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Entomology

Phylogenetic Analysis of Hazelnut Big Bud Mite - *Phytoptus avellanae* Nal. in the Black Sea Region of Georgia

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ABSTRACT. Hazelnut is one of the leading agricultural crops in Georgia. It is cultivated on 8% of the arable area in Georgia and is of great economic importance. Therefore, it is important to protect hazelnut plants from pests in order to produce high quality nuts and minimize losses. In recent years, the 'big bud mites' have become major pests of hazelnut plantations in Georgia. Their timely detection and diagnostics will enable the implementation of effective control measures. For this study, 20 orchards in the western Georgia regions of Guria, Samegrelo and Adjara were selected.

The species identification and phylogenetic analysis were carried through the sequencing of PCR fragments. The comparison of sequence results to reference isolates of *Phytoptus avellanae* in Genbank showed high percentage similarity (93-99%). Phylogenetic analysis were conducted in MEGA7 by using UPGMA methods. The phylogenetic trees show that Georgian isolates are grouped in two main clades. First clade contains 15 Georgian and 3 GenBank (KR149013.1, KR149017.1, KT070248.1) isolates of *P. avellanae*. Microhabitat of all these isolates were buds. The second clade contains 6 Georgian isolates and two isolates from GenBank (KR149026.1 and KR149027.1.), which were isolated from vagrant forms of *P. avellanae*. The phylogenetic trees show that Georgian isolate GUR1 is separate from Genbank and Georgian isolates. *© 2018 Bull. Georg. Natl. Acad. Sci.*

Key words: *Phytoptus avellanae, COI mtDNA, phylogenetic analysis*

The big bud mite (Eriophyoidea: Phytoptidae: *Phytoptus avellanae* Nal) is the most widespread arthropod pest of hazelnut throughout the world. The mite is generally found in hazelnut big buds [1]. These eriophyoid mites cause buds to become swollen, fleshy, deformed and pinkish ('big buds'). Infested vegetative buds develop weak and unhealthy shoots, damaged male catkins become stiff and brittle producing little pollen, and weakened female buds produce no nuts [2].

There are other secondary effects of the infestation: the deformed big buds provide a point of access for Eastern filbert blight [3], and *P. avellanae* is one of the chief transmitters of the fungal pathogen *Gloeosporium* sp. which causes twig desiccation. Although no natural vector of

Table 1. GPS coordinates of sites for sampling

Isolates	Host plant	Microhabitat	GPS coordinates	Locality
GUR5	*Corylus avellana*	Vagrant	N42°02.547 E042°13.786	Gogolesubani
GUR-17	*Corylus avellana*	Ex gall	N41°59.223 E042°11.200	Kvenobani
GUR 18	*Corylus avellana*	Vagrant	N41°59.223 E042°11,200	Kvenobani
GUR 19	*Corylus avellana*	Vagrant	N42°02.537 E042°13.678	Gogolesubani
GUR 52	*Corylus avellana*	Ex gall	N41°59.213 E042°11.186	Kvenobani
GUR 101	*Corylus avellana*	Ex gall	N42°01.537 E042°02.558	Lanchkhuti
GUR 142	*Corylus avellana*	Ex gall	N42°02.547 E042°13.786	Gogolesubani
GUR-143	*Corylus avellana*	Ex gall	N41°59.213 E042°11.186	Kvenobani
GUR-172	*Corylus avellana*	Ex gall	N41°59.214 E042°10.187	Kvenobani
GUR-182	*Corylus avellana*	Vagrant	N42°02.515 E042°13.708	Gogolesubani
GUR-192	*Corylus avellana*	Vagrant	N42°02.600 E042°13.596	Gogolesubani
GUR-193	*Corylus avellana*	Ex gall	N42°02.600 E042°13.596	Gogolesubani
ADCH-932	*Corylus avellana*	Ex gall	N41°52.780 E041°58.818	Chianeti
ADCH-751	*Corylus avellana*	Vagrant	N41°42.954 E041°43.542	Daba chaqvi
SAM-7	*Corylus avellana*	Ex gall	N42°09.505 E042°17.595	Marani
SAM-8	*Corylus avellana*	Ex gall	N42°09.822 E042°17.953	Abasha
SAM-10	*Corylus avellana*	Ex gall	N42°09.822 E042°17.553	Abasha
SAM-22	*Corylus avellana*	Ex gall	N45°34.999 E042°12.707	Napichkhua
SAM-32	*Corylus avellana*	Ex gall	N45°34.999 E042°12.707	Napichkhua
SAM-71	*Corylus avellana*	Ex gall	N42°09.505 E042°17.595	Marani
GUR-1	*Corylus avellana*	Ex gall	N42°02.515 E042°13.708	Gogoles ubani

apple mosaic virus in hazelnut is known eriophyoid mites (and more specifically members of the Eriophyidae) have been demonstrated to transmit plant viruses [4].

Phytoptus avellanae has two forms: a gall form and a vaegrant form. The two forms have different life cycle and each for caused a specific type of damage. The gall form lives inside big buds. It has simple life cycle and a single nymphal type. The vagrant form lives on vegetative and generative organs and the big buds. It has a different and rather complex life cycle and two nymphal types [5].

Later morphological comparisons of two forms of *P. avellanae* showed that the gall and vagrant forms were significantly different from each other in size, shape and color.

It is revealed that both forms of pests have different biology, based on this *P. avellanae* vagrant form is considered to be a new species [6,7]. The hypothesis that the forms of the *P.*

avellanae complex in *Corylus* spp. are two different types, is proved by the molecular surveys which indicates the different nucleotide sequences in both [8].

In recent years, *P. avellanae* have become major pests of hazelnut orchards in Georgia. Sometimes the loss caused by them reaches 90% [9]. That's why their timely detection and diagnostics will enable the implementation of effective control measures.

The aim of thise research was the phylogenetic analysis of the big bud mite (*P. avellanae*) in western Georgia by the sequencing of PCR fragments, which will facilitate the correct determination of species and planning of appropriate pest control measures.

Materials and Methods

For this study, 20 orchards in the western Georgia regions of Guria, Samegrelo and Adjara were selected (Table 1.). Shoots of 20-30 cm length with big buds and leaves were collected randomly from 30 branches in each orchard. The big buds and leaves were checked under a binocular microscope and slides were prepared for identification of the big bud mite species. Three species, namely *P. avellanae*, a vagrant cryptic species associated with *P. avellanae*, and *Cecidophyopsis vermiformis* (Nal.) were identified.

Phytoptus avellanae was removed from the buds and leaves with a fine needle and placed in 99.8% ethyl-alcohol for molecular analysis. The DNA was extracted with a DNA-extraction kit (DNeasy Blood & Tissue Kit_ Qiagen), with the lysis time increased to 24 hours. The DNA purity was 2.0 (A260/A280) and its concentration was 200 ng/µl. Increasing the period of cell lysis increased the amount of lysis products, including DNA.

These DNA were used to amplify a region of the mitochondrial cytochrome c oxidase I gene (COI) 658 bp long barcode region by polymerase chain reaction (PCR) [10] using the primer pair LCO1490 and HCO2198 [11]. Polymerase chain reaction (PCR) was conducted using Platinum PCR master mix (Invitrogen) in 25 µL final volume.

Thermocycler (SimpliAmp Thermal Cycler) was used for carrying out of polymerase chain reaction.

Cycling conditions were: one cyclet 94 °C for 2 minutes, followed by 33 cycles of 92 °C for 40 seconds, an 45°C for 40 seconds, and extension at 72 °C for 1 minutes 30 seconds. Extension phase 72°C for 5 minutes and cooling to 10 °C.

Obtained PCR products were studied by horizontal electrophoresis on agarose gel [12] Sequencing of PCR products was conducted commercially in: Laboratory Services Division of the University of Guelph (ON. Canada).

Obtained sequencing results were analyzed by computer programs BLASTN, MEGA 7. Phylogeny reconstruction was performed by using the UPGMA methods.

Results and Discussion

Phytoptus avellanae's mitochondrial cytochrome oxidase (COI) gene fragments from the samples collected on the *Corylus avellana* L. varieties in the Black See region of Georgia have been sequenced and the phylogenetic tree is built (Table 1). In western Georgia, specifically in the Adjara-Guria region were separated 15 isolates (GUR1, GUR5, GUR17, GUR18, GUR19, GUR52, GUR101, GUR142, GUR143, GUR172, GUR182, GUR192, GUR193, ADCH932, ADCH751), and in the Samegrelo region 6 isolates (SAM7, SAM8, SAM10, SAM22, SAM32, SAM 71).

BLAST Sequence analyzis show that mitochondrial cytochrome oxidase subunit 1 (COI) gene fragments of isolates have 93-99% similarity with the *P. avellanae's* isolates existing in the database (http://blast.ncbi.nlm.nih.gov/Blast) (Table 2), and the only isolate - GUR1 show similarity 78%. Phylogenetic tree show that the Georgian isolates of *P. avellanae* are grouped in two mainly clades. The first contained 15 Georgian isolates of *P. avellanae* (SAM 7, GUR101,

GUR172, SAM10, SAM71, SAM22, GUR143, GUR52, SAM32, ADCH932, GUR193, GUR17, GUR1A). This clade also contains three GenBank isolates: KR149013.1, KR149017.1, KT070248.1; in this case microhabitat of our isolates and GenBank isolates were buds. The second clade contains isolates GUR5, GUR192, GUR19, GUR182, GUR18, ADCH751 and the two isolates from GenBank (KR149026.1 and KR149027.1.) which were isolated from vagrant forms of *P. avellanae*. Phylogenetic tree shows that Georgian isolate GUR1 is separate from Genbank and Georgian isolates from the tow clades and to came out as unassociated isolates in the tree (Fig.1).

Table 2. The comparison of (COI) gene fragment from Georgian isolates and isolates from the database (http://blast.ncbi.nlm.nih.gov/Blast).

	Isolates	Identity (%)	Genbank No
1	GUR-5	94	KR149027.1
2	GUR-17	95	KT070248.1;
3	GUR-18	93	KR149027.1
4	GUR-19	93	KR149027.1
5	GUR-52	99	KR149013.1
6	GUR-101	99	KR149013.1
7	GUR-142	95	KT070248.1;
8	GUR-143	98	KR149013.1
9	GUR-172	98	KR149013.1
10	GUR-182	99	KT070248.1;
11	GUR-192	91	KR149027.1
12	GUR-193	99	KR149013.1
13	ADCH-932	99	KR149013.1
14	ADCH-751	94	KR149027.1
15	SAM-7	100	KR149013.1
16	SAM-8	94.	KT070248.1
17	SAM-10	99	KR149013.1
18	SAM-22	99	KR149013.1 KT070248.1
19	SAM 32	99	KR149013.1
20	SAM 71	99	KR149013.1;
21	GUR1	75	KR149013.1;

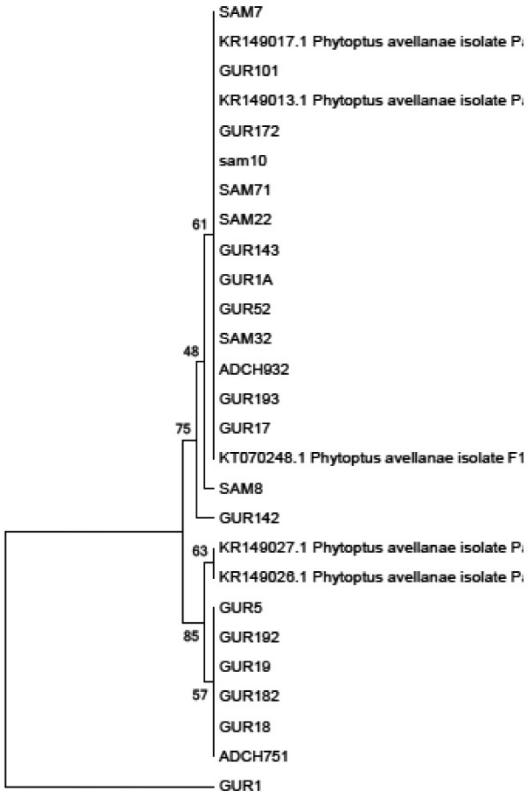

Fig. 1. UPGMA phylogenetic tree inferred from 711 bp of mtDNA COI gene sampled from *Phytoptus avellanae*. The evolutionary history was inferred using the UPGMA method [13]. The optimal tree with the sum of branch length = 0.26351073 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [14]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.. Codon positions included were 3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 127 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [15;16].

According to research held in Western Serbia, Phylogenetic analysis of mitochondrial DNA sequence of *P. avellanae* complex (gall and vagrant forms) isolated from same plant of *C. avellana* showed that high intergroup diversity 16,8 %, which confirms that we are dealing with different species [8].

Opinion of scientists, that *P. avellanae* vagrant form is a cryptic species, and exists only on *C. avellana* and not on *C. americana* and *C. colurna.* It needs further research in different regions, on different hazelnut species [8].

This hypothesis and existing data is confirmed by our research work: (COI) Phylogenetic analysis show that Georgian gall forms of *P. avellanae* get in the same clade as Genbank isolates from Serbia, Russia and India. The Georgian vagrant forms isolates get in the same clade of phylogenetic tree as Genbank isolates from Serbia [8]. However in order to strengthen the received data it is necessary to do Nuclear D2 sequence analyses.

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ენტომოლოგია

საქართველოს შავი ზღვისპირეთში გავრცელებული თხილის მავნებლის *Phytoptus avellanae* -ს ფილოგენეტიკური ანალიზი

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ბოლო წლებში, დასავლეთ საქართველოს თხილის ბაღებში თხილის კვირტის ტკიპა მნიშვნელოვანი მავნებელი გახდა. კვლევის მიზანს წარმოადგენდა მავნებლის დროული გამოვლენა და დიაგნოსტიკა, რაც ხელს შეუწყობს ეფექტური ბრძოლის ღონისძიებების განხორციელებას. კვლევის განხორციელებისათვის შეირჩა დასავლეთ საქართველოს რეგიონებში გურიის, სამეგრელოს და აჭარის 20 ბაღი.

მავნე სახეობების იდენტიფიკაცია მოხდა მიტოქონდრიული ციტოქრომ ოქსიდაზას (COI) გენის ფრაგმენტის სეკვენირებით და შემდეგ აიგო ფილოგენეტიკური ხე. იზოლატების სეკვენსის BLASTN ანალიზმა გვაჩვენა, რომ ყველა იზოლატი აჩვენებს 93-99% მსგავსებას მონაცემთა ბაზაში (http://blast.ncbi.nlm.nih.gov/Blast) არსებულ *Phytoptus avellanae*-ს იზოლატებთან. ფილოგენეტიკური ანალიზი განხორციელდა პროგრამა MEGA7-ში, UPGMA მეთოდით. ფილოგენეტიკური ხის ანალიზი აჩვენებს, რომ ქართული იზოლატები ორ ძირითად კლადშია გაერთიანებული: პირველი მოიცავს 15 ქართულ იზოლატს და სამ იზოლატს მონაცემთა ბაზიდან: KR149013.1; KR149017.1; KT070248.1; აღნიშნული იზოლატების მიკროჰაბიტატს წარმოადგენდა კვირტები. მეორე კლადი მოიცავს ექვს ქართულ იზოლატს და ორს მონაცემთა ბაზიდან - KR149026.1 და KR149027.1, რომლებიც გამოყოფილ იქნა *Phytoptus*

avellanae-ს მოხეტიალე ფორმებიდან. ხოლო მესამე კლადი მოიცავს მხოლოდ ერთ ქართულ იზოლატს **GUR1**, რომელიც განსხვავებულია როგორც დანარჩენი ქართული იზოლატებისგან, ასევე მონაცემთა ბაზის იზოლატებისგან. ფილოგენეტიკური ხის ანალიზი აჩვენებს რომ ***Phytoptus avellanae***-ს მოხეტიალე და კვირტში მცხოვრები ფორმები განსხვავდებიან ერთმანეთისგან და მოხეტიალე ფორმა წარმოადგენს კრიპტულ სახეობას.

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