Scientific Research Publishing

Advances in Entomology, 2021, 9, 113-121
https://www.scirp.org/journal/ae
ISSN Online: 2331-2017
ISSN Print: 2331-1991

Complete Nucleotide Sequence of the Mitochondrial DNA of *Halyomorpha halys* (Stal) (Hemiptera: Pentatomidae) Specimens Collected Across Georgia

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How to cite this paper: Gogniashvili, M., Kunelauri, N., Shanava, T., Tephnadze, N. and Beridze, T. (2021) Complete Nucleotide Sequence of the Mitochondrial DNA of *Halyomorpha halys* (Stal) (Hemiptera: Pentatomidae) Specimens Collected Across Georgia. *Advances in Entomology*, **9**, 113-121.
https://doi.org/10.4236/ae.2021.93010

Received: April 5, 2021
Accepted: June 7, 2021
Published: June 10, 2021

Abstract

The brown marmorated stink bug, *Halyomorpha halys* (Stal) (Hemiptera: Pentatomidae) is an invasive species native to East Asia that has spread across Asia, Europe, and North America. *H. halys* causes damages to various grains, fruits, and vegetables, which is exemplified by the significant damage to the hazelnut harvest in Georgia (during 2016). This report describes the first attempted genetic study of the spread of *H. halys* in Georgia. The first main goal of this research was to identify the haplotype of an invasive population in Georgia. For this purpose, the mitochondrial cytochrome c oxidase I subunit (*COI*) gene fragment from 65 samples *of H. halys* collected from different regions across Georgia was sequenced on an Applied Biosystems 3100 or 3700 genetic analyzer. In all cases, only the H1 haplotype, which is native to China, was identified. The second goal of this research was to determine the complete mitochondrial DNA sequence of *H. halys* (Stal) specimens collected across Georgia. The complete mitochondrial DNA of H1 haplotype sequenced on an Illumina MiSeq platform. The mitochondrial DNA of the Georgian H1 haplotype has a length of 15,478 base pairs. Using the sequence of the H22 haplotype of *H. halys* (native to Korea) as a reference, 62 single nucleotide polymorphisms (SNPs), three inversions, and four single T insertions were identified. Furthermore, 60 SNPs and four insertions in two tRNA and one rRNA genes were identified among 18 mitochondrial genes from the Georgian H1 haplotype. Nine of these SNPs resulted in amino acid substitutions. Furthermore, the detection of SNPs revealed many other polymorphic sites beyond the *COI* gene, which can be used to detect new haplotypes.

Keywords

Halyomorpha halys, Mitochondrial DNA, Illumina, Sequencing, Single

Nucleotide Polymorphism

1. Introduction

Halyomorpha halys (Stal) (Hemiptera: Pentatomidae) is an invasive insect pest that attacks crop species and causes substantial economic damage [1]. This insect family is native to various East Asian countries, including China, Japan, Korea, and Taiwan, where it is treated as a biological enemy and therefore, does not spread uncontrollably. Its rapid diffusion in other continents and the resulting damage of many agricultural crops, have led to global interest in this insect. In North America, *H. halys* is responsible for damages amounting to more than 21 billion dollars [2]. Since its first identification in the United States (US) in 1998, *H. halys* has spread in 34 states and represents a serious threat to agriculture. In Europe, this species was first identified in Switzerland in 2008. In 2014 it was identified in the Sochi region of Russia following spread from either Italy or Greece during preparations for the 2014 Winter Olympics [3]. In 2015, *H. halys* spread from Russia to the Abkhazia region of Georgia, and in 2016, these insects began to spread massively in different regions of west Georgia, where they significantly damaged nuts, corn, and citrus crops [4] [5]. By the end of 2017, *H. halys* had also spread to east Georgia.

Identifying the origin of a biological invasion has important implications for the effective control of the invasive species. Xu and colleagues [6] detected high levels of genetic divergence among native populations of *H. halys* and traced the origin of the invasive population in the US to the Beijing area of China. These researchers identified two mitochondrial *H. halys* haplotypes in the US and 43 haplotypes in native populations. In Italy, 20 previously unknown *H. halys* haplotypes were discovered through an analysis of the *COI* gene in mitochondrial DNA [3]. Another study involved a mitochondrial genome analysis to identify the genetic diversity of *H. halys.* For example, 45 *H. halys* haplotypes were detected in different ecoregions of the US, Europe, and Asia (10 Countries); the Korean *H. halys* populations exhibited the second highest level of diversity among the 10 countries and with only Greece exhibiting higher diversity. Haplotype H22 was prominent in Korea, H1 was prominent in China, Greece, Hungary, Italy, Canada, and USA, and H3 was prominent in France and Switzerland [7]. The identified haplotype diversity patterns revealed that the *H. halys* populations in Korea were genetically distinct from those in China, Europe, and North America. Additionally, the populations in Europe and North America were determined to have arisen through multiple invasions from China. *H. halys* haplotypes of both Chinese and Korean was spread only in Greece. In contrast, the Japanese haplotype is conserved in Japan and has not been identified elsewhere. According to these findings, only *H. halys* strains that originated from China have spread throughout Europe (with the exception of Greece) and North

America, whereas the spread of Korean strains has not been observed. Previously, the complete mitochondrial DNA sequence of only one *H. halys* H22 haplotype (native to Korea) had been determined (GenBank accession number NC_013272.1) [8]. Tracing the diffusion modes of species by analyzing the genetic structures and compositions of populations during the initial phase of colonization could enable the implementation of better pest control strategies. Furthermore, the reconstruction of geographical pathways can be used to design strategies for the management and prevention of pest invasions [2].

2. Materials and Methods

2.1. PCR Analyses

Sixty-five specimens of *H. halys* were collected from the western regions of Georgia, including Guria, Samegrelo, Imereti, Adjara, and Abkhazia. Genomic DNA was extracted from one hind leg of each specimen using a DNeasy Blood & Tissue Kit (Qiagen, Inc., Dusseldorf, Germany) according to the manufacturer's instructions. A 712 base pare (bp) section of the *COI* gene was amplified using the primer sequences designed by Folmer and colleagues [9] and modified according to the complete nucleotide sequence of H22 haplotype mitochondrial DNA (GenBank accession number NC_013272.1) [8]. The primer sequences were as follows: Forward, 5'-ATTCTACTAATCATAAAGATATTGG-3' and Reverse, 5'-TAAACTTCGGGGTGCCCAAAGAATCA-3'. PCR was performed using the following program: initial denaturation at 95˚C for 5 min; 34 cycles at 95˚C for 30 s, 45˚C - 50˚C for 30 s and 72˚C for 30 s; and final extension at 72˚C for 5 min. The PCR products were analyzed using 1.5% agarose gel electrophoresis and sequenced on an Applied Biosystems 3100 or 3700 genetic analyzer at the Laboratory Services Division of the University of Guelph (ON, Canada). Consensus files were aligned using Clustal X 1.83 [10]. The new *COI* sequences generated from the *H. halys* individuals were deposited in GenBank (LC581788-LC581792). The Mafft and Blast software programs were used for detection of SNPs and insertions [11] [12].

2.2. Evolutionary Analysis Using the Maximum Likelihood Method

The evolutionary history was inferred using the maximum likelihood method and Tamura-Nei model [13]. The tree with the highest log likelihood (−1055.47) is shown (**Figure 1**). The tree was drawn to scale, with branch lengths indicating the number of substitutions per site. This analysis involved 31 nucleotide sequences and the final dataset included 608 positions in the. Evolutionary analyses were conducted in MEGA X [14].

2.3. Genomic DNA Library Preparation and Sequencing on an Illumina MiSeq Platform

Genomic DNA libraries were constructed using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, MA, USA). Genomic DNAs

were quantified using the Qubit BR reagents (Qubit 3.0 Fluorometer, Life Technologies). Briefly, 1 μg of DNA was sheared into 500 bp fragments on a Covaris M220 focused ultra-sonicator (Covaris Inc) using SonoLabTM 7.1 software for 200 cycles/burst, 20.0 Duty factor, 50.0 Peak power, in screw-cap microtubes. After shearing, the DNAs were end-repaired using EndPrep master mix and ligated to indexed adaptors. The adaptor-ligated genomic DNAs were size selected with AMPure-beads using the gel free protocol described in the NEBNext Ultra DNA Library Prep manual. Size-selected DNAs were amplified by PCR to selectively enrich for fragments that have adapters on both ends. Final amplified libraries were run on an Agilent bioanlyzer DNA 2100 (Agilent, Santa Clara, CA, USA) to determine the average fragment size and to confirm the presence of DNA of the expected size range. The libraries were pooled in equimolar concentration and loaded onto a flowcell for cluster formation and sequenced on an Illumina MiSeq platform. The libraries were sequenced from both ends of the molecules to a total read length of 250 nt long from each end.

The raw bcl files were converted into demultiplexed compressed fastq files using Casava 1.8.2 (Illumina). FASTAQ (a text-based format for storing nucleotide sequence) files were trimmed using the computer program Sickle, a windowed adaptive trimming tool for FASTQ files using quality (https://github.com/najoshi/sickle). The reads were filtered by standard parameters (quality reads—20, cutoff length—20). The reads containing "N" were discarded. Reads were assembled into mitochondrial DNA molecules using the CLC Genomics Workbench 20.0.4 computer program (QIAGEN; https://digitalinsights.qiagen.com/). The contig was aligned to the reference mitochondrial genome sequence using BLASTN (http://www.ncbi.nlm.nih.gov). For detection of SNP (single nucleotide polymorphism) and Indels (insertion/deletion) computer programs Mafft and Blast were used [11] [12].

3. Results and Discussion

Mitochondrial genomes of the members of superclass Hexapoda are 14 - 16 kb in length and typically comprise 37 genes, 13 protein-coding genes (PCGs), 22 transfer RNA (tRNAs) genes and 2 ribosomal RNA (rRNAs) genes [8]. A phylogenetic tree of mitochondrial *COI* gene fragments (608 bp) from the 65 *H. halys* specimens collected across west Georgia was constructed (**Figure 1**). Thirty-one different haplotypes from GenBank [3] [7] [15] were used to construct the phylogenetic tree all Georgian *H. halys* samples were concentrates around the H1 haplotype, which is native to China.

This research also aimed to determine a complete mitochondrial DNA sequence for the *H. halys* (Stal) specimens collected in Georgia. The complete mitochondrial DNA sequence of only the *H. halys* H22 (native to Korea) haplotype is known (NC_013272.1) [8], and it contains two repeat regions: repeat 1, 15,178 - 15,469 bp and repeat 2, 15,638 - 16,513 bp. The complete mitochondrial DNA sequence of an *H. halys* specimen from Samegrelo, Georgia (H1 haplotype, HGS)

was sequenced in this study, and the sequence has been submitted to GenBank (LC579925). The mitochondrial DNA of HGS has a length of 15,478 bp. Using the sequence of the H22 haplotype as a reference, 62 SNPs, three inversions and four single T insertions were identified in the mitochondrial DNA of HGS. Out of 18 genes in the HGS mitochondrial DNA, 60 SNPs and four insertions were identified in two tRNA and one rRNA genes. Nine of these SNPs resulted in amino acid substitutions (Table 1, Table 2). The detection of these SNPs revealed many other polymorphic sites beyond the *COI* gene, which can be used to detect new haplotypes. The mitochondrial DNA of HGS also contains one repeat region, 15,182 - 15,473 bp.

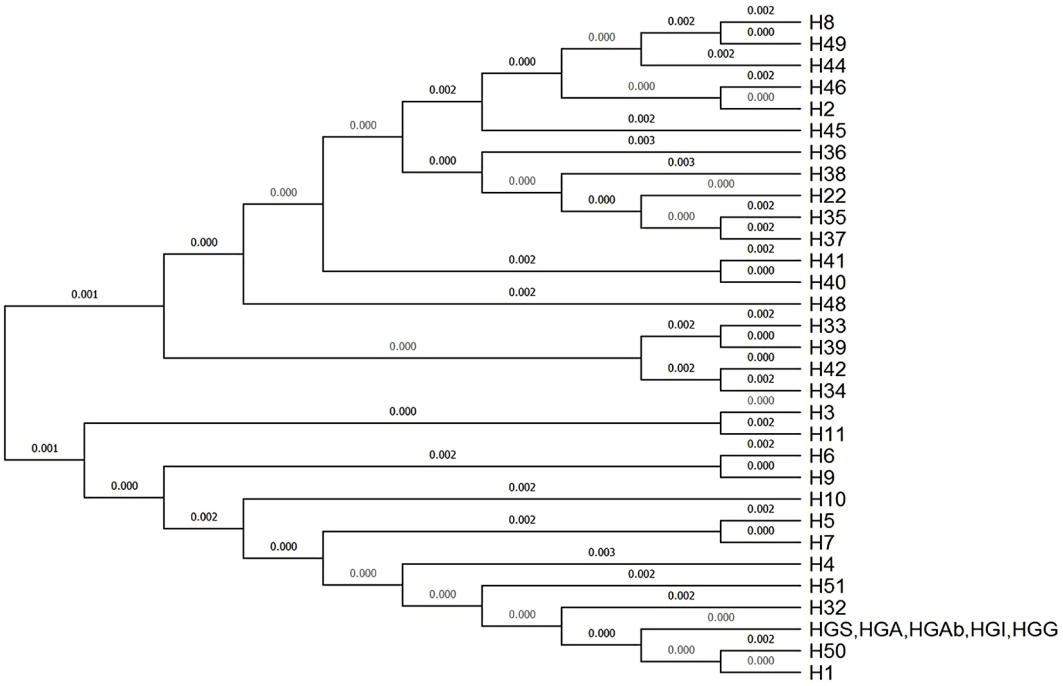

Figure 1. Mitochondrial genome (cytochrome c oxidase I subunit gene fragment) phylogeny of the *Halyomorpha halys* accessions.

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The following GenBank accessions were used for the analyses: H1_KY710348; H2_KX017350; H3_KM401490; H4_KF273383; H5_KF273384; H6_KF273385; H7_KF273386; H8_KY710311; H9_KF273388; H10_KF273389; H11_KF273390; H22_NC_013272; H32_KY710274; H33_KY710272; H34_KX01733; H35_KX017376; H37_KX017355; H39_KX017357; H36_KX017360; H38_KX017337; H40_KY710346; H41_KY710323; H42_KY710424; H44_KY710294; H45_KM401490; H46_KY710433; H48_KY710336; H49_KY710398; H50_KX017388; H51_KX017394.

Georgian *H. halys* samples by collection region: HGS, Samegrelo; HGI, Imereti; HGG, Guria; HGA, Adjara; HGAb, Abkhazia.

Table 1. SNPs in the mitochondrial DNA of *H. halys* (H1 haplotype) spread in Georgia.

Nucleotide positions according to H22 haplotype of *H. halys* (NC_013272.1)	Locus	H22 haplotype	HGS_Samegrelo	Amino acid substitution
462	Gene *ND2*	G	A	V-I
492	Gene *ND2*	C	T	Syn
509	Gene *ND2*	G	A	Syn
530	Gene *ND2*	A	G	Syn
699	Gene *ND2*	A	G	M-V
758	Gene *ND2*	C	T	Syn
833	Gene *ND2*	G	A	Syn
1049	Gene *ND2*	T	C	Syn
1789	Gene *COX1*	G	A	Syn
1777	Gene *COX1*	C	T	Syn
3368	Gene *COX2*	A	G	Syn
3494	Gene *COX2*	A	G	Syn
3936	Gene *ATP8*	C	T	S-L
4115	Gene *APT6*	G	A	V-M
4290	Gene *APT6*	T	C	Syn
4831	Gene *COX3*	A	G	Syn
5038	Gene *COX3*	T	C	Syn
5236	Gene *COX3*	R	A	Syn
5242	Gene *COX3*	T	C	Syn
5558	Gene *ND3*	A	G	Syn
5568	Gene *ND3*	A	G	M-V
5651	Gene *ND3*	C	T	Syn
5968	*tRNA-Arg*	T	C	
6371	Gene *ND5*	A	G	Syn
6587	Gene *ND5*	C	T	Syn
6771	Gene *ND5*	A	G	F-S
6893	Gene *ND5*	A	G	Syn
6941	Gene *ND5*	T	C	Syn
6956	Gene *ND5*	G	A	Syn
7006	Gene *ND5*	A	G	Syn
7134	Gene *ND5*	G	T	T-N
7847	Gene *ND5*	C	T	Syn
8344	Gene *ND4*	A	T	Syn
8383	Gene *ND4*	T	C	Syn
8527	Gene *ND4*	G	A	Syn
9546	Gene *ND4L*	T	C	Syn
9833	Gene *ND6*	T	C	Syn
10,116	Gene *ND6*	A	C	I-L
10,356	Gene *CYTB*	T	C	Syn
10,612	Gene *CYTB*	A	G	Syn
10,617	Gene *CYTB*	C	T	Syn

Continued

10,656	Gene *CYTB*	G	A	Syn
11,019	Gene *CYTB*	G	A	Syn
11,046	Gene *CYTB*	G	A	Syn
11,088	Gene *CYTB*	C	T	Syn
11,779	Gene *ND1*	G	A	Syn
11,791	Gene *ND1*	G	A	Syn
12,129	Gene *ND1*	T	C	I-V
12,199	Gene *ND1*	A	G	Syn
12,223	Gene *ND1*	A	G	Syn
12,319	Gene *ND1*	G	A	Syn
12,352	Gene *ND1*	G	A	Syn
13,715	*rRNA-16S*	G	A	
14,701	*rRNA-12S*	T	C	
14,956	Intergenic region *rRNA-12S*	G	A	

Table 2. Insertions and inversions in the mitochondrial DNA of *H. halys* (H1 haplotype) spread in Georgia.

Nucleotide positions According to H22 haplotype of *H. halys* (NC_013272.1)	Locus	H22 haplotype	HGS_Samegrelo
6077	*tRNA-Asn*	-	+1T
6233	*tRNA-Phe*	-	+1T
14,199	*rRNA-12S ribosomal RNA*	-	+1T
14,627	*rRNA-12S ribosomal RNA*	-	+1T
15,309 - 15,314	*Repeat region*	-	6 bp Invertion
15,317 - 15,318	*Repeat region*	-	2 bp Invertion
15,382 - 15,387	*Repeat region*	-	6 bp Inversion

4. Conclusion

This study represents the first attempted genetic study of the spread of *H. halys* in Georgia. Notably, only the H1 haplotype of Chinese origin was found to be widespread in Georgia. These results are consistent with the findings of Musolin *et al.* [5], who reported that the *H. halys* that distributed in Abkhazia, Georgia, was spread from Italy (an H1 haplotype dominant area) via Russia. The complete mitochondrial DNA sequence of HGS was determined (LC579925) in this study and its length was found to be 15,478 bp.

Data Availability Statement (DAS)

Cytochrome oxidase I sequences are openly available in GenBank at: https://www.ncbi.nlm.nih.gov/nuccore/?term=LC581788%3ALC581792%5BACCN%5D The mitogenome data supporting this study are openly available in

GenBank at: https://www.ncbi.nlm.nih.gov/nuccore/LC579925. The associated BioProject, SRA, and BioSample accession numbers are: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA678533, https://www.ncbi.nlm.nih.gov/sra/PRJNA678533, and SAMN16805180, respectively.

Authors' Contribution Statement

TB and MG conceived and designed research. TS and NT conducted experiments. NK, TB, and MG analyzed data. MG, TB, and NK wrote the manuscript. All authors read and approved the manuscript.

Acknowledgements

This work was supported by the Shota Rustaveli National Science Foundation of Georgia (SRNSFG) and the National Food Agency of Georgia (grant number: NFA-SRNSFG-18-403).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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