

## Genetic variation in East-Adriatic populations of the Asian tiger mosquito, *Aedes albopictus* (Diptera: Culicidae), inferred from *NADH5* and *COI* sequence variability

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**Abstract.** In the last few decades, *Aedes albopictus* (Skuse) (Diptera: Culicidae) (= *Stegomyia albopicta*), the so-called “Asian tiger mosquito”, has spread from its native range in southeast Asia to Africa, the Middle East, Europe, the Americas, and Pacific islands. The spread of this species poses a risk to human health as it is considered to be one of the main vectors of dengue and other arboviruses. *Aedes albopictus* was reported in Croatia in 2004, thereafter it was discovered at several coastal localities in 2005 and to date it has spread to most coastal areas and islands in Croatia. Here we investigate the genetic variability of *A. albopictus* based on 39 individuals collected during the summer of 2009 along the East-Adriatic coast and islands of Croatia and Montenegro and using two mitochondrial molecular markers: *cytochrome oxidase I (COI)* and *NADH dehydrogenase 5 (ND5)*. We identified a single *ND5* haplotype, corresponding to the previously reported and worldwide-distributed haplotype H3. The *COI* marker was more variable and we identified four *COI* haplotypes. In order to identify the geographic origin of the populations that colonized Croatia, we performed phylogenetic analyses of *ND5* and *COI* haplotypes in Croatian populations and other *A. albopictus* populations retrieved from the GenBank. The phylogenetic tree based on *ND5* haplotypes revealed two well supported clades where the unique Croatian *ND5* haplotype clustered with the majority of haplotypes originating from South-Asia, America, Europe, and Africa. Another smaller cluster consisted of only Brazilian haplotypes. The phylogenetic tree and haplotype network that resulted from the *COI* analysis also indicates that the three Croatian *COI* haplotypes cluster with European and American haplotypes. However the fourth Croatian *COI* haplotype was the only European haplotype that occurred in a separate clade (group) with Indian, South-Asian, and Brazilian haplotypes. This data suggests there have been several independent introduction events in Croatia.

### INTRODUCTION

In the last few decades, *Aedes albopictus* (Skuse) (Diptera: Culicidae) (= *Stegomyia albopicta*) (Reinert & Harbach, 2005), the so-called “Asian tiger mosquito”, has spread from its native range in southeast Asia to different parts of Africa, the Middle East, Europe, the Americas, and some Pacific islands (Gratz, 2004). The invasion of this species poses a risk to human health. Besides the nuisance caused by *A. albopictus* biting activity, it is a maintenance vector (occasionally epidemic) of dengue viruses in some parts of Asia (CDC, 2005) and plays an important role in the transmission of several other arboviruses, including chikungunya on the island of La Réunion (Reiter et al., 2006) and in Italy (Beltrame et al., 2007), eastern equine encephalitis (Mitchell et al., 1992), LaCrosse encephalitis (Gerhardt et al., 2001), Bunyaviridae (Francy et al., 1990), West Nile virus (Turell et al., 2001) and *Dirofilaria* spp. (Gratz, 2004). The first two cases of local dengue transmission in Europe were reported from Nice, southeast France, in 2010 (La Ruche, 2010). The same year, another autochthonous dengue transmission occurred via a traveller from Germany

returning from a trip to southern Croatia (Schmidt-Chanasit et al., 2010). It indicates that autochthonous transmission of dengue is also possible in the other parts of Europe where *A. albopictus* is established (La Ruche et al., 2010).

The usual way of dispersal of this invasive species is the transport of its eggs in used tires (Knudsen, 1995), so *A. albopictus* has been able to travel across very large distances and even between continents mainly via the international used tire trade (Reiter, 1998). Nevertheless, the international trade of a decorative plant, the Lucky Bamboo (*Dracaena* spp.), is also thought to have been important in the spread of this species (Madon et al., 2003). Due to its ability to colonize a wide range of natural and artificial breeding places together with the resistance of its eggs to desiccation and its relative lack of host specificity (Hawley, 1988), this species has been able to rapidly build up large stable populations in new geographic regions. Once established in a new area national trade and traffic has facilitated the subsequent rapid spread of this mosquito into other areas (Moore & Mitchell, 1997).

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Prior to 1985, it was known that *A. albopictus* was present from Madagascar, southern Asia northward to Beijing, China, Seoul, Korea, and Sendai, Japan (Hawley, 1988). *Aedes albopictus* was also established in Hawai'i, Guam, Indonesia, West Papua, Papua New Guinea and Solomon Islands eastward to Santa Cruz Island (Mitchell, 1995). In 1985 its presence was reported in the USA where it was probably arrived in tire shipments from Northern Asia (Hawley et al., 1987). This species is also spreading across the Pacific islands of Palau, Yap and Fiji (CDC, 2005) and Torres Strait Islands in Australia (Ritchie et al., 2006). Established populations of *A. albopictus* were detected in Brazil in 1986. This species colonized Mexico in 1988 and thereafter many countries of Central and South America: Argentina, Bolivia, Cuba, Honduras, Guatemala, El Salvador, Colombia, Cayman Islands (Rossi et al., 1999; CDC, 2005) and also some parts of Paraguay, Panama, Uruguay and Nicaragua (Cuéllar-Jiménez et al., 2007). In Africa, *A. albopictus* was discovered in Nigeria in 1991 and then spread to Cameroon (Fontenille & Toto, 2001), Bioko Island of Equatorial Guinea and Gabon (Toto et al., 2003; Krueger & Hagen, 2007). In the Middle East *A. albopictus* is present in Israel (Pener et al., 2003), Lebanon and Syria (Haddad et al., 2007).

In Europe, after the first detection of *A. albopictus* in Albania in 1979 (Adhami & Reiter, 1998), this species was found in Genoa in September 1990 and Padua in 1991 with a suggestion that latter introduction could have resulted from tire imports from the United States (Sabatini et al., 1990; Dalla Pozza & Majori, 1992; Dalla Pozza et al., 1994). Since then *A. albopictus* has spread throughout the entire mainland of Italy as well as some parts of Sicily and Sardinia. Apart from Albania and Italy, established homogenous populations of *A. albopictus* occur in Croatia, France, Greece, Monaco, Montenegro, San Marino, Slovenia, Spain and Vatican City (ECDC, 2008). *Aedes albopictus* is recorded as occurring in Germany but it is unknown whether it has established there (Pluskota et al., 2008). It has also been introduced into Belgium (Schaffner et al., 2004), but it has not established there (ECDC, 2008). In the Netherlands, it is recorded only inside greenhouses (Scholten et al., 2007; ECDC, 2008). In southern Switzerland, recent data suggest an ongoing spread (Flacio et al., 2004; ECDC, 2008). It has also been reported from isolated foci in Bosnia and Herzegovina, but no further details are available (ECDC, 2008).

*Aedes albopictus* was reported in Croatia in 2004 (Klobučar et al., 2006), thereafter it was discovered at several coastal localities in 2005 and to date it has spread to most coastal areas and islands in Croatia (Merdić et al., 2009). Little is known about the role of European *A. albopictus* in the pathogenesis of different diseases. In order to obtain a better understanding of the introduction, spread, evolution and local adaptation of *A. albopictus* it is necessary to study its genetic structure (ECDC, 2008).

The aims of this study were: (1) to determine the pattern of genetic variation within the Croatian *A. albopictus*

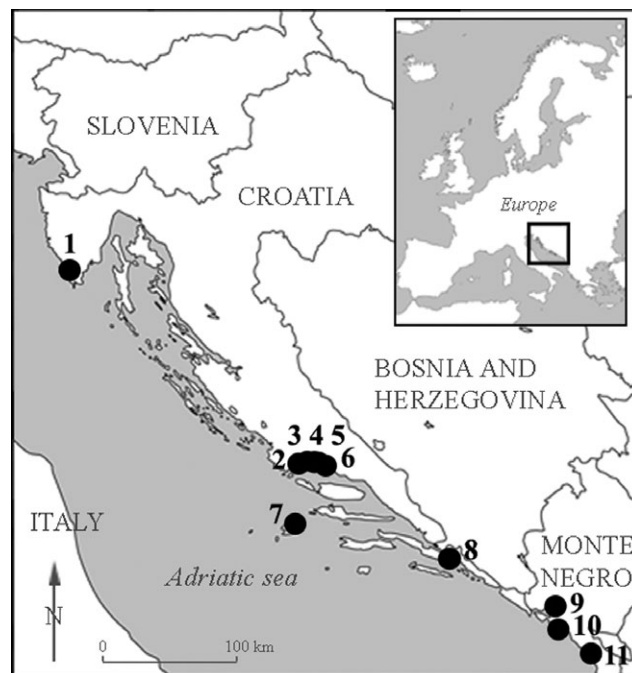


Fig. 1. Sites (numbered 1–11; see Table 1) at which the Asian tiger mosquito (*Aedes albopictus*) was sampled.

population based on analyses of the sequence diversity of two mitochondrial genes, *cytochrome oxidase I (COI)* and the *NADH dehydrogenase subunit 5 (ND5)*; and (2) to establish the evolutionary relationships of the Croatian population with other *A. albopictus* populations and determine the geographic origin of the populations that colonized Croatia.

## MATERIAL AND METHODS

### Sampling

We examined 39 specimens of *Aedes albopictus* collected at 11 locations along the East Adriatic Coast in September and October 2009 (Fig. 1; Table 1). Adult mosquitoes were captured with aspirators at eight locations on the Adriatic Coast of Croatia and their eggs collected using ovitraps at three locations on the Adriatic Coast of Montenegro (Fig. 1; Table 1). The eggs were hatched and the larvae reared to adults in the laboratory. Adult mosquitoes were identified based on their morphology and using a key (Becker et al., 2003). All samples were placed in plastic tubes with 80% ethanol until further processing.

### DNA extraction, amplification and sequencing

Prior to DNA extraction samples were thoroughly washed with 95% ethanol and left to dry in the air. Specimens were ground and DNA extraction was performed using a commercial kit for DNA isolation (Qiagen DNeasy Blood & Tissue kit, Hilden, Germany) according to the manufacturer's instructions. Two mitochondrial regions extracted from *Aedes albopictus* DNA were amplified, *cytochrome oxidase I (COI)* and *NADH dehydrogenase 5 subunit (ND5)*. PCR amplification of the *COI* subunit was first performed using the primers used by Mousson et al. (2005), 5' TGATCAAATTTATAAT 3' (COIF) and 5' GGTAATAATTAATAATAACTTC 3' (COIR), but no DNA was amplified. The analysis using a different set of primers (Patsoula et al., 2006) was successful; these primers were 5'-CGAGGATTTGGAAATTGATTAGTTC-3' (COI2F) and 5'-CCCGGTAAAATTAATAATAACTTC-3' (COI2R). In

TABLE 1. Sites sampled and data on the specimens collected. The four *COI* haplotypes, Cro1–Cro4, are identified. All the individuals analyzed had the unique *ND5* haplotype.

Sites sampled	Country / Locality	<i>COI</i> haplotypes, GenBank accession numbers (no. of individuals)	Date of collection	Stage of mosquito collected
Croatia				
1	Pula	Cro1, HQ906848 (11) Cro4, HQ906851 (1)	Oct 2009	Adult
2	Trogir	Cro1, HQ906848 (1)	Sep 2009	Adult
3	Split – Skalice	Cro1, HQ906848 (3)	Sep 2009	Adult
			Oct 2009	Adult
4	Split – Gripe	Cro1, HQ906848 (2) Cro2, HQ906849 (1) Cro3, HQ 906850 (1)	Sep 2009	Adult
5	Split – Meje	Cro1, HQ906848 (2) Cro2, HQ906849 (2)	Sep 2009	Adult
6	Podstrana	Cro1, HQ906848 (2)	Sep 2009	Adult
		Cro3, HQ 906850 (1)	Oct 2009	Adult
7	Vis	Cro1, HQ906848 (3)	Oct 2009	Adult
8	Ston	Cro1, HQ906848 (2)	Oct 2009	Adult
Montenegro				
9	Kotor	Cro1, HQ906848 (5)	Oct 2009	Egg
10	Budva	Cro1, HQ906848 (1)	Oct 2009	Egg
11	Bar	Cro1, HQ906848(1)	Oct 2009	Egg

order to amplify mitochondrial *NADH dehydrogenase 5 subunit* (*ND5*) the following primers designed by Birungi & Munstermann (2002) were used: 5'-TCCTTAGAATAAAATCCCGC-3' (*ND5F*) and 5' GTTCTGCTTTAGTTCATTCTTC 3' (*ND5R*). Each PCR reaction was performed using the TaqMan Master Mix solution (Qiagen, Hilden, Germany) in a final reaction volume of 25 µl containing 1 µM of each primer (Sigma-Aldrich, Steinheim, Germany) and 2 µl of template DNA. PCR reactions were conducted in a Mastercycler personal (Eppendorf, Hamburg, Germany) using, for *COI*, an initial denaturation step of 15 min at 95°C followed by 35 cycles of 94°C for 1 min, 50°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. For *ND5*, the PCR cycles consisted of an initial denaturation step at 95°C for 15 min, followed by 30 cycles at 94°C for 30 s, 46°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. The electrophoresis of the PCR products was done at 120 V on 1.5% agarose gel in TBE buffer with a 1000 bp DNA marker and afterwards purified with QIAquick gel extraction kit (Qiagen) according to manufacturer's specifications. The PCR products were sent to Macrogen (Seoul, Korea) for sequencing. Sequences were submitted to GenBank.

#### Data analysis

Alignment was performed manually on DNA sequences using Se-Al v2.0a11 (Rambaut A. 1996). Se-Al: Sequence Alignment Editor available at <http://evolve.zoo.ox.ac.uk/>, for a final length of 318 bp for *COI* and 369 bp for *ND5*. Numbers of variable sites, fixed differences, haplotype (gene) and nucleotide diversity were determined using DnaSPv5 (Librado & Rozas, 2009). Phylogenetic reconstructions were carried out using Bayesian inference with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003), with 4 chains of 1,000,000 generations, trees sampled every 100 generations and burnin value set to 20 % of the sampled trees. Sequences were analyzed using an evolutionary model designed for coding sequences taking the genetic code into account (Goldman & Yang, 1994; Muse & Gaunt, 1994; Shapiro et al.,

2006). Haplotype network was built from the same data and data from Table 3 using TCS (Clement et al., 2000).

## RESULTS

### Genetic variability of *Aedes albopictus* along the East Adriatic coast

All 39 samples collected along the East Adriatic coast and islands, from the North (Pula) to the South of the Adriatic region (Budva) (Fig. 1; Table 1), had a unique *ND5* haplotype, H3 (named by Birungi & Munstermann, 2002 and Mousson et al., 2005; Table 2). We deposited this 393 bp long sequence in GenBank under the accession number HQ906852.

*COI* sequences were also obtained for 39 individuals of *A. albopictus* (Fig. 1; Table 1). All sequences were 464 bp long. The total number of mutations was three. Two of them were parsimony informative and the third was a singleton. A total of four haplotypes were recorded. The haplotype (gene) diversity (*Hd*) in these specimens was very low ( $0.282 \pm 0.091$ ) and the corresponding nucleotide diversity ( $\pi$ ) was also very low ( $0.000064 \pm 0.004$ ) (Table 2). The most frequent *COI* haplotype, Cro1 (GenBank accession number HQ906848) was identified in 33 individuals collected at all 11 sampling sites along the entire East Adriatic coast and islands. Three individuals had the Cro2 haplotype (GenBank accession number HQ906849) and two the Cro3 haplotype (GenBank accession number HQ906850). A single individual had the Cro4 haplotype (GenBank accession number HQ906851) (Fig 1; Table 1). Our results indicate that the Tajima's *D* value (−1.2758) and *Fu's Fs* statistics (−2.216) for *COI* for the entire sample are both negative but not statistically significant ( $P > 0.10$ ). These results signify an excess of

TABLE 2. Variation in the sequences of *COI* and *ND5* of specimens of *Aedes albopictus* from the East Adriatic coast and islands and haplotypes retrieved from GenBank, including haplotypes recorded in this study.

	<i>COI</i>		<i>ND5</i>	
	Croatia and Montenegro (sequences)	GenBank (haplotypes)	Croatia and Montenegro (sequences)	GenBank (haplotypes)
Number of sequences/haplotypes	39	26	39	27
Number of sites	464	318	393	369
Variable sites	3	8	0	11
Singleton variable sites	1	7	0	8
Parsimony informative sites	2	1	0	3
G + C content	0.334	0.357	0.212	0.226
No. of haplotypes	4	9	1	10
Haplotype diversity: $Hd \pm sd$	$0.282 \pm 0.091$	$0.751 \pm 0.062$	0.00	$0.721 \pm 0.088$
Nucleotide diversity: $\pi \pm sd$	$0.00064 \pm 0.00022$	$0.00333 \pm 0.00051$	0.00	$0.00328 \pm 0.00081$

low frequency polymorphisms, confirming a recent expansion in population size (Tajima, 1989).

#### Phylogenetic relationships between *COI* and *ND5* haplotypes

In order to identify the geographic origin of populations that colonized Croatia, we performed phylogenetic analyses of *ND5* and *COI* haplotypes from Croatian populations and other *Aedes albopictus* haplotypes retrieved from GenBank. The analysis of these data revealed 9 haplotypes of *COI* and 10 of *ND5* with a low gene diversity value (Table 2). The analysis of these data showed that there were very few variations in *COI* (1 parsimony-informative site) and *ND5* (3 parsimony-informative sites). *COI* haplotypes were divided into 9 groups denoted as HC1-HC9 (Table 3). The most frequent Croatian *COI* haplotype Cro1 (HQ906848) belongs to the HC1 haplotype, whereas the second most frequent Croatian haplotype Cro2 (HQ906849) belongs to the HC2 haplotype. Two of the *COI* haplotypes: H3 and H4, are new.

The *ND5* phylogenetic tree revealed two well supported clades (posterior probabilities (pp) of 0.98) one containing all the Brazilian haplotypes and the other the American, European, African and South-Asian haplotypes (Fig. 2). The *COI* phylogenetic trees also revealed two weakly supported clades (pp of 0.59), where three Croatian haplotypes, including the most frequent Cro1 haplotype (HQ906848), clustered with other European and American haplotypes. However the fourth Croatian *COI* haplotype, Cro2 (HQ906849), was the only European haplotype that clustered into a separate clade together with Indian, Brazilian and South-Asian haplotypes (Fig. 3). The haplotype network illustrating relationships among nine *COI* haplogroups is shown in Fig. 4. The majority of the *A. albopictus* haplotypes clustered into two distinct groups HC1 and HC2 distinguished by a single mutation at nucleotide position 36 (Table 4). These two haplogroups formed an internal node in each grouping with immediate derivative(s) separated by a single mutation (Fig. 4).

#### DISCUSSION

In contrast to the high levels of allozyme variation within populations of Asian tiger mosquito, *Aedes albopictus*, documented by several independent studies (Black et al., 1988, Kambhampati et al., 1991, Urbanelli et al., 2000), the level of mtDNA variation based on molecular markers such as *Cytb*, *COI* and *ND5*, is notably low (Kambhampati & Rai, 1991; Birungi & Munsterman,

TABLE 3. Haplotype frequency and classification of populations of *Aedes albopictus* into 9 *COI* groups.

<i>COI</i> groups	Populations	Frequency/no. of individuals	Sample size
HC1	<b>HQ906848_Croatia_Spl</b>	0.85/33	39
	AY748238_Greece_Thes	0.95/19	20
	AY748239_Greece_Corf	0.05/1	20
	AJ971008_France_Mont	0.077/1	13
	AJ971013_Reunion_La	0.077/1	13
	AJ971007_Madagascar	0.077/1	13
	AJ971005_USA_Jackson	0.077/1	13
	AJ_971009_France_Nain	0.077/1	13
	AJ971011_USA_Hawaii	0.077/1	13
HC2	<b>HQ906849_Croatia_Spl</b>	0.08/3	39
	AY834241_India_Karna	0.25/1	4
	AY729984_India_Pondi	0.25/1	4
	DQ424959_India_Vihr	0.25/1	4
	FJ372983_India_Thiru	1.00/1	1
	AY971003_Brazil_Repr	0.077/1	13
	AJ971010_Viet_Nam_Nh	0.077/1	13
	AJ971014_Brazil_Sao	0.077/1	13
	AJ971004_Viet_Nam_Ha	0.077/1	13
HC3	<b>HQ906850_Croatia_Spl</b>	0.05/2	39
	<b>HQ906851_Croatia_Pul</b>	0.026/1	39
	AY072044_Taiwan_Taip	1.00/1	1
	EU259306_India_Manana	1.00/1	1
	DQ310142_India_Sahar	0.25/1	4
	AJ971012_Reunion_La	0.077/1	13
	AJ971006_Cambodia_Se	0.077/1	13

Haplotype data for populations from the native range; United States, Brazil, Madagascar and France, was obtained from Mousson et al. (2005). Haplotype data for populations from Greece was obtained from Patsoula et al. (2006) and from India from Kumar et al. (2007).

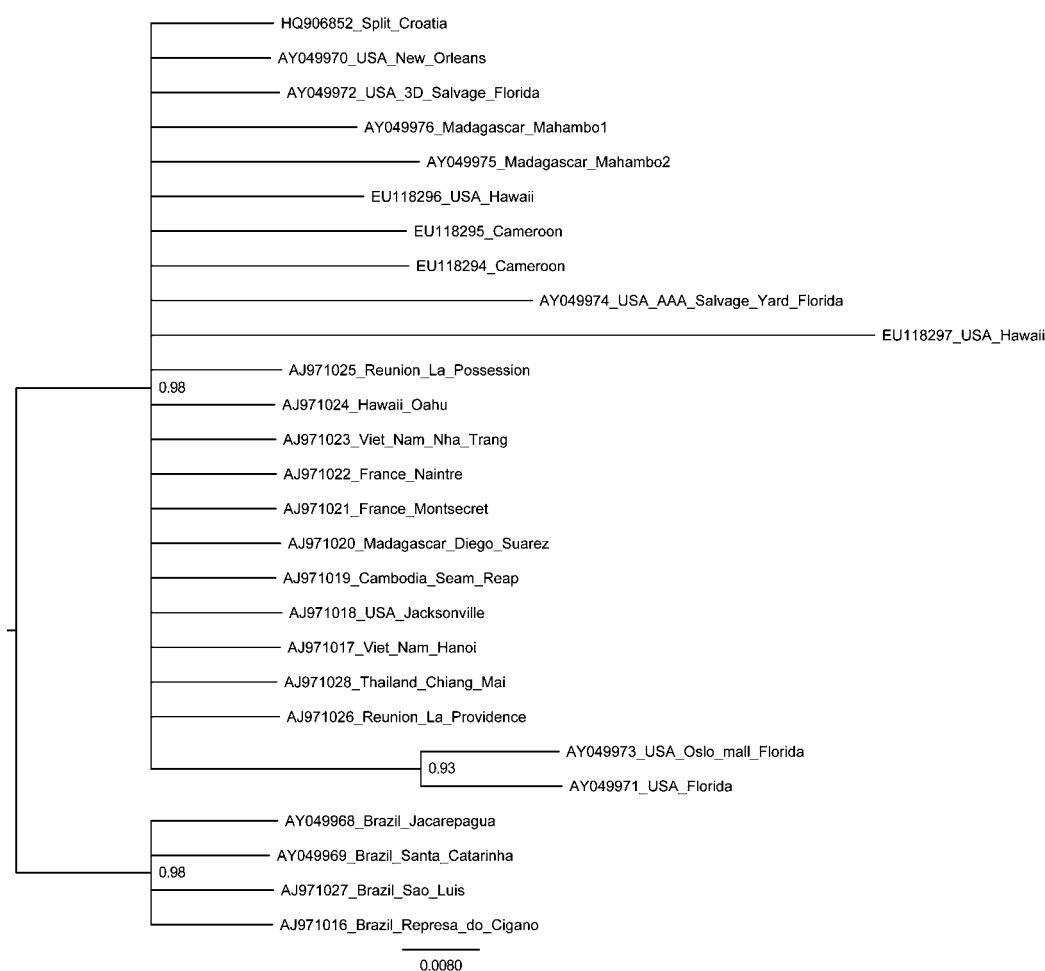


Fig. 2. Phylogenetic tree of *Aedes albopictus* ND5 haplotypes constructed using Bayesian inference. Numbers on nodes are posterior probabilities. Scale indicates expected number of substitutions per site.

2002; Mousson et al., 2005; Usmani-Brown et al., 2009; Maia et al., 2009; this study).

The observed pattern of genetic variation in populations of *A. albopictus* (high variation in the nuclear loci and low variation in mtDNA), which spread outside its native range, may be due to the small size of the founding populations, where genetic drift has had insufficient time to reduce the variation at nuclear loci and populations successively expanded and a few founder females became

established in new geographic areas (Birungi & Munstermann, 2002). Reduced levels of genetic variation could also be the consequence of extensive insect control measures, which involve source reduction and insecticide application leading to the reduction or even the eradication of *A. albopictus* populations (Birungi & Munstermann, 2002; Usmani-Brown et al., 2009).

Mousson et al. (2005) compared the variability in three mitochondrial markers (*Cytb*, *COI* and *ND5*) in 13 speci-

TABLE 4. Differences in the nucleotide sequences of the haplotypes of the mtDNA *cytochrome oxidase 1* (*COI*) gene of *A. albopictus* collected at locations along East-Adriatic coast.

COI haplotype	Nucleotide position *							
	18	19	36	79	102	180	198	318
HC1	G	T	C	G	G	T	G	G
HC2	—	—	T	—	—	—	—	—
HC3	—	—	T	—	—	—	A	—
HC4	—	—	T	—	—	—	—	A
HC5	—	—	T	—	—	C	—	—
HC6	—	—	—	A	—	—	—	—
HC7	—	G	—	—	—	—	—	—
HC8	A	—	T	—	—	—	—	—
HC9	—	—	—	—	A	—	—	—

\* Nucleotide position corresponding to the *A. albopictus* *COI* sequence in GenBank accession no. HQ906849; hyphens (—) denote nucleotide identity with haplotype HC1.

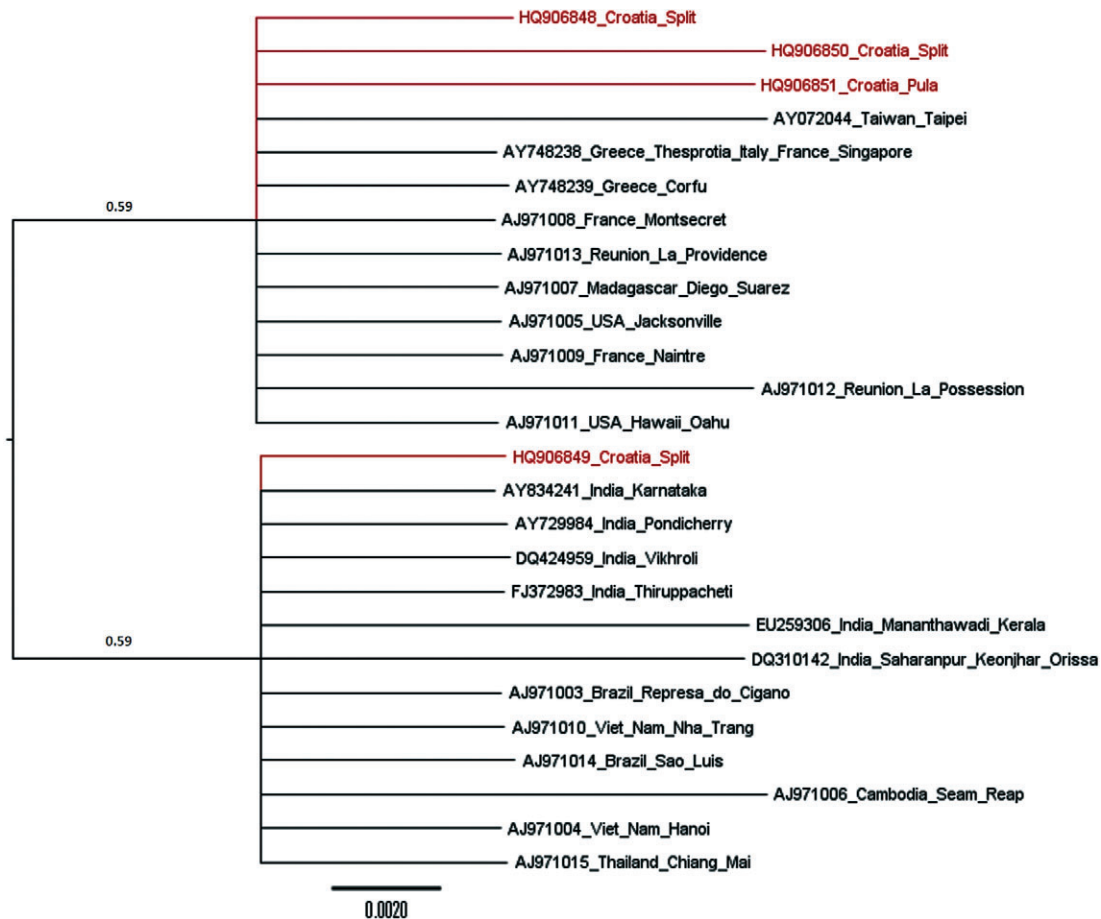


Fig. 3. Phylogenetic tree of *Aedes albopictus* based on *COI* and constructed using Bayesian inference. Numbers on nodes are posterior probabilities. Scale indicates expected number of substitutions per site.

mens of *A. albopictus* and concluded that *Cytb* was more variable and useful than *COI* and *ND5*. Our comparison of the sequence variability in *COI* and *ND5* markers based on 39 specimens of *A. albopictus* collected along the East Adriatic coast confirmed the overall low level of sequence divergence in these two genes. However, in our study, *COI* was more informative as a molecular marker

than *ND5* and revealed that there are four *COI* haplotypes but only one *ND5* haplotype.

Usmani-Brown et al. (2009) and Birungi & Munstermann (2002) combined all the available *ND5* haplotypes into 13 groups consisting of 13 different haplotypes, H1–H13. The two most frequent haplotypes were H1 and H3, which differ in a single C–T substitution. The most frequent *ND5* haplotype, H3, has been identified in the species' native range; continental United States, Hawaii, Madagascar, Cameroon, and Italy (Birungi & Munstermann 2002; Usmani-Brown et al., 2009; Maia et al., 2009). The populations of *A. albopictus* from Croatia and Montenegro analyzed in this study also belong to this haplotype. The second largest group, containing haplotype H1, contained only the Brazilian populations. Our phylogenetic analysis thus confirmed earlier studies indicating a separate introduction of this species into Brazil (Birungi & Munstermann, 2002; Usmani-Brown et al., 2009; Maia et al., 2009).

Similarly, we grouped all available *COI* sequences into 9 groups consisting of 9 *COI* haplotypes, HC1–HC9 (Table 3). The most frequent haplotypes are HC1 and HC2, which differ by a single C–T transition at position 36 according to our input file (Table 4). The most frequent *COI* group HC1 includes European and American haplotypes, while the HC2 group includes native range (Indian and South-Asian) and Brazilian haplotypes. The

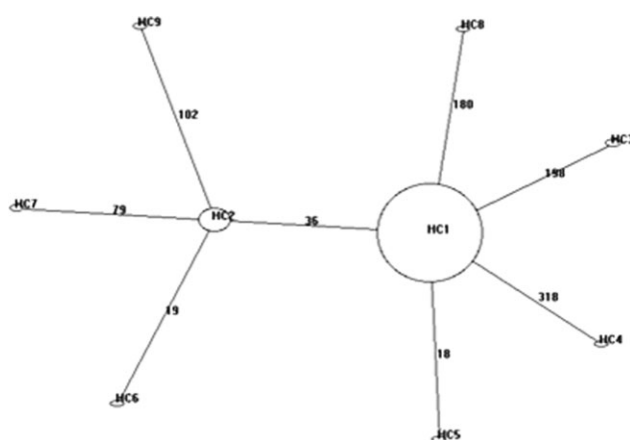


Fig. 4. Network of the *COI* haplotypes of *Aedes albopictus* generated using data from Table 3. The size of the ovals is proportional to the haplotype frequency. The number on line indicates the position of a single base substitution.

majority of specimens analyzed in this study and in a study of a Greek population (Patsoula et al., 2006) belong to the HC1 haplogroup, but we also found that three of the individuals collected at Split belong to the HC2 haplotype. This finding, and the two new previously unidentified *COI* haplotypes (HC3 and HC4), indicate that there are at least four *A. albopictus* haplotypes in Croatia (Table 3).

Such data might suggest that the populations of *A. albopictus* that colonized the East Adriatic coast of Croatia probably did not originate from a single source population. Instead, it is likely there were several source populations and introductions. At least two independent introductions are reported in neighbouring regions of Croatia: in Albania, the first European case of an *A. albopictus* infestation was reported in 1979 and probably arrived in tires imported from China (Adhami & Reiter, 1998); in Italy (Genoa and Veneto regions) this mosquito probably came in tires imported from the United States (Sabatini et al., 1990; Dalla Pozza & Majori, 1992; Dalla Pozza et al., 1994). Split has two harbours for international maritime transport and is also an important destination for tourists during summer when a large number of Italian tourists visit the Croatian coast. Further investigations should focus on the monitoring of *A. albopictus* in Croatia and neighbouring countries. Because of the rather small variation in mtDNA sequences, analyses using more variable neutral nuclear markers, such as microsatellites, along with a more extensive sampling of populations not only in this region but also in the native range are needed in order to obtain a better understanding of the biology and immigration patterns of this potentially dangerous invasive species.

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