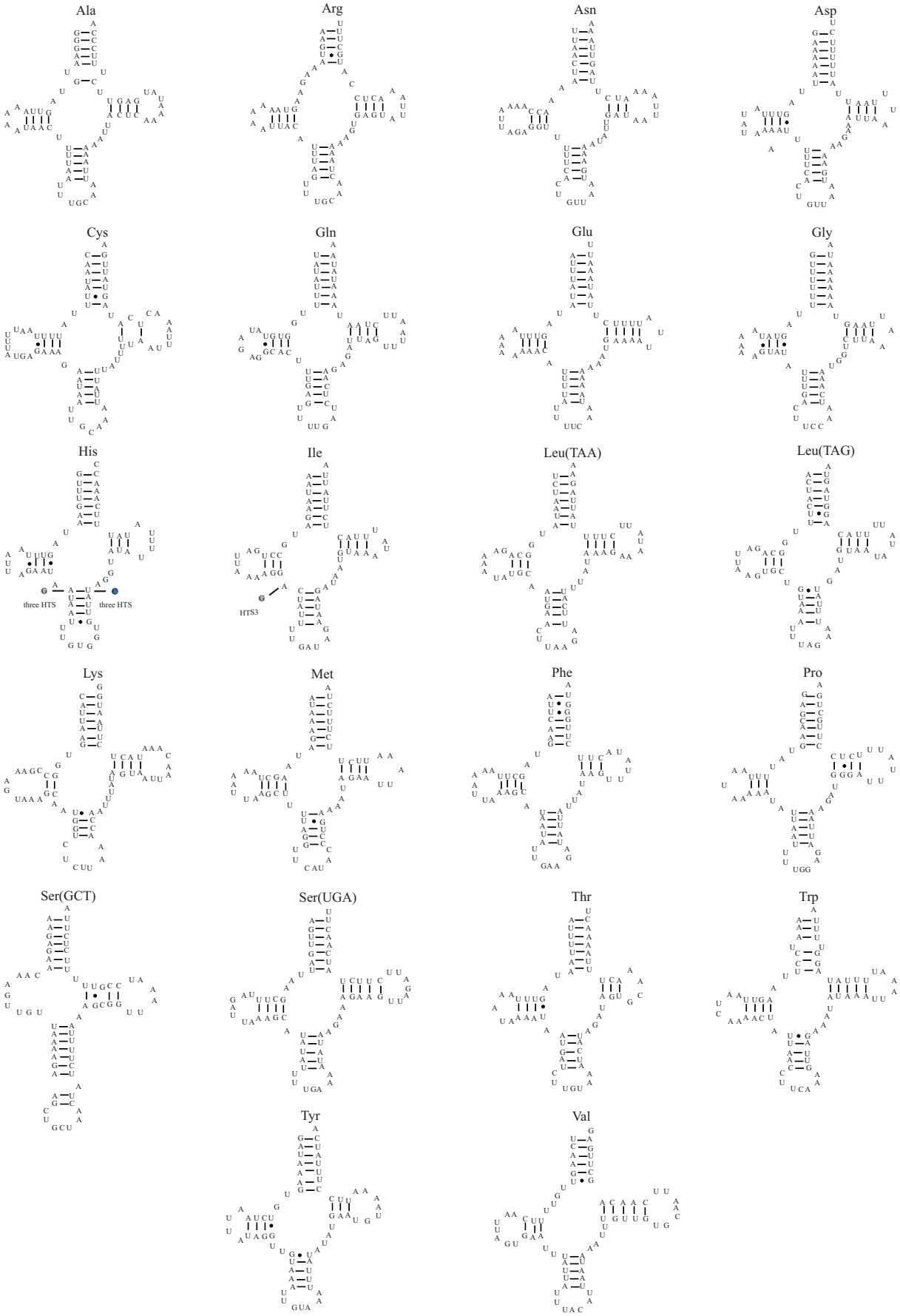

Figure S4. Inferred secondary structures of 22 tRNAs of *G. gigas* in the results of Sanger and HTS methods. The nucleotides in color stand for the results of HTS method. The tRNAs are labeled with the abbreviations of their corresponding amino acids.

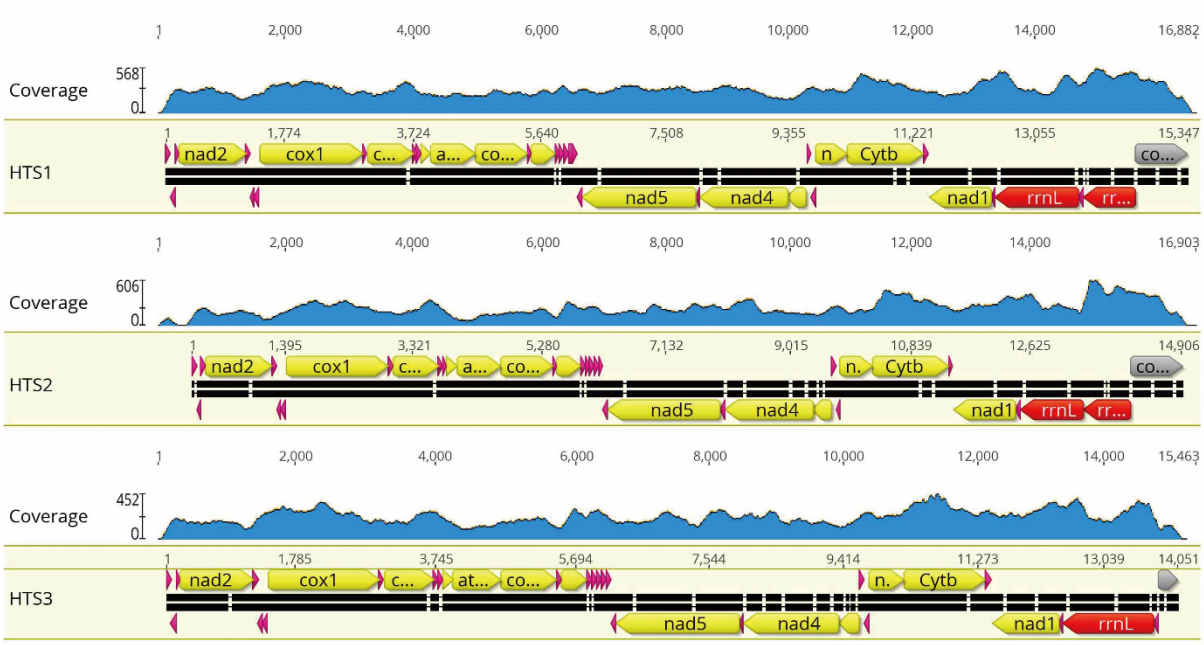

Figure S5. The coverage of short fragments at each position in the assembly results of HTS. The three results of HTS method were separately used as reference sequences to be mapped back onto the corresponding HTS scaffolds, and the mitochondrial genes were shown below the corresponding coverage. The scale bar had an indicator at the mean coverage level and the coverage for each nucleotide position was indicated by the height of the blue line.

Table S1. The primers used in Sanger method.

Fragment	Primer name	Nucleotide sequence (5'-3')
nad2-cox1	GP1	ATAAAAATCTCAACAAAAACTATAGCACTTTC
	GPA1	TGGATCTCCTCCACCAGCA
cox1-cox2	GP2	GTAAATTTTATTTCCACAATTATCAATATAG
	GPA2	TTAGTAGGTTTTATATATGATTCAAATTCAA
cox2-cox3	GP3	ATGAAATTATAAAAAATGAATTTCGC
	GPA3	TATTCCTCATTTTAATCCATTTGTTAC
cox3-nad5	GP4	CAAGCATATGTATTCTCAATTTTAACTACTC
	GPA4	TTGGTTTTAGTTAAAAGCATGGAG
nad5-nad4	GP5	TTATTATAACAATATGAATTATTGTCATAAAC
	GPA5	TAATTTTTCTTGTAATTATATCAATTATAAGAGGC
nad4-nad4L	GP6	ACTGAAACGAATTAATAAATAAACACCAG
	GPA6	CTGAACGTTTGTCTTCCGGGTT
nad4L-Cytb	GP7	CATAAACAATATTAGCTAAACAGAATAAACC
	GPA7	AATTTATAATTTCATTTGAAGCAATTCTGG
Cytb-nad1	GP8	TATCAACATTAATTATAATAATAAAACACCCA
	GPA8	CGGAAGGTGAGTCAGAGTTAGTTT
nad1-rrnL	GP9	TTTAGATTAAAGCATATATTTTGAAAATATAAG
	GPA9	CAGCGTAATTTTTTCGGAGAGTC
rrnL-rrnS	GP10	GTTATCCCTAAGGTAAGTTATTCTTATAATCAA
	GPA10	AGTATTTAATTTTGGTTTTTATTGAAAGA
rrnS-CR	GP11	AATTAAAATAATAGGGTATCTAATCCTAGTTT
	GPA11	TTGATCTTATTCTTTGGATAAGAAAA

Table S2. Data of heteropteran species downloaded from GenBank used in assembling sequences.

Mitogenome Infraorder-level	Family-level	Species	Accession number
Dipsocoromorpha	Schizopteridae	*Kokeshia xiei*	JN989542
Gerrormorpha	Gerridae	*Aquarius paludum*	NC012841
Gerrormorpha	Veliidae	*Entomovelias* sp.	KP400582
Gerrormorpha	Hydrometridae	*Hydrometra greeni*	FJ456945
Gerrormorpha	Gerridae	*Ptilomera tigrina*	KP400583
Nepomorpha	Aphelocheiridae	*Aphelocheirus ellipsoideus*	FJ456939
Nepomorpha	Belostomatidae	*Diplonychus rusticus*	FJ456940
Nepomorpha	Helotrephidae	*Helotrephes* sp.	FJ456951
Nepomorpha	Naucoridae	*Ilyocoris cimicoides*	FJ456947
Nepomorpha	Belostomatidae	*Lethocerus deyrollei*	JQ910985
Nepomorpha	Nepidae	*Nepa hoffmanni*	KT031802
Nepomorpha	Gelastocoridae	*Nerthra indica*	FJ456943
Nepomorpha	Ochteridae	*Ochterus marginatus*	FJ456950
Nepomorpha	Pleidae	*Paraplea frontalis*	KJ027516
Nepomorpha	Corixidae	*Sigara septemlineata*	FJ456941
Leptopodomorpha	Saldidae	*Saldula arsenjevi*	EU427345
Cimicomorpha	Miridae	*Adelphocoris lineolatus*	KU234537
Cimicomorpha	Cimicidae	*Cimex lectularius*	JQ739180
Cimicomorpha	Tingidae	*Pseudacysta perseae*	KM278221
Cimicomorpha	Tingidae	*Corythucha ciliata*	KC756280
Pentatomomorpha	Pentatomidae	*Erthesina fullo*	JQ743673

Table S2 (continued)

Mitogenome				
Infraorder-level	Family-level	Species	Accession number	
Pentatomomorpha	Tessaratomidae	*Eusthenes cupreus*	JQ910983	
Pentatomomorpha	Lygaeidae	*Geocoris pallidipennis*	EU427336	
Pentatomomorpha	Alydidae	*Riptortus pedestris*	EU427344	
Nuclear ribosomal DNA				
Infraorder-level	Family-level	Species	Accession number (18S)	Accession number (28S)
Enicocephalomorpha	Aenictopecheidae	*Aenictopecheidae* sp.	KJ461282	KJ461277
Enicocephalomorpha	Enicocephalidae	*Enicocephalus cubanus*	KJ461314	KJ461254
Enicocephalomorpha	Enicocephalidae	*Henschiella* sp.	KJ461295	KJ461284
Enicocephalomorpha	Enicocephalidae	*Stenopirates* sp.	KJ461304	JF719821
Dipsocoromorpha	Ceratocombidae	*Ceratocombus* sp.	KF781205	KJ461241
Dipsocoromorpha	Dipsocoridae	*Cryptostemma wygodzinskyi*	KJ461279	KJ461206
Gerromorpha	Hebridae	*Hebrus nipponicus*	KJ461283	KJ461253
Gerromorpha	Hebridae	*Hebrus* sp.	KJ461256	KJ461177
Gerromorpha	Hydrometridae	*Hydrometra greeni*	KJ461250	KJ461183
Gerromorpha	Mesoveliidae	*Mesovelia sp*	KJ461234	KJ461234
Gerromorpha	Veliidae	*Microvelia douglasi*	KJ461281	KJ461178
Gerromorpha	Gerridae	*Ptilomera tigrina*	JF719825	JF719825
Nepomorpha	Aphelocheiridae	*Aphelocheirus ellipsoideus*	KJ461184	KJ461297
Nepomorpha	Belostomatidae	*Diplonychus rusticus*	KJ461227	KJ461265
Nepomorpha	Naucoridae	*Heleocoris ovatus*	KJ461214	KJ461240
Nepomorpha	Gelastocoridae	*Nerthra indica*	KJ461313	KJ461276
Nepomorpha	Nepidae	*Ranatra chinensis*	KJ461186	JF719824
Nepomorpha	Corixidae	*Sigara* sp.	KJ461264	KJ461272
Leptopodomorpha	Saldidae	*Saldula saltatoria*	KJ461170	KJ461213
Leptopodomorpha	Leptopodidae	*Valleriola buenoi*	KJ461310	KJ461290
Leptopodomorpha	Leptopodidae	*Valleriola* sp.	KJ461202	KJ461204
Cimicomorpha	Miridae	*Adelphocoris lineolatus*	AY25229	KJ461163
Cimicomorpha	Anthocoridae	*Anthocoris pilosus*	KJ461226	KJ461268
Cimicomorpha	Miridae	*Argenis* sp.	KJ461231	KJ461244
Cimicomorpha	Reduviidae	*Cethera musiva*	KJ461289	KJ461185
Cimicomorpha	Tingidae	*Corythucha ciliata*	KJ461201	KJ461175
Pentatomomorpha	Lygaeidae	*Arocatus melanocephalus*	KJ461273	KJ461308
Pentatomomorpha	Pentatomidae	*Eurydema maracandica*	JX997807	JX997806
Pentatomomorpha	Alydidae	*Megalotomus ornaticeps*	KJ461221	KJ461236
Pentatomomorpha	Largidae	*Physopelta gutta gutta*	KJ461164	KJ461225

Table S3. Organization of mitogenome of *G. gigas* in the results of Sanger method.

Gene	Strand	Position	Anticodon	Size (bp)	Start codon	Stop codon	Intergenic nucleotides
trnI	J	1–64	GAT	64			
trnQ	N	62–130	TTG	69			-3
trnM	J	130–196	CAT	67			-1
nad2	J	197–1204		1008	ATA	TAA	0
trnW	J	1204–1272	TCA	69			-1
trnC	N	1265–1326	GCA	62			-8
trnY	N	1327–1391	GUA	65			0
cox1	J	1417–2961		1545	ATA	TAA	25

Table S3 (continued)

Gene	Strand	Position	Anticodon	Size (bp)	Start codon	Stop codon	Intergenic nucleotides
trnL1	J	2957–3023	TAA	67			-5
cox2	J	3030–3722		693	ATA	TAA	6
trnK	J	3703–3773	CTT	71			-20
trnD	J	3773–3835	GTC	63			-1
atp8	J	3836–3991		156	ATA	TAA	0
atp6	J	3985–4651		667	ATG	T	-7
cox3	J	4652–5435		784	ATG	T-tRNA	0
trnG	J	5436–5498	TCC	63			0
nad3	J	5496–5852		357	ATA	TAA	-3
trnA	J	5857–5922	TGC	66			4
trnR	J	5923–5987	TCG	65			0
trnN	J	5987–6053	GTT	67			-1
trnS1	J	6053–6121	GCT	69			-1
trnE	J	6123–6187	TTC	65			1
trnF	N	6187–6252	GAA	66			-1
nad5	N	6252–7964		1713	TTG	TAA	-1
trnH	N	7965–8027	GTG	63			0
nad4	N	8028–9351		1324	ATG	T	0
nad4L	N	9345–9629		285	ATA	TAA	-6
trnT	J	9638–9699	TGT	62			9
trnP	N	9700–9765	TGG	66			0
nad6	J	9769–10252		484	ATG	T	3
Cytb	J	10253–11389		1137	ATG	TAG	0
trnS2	J	11388–11459	TGA	76			-2
nad1	N	11475–12401		927	ATT	TAA	15
trnL2	N	12402–12466	TAG	65			0
rrnL	N	12467–13716		1250			0
trnV	N	13717–13787	TAC	71			0
rrnS	N	13788–14574		787			0
CR		14575–15347		773			0

Table S4. Nucleotide composition of mitogenome obtained by the results of Sanger and HTS methods.

Feature	Sequencing	Length	A%	C%	G%	T%	N%	A+T%	AT-skew	GC-skew
Whole	Sanger	15348	44.02	13.86	9.12	32.99	0	77.01	0.143	-0.206
	HTS1	15347	44.05	13.68	9.11	33.16	0	77.21	0.141	-0.201
	HTS2	14906*	43.93*	13.70*	9.13*	33.23*	0.01*	77.16*	0.139*	-0.200*
	HTS3	14051*	44.27*	13.34*	9.27*	33.04*	0.08*	77.31*	0.145*	-0.180*
PCGs	Sanger	11080	44.17	13.58	9.50	32.74	0	76.91	0.149	-0.177
	HTS1	11080	44.16	13.49	9.49	32.86	0	77.02	0.147	-0.174
	HTS2	11080	44.14	13.53	9.50	32.82	0	76.96	0.147	-0.175
	HTS3	11080	44.14	13.49	9.50	32.81	0.05	76.95	0.147	-0.174
PCGs_J	Sanger	6831	39.29	14.11	10.54	36.06	0	75.35	0.043	-0.145
	HTS1	6831	39.28	13.97	10.51	36.25	0	75.53	0.040	-0.141
	HTS2	6831	39.22	14.01	10.57	36.20	0	75.42	0.040	-0.140
	HTS3	6831	39.22	14.01	10.57	36.20	0	75.42	0.040	-0.140
PCGs_N	Sanger	4249	52.01	12.73	7.84	27.42	0	79.43	0.310	-0.238
	HTS1	4249	52.01	12.73	7.84	27.42	0	79.43	0.310	-0.238

Table S4 (continued)

Feature	Sequencing	Length	A%	C%	G%	T%	N%	A+T%	AT-skew	GC-skew
	HTS2	4249	52.06	12.76	7.79	27.39	0	79.45	0.311	-0.242
	HTS3	4249	52.06	12.66	7.79	27.35	0.14	79.41	0.311	-0.238
tRNAs	Sanger	1461	39.56	8.90	12.87	38.67	0	78.23	0.011	0.182
	HTS1	1461	39.49	8.97	12.94	38.60	0	78.09	0.011	0.181
	HTS2	1390*	39.34*	8.63*	13.03*	38.99*	0	78.33*	0.004*	0.203*
	HTS3	1461	39.43	8.97	13.00	38.60	0	78.03	0.011	0.183
tRNAs_J	Sanger	935	38.72	5.03	15.61	40.64	0	79.36	-0.024	0.513
	HTS1	935	38.72	5.03	15.61	40.64	0	79.36	-0.024	0.513
	HTS2	935	38.72	5.03	15.61	40.64	0	79.36	-0.024	0.513
	HTS3	935	38.61	5.03	15.72	40.64	0	79.25	-0.026	0.515
tRNAs_N	Sanger	526	41.06	15.78	7.98	35.17	0	76.23	0.077	-0.328
	HTS1	526	40.87	15.97	8.17	34.98	0	75.85	0.078	-0.323
	HTS2	455*	40.65*	16.04*	7.69*	35.60*	0	76.25*	0.066*	-0.352*
	HTS3	526	40.87	15.97	8.17	34.98	0	75.85	0.078	-0.323
rRNAs	Sanger	2038	47.69	14.43**	7.51	30.37**	0	78.06**	0.222**	-0.315**
	HTS1	2037	47.96	13.50**	7.51	31.03**	0	78.99**	0.214**	-0.285**
	HTS2	1667*	47.75*	13.38*	7.38*	31.43*	0.06*	79.18*	0.206*	-0.289*
	HTS3	1256*	48.65*	13.14*	7.32*	30.49*	0.40*	79.14*	0.229*	-0.284*
CR	Sanger	773	35.45	20.31	6.60	37.65	0	73.10	-0.030	-0.509
	HTS1	773	35.45	20.31	6.60	37.65	0	73.10	-0.030	-0.509
	HTS2	773	35.58	20.44	6.47	37.52	0	73.10	-0.027	-0.519
	HTS3	257*	40.08*	16.34*	5.06*	38.52*	0*	78.60*	0.020*	-0.553*
atp6	Sanger	667	40.18	13.64	8.55	37.63	0	77.81	0.033	-0.229
	HTS1	667	40.18	13.64	8.55	37.63	0	77.81	0.033	-0.229
	HTS2	667	40.03	13.79	8.55	37.63	0	77.66	0.031	-0.235
	HTS3	667	40.03	13.79	8.55	37.63	0	77.66	0.031	-0.235
atp8	Sanger	156	50.64	8.33	7.05	33.97	0	84.61	0.197	-0.083
	HTS1	156	50.64	8.33	7.05	33.97	0	84.61	0.197	-0.083
	HTS2	156	50.00	9.62	7.05	33.33	0	83.33	0.200	-0.154
	HTS3	156	50.00	9.62	7.05	33.33	0	83.33	0.200	-0.154
cox1	Sanger	1545	33.72**	15.99**	14.05**	36.25**	0	69.97**	-0.036**	-0.065**
	HTS1	1545	33.66**	15.28**	13.92**	37.15**	0	70.81**	-0.049**	-0.047**
	HTS2	1545	33.66	15.28	13.98	37.09	0	70.75	-0.048	-0.044
	HTS3	1545	33.66	15.28	13.98	37.09	0	70.75	-0.048	-0.044
cox2	Sanger	693	41.70	14.43	10.68	33.19	0	74.89	0.114	-0.149
	HTS1	693	41.70	14.57	10.68	33.04	0	74.74	0.116	-0.154
	HTS2	693	41.70	14.57	10.68	33.04	0	74.74	0.116	-0.154
	HTS3	693	41.70	14.57	10.68	33.04	0	74.74	0.116	-0.154
cox3	Sanger	784	36.22	15.94	13.14	34.69	0	70.91	0.022	-0.096
	HTS1	784	36.22	15.94	13.14	34.69	0	70.91	0.022	-0.096
	HTS2	784	35.97	16.07	13.39	34.57	0	70.54	0.020	-0.091
	HTS3	784	35.97	16.07	13.39	34.57	0	70.54	0.020	-0.091
Cytb	Sanger	1137	35.00	15.30	11.61	38.08	0	73.08	-0.042	-0.137
	HTS1	1137	35.00	15.30	11.61	38.08	0	73.08	-0.042	-0.137
	HTS2	1137	35.09	15.48	11.61	37.82	0	72.91	-0.037	-0.143
	HTS3	1137	35.09	15.48	11.61	37.82	0	72.91	-0.037	-0.143
nad1	Sanger	927	52.86	14.35	7.55	25.24	0	78.10	0.354	-0.311

Table S4 (continued)

Feature	Sequencing	Length	A%	C%	G%	T%	N%	A+T%	AT-skew	GC-skew
	HTS1	927	52.86	14.35	7.55	25.24	0	78.10	0.354	-0.311
	HTS2	927	52.86	14.35	7.55	25.24	0	78.10	0.354	-0.311
	HTS3	927	52.86	14.35	7.55	25.24	0	78.10	0.354	-0.311
nad2	Sanger	1008	46.33	12.10	6.55	35.02	0	81.35	0.139	-0.298
	HTS1	1008	46.33	12.10	6.55	35.02	0	81.35	0.139	-0.298
	HTS2	1008	46.23	11.90	6.65	35.22	0	81.45	0.135	-0.283
	HTS3	1008	46.23	11.90	6.65	35.22	0	81.45	0.135	-0.283
nad3	Sanger	357	42.86	14.01	8.40	34.73	0	77.59	0.105	-0.250
	HTS1	357	42.86	14.01	8.40	34.73	0	77.59	0.105	-0.250
	HTS2	357	42.86	13.73	8.40	35.01	0	77.87	0.101	-0.241
	HTS3	357	42.86	13.73	8.40	35.01	0	77.87	0.101	-0.241
nad4	Sanger	1324	53.63	12.46	7.63	26.28	0	79.91	0.342	-0.240
	HTS1	1324	53.63	12.46	7.63	26.28	0	79.91	0.342	-0.240
	HTS2	1324	53.78	12.39	7.48	26.36	0	80.14	0.342	-0.247
	HTS3	1324	53.78	12.08	7.48	26.21	0.45	79.99	0.345	-0.235
nad4L	Sanger	285	55.09	13.68	5.26	25.96	0	81.05	0.359	-0.445
	HTS1	285	55.09	13.68	5.26	25.96	0	81.05	0.359	-0.445
	HTS2	285	55.09	13.68	5.26	25.96	0	81.05	0.359	-0.445
	HTS3	285	55.09	13.68	5.26	25.96	0	81.05	0.359	-0.445
nad5	Sanger	1713	49.80	11.91	8.58	29.71	0	79.51	0.253	-0.163
	HTS1	1713	49.80	11.91	8.58	29.71	0	79.51	0.253	-0.163
	HTS2	1713	49.80	12.03	8.58	29.60	0	79.40	0.254	-0.167
	HTS3	1713	49.80	12.03	8.58	29.60	0	79.40	0.254	-0.167
nad6	Sanger	484	46.49	8.68	6.20	38.64	0	85.13	0.092	-0.167
	HTS1	484	46.49	8.68	6.20	38.64	0	85.13	0.092	-0.167
	HTS2	484	46.49	8.68	6.20	38.64	0	85.13	0.092	-0.167
	HTS3	484	46.49	8.68	6.20	38.64	0	85.13	0.092	-0.167
rrnS	Sanger	788	46.07**	15.10**	7.99**	30.84**	0	76.91**	0.198**	-0.308**
	HTS1	787	46.50**	14.36**	7.88**	31.25**	0	77.75**	0.196**	-0.291**
	HTS2	703*	48.51*	13.09*	6.54*	31.72*	0.14*	80.23*	0.209*	-0.333*
	HTS3	6*	50.00*	33.33*	0.00*	16.67*	0*	66.67*	0.500*	-1.00*
rrnL	Sanger	1250	48.72**	14.00**	7.20**	30.08**	0	78.80**	0.237**	-0.321**
	HTS1	1250	48.88**	12.96**	7.28**	30.88**	0	79.76**	0.226**	-0.281**
	HTS2	964*	47.20*	13.59*	7.99*	31.22*	0*	78.42*	0.204*	-0.259*
	HTS3	1250	48.64	13.04	7.36	30.56	0.40	79.20	0.228	-0.278

*The incomplete sequences of the genes in the results of Sanger and HTS methods.

**The notable differences in the results of Sanger and HTS methods.

Table S5. The different nucleotides of complete mitogenomic sequences in the results of Sanger and HTS methods. The amino acids are listed using single-letter amino acid abbreviations.

Gene	Site	Sanger	aa	HTS1	aa	HTS2	aa	HTS3	aa	Height of second-peak**	NL***
trnI	22	A		A		A		G		obvious	G
nad2	255*	C	T	C	T	T	M	T	M	×	
	550	C	N	C	N	T	N	T	N	unobvious	T
	627*	A	N	A	N	G	S	G	S	unobvious	G
cox1	1468	C	L	T	L	T	L	T	L	obvious	T
	1527	T	I	C	I	C	I	C	I	obvious	C

Table S5 (continued)

Gene	Site	Sanger	aa	HTS1	aa	HTS2	aa	HTS3	aa	Height of second-peak**	NL***
	1545	A	Q	A	Q	G	Q	G	Q	obvious	G
	1551	A	G	G	G	G	G	G	G	obvious	G
	1587	C	I	T	I	T	I	T	I	obvious	T
	1603*	A	I	T	F	T	F	T	F	obvious	T
	1623	G	M	A	M	A	M	A	M	obvious	A
	1635	C	I	T	I	T	I	T	I	obvious	T
	1650	C	F	T	F	T	F	T	F	obvious	T
	1704	C	R	A	R	A	R	A	R	obvious	A
	1734	C	P	C	P	T	P	T	P	obvious	T
	1750	C	L	T	L	T	L	T	L	obvious	T
	1753	C	L	T	L	T	L	T	L	obvious	T
	1773	T	D	T	D	C	D	C	D	obvious	C
	1782	A	A	C	A	C	A	C	A	obvious	C
	1812	T	L	C	L	A	L	A	L	obvious	A
	1822*	G	V	A	I	A	I	A	I	obvious	A
	1833	C	N	T	N	T	N	T	N	obvious	T
	1860	T	F	C	F	C	F	C	F	obvious	C
	1869	C	H	T	H	T	H	T	H	obvious	T
	1870	C	L	T	L	T	L	T	L	obvious	T
	1890	C	I	T	I	T	I	T	I	obvious	T
	1905	C	N	T	N	T	N	T	N	obvious	T
	1917	T	T	A	T	A	T	A	T	obvious	A
	1923	T	I	C	I	C	I	C	I	obvious	C
	1963	C	L	T	L	T	L	T	L	obvious	T
	1971	A	V	T	V	T	V	T	V	obvious	T
	2016	G	P	C	P	C	P	C	P	obvious	C
	2020	C	L	T	L	T	L	T	L	obvious	T
	2041	C	L	T	L	T	L	T	L	obvious	T
	2058	C	N	T	N	T	N	T	N	obvious	T
	2059*	C	L	T	F	C	L	C	L	×	
	2103	T	V	A	V	A	V	A	V	obvious	A
	2145	A	V	T	V	T	V	T	V	obvious	T
	2199	A	E	G	E	A	E	A	E	×	
	2205	A	G	T	G	T	G	T	G	obvious	T
	2210	G	S	A	S	A	S	A	S	obvious	A
	2355	A	A	A	A	G	A	G	A	obvious	G
	2604	A	W	A	W	G	W	G	W	obvious	G
	2727	G	P	G	P	A	P	A	P	obvious	A
cox2	3429	T	L	T	L	C	L	C	L	unobvious	**A****
	3473	C	I	C	I	T	I	T	I	obvious	T
	3602	T	Y	C	Y	T	Y	T	Y	unobvious	A/C
	3632	T	N	T	N	C	N	C	N	unobvious	C
atp8	3948*	A	N	A	N	C	T	C	T	unobvious	C
	3970	T	N	T	N	C	N	C	N	unobvious	C
atp6	4482	A	G	A	G	C	G	C	G	unobvious	C
cox3	4786	A	L	A	L	G	L	G	L	obvious	G
	4810	T	T	T	T	C	T	C	T	obvious	C

Table S5 (continued)

Gene	Site	Sanger	aa	HTS1	aa	HTS2	aa	HTS3	aa	Height of second-peak**	NL***
	5341	A	K	A	K	G	K	G	K	obvious	G
nad3	5801	C	I	C	I	T	I	T	I	unobvious	T
nad3	5801	C	I	C	I	T	I	T	I	unobvious	T
nad5	6897	C	G	C	G	T	G	T	G	unobvious	T/G
	7125	G	L	G	L	A	L	A	L	unobvious	A
	7149	A	L	A	L	G	L	G	L	unobvious	G
	7272	T	I	T	I	C	I	C	I	×	
	7353	T	P	T	P	C	P	C	P	obvious	C
	7539	T	T	T	T	C	T	C	T	obvious	C
	7720*	G	S	G	S	A	L	A	L	obvious	A
	7737	A	S	A	S	G	S	G	S	obvious	G
trnH	7988	A		G		G		G		obvious	G
	8004	T		C		C		C		obvious	C
nad4	8395	G	S	G	S	A	S	A	S	unobvious	A
	8422	G	C	G	C	A	C	A	C	obvious	A
	8722	A	S	A	S	G	S	G	S	obvious	G
	8905	C	P	C	P	T	P	T	P	obvious	T
	8944	G	G	G	G	A	G	A	G	obvious	A
	8985	A	L	A	L	G	L	G	L	unobvious	G
	9130	G	S	G	S	A	S	A	S	obvious	A
Cytb	10276	T	N	T	N	C	N	C	N	unobvious	C
	10612	A	M	A	M	G	M	G	M	unobvious	G
	10613	T	M	T	M	C	M	C	M	unobvious	C
	10681	G	G	G	G	A	G	A	G	unobvious	A
	10738	C	V	C	V	T	V	T	V	obvious	T
	10745	T	W	T	W	C	W	C	W	unobvious	C
	10972	C	F	C	F	T	F	T	F	obvious	T
	11074	T	Y	T	Y	C	Y	C	Y	obvious	C
	11225	T	M	T	M	C	M	C	M	obvious	C
	11324	C	I	C	I	A	I	A	I	obvious	A
rrnL	12470	C		T		T		T		obvious	T
	12521	C		T		T		T		obvious	T
	12676	A		G		G		G		obvious	G
	12678	A		G		G		G		obvious	G
	13055	-		A		A		A			
	13143	C		T		T		T		unobvious	T
	13169	T		C		C		C		unobvious	C
	13181	T		A		A		A		unobvious	A
	13182	C		T		T		T		unobvious	T
	13195	A		T		T		T		unobvious	T
	13237	C		T		T		T		unobvious	T
	13246	C		A		A		A		unobvious	A
	13270	G		A		A		A		unobvious	A
	13276	C		A		A		A		unobvious	A
	13297	C		T		T		T		unobvious	T
	13313	C		T		C		C		obvious	T
	13332	T		C		C		C		×	

Table S5 (continued)

Gene	Site	Sanger	aa	HTS1	aa	HTS2	aa	HTS3	aa	Height of second-peak**	NL***
	13345	C		T		T		T		unobvious	T
	13357	T		A		A		A		unobvious	A
	13426	C		T		T		T		unobvious	T
	13445	G		A		-		A		obvious	A
	13522	A		G		-		G		obvious	G
	13555	A		T		-		T		unobvious	T
	13588	A		T		-		T		obvious	T
	13594	G		A		-		A		unobvious	A
	13634	C		A		-		A		unobvious	A
	13678	C		T		-		T		×	
	13683	C		A		-		G		unobvious	G
	13686	A		T		-		T		unobvious	T
	13691	C		-		-		-		×	
	13696	T		C		-		C		unobvious	C
	13716	C		T		-		T		unobvious	T
rrnS	13803	C		T		-		-		obvious	T
	13808	G		A		-		-		unobvious	A
	13846	A		-		-		-		×	
	13943	C		T		T		-		obvious	T
	13951	C		C		T		-		obvious	T
	13954	C		A		A		-		obvious	A
	13966	C		A		A		-		obvious	A
	14006	C		G		G		-		obvious	G
	14032	C		T		T		-		obvious	T
	14135	G		A		G		-		unobvious	A
CR	14589	G		G		A		-		×	
	14639	C		C		T		-		×	
	14675	C		C		T		-		×	
	14720	T		T		C		-		×	
	14789	T		T		C		-		×	
	14796	C		C		T		-		×	
	14818	T		T		C		-		×	
	14822	T		T		C		-		×	
	14920	T		T		C		-		×	
	14944	C		C		T		-		×	
	15121	T		T		T		C		×	
	15174	T		T		T		C		×	
	15178	C		C		C		T		×	
	15179	C		C		C		T		×	
	15180	T		T		T		-		×	
	15201	T		T		T		C		×	
	15226	T		T		T		A		×	
	15232	C		C		C		T		×	
	15234	A		A		A		C		×	
	15239	T		T		T		C		×	
	15242	A		A		A		T		×	
	15245	-		-		-		C			

Table S5 (continued)

Gene	Site	Sanger	aa	HTS1	aa	HTS2	aa	HTS3	aa	Height of second-peak**	NL***
	15256	G		G		G		A		×	
	15257	G		G		G		A		×	
	15262	A		A		A		T		×	
	15294	A		A		A		G		×	
	15311	G		G		G		A		×	
	15315	A		A		A		T		×	
	15326	T		T		T		A		×	

*The heteroplasmic sites which may lead to heteroplasmic amino acids.

**The meaning of obvious and unobviously are corresponding to Figure 5; the X mark means the site does not exist second-peak.

***NL stands for the nucleotide shown by second-peak; the letter with asterisks stands for nucleotied which is not in the results of Sanger and HTS methods; the slash stands for the two nucleotides with same fluorescence intensity.

Table S6. Base states of the heteroplasmy sites between the two sequencing methods in the assembly results of HTS.

Gene	Site	Sanger	HTS1				HTS2				HTS3			
			A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)
trnI	22	A	79.60	1.50	19.00	0.00	58.10	3.90	38.00	0.00	48.70	2.60	48.70	0.00
nad2	255	C	0.00	66.10	1.00	32.90	0.00	0.00	0.00	100.00	0.00	0.00	0.00	100.00
	550	C	0.30	97.90	0.00	1.70	0.60	25.90	0.00	73.50	0.00	35.50	0.00	64.50
	627	A	100.00	0.00	0.00	0.00	43.10	0.60	56.30	0.00	41.90	0.00	58.10	0.00
cox1	1468	C	0.00	23.70	0.40	75.90	0.00	37.90	0.00	62.10	0.00	36.70	0.40	63.00
	1527	T	0.00	77.30	0.00	22.70	0.00	58.90	0.00	41.10	0.00	59.90	0.30	39.80
	1545	A	99.70	0.00	0.00	0.30	43.80	0.40	54.40	1.30	45.20	0.00	54.10	0.70
	1551	A	22.00	0.00	78.00	0.00	40.60	0.80	57.70	0.80	36.30	0.00	62.40	1.40
	1587	C	0.30	20.80	0.60	78.30	0.00	35.50	0.00	64.50	0.30	28.00	0.00	71.60
	1603	A	18.20	0.00	0.00	81.80	31.80	0.00	0.00	68.20	26.30	0.00	0.30	73.40
	1623	G	82.60	0.00	17.10	0.30	66.80	0.00	33.20	0.00	75.10	0.30	24.60	0.00
	1635	C	0.30	26.70	0.00	73.00	0.40	40.10	0.40	59.10	0.00	37.90	0.30	61.80
	1650	C	0.00	22.70	0.00	77.30	0.00	38.60	0.00	61.40	0.70	31.60	0.30	67.40
	1704	C	78.30	18.20	0.00	3.40	64.90	28.80	0.00	6.30	68.50	29.50	0.00	2.00
	1734	C	0.60	98.00	0.00	1.40	1.10	39.40	0.40	59.20	0.00	36.80	0.70	62.60
	1750	C	0.30	26.00	0.00	73.70	1.30	37.50	0.00	61.20	1.40	36.20	0.00	62.40
	1753	C	0.30	26.10	0.30	73.30	1.30	39.30	0.00	59.30	0.70	38.00	0.00	61.30
	1773	T	0.00	0.80	0.60	98.60	0.90	64.70	0.00	34.40	0.40	63.00	0.00	36.70
	1782	A	21.00	72.50	1.10	5.30	25.50	65.70	0.60	8.20	35.90	61.10	0.40	2.60
	1812	T	2.20	73.90	0.00	24.00	70.00	0.00	0.00	30.00	65.10	1.10	0.00	33.80
	1822	G	74.00	0.30	25.70	0.00	69.20	0.00	30.80	0.00	64.90	0.40	34.80	0.00
	1833	C	0.00	24.10	0.30	75.60	0.00	31.20	0.60	68.20	0.40	34.40	0.40	64.80
	1860	T	0.30	74.60	0.00	25.10	0.30	66.70	0.00	33.00	0.00	65.30	0.00	34.70
	1869	C	0.00	23.20	0.30	76.50	0.00	30.10	0.00	69.90	0.00	31.50	0.00	68.50
	1870	C	0.00	20.60	0.30	79.20	0.00	24.60	0.00	75.40	0.00	31.20	0.00	68.80
	1890	C	0.00	24.50	0.00	75.50	0.00	29.50	0.00	70.50	0.00	33.60	0.00	66.40
	1905	C	0.30	24.00	0.00	75.70	0.00	27.70	0.30	71.90	0.40	33.10	0.00	66.50
	1917	T	74.90	1.50	0.30	23.30	73.10	0.70	0.00	26.20	65.30	0.40	0.40	33.90
	1923	T	0.00	74.30	0.30	25.40	0.00	70.30	0.30	29.40	0.40	64.80	0.00	34.80
	1963	C	0.30	23.80	0.00	75.90	0.00	29.30	0.00	70.70	0.00	35.00	0.70	64.30
	1971	A	24.00	0.00	0.60	75.40	29.70	0.00	1.40	68.90	33.50	0.00	0.00	66.50
	2016	G	1.90	78.40	18.90	0.80	2.30	71.80	25.10	0.80	2.10	62.30	34.90	0.70

Table S6 (continued)

Gene	Site	Sanger	HTS1				HTS2				HTS3			
			A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)
	2020	C	0.00	20.40	0.30	79.30	0.00	26.10	0.00	73.90	0.00	35.00	0.00	65.00
	2041	C	0.00	20.90	0.00	79.10	0.00	26.50	0.00	73.50	0.00	36.70	0.00	63.30
	2058	C	0.00	22.30	0.00	77.80	0.00	26.20	0.00	73.80	0.00	33.90	0.30	65.80
	2059	C	0.00	21.10	0.30	78.70	0.00	96.40	0.00	3.60	0.00	95.10	0.00	4.90
	2103	T	80.30	1.50	0.30	18.00	73.20	3.00	0.00	23.80	67.50	1.20	0.30	31.10
	2145	A	21.10	0.00	0.30	78.60	33.70	0.00	0.00	66.30	34.50	0.30	0.50	64.70
	2199	A	22.20	0.00	77.60	0.30	100.00	0.00	0.00	0.00	99.10	0.00	0.30	0.00
	2205	A	2.80	0.00	0.00	77.20	36.30	0.60	0.30	62.70	32.90	0.00	0.00	67.10
	2210	G	77.80	0.30	21.70	0.30	65.90	0.00	34.10	0.00	69.10	0.00	30.90	0.00
	2355	A	99.70	0.00	0.00	0.30	37.80	0.00	61.20	1.00	40.00	0.00	58.90	0.10
	2604	A	99.40	0.60	0.00	0.00	37.20	0.40	62.30	0.00	43.30	0.00	55.90	0.80
	2727	G	19.80	0.00	80.20	0.00	98.00	1.00	0.00	1.00	98.90	0.50	0.00	0.50
cox2	3429	T	0.00	1.40	0.00	98.60	0.00	64.40	0.00	35.60	0.00	64.00	0.50	35.40
	3473	C	0.30	81.50	0.00	18.20	0.00	0.40	0.00	99.60	0.00	0.00	0.00	100.00
	3602	T	0.00	83.20	0.00	16.80	0.00	9.80	0.30	89.90	0.00	9.90	0.00	90.10
	3632	T	0.00	17.70	0.00	82.30	0.30	97.50	0.00	2.20	0.00	95.00	0.00	5.00
atp8	3948	A	79.80	19.70	0.00	0.40	0.00	100.00	0.00	0.00	0.60	96.60	0.00	2.80
	3970	T	0.00	0.40	0.00	99.60	0.00	70.50	0.00	29.50	2.30	66.90	0.00	30.80
atp6	4482	A	94.70	0.00	0.00	5.30	1.50	93.40	2.90	2.20	0.80	92.20	0.80	6.20
cox3	4786	A	0.40	99.60	0.00	0.00	7.90	0.60	91.60	0.00	11.00	0.00	89.00	0.00
	4810	T	0.00	0.90	0.00	99.10	1.10	92.20	0.00	6.70	1.20	87.30	0.00	11.60
	5341	A	99.20	0.80	0.00	0.00	22.30	0.50	76.20	1.00	12.30	1.20	86.40	0.00
nad3	5801	C	0.00	73.70	0.00	26.30	0.00	1.20	0.00	98.80	0.40	2.50	0.00	97.10
nad5	6897	C	1.70	81.70	0.90	15.70	2.50	9.70	0.80	86.90	2.00	4.70	0.00	93.30
	7125	G	25.00	0.00	75.00	0.00	98.10	0.50	1.40	0.00	100.00	0.00	0.00	0.00
	7149	A	96.70	0.00	3.30	0.00	28.40	0.50	70.60	0.50	34.90	0.00	65.10	0.00
	7272	T	1.10	3.30	0.40	95.30	0.00	76.60	0.00	23.40	0.00	73.00	0.00	27.00
	7353	T	0.00	0.70	0.70	98.60	0.50	69.90	1.60	28.00	0.00	66.40	0.00	33.60
	7539	T	0.30	26.70	0.00	73.00	0.00	99.30	0.00	0.70	0.40	96.30	0.00	3.30
	7720	G	5.50	0.00	94.50	0.00	46.60	0.00	53.40	0.00	42.70	0.00	56.90	0.30
	7737	A	98.40	0.00	1.60	0.00	57.00	0.00	43.00	0.00	65.50	0.00	34.50	0.00
trnH	7988	A	34.20	0.00	65.80	0.00	70.20	0.00	29.30	0.40	71.60	0.00	28.40	0.00
	8004	T	1.50	64.30	0.00	34.20	0.00	30.20	0.00	69.80	99.60	0.40	0.00	0.00
nad4	8395	G	43.10	0.30	56.60	0.00	99.70	0.00	0.00	0.30	100.00	0.00	0.00	0.00
	8422	G	41.50	0.00	58.20	0.30	98.60	0.00	1.40	0.00	98.50	0.00	1.50	0.00
	8722	A	100.00	0.00	0.00	0.00	43.10	0.00	56.90	0.00	45.30	0.00	54.20	0.50
	8905	C	0.80	76.40	0.00	22.80	0.00	5.60	0.50	93.80	0.00	7.00	0.50	92.50
	8944	G	0.80	0.00	79.20	20.00	47.60	0.00	4.70	47.60	50.90	0.60	6.70	41.70
	8985	A	99.60	0.40	0.00	0.00	50.90	0.00	49.10	0.00	45.80	0.70	53.60	0.00
	9130	G	25.60	1.10	69.40	3.90	98.10	0.00	1.90	0.00	96.10	0.00	2.20	1.70
Cytb	10276	T	0.70	0.30	0.00	99.00	0.40	45.00	0.00	54.50	0.30	43.80	0.00	55.90
	10612	A	99.10	0.00	0.90	0.00	53.20	0.00	46.80	0.00	53.30	0.00	46.70	0.00
	10613	T	0.00	1.20	0.00	98.80	0.00	45.20	0.00	54.80	0.00	45.90	0.00	54.10
	10681	G	2.00	0.00	98.00	0.00	47.60	0.30	51.90	0.30	46.90	0.00	53.10	0.00
	10738	C	0.00	99.00	0.00	1.00	0.20	50.80	0.00	48.90	0.00	56.00	0.20	43.70
	10745	T	0.50	0.00	0.00	99.50	0.00	46.40	0.00	53.60	0.20	40.00	0.00	59.70

Table S6 (continued)

Gene	Site	Sanger	HTS1				HTS2				HTS3			
			A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)
	10972	C	0.00	99.40	0.30	0.30	0.30	51.60	0.00	48.00	0.00	53.20	0.00	46.80
	11074	T	0.00	40.80	0.00	59.20	0.00	86.50	0.00	13.50	0.00	88.30	0.00	11.70
	11225	T	0.00	3.40	0.30	96.30	3.00	45.40	0.00	51.70	0.40	47.90	0.00	51.70
	11324	C	0.00	90.00	0.40	9.60	43.20	47.70	1.10	8.00	44.80	45.90	0.00	9.30
rrnL	12470	C	2.20	37.90	0.00	59.90	2.80	58.60	0.60	38.00	4.90	60.50	0.00	34.60
	12521	C	0.00	43.50	0.00	56.50	0.30	64.60	0.50	34.60	0.60	62.80	0.60	35.90
	12676	A	2.20	0.00	97.80	0.00	1.90	0.00	96.80	1.30	2.00	0.00	97.20	0.80
	12678	A	4.80	0.00	94.70	0.40	3.70	0.30	96.00	0.00	4.20	0.50	95.30	0.00
	13055	-	99.70	0.00	0.00	0.30	99.00	0.70	0.30	0.00	99.40	0.00	0.00	0.60
	13143	C	2.80	3.60	0.80	92.80	1.60	56.20	0.00	42.20	3.00	29.50	0.00	67.50
	13169	T	3.30	82.70	1.60	12.30	0.90	67.10	0.00	32.10	1.60	50.50	0.00	47.90
	13181	T	69.80	0.90	0.00	29.30	41.70	2.50	0.00	55.80	59.60	3.90	0.50	36.00
	13182	C	2.70	27.20	0.00	70.10	3.00	55.30	0.00	41.80	3.50	34.70	0.00	61.90
	13195	A	25.30	0.50	0.00	74.20	55.30	1.90	1.00	41.80	30.80	1.50	9.00	67.70
	13237	C	3.90	26.80	0.40	68.90	1.40	44.30	0.00	54.30	0.90	44.30	0.00	54.80
	13246	C	68.80	30.80	0.40	0.00	42.90	55.30	0.90	0.90	69.90	30.10	0.00	0.00
	13270	G	77.70	0.00	21.90	0.40	49.10	0.00	50.90	0.00	57.90	0.80	41.30	0.00
	13276	C	67.50	29.10	0.00	3.40	40.50	52.70	0.00	6.80	66.00	29.40	0.00	4.60
	13297	C	1.30	31.40	0.00	67.40	0.50	1.80	0.50	97.30	0.00	33.00	0.00	67.00
	13313	C	0.80	22.00	0.00	77.20	0.00	79.00	0.00	21.00	0.00	77.70	0.00	22.30
	13332	T	1.20	79.40	0.40	19.00	0.00	62.50	0.00	37.50	0.00	58.30	0.00	41.70
	13345	C	1.20	0.80	0.40	97.90	0.50	62.40	0.00	37.00	0.40	27.80	0.00	71.80
	13357	T	77.60	0.70	1.50	20.20	62.40	0.00	2.20	35.40	60.10	0.00	0.00	39.90
	13426	C	0.80	48.00	0.00	51.20	0.00	90.40	0.00	9.60	0.60	25.80	0.30	73.20
	13445	G	87.30	0.00	12.40	0.30	-	-	-	-	75.90	0.00	23.50	0.60
	13522	A	43.80	0.20	56.00	0.00	-	-	-	-	38.90	0.30	60.30	0.50
	13555	A	18.40	0.60	0.40	80.50	-	-	-	-	27.90	0.30	0.00	71.80
	13588	A	59.60	0.00	0.00	40.40	-	-	-	-	25.40	0.00	0.00	74.60
	13594	G	25.70	0.50	0.50	73.40	-	-	-	-	55.30	0.30	43.80	0.60
	13634	C	54.10	24.40	0.00	21.50	-	-	-	-	44.80	35.10	0.00	20.10
	13678	C	2.90	42.30	1.00	53.80	-	-	-	-	0.30	42.70	0.30	56.70
	13683	C	46.30	26.10	13.90	13.70	-	-	-	-	30.00	15.80	42.90	11.30
	13686	A	84.90	0.30	0.30	14.60	-	-	-	-	78.50	0.00	0.70	20.80
	13691	C	-	-	-	-	-	-	-	-	-	-	-	-
	13696	T	3.50	38.20	0.00	58.30	-	-	-	-	3.80	74.50	0.00	21.70
	13716	C	0.30	57.50	0.00	42.20	-	-	-	-	0.00	19.80	0.00	80.20
rrnS	13803	C	1.30	62.50	0.00	36.10	-	-	-	-	-	-	-	-
	13808	G	52.90	0.80	45.00	1.30	-	-	-	-	-	-	-	-
	13846	A	-	-	-	-	-	-	-	-	-	-	-	-
	13943	C	3.10	49.70	0.80	46.40	2.60	58.50	0.80	38.00	-	-	-	-
	13951	C	0.20	98.00	0.00	1.80	0.40	26.70	0.00	72.90	-	-	-	-
	13954	C	43.30	52.00	0.20	4.40	59.30	35.00	0.50	5.20	-	-	-	-
	13966	C	44.70	46.90	0.00	8.40	33.60	59.40	0.00	7.00	-	-	-	-
	14006	C	0.90	17.50	80.50	1.10	1.40	21.40	77.20	0.00	-	-	-	-
	14032	C	0.70	44.40	0.20	54.60	0.90	35.30	0.50	63.30	-	-	-	-
	14135	G	12.20	0.40	86.10	1.30	23.60	0.90	75.30	0.20	-	-	-	-

Table S6 (continued)

Gene	Site	Sanger	HTS1				HTS2				HTS3			
			A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)
CR	14589	G	2.60	1.10	95.70	0.60	73.70	0.70	25.60	0.00	-	-	-	-
	14639	C	1.90	84.70	0.00	13.40	0.90	28.30	0.00	70.80	-	-	-	-
	14675	C	0.00	90.30	0.00	9.70	0.50	27.90	0.20	71.40	-	-	-	-
	14720	T	0.00	58.70	0.00	41.30	0.20	98.30	0.00	1.40	-	-	-	-
	14789	T	42.80	22.50	0.00	34.80	46.00	54.00	0.00	0.00	-	-	-	-
	14796	C	0.30	96.70	0.30	2.80	0.00	23.20	0.30	76.50	-	-	-	-
	14818	T	0.80	61.90	0.00	37.40	0.50	98.20	0.30	1.00	-	-	-	-
	14822	T	0.30	41.30	0.00	58.50	0.50	70.00	0.50	29.00	-	-	-	-
	14920	T	0.30	22.80	0.00	77.00	0.00	51.60	2.30	46.10	-	-	-	-
	14944	C	1.20	86.40	0.00	12.40	0.30	42.90	0.00	56.90	-	-	-	-
	15121	T	7.20	31.20	0.00	61.60	14.20	34.30	0.00	51.50	91.40	0.00	0.00	8.60
	15174	T	0.50	14.60	0.00	85.00	0.00	35.40	0.00	64.60	0.00	100.00	0.00	0.00
	15178	C	3.60	74.40	6.70	15.40	7.30	43.30	10.00	39.30	0.00	0.80	0.00	99.20
	15179	C	3.80	94.90	0.00	1.30	13.60	80.20	1.20	4.90	0.00	45.90	0.00	54.10
	15180	T	2.60	0.50	0.00	96.80	7.00	0.00	0.00	93.00	-	-	-	-
	15201	T	0.00	23.10	0.00	76.90	0.60	47.40	0.60	51.30	0.00	0.00	100.00	0.00
	15226	T	21.90	1.60	0.00	76.60	44.60	1.80	0.00	53.60	0.00	0.00	0.00	100.00
	15232	C	0.50	97.40	0.00	2.10	0.60	94.50	0.00	4.80	0.00	0.00	0.70	99.30
	15234	A	97.90	2.10	0.00	0.00	96.90	1.90	0.00	1.20	0.00	100.00	0.00	0.00
	15239	T	0.00	2.00	0.00	98.00	0.00	0.60	0.00	99.40	100.00	0.00	0.00	0.00
	15242	A	99.50	0.50	0.00	0.00	99.40	0.60	0.00	0.00	0.00	6.40	0.00	93.60
	15245	-	-	-	-	-	-	-	-	-	100.00	0.00	0.00	0.00
	15256	G	20.50	0.50	78.50	0.50	48.00	0.00	52.00	0.00	100.00	0.00	0.00	0.00
	15257	G	19.90	0.00	80.10	0.00	48.10	0.00	51.90	0.00	56.00	0.00	44.00	0.00
	15262	A	83.60	0.00	0.00	16.40	55.20	0.00	0.00	44.80	0.00	93.30	0.00	6.70
	15294	A	79.70	0.00	20.30	0.00	54.40	0.60	44.90	0.00	99.10	0.00	0.00	0.90
	15311	G	19.30	1.30	79.30	0.00	47.40	0.00	52.60	0.00	99.00	1.00	0.00	0.00
	15315	A	80.60	0.00	0.00	19.40	1.60	0.00	0.80	97.70	1.10	0.00	0.00	98.90
	15326	T	18.20	0.00	0.00	81.80	47.50	0.00	1.70	50.80	0.00	0.00	0.00	100.00

Table S7. Intraspecific pairwise K2P distances of *Gigantometra gigas* using *cox1* sequences (Sanger) in three collecting sites.

HNDL1																						
HNDL2	0.012																					
HNDL3	0.008	0.005																				
HNDL4	0.006	0.009	0.005																			
HNDL5	0.012	0.006	0.008	0.006																		
HNDL6	0.000	0.012	0.008	0.006	0.012																	
HNDL7	0.008	0.005	0.000	0.005	0.008	0.008																
HNDL8	0.002	0.014	0.009	0.008	0.014	0.002	0.009															
HNDL9	0.011	0.002	0.003	0.008	0.005	0.011	0.003	0.012														
HNDL10	0.017	0.005	0.009	0.014	0.008	0.017	0.009	0.019	0.006													
HNYG1	0.005	0.011	0.006	0.002	0.008	0.005	0.006	0.006	0.009	0.016												
HNYG2	0.006	0.012	0.008	0.003	0.009	0.006	0.008	0.008	0.011	0.017	0.002											
HNYG3	0.006	0.012	0.008	0.003	0.009	0.006	0.008	0.008	0.011	0.017	0.002	0.000										
HNYG4	0.005	0.011	0.006	0.002	0.008	0.005	0.006	0.006	0.009	0.016	0.000	0.002	0.002									
HNYG5	0.006	0.012	0.008	0.003	0.009	0.006	0.008	0.008	0.011	0.017	0.002	0.000	0.000	0.002								
HNYG6	0.005	0.014	0.009	0.008	0.014	0.005	0.009	0.006	0.012	0.019	0.006	0.008	0.008	0.006	0.008							
HNYG7	0.008	0.008	0.003	0.002	0.008	0.008	0.003	0.009	0.006	0.012	0.003	0.005	0.005	0.003	0.005	0.009						
HNYG8	0.006	0.012	0.008	0.003	0.009	0.006	0.008	0.008	0.011	0.017	0.002	0.000	0.000	0.002	0.000	0.008	0.005					
HNYG9	0.020	0.008	0.012	0.017	0.011	0.020	0.012	0.019	0.009	0.003	0.019	0.020	0.020	0.019	0.020	0.022	0.016	0.020				
HNYG10	0.005	0.011	0.006	0.002	0.008	0.005	0.006	0.006	0.009	0.016	0.000	0.002	0.002	0.000	0.002	0.006	0.003	0.002	0.019			
VIET1	0.008	0.014	0.009	0.005	0.011	0.008	0.009	0.009	0.012	0.019	0.003	0.005	0.005	0.003	0.005	0.009	0.006	0.005	0.022	0.003		
VIET2	0.002	0.014	0.009	0.008	0.014	0.002	0.009	0.000	0.012	0.019	0.006	0.008	0.008	0.006	0.008	0.006	0.009	0.008	0.019	0.006	0.009	
VIET3	0.012	0.017	0.014	0.009	0.014	0.012	0.014	0.014	0.016	0.022	0.008	0.009	0.009	0.008	0.009	0.014	0.011	0.009	0.025	0.008	0.008	0.014

Abbreviation. HNDL—Diaoluoshan Nature Reserve, Hainan; HNYG—Yinggeling Nature Reserve, Hainan; VIET—Northern Vietnam.