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Diploma dissertation title

Genetic analysis of Greek *Aedes albopictus* populations using the mitochondrial gene *COI* as a marker

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The current diploma dissertation was carried out from September 2018 until June 2019 in the laboratory of Molecular Biology and Genomics under the supervision of Professor Dr. Mathiopoulos.

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SUMMARY

Aedes albopictus, also known as the Asian tiger mosquito, is one of the 100 worst invasive alien species of the world (Courchamp, 2013). During the last 30 years it has spread through every single continent except Antarctica (Lounibos, 2002). It is mosquito species of public health significance known to be a competent vector of Chikungunya (CHIKV), Zika (ZIKV), West Nile (WNV), Dengue (DENV) and many other types of arboviruses (Gratz, 2004). It is known to have invaded Europe, and more specifically the country of Albania, for the first time in 1979 (Adhami & Reiter, 1998), with North Italy following in 1990 (Sabatini et al., 1990). In Greece, there were no reports until 2003, when it is considered that the country was invaded by it (Samanidou-Voyadjoglou et al., 2005). There are a few population genetic studies that have included Greek specimens, however almost every study has included only one region of Greece (Athens) and in total only specimens from three regions (Athens, Corfu and Thesprotia) have been studied. These studies correlated the Greek *Aedes albopictus* populations with Japanese and Italian (Shaikevich & Talbalaghi, 2013), French, La Reunion, USA, Madagascar and Central African (Kamgang et al., 2011; Kamgang et al., 2013), Chinese, Albanian, Italian, Thai and Brazilian (Battaglia et al., 2016), Thai (Manni et al., 2017) as well as with African and Asian samples (Kotsakiozi et al., 2017). As shown already, these studies are associated with often contradicting results, most likely due to the chaotic propagule distribution that the tiger mosquito has followed during its expansion (Manni et al., 2017). In an attempt to shed more light in determining the population structure of *Aedes albopictus* in Greece, an extensive and therefore more thorough sampling has been carried out, in the present study, including 8 separated geographical regions of Greece. The gene of cytochrome c oxidase I (*COI*) was used as the genetic marker to determine the variation of the Greek populations. Our results verify most of the aforementioned genetic proximities of the Greek populations and also identify some novel ones, namely with Chinese, Hawaiian, Russian, Canadian, Taiwanese, Panama and Malaysian populations.

1. Introduction

1.1. Asian tiger mosquito – General information

Aedes albopictus (from Greek άηδής, meaning 'loathful', and from Latin albopictus, meaning 'white-stained') is an invasive mosquito species which is known to have originated from south East Asia. Very rapidly, it has expanded and successfully established in almost every continent except Antarctica. Interestingly, this expansion of its geographical distribution occurred mostly during the last 30 years (Lounibos, 2002).

A. albopictus is not a migratory species, and its flight range can reach up to 1 km (Bonnet & Worcester, 1946; Gubler, 1971). The main reason behind its swift spread lies in its connection with human activities. International trade of many types of products (such as lucky bamboo, tyres, containers etc.) may act in its favor, due to the formation of small water pools, where the mosquito can lay its eggs. Even if the environmental conditions become inappropriate for their development (e.g. due to winter conditions), the eggs of this mosquito, signaled through the change in day length, have the ability to enter a diapause phase which could last up to several months (Medlock et al., 2006). Taking these facts into consideration, the spread of *A. albopictus* in different latitudes comes to no surprise, while it includes both populations living in temperate and tropical regions which do and do not display the diapause trait respectively.

Aedes albopictus is also a competent vector for various viruses. These include the three flaviviruses of Japanese encephalitis, West Nile, and yellow fever which can be transmitted and developed in humans (Mitchell, 1995b; Shroyer, 1986) as well as from parent mosquito to offspring mosquito. The latter fact applies not only to these viruses, but also to dengue (Estrada-Franco et al., 1995). Moreover, it has been shown to be as efficient vector as *Aedes aegypti is* in terms of infection and dissemination of chikungunya and Dengue viruses (Paupy et al., 2010), while the contrary seems to happen with regards to Zika virus, where *Aedes aegypti* is slightly more competent (Lozano-Fuentes et al., 2019). There is also some evidence suggesting that it is a possible vector of the Usutu virus (USUV) (Calzolari et al., 2012). As for animal diseases, it is a known vector of *Dirofilaria immitis* and *Dirofilaria repens* (Gratz, 2004), parasites which primarily affect dogs as well as other animals and in some very rare cases, humans.

1.2. Geographical distribution

As mentioned above, this species is highly invasive. In fact, it is included in the list of the International Union for Conservation of Nature (IUCN) for the top 100 worst invasive alien species worldwide (Courchamp, 2013). Therefore, it has spread rapidly from its indigenous areas in Southeast Asia, western Pacific and Indian Ocean islands to almost all over the world, as shown in Table 1.

Continent	Countries/Regions
Europe	Albania, Austria (not established to date), Belgium (not established to date), Bosnia & Herzegovina, Bulgaria, Croatia, Czech Republic (not established to date), France (including Corsica), Georgia, Germany, Greece, Hungary, Italy (including Sardinia, Sicily, Lampedusa, and other islands), Malta, Monaco, Montenegro, the Netherlands (not established to date), Romania, Russia, San Marino, Serbia (not established to date), Slovakia (not established to date), Slovenia, Spain, Switzerland, Turkey and Vatican City
Middle East	Israel, Lebanon, Saudi Arabia (to be confirmed), Syria, Yemen (to be confirmed)
Asia &	Australia (established only in the Torres Strait, the region that

Australasia	separates mainland Australia from Papua New Guinea), Japan, New Zealand (not established), numerous Pacific Ocean and Indian Ocean islands, southern Asia
North, Central America & Caribbean	Barbados (not established), Belize, Cayman Islands, Costa Rica, Cuba, Dominican Republic, El Salvador, Guatemala, Haiti, Honduras, Mexico, Nicaragua, Panama, Trinidad (not established to date), USA
South America	Argentina, Bolivia (not confirmed), Brazil, Colombia, Paraguay, Uruguay, Venezuela
Africa	Algeria, Cameroon, Central African Republic, Equatorial Guinea, Gabon, Madagascar, Nigeria, Republic of Congo, South Africa (not established)

Table 1: *A. albopictus* has established itself successfully in every single continent. The exact countries where this event has occurred are shown, as well as some regions where the invasion has not been established yet. *References can be found at the end of chapter 1.2.

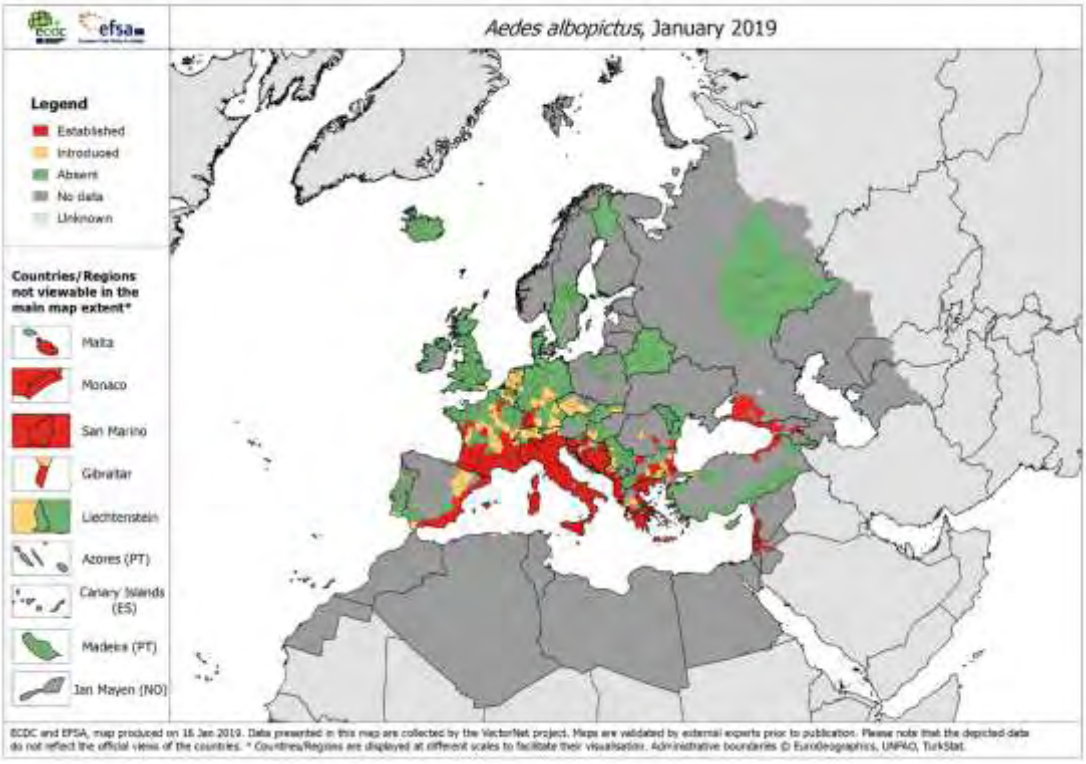


Figure 1: Current distribution of *Aedes albopictus* in Europe (ECDC 2019/VBORNET/VECTORNET)

A. albopictus primarily feeds on humans (anthropophily), although initially it probably fed on animals (zoophily), since its primary habitat were the forests of Southeast Asia. Human outspread gave the species the chance to broaden its targets and it is now known to be a daytime opportunistic feeder with a preference on human if it gets the chance to choose (Kamgang et al., 2012). It can be found both in rural and urban areas, but it is mainly exophilic and exophagous, which means that it prefers to rest and bite in open areas but that does not exclude indoor biting (Valerio et al., 2010).

The expansion of *A. albopictus* around the world, didn`t have the characteristics of a progressive one, but it was determined by the international trade routes followed by humans (Medlock et al., 2012; Roche et al., 2015). In USA, it was first introduced in the island of Hawaii and became established during the second half of the nineteenth century (HARDY, 1960), while around 1985 it was found in Harris county, Texas, denoting its introduction in the continental country as well (Sprenger & Wuithiranyagool, 1986). With regards to Central and South America, it was first found in Brazil in 1986, then in Mexico in 1988, while invasion of a lot of other countries followed later (Rossi et al., 1999). In Europe, the species was detected first in Albania in 1979 (Adhami & Reiter, 1998), and it was suspected to have been introduced from China (one of the few trading partners of Albania at that time in the 1970s). The next European country where it was detected was North Italy in 1990 (Sabatini et al., 1990), then in France around 1999 (Schaffner & Karch, 2000a) and Belgium in 2000 (Schaffner et al., 2004). Thereafter, many other European regions have been infested, like the Balkan countries, Malta, Spain, Switzerland, Netherlands, Germany, Czech Republic, Slovakia, Slovenia, Romania and Russia (Scholte & Schaffner, 2007). The first record of the species presence in Greece was between the years 2003 and 2004 in the regions of Corfu and Thesprotia (Samanidou-Voyadjoglou et al., 2005). In Africa, *A. albopictus* larvae were recorded for the first time in 1989 in Cape Town (Cornel & Hunt, 1991), while in 2000 it was

detected in Cameroon, and later found to be established in various localities (Simard et al., 2005).

*Table 1 references: (Adeleke et al., 2015; Akiner et al., 2016; Aranda et al., 2006; Becker et al., 2013; Benedict et al., 2007; Bocková et al., 2013; Bonizzoni et al., 2013; Buhagiar, 2009; Carrieri et al., 2011; Carvalho/ et al., 2014; Contini, 2007; E.C.F.D., 2009; Eritja et al., 2005; Gatt et al., 2009; Haddad et al., 2007; Haddad et al., 2012; Izri et al., 2011; Kraemer et al., 2015; Le Maitre & Chadee, 1983; Lounibos, 2002; Madon et al., 2002; Marquetti et al., 2012; Medlock et al., 2015; Ngoagouni et al., 2015; Oter et al., 2013; Paupy et al., 2009; Prioteasa et al., 2015; Schaffner & Karch, 2000b; Schaffner et al., 2004; Scholte & Schaffner, 2007; Šebesta et al., 2012; Seidel et al., 2012; van den Hurk et al., 2016).

1.3. The mosquito`s biology

1.3.1. Physiological aspects

Aedes albopictus owes its successful expansion to its ability to tolerate lower temperatures. As mentioned, it can enter a photoperiodic diapause during the winter of temperate regions (Hanson & Craig, 1994). Japanese (Kobayashi et al., 2002) and La Reunion (Delatte et al., 2009) populations have been found to survive and breed prosperously in mean annual temperatures above 10^{o}C, or even above -5^{o}C in the USA (Mitchell, 1995a). High survival at low temperatures may be attributed to increase in lipogenesis in lower temperatures (Briegel & Timmermann, 2001).

The species is indigenous to tropical forests of Southeast Asia, but even so, through its interaction with human, it has come to adapt to habitats fairly different, like densely populated cities and suburban regions [many examples like Rome (Valerio et al., 2010), the urban region of Athens and the suburban region of Karditsa sampled for the current study]. Initially, it laid its eggs near natural small stagnant water pools (e.g. tree holes, bromeliads), but now any small water container will do. Nevertheless, it may use non-stagnant water sources in order to breed as well. In any case, it was found that, regardless of the water type, the probability for it to serve as a breeding site increases as long as there are flowering plants nearby (Davis et al., 2016).

Since it exhibits anthropophilic feeding behavior, it could transmit viruses among humans. Also, due to its opportunistic zoophily, it could serve as a bridge-vector between animals and humans (a phenomenon which is much riskier to happen in areas with high virus heterogeneity like Central Africa), thereby increasing the total number of viruses it can transmit. The “bridge-vector” feature, arises from the utilization of blood as nutrient source and typically means that pathogens are received during feeding from an infected host and are then transmitted between hosts through sequential blood feedings.

1.3.2. Ecology and competition

Like every other living organism, *A. albopictus* does not live on its own, but it coexists with other mosquito species. Its relationship with them often depends on the requirements they have from the natural environment in terms of breeding water (Cole, 1949). *Aedes aegypti* is one of the most important mosquito species which coexists with *A. albopictus* and is also a significant disease vector, (Knudsen, 1995) which ranks first before *A. albopictus* with regards to the transmission of dengue and dengue haemorrhagic fever (DHF). *Aedes aegypti*, in contrast to *Aedes albopictus,* originated in sub-Saharan Africa (Mousson et al., 2005), but it has invaded much of the tropical and subtropical world where *A. albopictus* is also found (Tabachnick, 1991). Due to the coexistence of these two species (Chen et al., 2006), a competition between them exists and is based on the fact that each species is

observed in slightly different areas (*A. albopictus* in suburban and rural outdoor areas, *A. aegypti* in urban areas and both of them in roughly the same numbers in small urban gardens). Therefore, one can speculate that on many occasions one species could be displaced by the other (Gilotra et al., 1967). Juliano et al., report that in North America, *Aedes albopictus* was able to replace *Aedes aegypti* due to its superiority in larvae resource competition (Juliano, 1998). Also, *A. albopictus* replaced *A. aegypti* in Indian peripheral towns but it also expanded to eastern plateaus, avoiding the competition with the latter (Kalra et al., 1997).

On the other hand, there are cases where the reverse happened, and *Aedes aegypti* replaced (Service, 1992) or significantly suppressed *Aedes albopictus* numbers in urban areas (Rudnick & Hammon, 1960). Many factors have been shown to affect Aedes larval development (either directly like pupating failure, or indirectly like longer development times and small size adults) and therefore might determine the prevalence of certain larvae over others when they occur in larvae communities. Some of these factors are climate (temperature, humidity, rainfall, pH), food resources, differential tolerance of water salinity (although not clear how it affects the interactions between the species), land use (e.g. transformation of forests to residential areas), biotic invasions (parasitism, predation, competition) and the bacterial composition of the water used for larval development or oviposition, which are important for the trophic interactions as well as for the attraction or repulse of the gravid females and the oviposition stimulation (Benzon & Apperson, 1988; Clements, 2011; Lounibos et al., 2010; Tabachnick, 1991; Trexler et al., 2003; YEE et al., 2014).

1.3.3. Symbiotic microorganisms

Aedes albopictus, like every other studied multicellular organism from plants to animals, contains symbiotic microbiota (Mendes & Raaijmakers, 2015). Insects which follow diets limited in nutrients (vertebrate blood, plant phloem, wood) benefit from their symbiotic microorganisms by provision of nutritional substances that are not contained in their food or that they are not able to generate by themselves (Weiss & Aksoy, 2011). These types of associations are called mutualistic and the microorganisms (primary symbionts), being essential for the insect`s survivorship, are passed on vertically to its offspring. Another type of association is commensalism. In this type of symbiosis, which is commonly temporary, none of the involved species is harmed or benefited. The commensal bacteria (or secondary symbionts) can be obtained by their host in various ways such as vertical and horizontal transmission or even from the environment. An additional type often encountered in insect symbiosis is parasitism, of maternally inherited parasites that not only benefit from the association with the host and also provoke harm to insect.

1.3.3.1. The case of CI-inducing *Wolbachia*

One of the most common microbes which belongs to this type is *Wolbachia* (Werren, 1997; Werren et al., 1995b), one of the most widespread endosymbionts in the biosphere estimated to have reached infection rates from 20% (Werren et al., 1995a; Werren & Windsor, 2000) to 76% (Jeyaprakash & Hoy, 2000). It is inherited maternally and horizontally through the egg cytoplasm and may affect the reproductive capacity of its hosts in many ways (reproductive incompatibility, parthenogenesis, and feminization). Cytoplasmic incompatibility (CI), is a phenomenon where *Wolbachia*-infected males produce offspring which die during the embryonic stages (Tram et al., 2006), when mating with uninfected females (unidirectional CI) or when mating with females infected by different *Wolbachia* strains (bidirectional CI). Thus, mating of infected females with infected (only by the same *Wolbachia* strain) or uninfected males is compatible and produces infected offspring, biasing in this way the population towards the genotype of the associated females (Figure 2).

CI, especially bidirectional, might be able to guide new species formation as the populations are reproductively isolated. Generally (but also for the *Wolbachia* strains), the CI phenotype is affected by several factors such as double versus single infections, bacterial density, host genotype and age, as well as environmental factors (Stouthamer et al., 1999). Although, there are some cases where the inheritance of the infection might be flawed, which means that infected females might not be able to transmit the infection to all their offspring, thereby producing uninfected daughters as well. Possible reasons for this could be exposure to high temperatures (Stevens, 1989), natural antibiotics (Stevens & Wicklow, 1992) or sometimes genetic factors. Also, the infection could impact the fecundity of the female insect and, as a result, the available time for reproduction is reduced compared to the uninfected one`s.

1.3.3.2. *Wolbachia* dynamics

When the presence of the bacteria is not very extensive in the insect populations, the uninfected females are not very likely to encounter and breed with infected males, and combined with the probability of a failed *Wolbachia* transmission, they have the advantage over the infected females. On the contrary, when their presence is more dominant, the balance is shifted towards the infected females since the uninfected ones are very likely to lose their offsprings due to CI. Therefore, there is a certain threshold that the bacteria need to overcome in order to expand further inside a population (Stouthamer et al., 1999). The molecular mechanism through which CI is achieved by *Wolbachia* bacteria associates with the production of the ankyrin (ANK) domain proteins (Sinkins et al., 2005; Walker et al., 2007) or through the WO phages infecting almost 90% of the *Wolbachia* bacterial strains which are found in insects (Bordenstein & Wernegreen, 2004; Gavotte et al., 2004)

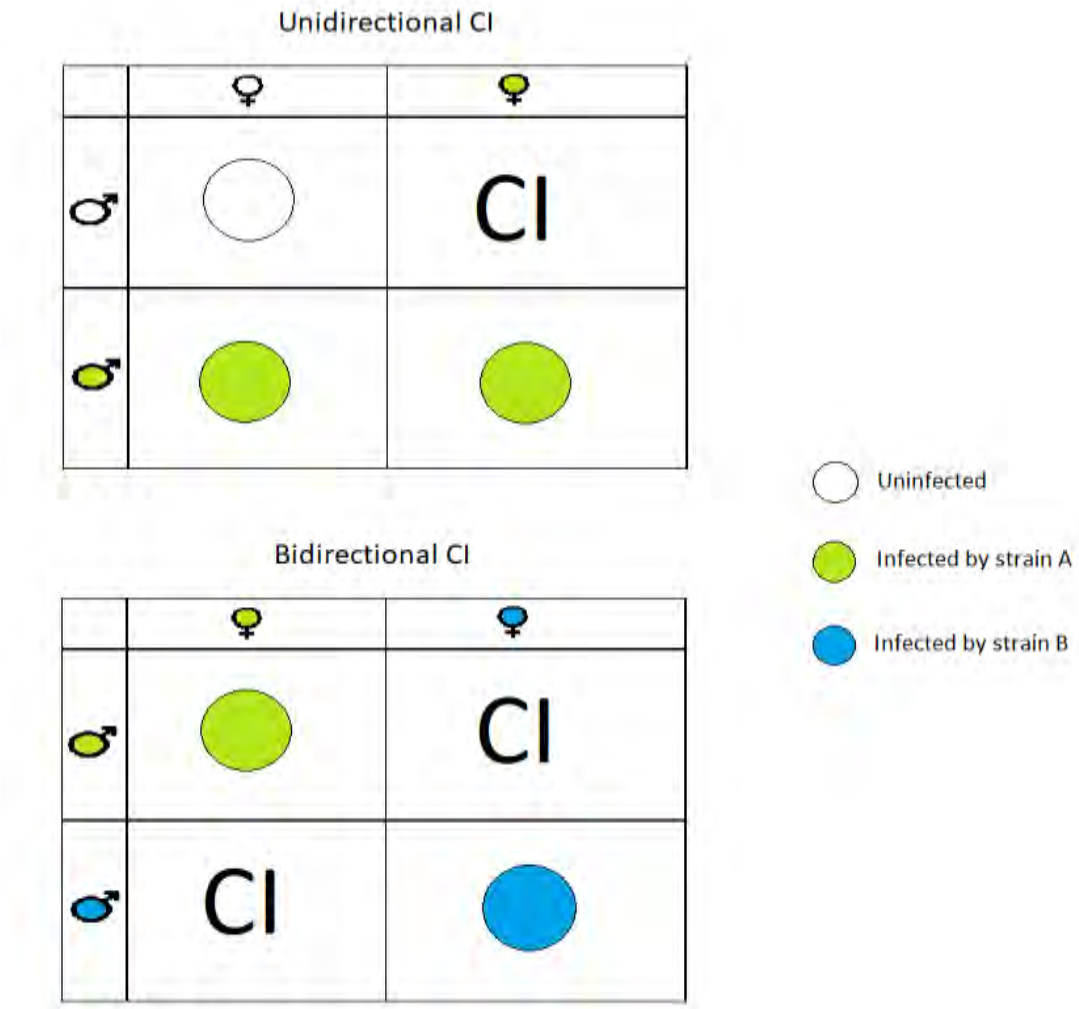


Figure 2: Explanation of how unidirectional and bidirectional cytoplasmic incompatibility occurs. When a female mosquito infected with *Wolbachia* strain A breeds with an uninfected, or infected with strain B male, CI occurs. The only possibility for the infected female to produce viable (but also infected) offspring, is to breed with an infected male. Another possibility is the breeding of an infected male with an uninfected female, in which case viable infected offspring is produced. Figure adapted from Hu.johannes (Wikipedia.org)

Also interesting is the fact that some *Wolbachia* strains force their hosts to premature death due to the overly increased bacterial cell replication in comparison to the host`s cells. A strain found in *Drosophila* may reduce the lifespan up to 50% (Min & Benzer, 1997) and for that reason it was speculated that it could function as a tool for insect population control (Sinkins & O'Neill, 2000).

1.4. Vector status and disease transmission

1.4.1. Disease cases and competence studies

A. albopictus has caused a chikungunya virus outbreak, the first considerable mosquito-borne disease (MBD) outbreak in Europe, during the years 2006-2007 (Angelini et al., 2007). It started in the two villages of Castiglione di Cervia and Castiglione di Ravenna and then spread out causing two more, weaker outbreaks in the same area (Angelini et al., 2008). The introduction of the virus was carried out involuntarily by a traveler from India, a fact that was

confirmed later due to the genetic association of the strain that caused the outbreak with strains from India, which were also, interestingly, found to contain the mutation that shows to be best adapted to *A. albopictus* (Rezza et al., 2007). Screening of the mosquitoes as well as of blood samples, incriminated *Aedes albopictus* as the vector. CHIKV was re-transmitted from India (this time from a different state) in 2010. In this occasion, the two disease incidences occurred in France (Fréjus) (Marc et al., 2011). Also another local transmission of DENV in Europe occurred in Nice of France in September 2010, after the massive outbreak of 1927-1928 in Athens, Greece and its nearby areas (in the Greek outbreak, *A. aegypti* was the incriminated vector) (Rosen, 1986) (La Ruche et al., 2010). The next local transmission was detected, again in 2010, in the country of Croatia (Gjenero-Margan et al., 2011), in which two cases of infected tourists were reported. In 2013, DENV local transmission was reported in France Aix-en-Provence, Bouches-du-Rhone department (Marchand et al., 2013), an area where *Aedes albopictus* was known to be established.

Pathogen	Genus	Clinical importance	Disease occurence
Chikungunya virus (CHIKV)	Alphavirus	Low chances to be asymptomatic (Thiberville et al., 2013). High fever, headache, insomnia, extreme exhaustion, joint pain (Thiberville et al., 2013). There is no vaccine available (Moutailler et al., 2009).	Endemic in Africa and Asia; Frequently imported into Europe
Eastern Equine encephalitis (EEV)	Alphavirus	Encephalitic – brain swelling and might be asymptomatic or Systematic. Symptoms: high fever, myalgia, headache meningeal irritation and more (Pfeffer & Dobler, 2010).	Americas; No evidence of introduction into Europe to date
Venezualan Equine encephalitis (VEE)	Alphavirus	Usually no severe symptoms but in humans might range from unapparent to acute encephalitis. Children are more likely to develop fatal encephalitis and suffer permanent neurological sequelae than adults (Scott C. Weaver et al., 2004).	Endemic in the Americas; No evidence of introduction into Europe to date
Jamestown Canyon virus (JCV)	Bunyavirus	Summertime illness symptms with sore throat, rhinorrhea and cough, followed by fever, headache, nausea and vomiting can occur. Neuroinvasive diseases have been reported in the majority of cases (Lindsey et al., 2014).	Endemic in North America; No evidence of introduction into Europe to date
Dengue virus (DENV)	Flavivirus	Dengue fever: Vast majority asymptmatic or with mild symptoms. A small percentage might have more severe symptoms but even less contain risk of lethality (Reiter, 2010; Whitehorn & Farrar, 2010)	Endemic in Africa, Americas and Caribbean, Asia; Frequently imported into Europe
Japanese Encephalitis virus (JEV)	Flavivirus	Most cases are aymptomatic, but in 1 out of 250. Encephalitis might occur with symptoms like headache, high fever, disorientation, coma,	Endemic in Asia; No clear evidence of introduction into Europe to date

		tremors and convulsions (Simon & Kruse, 2019). Life threatening mostly in children. Most common viral encephalitis cause in Asia (Campbell et al., 2011).	
West Nile virus (WNV)	Flavivirus	80% of WNV infection cases go unnoticed, 20% develop mild symptoms and in less than 1% meningitis or encephalitis might be caused with a mortality occurring in up to 20% of these cases (Gubler, 2002).	Worldwide
Zika virus (ZIKV)	Flavivirus	Zika fever: might be like a mild form of dengue fever with no or mild symptoms. There is no special treatment and prevention is not possible with vaccines or medication (Gubler, 2002).	Epidemic in Africa, Asia
La Crosse virus (LACV)	Orthobunyavirus	LACV disease with symptoms of fever (usually lasting 2-3 days), headache, nausea, vomiting, fatigue and lethargy (Borucki et al., 2002). Severe neuroinvasive usually exhibited in children under 15 (McJunkin et al., 2001).	Endemic in northern America; No evidence of introduction into Europe to date
Dirofilaria immitis, D. repens	*Dirofilaria*	Dirofilariasis in humans: pulmonariasis which might be asymptomatic. Other cases might infect the skin or the eye (Kim et al., 2002).	Endemic in southern Europe and the Americas

Table 2: The features of diseases, which *Aedes albopictus* is known to be a competent vector of. Table adapted from (Schaffner et al., 2013).

From the total number of viruses for which *Aedes albopictus* is considered a competent vector, some of them have been found in mosquitoes captured in the wild in a number of countries, while in laboratory tests the mosquito exhibited a successful vector capacity (Paupy et al., 2009). Examples of such viruses are eastern equine encephalitis virus (Mitchell et al., 1992; Turell et al., 1994), La Crosse virus (Gerhardt et al., 2001; Grimstad et al., 1989), Venezuelan equine encephalitis virus (Beaman & Turell, 1991; Turell & Beaman, 1992), West Nile virus (Holick et al., 2002; Roiz et al., 2010; Sardelis et al., 2002), Japanese encephalitis virus (Paupy et al., 2009), as well as Usutu virus (Calzolari et al., 2010).

With the exception of health risks, the establishment of *Aedes albopictus* involves nuisance and has a considerable impact on people`s quality of life (Curco et al., 2008). Adding to that, children have been shown to spent less time outdoors, reducing the amount of time they are physically active which implies an indirect connection of the mosquito`s nuisance activity with child obesity (Worobey et al., 2013).

1.5. Population Genetics

All the aforementioned facts about *Aedes albopictus* highlight the need for better understanding of its biology, vectorial capacity and population administration strategy. As stated by (Goubert et al., 2016), through the analysis of population genetics, we can understand the origins and the frequency of introductions of invasive populations, which will

be of great value in estimating the probability of the dissemination of various traits (such as environmental adaptation, insecticide, disease traits). It will also greatly aid in linking specific DNA polymorphisms or haplotypes with vector competence for certain viruses as was carried out by (Tsetsarkin et al., 2007) for CHIKV, which was associated with a single mutation that, in turn, resulted in a single amino acid substitution and increased vector specificity. The outcome of these events was the 2005-2006 epidemic of La Reunion island which, primarily, lacked the typical vector. Except of vector competence, a very crucial association would be between DNA polymorphisms and insecticide resistance. An example of research of this kind is the connection between Knockdown Resistance (*kdr*) mutations of the voltage sensitive sodium channel (*Vssc*) gene with target-site resistance (a type of resistance in which the site of action of the insecticide is modified, resulting in the distortion of the insecticide`s binding efficiency) in DDT and pyrethroids in *Aedes albopictus* (Auteri et al., 2018). Such knowledge will render more feasible answering questions like the spreading probability of these traits.

For an invasive species like *Aedes albopictus*, which is dispersed in high frequencies due to anthropogenic activities, population structure analysis could also be useful in determining source(s) of introduction as well as rates at which this occurs (or has occurred), or adaptation of mosquito populations in different invaded environments. It would be very useful to combine genetic data with behavioral studies. As observed during the sampling procedure of the present study, reaction time and agility of the targeted mosquitoes differ. It is also expected to expand existing knowledge regarding mating behavior of these mosquitoes, which might be useful in order to increase boundary resolution between populations (Goubert et al., 2016).

Analysis of genetic variability for different *Aedes albopictus* populations has shown that the intrapopulation variability is the most genetically diverse. This result was observed with the use of various genetic markers (allozymes, mtDNA, microsatellites, RAPD and ITS2) and they all revealed that less than 50% of genetic variability was found between populations. This observation might result from genetic drift through which the, probably small, founder populations underwent and is consistent with their low dispersal capacity which implies scanty gene flow with other populations (Goubert et al., 2016).

Invasive populations contain lower genetic variability compared to native ones, due to the founder effect they are subjected to, which associates with small invading population size and the process of adaptation. Actually, these facts alongside with their consequences such as impotence for tolerating selective pressure (Goodnight, 1988), as well as augmented chance of inbreeding complicated scientific understanding of how an invasion could actually end up successful (also called “the paradox of invasion biology” (Dlugosch & Parker, 2008; Roman & Darling, 2007). We now know, however, that propagule pressure, i.e. the number and frequency of repeated invasions, could actually function as a balancing factor allowing adaptation of the organism (even though the ratio of propagule pressure to genetic diversity for successful invasion is not yet known) (Bock et al., 2015). This seems to be the case for the Asian tiger mosquito, whose expansion does not appear to be accompanied by reduction of genetic variability, but there is data to support recurrent and immense introductions. Also, due to the direct relationship of its dissemination with human activities, the invasion process does not exhibit a gradient progress from region to region as it applies for *Aedes aegypti* (Powell & Tabachnick, 2013).

1.5.1. Genetic variability in the indigenous region

The origins of the Asian tiger mosquito are suggested to include Southern and tropical Eastern Asia (Bonizzoni et al., 2013; Hawley, 1988). In studies carried out with extensive

sampling from these areas alongside with Japan, significant genetic diversity was found. 62 haplotypes in 174 individuals from 16 localities in Japan, China, Taiwan, Bhutan, Thailand and Vietnam were found by Porretta et al. (2012), 66 in 346 individuals from China, Japan, Taiwan and Singapore (those represented the native home range) by (Zhong et al., 2013) and 48 in 304 individuals from Bhutan, Thailand, Malaysia, Vietnam, China and Japan in (Ismail et al., 2017). (Porretta et al., 2012) make an effort to reconstruct the history of the mosquito during the period of the Last Glacial Medium (LGM, around 21,000 years BP). During this period, the Malay Peninsula, the islands of Borneo, Java, Sumatra and their surrounding ones comprised the greater landmass of Sundaland due to the drop in the sea levels. These studies support the hypothesis that *Aedes albopictus* continued to exist throughout that area, an aftereffect of their wide ecological plasticity, in populations which displayed a regular gene flow. In fact, they also experienced a demographical expansion and the populations that were maintained also expanded northwards to the regions that represent its current distribution (Central and Northern China and Japan) after the LGM due to the improvement in the climatic conditions. As (Goubert et al., 2016) suggest, the studies which investigate the genetics of the native range populations, should include more samples from the southern part (Java, Borneo, Sumatra).

1.5.2. Genetic variability in the Indian Ocean islands

In their study, (Delatte et al., 2011) found two genetic clusters in the area with *COI* polymorphism analysis. One cluster contained haplotypes from samples of 2006-2007 and the other contained samples from the past (1956 and 2002). Based on these results, they report that the Southwest Indian Ocean islands were invaded at least twice by *Aedes albopictus*, with the first one occurring a few hundred years ago when human (mainly Westerners) colonized the islands of Mauritius, Madagascar and La Reunion, while the second one, which is still under way, happened from 1990 or later and the mosquito reached almost every island in the Southwest Indian Ocean. Previously, (Kambhampati et al., 1991), genotyping 24 samples from one site in 1988 for enzymatic loci, showed that they were grouped separately from native area samples.

1.5.3. Genetic variability in the island of Hawaii

Hawaii is considered a “bridge” through which *Aedes albopictus* might have been transported to USA. Samples from Hawaii, China, Taiwan and Japan were grouped together while samples from Singapore were grouped separately in the study of (Zhong et al., 2013). In the study of (Usmani-Brown et al., 2009) who used the, supposed lowly variable, *ND5* marker gene, the Hawaiian samples scored the highest overall haplotype diversity in comparison to Brazil, continental United States and native range. High haplotype diversity was also found for Madagascar and La Reunion, which led them to speculate that geographic isolation, which means lack of colonizing insects, and broad ecological space (Gillespie & Roderick, 2002) in which it could adapt with the passage of time, were the main reasons that accounted for this increased genetic variability. Similar results were obtained by a previous study of (Mousson et al., 2005) who used *COI*, *ND5* and *Cytb* as markers and Hawaiian samples along with La Reunion ones once again showed the highest amounts of genetic diversity.

1.5.4. Genetic variability in the Americas

For continental America, as mentioned above, the species was introduced for the first time at around 1985 when it was detected in Harris county, Texas, (Sprenger & Wuithiranyagool, 1986). Around the same period, it was found in Brazil (1986), then Mexico in 1988 and later in many other countries (Rossi et al., 1999). (Kambhampati et al., 1991) found that Brazilian

samples were genetically close to Chinese and closest to Japanese ones. (Maia et al., 2009) found two total haplotypes which co-existed in Brazil and originating from the rest of the world and they hypothesize that two different introduction events were carried out in Brazil from separate regions. (Usmani-Brown et al., 2009) found that the Brazilian haplotype was unique suggesting independent colonization of the species in the country. Using mtDNA markers (Mousson et al., 2005) found that samples from Brazil formed a separate clade from Vietnam, Thailand and Cambodia, again supporting the hypothesis that the Brazilian population differs genetically.

For USA, due to sea trade that was carried out, multiple introductions allowed *Aedes albopictus* to settle (Moore, 1999; Reiter & Sprenger, 1987). In most analyses, US samples show increased genetic similarity with the Northern Asian and Japanese samples (Kambhampati et al., 1991; Morales Vargas et al., 2013; Mousson et al., 2005; Urbanelli et al., 2000). However, samples collected in 2001 from Los Angeles were found to be closely genetically associated with Singapore ones, yet subsequent sampling of 2011 in Los Angeles did not confirm these results. These results imply that USA was successfully colonized only by temperate genotypes(Zhong et al., 2013).

1.5.5. Genetic variability in Europe

As already mentioned, the first European country that was invaded by *Aedes albopictus* was Albania in 1979 (Adhami & Reiter, 1998), which was then followed by invasion of North Italy in 1990 (Sabatini et al., 1990), then France at around 1999 (Schaffner & Karch, 2000a) and Belgium in 2000 (Schaffner et al., 2004). Italian with Japanese and US samples have been found to resemble genetically according to information derived by various markers like allozymes from (Urbanelli et al., 2000), *COI* (Zhong et al., 2013) and *ITS2* (Shaikevich & Talbalaghi, 2013). Nevertheless, these results should be taken with a grain of salt due to the underrepresentation of the Italian populations in *COI* and *ITS2* studies. Another study was conducted by (Manni et al., 2015), who analyzed sequences from Thailand, Italy and La Reunion and found them highly polymorphic, but no genetic structure associated with different continent was found. Microsatellite polymorphisms showed that between French and Vietnamese populations there are genetic differences, suggesting continental differentiation (Minard et al., 2015). (Urbanelli et al., 2000) found genetic similarity of Italian, US and Japanese samples which altogether differed from Indonesian samples. Based on these latter results, (Goubert et al., 2016) report the need to sample and analyze sequences from both North and Southeast Asian populations due to the fact that many of the understudied populations (especially northern and continental native ones) are very probable to colonize European regions in the view of their similar climates.

1.5.6. Genetic variability in Africa

A. albopictus was first found in 1989 in Cape Town (Cornel & Hunt, 1991), in Cameroon in 2000, and later it was perceived that it had settled in many other regions (Diallo et al., 2010; Simard et al., 2005; Toto et al., 2003). (Kamgang et al., 2011) did not find high levels of polymorphism with the use of *ND5* and *COI* when studying populations from Cameroon, which is relatable to the recent invasion of *Aedes albopictus* in the country, while with the majority of the microsatellite markers, they found high genetic variability. In total, their results pointed to multiple introduction events as two genetic clusters were present in most samples from Cameroon, but they were unable to pinpoint the exact geographical origins of the invasions. Nevertheless, using *COI* polymorphism they were able to infer most of the genetic kinship of Cameroonian populations with tropical ones compared to the temperate and subtropical ones. However, (Usmani-Brown et al., 2009) using *ND5* polymorphism, genetically associated Cameroon samples with Hawaiian, US and Italian ones. (Kamgang et

al., 2013) found that central African populations are linked both to tropical and temperate or subtropical lineages, suggesting that Western African population are resulting from multiple introductions or through a mixture of different populations.

1.6. The use of haplotypes in population genetics

The term haplotype is used to describe alleles that collectively contain different sets of SNPs at nearby sites that are statistically associated, which means that they tend to be inherited together inside a population, that complies with the Hardy-Weinberg equilibrium requirements (Gibbs et al., 2003). When new mutations arise or recombination events occur between the corresponding maternal and paternal chromosomal regions, new haplotypes are generated, and the outcome is a mosaic chromosome between the two parental (Pääbo, 2003). Due to haplotype coinheritance, these alleles are genetically linked among individuals within a population (called linkage disequilibrium). Loci on the same chromosome are inherited together unless a recombination event separates them into different haplotypes. In populations, this coinheritance causes linkage disequilibrium (LD) between physically nearby loci and signifies a shared ancestry. Thus, identification of interlinked variation in populations, at sets of physically closely positioned markers is a valuable tool for inferring their historical descent (Lawson et al., 2012).

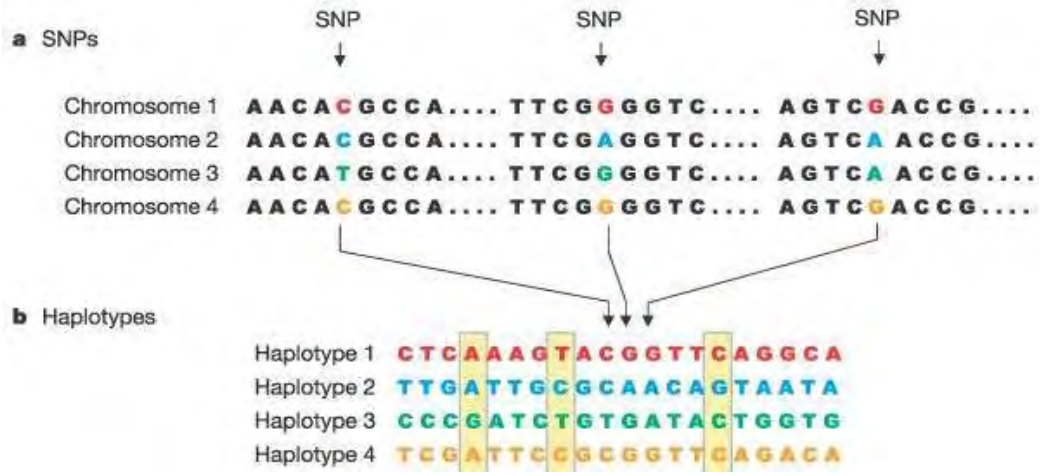


Figure 3: a) Four corresponding chromosomal regions of different individuals. There are three sites in which SNPs are present. In the specific example, there are two possible alleles for each SNP. **b**) As mentioned, the alleles with SNPs at nearby sites constitute a haplotype. In this example 20 SNPs part of a genotype are shown which are present in a region of 6000 bases of DNA (only the variable sites are shown, while the SNPs shown in **a** are also included). Figure adapted from (Gibbs et al., 2003).

1.7. Genetic markers as tools for population genetics studies

Genetic markers can be described as variants of known chromosomic location in the DNA that allow the discrimination between species and individuals via the detection of alternative polymorphic versions of genotypes or gene alleles. The genetic markers might be related with genes that contain known function (type I) or with anonymous parts of the genome (type II) (O'Brien, 1991). Most commonly used types of genetic markers include the restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), microsatellites or simple sequence repeat (SSR), and single nucleotide polymorphism (SNP). Type I markers are useful for studies concerning comparative genomics, genome evolution and candidate gene identification. Type

II are considered non coding and their use is extensive in population genetics studies (Raza et al., 2016).

Mitochondrial DNA is maternally inherited and its high mutation rates in relation to nuclear genome accounts for its use as a marker for phylogeny construction and evolutionary history deduction and, as a result, for inter- and intraspecies comparisons (Al-Samarai & A. Al-Kazaz, 2015). There are several limitations in the use of mtDNA as a marker, which, as discussed by (Zhang & Hewitt, 2003), arise from its maternal inheritance and its small effective population size in comparison to the nuclear sequences that results in faster lineage sorting rate and higher allele extinction rate for mtDNA. These limitations result in the simplified image obtained for the evolutionary history, the undervalued calculation of genetic diversity, ambiguities in genealogical analysis due to likely missing links in mitochondrial haplotypes and the difficulty in identifying distant population processes (Zhang & Hewitt, 2003).

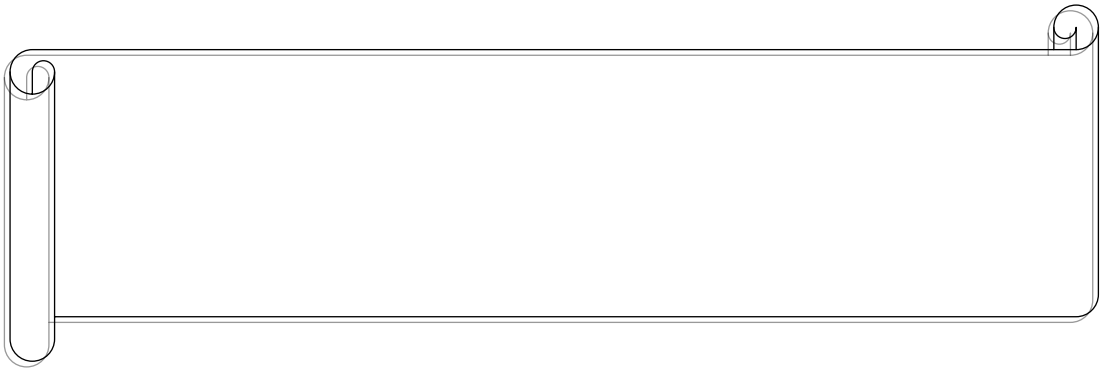
However, the use of nuclear DNA markers is not without difficulties either. In nuclear genomes, recombination occurs with different rates depending on several factors (like chromosomal locations of the loci) and it can twist the evolutionary history derived by haplotype data. Also, a few or even a single nucleotide substitution under selection will influence whole regions that exhibit low recombination rates and, as a result, genetic variability is also affected [hitchhiking effect (Aldridge et al., 1998)]. The contrary seems to apply for regions of high recombination rates. Moreover, noncoding nuclear regions are commonly used as markers due to their high amount of variability owing to the presence of, amongst other, indels. However, when used for phylogenetic analysis, indels appear as gaps which have been proven to be challenging to handle. One additional problem is the "gene tree and species tree" problem, where the results obtained from the use of a genetic marker concern only the region used and that contains the marker. This complication is even more intense for nuclear DNA due to the differential influence of evolutionary forces (e.g. random drift, population bottleneck) in the genome (genome-wide or region specific). These difficulties, along with others (low divergence and polytomy, heterozygosity and allele discrimination) are approached by (Zhang & Hewitt, 2003)

Microsatellites are repetitive DNA motifs that can be found in a large number of locations in the genome of an organism (Richard et al., 2008). Genetic structure of populations, parentage and relatedness tests, assessment of genetic diversity, and recent population history elucidation can be elucidated with the use of these markers. However, their use should be made while keeping in mind several issues (Zhang & Hewitt, 2003). One issue is that it is not certain if size differences between these markers always implies evolutionary relationships. Also, the mutation rates of microsatellite loci is not homogenous between microsatellite loci (Brohede et al., 2002). Finally, some studies have shown that some microsatellite loci to be conserved (FitzSimmons et al., 1995; Martin et al., 2002).

Aim of the Study

The aim of this study was to conduct a population genetics analysis with a more extensive, compared to previous studies, sample range of *A. albopictus* populations in Greece. As a result, a greater resolution should be achieved with the use of *COI* gene as a genetic marker to determine the genetic variability of the populations from the sampled regions. Whole genomic DNA was extracted from mosquito bodies and large *COI* fragments (>90%) were amplified via PCR and subsequently sequenced. The obtained Greek sequences together with a worldwide collection of *A. albopictus* COI sequence from GenBank which was of comparable length to the Greek sequences, were used for the phylogenetic analysis of the specific *COI* fragments.

2. Materials & Methods



2.1. Mosquito sampling

We sampled wild adult mosquitoes from eight different sites in Greece. In Figure 4 from (Badieritakis et al., 2018), we can see Greek regional units in which presence of *Aedes albopictus* was determined via a number of different sources up until 2016. We can also see the

Regional Units where we caught the adult mosquitoes. The method through which they were caught was by using an aspirator (Figure 5) and a human bait. After sampling, the

Figure 5: The aspirator used for the sampling of the mosquitoes.

Photo credits to the author

mosquitoes were kept at -20^{o}C or in 80% ethanol until the time they were to be used for DNA extraction.

Regional Unit	Spot	Coordinates	Number of mosquitoes caught	Collection Date
N. Karditsas	Gheorghikon village, Private property	39.337248, 21.866327	7	16-8-2018

Figure 4: Map adapted from (Badieritakis et al., 2018). The Regional Units where different sources have reported the presence of *Aedes albopictus*. Red color: Regional Units where samples have been obtained from public services (Benaki Phytopathological Institute and the National School of Public Health) as well as from citizens and pest management companies, Orange color: Samples obtained from public services, Yellow color: Samples obtained by pest management companies and/or citizens, Gray color: No *Aedes albopictus* individuals found, so the presence cannot be confirmed officially, Green color: The capture has been attempted using ovitraps but was not successful. We can additionally see the sites where we sampled adult *A. albopictus* mosquitoes for the needs of the current research. Exact places and number of samples shown in Table 1.

: Regional Units sampled

N. Trikalon	City of Trikala, Private property	39.553987, 21.786793	7	17-8-2018
N. Fthiotidas	City of Lamia, Public Cemetery	38.893000, 22.404818	7	18-8-2018
N. Larisas	City of Larisa, Public spot	39.619745, 22.405297	7	20-8-2018
N. Magnisias	City of Volos, N. Ionia region, Private property	39.383328, 22.928822	11	8-8-2018
N. Achaias	Arachovitika village, Private property	38.324394, 21.837267	8	15-8-2018
N. Anatolikis Attikis	City of Athens, Zografou Park	37.975508, 23.760252	11	9-9-2018
N. Ioanninon	City of Ioannina	39.653565, 20.848080	8	23-8-2018

Table 3: Exact sampling sites with coordinates shown, along with number of samples taken and the sampling date. Coordinates found on Google Maps. All of the samples were taken by the author except N. Magnisias and N. Achaias ones, which were a kind offer from professor Mathiopoulos.

2.2. DNA extraction

The whole genomic DNA was extracted from the *A. albopictus* mosquitoes using the CTAB method. For the record, the first scientist to isolate nucleic acid from white blood cells was Johannes Friedrich Miescher (13 August 1844 – 26 August 1895) in 1871 (Dahm, 2008). In general, DNA extraction involves the following steps:

1. The cells from which DNA isolation is to be carried out must be obtained. Therefore, in the current case, the tissues must be grinded.
2. The cell membranes have to be disrupted, in order for the cell content (DNA included) to be released into the solution. This is achieved with the addition of a surfactant or a detergent which binds to the lipids and proteins, separating them from the membrane.
3. RNA and proteins must be disposed of. Leftover proteins and ones that are bound to DNA are digested with the use of proteases. RNA is also degraded with the addition of an RNase.
4. Salt must be added to the solution to cause the unwanted cell debris (lipids, proteins, RNA etc.) to assemble.
5. Centrifugation of the solution will cause the formation of a two different phases, making it possible to separate the DNA from the cell debris.
6. DNA must be isolated from the solution. In the current protocol ethanol was used to precipitate the DNA out of solution. Cold ethanol is added to the solution along with salt. Ethanol makes the DNA insoluble in water and the salt neutralizes its charge, stops the repulsion between the DNA molecules. This allows DNA aggregation and thereby makes it possible for it to drop out of the solution and form a pellet after centrifugation.

Materials:

- Pulverized mosquito
- Freshly prepared CTAB buffer (CTAB 10%, 5M NaCl, 0.5M EDTA, 1M Tris-HCl pH=8, Polyvinylpyrrolidone, H_2O)
- RNase A
- Proteinase K
- Cold 100% Ethanol
- 3M CH_3COONa pH=3.4
- Chloroform
- Ice cold 70% Ethanol

Protocol:

1. Mosquitoes that are stored in ethanol should be taken out them and left them on a paper towel for 15.
2. Transfer each mosquito to a 1,5 mL tube
3. Add 200 μL (progressively – 20 μL first) CTAB and grind with a plastic pestle.
4. Add 20 μL RNase A and incubate for 30 min in 37^{o}C
5. Add 0,4 μL proteinase K (stock: 1 mg/mL) and vortex
6. Incubate at 55-60^{o}C for 2 hr (can be overnight)
7. Add CTAB up to 400 μl. Then add equal volume (400 μL) of chloroform (no isoamylic alcohol used).
8. Mix phase by vigorous vortexing (1-2 min)
9. Centrifuge at 14000 rpm for 5 min
10. Transfer the upper, aquatic phase to a new 1,5 mL tube. Avoid debris and organic phase
11. Add 1 ml (2.5 x volume of the upper phase) of cold 100% ethanol and then 1/10 (again, 1/10 of the upper phase volume) 3 M CH_3COONa, pH 3.4. Invert tubes several times to mix
12. (Optional) One more step of 150 μL CTAB and 200 μL chloroform can be added in order to get a better upper phase yield. So, the upper phase of step 13 is placed it in a 1.5 mL tube. Then CTAB and chloroform is added in the rest (lower and intermediate phase) and centrifuge follows at 14000 rpm for 5 minutes. The new upper phase is joined with the previous one in the 1,5 mL.
13. Place at -80^{o}C for 1 hr (can stay overnight)
14. Centrifuge at maximum speed (14000 rpm) for 30 min at 4 ^{o}C.
15. Discard supernatant
16. Add 500 μL ice cold 70% ethanol in the tubes (no inversion!).
17. Centrifuge at max speed, this time for 10 min, 4 ^{o}C.
18. Discard supernatant without disturbing DNA pellet.
19. Let dry for 15-20 minutes
20. Add 30-50 μL sterile water and incubate at 60^{o}C for 5 min. Vortex vigorously for 1 min and spin down.
21. Store at -20^{o}C

2.3. Polymerase Chain Reaction (PCR)

Coined by Karl Mullis (Mullis et al., 1989), PCR is one of the most used and well known techniques in molecular biology research. With PCR, one can target a specific DNA fragment and then use primers to amplify it exponentially after each round. Starting with only a small amount of initial DNA sequences, after each round new sequences are synthesized and they serve as templates in the amplification of the next round. In this way, the required quantity of DNA sample can be easily achieved, let alone the fact that only the particular wanted sequences can be studied. In general the procedure is as follows:

1. **Denaturation:** The DNA strands are separated (or melted) by applying high temperature
2. **Annealing:** The temperature is lowered and the primers, which are short single stranded sequences complementary to certain sequences of the DNA, are annealed to their target
3. **Elongation:** The temperature is elevated again (not as high as the denaturing temperature) in order for the DNA polymerase to synthesize DNA starting from the primers and with a 5'→3' direction
4. The procedure is repeated until the desired DNA quantity is obtained. To determine the number of cycles, one may calculate how much DNA is generated after each round. The mathematical operation showing the latter is 2^n, where n is the number of samples.

Materials:

- DNA template
- Primers
- DNA polymerase
- $MgCl_2$
- dNTPs
- Polymerase Buffer
- PCR H_2O

Protocol – From KAPA Biosystems (KAPA Taq PCR kit):

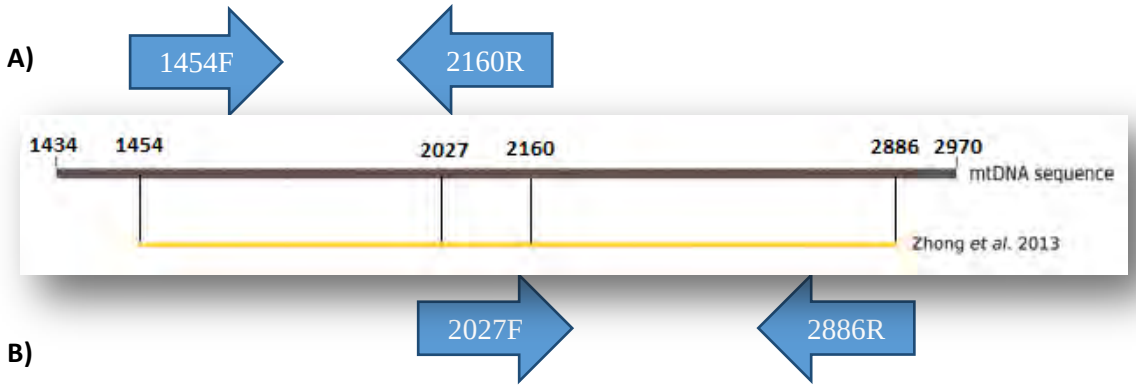
- Primers, DNA, Polymerase Buffer, $MgCl_2$ must be agitated before being added to the reaction mixture
- For dNTPs, gently flicking the tube will suffice
- Every component must be left to thaw before use

Component	25 µL reaction	Final conc.
PCR-grade water	Up to 25 µL	N/A
10X Taq Buffer	2.5 µL	1X
25 mM $MgCl_2$	1.5 mM at 1X Taq Buffer	≥1.5 mM
10 mM dNTP Mix	0.5 µL	0.2 mM each
10 µM Forward Primer	1.0 µL	0.4 µM
10 µM Reverse Primer	1.0 µL	0.4 µM
5 U/µL Taq DNA Polymerase	0.1 µL	0.5 U
Template DNA	≤250 ng for genomic DNA; ≤25 ng for less complex DNA (e.g. plasmid, lambda)	

After transferring every single component to the PCR tubes, the latter are inserted in the PCR thermocycler. The conditions are adjusted and the reaction may begin. The PCR conditions of the current study were adapted from (Zhong et al., 2013).

Conditions:

Step	**Temperature**	**Duration**	**Cycles**
Initial denaturation	94 °C	3 min*	1
Denaturation	94°C	30 sec	35
Annealing	55°C	30 sec	
Extension	72°C	1 min/kb	
Final extension (optional)	72°C	6 min/kb	1
Hold	4-10°C	∞	1



Primer	**Sequence**	**Tm (°C)**
1454F	5’ GGTCAACAAATCATAAAGATATTGG 3’	50.5
2160R	5’ TAAACTTCTGGATGACCAAAAAATCA 3’	53.4
2027F	5’ CCCGTATTAGCCGGAGCTAT 3’	56.2
2886R	5’ ATGGGGAAAGAAGGAGTTCG 3’	54.5

Figure 6: A) The range of *COI* covered for the analysis. The primers and the selected sequence are exactly the way described by (Zhong et al., 2013). As shown, the primer pairs

are 1454F/2160R and 2027F/2886R and they amplify 706 and 859 bp fragments correspondingly. B) The nucleotide sequences and melting temperatures of every primer used

2.4. Gel electrophoresis

The concept of electrophoresis started in 1931 by Arne Tiselius (Kyle & Shampo, 2005). From then on and to this day, many developments for the analysis and separation have been generated. The use of gel for electrophoresis is very widespread. Many types of gel are being used (agarose, polyacrylamide, starch). For the needs of DNA electrophoresis in this study we used agarose gel. The principle of the method is based on the fact that DNA is negatively charged due to the phosphate group it contains. The DNA is loaded in the wells of the gel, along with a loading buffer which contains, among others, a dye that gives a color to help the process monitoring and glycerol (or some other high density reagent) to assist in the submergence of DNA. With the application of positive charge on one end of the gel apparatus and negative charge on the other, the negative charge of DNA is actually used to make the DNA travel through the pores of the gel, from the negative to the positive end. That way, the DNA molecules are separated based on their length (the smaller ones pass more easily through the pores).

Materials:

- Agarose
- TBE 5X (1L: 54g Tris-base, 20mL EDTA 0,5M (pH:8), 27,5g Boric acid)
- DNA
- Loading buffer 10X (orange G, bromophenol blue, xylene cyanol FF or bromocresol green)
- Ladder
- Ethidium Bromide

Protocol:

Create an agarose gel:

1. Measure agarose by mixing TBE 0,5X with 1% agarose and 4 μL BrEt
2. Mix agarose powder with 0,5X TBE
3. Microwave for 1-3 min until the agarose is completely dissolved (without overboiling the solution)
4. Let agarose solution cool down to about 50 °C (or cool it by pouring water on the walls of the flask it is contained in while agitating it)
5. Add 4 μL ethidium bromide (EtBr)
6. Pour the agarose into a gel tray with the well comb in place (slowly, to avoid bubbles)
7. Place newly poured gel at 4 °C for 10-15 mins or let it solidify at room temperature for 20-30 mins

Load the samples and run the agarose gel:

1. Add 1-5 μL of DNA sample into an 1,5 mL tube (1 μg DNA in total)
2. Add H_2O to 10 μl final volume and add 1 μL loading dye 10X
3. Once solidified, place the agarose gel into the gel box
4. Pour TBE 0,5X buffer in the gel box until the gel is covered
5. Load the molecular weight ladder in the first lane of the gel

6. Load the samples into the other wells
7. Close the gel box and connect the electrodes to the power source
8. Run the gel at 80 (small gels)- 150 V (big gels) for 1 – 1,5 hr (depends on the gel)
9. Turn off the power, disconnect the electrodes and take the gel out of the box
10. Use the device with the UV light to visualize the results (DNA bands)

2.5. PCR cleanup

In order to use the PCR product for downstream analyses, it first needs to be cleaned from every unneeded leftover component that might impede further reactions, in our case, sequencing. These components include enzymes, primers, primer dimers, residual salts, nucleotides, polymerase buffer, proteins, RNA etc. Various methods are used in order to purify PCR products (column based, ethanol precipitation, enzymatic). In the enzymatic method, alkaline phosphatases and exonucleases are used to deactivate dNTPs by dephosphorylating them and to get rid of primers or other DNA sequences. The column based purification is based on the fact that in certain salt concentration conditions the PCR product will bind to the column membrane. The DNA washing steps are carried out with ethanol solutions while the cleaned up DNA is unbound from the membrane with the use of low salt solutions. The NucleoSpin® PCR Clean-up kit from Macherey-Nagel was used and the procedure was carried out according to the manufacturer`s instuctions.

Materials:

- Buffer NTI
- Buffer NT3
- PCR grade H_2O

Protocol:

1. Calculate the double volume for NTI in relation to the sample volume and add it in the tube containing the sample in order to combine them into a single mixture. This will establish the suitable conditions for the DNA to bind on the silica membrane
2. Place the column in a 2 mL collection tube provided by the kit. Transfer the mixture of the NTI buffer with the sample inside the column and centrifuge for 1 minute at 11000 x g. After centrifugation, get rid of the flow-through and return the column in the collection tube
3. Add 700 μL from buffer NT3 into the column and centrifuge for 1 minute at 11000 x g. Get rid of the flow-through and return the column in the collection tube
4. Repeat step 3 for finer salt removal
5. For the total removal of buffer NT3, carry out a centrifugation at 11000 x g for 2 minutes. Be careful not to touch the column to the collection tube
6. Let the column dry from ethanol for 5 minutes in room temperature
7. Get a new 1.5 mL tube in order to place the column into it. Add 20 μL PCR grade H_2O and incubate at room temperature (18-25^{o}C) for 1 minute before centrifuging for 2 minutes at 11000 x g

2.6. PCR cleaned DNA quantification

In order for the PCR product to be sequenced, there are certain requirements from each company offering sequencing services for the solution volume and DNA concentration (e.g. I

had to send 10 μL of 20-50 ng/μL for each primer for *COI* sequencing). For that reason it is necessary to quantify the clean PCR product before sending it. Qubit fluorometer and gel quantification were the two methods used independently for that cause.

- **Qubit fluorometer 2.0:**

The principle of Qubit quantification is based on the use of fluorescent dyes which produce meager signal when not bound to the nucleic acid or protein (depending on the assay someone wants to carry out). When intercalated between the DNA bases, it changes in form and produces an immensely more powerful signal when excited, which is directly correlated with the amount of DNA in the sample. The process of quantification involves the creation of a working solution containing the buffer with the reagent (dye) and then the mix of the working solution with the sample.

Materials:

- DNA sample
- Qubit Buffer
- Qubit Reagent

Protocol:

In order to quantify with Qubit, the Qubit reagents need to be added in order to create the solution, where samples will then be added for quantification. How to do it:

1. Create the Working solution by diluting the Qubit reagent 1:200 in Qubit buffer. Prepare 200 μL of Working Solution for each standard and sample.
2. For each sample, 190 μL (for Standard calculation) are needed or 199 μL (for User Sample) of working solution (it comes with the Qubit machine). In correspondence, 10 μL or 1μL of user sample (respectively) are needed to achieve a total volume of 200 μL.
3. Vortex all tubes for 2-3 sec.
4. Incubate the tubes for 2 minutes at room temperature (15 minutes for Qubit protein assay).
5. Insert the tubes and take readings.

- **GelAnalyzer quantification:**

The gel quantification process involves the analysis of the image obtained from a completed gel electrophoresis of a certain DNA sample volume. The amount of DNA of the sample is calculated in relation to the known amount of DNA that is contained in the bands of the DNA ladder.

Materials:

- Gel electrophoresis image containing the sample for quantification
- GelAnalyzer software

Method:

1. Click on “New analysis” and select the gel image (.jpeg file)
2. While on “Lanes mode” click on “Detect Lanes”. Delete the extra lanes found

3. While on “Bands mode” click on “Detect bands on every lane”. Delete the extra bands found

4. Go to “Background subtract mode” and carry out the “Rolling Ball” subtraction with a ball radius of 25 for every lane

5. Go to “Rf calibration mode” and “add default start and end rf curves”. Start should be approximately on the wells were the sample was loaded and the end approximately on the last band which reached the furthest downstream

6. Go to “Quantity calibration mode”. Click on every known band (ladder bands) and add the amount of DNA contained

7. By clicking on each different lane the amount of DNA contained in each band should be shown in the “Analysis info” window and in the “Calibration volume lane”

2.7. Sequencing

The amplified DNA sequences were then sequenced with the Sanger method. Its name is derived by Frederick Sanger who introduced this method along with his group in 1977 (Sanger & Coulson, 1975; Sanger et al., 1977).

The principle of Sanger sequencing is based on the PCR amplification of the sample sequence. The reaction contains all the needed components, i.e. polymerase, primer, DNA template, deoxynucleotides (dNTPs) as well as a modified type of dNTPs called dideoxynucleotides (ddNTPs) which are short of the 3’-OH and end the sequence elongation as they cannot form phosphodiester bonds with a next nucleotide. The ratio of dNTPs to ddNTPs must be at least 100:1 for the sufficient synthesis of sequences and the production of a capable number of ddNTP-terminated ones.

An electrophoresis (gel or capillary) is ran for the synthesized sequences in order for them to be sorted by size. The sequence determination can be carried out in two ways. First, if the reactions are carried out separately for each of the four different ddNTPs, then the electrophoresis will be carried out for the fragments produced for each ddNTP. In that manner, the identification of each nucleotide contained in the sequence will be done separately. In the second way, each ddNTP is labelled with a different fluorescent dye. Thus, every ddNTP is used in a single reaction on account of the different wavelengths at which each dye emits.

Our clean PCR products were sent for sequencing to Cemia SA (Cellular and molecular immunological applications, 31 Str Makrigianni, Larissa, Greece). Cemia uses the Sanger method of sequencing and ABI3730xl DNA analyzer to offer sequencing services. We used two primers for the sequencing of our samples (2027F and 2160 R) and we sent 10 μL of 5 pmol/μL for each primer. Since our samples were longer than 1000 bp, we sent 20 μL of 20 ng/μL (10 μL for each primer). The sequencing results were obtained as electropherograms and were viewed by using SnapGene software (from GSL Biotech; available at snapgene.com). The electropherograms represent records of the light emission over time where, ideally, each different base is corresponded with a different well-defined peak.

2.8. Sequence analysis

- Fragment merging

Due to the fact that two primers were sent per sequence, the results obtained were two fragments for each one. For that reason, these two fragments had to be joined together into a single sequence. The software that facilitates this kind of manipulation is FragmentMerger

(Bell & Kramvis, 2013). This tool is freely available online (http://hvdr.bioinf.wits.ac.za/fmt/) and can accept either chromatograms (which are trimmed) or FASTA files. It is used in order to automatically detect the overlapping regions of the sequences and assemble them into a single continuous one. It is rather simple in using, demanding only the sequence uploading and the specification of the sequence type and direction.

- **Sequence Alignment**

CLC Sequence Viewer 8.0 (https://www.qiagenbioinformatics.com/) was used for this goal. Multiple sequence alignment was carried out including every sequencing output. The software method by which the software carries out multiple sequence alignment is "progressive sequence alignment" (Feng & Doolittle, 1987) which practically means that it is the outcome of separate pairwise alignments.

Method:

1. Import the sequences
2. Go to Toolbox → Alignments and Trees → Create Alignment
3. Select the desired sequences
4. Carry out the very accurate (slow) alignment with the default parameters (Gap open cost: 10.0, Gap extension cost: 1.0 etc.)
5. Export as a FASTA alignment (fa, fsa, fasta) for downstream analysis

2.9. Variation analysis

The ultimate purpose of the current study was to study the polymorphisms of the sequences obtained. Finding the SNPs that differentiate the sequences between them or with sequences that will be downloaded from GenBank. For this population analysis, DnaSP6 (Rozas et al., 2017; Rozas & Rozas, 1995) was used, which is a program that provides the ability to assess the sequence polymorphism that contributes to the variation between and within populations, employing an efficient number of parameters. For the construction of the haplotype network, popart (Leigh & Bryant, 2015) was used.

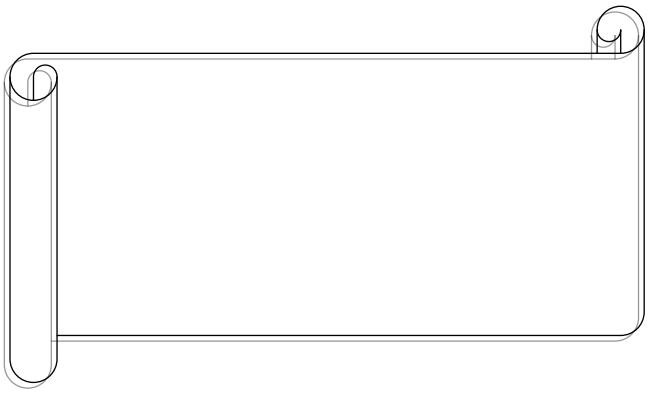
- **DnaSP6 analysis**

1. Open the sequence alignment file with DnaSP6
2. Go to "Analysis" and click on "Polymorphic Sites". This command gives some information about the polymorphisms of the sequences
3. In "Analysis", click on "DNA Polymorphism" to measure the DNA polymorphism
4. In "Analysis", click on "Fu and Li's (and other) Tests" to carry out Fu`s and Li`s D* and F* tests which assess the selective neutrality of the mutations
5. In "Analysis", click on "Tajima's Test", to carry out the D test in order to test the hypothesis that the sequences are evolving in a neutral way
6. Go to "Data" and click on "Define Sequence Sets" in order to apportion the sequences to the different populations
7. In "Analysis", click on "DNA Divergence Between Populations" to get an evaluation of the divergence between the population based on the observed polymorphism
8. Go to "Analysis" and click on "Gene Flow and Genetic Differentiation" in order to obtain measures about the divergence and the gene flow between the populations
9. Go to "Overview" and click on "Polymorphism/Divergence Data'' to get some measures of polymorphisms between and within the populations and some neutrality test scores

10. Go to “Generate” and click on “Haplotype Data File” to obtain the file with the haplotypes found in the population

- **popart analysis**

1. Open the alignment file with DnaSP6
2. Select “File”, click on “Save / Export Data As” and select NEXUS File Format
3. Open popart software, click on “Open Nexus File” and select the NEXUS file that was exported from DnaSP6
4. Click on “Network” and select “TCS Network” to obtain the haplotype network in order to have a visual representation of the sequences` relationships



3. Results

3.1. DNA extraction and fragment amplification

Whole genomic DNA was extracted from 42 whole mosquito samples. Table 4 shows the exact numbers of DNA extractions that were carried out for each region, as well as for some laboratory strains that were used as positive controls, due to the fact that, after the sampling it was not certain whether the individuals were *Aedes albopictus* or some related species that is hard to discriminate phenotypically [e.g. *Aedes cretinus* (YEE et al., 2014)]. The number of individuals for each region whose *COI* was used for the analysis is also shown.

Region	**DNA extraction from No of mosquitoes**	**Used for *COI* variation analysis**
Karditsa	4	2
Trikala	4	2
Larisa	5	2
Volos	4	2
Patras	4	2
Ioannina	6	3
Athens	6	2
Lamia	4	3
Laboratory	5	-
Total	42	18

Table 4: Number of DNA extractions carried out and number of sequences used from each region.

The isolated DNA was used in order to amplify the gene of cytochrome c oxidase subunit I (*COI*). PCR was carried out with specific primers under the conditions described by (Zhong et al., 2013). In some occasions in which the desired amount of PCR product was not obtained, an increase in cycles and amount of template DNA were carried out, together with duplicated reactions for the same sample which were then combined before PCR cleanup. Cytochrome c oxidase is responsible for the reduction of oxygen to water catalysis. *COI* encodes for one out of the three total subunits that constitute the respiratory complex IV (or cytochrome c oxidase), the third and final enzyme of the electron transport chain of mitochondrial oxidative phosphorylation (Consortium, 2018).

The use of mtDNA as a genetic marker for the evaluation of genetic variability of different *Aedes albopictus* populations has been ample. Different lengths of the mitochondrial COI gene can be found in GenBank (415-1510 bp) and many of them have been used for population genetics analyses of *Aedes albopictus*. When a small part of the coding sequence was used (Beebe et al., 2013; Birungi & Munstermann, 2002; Delatte et al., 2011; Kamgang et al., 2011; Mousson et al., 2005; Porretta et al., 2012; Shaikevich & Talbalaghi, 2013; Usmani-Brown et al., 2009; Zawani et al., 2014; ŽITKO et al., 2011), a limited number of haplotypes was identified compared to the use of longer parts of coding sequence (Ismail et al., 2015; Porretta et al., 2012; Zhong et al., 2013), where higher amounts of variability in both native and invasive populations were detected.

The *COI* gene part that was used in the current study was the one of (Zhong et al., 2013). The full length of the gene and the length of the part used in the analysis are 1536 and 1433 bp correspondingly. It is obvious that the latter encompasses more than 90% of the gene`s full range, while at the of this study, it was the largest part that had been used for population genetics studies of *Aedes albopictus,* containing most of the fragments that were used in other studies. In the study of Zhong et al., 2013, four primers were used and two fragments were produced which were subsequently cloned and sequenced. In the current study, it was observed that the full length amplicon could be produced only with 1454F and 2886R primer

pair, hence every amplicon used in this study was produced with this pair. It should be noted that the primers were (Altschul et al., 1990) analysed with BLAST before use in order to verify their specificity for *COI* of *Aedes albopictus*.

It is worth mentioning that an attempt was made to sequence the *ITS2* region of the specimens sampled. The PCR conditions and the primers used were as described by (Manni et al., 2015). PCR amplification of the specific primers was successful. However sequencing of the clean PCR products turned out to be troublesome. With the forward primer approximately a 350 bp long product was constantly obtained instead of the expected 502-588 bp and with the reverse primer the product was longer that 500 bp, nonetheless it constitutively contained high background noise and double peaks. The duration of this study would not suffice for troubleshooting of this sequencing, so it was abandoned, at least for the time being.

3.2. GenBank sequence sampling

In addition to the amplified sequences from Greek samples, additional COI sequences (Clark et al., 2016) were also downloaded from GenBank, in order to compare the variability of the Greek sequences to the one of other regions or countries. Every *COI* sequence of *Aedes albopictus* that was deposited in GenBank up until July 2019 and was longer than 1300 bp was downloaded and used for downstream analysis. In Table 5, all sequences that were sampled from GenBank are shown.

Country	Localities	No of Sequences	Length (bp)	Reference	Source
USA	Ohio	6	1433	Giordano et al., 2017, Unpublished	GenBank
USA	Virginia	2	1536	(Battaglia et al., 2016)	GenBank
Central America	Panama, Costa Rica	6	1390	(Futami et al., 2015)	GenBank
China	Foshan	1	1536	(Battaglia et al., 2016)	GenBank
China	Changsha, Chenzhou, Dalian, Foshan, Guangzhou, Haikou, Hangzhou, Jiangmen, Nanchang, Qiongzhong, Sanya, Shanghai, Tianshui, Wenchang, Xi`an, Zhongshan	24	1537	Hu et al., 2016, Unpublished	GenBank
Italy	Cassino, Cesena,	8	1536	(Battaglia	GenBank

	Pavia, Reggio Calabria, Rimini			et al., 2016)	
Japan	Wakayama	1	1536	(Battaglia et al., 2016)	GenBank
Malaysia	Perak, Pulau Pinang, Sabah, Selangor	33 (haplotypes)	1510	Amrannudin et al., 2017, Unpublished	GenBank
Philippines	Laguna, Los Banos	5	1536	(Battaglia et al., 2016)	GenBank
Taiwan	Taipei	1	1536	Ho et al., 2005, *Aedes albopictus* full mtDNA sequencing	GenBank
Thailand	Chumphon, Lampang, Uthai Thani	3	1536	(Battaglia et al., 2016)	GenBank
Albania	Tirana	2	1536	(Battaglia et al., 2016)	GenBank
Greece	Athens	2	1536	(Battaglia et al., 2016)	GenBank
Canada	Windsor	8	1433	Giordano et al., 2017, Unpublished	GenBank
Russia (Black Sea coast)	Adler, Apheronsk, Chadyschinsk, Goryachy Klyuch, Natukhayevskaya, Neberdzhayevskaya, Novorossiysk, Sochi, Tupse, Vladimirovka	62	1317-1433	Fedorova and Shaikevich, 2018, Unpublished	GenBank
China, USA, Italy, Taiwan, Hawaii, Japan, Singapore	CHI-Guangzhou, Xiamen, Wuxi, Xinzhu, JPN-Nagasaki, SG-Serangoon, ITA-Trentino, USA-Los Angeles 2001,	66 (haplotypes)	1433	(Zhong et al., 2013)	GenBank

	USA-Los Angeles 2011, USA-New Jersey, Texas, HW-O'ahu				

Table 5: The sequences that were downloaded from GenBank are listed along with the country and region from which the *Aedes albopictus* samples originated, the number of sequences downloaded and the publication from which they were obtained. Total number of sequences: 230.

3.3. Sequencing of the amplified fragments

3.3.1. Sequences obtained

After PCR amplification, sequencing followed. The PCR product was cleaned and the primers used were the two that annealed in the middle of the amplicon, i.e. 2027F and 2160R. The obtained sequences from 2027F had lengths which ranged from 793 to 845 bp, with the exception of two cases of 546 and 1198 bp (the 546 bp one was obviously not useful) while for 2160R lengths ranged from 665 to 668 bp. One attempt was made to use 2886R primer for resulting into a 519 bp long sequence. The final merged sequences of the longer products ranged between 1441 and 1446 bp.

3.3.2. Chromatogram assessment

After obtaining the raw sequencing products and before merging them into a consensus sequence, chromatograms were inspected in order to find bases of ambiguous quality. Sequencing products of low quality were discarded. Out of 49 (24 for 2027F primer, 24 for 2160R and 1 for 2886R) obtained sequencing products, 36 were kept and were merged into 18 *COI* sequences in order to be used for downstream analysis.

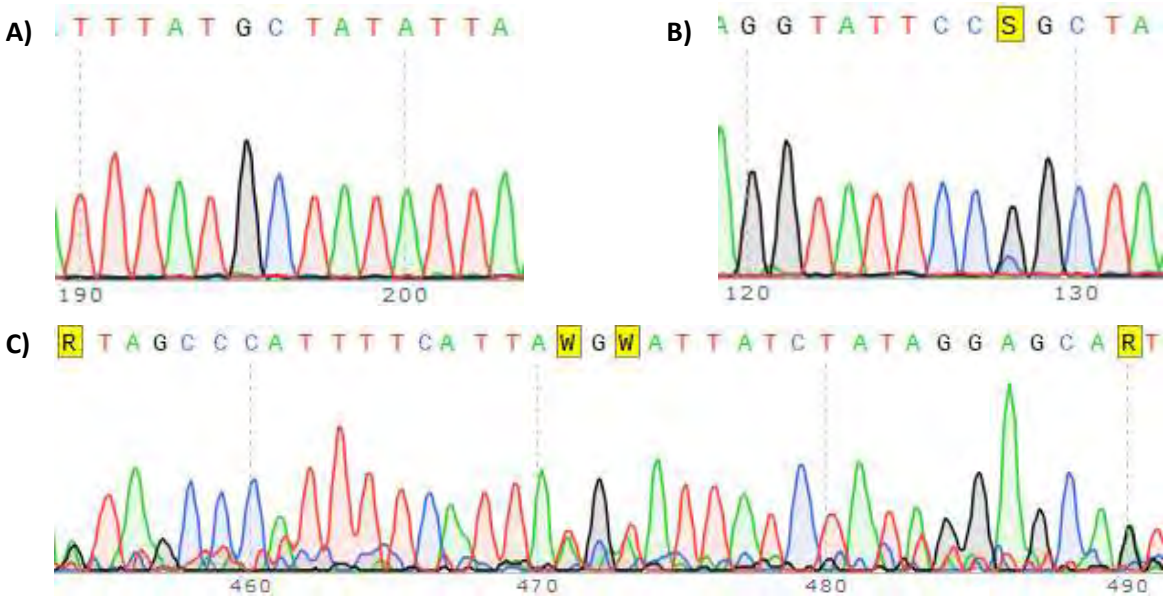


Figure 7: Examples of sequencing products obtained. A) A good quality region of the sequencing product, with evenly spaced, single colored, well-defined peaks of good height and no background noise. B) An also of good quality region of the sequencing product that contains one ambiguous base call. In the specific case it is safe to say that this base is a G. C) An example of low quality, with high background noise and in many sites secondary peaks can be observed. Also, the bases of ambiguous quality cannot be determined by inspecting the chromatogram peaks.

3.4. Alignment with the rest of the sequences

The next step was to align every sequence that was selected for variation analysis, including the Greek sequences that were produced for the current study as well as the sequences downloaded from GenBank. As shown in Table 5, sequences are of different lengths while the Greek sequences to be used contain some bases of low quality in their beginning and end, typical of any raw Sanger sequencing product. This bias had to be corrected in order to end up with the merged sequences. Due to the fact that the sequences produced by the two primers have an overlap of 133 bp, any low quality bases that appeared in the intermediate part of the merged sequence are compensated.

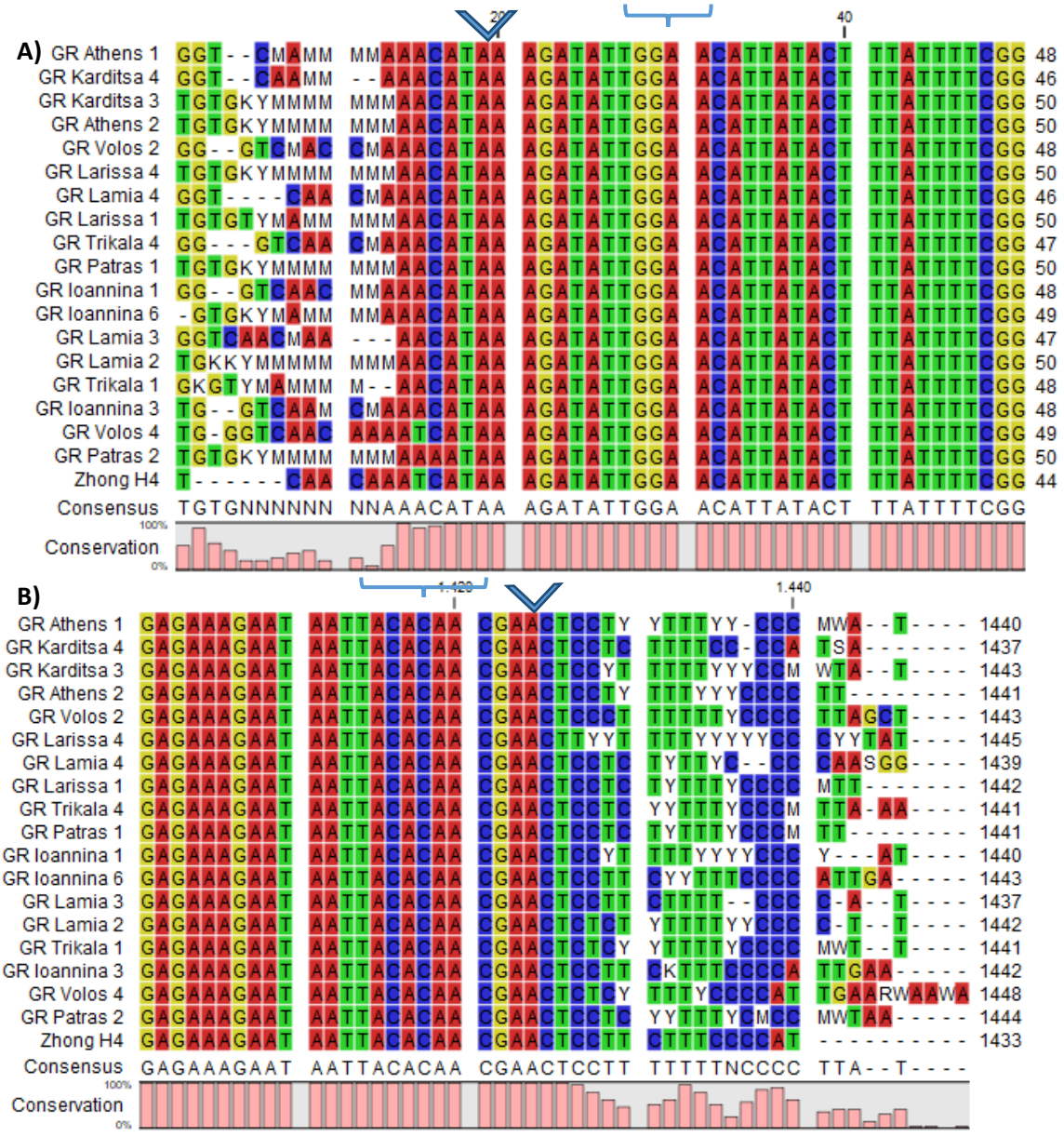


Figure 8: The merged sequences contained low quality base calls at their starts (A) and ends (B). As can be seen in the figure, there are many bases of ambiguous quality and there are many mismatches in the alignment due to, probably, falsely called bases. After the inspection of the chromatograms, the consensus good quality start for all sequences is site 19 and the consensus good quality end is site 1424 (shown in the figure with the blue arrows) making up a total of 1405 bp that are available to be used for the analysis. Additionally, after

the alignment of the total number of sequences, due to their different lengths, they were shortened in such way that they all had the same length. The used sequence start site is indicated in the figure with the blue braces (start: site 30, end: site 1418, total length of used sequence: 1388 bp).

3.5. Variation analysis

Subsequently, the analysis of the sequences` polymorphism was carried out. The total number of sequences used for DnaSP6 analysis was 248, which contained 1388 nucleotide sites of which 1056 (76%) were invariable and 332 (23.9%) were polymorphic. Out of the polymorphic, 38 were singleton variable and 294 were parsimony informative sites (more details in Table 6).

Singleton variable sites (two variants)	36
Site positions	33, 46, 52, 58, 60, 64, 104, 133, 189, 229, 267, 317, 326, 374, 397, 413, 431, 451, 490, 506, 568, 574, 577, 587, 600, 635, 837, 956, 1050, 1055, 1095, 1106, 1177, 1265, 1291, 1359
Parsimony informative sites (two variants)	260
Site positions	4, 10, 13, 18, 22, 23, 24, 25, 26, 31, 34, 40, 43, 48, 50, 55, 56, 59, 67, 68, 69, 70, 73, 74, 76, 85, 88, 89, 100, 106, 121, 127, 130, 139, 154, 166, 169, 172, 174, 181, 193, 197, 205, 212, 214, 220, 232, 235, 238, 244, 260, 263, 266, 271, 274, 277, 279, 280, 283, 289, 290, 292, 298, 299, 300, 301, 302, 313, 316, 319, 320, 325, 334, 336, 346, 352, 355, 356, 359, 364, 367, 373, 379, 391, 394, 396, 401, 403, 418, 421, 424, 433, 445, 450, 454, 462, 468, 475, 476, 484, 485, 486, 487, 492, 494, 496, 499, 514, 516, 519, 523, 526, 530, 532, 533, 534, 537, 543, 549, 550, 556, 560, 578, 589, 591, 595, 596, 604, 607, 610, 616, 622, 623, 626, 628, 633, 638, 642, 644, 647, 653, 665, 671, 674, 692, 696, 698, 701, 731, 740, 755, 761, 785, 789, 791, 797, 803, 806, 815, 830, 833, 839, 842, 848, 851, 866, 875, 878, 881, 896, 929, 933, 935, 938, 944, 952, 953, 958, 959, 966, 968, 969, 977, 989, 1001, 1004, 1007, 1010, 1011, 1019, 1022, 1023, 1025, 1037, 1040, 1052, 1058, 1064, 1070, 1073, 1076, 1079, 1082, 1085, 1091, 1094, 1109, 1127, 1134, 1136, 1145, 1148, 1157, 1161, 1164, 1166, 1172, 1176, 1187, 1188, 1189, 1198, 1199, 1211, 1217, 1221, 1226, 1229, 1232, 1235, 1251, 1256, 1271, 1277, 1283, 1286, 1289, 1292, 1295, 1296, 1300, 1301, 1311, 1316, 1319, 1325, 1328, 1330, 1337, 1338, 1346, 1347, 1350, 1352, 1358, 1379, 1382, 1383, 1386, 1388
Singleton variable sites (three variants)	2

Site positions	65, 230
Parsimony informative sites (three variants)	32
Site positions	19, 29, 49, 62, 79, 82, 202, 286, 295, 328, 343, 349, 412, 415, 466, 472, 473, 520, 547, 553, 562, 598, 629, 821, 872, 893, 962, 965, 1016, 1100, 1202, 1266
Singleton variable sites (four variants)	0
Site positions	2
Parsimony informative sites (four variants)	478, 508

Table 6: Detailed output of the polymorphic sites analysis with DnaSP 6.

- Disambiguation: *Singleton variable sites* are sites which contain at least two types of nucleotides with, at most, one occurring multiple times (and Nei & Eds.) Kumar, 2000). *Parsimony informative sites* are sites which contain at least two types of nucleotides, and each represented at least twice (Aluru, 2005; Mount, 2008).

G+C content	29.9%
Total number of mutations (Eta)	370
Number of haplotypes (h)	113
Haplotype diversity (Hd)	0.923
Variance of haplotype diversity	0,00018
Standard Deviation of Haplotype diversity	0,014
Nucleotide diversity per site (Pi)	0,01364
Theta (per site) from Eta	0,04378
Theta (per sequence) from Eta	60,76904
Average number of nucleotide differences (k)	18,93
Fu and Li's D* test statistic (FLD*) (using biallelic positions)	0,84255
Fu and Li's F* test statistic (FLF*) (using biallelic positions)	-0,78733
Fu's Fs statistic	-32,488
Tajima's D (Statistical significance: **, P < 0.01)	-2,16679

Table 7: Polymorphism and neutrality tests of the total number of sequences and positions, data using DnaSP 6.

- Disambiguation: *Haplotype diversity (Hd)* is a measure of the uniqueness of a particular haplotype in a given population (Nei & Tajima, 1981). *Nucleotide diversity (Pi)* represents the number of nucleotide differences per nucleotide site and thus, it can be used as a measure for genic variation by comparing the average number of nucleotide differences per site between two randomly chosen populations (Nei & Li, 1979). *Theta* represents the population mutation rate θ=4*Ne*μ, Ne=effective population size, μ=per generation mutation rate (Watterson, 1975). *Tajima`s D* is based on the fact that, in neutrally evolving populations, the number of segregating sites and the average number of nucleotide differences are correlated. In that sense, the difference between these two measures of genetic diversity is estimated in order to determine whether a DNA sequence is evolving neutrally or under a non-random process, i.e. it is under selection. (Tajima, 1989). When evolution is neutral, Tajima`s D equals zero, is positive when there is a surplus of rare polymorphism, indicative of positive selection and negative when there is a surplus of high frequency variants, indicative of balancing selection (Oleksyk et al., 2010). *Fu and Li`s D* and F* test statistics* compare the number of derived singleton mutations and the mean pairwise difference between sequences (the asterisk means

that the tests are carried out without an outgroup and thus, only intraspecific polymorphism is required) in order to identify evidence of deviation from neutrality (Fu & Li, 1993). *Fu`s Fs statistic* measures the probability of observing a certain number of haplotypes for a given value of theta (θ). The test recognizes discrepancies between the values of θ derived from a number of haplotypes and average pairwise sequence divergence. When the values are negative, it means that there is an excess number of alleles indicative of genetic hitchhiking or else as demographic expansion, when positive, it means that there is a deficiency of alleles interpreted as a recent population bottleneck or overdominant selection (i.e. higher fitness of heterozygotes) (Fu, 1997).

The number of segregating sites (S) ranged from 0 (Albania) to 316 (Malaysian haplotypes), while haplotype diversity (Hd) ranged from 0.000 (Albania) to 1 (Japan, Thailand, Los Angeles 2001 sequences, Taiwan, Canada, Central America, Singapore. It should be noted that these populations were represented by a low number of sequences, i.e. from 1 to 10 sequences), nucleotide diversity (Pi) ranged from 0,000 (Albania) to 0,06513 (Malaysia). For the Greek sequences, S was 5, Hd was 0,09524 and Pi was 0.00034.

	China	**Albania**	**Italy**	**Japan**	**Thailand**
Greece	0.2616	0.000	0.3553	n/c	0.7674

	Malaysia	**Philippines**	**Hawaii**
Greece	0.2867	0.8771	0.2505

	USA	**LA2011_Zhong2013**	**LA2001_Zhong2013**	**Central America**
Greece	0.3644	0.42066	0.32840	0.4407

	Taiwan	**Russia**	**Canada**	**Singapore**
Greece	0.2899	0.2282	0.3964	0.4385

Table 8: Pairwise Fst values of the Greek sequences with the sequences of the rest of the populations.

- Disambiguation: *Fst* or fixation index is a measure of genetic distance between populations. The average subpopulation heterozygosity and the total population expected heterozygosity are needed for its calculation. Fst can only have positive values. It ranges between 0 (panmictic populations with no division to subpopulation, the mating is random and no genetic divergence within the population) and 1 (complete isolated subpopulations - extremely subdivided population). Fst values up to 0.05 indicate negligible genetic differentiation whereas >0.25 means very great genetic differentiation within the population analyzed. (Wright, 1971)

The Nm value, an estimate of the gene flow, product of the effective size of individual populations (N) and the rate of migration among them (m) between the populations was calculated at 1.66 (Nm > 1), indicating that there is enough gene flow between the populations to compensate the effects of genetic drift (Larson et al., 1984; Tiffin & Ross-Ibarra, 2014; Wright, 1931).

Due to the inability to calculate the Fst value between Greece and Japan, the Polymorphism/Divergence Data were calculated. Number of variable sites, S = 7 and number of mutations Eta = 7.

3.6. Haplotypes

From the 113 haplotypes, only 37 were kept due to difficulties associated with the legibility of the produced haplotype network. The haplotypes that were excluded were the ones that were represented only by sequences of the Malaysian haplotypes or by haplotypes of Zhong et al. 2013.

Haplotype No.	Regions of corresponded sequences	No. of Sequences
1	China (NanJing, Chenzhou, Jiangmen, Jiangsu), Albania (all), Italy (Pavia, Rimini), USA (Ohio), Russia (Adler, Aphseronsk, Chadyschinsk, Goryachy Klyusk, Neber, Novor, Sochi, Tupse) Canada, Haplotype 3 – Zhong et al. 2013 (Guangzhou, Xiamen, Jiangsu, Xinzhu-Taiwan, Japan, Trentino-Italy, LA2001, LA2011, O'ahu-Hawaii, **Greece [all except Ath1 from (Battaglia et al., 2016)**]	63
2	Japan (Wakayama), USA (Ohio), Russia (Chadyschinsk, Natu, Neber, Novor, Sochi, Vlad)	15
3	China (Foshan), Panama	2
4	China (Dalian)	1
5	China (Zhongshan), Haplotype 1 – Zhong et al. 2013 (Guangzhou, Xiamen, LA2001)	2
6	China (Hangzhou), Thailand, Malaysian haplotypes 9, 22	4
7	China (Hangzhou)	1
8	Italy (Cassino, Pavia, Rimini), USA (Ohio), Canada, Haplotype 37 – Zhong et al. 2013 (Italy-Trentino, USA-New Jersey, Texas)	6
9	China (Xi`an), Thailand, Malaysian haplotypes 1, 21, Haplotype 46 – Zhong et al. 2013 (LA2001)	5
10	China (Guangzhu)	1
11	Italy (Cesena), USA (Ohio), Russia (Chadyschinsk, Goryachy Klyusk, Novor, Sochi, Haplotype 17 – Zhong et al. 2013 (Xinzhu-Taiwan, Trentino-Italy, LA2011, USA-Texas, O'ahu-Hawaii)	16
12	Philippines (Laguna), Malaysian haplotypes 4,6	5
13	Philippines (Laguna)	1
14	China (Tianshui)	1
15	Italy (Calabria, Cesena), USA (Virginia), Canada, Haplotype 39 – Zhong et al. 2013 (Trentino-Italy, USA-Texas)	6
16	Taiwan (Taipei)	1
17	China (Hangjiang, Jimei, Liangjian, Fuzhou, Xiapu), Haplotype 8 – Zhong et al. 2013 (China-Xiamen)	9
18	Thailand	1
19	Philippines (Laguna)	1
20	**Greece [Athens – Ath1 from (Battaglia et al., 2016)]**, Haplotype 56 – Zhong et al. 2013 (USA-Texas), Malaysian haplotypes 20, 24	4
21	China (Liangjian), Haplotype 2 – Zhong et al. 2013 (China-Guangzhou)	2
22	Brazil, Haplotype 45 – Zhong et al. 2013 (LA2001)	2
23	Russia-Goryachy Klyusk	1

24	Canada, Costa Rica	2
25	USA (Ohio), Haplotype 25 – Zhong et al. 2013 (Japan-Nagasaki)	2
26	Russia (Goryachy Klyusk, Natu, Novor), Haplotype 54 – Zhong et al. 2013 (USA-New Jersey)	4
27	Russia (Vlad)	1
28	USA (Ohio)	1
29	Canada	1
30	Canada	1
31	Canada	1
32	Canada	1
33	Costa Rica	1
34	Costa Rica	1
35	Costa Rica	1
36	Costa Rica	1

Table 9: The haplotype data generated by DnaSP6. The Greek sequences are shown in bold letters.

The Polymorphism/Divergence data between the Albanian sequences and the Greek ones from Haplotype 1 showed segregating sites, S = 0 and total number of mutations, Eta = 0.

3.7. Haplotype network

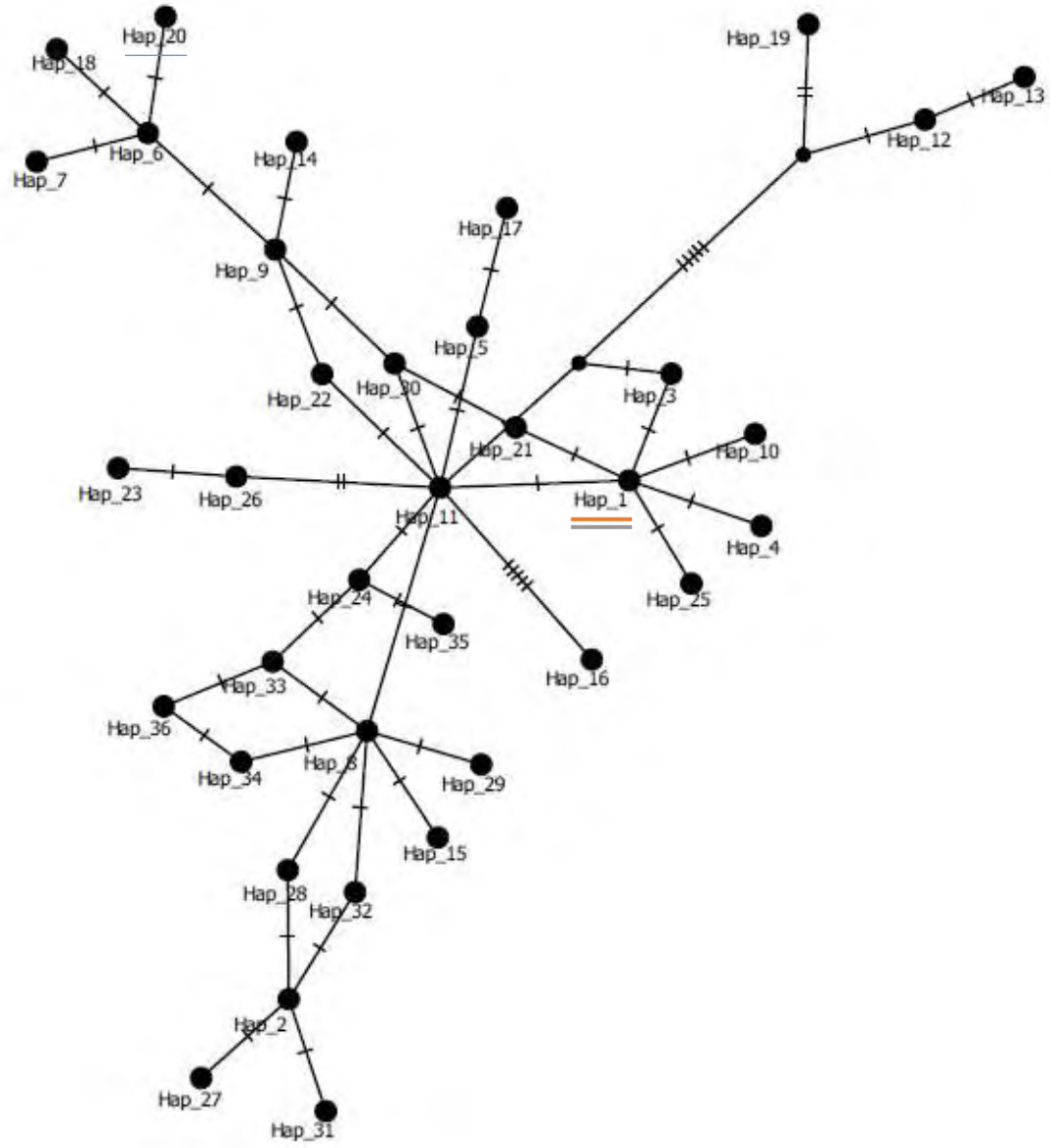
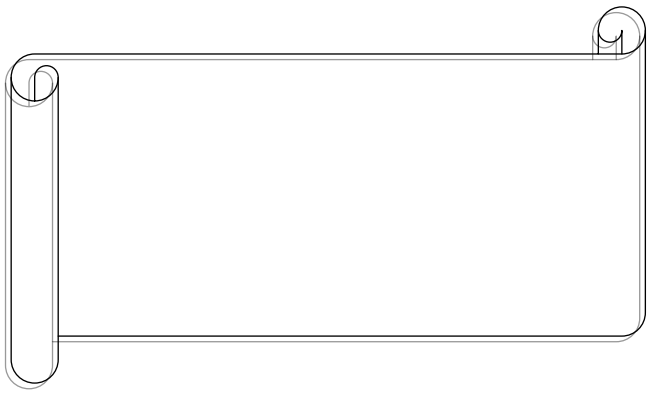


Figure 9: Haplotype network of the 37 haplotypes, generated using PopART. The mutation steps between haplotypes are shown as hatch marks. Haplotype 1 (underlined with a glowing orange color) is the one that contains all the Greek sequences except Ath1, while Haplotype 20 contains the one remaining sequence "Ath1" (underlined with glowing blue color).



4. Discussion

Aedes albopictus is one of the most invasive mosquito species with significant worldwide distribution. Severe concerns accompany this invasiveness, due to its association with mosquito-borne diseases. Population genetics analyses are thus very important in order to identify which populations are genealogically related and hence are more probable to carry disease vector or insecticide resistance traits. *Aedes albopictus* exhibits a very short flight range that limits its autonomous expansion. Passive dispersal of adult mosquitoes or their eggs due to anthropogenic activities like global tire trade is the main reason behind the chaotic dispersal of the species (Manni et al., 2017; Paupy et al., 2009; Reiter & Sprenger, 1987).

The aim of the present study was to carry out a thorough and systematic genetic analysis of Greek *Aedes albopictus* populations. Up until now, Greek populations had been included only a few times in this kind of studies. Nevertheless, these previous studies only included samples that were either limited to one region (Athens, Greece) or not specified while sampling was not as extensive as in the current study. For that cause, samples ranging from Northwestern, Central, Central-Western and Central-Eastern Greece were collected and *COI* gene was used as a genetic marker to determine the variability of these populations.

4.1. Previous work on Greek *Aedes albopictus* populations

There are several studies that have included Greek samples in the population genetics analysis of the tiger mosquito. One of the studies was from (Shaikevich & Talbalaghi, 2013) who used partial *COI* sequences of 619 bp (Athens) and 473 bp (Corfu and Thesprotia) length, while for *ITS2* they used 468 and 467 bp (Corfu and Thesprotia respectively) long sequences. Based on the *COI* results, they found three total haplotypes. The second haplotype had one mutation in relation to the first and the third had one mutation in relation to the second. This haplotype was found in individuals which were sampled from three sites in Piedmont region but not in individuals from Liguria region. This haplotype was identical to Greek and Japanese haplotypes. Based on the *ITS2* region variation, two clusters were formed with the first one containing specimens originating from the subtropical regions of Greece, Japan and Italy while the second one contained the specimens from tropical China.

The next two studies were from (Kamgang et al., 2011; Kamgang et al., 2013). They also used the *COI* sequences from (Patsoula et al., 2006). In the 2011 study, using Bayesian inference the *COI* sequences were separated into two lineages. The first one contained temperate and subtropical populations (France, Greece, USA, Reunion Island and Madagascar) and the second tropical populations (Brazil, India, Cambodia, Thailand, Vietnam and Cameroon). In the 2013 study, again using Bayesian inference, the *COI* sequences were split into two lineages. The first lineage included tropical populations (Brazil, Cambodia, India, Thailand, Viet Nam, Cameroon and two haplotypes from Central African Republic), while the second included temperate and subtropical populations (France, Greece, Madagascar, Reunion, the USA and two other haplotypes from Central African Republic).

An additional study was carried out by (Battaglia et al., 2016). In this research, whole mitogenomes of *Aedes albopictus* samples were sequenced. The countries sampled were Italy, USA, Brazil, Thailand, Greece (Athens), Albania, China, Philippines, Japan and Taiwan. Three major clusters were formed from these mitogenomes which the researchers called

Haplogroup A1, A2, A3. Haplogroup A1 is divided to A1a and A1b and A1 is further subdivided to A1a1 and A1a2. The branch A1 contains the mitogenomes of Greek, Albanian, Italian samples as well as two mitogenomes from China (one wild type and one laboratory). Also, Haplogroup A1b included every mitogenome from Thailand, one from Brazil and one Greek.

Another study was carried out by (Manni et al., 2017), including sampling of ten populations from Southeast Asia (Japan, China, Thailand), Indian Ocean (La Reunion), Mediterranean Basin (Greece, Albania, Italy), Pacific Ocean Islands (Hawaii) and North America (USA). Greece was represented by 29 mosquito individuals sampled from Athens. Variation analysis was based on seventeen microsatellite loci that were previously generated (Manni et al., 2015). These results indicated that Albanian and Greek samples, despite geographic proximity, were maximally differentiated (Fst = 0.187). On the other hand, Greek samples were genetically very close (Fst = 0.083) to the Thai ones, while the Albanian ones to the Chinese (Fst = 0.075). Generally, it was also found that Southeast Asia, and mainly China, is the region of origin of *Aedes albopictus*, and that its dispersion has been carried out by a chaotic propagule distribution which plays a key role in preserving genetic variability of the invasive populations as well as in their effective establishment.

The final study including Greek *Aedes albopictus* populations was conducted by (Kotsakiozi et al., 2017). They used double-digest Restriction site-Associated (ddRAD) DNA sequencing generating approximately 58000 SNPs on samples taken from 20 worldwide native (DRC, Brazil, Gabon, Greece, Italy, USA, Bermuda) and invasive (Japan, Malaysia, Singapore, Vietnam) populations. The origin of sampling is not specified for the Greek specimens. With regards to genetic diversity, a mean observed heterozygosity (Ho) slightly lower than the native range`s specimens was observed, but with no statistical significance. In congruency with (Manni et al., 2017), the same study concluded that Greek and Italian samples differed on the genetic level. Greek specimens clustered with specimens from Africa and Asia. In the phylogenetic tree, native populations from Southeast Asia did not form a single clade, but each of them formed a separate clade with an invasive population and in the case of the Greek populations, they clustered with those from Singapore.

4.2 Comparison with our results

From the haplotypes generated, the most common one containing 64 out of 248 sequences, included the Albanian and 19 out of 20 Greek samples. This haplotype also contained Chinese, Italian, Russian (from the Black Sea coast), Canadian, Hawaiian, Japanese, Taiwanese and sequences from the USA [Ohio, Los Angeles 2001 and 2011 from (Zhong et al., 2013)]. Many of these correlations have already been shown in the previously mentioned studies, namely Greek, Japanese and Italian in (Shaikevich & Talbalaghi, 2013), Greek with USA in (Kamgang et al., 2011; Kamgang et al., 2013) and Greek with Italian, Albanian and Chinese in (Battaglia et al., 2016). (Kotsakiozi et al., 2017) related the Greek population with the population from Singapore. From the haplotypes of (Zhong et al., 2013) included in the analysis, some were also found in populations from Singapore but were not related to the Greek samples. Nevertheless, sequences from the same study, sampled in 2001 from Los Angeles belonged to specimens genetically close to haplotypes from Singapore, which didn`t manage to settle in the region as they were not found in 2011. These sequences shared the same haplotype with the Greek samples of Haplotype 1 (Table 9, Figure 9).

The most closely related haplotypes to Haplotype 1 (Figure 9), separated by one mutation step, are Haplotypes 3, 4, 10, 11, 21 and 25. These haplotypes contain sequences from China, Panama, Italy, USA, Russia, Taiwan and Hawaii. The relatedness, on the genetic level, of the Greek populations with those of Hawaii, China, Russia, Canada, Taiwan and, a bit more

distantly, Panama are reported for the first time in this study. With respect to the remaining Greek sequence, "Ath1", it shares Haplotype 20 with Malaysian and USA haplotypes. The closest haplotypes to Haplotype 20, with a distance of 1 mutation step are Haplotypes 6 and 18 which include sequences from China, Thailand and Malaysia. The genetic relatedness of Greek to Chinese and Thai sequences was also reported by (Battaglia et al., 2016) (from whom the sequence was derived in the first place), although the affinity to Malaysian haplotypes is reported in this study for the first time.

As shown in the results, all the Greek *COI* fragments of Haplotype 1 obtained showed 100% identity (number of segregating sites, S = 0) with one another as well as with the Albanian ones. This is in contrast with the results of (Manni et al., 2017), which showed that Greek and Albanian specimens were highly differentiated and the first showed highest genetic affinity to Thai specimens, while the second to Chinese ones. Although the genetic affinity to the Thai specimens was not supported by this study (with the exception of the "Ath1" sequence), the possibility that this happened due to scarce representation of the Thai samples cannot be ruled out. As mentioned, Greek samples were also close the Italian ones, in contrast to the results of (Kotsakiozi et al., 2017) who supported dissimilarity of the two. However, the Italian samples were rather diverse themselves, since 18 Italian sequences (haplotypes included amongst them) were present in 11 haplotypes and their Haplotype diversity (Hd) was 0.928. Moreover, the Greek sequences showed genetic affinity to Pavia, Rimini and Trentino ones from the 6 total regions sampled, while even sequences from these regions were part of other haplotypes too. It is noteworthy that, having found such low diversity in the Greek *COI* sequences, the possibility that the Greek *Aedes albopictus* populations contain low amount of genetic variation cannot be ruled out. This could be the result of *Wolbachia* infection which due to cytoplasmic incompatibility (CI) cause the reduction of the mtDNA diversity (see 1.3.3.1) and the contrast with the claim of (Goubert et al., 2016) about the majority of genetic variation residing 'within populations' is apparent. In this manner, the presence of *Wolbachia* needs to be determined in the mosquito samples that were used [e.g. as carried out by (Ahmad et al., 2017)]. Also, the reason for this reduced genetic diversity could be merely the fact that the introduction is recent when compared to other introductions (e.g. the highly diverse Italian populations which were introduced in 1990).

5. Supplementary Material

The 18 merged sequences used for the analysis (see Table 4)

Athens 1 (1441 bp)

GGTCMAMMMMAAACATAAAGATATTGGAACATTATACTTTATTTTCGGTATTTG
ATCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGACAT
CCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCATGC
TTTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAAACT
GACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAA
TATAAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTATAGT
AGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGAACA
GCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGGAAT
CTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCAG
CTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACAGCT
ATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTAACA
GACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTATTTT
ATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTCTGCC
AGGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGGAAAC
TTTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGATTTA
TTGTATGAGCCCATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGCTTAT
TTTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTAGTTG
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TAGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGCTAAT
TCATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTCATTA
TGTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGATATC
CATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTACAAT
AATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAGCCG
GAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATTATT
TCTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTATTT
GAGAAAGAATAATTACACAACGAACTCCTYYTTTYYCCCMWAT

Athens 2 (1442 bp)

TGTGKYMMMMMMMAACATAAAGATATTGGAACATTATACTTTATTTTCGGTAT
TTGATCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGA
CATCCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCA
TGCTTTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAA
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AGTAGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGA
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AATCTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGAT
CAGCTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACA
GCTATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTA
ACAGACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTA
TTTTATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTC
TGCCAGGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGG
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TTATTTTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTA

GTTGATTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGA
TCTTTAGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGC
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ATTATGTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGA
TATCCATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTAC
AATAATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAG
CCGGAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATT
ATTTCTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTA
TTTGAGAAAGAATAATTACACAACGAACTCCTYTTTYYYCCCCTT

Ioannina 1 (1441 bp)

GGGTCAACMMAAACATAAAGATATTGGAACATTATACTTTATTTTCGGTATTTGA
TCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGACATC
CTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCATGCT
TTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAAACTG
ACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAAT
ATAAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTATAGT
AGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGAACA
GCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGGAAT
CTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCAG
CTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACAGCT
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GACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTATTTT
ATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTCTGCC
AGGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGGAAAC
TTTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGATTTA
TTGTATGAGCCCATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGCTTAT
TTTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTAGTTG
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CATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTACAAT
AATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAGCCG
GAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATTATT
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GAGAAAGAATAATTACACAACGAACTCCYTTTTYYYYCCCYAT

Ioannina 3 (1443 bp)

TGGTCAAMCMAAACATAAAGATATTGGAACMTTATACTTTATTTTCGGTATTTGA
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CTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCATGCT
TTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAAACTG
ACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAAT
ATAAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTATAGT
AGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGAACA
GCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGGAAT
CTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCAG
CTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACAGCT
ATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTAACA
GACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTATTTT
ATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTCTGCC
AGGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGGAAAC
TTTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGATTTA
TTGTATGAGCYCATCATATRTTCACAGTTGGTATAGATGTTGATACTCGAGCTTAT
TTTACRTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTAGTTG

ATTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGATCTT
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TGTATTATCTATAGGAGCAGTATTTGCTATTATAGCARGATTTATTCATTGATATC
CATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTACAAT
AATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAGCCG
GAATACCTCGACGTTACTCTGATTKTCCAGATAGTTATTTAACTTGAAATATTATT
TCTTCTTTAGGAAGAATAAYTTCTTTATTTGCTATTATTKTCTTTTTATTTATTATT
KGAGAAAGAATAATTACACAACGAACTCCTTCKTTTCCCCATTGAA

Ioannina 3 2886r (525 bp) raw sequencing product (used to determine ambiguous bases of Ioannina 3)

TCCAGTCCGACACCAGTGGTGYKATWTASYTKAYCATASCAAATAAAGAAATTA
TTCTTCCTAAAGAAGAAATAATATTT
CAAGTTAAATAACTATCTGGAAAATCAGAGTAACGTCGAGGTATTCCSGCTAATC
CTAAAAAATGTTGAGGAAAAAAAGT
TAAATTAACTCCGATAAATATTATTGTAAATTGAGCTTTTAATCATGAAGGATTTA
TAATTATTCCTGTTAATAATGGAT
ATCAATGAATAAATCCTGCTATAATAGCAAATACTGCTCCTATAGATAATACATA
ATGAAAATGGGCTACTACATAATAT
GTATCGTGAAGTACAATATCAATTGATGAATTASCTAAAATTACTCCGGTTAATC
CCCCAACTGTAAATAAAAAAACAAA
TCCTAAAGATCATAAAAGTGCAGGGCTATATGTTAATTGTGTWCCATGAARAGTA
ACTAWTCAACTAAGAATTATAATTC
CTGTAMGTACMGCAAYAACTATAGTTGCAGACGTGKAAA

Ioannina 6 (1444 bp)

GTGKYMAMMMMAAACATAAAGATATTGGAACATTATACTTTATTTTCGGTATTT
GATCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGACA
TCCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCATG
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TGACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATA
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CCGGAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATT
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Karditsa 3 (1444 bp)

TGTGKYMMMMMMMAACATAAAGATATTGGAACATTATACTTTATTTTCGGTAT
TTGATCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGA

CATCCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCA
TGCTTTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAA
ACTGACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAA
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AATCTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGAT
CAGCTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACA
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GTTGATTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGA
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ATTATGTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGA
TATCCATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTAC
AATAATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAG
CCGGAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATT
ATTTCTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTA
TTTGAGAAAGAATAATTACACAACGAACTCCYTTTTTYYYCCMWTAT

Karditsa 4 (1438 bp)

GGTCAAMMAAACATAAAGATATTGGAACATTATACTTTATTTTCGGTATTTGATC
TGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGACATCCT
GGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCATGCTTT
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AAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGAACAGC
TCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGGAATCT
CATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCAGCT
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CATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTCATTAT
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ATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTACAATA
ATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAGCCGG
AATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATTATTT
CTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTATTTG
AGAAAGAATAATTACACAACGAACTCCTCTTTTCCCCATSA

Lamia 2 (1443 bp)

TGKKYMMMMMMMMAACATAAAGATATTGGAACATTATACTTTATTTTCGGTAT
TTGATCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGA

CATCCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCA
TGCTTTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAA
ACTGACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAA
TAATATAAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTAT
AGTAGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGA
ACAGCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGG
AATCTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGAT
CAGCTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACA
GCTATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTA
ACAGACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTA
TTTTATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTC
TGCCAGGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGG
AAACTTTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGA
TTTATTGTATGAGCCCATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGC
TTATTTTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTA
GTTGATTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGA
TCTTTAGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGC
TAATTCATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTC
ATTATGTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGA
TATCCATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTAC
AATAATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAG
CCGGAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATT
ATTTCTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTA
TTTGAGAAAGAATAATTACACAACGAACTCTCTYTTTTYYCCCCTT

Lamia 3 (1438 bp)

GGTCAACMAAAACATAAAGATATTGGAACATTATACTTTATTTTCGGTATTTGAT
CTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGACATCC
TGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCATGCTT
TTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAAACTGA
CTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAATAT
AAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTATAGTAG
AAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGAACAGC
TCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGGAATCT
CATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCAGCT
GGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACAGCTAT
TTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTAACAG
ACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTATTTTA
TATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTCTGCCA
GGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGGAAACT
TTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGATTTAT
TGTATGAGCCCATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGCTTATT
TTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTAGTTGA
TTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGATCTTT
AGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGCTAATT
CATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTCATTAT
GTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGATATCC
ATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTACAATA
ATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAGCCGG
AATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATTATTT
CTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTATTTG
AGAAAGAATAATTACACAACGAACTCCTTCTTTTCCCCAT

Lamia 4 (1440 bp)

GGTCAACMAAACATAAAGATATTGGAACATTATACTTTATTTTCGGTATTTGATC
TGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGACATCCT

GGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCATGCTTT
TATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAAACTGAC
TAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAATAT
AAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTATAGTAG
AAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGAACAGC
TCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGGAATCT
CATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCAGCT
GGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACAGCTAT
TTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTAACAG
ACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTATTTTA
TATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTCTGCCA
GGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGGAAACT
TTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGATTTAT
TGTATGAGCCCATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGCTTATT
TTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTAGTTGA
TTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGATCTTT
AGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGCTAATT
CATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTCATTAT
GTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGATATCC
ATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTACAATA
ATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAGCCGG
AATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATTATTT
CTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTATTTG
AGAAAGAATAATTACACAACGAACTCCTCTYTTYCCCCAASGG

Larisa 1 (1443 bp)

TGTGTYMAMMMMMAACATAAAGATATTGGAACATTATACTTTATTTTCGGTATT
TGATCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGAC
ATCCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCAT
GCTTTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAAA
CTGACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAAT
AATATAAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTAT
AGTAGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGA
ACAGCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGG
AATCTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGAT
CAGCTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACA
GCTATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTA
ACAGACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTA
TTTTATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTC
TGCCAGGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGG
AAACTTTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGA
TTTATTGTATGAGCCCATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGC
TTATTTTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTA
GTTGATTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGA
TCTTTAGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGC
TAATTCATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTC
ATTATGTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGA
TATCCATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTAC
AATAATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAG
CCGGAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATT
ATTTCTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTA
TTTGAGAAAGAATAATTACACAACGAACTCCTCTYTTTYCCCCMTT

Larisa 4 (1446 bp)

TGTGKYMMMMMMMAACATAAAGATATTGGAACATTATACTTTATTTTCGGTAT
TTGATCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGA

CATCCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCA
TGCTTTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAA
ACTGACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAA
TAATATAAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTAT
AGTAGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGA
ACAGCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGG
AATCTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGAT
CAGCTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACA
GCTATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTA
ACAGACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTA
TTTTATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTC
TGCCAGGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGG
AAACTTTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGA
TTTATTGTATGAGCCCATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGC
TTATTTTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTA
GTTGATTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGA
TCTTTAGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGC
TAATTCATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTC
ATTATGTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGA
TATCCATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTAC
AATAATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAG
CCGGAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATT
ATTTCTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTA
TTTGAGAAAGAATAATTACACAACGAACTTYYTTTTYYYYYCCCYYTAT

Trikala 1 (1442 bp)

GKGTYMAMMMMAACATAAAGATATTGGAACATTATACTTTATTTTCGGTATTTG
ATCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGACAT
CCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCATGC
TTTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAAACT
GACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAA
TATAAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTATAGT
AGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGAACA
GCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGGAAT
CTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCAG
CTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACAGCT
ATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTAACA
GACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTATTTT
ATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTCTGCC
AGGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGGAAAC
TTTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGATTTA
TTGTATGAGCCCATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGCTTAT
TTTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTAGTTG
ATTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGATCTT
TAGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGCTAAT
TCATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTCATTA
TGTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGATATC
CATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTACAAT
AATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAGCCG
GAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATTATT
TCTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTATTT
GAGAAAGAATAATTACACAACGAACTCTCYYTTTTYCCCCMWTT

Trikala 4 (1442 bp)

GGGTCAACMAAACATAAAGATATTGGAACATTATACTTTATTTTCGGTATTTGAT
CTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGACATCC

TGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCATGCTT
TTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAAACTGA
CTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAATAT
AAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTATAGTAG
AAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGAACAGC
TCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGGAATCT
CATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCAGCT
GGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACAGCTAT
TTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTAACAG
ACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTATTTTA
TATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTCTGCCA
GGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGGAAACT
TTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGATTTAT
TGTATGAGCCCATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGCTTATT
TTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTAGTTGA
TTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGATCTTT
AGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGCTAATT
CATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTCATTAT
GTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGATATCC
ATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTACAATA
ATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAGCCGG
AATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATTATTT
CTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTATTTG
AGAAAGAATAATTACACAACGAACTCCTCYYTTTYCCCMTTAAA

Volos 2 (1444 bp)

GGGTCMACCMAAACATAAAGATATTGGAACATTATACTTTATTTTCGGTATTTGA
TCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGACATC
CTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCATGCT
TTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAAACTG
ACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAAT
ATAAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTATAGT
AGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGAACA
GCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGGAAT
CTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCAG
CTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACAGCT
ATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTAACA
GACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTATTTT
ATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTCTGCC
AGGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGGAAAC
TTTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGATTTA
TTGTATGAGCCCATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGCTTAT
TTTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTAGTTG
ATTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGATCTT
TAGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGCTAAT
TCATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTCATTA
TGTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGATATC
CATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTACAAT
AATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAGCCG
GAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATTATT
TCTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTATTT
GAGAAAGAATAATTACACAACGAACTCCCTTTTTTYCCCCTTAGCT

Volos 4 (1805 bp)

TGGGTCAACAAAATCATAAAGATATTGGAACMTTATACTTTATTTTCGGTATTTG
ATCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGACAT

CCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCATGC
TTTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAAACT
GACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAA
TATAAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTATAGT
AGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGAACA
GCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGGAAT
CTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCAG
CTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACAGCT
ATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTAACA
GACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTATTTT
ATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTCTGCC
AGGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGGAAAC
TTTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGATTTA
TTGTATGAGCCCATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGCTTAT
TTTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTAGTTG
ATTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGATCTT
TAGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGCTAAT
TCATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTCATTA
TGTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGATATC
CATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTACAAT
AATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAGCCG
GAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATTATT
TCTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTATTT
GAGAAAGAATAATTACACAACGAACTCTCYTTTYCCCCATTGAARWAAWAAAA
WAGCTGTWAWTTWCYACYGATCACMCAATAAAGGTAGTCGATCAAGAGTAATA
SCAGCTGATCSGAWATTAATACWGTTGTAATAAAWTTACTGCTCCTAARATASAT
GAAATTCCCGCTMMYGTAAGAAAAATTGCTAAATCAACTGAGYCCCAGCATGAS
YGGTGCRGASAAGAGGARGATAAACGTCACCTGTCAGCTCGTTTCTCTATGACTA
WAGCAGCATGTCAAGAGGGGTARAGTCAGACTATTATTWTTYGAGTAAGCTAGT
CAGCTCCTAGMTAGGTMAGYAGTGCAATCCCCATTGAGCTACAGAGATCATAAG
CCWGACAGTACMGTTGMCCTGTACATSA

Patras 1 (1442 bp)

TGTGKYMMMMMMMAACATAAAGATATTGGAACATTATACTTTATTTTCGGTAT
TTGATCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGA
CATCCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCA
TGCTTTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAA
ACTGACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAA
TAATATAAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTAT
AGTAGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGA
ACAGCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGG
AATCTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGAT
CAGCTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACA
GCTATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTA
ACAGACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTA
TTTTATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTC
TGCCAGGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGG
AAACTTTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGA
TTTATTGTATGAGCCCATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGC
TTATTTTACGTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTA
GTTGATTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGA
TCTTTAGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGC
TAATTCATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTC
ATTATGTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGA
TATCCATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTAC

AATAATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAG
CCGGAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATT
ATTTCTTCTTTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATTA
TTTGAGAAAGAATAATTACACAACGAACTCCTCTYTTTYCCCMTT

Patras 2 (1446 bp)

TGTGKYMMMMMMMAAAATAAAGATATTGGAACATTATACTTTATTTTCGGTAT
TTGATCTGGAATAGTCGGAACTTCACTAAGAGTTTTAATTCGTATTGAACTTAGA
CATCCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGCTCA
TGCTTTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAA
ACTGACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTYCCTCGAATAAA
TAATATAAGTTTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTAT
AGTAGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCTCTTTCTTCTGGA
ACAGCTCATGCTGGRGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGG
AATCTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGAT
CAGCTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACA
GCTATTTTATTACTTCTTTCYCTACCCGTATTAGCCGGAGCTATTACTATATTATTA
ACAGACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTA
TTTTATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTC
TGCCAGGATTTGGAATAATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGG
AAACTTTTGGTACTTTAGGAATAATTTATGCTATATTAACAATTGGCTTATTAGGA
TTTATTGTATGAGCYCATCATATRTTYACAGTTGGTATAGATGTTGATACTCGAGC
TTATTTYACRTCTGCAACTATAATTATTGCGGTACCTACAGGAATTAAAATTTTTA
GTTGATTAGCTACTCTTCATGGAACACAATTAACATATAGCCCTGCACTTTTATGA
TCTTTAGGATTTGTTTTTTTATTTACAGTTGGGGGATTAACCGGAGTAATTTTAGC
TAATTCATCAATTGATATTGTACTTCACGATACATATTATGTAGTAGCCCATTTTC
ATTATGTATTATCTATAGGAGCAGTATTTGCTATTATAGCAGGATTTATTCATTGA
TATCCATTATTAACAGGAATAATTATAAATCCTTCATGATTAAAAGCTCAATTTAC
AATAATATTTATCGGAGTTAATTTAACTTTTTTTCCTCAACATTTTTTAGGATTAG
CCGGAATACCTCGACGTTACTCTGATTTTCCAGATAGTTATTTAACTTGAAATATT
ATTTCTTCTWTAGGAAGAATAATTTCTTTATTTGCTATTATTTTCTTTTTATTTATT
ATTTGAGAAAGAATAATTACACAACGAACTCCTCYYTTTYCMCCMWTAAA

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