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Original scientific paper

MITOCHONDRIAL 16S rDNA PROFILING AND PHYLOGENETIC ANALYSIS SUGGEST GENETIC DIVERSITY OF ASH WEEVIL (*Stereonichus fraxini* De Geer) IN SERBIA

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This study contributes to knowledge of ash weevil (*Stereonychus fraxini* De Geer) molecular taxonomy, phylogeny and genetic diversity. Adult and larvae stages of insect were collected from several locations covering northern and central part of Serbia cojoined with homologous sequences with respect to their different geographic origin and hypothesis of their evolutionary relationships. Due to its slow rates of evolution the gene region that covers mitochondrial 16S rDNA, was choice for sequence profiling and phylogenetic reconstruction of ash weevil in correspondence with sequences of related tribes Cionini. Phylogenetic analyses demonstrating clear separation of the native weevil populations and the Cionini tribes. Even though bioinformatic tools confirm that all native specimens belong to species *Stereonychus fraxini*, different profile of the mitochondrial 16S rDNA in the clade of Serbian specimens indicate intraspecific genomic rearrangement in one specimen detached it to northern geographic position. Those particular specimens invade also different *Fraxinus* species. Genetic distinctness of other imagos from this particular individual proved by indels and point mutations found in their sequences. By screening the mitochondrial 16S rDNA, molecular evidence suggests the existence of the specimen with rearranged genome that indicate genetic variability in native populations of ash weevil species.

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INTRODUCTION

The weevils (superfamily-Curculionoidea), order Coleoptera 'Phytophaga' are the most species-rich superfamily of beetles comprising about 130 000 taxa that have been described worldwide (MARVALDI *et al.*, 2009) with several thousand species only in Europe (HUNDSDOERFER *et al*., 2009). One of the most harmful insect defoliator, feeding on ash species in South East Europe *Stereonychus fraxini* (De Geer 1775), is common not only in Europe but also found in North Africa and Asia Minor (WINGELMÜLLER, 1921; DREKIĆ *et al.*, 2014). Ash weevil was considered monophagous, but with recent investigations it belongs to oligophagous species feeding on species like *F. excelsior* L., *F. angustifolia* Vahl., *Olea europea* L. and *Phyllirea media* L. (MIKLOŠ, 1954; SCHERF, 1964; HRAŠOVEC and HARAPIN, 1999; DREKIĆ *et al*., 2014). Feeding on the leaves, ash weevil causes physiological weakness of the trees and in heavy attacks it could completely destroy terminal buds preventing its spring development. That opens the opportunity for invasion of secondary insects (VAJDA, 1974; MEDAREVIĆ *et al*., 2009). Phylogenetic studies based on morphological evidence were undertaken to reveal relationships within weevils and/or chrisomeloids (NAPP, 1994; REID, 2000; MARVALDI *et al*., 2002). Besides morphological, data based on biology of the insect were published by numerous authors (NÜSSLIN and RHUMBLER, 1927; MIKLOŠ, 1954; SCHREF, 1964; BLANDO and MINEO, 2004). In Serbia, research of *S. fraxini* has been conducted by AVRAMOVIĆ *et al*. (2008), DREKIĆ *et al*. (2011, 2013, 2014), and GLAVENDEKIĆ (2010) who recorded and confirmed its existence in Serbia on various plant species. Significant contribution to lacking data in its biology were provided by DREKIĆ *et al*. (2014) filling the gap that has been missing in this area of research, like fertility and female fecundity, number of larval stages and quantity of food consumed by larvae and the adults that existing in Serbia**.**

Since wide diversity of the species creates difficulties in the reconstruction of the phylogenetic relationships and individual morphological characterization, application of molecular tools has been added to morphological research in this study with aim to get better insights into genetic diversity of ash weevil population in Serbia.

MATERIALS AND METHODS

Material

Imago stage of ash weevils were collected in 17 locations exclusively in Serbia, in its central and northern part. Most specimens were hosted by *Fraxinus angustifolia* while one was found feeding on *F. pensylvanica* in Ristovača, Bač in the northern part, and additional one was collected from *F. excelsior* in the Juhor Oblast 12, Jagodina locality on the southern part of the country. After BLAST searching tool 7 sequences that showed more than 95% identity were added from GenBank/EMBL database and included in the analyses: *Cionus thapsus* (Fabricius 1792), *Cionus sp*., *Cionus olens* (Fabricius 1792), *Stereonychus fraxini* (De Geer 1775) derived from Germany as considered as referent sequence with 100% identity, *Adexius scrobipennis* (Gyllenhal 1834), and *Chrysolina fastuosa* (Scopoli 1763) and *Antarctobius vulsus* (Enderlein 1907) as the outgroup. A phylogeny of total of 24 sequences from Phytophaga including families Curculionoidea and Chrysomeloidea groups were recovered using mt 16S rDNA. Morphological identification keys (FREUDE *et al*., 1983) of weevil taxa and genomic studies were done in

collaboration of the Laboratory for Entomology and the Laboratory for Molecular Research of the Institute of Lowland Forestry and Environment of the University of Novi Sad.

DNA isolation, amplification and sequencing

Genomic DNA isolation, PCR amplification using master mixes and cycling parameters was done using protocol by HUNDSDOERFER *et al*. (2009). Amplification of about 500 bp of the mt 16S rDNA was performed using forward and reverse specific primers (originally designed by KOCHER *et al*. (1989); XIONG and KOCHER (1991) modified after primer LR-N-13398 and LR-J-12887 by SIMON *et al*. (1994). PCR amplifications were performed using Eppendorf Thermal cycler. The concentration and purity of the isolated DNA were determined by UV BioSpec-nano spectrophotometer. Amplicons were separated on 2% agarose gel stained with ethidium bromide (EtBr) using horizontal electrophoresis apparatus and visualized by DOC print system. Amplified products were extracted from agarose gel using QIAquick Gel Extraction Kit and purified with QIAquick PCR Purification Kit (Qiagen, www.qiagen.com). Sample sequences were prepared by manufacturer instructions and sequenced in forward and reverse directions using ABI3730XL Automated Sequencers and MACROGEN Europe services.

Sequence analysis and dendrogram construction

All 17 obtained sequences from Serbian weevil taxa were deposited in GenBank of National Centre for Biotechnology Information (Table 1). They were compared with 5 sequences from NCBI database using BLASTn searching tool. The analysis involved 24 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Evolutionary analyses were conducted in MEGA software (KUMAR *et al*., 2016; TAMURA *et al*., 2011). Sequences were examined and hand refined and then used for further phylogenetic and polymorphic analysis. Sequence alignment was performed using SeaView 4.6 software (SeaView - Multiplatform GUI for molecular phylogeny. For multiple alignments simple MUSCLE method was preferable since sequence wasn't DNA coding nature. Dendrograms were constructed using Neighbour-Joining Method in MEGA 7 (Molecular Evolutionary Genetics Analysis software based on the sequences from this study and the sequences obtained from NCBI database. In building phylogenetic tree, the attention was focused on three parameters: 1. Model/Method, 2. Rates among Sites and 3. Gap/Missing Data Treatment. The evolutionary history was inferred using the Neighbour-Joining method. 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. The evolutionary distances were computed using the Maximum Composite Likelihood method (TAMURA and KUMAR, 2002; TAMURA *et al*., 2004) and are in the units of the number of base substitutions per site.

Maximum Likelihood Method (ML) was chosen as it provides a variety of substitution models to correct for multiple changes at the same site during the evolutionary history of the sequences. Each entry shows the probability of substitution (r) from one base (row) to another base (column) (TAMURA *et al*., 2004). For the rates among sites the Gamma distributed with Invariant Sites (G+I) was selected including Transitions + Transversions mutation rates. For Gaps/Missing data treatment Partial Deletion option was preferable in case not to lose a lot of information by removing too much informative sites. Bootstrap values were calculated over 1000 replicates in order to assess branch topology. While *Chrysolina fastuosa* (Scopoli 1763) and

Antarctobius vulsus (Enderlein 1907) were used as outgroups in dendrogram construction, *Stereonychus fraxini* (De Geer 1775) (Accession No. in NCBI database AJ495552.1) was used as a reference sequence for polymorphism studies.

RESULTS

Sequences collected from 17 locations were successfully sequenced in both directions. Out of total *24* sequences analysed, 17 were generated in this study and compared with the aligned sequences from NCBI database. As the only match with 100% identity in all 17 cases, reference sequence of German geographical origin (Acc. No. AJ495552.1) confirmed taxonomical belonging of all Serbian specimens to the species *Stereonychus fraxini*. The rest six sequences including in this study were with lower discrimination rate but not lesser than 95%. Obtained data set of aligned mt16S rDNA from 24 sequences ranges from 465bp to 544bp total positions in the final dataset. After submission of 17 sequences to NCBI and together with sequences from the same data base total of 24 sequences (Table 1) were aligned.

Table 1. Gen Bank accession numbers, species, laboratory designation of each taxon, location and coordinates

GenBank accession number	Species	Laboratory designation	Location	GPS coordinates	
KX708918*	*S. fraxini*	1320ZAB027	Protected forests of Apatin, Serbia	N 45^{0}34′27″	E 18^{0}55′42″
KX708919*	*S. fraxini*	1320ZAB029	Village Gola Glava Valjevo, Serbia	N 44^{0}22′25″	E 19^{0}54′16″
KX708920*	*S. fraxini*	1320ZAB031	Sremska Mitrovica (Raškovica-Smogvica), Serbia	N 44^{0}57′20″	E 19^{0}09′56″
KX708921*	*S. fraxini*	1320ZAB041	Village Gornji Stepoš Kruševac, Serbia	N 43^{0}30′08 ″	E 21^{0}17′39″
KX708922*	*S. fraxini*	1320ZAB044	Morovic-(Vinična, Oblast 29), Serbia	N 44^{0}55′44″	E 19^{0}09′48″
KX755280*	*S. fraxini*	1320ZAB030	Village Zlot Bor, Serbia	N 44^0 01′ 27″	E 21^0 59′ 36″
KX755281*	*S. fraxini*	1320ZAB032	Odzaci-Branjevina Oblast12, Serbia	N 45^0 27′ 16″	E 19^0 12′ 11″
KX755282*	*S. fraxini*	1320ZAB033	Ristovaca – Bač, Serbia	N 45^0 25′ 36″	E 19^0 14′ 11″
KX755283*	*S. fraxini*	1320ZAB034	Rogot – Batočina, Serbia	N 44^0 08′ 46″	E 21^0 06′ 4″
KX755284*	*S. fraxini*	1320ZAB035	Village Preljina Čačak, Serbia	N 43^0 55′ 54″	E 20^0 25′ 9″
KX755285*	*S. fraxini*	1320ZAB036	Village Stepojevac, Serbia	N 44^0 37′ 29″	E 20^0 13′ 29″
KX755286*	*S. fraxini*	1320ZAB037	Village Osnic Zaječar, Serbia	N 43^0 53′ 02″	E 22^0 06′ 13″
KX755287*	*S. fraxini*	1320ZAB038	Juhor Oblast 12 Jagodina, Serbia	N 43^0 51′ 45″	E 21^0 16′ 03″
KX755288*	*S. fraxini*	1320ZAB040	Mlava Požarevac, Serbia	N 44^0 35′ 31″	E 21^0 14′ 41″
KX755289*	*S. fraxini*	1320ZAB042	Morovic (Blata-Malovanci, Oblast 29), Serbia	N 44^0 59′ 56″	E 19^0 9′ 54″
KX755290*	*S. fraxini*	1320ZAB043	S.Mitrovica (Vinična- Žeravinac–Puk), Serbia	N 44^0 56′ 28″	E 19^0 11′ 35″
KX755291*	*S. fraxini*	1320ZAB045	Village Vitojevac-Kraljevo, Serbia	N 43^{0}44′4″	E 20^0 48′ 13″
AJ495552.1	*S.fraxini* Ref. seq	-	Germany	-	-
GU988184.1	*Cionus* sp.	-	Germany	-	-
LN888401.1	*Cionus thapsus*	-	Germany	-	-
AJ495551.1	*Cionus olens*	-	Germany	-	-
EU286289.1	*Adexius scrobipennis*	-	-	-	-
AF097084.1	*Chrysolina fastuosa* Outgroup	-	USA	-	-
EF213979.1	*Antarctobius vulsus*	-	UK	-	-

*NCBI sequences Accessions numbers revealed from this study

Genetic distances were measured within and between groups of analysed sequences. According to ML pairwise comparison between sequences, the phylogenetic tree was constructed (Fig. 1).

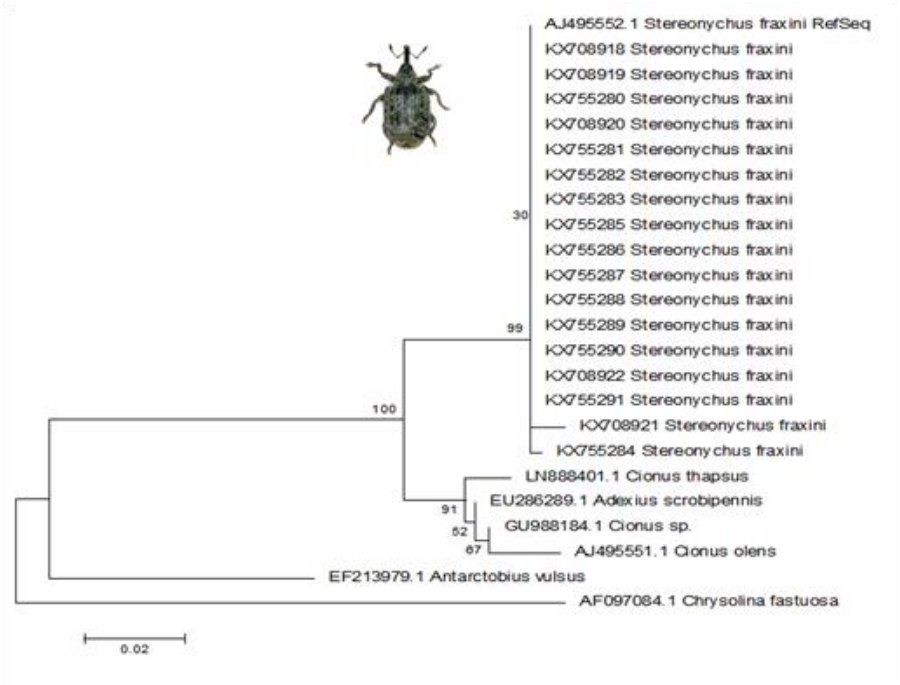

Fig. 1 Reconstruction of the phylogeny of the Curculionidae based on 16S mtDNA sequence data presented with branch lengths. Numbers of the left of nodes represent maximum likelihood bootstrap values. Scale line represents 0.02 evolutionary changes.

All Serbian specimens exhibited low level of intraspecific variations (Table 2) and polymorphic sites in comparison to referent sequence, while other species (*Cionus* and *Adexius*) revealed higher genetic variability.

After data processing and according to genetic distance of sequences, phylogenetic clustering revealed two main clades. All of the *Stereonychus fraxini* sequences including the reference sequence were grouped together within one clade and supported by the bootstrap value of 99%. This clade was clearly separated from the second clade (bootstrap value of 100%) that involved sequences derived from species from Curculionidae family like *Cionus thapsus*, *Cionus sp*., *Cionus olens*, and *Adexius scrobipennis*. Outgroup species from two different families, including Chrysomelidae with *Chrisolina fastuosa* and Curculionidae family represented by *Antarctobius vulsus* were also supported by bootstrap value of 100% in both clades and were different from all the sequences included in this study. First clade comprising all sequences of Serbian *S. fraxini* is showing slight deviation in genetic distinctness in three sequences, including KX708921 with 0.007 genetic distance originated from Village Gornji Stepoš Kruševac, and

0.002 genetic distance for KX755284 originated from Village Preljina Čačak and KX708920 originated from Sremska Mitrovica (Raškovica-Smogvica). It is also worthy to add that first two locations are geographically close, while the third one is oppositely located, in northern part of the country (Table 1).

Table 2. Informative nucleotide sites from the alignment of the 5' and 3' end ofthe partial mitochondrial 16S rRNA sequences for S. fraxini

Accession number	Vertical numbering* by the position in the sequence alignment (number of bp) regarding to the beginning of the referent sequence**																												
																4	4	4	4	4	4	4	5	5	5	5	5	5	5
	1*	1	1	1	2	2	2	2	2	2	3	3	3	3	3	6	7	7	8	8	8	8	0	1	3	3	4	4	4
	1	4	6	9	0	1	2	3	5	8	1	3	4	8	9	6	3	5	4	7	8	9	2	9	1	9	1	3	4
AJ495552.1**	A	T	A	T	A	A	G	G	C	G	C	G	-	T	A	T	T	T	T	-	-	-	-	-	-	-	-	-	-
KX708918	-	-	-	-	-	-	-	G	C	G	C	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX708919	-	-	-	-	-	-	-	-	-	G	C	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX755280	-	-	-	-	-	A	G	G	C	G	C	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX708920	-	-	-	-	-	-	G	G	C	G	C	G	C	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX755281	-	-	-	-	-	-	-	G	C	G	C	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX755282	-	-	-	-	-	G	G	A	A	G	C	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX755283	-	-	-	-	A	A	G	G	C	G	C	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX755284	-	-	-	-	-	-	-	-	-	-	-	C	T	G	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX755285	-	-	-	-	-	-	-	C	C	G	A	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX755286	-	-	-	-	-	-	-	-	-	-	-	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX755287	-	-	-	-	-	-	-	-	-	G	C	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX755288	-	-	-	-	-	-	-	G	C	G	C	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX708921	-	-	-	T	T	A	G	G	C	G	C	G	C	T	A	C	G	C	T	A	G	A	T	T	-	-	-	-	-
KX755289	-	-	T	A	A	G	G	G	C	G	C	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	C	-
KX755290	-	-	-	T	A	A	A	G	C	G	C	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX708922	-	-	-	-	-	-	-	G	C	G	C	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
KX755291	-	-	T	T	A	A	G	G	C	G	C	G	-	T	A	T	T	T	T	A	G	A	G	T	C	A	A	-	-
GU988184.1	-	T	A	T	A	A	G	G	C	G	C	G	-	T	T	T	T	T	A	T	A	-	G	A	T	T	T	A	G
LN888401.1	-	-	-	-	-	-	-	-	-	G	C	G	-	T	T	T	T	T	A	T	A	-	G	-	-	-	-	-	-
AJ495551.1	T	T	A	T	A	A	G	G	C	G	C	G	-	T	T	T	T	T	A	-	-	-	-	-	-	-	-	-	-
EU286289.1	C	T	A	T	A	A	G	G	C	G	C	G	-	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AF097084.1	-	A	A	T	A	A	G	G	C	A	C	G	-	A	A	T	T	T	A	T	A	-	G	-	-	-	-	-	-
EF213979.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	T	T	A	T	A	T	G	A	T	-	-	-	-

** Referent sequence; Underlined – sequences generated in this study

The optimal tree with the sum of branch length = 0.31151962 is shown (Figure 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (FELSENSTEIN, 1985).

Structural variation of the sequences

Structural profile of mt16S rDNA sequence of Serbian weevil against referent sequence of German origin comprised 13-point mutations, where transversion mutations prevailed to transition ones and 3 insertions.

Table 3. Gene Bank Accessions, species and family designation, country of origin with locations and host species

Accession No. NCBI	Species	Familly	Country origin	Host species	Location/Source
KX708918	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Protected forests of Apatin, Serbia
KX708919	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Village Gola Glava Valjevo, Serbia
KX708920	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Sremska Mitrovica (Raskovica-Smogvica), Serbia
KX708921	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Village Gornji Stepos Krusevac, Serbia
KX708922	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Morovic-(Vinična, Oblast 29), Serbia
KX755280	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Village Zlot Bor, Serbia
KX755281	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Odzaci-Branjevina Oblast12,Serbia
KX755282	*S. fraxini*	Curculionidae	Serbia	*Fraxinus pennsylvanica* Marsh.	Ristovaca – Bač, Serbia
KX755283	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Rogot – Batočina, Serbia
KX755284	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Village Preljina Čačak, Serbia
KX755285	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Village Stepojevac, Serbia
KX755286	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Village Osnic Zaječar, Serbia
KX755287	*S. fraxini*	Curculionidae	Serbia	*Fraxinus excelsior* L.	Juhor Oblast 12 Jagodina, Serbia
KX755288	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Mlava Požarevac, Serbia
KX755289	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Morovic (Blata-Malovanci, Oblast 29), Serbia
KX755290	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	S.Mitrovica (Vinična- Žeravinac–Puk), Serbia
KX755291	*S. fraxini*	Curculionidae	Serbia	*Fraxinus angustifolia* Vahl.	Village Vitojevac-Kraljevo, Serbia
AJ495552.1	*S. fraxini RefSeq*	Curculionidae	Germany	-	Hundsdoerfer A. et al., Institute of Pharmaceutical Biology, University of Heidelberg, Germany
GU988184.1	*Cionus sp.* (Fabricius 1792)	Curculionidae	Germany	Quercus, Carpinus	Lat_lon: 50.158611 N 7.152500 E
LN888401.1	*Cionus thapsus* (Fabricius 1792)	Curculionidae	Germany	-	Taenzler,R. et al., Entomology, Zoological State Collection, Munich, Germany
AJ495551.1	*Cionus olens* (Fabricius 1792)	Curculionidae	Germany	-	Hundsdoerfer A., Institute of Pharmaceutical Biology, University of Heidelberg, Germany
EU286289.1	*Adexius scrobipennis* (Gyllenhal 1834)	Curculionidae	France	-	Zoologisches Forschungsmuseum AlexandeKoenig (ZFMK), Bonn Germany
AF097084.1	*Chrysolina fastuosa* (Scopoli, 1763)	Chrysomelidae	USA	-	Hsiao,T.H. and Pasteels,J.M. Utah State Univ., Logan, UT 84322,USA
EF213979.1	*Antarctobius vulsus* (Enderlein, 1907)	Curculionidae	UK	-	Papadopoulou,A. et al., Department of Entomology, The Natural History Museum, London SW7 5BD, UK

Point mutations were found in sequences Acc. No. KX708921, KX755282, KX755284 and KX755285 where 6 transversions were revealed in 4 populations named: Village Gornji Stepoš Kruševac, Ristovaca-Bač, Village Preljina Čačak and Village Stepojevac. All sequences

were from the weevil hosted the same taxon (Table 3), except one from Ristovaca-Bač that was collected from *F. pennsylvanica*, where 3 mutations (2 transitions and 1 transversion) were found.

Three indels, particularly insertions, were edited in three sequences Acc. No. KX708920, KX708921, and KX755284 of the weevil found in native populations (Sremska Mitrovica (Raškovica-Smogvica), Village Gornji Stepoš Kruševac, Village Preljina Čačak). Higher sequence polymorphisms were found in *Cionus* and *Adexius* taxons. For simplicity, the sum of r values has been made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in *italics*. (Table 4). The nucleotide frequencies are 35.88% (A), 39.44% (T/U), 16.22% (C), and 8.47% (G). The transition/transversion rate ratios are k_1 = 1.089 (purines) and k_2 = 1.691 (pyrimidines). The overall transition/transversion bias is R = 0.48, where R = [A*G*k_1 + T*C*k_2] / [(A+G) *(T+C)] (Table 4).

Table 4. Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	T	C	G
A	-	11.68	2.51	5.23
T	10.62	-	4.24	4.8
C	10.62	19.75	-	4.8
G	11.56	11.68	2.51	-

Legend: Each entry shows the probability of substitution (r) from one base (row) to another base (column). Rates of different transitional substitutions are shown as underlined and all others are transversional substitutions.

DISCUSSION

Based on 16S rDNA profiling and phylogenetic analyses of individuals from 17 different locations in central and northern Serbia, genetic variability was recorded in native populations of ash weevil. In addition, sequences from Curculioninae and Cionini were included in this study to recover phylogenetic relationship with these genetically similar groups. According to HUNDSDOERFER *et al*. (2009) and WINK *et al*., (1997), mt16SrDNA marker was more variable than nuclear 18S rDNA and could provide higher resolution within Curculionidae in respect to relationships within the families and subfamilies as showed in this research. All specimens from 17 locations in Serbia were designated as species *Stereonychus fraxini* compared against *S. fraxini* with 100% identity derived from Germany and outlined as referent sequence. Other six blasted sequences were with more than 95% identity in comparison to native specimens taxonomically belonging to family Curculionidea as species *Cionus thapsus*, *Cionus olens*, Cionus sp. all from Germany, *Adexius scrobipennis* from France and *Anctarctobius vulsus* from the UK. *Chrisolina fastuosa* (Chrisomelidae, USA) was introduced as outgroup. Based on ML method the phylogenetic tree was constructed. Tree comprised two clades, one consisted of *Sreonychus fraxini* from native populations together with referent sequence from Germany indicating, in spite geographical distinctness, their genetic similarity. In this clade there are three sequences that are distinguished from others affiliated, one from northern part of Serbia and two from central part. Differentiation of the distinguished individuals was derived from their specific

sequence structure. The other clade was separated as genetically close and involved *Cionus* and *Adexius* species. As the most distant, third clade were *Antarctobius vulsus* from Curculionidea and *Chrisolina fastuosa* as species from sister group Chrisomelidae as outgroup in this research. Based on these intraspecific sequence polymorphisms observed in this study, additional morphological and ecological research should be introduced in the future.

Numerous genomic variations were considered in order to gather more knowledge about genetic divergence between populations of ash weevil found in Serbia. The focus was on low-level genomic variations such as Single Nucleotide Polymorphisms (SNPs), Insertion/Deletion, transitions and transversions. Those are revealing small differences between genomes but are important drivers of the evolution processes, by modifying how or whether genes are transcribed and translated. Those mutations, especially transversions prevails in noncoding regions. Point mutations also cover a single nucleotide insertions or deletions as types of frameshift mutations. This kind of mutations can make the rearrangement in the sequence and implicate a frame shift that could lead to structural rearrangement in the genome of the individual itself. (BECKENBACH *et al*., 2005). Also trigger of the shift in the gene expression, rearranging the structure and function of the protein product. Those changes can possibly change morphology of the organism and thus are very important in expression of the morpho-anatomic characteristics of the individual. This kind of low level genomic variation was observed in all sequences in this study. Out of 24 point mutations 13, together with 3 insertions, were observed in the first clade of native weevil. While 11 were found in six sequences comprised by second clade (mainly A---T type) and without indels. Insertions were found only in native weevils in the first clade of the phenogram. Insertions were observed as single nucleotide insertions of C and G bases, which are possible to found in noncoding regions in three sequences from Sremska Mitrovica (Raškovica-Smogvica), Acc. No. KX708920, Village Gornji Stepos Kruševac, Acc. N. KX708921 and Village Preljina Čačak, Acc. N. KX755284. The other point mutations observed in five sequences of *S. fraxini* were of transversions nature (6) that prevailed transition ones (4). Similar results were found by XIONG and KOCHER (1991) during the using mt 16S rDNA marker in several morphotypes of black flies. Most transversions differences were A---C and T---G type. It was interesting that both types of mutations (transitions and transversions) were found in the specimen that hosted by *F. pennsilvanica*. The assumption could be that those several individuals were started adaptation of their genomes to either of northern climatic zone or to different plant host of *Fraxinus* species. Due to structural rearrangements in the ash weevil mt 16S rDNA region it could be concluded that genetic differences exists on the genom bases between populations from different location of *Stereonychus fraxini* in Serbia.

There are several aspects that could be interesting to elucidate in future research regarding ash weevil in Serbia like determining the degree of its preferences to *F. pennsylvanica*, *F. excelsior* and *F. pennsilvanica* var. *lanceolata*. Possible differences in biology, morpho-anatomic characters (DREKIĆ, 2017), extent of preferences to other ash species and molecular profiles as well of different recently observed forms of ash weevil should be in the focus of the future research interest.

CONCLUSIONS

This study was conducted by using SNPs and mt 16SrDNA marker to reveal taxonomy, phylogenetic analyses and genetic variability between populations of ash weevil in Serbia. Results prooved taxonomical belonging of native ash weevel to *Stereonychus fraxini* species and

its genetic similarity to European origin. This research work represents significant addition to the knowledge about genetic variability and profile of autochtonous insect species in Serbia, but even more important they are a valuable contribution to the understanding of phylogenetic relationships within selected imagos of ash weevel genera in the country. Analysis of constructed dendrograms pointed out that low genetic variations observed between native populations of ash weevils comprised all collected serbian specimens into one clade with exception of three genera from geogafically distant locations and with a specific genetic profile. The second, genetically distinct clade, was highly differentiate, included sequences of representative of Cionini tribes of subfamily Curculioninae. Resolving the suficient variability to distinguish between species, mt 16S rDNA marker was found suitable for the research of interspecific studies as well as phylogenetic analysis in insect community. This research data can be a valuable contribution to the present knowledge in taxonomy, as well as phylogeny and genetic variability, of the species belonging to Curculionidae family in this region.

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http://www.megasoftware.net MEGA 7 (Molecular Evolutionary Genetics Analysis software)

PROFIL MITOHONDRIJALNE 16S rDNA I FILOGENETSKA ANALIZA UKAZUJU NA GENETIČKU VARIJABILNOST JASENOVE PIPE (*Stereonychus fraxini* De Geer) U SRBIJI

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Izvod

Jasenova pipa (*Stereonychus fraxini* De Geer) egzistira u velikom broju vrsta i ekstremnom bogatstvu oblika koji su uzrok dosadašnje nezadovoljavajuće klasifikacije isključivo morfo-anatomskim pristupom. Da bi se poboljšala klasifikacija, prethodne studije obuhvataju molekularne tehnike koje uključuju nuklearne i organelarne markere. Koristeći molekularne metode poput sekvenciranja, analiziranja profila sekvence mitohondrijalnog 16S rDNA, rezultati sugerišu postojanje individua sa preuređenim genomom koji ukazuju na genetsku varijabilnost kod prirodnih populacija pipe u Srbiji. Podaci dobijeni u ovoj studiji doprineli su utvrđivanju taksonomije jasenove pipe, njene filogenetske pripadnosti i postojanja genetičke varijabilnosti ove vrste u Srbiji.

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