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Phylogeography of the Land Snail *Eobania vermiculata* (O.F. Müller, 1774) (Gastropoda: Pulmonata) along the Croatian Coast and Islands

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ABSTRACT

Eobania vermiculata [11], is a typical Mediterranean species of large land snails. Nonindigenous populations of this species, however, are already established in the USA, Australia and elsewhere in the world, where this species is considered to represent a potentially serious threat as a pest and invasive species. The aims of this study were: 1) to determine the pattern of genetic variation within the Croatian *E. vermiculata* populations based on analyses of sequence diversity of two mitochondrial genes, 16S rDNA and the cytochrome oxidase I (COI), and 2) to shed more light upon the phylogeography of *E. vermiculata* in this area. Seventy-seven specimens of land snail *Eobania vermiculata* were sampled at 19 sampling sites along Croatian coastal region and islands. The partial 16S rRNA gene sequences (379 bp) grouped into 14 haplotypes, whereas the partial COI gene sequences (523 bp) grouped into 13 haplotypes. The overall population is characterized by relatively high haplotype (gene) diversity (0.719±0.042 for 16S rDNA and 0.869±0.020 for COI). Demographic Fu F's tests and Tajima's D value indicated no significant change in the population size, thus suggesting long historical presence of *E. vermiculata* in this region. Maximum likelihood phylogenetic analysis, Bayesian inference and median joining haplotype network showed a genetic splitting of Croatian 16S rRNA and COI sequences, with a clear distinction between south-Adriatic and north-Adriatic haplotypes. A possible explanation for the observed phylogeography of *E. vermiculata*, could be related to the climate change, glaciations and the Adriatic Sea level oscillations during the Quaternary.

Keywords: *Eobania vermiculata*, Gastropoda, Snails, Phylogeography, Molecular Phylogeny, 16S rRNA, COI.

1. Introduction

Eobania vermiculata [11] syn. *Helix vermiculata* [11], known as the chocolate-band snail, is a terrestrial, air-breathing mollusk from the family Helicidae [1, 2]. It is one of the main representatives of the helicid land snails in the Mediterranean area where it ranges from eastern Spain to the Crimea, including Italy, Croatia, Albania, Greece, Turkey and Bulgaria [3-8]. *E. vermiculata* has been reported in Egypt, Israel and Saudi Arabia [9-11]. Use of this snail in the diet is often in the Mediterranean region and is considered as a major factor to its global spread [12]. Nonindigenous distribution of *E. vermiculata* includes Australia, United States and other parts of the world. Its populations are already established in the USA, therefore this species is considered to represent a potentially serious threat as a pest, an invasive species which could negatively affect agriculture, natural ecosystems, human health or commerce. Therefore it has been suggested that this species be given top national quarantine significance in the USA [13].

E. vermiculata lives in very diverse habitats, usually in dry vegetation but also in gardens and city parks. It is frequently found in the coastal vicinity, vineyards and other agricultural crops. With having a long lifespan (2-5 years) an established population can continually grow and form dense populations. Detailed studies on biology, ecology and growth have been presented [14-16]. This snail has been frequently recorded along Croatian coastal part and islands [17-23].

The aim of this paper was to explore the genetic variability among *E. vermiculata* populations along Croatian coastal part and to shed more light upon the phylogeography of the species. To this end, we combined our previously published sequence data on two mitochondrial genes (16S rDNA and cytochrome oxidase subunit I, COI) and new sequences obtained in this study.

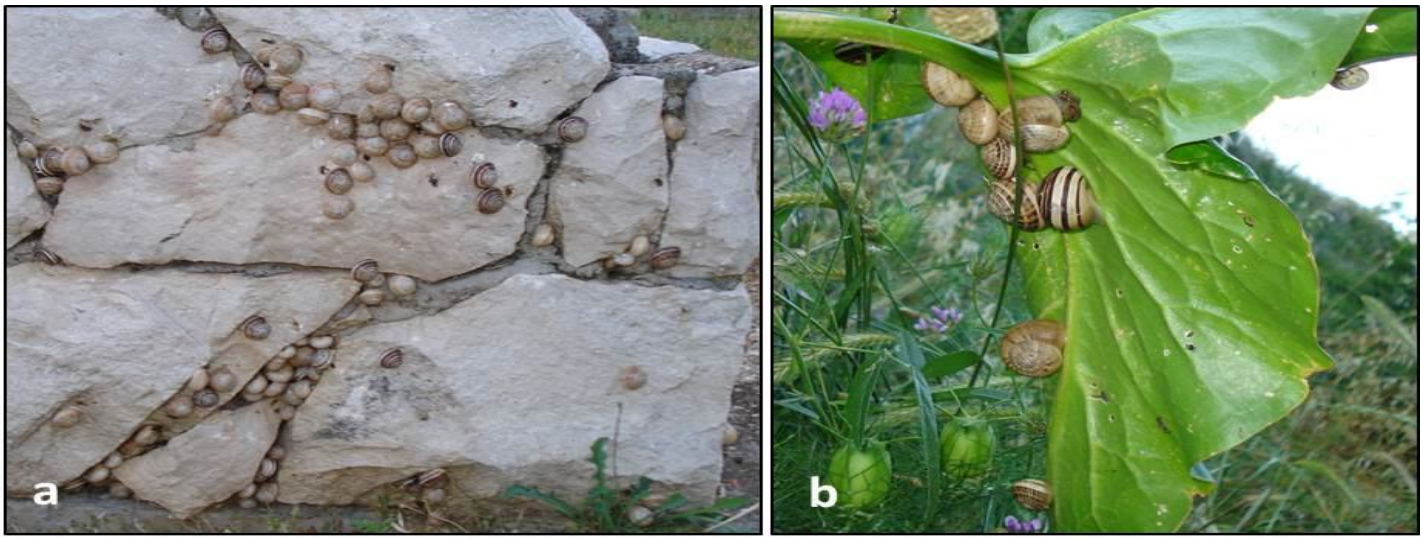

Fig 1: *Eobania vermiculata* on natural habitats. a) drystone wall, b) plant leaf.

2. Materials and Methods

2.1 Sample collection, DNA-isolation, PCR-amplification and sequencing of DNA

Adult individuals of *E. vermiculata* have been sampled at 19 locations along the Croatian coastal part and islands (table 1, figures 1, 2). Two mitochondrial genes (16S rRNA and COI) were PCR amplified with the primers described [2]. The specific PCR products were purified using "QIAEX II Gel Extraction Kit 150" (Quiagen, Hilden, Germany). Samples were subjected to cycle sequencing using the ABI PRISM® 3100-Avant Genetic Analyzer.

2.2. Sequence analysis

DNA sequences were assembled and prealigned using BioEdit ver. 7.0.5.3[25], aligned in ClustalW [26] implemented in MEGA5 [27] and the alignment was refined manually. The sequences were deposited in GenBank: KF289759- KF289761 (this study), JF277380 – JF277390 [24] for 16S and KF289762- KF289764 (this study), JF277391- JF277396, JF802030 - JF802033 [24] for COI. In order to avoid multiple submissions of identical sequences, we sent only one sequence of each haplotype. The number of haplotypes, the haplotype and nucleotide diversity within populations (Table 3) were determined using DnaSPv5 [28].

Phylogenetic analyses were carried out using maximum likelihood (ML) and Bayesian-based inference (BI) methods. The most suitable model of nucleotide evolution was determined by the Akaike Information Criterion, AIC, [29] as implemented in JModelTest [30]. Bayesian analysis was performed with MrBayes 3.1 [31] with 4 chains of 1,000,000 generations, trees sampled every 100 generations and burn in value set to 25 % of the sampled trees. Maximum-likelihood analysis using starting trees obtained by neighbor-joining and TBR branch swapping with model parameters was performed using PAUP* 4.0b10 [32]. The number of bootstrap replicates was set to 1000. Phylogenetic trees were displayed in FigTree v1.3.1.The median joining (MJ) algorithm implemented in Network v4.6.1.0. Software [33] was used with for constructing networks (weight=10 and ε = 10). To infer the demographic history of populations i.e. to detect possible past population expansion, we used Fu's F_S statistics [34], and Tajima's D value [35] using DnaSPv5 [28].

To infer the evolutionary origin and relationship of the 16S RNA haplotypes originating from Croatia, we made comparison with sequences available at GenBank: AY546357 from St. Georges, Switzerland [2], AY741409 form Sienna, Italy [36], HM147182 from Canary Islands, Spain (Neiber 2010, unpublished). We also made comparison of the Croatian sequences with sequences AY546277 from Marsej, France [2], GU598216 from Canary Island, Spain (Neiber 2010, unpublished) and JX911300 from France (Ansart *et al*. 2012, unpublished). Sequences (JF807626-JF807633) published by Desouky and Busais [11], and assigned to belong to *E. vermiculata* from Saudi Arabia and Egypt, were too divergent from Croatian and all other *E. vermiculata* sequences and exhibited a significant sequence similarity to species from the genus *Marmorana*.

Table 1: Sites sampled and data on the specimens collected

S.No	Sites sampled/Locality	16S rDNA haplotype	No. of individuals	COI haplotype	No. of individuals
1	**Rab**	H12	4	H8 H9	3 1
2	**Losinj**	H13 H14	1 3	H8 H11	2 1
3	**Karlobag**	H12	2	H8	2
4	**Paklenica**	H1 H11	4 1	H12	5
5	**Zadar**	H1	3	H1 H2	1 4
6	**Rogoznica**	H1 H3	1 4	H1 H2 H3 H7	2 2 1 1
7	**Okrug**	H1 H3	2 1	H2 H3 H5 H6 H7	1 1 1 2 1
8	**Šolta**	H1 H3	3 1	H1 H3 H4 H5 H6	1 1 1 1 1
9	**Brač**	H1	5	H1 H7	2 1
10	**Split - west**	H1 H3 H7 H8	1 3 1 1	H2 H7	2 1
11	**Split - east**	H1 H3	1 6	H2 H13	6 1
12	**Omiš**	H1 H3	2 2	H1 H2 H3	1 2 1
13	**Korčula**	H1 H7	1 1	H2 H6	1 1
14	**Vis**	H1 H2	1 1	H3 H2 H4	3 1 1
15	**Sušac**	H1 H3 H9 H10	4 1 1 1	H7 H10	1 2
16	**Palagruža**	H1	4	H7	3
17	**Lastovo**	H1	2	H7	3
18	**Makarska**	H1 H3 H4	1 1 1	H1	1
19	**Dubrovnik**	H1 H6	2 3	H1	4
	Total: 19	14 haplotypes, 77 specimens		13 haplotypes, 74 specimens	

Table 2: Genetic diversity data and results of neutrality tests of the 16S rRNA and COI sequences of the analyzed population of *Eobania vermiculata*

	16S rRNA	COI
No. of individuals sequenced	77	74
Fragment size (bp)	370	523
No. of variable sites	15	24
No. of parsimony informative sites	7	22
No. of haplotypes	14	13
Haplotype diversity (±SD)	0.719±0.042	0.869±0.020
Nucleotide diversity (±SD)	0.0039±0.0005	0.0084±0.0013
Neutrality tests		
Tajima's D	-1.4711	-0.4363
Fu's Fs	-6.599**	0.323

3. Results

3.1 Genetic variability of Eobania vermiculata along the East Adriatic coast

The genetic diversity information in the mtDNA sequences of Croatian populations of *E. vermiculata* were given in table 2. Partial 16S rRNA gene sequences (379 bp) were obtained for 77 *E. vermiculata* individuals and grouped into 14 haplotypes. The most frequent 16S haplotype H1 has been identified in 36 individuals (nearly one half of the specimens analyzed), whereas the second frequent haplotype has been H3 identified in 19 individuals. Eight haplotypes were unique (private) (table 2). The haplotype diversity (Hd) for all analyzed specimens was relatively high 0.719±0.042, whereas the corresponding nucleotide diversity (π) was quite low 0.0039±0.0005 (table 2). Partial COI gene sequences (523 bp) were obtained for 74 individuals and grouped into 13 haplotypes. The most frequent COI haplotypes were H1, H2 and H7 and only three haplotypes were unique (table 2). The haplotype (gene) diversity (Hd) for COI in all analyzed specimens was rather high 0.869±0.020, and the corresponding nucleotide diversity (π) was 0.00842 ± 0.00128 (table 2). Demographic parameters used to test for demographic events were estimated for each gene. Tajima's D value was not significant either for COI or for 16S, whereas the Fu's test was significant only for 16S rDNA (Fs=-6.599 P<0.01, table 2).

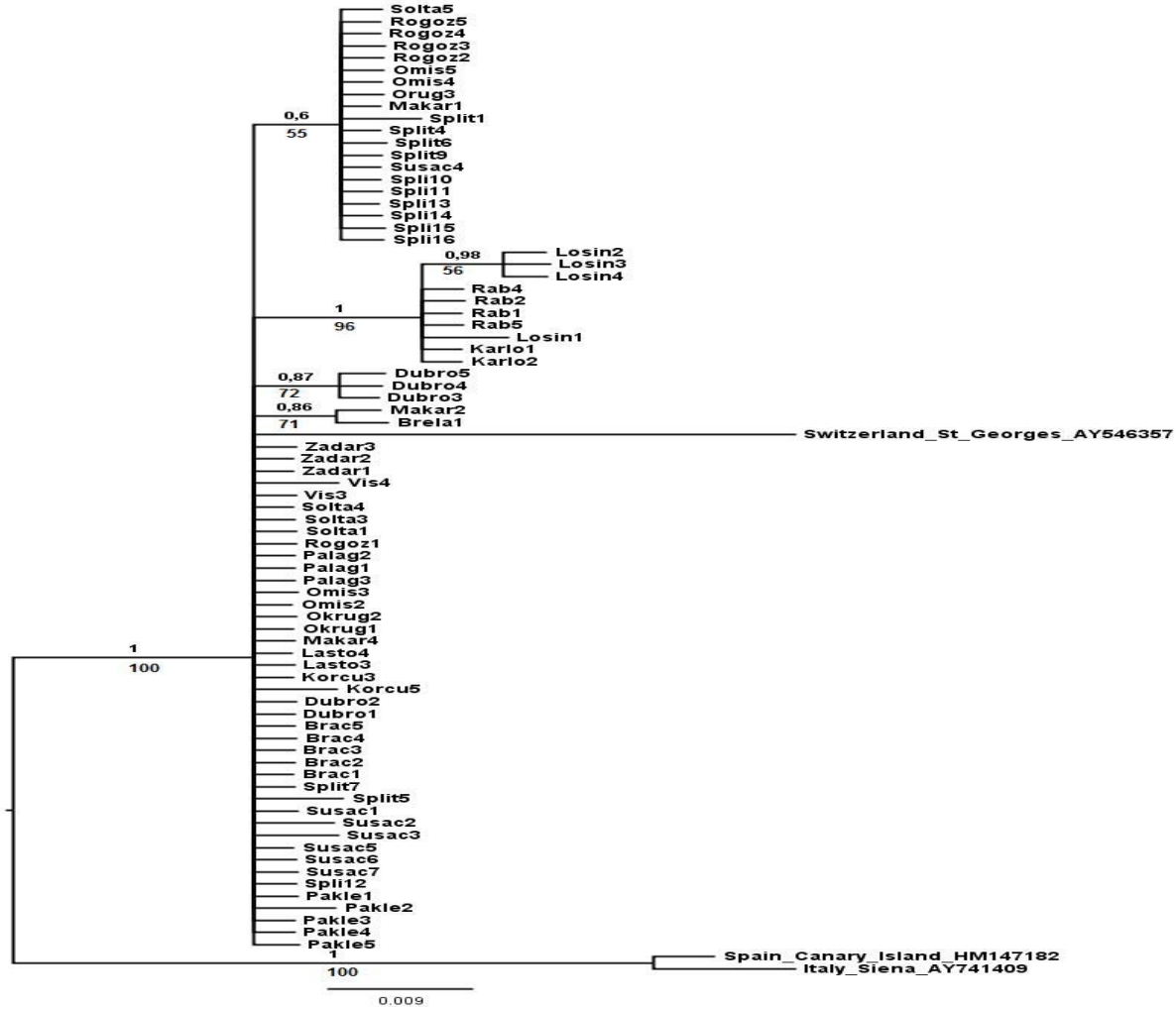

Fig 2: Phylogenetic tree resulting from Bayesian analysis of 16S rDNA sequences. The numbers above the branches depict Baysian posterior probabilities and the numbers below the branch indicate bootstrap support values from Maximum likelihood analysis. Scale indicates expected number of substitutions per site

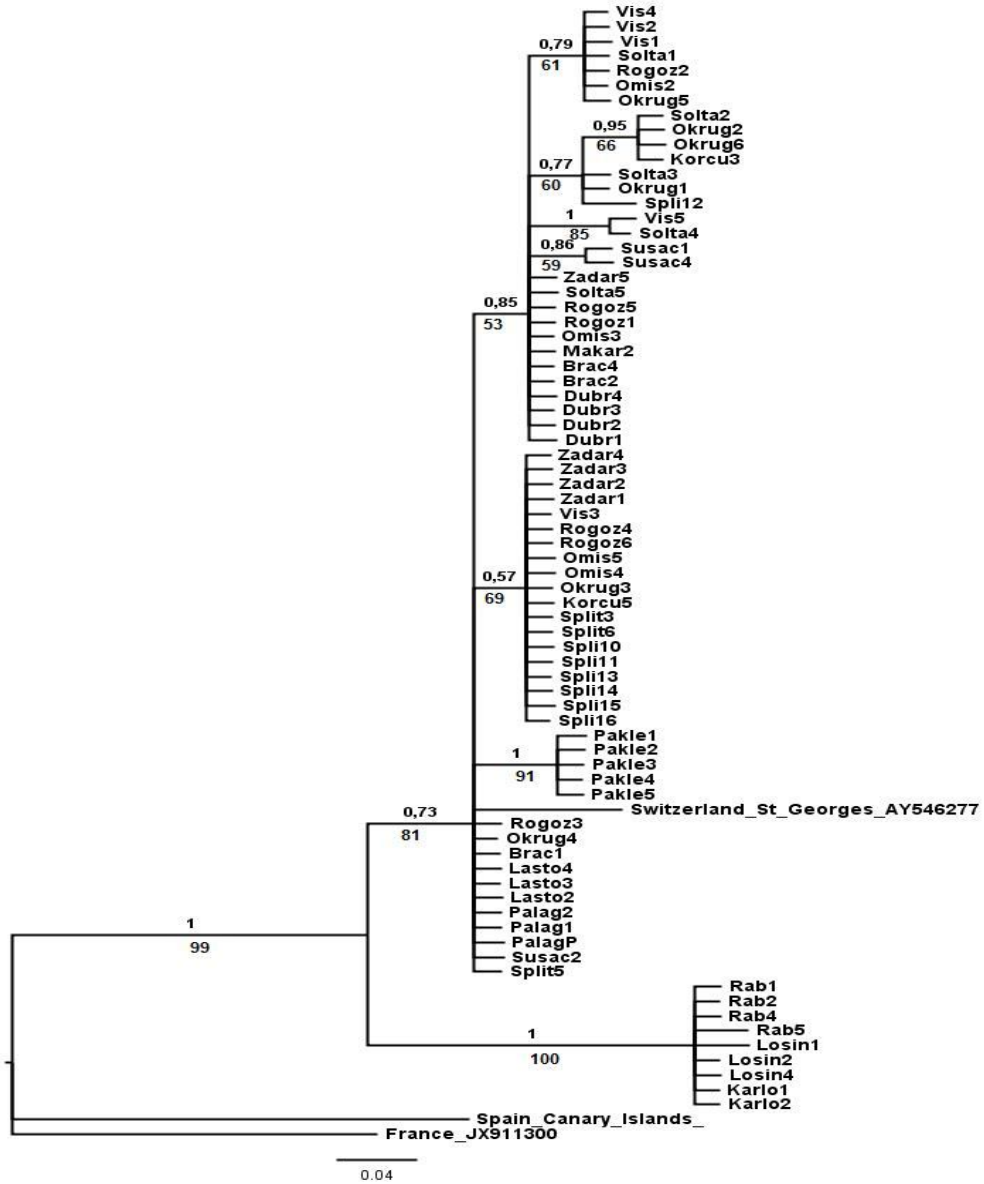

Fig 3: Phylogenetic tree resulting from Bayesian analysis of COI sequences. The numbers above the branches depict Baysian posterior probabilities and the numbers below the branch indicate bootstrap support values from Maximum likelihood analysis. Scale indicates expected number of substitutions per site.

Table S1: Labelling of the haplotypes. Haplotypes from Rađa *et al*. (2012) have been here renamed.

16S rDNA			COI		
GenBank Accession number	**Haplotype (Rađa *et al.* 2012)**	**Haplotype (new)**	**GenBank Accession number**	**Haplotype (Rađa *et al.* 2012)**	**Haplotype (new)**
JF277380	H1	H1	JF277391	H1	H7
JF277381	H2	H3	JF277392	H2	H8
JF277382	H3	H12	JF277393	H3	H9
JF277383	H4	H6	JF277394	H4	H1
JF277384	H5	H4	JF277395	H5	H10
JF277385	H6	H2	JF277396	H6	H2
JF277386	H7	H5	JF802030	H7	H3
JF277387	H8	H7	JF802031	H8	H4
JF277388	H9	H8	JF802032	H9	H5
JF277389	H10	H9	JF802033	H10	H6
JF277390	H11	H10	KF289762		H11
KF289759		H11	KF289763		H12
KF289760		H13	KF289764		H13
KF289761		H14			

3.2 Phylogenetic analyses of 16S rDNA and COI haplotypes and fine-scale phylogeography

Both phylogenetic algorithms Maximum-Likelihood (ML) and Bayesian Inference (BI) resulted in similar tree topology. Only the BI trees are shown (figures 2 and 3), which summarize the topology and posterior probabilities (PP) from BI plus bootstrap support (BS) from the ML analysis. All 16S rDNA sequences clustered into the single clade strongly supported by both analyses (1.0 PP, 100 % BS) (figure 2). Within this large clade several sub-clades were formed, but moderately to weakly supported. The exception was the clade comprised of north-Adriatic populations from islands of Rab and Lošinj and city of Karlobag which formed separate, but closely related clade with high support (1.0 PP, 96 % BS). A single sequence from Switzerland grouped also within this clade, in contrast to sequences from Italy and Spain which separated outside of this clade. COI sequences clustered into two separate clades, of which only the one comprised of north-Adriatic sequences was maximally supported (1.0 PP, 100 % BS) (figure 3). The other clade comprised of all remaining Croatian and a single Swiss COI sequences was not well supported (0.73 PP, 81 % BS).

Alternative procedure to phylogenetic tree reconstruction was also conducted to depict the relationships among the *E. vermiculata* haplotypes. We used the network-based approach which seems better suited for representing relationships among closely related sequences [37]. The haplotype network illustrating relationships among 14 *E. vermiculata* 16S rDNA haplotypes is shown in figure 4b. The most common 16S haplotype H1 formed a central node with immediate derivatives separated by a single mutation in the case of all but H2 and H12 haplotypes. Two mutations occurred in the case of H2 haplotype originating from island of Vis, a remote island in the open Adriatic Sea. Three mutations distinguished the haplotype H12 originating from the northernmost locality of islands of Rab and Lošinj and city of Karlobag. Figure 4a shows geographic distribution of 16S rDNA haplotypes. Haplotype H1 is found in all sampling sites in south-Adriatic mainland and islands, including southernmost Dubrovnik. The haplotype network illustrating relationships among 13 COI haplotypes is shown in figure 5b The separation of north-Adriatic populations of south-Adriatic is even better visible than in 16S haplotype network. The biggest difference can be seen between the northern haplotype H8 and southern haplotype H7, which are separated by 12 mutational steps. Figure 5a shows geographic distribution of *E. vermiculata* COI haplotypes along the Croatian coast and islands.

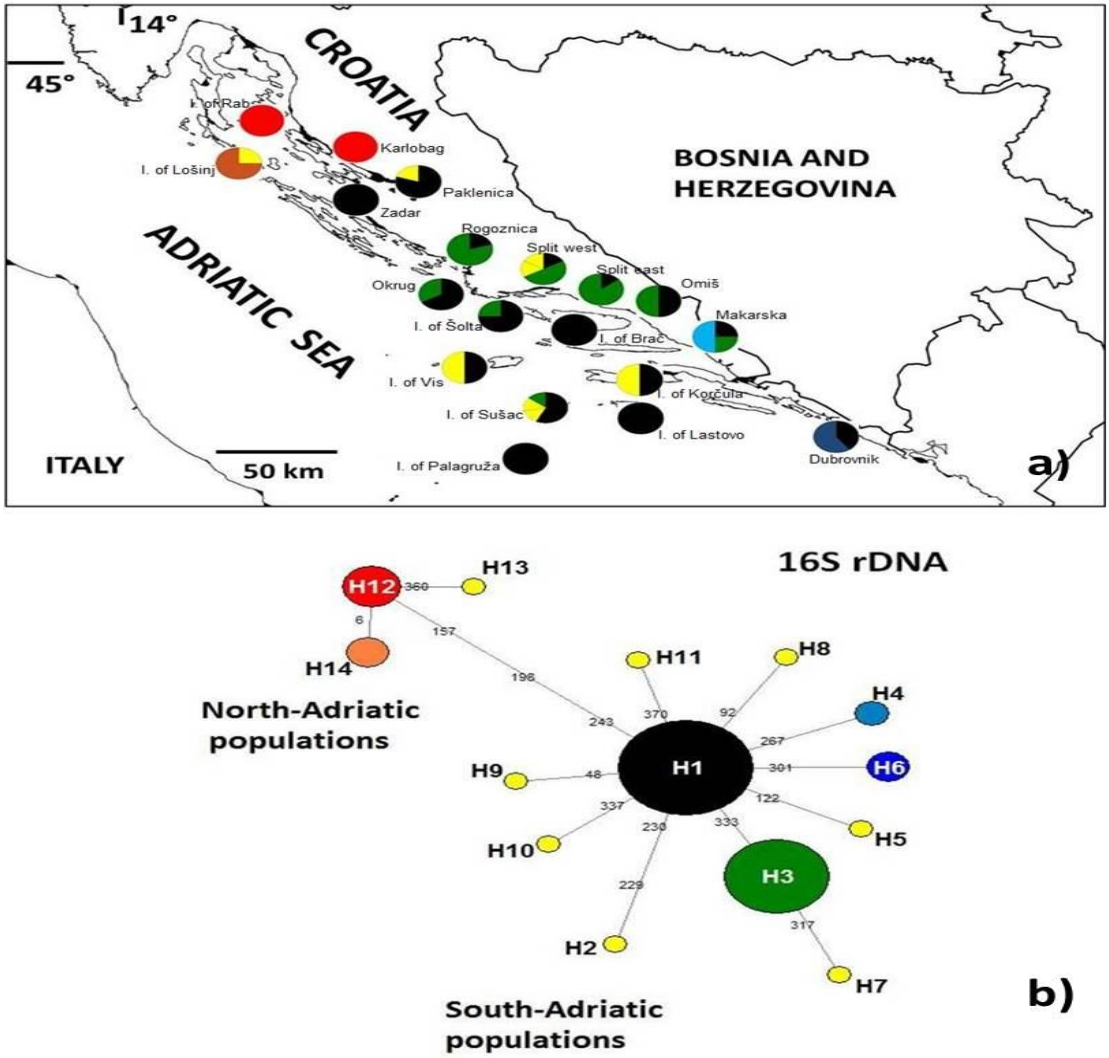

Fig 4: a) Distribution of *Eobania vermiculata* 16S rDNA haplotypes. Pie chart represents the location of the sample sites. Haplotypes identified in each population are shown in different colors. b) Median-joining network for the 16S rDNA haplotypes. Each circle represents a haplotype, and circle size is shown proportional to haplotype frequency. Colors indicate the geographic origin of haplotypes. The number on line indicates the position of a single base substitution and branch lengths are approximately equal to inferred mutational steps. Haplotype codes according to those in Table

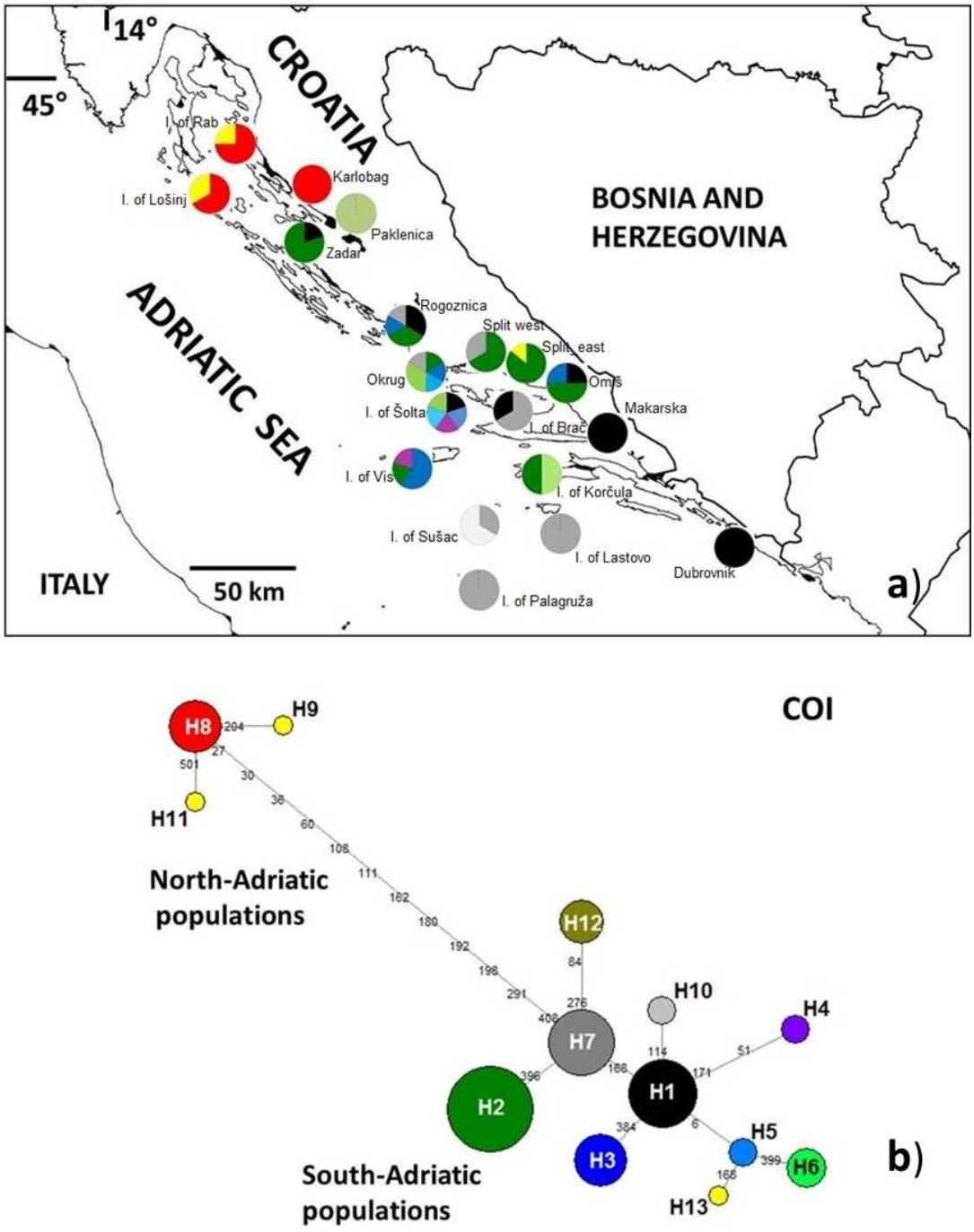

Fig 5: a) Distribution of *Eobania vermiculata* COI haplotypes. Pie chart represents the location of the sample sites. Haplotypes identified in each population are shown in different colors. b) Median-joining network for the 16S rDNA haplotypes. Each circle represents a haplotype, and circle size is shown proportional to haplotype frequency. Colors indicate the geographic origin of haplotypes. The number on line indicates the position of a single base substitution and branch lengths are approximately equal to inferred mutational steps. Haplotype codes according to those in Table 1.

4. Discussion

Here we reported the combined phylogenetic and phylogeographic analysis of our previously published sequence data [24] and newly obtained sequence for two mitochondrial genes (16S rDNA and COI) for 77 specimens of land snail *Eobania vermiculata* sampled along 19 sampling sites along Croatian coastal region and islands. The analyzed population is characterized by relatively high haplotype (gene) diversity (0.719±0.042 for 16S rDNA and 0.869±0.020 for COI) in combination with relatively low nucleotide diversity. These findings and the results of demographic Fu F's tests and Tajima's D value indicate no significant changes in sizes of Crotian populations and likely suggest its long historical presence in this region, which is consistent with current opinion about the authentic Mediterranean origin of *E. vermiculata.*

We did not detect major differences in 16S rDNA and COI haplotype distribution between the continental and island populations of *E. vermiculata*, although previous studies on its shell characteristics envisaged such differences [18, 19, 24]. It is probably due to the fact that the Adriatic islands are by its

formation relatively young; they formed after the last glacial period (app. 10.000 - 30.000 years ago), after the rising of the sea level by approximately 100 meters [38-41]. Instead, differences in mtDNA nucleotide composition were observed between the north-Adriatic and south-Adriatic *E. vermiculata* populations. These differences were weakly expressed in the tree-based phylogenetic analysis. Both maximum likelihood and Bayesian trees for both 16S and COI phylogenetic trees revealed a rather weak genetic differentiation between the north-Adriatic and south-Adriatic populations. However, differences between these populations were much better visible in the 16S and COI haplotype networks. Differences between the north-and south-Adriatic Adriatic populations are particularly well expressed in the COI marker; the south haplotype H7 was separated by 12 mutational steps from the north haplotype H8.

E. vermiculata is a typical Mediterranean species and can often be found in similar habitats as another Mediterranean brown garden snail, *Cornu aspersum* [11], (syn. *Helix aspersa*). These two species are also genetically closely related [2]. In contrast to *E. vermiculata*, biogeography and molecular phylogeny of *C. aspersum* has been extensively studied (for review see [42]). Several morpho-anatomical and molecular studies indicated the north-African origin and center of dispersal of *C. aspersum* and confirmed two morphologically and genetically different *C. aspersum* lineages (ancestral eastern and younger western). Our results of phylogeography analyses of the Croatian populations of *C. aspersum* (Puizina *et al*., submitted) indicate that the putative ancestral eastern *C. aspersum* haplotype lineage, which disappeared in most of Mediterranean countries, persisted along Croatian islands and coast and it is widespread, whereas younger western lineage has been confined to the South-Adriatic region. Results of fine-scale phylogeography analysis of the Croatian populations of *E. vermiculata* obtained in this study indicate that *E. vermiculata* could be the one of the co-distributed species along with *C. aspersum* as Guiller and Madec [42] proposed. Collecting of more specimens from different localities both in the north-Adriatic region as well as in the Mediterranean, and their comparative molecular-phylogenetic analysis could shed light on center of origin, possible colonization routes and evolutionary history of this nowadays widespread snail.

5. Acknowledgement

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