Eurasian Journal of Forest Science

2023 11(2): 66-78

https://dergipark.org.tr/en/pub/ejejfs

A Preliminary mitochondrial cytochrome *c* oxidase-I-based phylogeographic and phylogenetic analysis of Eurasian *Acanthocinus griseus* (Coleoptera, Cerambycidae)

Aynur ABBASZADA[1], Fevzi UÇKAN[1], Havva Kübra SOYDABAŞ-AYOUB[1,*]

[1)]*Department of Biology, Faculty of Arts and Science, Kocaeli University, Kocaeli, 41100, Turkey.*

Corresponding author: havvasoydabas@gmail.com

Abstract

Acanthocinus griseus (Fabricius, 1792) (Coleoptera: Cerambycidae, Lamiinae, Acanthocinini) has long been known for its role in the decay process of the wood in the forest ecosystem, and two critical features of the species, inhabiting standing trees and being a vector of pine wood nematodes *Bursaphelenchus* spp., have been noted recently. Therefore, understanding the current relationships and possible migration scenarios has been further required to assess invasion risks. The present work provided a preliminary comprehension of the phylogeographic and phylogenetic relationships of *A. griseus* based on the mitochondrial cytochrome *c* oxidase-I (COI) gene region (658 bp), with sequences produced in the present study, from the specimens collected from timberyards, ports and forests of Kocaeli Province, Turkey, and with available sequences in GenBank of inhabitants of Eurasia, and of intercepted specimens in ports. The intraspecific genetic distance of *A. griseus* was 1.37-0,3%, while the interspecific distance was 10,79-13,37%, except the closeness of an *A. griseus* haplotype (AGR1) to *A. sachalinensis* (0,3%) more than its conspecifics (4,71-5,47%). The ML and BI analyses suggested identical topologies. The statistical parsimony network drew a reticular branching diagram without grouping across countries, which addresses ongoing gene flow. Most haplotypes from Turkey were clustered around a central haplotype (AGR11), which may indicate a bottleneck effect. A haplotype previously intercepted in USA ports was identical to one sampled in Kocaeli. The present study suggests the possible ongoing intraspecific gene flow within *A. griseus* might be due to facilitated migration by the international wood trade, and the relationship between *A. griseus* and *A. sachalinensis* should be reconsidered from both morphological and molecular points of view.

Keywords: DNA Barcoding, biosecurity, international wood trade, migration, vector, pine wilt nematode.

Introduction

The intensification of international wood trade traffic in the last decades has led to an increase in threats posed by pests, diseases, and invasive species. The adopted measures and precautions, such as ISPM No. 15 (international standard for phytosanitary measures), border surveillance, and containment, successfully reducing transferring of non-native organisms at the arrival point (Allen et al. 2017). However, none of the measures and implementations can prevent invasions entirely (Hulme 2009, Haack et al. 2014). Therefore, post-border surveillance is indispensable, especially for the forests located around ports. Kocaeli province is a forested port city between the Balkan Peninsula and Asia Minor, in which a high volume of industrial wood, timber and wooden packaging materials have been

transferred, stored and processed (Çakmak et al. 2019). One of the wood-boring species that has been reported repeatedly from Kocaeli ports, timberyards and forests in the last few years is *Acanthocinus griseus* (Fabricius, 1792) (Soydabaş et al. 2017; Atak et al. 2021; Soydabaş-Ayoub and Uçkan, 2022). *A. griseus* has an essential role in the forest ecosystem by contributing to the decay process of weakened, burned, uprooted or recently felled trees. It had been a golden specimen for collectors due to its rarity during the 20th century (Lindhe et al. 2010, Cocoş et al. 2017). However, for the last two decades, *A. griseus* has fallen from grace because it is more abundant than expected (Martikainen 2002) and more harmful than previously thought (Wang et al. 2021). Nowadays, it is known that this saproxylic cerambycid has a wide range of natural distribution in Europe, from Spain, Italy, and Greece in the south to Norway, Sweden, and Finland in the north, wherever coniferous species occur (Bense 1995). It mainly condenses in weakened spruce but also inhabits standing trees. It has been noted that the beetle is a vector of pine wood nematode, *Bursaphelenchus xylophilus* (Linit 1988, Ryss et al. 2005, Wang et al. 2021), which causes critical physiological changes such as reduced photosynthesis levels and cambium destruction (Fukuda 1997). Also, it has been associated with another nematode, *Bursaphelenchus mucronatus*, sampled on the imported logs from Bulgaria at the border of Turkey and Bulgaria (Tülek et al. 2019). Moreover, the species has been reported with several port interceptions from the United States (Wu et al. 2017). However, neither intraspecific relationships within a nor migrations across countries had not been studied up to date.

One of the practical tools for post-border biosecurity surveillance is DNA barcoding using cytochrome *c* oxidase I (*COI*) gene region (Armstrong and Ball 2005, Collins et al. 2012). Furthermore, this gene region is useful for monitoring migration routes (Moritz 1994, Roderick 1996, Hurst and Jiggins 2005) and revealing inter and intra-specific phylogenetic relationships (Simon et al. 1994, Hebert et al. 2004a, Hernández-Triana et al. 2015, Paterson et al. 2016).

Therefore, in the present work, we aimed to determine native and introduced haplotypes of *A. griseus* and their phylogenetic and phylogeographic relationships, and to unveil likely cryptic speciation using the COI gene region.

Materials and methods

Sampling and Identification

The samples collected from timber yards and forests using three-funnel traps, ipsdienol and α-pinene binary combination were held in (Table 1). Morphological identification was conducted according to Bíly and Mehl (1989) and Bense (1995). The specimens were preserved at -20 °C in 99% ethanol and deposited at Kocaeli University, Turkey, Department of Biology.

DNA Isolation, PCR, and Sequencing

A piece of femur muscle of each specimen was rested overnight in the lysis buffer (2% sodium dodecyl sulfate (SDS), 3 mM $CaCl_2$, 250 µg/ml proteinase K, 8 mM dithiothreitol (DTT), 100 mM Tris buffer pH 8, and 100 mM NaCl) (Soydabaş-Ayoub 2021) and DNA isolation was performed according to Sambrook and Russell (2006). The PCRs (polymerase chain reactions) were performed for the HCO2198 – LCO1490 primer pair (Folmer et al. 1994) to amplify cytochrome *c* oxidase I (COI) gene region by BioRad CFX Connect™ thermal cycler. Quick-Load® Taq 2X Master Mix (New England Biolabs Inc.) was used with the addition of 0.08 mg/mL bovine serum albumin (Soydabaş-Ayoub 2021), 0.2 µM primer for each and around 50 ng of template DNA dissolved in ddH_2O. The thermocycling parameters were 95 °C for 1 min, 5 cycles of 95 °C for 30 s, 46 °C for 1 min and 72 °C for 1 min; then, 30 cycles of 95 °C for 30 s, 51 °C for 60 s, 72 °C for 1 min, and 72 °C for 10 min ending (Aksöyek et al. 2017).

Table 1. Voucher codes and GenBank accession numbers of *Acanthocinus griseus* specimens, their sampling dates, geographic coordinates, altitudes, and localities.

#	Voucher Code	Accession Number	Date	Coordinate	Altitude (m)	Locality
1	KOU-AG119	OP342792	01.06.2016	Lat: 40.819576, Lng: 29.493635	173	Gebze
2	KOU-AG101	OP342789	25.09.2017	Lat: 40.829273, Lng: 29.918154	460	İzmit
3	KOU-AG103	OP342791	14.05.2016	Lat: 40.827546, Lng: 29.913539	484	İzmit
4	KOU-AG130	OP342790	07.06.2017	Lat: 40.825921, Lng: 29.497796	208	Gebze
5	KOU-AG71	OP342788	28.05.2016	Lat: 40.828907 , Lng: 29.917394	426	İzmit
6	KOU-AG117	OP342794	05.06.2016	Lat: 40.788767, Lng: 29.845592	298	Derince
7	KOU-AG24	OP342793	18.07.2017	Lat: 40.827546 , Lng: 29.913539	484	İzmit

PCR products were purified using ExoSAP-IT™ PCR Product Cleanup Reagent and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) by ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA) at Macrogen Netherlands Laboratory. The sequences were uploaded to GenBank under OP342788-OP342794 accession numbers.

Genetic Diversity, Phylogenetic Analysis and Network

The chromatograms obtained by bidirectional reads were checked and assembled in Geneious Prime v2019 (Kearse et al. 2012, Biomatters Inc., USA) and blasted on GenBank. The sequences obtained in the present study (Table 1) and retrieved from GenBank and BOLD (Barcode of Life) taxonomy browsers (Table 2, Table 3) were aligned by MUSCLE v3 (Edgar 2014). The aligned dataset (658 bp) was analysed in DnaSP v6 (Rozas et al. 2017) to calculate the total number of mutations (Eta), the number of segregating sites (S), nucleotide diversity (Pi), and haplotype diversity (Hd). The uncorrected p-distances between haplotypes were calculated in MEGA X v10.0.5 (Kumar et al. 2018). The best-fitting model was determined using greedy search by PartitionFinder v2 (Stamatakis 2014, Lanfear et al. 2017) according to AICc (Akaike's information criteria corrected) for the phylogenetic analyses. The outgroups were selected among the sequences in the BOLD taxonomy browser, representing each available species of the genus *Acanthocinus* (Table 3). Maximum Likelihood (ML) analysis was performed in PhyML (Guindon et al. 2010) with the TN93 nucleotide substitution model and 10,000 bootstrap replicates. Bayesian Inference (BI) analysis was performed in MrBayes (Ronquist and Huelsenbeck 2003) with a GTR+G+I model of nucleotide substitution, 10,000,000 chain length and 10,000 subsampling frequency. The first 25% of the states were discarded as burn-in. Statistical parsimony network (TCS) (Templeton et al. 1992) was performed in PopART v1.7 (Leigh and Bryant 2015).

Results

Genetic diversity of Acanthocinus griseus

A total number of 19 haplotypes was determined among 27 sequences, 658 bp in length; seven of them were produced in this study. The guanine and cytosine (G+C) rate was 32.3%, compatible with protein-coding DNA sequences. A summary of genetic diversity statistics is presented in Table 4.

Table 2. Haplotype codes, accession IDs, and sampling localities of *Acanthocinus griseus* specimens obtained from this study and retrieved from databases

#	Haplotype Code	GenBank Accesion ID	Locality	References
1	AGR1	KY357618	Intercepted in a USA Port	Wu et al. 2017
2	AGR2	KM450656	Italy, Veneto	Rougerie et al. 2015
3	AGR3	KJ966915	Finland, Nylandia	Pentinsaari et al. 2014
4	AGR4	OP279163	Turkey, Marmara Basin	Soydabaş-Ayoub and Uçkan 2022
5	AGR5	OP342789	Turkey, Kocaeli Izmit	This study
6	AGR6	OP279164	Turkey, Marmara Basin	Soydabaş-Ayoub and Uçkan 2022
7	AGR7	KJ963896	Finland, Kainuu	Pentinsaari et al. 2014
8	AGR8	OP342788	Turkey, Kocaeli Izmit	This study
9	AGR9	KY357621	Intercepted in a USA Port	Wu et al. 2017
10		KY357620	Intercepted in a USA Port	Wu et al. 2017
11		OP279167	Turkey, Marmara Basin	Soydabaş-Ayoub and Uçkan 2022
12		OP279168	Turkey, Marmara Basin	Soydabaş-Ayoub and Uçkan 2022
13		OP279169	Turkey, Marmara Basin	Soydabaş-Ayoub and Uçkan 2022
14	AGR10	KM450534	Italy, Veneto	Rougerie et al. 2015
15	AGR11	OP342792	Turkey, Kocaeli Izmit	This study
16		OP279170	Turkey, Marmara Basin	Soydabaş-Ayoub and Uçkan 2022
17	AGR12	OP342790	Turkey, Kocaeli Gebze	This study
18	AGR13	OP342791	Turkey, Kocaeli Izmit	This study
20	AGR14	OP342793	Turkey, Kocaeli Izmit	This study
21		OP279165	Turkey, Marmara Basin	Soydabaş-Ayoub and Uçkan 2022
22		OP279166	Turkey, Marmara Basin	Soydabaş-Ayoub and Uçkan 2022
23	AGR15	OP342794	Turkey, Kocaeli Derince	This study
24	AGR16	KM441146	Italy, Veneto	Rougerie et al. 2015
25	AGR17	KU918495	Germany, Bavaria	Rulik et al. 2017
26	AGR18	KU915734	Germany, Thuringia	Rulik et al. 2017
27	AGR19	HQ559242	Finland, South Karelia	Pentinsaari et al. 2014

The interspecific distances ranged from 10.79 to 13.37%, with one exception. The distance between the AGR1 (*A. griseus*, KY357618, Wu et al. 2017) and ASA (*A. sachalinensis*, KY683654, Grebennikov et al. 2017) haplotypes was 0.3%. Their distances to other *A. griseus* haplotypes were similar and ranged between 4.71-5.47%. The intraspecific distances among remaining *A. griseus* haplotypes (ASR2-ASR12) were between 1.37-0.3%

Table 3. Binomial names, BOLD IDs and GenBank accession numbers of outgroups are used in phylogenetic analyses.

#	Species	BOLD-ID	GenBank	References
1	*Acanthocinus obliquus*	CERNO032-08		Anonymous
2	*Acanthocinus reticulatus*	PSFOR030-13	KM285803	Rougerie et al. 2015
3	*Acanthocinus aedilis*	PSFOR029-13	KM286078	Rougerie et al. 2015
4	*Acanthocinus pusillus*	CERGL150-08		Anonymous
5	*Acanthocinus nodosus*	BBCCA042-12		Anonymous
6	*Acanthocinus princeps*	CERPA269-08		Anonymous
7	*Acanthocinus sachalinensis*	VVGPL2946-15	KY683654	Grebennikov et al. 2017

Table 4. Summary of genetic diversity statistics of COI gene region (658 bp) of *Acanthocinus griseus*

Samples	n	H	Hd	SD(Hd)	Pi	SD(Pi)	S	Pin	Eta	G+C (%)
This study	7	7	1	0.076	0.00420	0.00081	8	2	8	32.3
Overall	27	19	0.957	0.028	0.00948	0.00334	50	12	53	32.3

n, the number of samples; H, the number of haplotypes; Hd, the haplotype diversity; Pi, the nucleotide diversity; Eta, the total number of mutations; S, the number of segregating sites, Pin Parsimony informative sites and SD standard deviation.

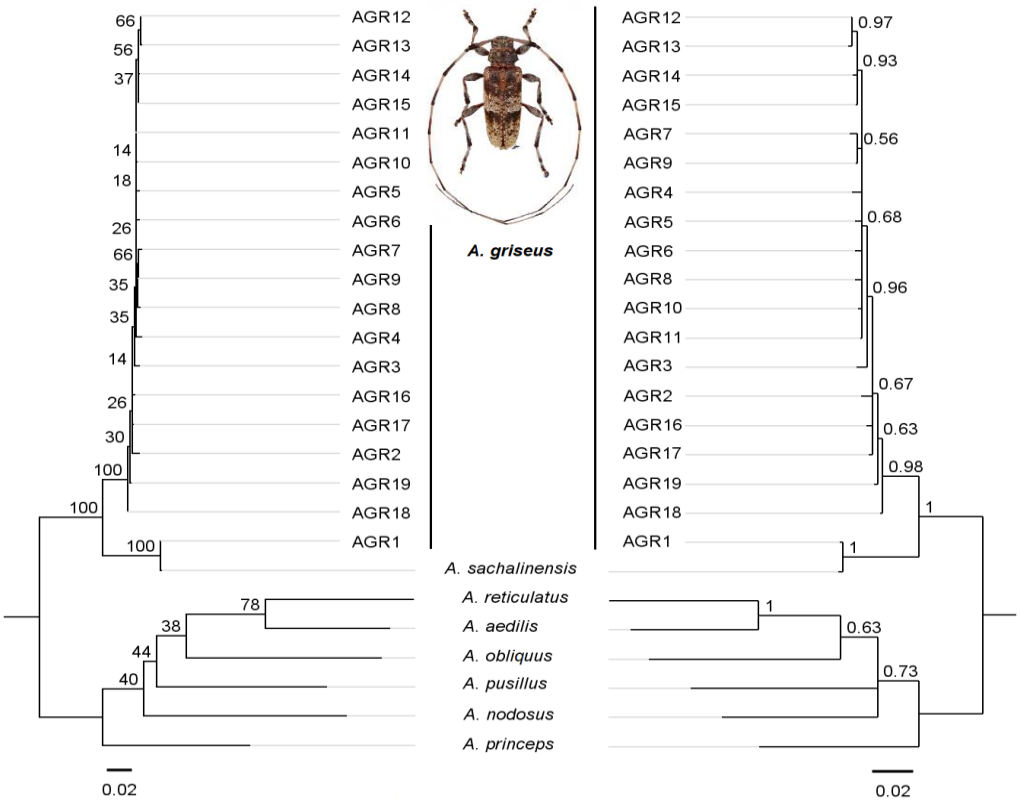

Figure 1. The phylograms inferred from Maximum Likelihood (ML) (left) and Bayesian Inference (BI) (right) analyses of 658 bp mitochondrial COI gene region of *Acanthocinus griseus* samples. The other members of the genus were used as an outgroup. Scale bars represent substation per site. Bootstrap supports of ML and posterior probabilities of BI are shown beside nodes. The photograph of *Acanthosinus griseus* is courtesy of Jean-Philippe Roguet (lamiinae.org).

.

Table 5. Uncorrected p-distances (%) between COI haplotypes of *Acanthocinus griseus* and other *Acanthocinus* species (average 5.8%)

	AOB	ARE	AAE	APU	ANO	APR	AGR1	ASA	AGR2	AGR3	AGR4	AGR5	AGR6	AGR7	AGR8	AGR9	AGR10	AGR11	AGR12	AGR13	AGR14	AGR15	AGR16	AGR17	AGR18	AGR19
AOB																										
ARE	**12.77**																									
AAE	**11.25**	**9.57**																								
APU	**12.31**	**12.01**	**12.01**																							
ANO	**11.70**	**10.79**	**12.01**	**12.46**																						
APR	**12.46**	**13.07**	**12.77**	**12.01**	**11.09**																					
AGR1	**13.22**	**13.07**	**12.77**	**12.01**	**12.16**	**11.25**																				
ASA	**13.37**	**12.92**	**13.07**	**12.31**	**12.16**	**11.25**	**0.30**																			
AGR2	12.46	11.85	11.85	11.09	10.94	10.94	**5.02**	**5.02**																		
AGR3	12.77	12.31	12.31	11.70	11.25	11.25	**5.17**	**5.17**	1.22																	
AGR4	12.61	11.70	12.16	11.09	11.25	11.25	**5.47**	**5.47**	1.37	1.06																
AGR5	12.77	12.16	12.16	11.40	11.40	11.40	**5.62**	**5.62**	1.22	0.91	0.76															
AGR6	12.31	11.70	11.70	10.94	11.25	10.94	**5.17**	**5.17**	1.22	0.91	0.76	0.61														
AGR7	12.77	11.85	11.85	11.25	11.40	11.09	**5.47**	**5.47**	1.06	1.06	0.91	0.76	0.76													
AGR8	12.31	11.55	11.55	11.25	10.79	11.09	**5.47**	**5.47**	1.06	0.91	0.76	0.61	0.61	0.61												
AGR9	12.46	11.85	11.85	10.94	11.09	11.09	**5.17**	**5.17**	0.76	0.76	0.61	0.46	0.46	0.30	0.30											
AGR10	12.61	12.01	12.01	11.25	11.25	11.09	**5.17**	**5.17**	1.06	0.76	0.61	0.46	0.46	0.61	0.46	0.30										
AGR11	12.46	11.85	11.85	11.09	11.09	11.09	**5.32**	**5.32**	0.91	0.61	0.46	0.30	0.30	0.46	0.30	0.15	0.15									
AGR12	12.46	12.01	12.01	11.40	11.25	11.09	**5.62**	**5.62**	0.91	0.91	0.76	0.61	0.61	0.76	0.61	0.46	0.46	0.30								
AGR13	12.61	12.16	12.16	11.55	11.40	11.25	**5.78**	**5.78**	1.06	1.06	0.91	0.76	0.76	0.91	0.76	0.61	0.61	0.46	0.15							
AGR14	12.77	12.16	12.01	11.40	11.25	11.09	**5.62**	**5.62**	1.22	0.91	0.76	0.61	0.61	0.76	0.61	0.46	0.46	0.30	0.30	0.46						
AGR15	12.61	12.01	12.01	11.25	11.09	10.94	**5.47**	**5.47**	1.06	0.76	0.61	0.46	0.46	0.61	0.46	0.30	0.30	0.15	0.15	0.30	0.15					
AGR16	12.77	11.85	12.16	11.40	10.94	10.79	**5.02**	**5.02**	0.91	0.91	1.06	0.91	0.91	1.06	0.91	0.76	0.76	0.61	0.91	1.06	0.91	0.76				
AGR17	12.31	12.01	12.01	11.25	10.64	10.94	**5.17**	**5.17**	0.76	0.76	0.91	0.76	0.76	0.91	0.76	0.61	0.61	0.46	0.76	0.91	0.76	0.61	0.46			
AGR18	12.31	11.55	11.70	10.79	10.94	10.64	**4.71**	**4.71**	0.91	0.91	0.76	0.91	0.61	1.06	0.91	0.76	0.76	0.61	0.91	1.06	0.91	0.76	0.61	0.46		
AGR19	12.46	11.70	11.85	10.79	10.79	10.79	**4.71**	**4.71**	0.61	0.91	0.76	0.91	0.91	0.76	0.76	0.46	0.76	0.61	0.91	1.06	0.91	0.76	0.61	0.46	0.30	

AOB: *Acanthocinus obliquus*, ARE: *A. reticulatus*, AAE: *A. aedilis*, APU: *A. pusillus* ANO: *A. nodosus*, APR: *A. princeps*, AGR. *A. griseus*, ASA: *A. sachalinensis*

Phylogenetic Analysis and Network

The phylograms inferred from ML and BI analyses of the 658 bp mitochondrial COI gene region of *Acanthocinus* samples were compatible. The outgroups showed the same clusterings. *A. reticulatus* was retrieved as a sister to *A. aedilis*. *A. princeps* was stated at the basal-most branch (Figure 1). *A. sachalinensis* were retrieved as a sister to AGR1 haplotype of *A. griseus*. Both were clustered under the *A. griseus* species group. All the remaining haplotypes were clustered together without a distinctive grouping but with polytomies.

The statistical parsimony network provided a profound understanding of intraspecific relationships of *A. griseus* haplotypes. AGR1 was 30 mutational steps away from the closest one, and a hypothetical haplotype connected AGR19 from Finland and AGR18 from Germany. Also, three more hypothetical haplotypes built a bridge to connect the haplotypes AGR 18-AGR19 to AGR3, AGR2 and AGR16, AGR17, and AGR 11 from Finland, Italy, Germany, and Turkey, respectively.

The haplotype AGR11 was at the centre of other haplotypes from Turkey, and a radial branching appeared around this haplotype. All of the haplotypes around AGR11 were from Turkey, with some exceptions. The AGR10 was only one mutational step from the central haplotype and was from Italy. In addition, the haplotype named AGR9 was sampled in Turkey in this study. It was previously reported as an intercepted haplotype in a USA port by Wu et al. (2017). Also, there were only two mutational steps between AGR9 and AGR7 from Finland.

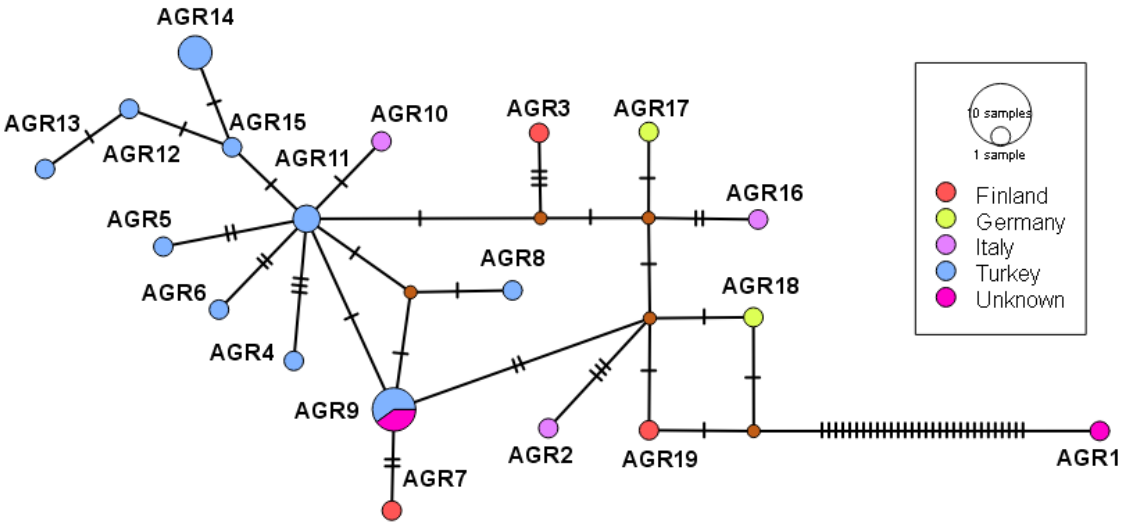

Figure 2. Statistical parsimony network of 658 bp mitochondrial COI gene region of *Acanthocinus griseus* samples. Brown points are hypothetical haplotypes. Notches on the lines indicate the number of mutations between haplotypes.

Discussion

Most wood-boring cerambycids are dispensable members of the forest ecosystem due to their role in wood decay. However, certain circumstances may turn them into invaders, such as a decrease in the

population of their predators or competitors. Also, they can be devastating pests outside their native distribution ranges if they cannot be detected, controlled and eradicated on time. For example, the polyphagous long-horned beetle *Anoplophora glabripennis* is a pest in its native range, Asia; and it has been a challenging invader in Europe, the United States and Canada since the 1990s (Wang et al. 2023). Moreover, it was detected in Zeytinburnu Istanbul province on *Acer negundo* (Ayberk et al. 2014). Another invader cerambycid, *Aromia bungii*, is a pest in its native range, east Asia, and an invader in Europe and Japan (Tamura and Shoda-Kagaya 2022). *Tetropium fuscum* is an interesting example, which is native to Europe and Northern Asia, has dominated its native congener *Tetropium cinnamopterum* in Nova Scotia, Canada (Dearborn et al. 2016). *Arhopalus rusticus*, a vector of the nematodes *Bursaphelenchus* spp., responsible for the pine-wilt disease (Ryss et al. 2005; Wang et al. 2021), inhabits both the Old and the New World naturally; however, it has been distributed to the Afrotropical Neotropical and Australian regions by human-mediated transport (Wang and Leschen 2003).

A. griseus, the guest of *Pinus, Picea, Abies*, has been evaluated as a forest pest after it had been determined as a vector of pine wilt nematode, *Bursaphelenchus xylophilus* (Linit 1988, Ryss et al. 2005, Wang et al. 2021). Moreover, it is known that this species can escape from phytosanitary measures and travel through industrial woods (Wu et al. 2017). The recent reports (Soydabaş et al. 2017; Wu et al. 2017; Tülek et al. 2019; Atak et al. 2021; Wang et al. 2021; Soydabaş-Ayoub and Uçkan 2022) revealed the potential threat posed by *A. griseus*, which point to the necessity of the post-border surveillance for early detection and rapid response. The present study is the first post-border assessment of *A. griseus*, dealing with determining native and non-native haplotypes and their relationships in the forested port city, Kocaeli Province.

The results have shown that all *A. griseus COI*-haplotypes reported from previous barcoding studies up to date from Finland (Pentinsaari et al. 2014), Italy (Rougerie et al. 2015), Germany (Rulik et al. 2017), USA (Wu et al. 2017) and Turkey (Soydabaş-Ayoub and Uçkan 2022) (Table 2) were close to each other. The intraspecific genetic distance of *A. griseus* was 1.37-0.3%, within the expected intraspecific genetic distance range of 0-3% (Hebert et al. 2003, Hebert et al. 2004a, Ward et al. 2005, Hajibabaei et al. 2006). The inter- to intraspecific distance ratio was compatible with the 10X rule (Hebert et al.2004b) because the interspecific distances ranged from 10.79 to 13.37% (Table 5).

An unexpected genetic distance value estimated between the AGR1 haplotype of *A. griseus* declared by Wu et al. (2017) and the ASA haplotype representing *A. sachalinensis* declared by Grebennikov et al. (2017), which was equal to the lowest intraspecific value, 0.3%. This estimation point to a discrepancy between morphological identification and COI barcode sequence of the AGR1 haplotype of *A. griseus*, which might be due to misidentification or cross-contamination. Nevertheless, hybridisation between two species is also possible; because both species occur in Russia. The origin of AGR1 is unclear since it was intercepted in a port in the USA (*A. griseus*, KY357618, Wu et al. 2017), but ASA (*A. sachalinensis*, KY683654, Grebennikov et al. 2017) was reported from Russia. Interspecific fertilisation cases are known for other cerambycid species, such as in *Morimus asper* complex (Solano et al. 2013), between *Cerambyx cerdo* and *C. welensii* Torres-Vila and Bonal (2019) and between *Arhopalus rusticus* and *Ar. syriacus* (Soydabaş-Ayoub et al. 2022). Or, it might be a sign of cryptic speciation, similar to other cerambycids previously revealed by Wallin et al. (2009), 11.5% distance between the siblings *Leiopus linnei* and *L. nebulosus*, and by Çakmak et al. (2020) 10% genetic distance between American and Eurasian haplotypes of *Rhagium inquisitor*, that point to allopatric speciation. Beyond all speculations, to unveil the main reasons for the unexpected genetic closeness between *A. sachalinensis* and *A. griseus* haplotypes, further studies are needed in vitro and in nature.

The polytomies were remarkable within the *A. griseus* species group at the phylograms resulting in both ML and BI analyses. Although the reasons for these polytomies could be the insufficient synapomorphic characters of the COI barcode sequences (Simon et al. 1994) or missing haplotypes of common ancestors of haplotypes (Townsend and Lopez-Giraldez 2010), a strong possibility is that their evolution occurred in a non-dichotomous manner. Because the network diagram suggested by statistical parsimony, which allows the reticular and radial branchings, provided further insight into the evolution and the phylogeographic structure of *A. griseus.* A radial grouping of Kocaeli haplotypes appeared in the network, indicating a bottleneck effect (Richards et al. 2019), and AGR11 seems to be the founder of the Kocaeli population due to its positioning at the centre of all other haplotypes. Any grouping has not appeared across European countries. The haplotypes from Finland, Italy, Germany, and Turkey were connected directly or through hypothetical haplotypes with a reticular pattern which indicates ongoing gene flow (Wollenberg et al. 2019). The shared haplotype between Turkey and USA (intercepted in the USA by Wu et al. 2017), the most sampled in this study, also supports the ongoing transfer overseas.

It is possible the conclude from our study that the ongoing gene flow occurs in *A. griseus*, which is probably facilitated by international wood trade. Therefore, regular post-border surveillance is required to recognise this potential invader and pine-wilt nematode vector before it becomes catastrophic. We also suggest further studies on the relationship between *A. griseus* and *A. sachalinensis*.

References

Allen, E., Noseworthy, M., Ormsby, M. (2017). Phytosanitary measures to reduce the movement of forest pests with the international trade of wood products. Biological invasions, 19, 3365-3376. https://doi.org/10.1007/s10530-017-1515-0

Aksöyek, E., İbiş, O., Özcan, S., Moradi, M., Tez, C. (2017). DNA barcoding of three species (*Canis aureus*, *Canis lupus* and *Vulpes vulpes*) of Canidae. Mitochondrial DNA Part A 28(5), 747-755. doi.org/10.1080/24701394.2016.1180512

Armstrong, K.F., Ball, S.L. (2005). DNA barcodes for biosecurity: invasive species identification. Philosophical Transactions of the Royal Society B: Biological Sciences, 360(1462), 1813-1823.

Atak, Ş., Uçkan, F. Ayoub, H.K.S. (2021). A faunistic study on Cerambycidae (Coleoptera) of Kocaeli province (Turkey). Bitki Koruma Bülteni 61(2), 33-43. doi.org/10.16955/bitkorb.796414

Ayberk, H., Ozdikmen, H., Cebeci, H. (2014). A serious pest alert for Turkey: a newly introduced invasive longhorned beetle, *Anoplophora glabripennis* (Cerambycidae: Lamiinae). Florida Entomologist, 97(4), 1852-1855.

Bense, U. (1995). Longhorn Beetles: Illustrated Key to the Cerambycidae and Vesperidae of Europe=Bockkäfer (117). Weikersheim, Germany, Margraf Verlag.

Bílý, S., Mehl, O. (1989). Longhorn Beetles (Coleoptera, Cerambycidae) of Fennoscandia and Denmark (22). Leiden, Brill.

Cocoş, D., Etxebeste, I., Schroeder, M. (2017). An efficient detection method for the red-listed beetle *Acanthocinus griseus* based on attractant-baited traps. Insect Conservation and Diversity, 10(4), 294-301. https://doi.org/10.1111/icad.12224s

Collins, R.A., Armstrong, K.F., Meier, R., Yi, Y., Brown, S.D., Cruickshank, R.H., Keeling, S., Johnston, C. (2012). Barcoding and border biosecurity: identifying cyprinid fishes in the aquarium trade. PloS one, 7(1), e28381.

Çakmak, Y.E., Ayoub, H.K.S. Uckan, F. (2019). Size divergence of *Rhagium inquisitor*: sexual similarity versus environmental variability. Feb-Fresenius Environmental Bulletin 28, 7593-7602.

Çakmak, Y.E., Soydabaş-Ayoub, H.K. Uckan, F. (2020). A preliminary phylogenetic analysis of ribbed-pine-borer (*Rhagium inquisitor*) based on mitochondrial COI sequences. Journal of Asia-Pacific Entomology 23(3), 809-815. doi.org/10.1016/j.aspen.2020.06.008

Dearborn, K. W., Heard, S. B., Sweeney, J., Pureswaran, D. S. (2016). Displacement of *Tetropium cinnamopterum* (Coleoptera: Cerambycidae) by its invasive congener *Tetropium fuscum*. Environmental Entomology, 45(4), 848-854.

Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research 32(5), 1792-1797. doi.org/10.1093/nar/gkh340

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3(5), 294-299.

Fukuda, K. (1997). Physiological process of the symptom development and resistance mechanism in pine wilt disease. Journal of forest research, 2(3), 171-181.

Grebennikov, V.V., Jendek, E., Smirnov, M.E. (2017). Diagnostic and phylogenetic utility of the first DNA barcode library for longhorn beetles (Coleoptera: Cerambycidae) from the Russian Far East. Zootaxa, 4276(3), 441-445.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic Biology, 59(3), 307-321. doi.org/10.1093/sysbio/syq010

Haack, R.A., Britton, K.O., Brockerhoff, E.G., Cavey, J.F., Garrett, L.J., Kimberley, M., Lowenstein, F., Nuding, A., Olson, L.J., Turner, J., Vasilaky, K.N. (2014). Effectiveness of the International Phytosanitary Standard ISPM No. 15 on reducing wood borer infestation rates in wood packaging material entering the United States. PLoS One 9(5).

Hajibabaei, M., Smith, M. A., Janzen, D. H., Rodriguez, J. J., Whitfield, J. B., Hebert, P. D. (2006). A minimalist barcode can identify a specimen whose DNA is degraded. Molecular Ecology Notes, 6(4), 959-964.

Hebert, P. D., Ratnasingham, S., De Waard, J. R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270(suppl_1), S96-S99.

Hebert, P.D., Penton, E.H., Burns, J.M., Janzen, D.H., Hallwachs, W., (2004a). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proceedings of the National Academy of Sciences, 101(41), 14812-14817.

Hebert, P.D., Stoeckle, M.Y., Zemlak, T.S., Francis, C.M., (2004b). Identification of birds through DNA barcodes. PLoS Biol. 2 (10).

Hernández-Triana, L.M., Chaverri, L.G., Rodriguez-Perez, M.A., Prosser, S.W., Hebert, P.D., Gregory, T.R., Johnson, N. (2015). DNA barcoding of Neotropical black flies (Diptera: Simuliidae): Species identification and discovery of cryptic diversity in Mesoamerica. Zootaxa, 3936(1), 93-114.

Hulme, P.E. (2009). Trade, transport and trouble: managing invasive species pathways in an era of globalisation. Journal of Applied Ecology, 46(1), 10-18.

Hurst, G.D., Jiggins, F.M. (2005). Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. Proceedings of the Royal Society B: Biological Sciences, 272(1572), 1525-1534.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., ... Drummond, A. (2012) Geneious Basic: an integrated and extendable desktop software platform for the organisation and analysis of sequence data. Bioinformatics, 28(12), 1647-1649. doi.org/10.1093/bioinformatics/bts199

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution, 35(6), 1547. doi.org/10.1093/molbev/msy096

Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T., Calcott, B. (2017). PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. Molecular Biology and Evolution, 34(3), 772-773.

Leigh, J. W., Bryant, D. (2015) POPART: full-feature software for haplotype network construction. Methods in Ecology and Evolution 6(9), 1110-1116. doi.org/10.1111/2041-210X.12410

Lindhe, A., Jeppsson, T., Ehnström, B. (2010). Longhorn beetles in Sweden-changes in distribution and abundance over the last two hundred years. Entomologisk Tidskrift, 131(4), 241-508.

Linit, M. J. (1988). Nematode-vector relationships in the pine wilt disease system. Journal of Nematology, 20, 227-235.

Martikainen, P. (2002). Ecology and conservation status of *Acanthocinus griseus* (Fabricius, 1792)(Coleoptera: Cerambycidae) in Finland. Entomologica Fennica, 13(1), 41-50.

Moritz, C., Cicero, C. Godfray, C. (2004) DNA barcoding: promise and pitfalls. PLoS biology 2(10), e354. doi.org/10.1371/journal.pbio.0020354

Paterson, I.D., Mangan, R., Downie, D.A., Coetzee, J.A., Hill, M.P., Burke,A. M., ... Compton, S.G. (2016). Two in one: cryptic species discovered in biological control agent populations using molecular data and crossbreeding experiments. Ecology and Evolution, 6(17), 6139-6150.

Pentinsaari, M., Hebert, P.D., Mutanen, M. (2014) Barcoding beetles: a regional survey of 1872 species reveals high identification success and unusually deep interspecific divergences. PLoS One 9(9): e108651. doi.org/10.1371/journal.pone.0108651

Richards, E.J., Servedio, M.R., Martin, C.H. (2019) Searching for sympatric speciation in the genomic era. BioEssays, 41(7), 1900047. doi.org/10.1002/bies.201900047

Roderick, G. K. (1996). Geographic structure of insect populations: gene flow, phylogeography, and their uses. Annual Review of Entomology, 41(1), 325-352.

Ronquist, F., Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19(12), 1572-1574. doi.org/10.1093/bioinformatics/btg180

Rougerie R., Lopez-Vaamonde C., Barnouin T., Delnatte J., Moulin N., Noblecourt T., Nusillard B., Parmain G., Soldati F. Bouget C. (2015) PASSIFOR: A reference library of DNA barcodes for French saproxylic beetles (Insecta, Coleoptera). Biodiversity Data Journal, 3: e4078. doi: 10.3897/BDJ.3.e4078

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., Sánchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. Molecular Biology and Evolution, 34(12), 3299-3302.

Rulik, B., Eberle, J., von der Mark, L., Thormann, J., Jung, M., Köhler, F., Apfel, W., Weigel, A., Kopetz, A., Köhler, J. Fritzlar, F. (2017) Using taxonomic consistency with semi-automated data pre-processing for high quality DNA barcodes. Methods in Ecology and Evolution 8(12), 1878-1887. doi.org/10.1111/2041-210X.12824

Ryss, A., Vieira P., Mota, M., Kulinich, O. (2005). A Synopsis of the genus *Bursaphelenchus* Fuchs, 1937 (Aphelenchida: Parasitaphelenchidae) with keys to species. Nematology 7, 393-458.

Sambrook, J., Russell, D.W. (2006). Purification of nucleic acids by extraction with phenol: chloroform. Cold Spring Harbor Protocols, 2006(1), pdb-prot4455. doi:10.1101/pdb.prot093450

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P. (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87(6), 651-701. doi.org/10.1093/aesa/87.6.651

Solano, E., Mancini, E., Ciucci, P., Mason, F., Audisio, P., Antonini, G. (2013). The EU protected taxon *Morimus funereus* Mulsant, 1862 (Coleoptera: Cerambycidae) and its western Palaearctic allies: systematics and conservation outcomes. Conservation Genetics, 14(3), 683-694. doi.org/10.1007/s10592-013-0461-3

Soydabaş, H.K., Uçkan, F., Atak, Ş. (2017). Phylogenetic status of Lamiinae (Cerambycidae: Coleoptera) species from Kocaeli province based COI gene sequences. In: XIII. Congress of Ecology and Environment with International Participation-UKECEK 2017, pp.136.

Soydabaş Ayoub, H.K. (2021). Nüklear ve mitokondriyal DNA dizileri kullanılarak Kocaeli ilindeki Cerambycidae (Coleoptera) türlerinin filogenetik analizi. [Doktora Tezi], Kocaeli Üniversitesi, Kocaeli, Türkiye.

Soydabaş-Ayoub, H. K., Uckan, F. (2022). Time-scaled phylogenetic analysis of extant Lamiinae (Coleoptera, Cerambycidae) species of East of Marmara Basin, Türkiye, and their evolutionary similarity with the Eurasian congeners. bioRxiv, 2022-09. https://doi.org/10.1101/2022.09.10.507409

Soydabaş-Ayoub, H. K., Uçkan, F., Atak, Ş., Şimşek, B. Ş., İbiş, O. (2022). Assessment of the phylogenetic relationships within the spondylidine branch of Spondylidinae (Coleoptera, Cerambycidae). Insect Systematics & Evolution, 54(3), 281-311. https://doi.org/10.1163/1876312X-bja10042

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics, 30(9), 1312-1313.

Tamura, S., Shoda-Kagaya, E. (2022). Genetic Differences among Established Populations of Aromia bungii (Faldermann, 1835)(Coleoptera: Cerambycidae) in Japan: Suggestion of Multiple Introductions. Insects, 13(2), 217. https://doi.org/10.3390/insects13020217

Templeton, A.R., Crandall, K.A., Sing, C.F. (1992). A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132(2), 619-633. https://doi.org/10.1093/genetics/132.2.619

Torres-Vila, L. M., Bonal, R. (2019). DNA barcoding of large oak-living cerambycids: Diagnostic tool, phylogenetic insights and natural hybridisation between Cerambyx cerdo and Cerambyx welensii (Coleoptera: Cerambycidae). Bulletin of entomological research, 109(5), 583-594. doi:10.1017/S0007485318000925

Townsend, J.P., Lopez-Giraldez, F. (2010). Optimal selection of gene and ingroup taxon sampling for resolving phylogenetic relationships. Systematic Biology 59(4), 446-457. doi.org/10.1093/sysbio/syq025

Tülek, A., Kepenekçi, İ., İmren, M., Akbulut, S., Tülek, B., Koca, A. S., Özdikmen, H. (2019). Detection of *Bursaphelenchus mucronatus* associated with *Acanthocinus griseus* on *Pinus nigra* in the northwestern Turkey. Nematropica, 49(2), 220–228.

Wallin, H., Nylander, U., Kvamme, T. (2009). Two sibling species of *Leiopus* Audinet-Serville, 1835 (Coleoptera: Cerambycidae) from Europe*: L. nebulosus* (Linnaeus, 1758) and *L. linnei* sp. nov. Zootaxa, 2010(1), 31-45.

Wang L, Li C, Luo Y, Wang G, Dou Z, Haq IU, Shang S, Cui M. (2023). Current and future control of the wood-boring pest *Anoplophora glabripennis*. Insect Science. https://doi.org/10.1111/1744-7917.13187

Wang, Q., Leschen, R. A. (2003). Identification and distribution of *Arhopalus* species (Coleoptera: Cerambycidae: Aseminae) in Australia and New Zealand. New Zealand Entomologist, 26(1), 53-59.

Wang, Y., Chen, F., Wang, L., Li, M. (2021). Investigation of beetle species that carry the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, in China. Journal of Forestry Research, 32(4), 1745-1751. https://doi.org/10.1007/s11676-020-01146-2

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., Hebert, P.D. (2005). DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society B: Biological Sciences, 360(1462), 1847-1857.

Wollenberg Valero, K.C., Marshall, J.C., Bastiaans, E., Caccone, A., Camargo, A., Morando, M., M.L., Pabijan, M., Russello, M.A., Sinervo, B., Werneck, F.P. (2019). Patterns, mechanisms and genetics of speciation in reptiles and amphibians. Genes 10(9), 646. doi.org/10.3390/genes10090646

Wu, Y., Trepanowski, N.F., Molongoski, J.J., Reagel, P.F., Lingafelter, S.W., Nadel, H. (2017). Identification of wood-boring beetles (Cerambycidae and Buprestidae) intercepted in trade-associated solid wood packaging material using DNA barcoding and morphology. Scientific Reports, 7, 40316.

Submitted: 05.03.2023 Accepted: 12.06.2023