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Molecular Genetics

Re-Identifying *Halyomorpha halys* (Stal) (Hemiptera: Pentatomidae) Haplotype Spread in Georgia by Mitochondrial DNA Sequences after Three Years of Initial Identification

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The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is a global invasive species, which is native to East Asia, that is threatening agriculture in invaded regions. In 2016, this species significantly damaged the hazelnut harvest in Georgia. In 2018 there was a first attempt to genetically study *Halyomorpha halys* spread in Georgia in the Institute of Molecular Genetics (Agricultural University of Georgia). By sequencing of mitochondrial cytochrome c oxidase, I subunit gene fragment of 65 samples spread in different regions of Georgia, was identified the haplotype of an invasive population. In all cases only H1 haplotype that is native to China was identified and the complete mitochondrial DNA of *H. halys* H1 haplotype was sequenced. Three years later, in 2021, 25 *H. halys* samples from West Georgia were collected and checked the haplotype diversity again using the mitochondrial cytochrome c oxidase I and II subunit gene fragments sequencing. All 25 samples of *H. halys* spread in West Georgia were again identified as H1 Chinese haplotype three years later. Afterwards, the complete mitochondrial DNA of five *H. halys* samples from different regions of West Georgia were sequenced on an Illumina NovaSeq 6000 Platform and assembled by CLC Genomics Workbench 20.0.4 computer program. As a result, complete mitochondrial DNA sequences were identical with those from 2018 (Gene Bank number: LC579925). Only identifying the same H1 haplotype in 2021 that is dominant and native to China indicates that new haplotypes of *H. halys* have not spread in Georgia since 2018. *© 2022 Bull. Georg. Natl. Acad. Sci.*

brown marmorated stink bug, *coxI* gene fragment, complete mitochondrial DNA, Illumina, sequencing

Halyomorpha halys is a global invasive species, which feeds on more than 300 host plants. It is originally from Southeast Asia, namely from Japan, the Republic of Korea, and China. Over time, it spread and first invaded the United States, where it caused significant and extensive agricultural damage over the past decade. Next, it invaded Canada [1-5], 12 countries in Europe, Russia, and the rest of Georgia [6]. Now it is also established in South America. Due to the significant damage that

BMSB has been causing to a wide variety of agricultural crops in the invaded countries and regions, it poses a global economic threat for the agricultural and horticultural industry [3]. Vast amount of reports show that the economic losses caused by BMSB are valued at more than 37 million USD in North America. In 2016, it also caused serious damage to the hazelnut harvest in Georgia. In 2014 it was found [7] that there are significant genetic differences among native populations of *H. halys* and therefore, were able to trace the origin of US *H. halys* to Beijing, China.

mitochondrial cytochrome c oxidase I subunit gene fragment of 65 samples spread in different regions of Georgia [8-10]. In all cases only H1 haplotype that is native to China was identified and the complete mitochondrial DNA of *H. halys* H1 haplotype was sequenced. None of these complete mitochondrial genomes of any Chinese haplotypes have been described before. Using the sequence of H22 haplotype of *H. halys* (Native to Korea) as a reference, 62 SNPs, three inversions, and four one T insertions were identified. In 18 genes of mitochondrial DNA of Georgian H1 haplotype, 60

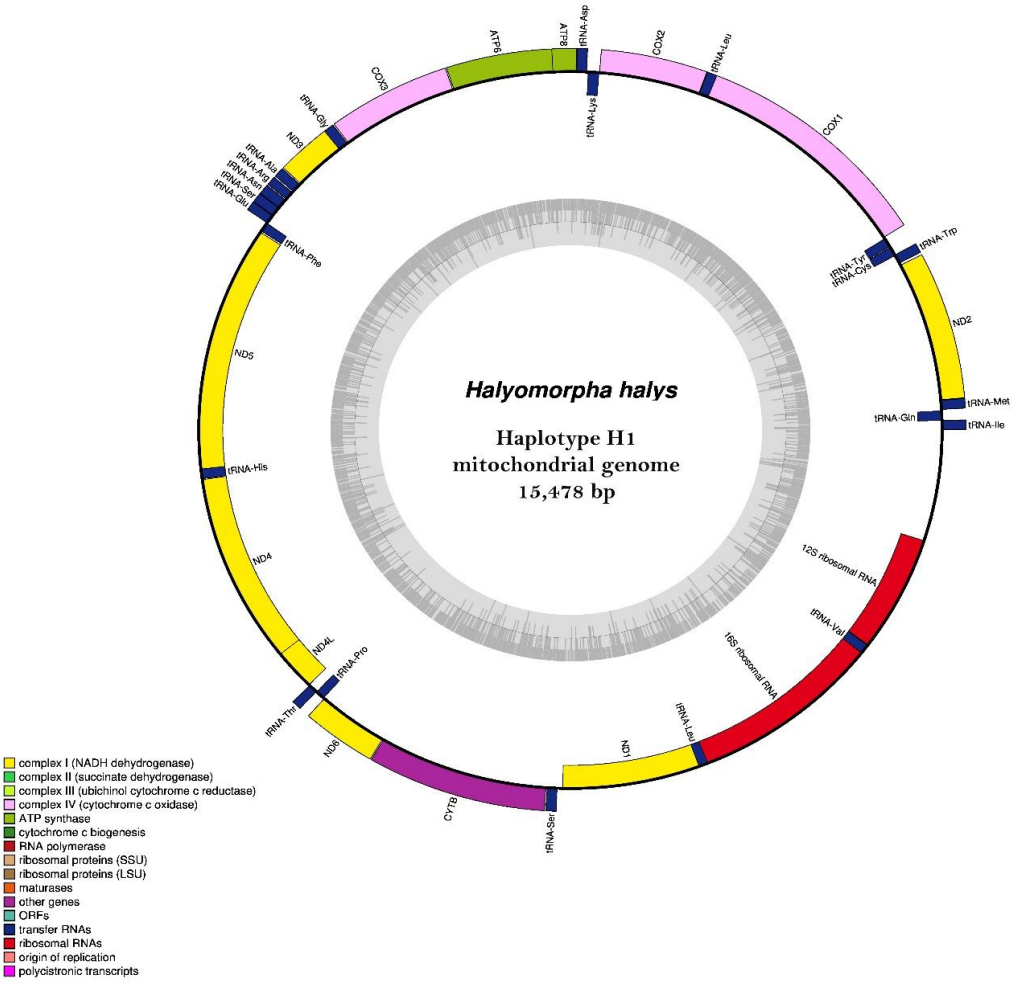

Fig. Map of complete mitochondrial genome of *H.halys* (H1 Haplotype) spread in West Georgia.

The genetic study of *H. halys* spread in Georgia was attempted at the Institute of Molecular Genetics, where we identified the haplotype of an invasive population in Georgia by sequencing the

SNPs, and four insertions in two *tRNA* and one *rRNA* genes were found. Nine SNPs resulted in amino acid substitutions.

Table. SNPs, Indels and repeat region in the mitochondrial DNA of *H. halys* samples (G2, G3, G8, G10, G16, H1 haplotype (LC579925)) spread in Georgia

Nucleotide positions according to H22 haplotype of *H. halys* (NC_013272.1)	Locus	H22 haplotype	H1 haplotype (LC579925), G2, G3, G8, G10, G16	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	
6077	*tRNA-Asn*	-	+1T	
6233	*tRNA-Phe*	-	+1T	
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
10116	Gene *ND6*	A	C	I-L
10356	Gene *CYTB*	T	C	Syn
10612	Gene *CYTB*	A	G	Syn
10617	Gene *CYTB*	C	T	Syn
10656	Gene *CYTB*	G	A	Syn
11019	Gene *CYTB*	G	A	Syn
11046	Gene *CYTB*	G	A	Syn
11088	Gene *CYTB*	C	T	Syn
11779	Gene *ND1*	G	A	Syn
11791	Gene *ND1*	G	A	Syn
12129	Gene *ND1*	T	C	I-V
12199	Gene *ND1*	A	G	Syn
12223	Gene *ND1*	A	G	Syn
12319	Gene *ND1*	G	A	Syn
12352	Gene *ND1*	G	A	Syn
13715	*rRNA-16S*	G	A	
14199	*rRNA-12S ribosomal RNA*	-	+1T	
14627	*rRNA-12S ribosomal RNA*	-	+1T	
14701	*rRNA-12S*	T	C	
14956	Intergenic region *rRNA-12S*	G	A	
15309-15314	*Repeat region*	-	6bp Invertion	
15317-15318	*Repeat region*	-	2bp Invertion	
15382-15387	*Repeat region*	-	6 bp Inversion	

In 2018 twenty new *H. halys* haplotypes were detected in Italy [11]. Additionally, 45 haplotypes were identified in 10 countries across the United States, Europe, and Asia, where Greek populations showed highest diversity followed by Korean populations [12]. In 2021, [13] also found haplotype diversity in China with main haplotypes H1, H33, H22, and H3, H23, H45, N22, N40 were the primary Japanese haplotypes detected. Unlike the native regions, low haplotype diversity was observed in the invaded regions where the main haplotypes were H1 and H3. In Georgia, Romania, Turkey and the USA only one, H1 haplotype, was identified. Therefore, [13] concluded that there might have been a secondary invasion from the USA to Europe (Georgia, Hungary, Italy, Romania, and Turkey) and then to Chile. It is likely that *H. halys* originated from China, after which the invasion took place to USA and from there to Canada. By contrast, Europe was invaded from multiple directions and therefore has multiple introductions: for example, *H. halys* entered from Asia directly in France and Switzerland and also possibly through the United States and secondary invasions in Italy, Hungary, and Greece. H157 was found for the first time in Switzerland. The highest diversity was identified in specimens that were collected in Greece, where H158, H159, and H160 were unique to Greece as they have not been detected elsewhere [13].

Analyzing the genetic structure and composition of populations in their beginning stage of colonization in order to trace the spread of the species will contribute to implementing better pest control strategies. Similarly, the reconstruction of geographical pathways will add great value to the management and prevention of future pest invasions as they can be used to design effective strategies for pest invasion.

Materials and Methods

Materials, DNA isolation, PCR analysis. 25 samples of *H. halys* were selected in western regions of Georgia: Guria, Samegrelo, Imereti, Adjara and Abkhazia in 2021. Genomic DNA was extracted from each specimen using a DNeasy Blood & Tissue Kit (Qiagen, Inc., Dusseldorf, Germany) as described by the manufacturers. Two sections of the *coxI* gene (712 bp and 800 bp in lengths) was amplified using the primers given in [14], which was modified according to complete nucleotide sequence of H22 haplotype mitochondrial DNA (GenBank accession number NC_013272.1) [15]: For the first *coxI* gene region: Forward,
5′ - ATTCTACTAATCATAAAGATATTGG - 3′ and Reverse,
5′ - TAAACTTCGGGGTGCCCAAAGAATCA - 3′ and for second *coxI* gene region: Forward, 5′ - TTGGGCACCCCGAAGTTTAT - 3′ and Reverse, 5′- ATGAATGTTCGGCTGGAGGT - 3′. PCR was performed using the following cycles: 95°C for 5 min (initial denaturation); 34 cycles at 95°C for 30 s, 45 to 50°C for 30 s, 72°C for 30 s; and 72°C for 5 min (final extension). PCR products were analyzed using 1.5% agarose gel electrophoresis. PCR products were sequenced on Applied Biosystems 3100 or 3700 genetic analysers at the Laboratory Services Division of the University of Guelph (ON, Canada). Consensus files were aligned using Clustal X 1.83 [16]. For detection of SNPs and insertion the Mafft and Blast software programs were used [17, 18].

DNA library preparation, sequencing on an illumina novaSeq 6000 platform. Construction of 5 shotgun genomic libraries and sequencing on the NovaSeq 6000 was carried out at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana Champaign (UIUC). The shotgun genomic DNA libraries were constructed from 50 ng of DNA after sonication with a Covaris ME220 (Covaris, MA, USA) to an average fragment size of 400 bp with the Hyper Library Preparation Kit from Kapa Biosystems (Roche, CA, USA). To prevent index switching, the libraries were constructed using unique dual-indexed adaptors from Illumina (San

Diego, CA, USA). The individually barcoded libraries were amplified with 6 cycles of PCR and run on a Fragment Analyzer (Agilent, CA, USA) to confirm the absence of free primers and primer dimers and to confirm the presence of DNA of the expected size range. Libraries were pooled in equimolar concentration and the pool was further quantitated by qPCR on a BioRad CFX Connect Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The pooled, barcoded, shotgun libraries were loaded on a NovaSeq SP lane for cluster formation and were sequenced for 150 cycles from each side of the DNA fragments. The fastq read files were generated and demultiplexed with the bcl2fastq v2.20 Conversion Software (Illumina, San Diego, CA, USA).

Assembly and annotation of complete mitochondrial DNA. Reads were assembled into mitochondrial DNA molecules using the CLC Genomics Workbench 20.0.4 computer program (QIAGEN). The contig was aligned to the reference mitochondrial genome sequence using BLASTN [18].

Results

Two 712 bp and 800 bp regions of the *coxI* gene were amplified from 25 *H. halys* specimens from West Georgia. Only Haplotype H1 (Native for China) was detected in West Georgia as it was in 2018. The complete mitochondrial DNA was sequenced, assembled and annotated of five *H. halys* samples for the different regions of West Georgia: Guria, Samegrelo, Imereti, Adjara and Abkhazia (Fig.). Using the sequence of H22 haplotype of *H. halys* (GenBank Accession number NC_013272.1) (Native for Korea) and H1 haplotype (GenBank accession number LC579925) as references, 62 SNPs, three inversions and four one T insertions were identified. 60 SNPs, four insertions in two *tRNA*, and one *rRNA* genes were found in 18 genes of mitochondrial DNA of Georgian H1 haplotype. Nine SNPs resulted in amino acid substitutions. Detection of SNPs revealed many other polymorphic sites besides COI gene, allowing these sites to be used to detect new haplotypes. The mitochondrial DNA of H1 contains one repeat region 15182-15473. The sequenced samples were identical to H1 haplotype samples from Georgia that were sequenced in 2018 (GenBank Accession number LC579925) (Table).

In 2018, it was our first attempt to study the genetic diversity of *H. halys* that was spread in Georgia. In 2021, we replicated the study to see if we could identify the invasion of different or new haplotypes. As a result of our later study, we found that all the samples collected from the Western Georgian regions belong to H1 haplotype. Therefore, it seems that new invasions have not taken place since 2018.

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მოლეკულური გენეტიკა

საქართველოში გავრცელებული *Halyomorpha halys* (Stal) (Hemiptera: Pentatomidae) ჰაპლოტიპის ხელახალი იდენტიფიკაცია მიტოქონდრიული დნმ-ის თანმიმდევრობით საწყისი იდენტიფიკაციიდან სამი წლის შემდეგ

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(წარმოდგენილია აკადემიის წევრის თ. ბერიძის მიერ)

აზიური ფაროსანა, ***Halyomorpha halys*** (Stål) (Hemiptera: Pentatomidae), არის გლობალური ინვაზიური სახეობა, რომელიც ბუნებრივია აღმოსავლეთ აზიაში. ის საფრთხეს უქმნის სოფლის მეურნეობას ინვაზიურ რეგიონებში. 2016 წელს ამ სახეობამ საგრძნობლად დააზიანა თხილის მოსავალი საქართველოში. 2018 წელს საქართველოში გავრცელებული *H. halys* გენეტიკური მრავალფეროვნების შესწავლის მცდელობა იყო საქართველოს აგრარული უნივერსიტეტის მოლეკულური გენეტიკის ინსტიტუტის ლაბორატორიაში ჩვენ მიერ. საქართველოს სხვადასხვა რეგიონში გავრცელებული 65 ნიმუშის მიტოქონდრიული ციტოქრომ c ოქსიდაზა I სუბერთეულის გენის ფრაგმენტის თანმიმდევრობით, გამოვლინდა ინვაზიური პოპულაციის ჰაპლოტიპი. ყველა შემთხვევაში იდენტიფიცირებული იყო მხოლოდ H1 ჰაპლოტიპი, რომელიც ბუნებრივია ჩინეთში და სეკვენირებულ იქნა *H. halys*-ის H1 ჰაპლოტიპის სრული მიტოქონდრიული დნმ. სამი წლის შემდეგ, 2021 წელს, დასავლეთ საქართველოში შეგროვებულ იქნა *H. halys*-ის 25 ნიმუში ჰაპლოტიპების მრავალფეროვნების ხელახლა შესამოწმებლად (მიტოქონდრიული ციტოქრომ c ოქსიდაზას I და II ქვედანაყოფის გენის ფრაგმენტების თანმიმდევრობის გამოყენებით). *H. halys*-ის ოცდახუთივე ნიმუში, რომელიც დასავლეთ საქართველოშია გავრცელებული, სამი წლის შემდეგ კვლავ H1 ჩინური ჰაპლოტიპი აღმოჩნდა. ამის შემდეგ, დასავლეთ საქართველოს სხვადასხვა რეგიონიდან *H. halys*-ის ხუთი ნიმუშის სრული მიტოქონდრიული დნმ დავასეკვენირეთ Illumina NovaSeq 6000 პლატფორმაზე და ავაწყეთ კომპიუტერული პროგრამის (CLC Genomics Workbench 20.0.4) საშუალებით. შედეგად, სრული მიტოქონდრიული დნმ-ის თანმიმდევრობები 2018 წლის (LC579925) იდენტური იყო. მხოლოდ 2021 წელს იმავე H1 ჰაპლოტიპის იდენტიფიცირება, რომელიც დომინანტი და მშობლიურია ჩინეთში, მიუთითებს იმაზე, რომ *H. halys*-ის ახალი ჰაპლოტიპები საქართველოში 2018 წლიდან არ გავრცელებულა.

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