

Research Article

Molecular Characterization of the Asian Tiger Mosquito *Aedes albopictus* (Skuse) (Diptera: Culicidae) in Northern Italy

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Polymorphism of the gene *COI* encoding cytochrome C oxidase subunit I and of the rRNA second internal transcribed spacer (ITS2) was measured in *Aedes albopictus* collected in three locations in Piedmont region and one location in Liguria region in Italy. According to our data, besides the middle part of the *COI* gene usually used for analysis, there are also SNPs in the 5' half of the gene, which can provide additional information about the polymorphism of *A. albopictus* populations. Based on *COI* gene, three various mitochondrial haplotypes were identified in *A. albopictus* from four north Italian sampling sites. Different sources of introductions can be considered. The variability between *COI* sequences of the north Italian *A. albopictus* is 0.001 while the variability between them based on the pairwise analysis of ITS2 sequences is 10 time more—0.011. For the first time, a 4% divergence between the populations of *A. albopictus* of tropical and subtropical origins was found on the basis of analyzing the ITS2. A relatively high level of variability of ITS2 between tropical and subtropical populations can be used to develop new markers for phylogenetic studies. No individuals with mitochondrial and nuclear haplotypes specific to tropical populations were found in the four north Italian sampling sites.

1. Introduction

Aedes albopictus was first detected in Italy in Genova (Liguria) in 1990 [1]. In 2007, it has been incriminated as the main vector of the first European outbreak of chikungunya virus (CHIKV) in northern Italy [2]. *A. albopictus* from Northern Italy was also demonstrated to efficiently transmit in laboratory CHIKV and to a lesser extent, dengue virus (DENV) [3]. Its expansion all over the world may contribute to obtain a mosaic of genetically distinct populations expressing different abilities to transmit arboviruses [4–7]. Studies using cytochrome C oxidase subunit I (*COI*) and NADH dehydrogenase subunit 5 (*ND5*) genes showed a low level of polymorphism of the mitochondrial DNA (mtDNA), but revealed differences between tropical and subtropical populations [8–12]. Low levels of mtDNA polymorphism can be associated with the founder effect [8], and also with the presence of maternally inherited symbiotic bacterium

Wolbachia in *A. albopictus* [13]. It is considered that the *Wolbachia*-infected individuals are favoured owing to the phenomenon of cytoplasmic incompatibility. Unlike the mtDNA, the nuclear DNA is not exposed to the selective sweep of the *Wolbachia*-infected mitochondrial haplotype. Therefore, genetic variations in nuclear DNA must be higher. Moreover, it was previously shown that the intergenic spacer region of rRNA varied in size from 2 to 10 kb in *A. albopictus* populations [14]. The relatively high level of polymorphism was also observed using allozyme markers for north American, Brazilian [15] and Italian populations [16].

As DNA analysis was conducted only for two *A. albopictus* mosquitoes from Italy [10], we undertook a genetic study of these mosquitoes in northern Italy. We studied genetic diversity within *A. albopictus* from four sampling sites based on analyses of the mitochondrial and nuclear sequences. Since in Croatia, only one common mitochondrial haplotype for the *ND5* gene and four different mitochondrial haplotypes

for the *COI* gene were detected [12] and in Cameroon four other mitochondrial haplotypes of the *COI* gene were found in the absence of polymorphism of the *ND5* gene [11], we selected *COI* as the most informative gene for analysis of *A. albopictus* from different sampling sites. *COI* is also used for Barcoding and published sequence data of different geographical origin are available for analysis. To study nuclear gene diversity within and among of *A. albopictus* from different sampling sites, we investigated the second internal transcribed spacer (ITS2) of rRNA that is traditionally used to identify interspecific differences. ITS2 was successfully used to differentiate the *Aedes* species [10].

Using *COI* and ITS2 as genetic markers, we determined the geographic origin of *A. albopictus* invading northern Italy. These data will help to predict further expansion of *A. albopictus* on the European continent and to develop measures to counteract.

2. Materials and Methods

A. albopictus mosquitoes were collected in four locations in northern Italy during the summer of 2009 and 2011: Piovera 44°57'35.14"N, 8°44'14.23"E (2 individuals), Tortona 44°53'39"N, 8°56"E (2 individuals), Alessandria 44°55'21.47"N, 8°38'00.91"E (11 individuals) from Piedmont region, and Varazze 44°21'37.79"N, 8°34'33.88"E (19 individuals) from Liguria region. Altogether, 34 specimens were investigated.

For identification were used Identification Key by Stojanovich and Scott and the Key proposed by Michele M. Cutwa-Francis and George F. O'Meara. A further, certainty in the eggs identification is based on the multiannual experience yet unpublished, in proposed, by Asghar Talbalaghi, for identification of eggs of *A. albopictus* which are very similar to the species of *Ochlerotatus geniculatus* (<http://www.zanzare.eu/pdf/Talbalaghi.Italy.2010.pdf>).

Part of a project mosquitoes control—Alessandria active since 1996 in 30 municipalities in the county of Alessandria, the first ovitraps were positioned in 2004 to monitor eventual arrival from untreated areas outside our jurisdiction. The first record of positive dates back to 2007 with 3 positive cases on 20 ovitraps positioned. To highlight the capacity of *A. albopictus* to spread everywhere once colonized in a certain area, we recorded of 250 ovitraps positioned during the campaign of 2012 from April to November in Province of Alessandria, 150 of ovitraps resulted positive at least a week. The black glass of PVC used by ovitraps are installed from May until the end of November and checked every week by changing the wooden strips and observed in laboratory of Mosquito Control by Talbalaghi by using Microscope 60x.

Besides, further observations of *A. albopictus* were carried out in occasions of first evaluation of BG traps. The ovitrap installed in Varazze, a few kilometers far from Genoa port in the Liguria Region, are installed to sate most in-depth and comparative genetic studies and for the comparison of vector competence of the population on the other side of the Apennines that separates Alessandria from Genoa 80 km far.

DNA was isolated from ethanol-preserved adult mosquitoes. PCR amplification of the *COI* gene was performed

using the primers TY-J-1460 [17] and COIR2 [18]. The mosquitoes were tested for *Wolbachia* infection using PCR with primers wsp81F and wsp691R complementary to the gene wsp [19]. The ITS2 region was amplified using primers complementary to the 5,8S and 28S rRNA [20]. The DNA extraction and PCR were performed according to previously described methods [18].

Fourteen PCR products of *COI* gene and twelve PCR products of ITS2 were sequenced from samples of *A. albopictus* from four native sampling sites. The newly determined nucleotide sequences of the *COI* gene and ITS2 region were submitted to GenBank under accession numbers JX679373-JX679398.

The obtained sequences were analyzed using software Chromas (<http://www.technelysium.com.au/>), phylogenetic analyses were conducted in MEGA5 [21]. Estimates of Average Evolutionary Divergence over all Sequence Pairs were conducted using the Maximum Composite Likelihood method in MEGA5. The average evolutionary divergence estimated as a number of base substitutions per site from averaging over all sequence pairs within and between each group. All results are based on the pairwise analysis of 24 known to date sequences of ITS2. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 346 positions in the final dataset.

Phylogenetic tree was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length of 0.11842934 is shown (Figure 2). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are given in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

3. Results and Discussion

Polymorphism of the *COI* gene mtDNA was examined in a 686 bp fragment of sequences of the 9 specimens of *A. albopictus* from three locations in Piedmont region and 5 specimens from one location in Liguria region, Italy. In total, we found only 2 variable nucleotide sites (0.3%) in the 14 DNA sequences studied; both were considered informative under the conditions of parsimony (Figure 1). Among the sequences analyzed, all detected substitutions were found to be transitions A ↔ G located at the first codon position and affecting the amino acid sequence. The number of base substitutions per site from averaging over all sequence pairs is 0.001 based on the pairwise analysis of 14 sequences. Intrapopulation polymorphism was detected in three of the four studied sampling sites. All of them were sampling sites from Piedmont. Individuals from Varazze, Liguria, were monomorphic in nucleotide composition of *COI*.

In order to determine the geographic origin and phylogenetic relationships of *A. albopictus* invading northern

[1223333	4556]	[1]
[4782241469	6024]	[122480]
[7402081641	3866]	[657134]
#Piovera1	TGGGAGTGTG	TTGG	SVVDAS	
#Piovera2	. . A I	
#Tortona1	
#Tortona2	. . A I	
#Alessandria1	. AA I I	
#Alessandria2	. . A I	
#Alessandria3	
#Alessandria4	. . A I	
#Alessandria5	. AA I I	
#Varazze1	
#Varazze2	
#Varazze3	
#Varazze4	
#Varazze5	
#AY748238.Italy	NNNNN	NNNN	
#JF810659.Greece	N . A	N . I	
#AY748238.Greece	NNNNN	NNNN	
#AY748239.Greece	NNNNN . C	NNNN . P	
#JQ388786.Germany	
#JQ004524_haplotypeH1	
#JQ004525_haplotypeH2	. . A I	
#HE820720.Russia	NNNNNN . . R A	NNNNN	
#AJ971008.France	NNNN	NNNN	
#AJ971012.Reunion	NNNN . . . A	NNNN	
#JN406675 .Madagascar	NNNN	NNNN	
#AJ971005.USA	NNNN	NNNN	
#GQ143719 .Australia	C . A C C A C	P . I H T	
#AB690835.Japan	. . A I	
#HQ398901 .Vietnam	. . A	A I	
#HQ906848 .Croatia_CRO1	NNNNN	NNNN	
#HQ906849 .Croatia_CRO2	NNNNN . . . C	NNNN	
#HQ906850 .Croatia_CRO3	NNNNN A	NNNN	
#HQ906851.Croatia_CRO4	NNNNN . . . C	NNNN	
#AJ971014 .Brazil	NNNN . . . C	NNNN	
#JQ235749.China	. . A	C I	
#JF309317.Cameroon_H1	NNNNN . . . C A	NNNN	
#JF309318 .Cameroon_H2	NNNNN . . . C	NNNN	
#JF309319.Cameroon_H3	NNNNN . . . C . . C	NNNN	
#JF309320.Cameroon_H4	NNNNN . . . C A	NNNN	

FIGURE 1: Alignment showing variable nucleotide and variable amino acid sites of the gene *COI* in *A. albopictus*. The numbers on the top of the alignment indicate the position number of variable sites in the sequence. N represents unknown sites that fall out of the analysis in the study of only the middle part of the gene.

Italy, *COI* sequences recorded in northern Italy and sequence data previously published in GenBank were aligned (Figure 1). In all investigated sequences T (A in the complementary strand) is located at position 364, which distinguishes temperate/subtropical populations from all tropical populations characterized by C (G) in this position. The most frequent studied mitochondrial haplotype (57%) was detected in all

populations. This variant is identical to the mitochondrial haplotype found in southern Germany. A second mitochondrial haplotype was detected in all three sampling sites in Piedmont region and was absent in Liguria region. This mitochondrial haplotype differs from the first by one nucleotide substitution at position 80 and is identical to those found in Japan and Greece. The third mitochondrial haplotype

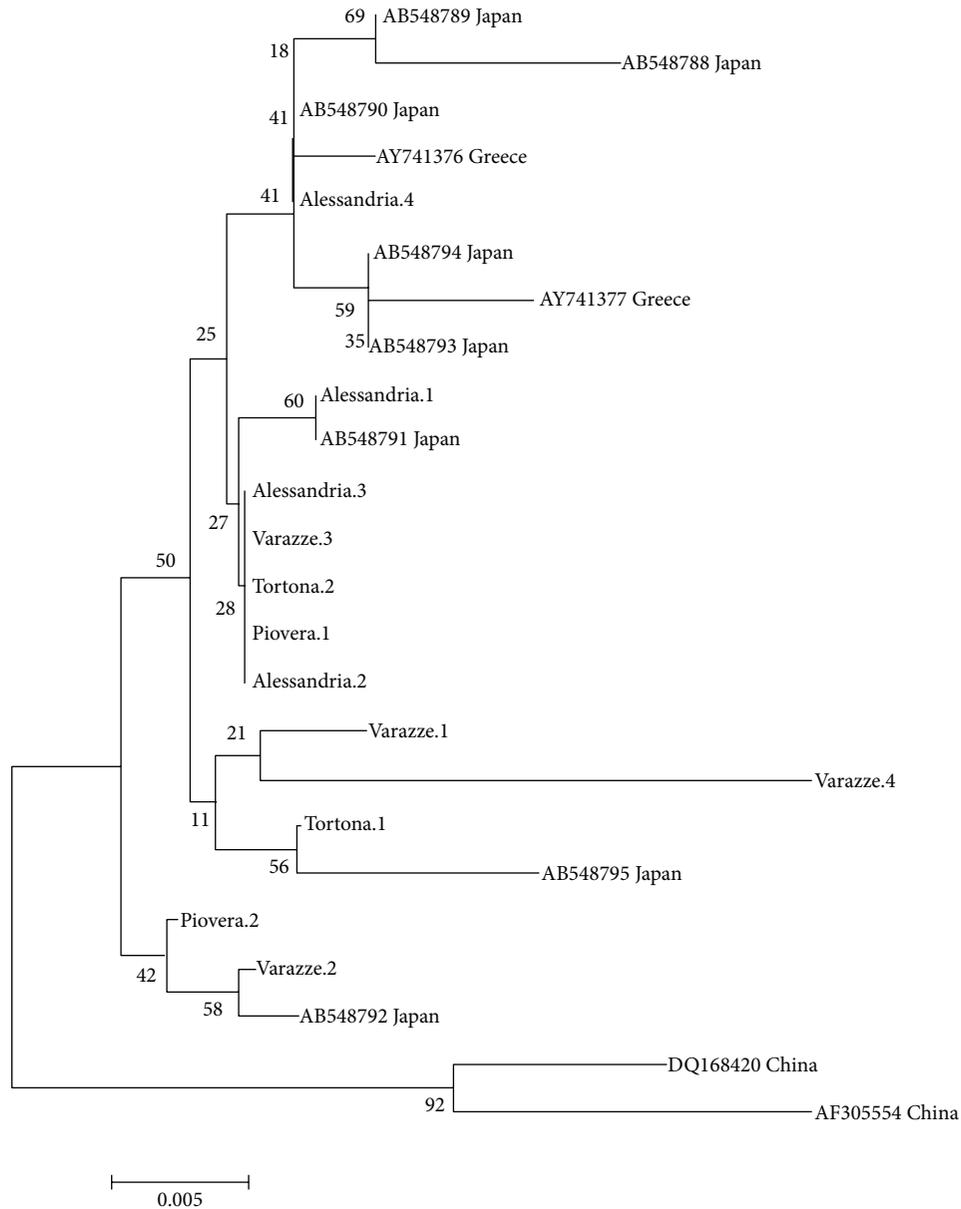


FIGURE 2: Evolutionary relationships of *A. albopictus* based on the ITS2 analysis. The evolutionary history was inferred using the Neighbor-Joining method. There were a total of 346 positions in the final dataset. Phylogenetic analyses were conducted in MEGA5. Previously published sequence data are shown together with their GenBank accession numbers.

observed by us for the first time differs from the second by one transition $A \leftrightarrow G$ at position 74 and was found only in the sampling site of Alessandria in Italy.

As shown in Figure 1, many already known mitochondrial haplotypes vary in this 5' part of the *COI* gene and it could provide additional information about the polymorphism of European populations and save as a new marker sequence. This part of the sequences (approximately 240 bp) was lost when only the middle part of the gene is sequenced.

To study nuclear gene diversity within and among of *A. albopictus* from various sampling sites, we investigated ITS2 region used to differentiate the *Aedes* species. Typically, about 515-bp amplification products were obtained after

amplification with primers 5.8S and 28S for all 34 mosquitoes studied. 4 samples from Alessandria, 4 samples from Varazze, 2 samples from Piovera, and 2 samples from Tortona were sequenced. Conservative DNA sequences of the 5.8S and 28S rRNA genes were identical in all studied mosquitoes and were removed from the analysis. The obtained ITS2 sequences were 391–397 bp in length. 16 variable sites (4.8%) and 5 indels were found. In 3' region of the ITS2, we detected TAGTCGC duplication in one sample from Alessandria and one sample from Varazze (data not shown). We have compared the genetic diversity of ITS2 within and between our studied sampling sites and with that of *A. albopictus* from northwestern Greece, Japan, and China. Only these

TABLE 1: Estimates of average evolutionary divergence in the ITS2 over sequence pairs within and between *A. albopictus* populations.

	Italy	Greece	Japan	China
Within				
between	0.009	0.012	0.013	0.020
Italy				
Greece	0.013			
Japan	0.011	0.012		
China	0.037	0.043	0.041	

sequences of ITS2 of *A. albopictus* are known to date and are available in GenBank (Table 1). The average evolutionary divergence was estimated as a number of base substitutions per site from averaging over all sequence pairs within and between each group. Intrapopulation diversity is similar in all compared populations. The differences between the populations of Italy, Greece, and Japan are comparable to variability within these populations. The population of China is different from the others.

The dendrogram shows clearly that based on the ITS2 analysis, *A. albopictus* specimens are clustered into two groups. The first consists of Italian, Greek, and Japanese mosquitoes, all of subtropical origin. The second cluster includes mosquitoes from China, which have a tropical origin. Within the first cluster, there is no population specific haplotypes and individuals from different populations are not grouped together (Figure 2).

The overall average variability between *COI* sequences of north Italian *A. albopictus* is 0.001 while the overall average variability between them based on the pairwise analysis of ITS2 sequences is 10 times more—0.011. As expected, the polymorphism in nuclear DNA is higher than in mtDNA. Nucleotide differences in ITS2 did not allow us to detect differences between *A. albopictus* from different sampling sites in northern Italy. At the same time, based on the analysis of ITS2, there was a 4% divergence between Chinese mosquitoes of tropical origin and all other mosquitoes of subtropical origin investigated until now on the basis of their mtDNA. However, the sequences from more individuals of geographically remote habitats should be studied. A relatively high level of genetic variability of ITS2 between tropical and subtropical populations can be used to develop markers that will provide additional information about spreading and possible hybridization of *A. albopictus* with different origins.

All 34 studied mosquitoes were also tested for *Wolbachia* infection using PCR with primers for *Wolbachia* surface protein (*wsp*) gene. PCR products of the expected size about 620 bp were obtained from individuals from two sampling sites—Piovera and Tortona. The PCR showed no positive results for individuals from two other sampling sites—Alessandria and Varazze. The DNA of all uninfected individuals was positive for PCR amplification using primers for *COI* and ITS2, indicating satisfactory of DNA template quality. Polymorphism of the *COI* gene in different sampling sites together with various *Wolbachia* infection status may indicate that the studied *A. albopictus* did not originate from a single

ancestor population. Different sources of introductions can be considered.

It is important that to date no individuals with mitochondrial and nuclear haplotypes specific to tropical populations were detected in the study area. Individuals with tropical mitochondrial haplotypes were found in 2009 in Croatia [12]. The identity of the corresponding nuclear DNA remained unknown. It is not known whether there are barriers to genetic exchanges between *A. albopictus* of the subtropical and tropical origins in Europe, although the absence of genetic exchange between the Brazil (tropical origin) and the United States (subtropical) populations was proved [8]. Biological features such as the ability to entering in diapause and feeding preferences may affect the vector capacity. Study of mtDNA allows to determine the origin through the maternal line, whereas investigation of nuclear DNA can provide information on hybridization. Determination of the genetic structure of vector populations will allow developing strategies only targeting competent vectors.

4. Conclusions

The present study revealed three mitochondrial haplotypes based on *COI* gene and various *Wolbachia* infection status in *A. albopictus* from four sampling sites in northern Italy. Different sources of introductions can be considered.

For the first time, a 4% divergence between the populations of *A. albopictus* of tropical and subtropical origins was found on the basis of analyzing the ITS2.

No individuals with mitochondrial and nuclear haplotypes specific to tropical populations were found in the studied northern Italian populations.

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