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Assessment of genetic variation in the Anatolian populations of *Andricus gallaetinctoriae* (Hymenoptera: Cynipidae)

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Abstract

In this study PCR-RFLP method was conducted to reveal intraspecific mitochondrial DNA variation of oak gall wasp species *Andricus gallaetinctoriae* (Oliver, 1791) (Hymenoptera: Cynipidae) collected from the Anatolian part of Turkey. A total of 104 specimens from 13 localities produced 15 distinct haplotypes. Data analyses estimated average haplotype diversity as 0. 3415 and mean nucleotide diversity as 0. 02572. Pairwise comparisons of populations supported the presence of genetic divergence between eastern and western populations. Our current results may also be highly correlated with well-marked geographic barriers dividing species/lineage distribution in Anatolia.

Keywords: Anatolia, *Andricus gallaetinctoriae*, Cynipidae, genetic variation.

INTRODUCTION

In the last several decades, the field of molecular systematics has been revolutionized through PCR based DNA studies. Implementation of such molecular techniques as restriction fragment length polymorphism of PCR products (PCR-RFLP) has led to increased interest on the population genetic structure and distribution of the genetic variation across the range of a species [1,2,3]. PCR based RFLP studies have been frequently applied to mitochondrial DNA that is known with its rapid rate of evolution, absence of recombination, and maternal inheritance [4]. In diverse animal groups, mtDNA has proven as a molecular marker to reveal genetic structuring and organismal phylogenies [5,6,7,8]. Moreover, it unveiled historical processes such as dispersal routes and recolonization events [9]. In particular, studies through using mtDNA carried on the Palearctic region indicated clearly that many of the European taxa were enriched by genetic sources originated from Turkey and more eastern parts [10].

Located in the Palearctic area, particular attention has been given to Anatolia due to its role as a shelter for many refugees and a corridor for trespassing species. Because of topographic diversity, various climatic regimes and being placed at the junction of Euro-Siberian, Mediterranean and lrano-Turanian phytogeographical regions, Turkey is rich in species and genetic diversity [11,12]. Among other studied species, gall wasps showed conspicuous level of genetic variation and structuring of the genetic diversity with respect to geographic locations of the sampled population across their distribution range [13].

Andricus gallaetinctoriae (Oliver, 1791) (Hymenoptera: Cynipidae) shows a wide distribution in the Holarctic region from Hungary, Transcarpathia, Balkans, through Turkey to Mesopotamia. It is found most commonly on *Quercus infectoria* Oliv., *Q. pubescens*, and *Q. petraea*. Galls of A. *gallaetinctoriae* can also be formed on *Q. ithaburensis* Decne. and *Q. robur* L. [14,15,16]. Its monolocular agamic generation galls are spherical with stiff spines, petroleum green color early in development and off-white color in maturation, and located on the buds of branches. Gall formation begins through mid-June to early July and matures in September. In this study, we used asexual generation individuals of *A. gallaetinctoriae* and employed PCR-RFLP method i) to reveal overall genetic diversity across the sampled populations, ii) to test if there is a general distributional pattern of genetic variation present in oak gall wasps, and to compare the observed structure with that of other gall wasp species from Turkey.

MATERIALS AND METHODS

Sampling and Laboratory Protocols

Parthenogenetic generation galls of A. *gallaetinctoriae* were collected during the summer of 2011 and 2012. Collection sites and distribution areas of total 104 individuals used in this study are shown in Table 1.

Total genomic DNA of single *A. gallaetinctoriae* specimens was extracted using DNeasy Tissue Kit (QIAGEN). Two mitochondrial DNA regions were preferred for PCR-RFLP analysis; the first region (2560 bp) which covers ND4, ND6 and cyt b gene fragment was amplified using mt24 5'- GGAGCTTCAACATGAGCTTT-3' and mt28 5'-ATTACACCTCCTAATTTATTAGGAAT-3' primers. The second region (1800 bp) comprising the ATPases (6, 8) and COIII genes was amplified using universal insect primers mt19 5'-GAAATTTGTGGAGCAAATCATAG-3', mt22 5'-TCAACAAAGTGTCAGTATCA-3' [17,7].

Amplifications were carried out in a final volume of 25µl containing 0.5µl of the total DNA extraction, 2µl 10X Buffer A (Vivantis), 0.8µl MgCl2 (50mM), 0.8µl dNTPs (2mM each), 0.32µl of each primer (20µM) and 0.25 U of Taq DNA Polymerase (Vivantis). Thermocycling condition was 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min 20 sec at 44 °C, 4 min at 64 °C, and a final extension of 5 min at 64 °C.

Amplified products were visualized on 1% agarose gel buffered with Tris-Boric acid- EDTA (TBE), stained with ethidium bromide. Restrictions enzymes for digestion of amplified PCR products were previously used in other Hymenoptera species [17,13,8]. Amplicons of the both regions were digested separately with HinfI, HaeIII, HinIII and EcoRI following the manufacturer's instructions. Digested products were checked under UV light with a 1kb DNA ladder (Sigma, D0428), and photographed.

Table 1. Sampled populations of *Andricus gallaetinctoriae* with their abbreviations and coordinates

Locality	Abbreviation	Coordinates
1.Bolu	BOL	N 40°40.380' E 31°25.991'
2.Erzincan	ERI	N 39°54.650' E 38°28.483'
3.Çankırı	CKR	N 40°48.446' E 33°19.628'
4.Çanakkale	CAN	N 40°32.166' E 26°65.754'
5.Isparta	ISP	N 37°62.133' E 30°85.744'
6.Karabük	KAR	N 40°89.558' E 32°57.997'
7.Gümüşhane	GUM	N 40°17.639' E 39°13.861'
8.Kahramanmaraş	KAH	N 37°48.467' E 37°44.109'
9.Siirt	SIR	N 38°12.710' E 41°67.598'
10.Batman	BAT	N 38°16.957' E 41°45.983'
11.Erzurum	ERZ	N 39°86.495' E 40°62.626'
12.Elazığ	ELA	N 38°57.990' E 38°86.239'
13.Adıyaman	ADI	N 37°72.166' E 37°96.444'

Data Analysis

Obtained restriction patterns of two mtDNA fragments for each enzyme were coded alphabetically and composite haplotypes were generated based on each observed pattern. Haplotype and nucleotide diversity for each population were determined using DA program implemented under the REAP software package [18]. A pairwise population comparison using a Monte Carlo randomization procedure with 1000 dememorization steps was carried out [19]. The average number of nucleotide substitutions per site between haplotypes was used to reconstruct a Dollo parsimony majority rule consensus tree using the DOLLOP program present under PHYLIP package [20].

RESULT AND DICUSSION

A total of 15 composite haplotype was determined from all specimens of *A. gallaetinctoriae* collected from 13 populations (Table 2). The mtDNA fragments from 104 individuals had 25 recognition sites for the restriction enzymes used in this study. The present analysis of PCR-RFLP site variation of two mtDNA regions of *A. gallaetinctoriae* is congruent with other Hymenopteran species studied so far [21,17,13,7,8,7]. The composite haplotypes and their frequencies in each of the studied population are given in Table 2. Collection sites and haplotype frequencies represented in pie charts are shown on a map in Figure 1.

Among all composite haplotypes, the frequency of haplotype 4 was the highest and represented by 21 individuals collected from three localities (Erzincan, Bolu and Karabük). Two other most frequent haplotypes were H7 and H8, each detected in 20 individuals. Haplotype 8 was observed in only two localities (Kahramanmaraş and Siirt). However, H7 was found in five localities (Çankırı, Çanakkale, Isparta, Gümüşhane, and Batman), and in fact it was the most geographically widespread haplotype. Out of 15 detected haplotypes three of them (H2, H10 and H14) were found as private haplotypes in only a single individual. Interestingly, H2 as one of the private haplotypes found in Bolu where the highest number of haplotypes was detected (Nhap= 6). Batman was the second locality with high haplotype number (Nhap= 5), of which three haplotypes were private. Overall, among all examined populations seven localities had only a single type of haplotype (Erzincan, Çankırı, Çanakkale, Isparta, Karabük, Gümüşhane, and Kahramanmaraş, see Figure 1) although frequency of some of these haplotypes was fairly high (see Table 2).

When haplotype and nucleotide diversity for each *A. gallaetinctoriae* population was analyzed we observed no haplotype variation in 7 populations (Erzincan, Çankırı, Çanakkale, Isparta, Karabük, Gümüşhane, and Kahramanmaraş) due to the presence of only a single haplotype in these locations (Table 3). Most of the remaining populations showed conspicuously high level of haplotype polymorphism. Among the localities with high diversity, Elazığ population (1.0) had the greatest genetic variation followed by Batman (0.8571), Erzurum (0.8333), and Bolu (0.8095). Some of these localities also showed high amount of genetic diversity in other gall wasp species [7,13,8]. Adıyaman (0.500) and Siirt (0.4396) populations showed intermediate level of variation. Overall mean haplotype diversity of *A. gallaetinctoriae* was 0.3415. Nonetheless, mean nucleotide diversity for the studied species was 0.0257 with the highest variation estimated for Bolu (0.099), followed by Batman (0.0784). Two other populations, namely Elazığ (0.0463) and Erzurum (0.0451), showed almost similar estimates. Except the aforementioned seven localities, the lowest diversity was observed in Siirt (0.0211) (see Table 3).

Few other gall wasp species have been examined thoroughly in Turkey so far [7,13,8]. Of these, haplotype and nucleotide diversity was 0.8089 and 0.115 for *A. lucidus*, 0.45 and 0.05 for *A. quercustozae*, and 0.46 and 0.101 for *A. caputmedusae*.

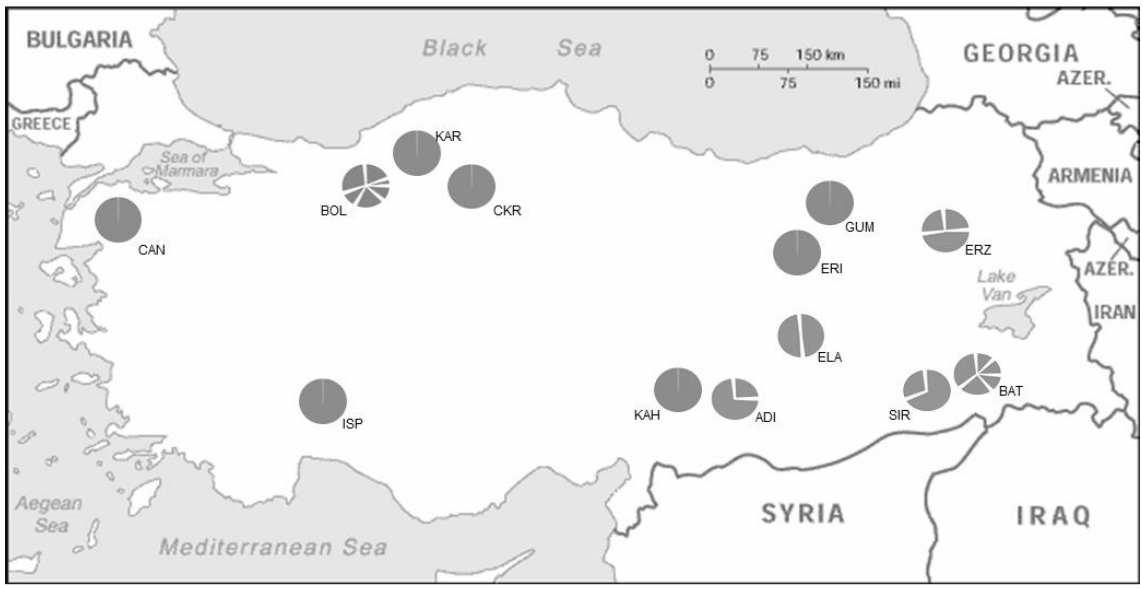

Figure 1. Collection localities of *A. gallaetinctoriae* and associated haplotypes are shown as pie charts in each sampled location. Haplotype richness in each population is correlated with pie chart representation. Population abbreviations are provided in Table 1.

Compared to these species, *A. gallaetinctoriae* seems to have lower haplotype and nucleotide diversity (0.341 and 0.0257, respectively). However, more region-wide studies conducted in the Holarctic area revealed that the Turkish populations had noticeably high level of genetic variation compared to the European populations. In a closely related gall wasp species, *A. quercustozae* the greatest nucleotide diversity was detected in Turkey (0.2-4.2%), followed by the lower diversity in the Balkan populations (0.2-1.4%), Italy (0.2-0.7%), and Iberia (0.2-1.0%) [10]. Compared to the nucleotide sequence data PCR-RFLP method can reveal less amount of genetic variation present in a species, however the genetic variation observed through the application of RFLP in *A. gallaetinctoriae* is still notorious and deserves attention.

When nucleotide divergence was compared in a pairwise manner, the highest divergence was detected between Çankırı, Çanakkale, Isparta, Gümüşhane, and Adıyaman population (0.253566), followed by the Kahramanmaraş (0.212373), and Siirt population (0.188639). Erzurum and Kahramanmaraş populations differed from each other with a value of 0.150605. Interestingly, the four populations (Çankırı, Çanakkale, Isparta, and Gümüşhane), which shared haplotype 7 showed the highest amount of nucleotide divergence from eastern populations. The remaining comparisons of nucleotide divergence estimates ranged between 0.0 and 0.119918. On the other hand, highest nucleotide diversity was observed between Çankırı, Çanakkale, Isparta, Gümüşhane, and Adıyaman population (0.275287) followed by Kahramanmaraş (0.212373), and Siirt populations (0.199225) (see Table 4). Taken as a whole, pairwise comparisons indicated some levels of genetic divergence between eastern and western populations. Indeed, geographically east and west division in the distribution of the genetic variation has been shown in other gall wasp species from Anatolia marking the significance of geographic barriers which divide species/lineage distribution [10,13]. Likewise, several other insect species have pointed out similar patterns in Orthoptera [22].

All composite haplotypes detected in *A. gallaetinctoriae* have been used to construct an unrooted Dollo parsimony majority rule consensus tree (Figure 2). Three haplogroups marked as A, B, and C are apparent in the tree. In the A cluster H7 found in Batman, Çankırı, Çanakkale, Isparta and Gümüşhane, and H10 found in Batman are grouped together. The second group covers H8 from Siirt and Kahramanmaraş, and H9 from Siirt. The third group is consisted of H4 from Bolu, Erzincan, and Karabük and H5 found only in Bolu. B cluster includes two small groupings, of which the first is composed H15 from Adıyaman,and H13 from Erzurum and Elazığ, and the second includes H14 from Adıyaman and H3 from Batman, Bolu and Erzurum. The C cluster, on the other hand, is composed of mostly Bolu haplotypes where they form a distinct small grouping. A second small grouping covers H11 from Batman and H12 from Batman, Erzurum and Elazığ. When the tree is overall evaluated the A and C clusters include a mixture of haplotypes distributed in both east and west populations of *A. gallaetinctoriae*, however B cluster is composed of haplotypes found in eastern distributional range of the species except H3 detected as a shared haplotype among Batman, Erzurum and Bolu populations. Similar studies conducted in gall wasp species, *A. caputmedusae* [13], and *A. quercustozae* [7] clearly indicated a genetic break between eastern and western populations besides a mixed haplogroup. Compared to these gall wasp species from Turkey our current findings on *A. gallaetinctoriae* indicated not such a strong division of the genetic variation; however there is still explicit east and west break across the sampled area.

Current structure of the populations and the allocation of genetic variation throughout the range of a species are overall shaped both by historical and contemporary factors. Studies carried particularly on the species showing distribution in Turkey are extremely useful for the examination of Anatolia providing genetic source for the European populations. Thus more detailed studies both in gall wasps and other unrelated groups of organisms from Anatolia may shed further light into the importance of Turkish genetic diversity present in distinct species.

Table 2. Composite haplotypes and their frequencies in *Andricus gallaetinctoriae* populations (see Table 1). Composite haplotypes based on digestion pattern are represented by capital letters in the following order for both mtDNA fragments ND4, ND6, Cyt B and ATPases, COIII: HinfI, HaeIII, HindII, EcoRI.

		Populations													
Haplotype	**Composite Haplotype**	**BOL**	**ERI**	**CKR**	**CAN**	**ISP**	**KAR**	**GUM**	**KAH**	**SIR**	**BAT**	**ERZ**	**ELA**	**ADI**	**Total**
H1	AAAAAAAA	6													**6**
H2	BAAAAAAA	1													**1**
H3	BAAABABA	3									1	1			**5**
H4	AAAABAAA	6	10				5								**21**
H5	AAAACAAA	3													**3**
H6	AAAADAAA	9													**9**
H7	AAAABABA			7	2	2		8			1				**20**
H8	AABABABA								10	10					**20**
H9	AABADABA									4					**4**
H10	AAAABABB										1				**1**
H11	AAAADABA										2				**2**
H12	BAAADABA										3	2	1		**6**
H13	BAAADBBA											1	1		**2**
H14	BAAACABA													1	**1**
H15	BAAABBBA													3	**3**
Sample Size		**28**	**10**	**7**	**2**	**2**	**5**	**8**	**10**	**14**	**8**	**4**	**2**	**4**	**104**

Table 3 . Haplotype and nucleotide diversity for each *A. gallaetinctoriae* population with the average values and mean ± SE.

Population	**Haplotype Diversity**	**Nucleotide Diversity**
1.Bolu	0.8095±0.03811	0.099802
2.Erzincan	0.0000±0.00000	0.000000
3.Çankırı	0.0000±0.00000	0.000000
4.Çanakkale	0.0000±0.00000	0.000000
5.Isparta	0.0000±0.00000	0.000000
6.Karaman	0.0000±0.00000	0.000000
7.Gümüşhane	0.0000±0.00000	0.000000
8.Kahramanmaraş	0.0000±0.00000	0.000000
9.Siirt	0.4396±0.11198	0.021171
10.Batman	0.8571±0.10825	0.078412
11.Erzurum	0.8333±0.22244	0.045148
12.Elazığ	1.0000±0.50000	0.046388
13.Adıyaman	0.5000±0.26517	0.043443
Average	0.3415±0.01289	0.025720±0.0000900

Table 4. Pair-wise nucleotide diversity (above diagonal) and net nucleotide divergence (below diagonal) among the populations of *A. gallaetinctoriae.*

	BOL	ERI	CKR	CAN	ISP	KAR	GUM	KAH	SIR	BAT	ERZ	ELA	ADI
BOL		0.093783	0.117955	0.117955	0.117955	0.093783	0.117955	0.170653	0.182784	0.143233	0.183974	0.157615	0.193907
ERI	0.043882		0.119918	0.119918	0.119918	0.000000	0.119918	0.045615	0.061027	0.090346	0.131924	0.097460	0.102297
CKR	0.068054	0.119918		0.000000	0.000000	0.119918	0.000000	0.212373	0.199225	0.102983	0.167166	0.168187	0.275287
CAN	0.068054	0.119918	0.000000		0.000000	0.119918	0.000000	0.212373	0.199225	0.102983	0.167166	0.168187	0.275287
ISP	0.068054	0.119918	0.000000	0.000000		0.119918	0.000000	0.212373	0.199225	0.102983	0.167166	0.168187	0.275287
KAR	0.043882	0.000000	0.119918	0.119918	0.119918		0.119918	0.045615	0.061027	0.090346	0.131924	0.097460	0.102297
GUM	0.068054	0.119918	0.000000	0.000000	0.000000	0.119918		0.212373	0.199225	0.102983	0.167166	0.168187	0.275287
KAH	0.120752	0.045615	0.212373	0.212373	0.212373	0.045615	0.212373		0.013761	0.137090	0.173179	0.135565	0.153625
SIR	0.122298	0.050442	0.188639	0.188639	0.188639	0.050442	0.188639	0.003176		0.128286	0.162570	0.137375	0.173232
BAT	0.054125	0.051140	0.063777	0.063777	0.063777	0.051140	0.063777	0.097884	0.078495		0.074061	0.070299	0.127147
ERZ	0.111498	0.109350	0.144592	0.144592	0.144592	0.109350	0.144592	0.150605	0.129410	0.012280		0.033958	0.067382
ELA	0.084520	0.074266	0.144993	0.144993	0.144993	0.074266	0.144993	0.112371	0.103596	0.007898	0,011811		0.063214
ADI	0.122284	0.080576	0.253566	0.253566	0.253566	0.080576	0.253566	0.131904	0.140925	0.066220	0.023086	0.018299	

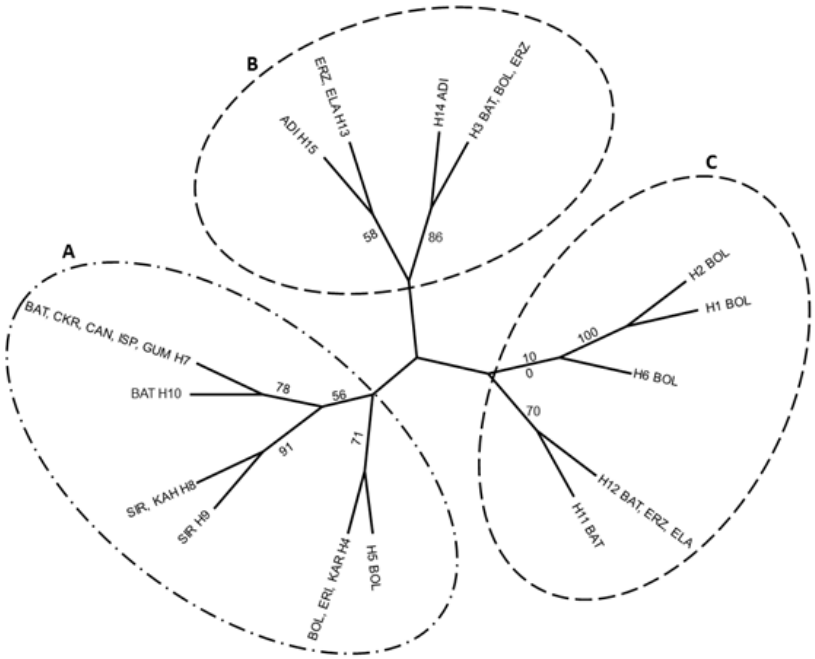

Figure 2. Unrooted Dollo parsimony majority- rule consensus tree of *Andricus gallaetinctoriae* haplotypes. Bootstrap values >50 are shown the branches.

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