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Invasive mosquito species *Aedes albopictus* and *Aedes aegypti* on the Black Sea coast of the Caucasus: genetics (*COI*, ITS2), *Wolbachia* and *Dirofilaria* infections

E.V. Shaikevich[1] ✉, I.V. Patraman[2], A.S. Bogacheva[3], V.M. Rakova[2], O.P. Zelya[2], L.A. Ganushkina[2]

[1] Vavilov Institute of General Genetics, RAS, Moscow, Russia
[2] Institute of Medical Parasitology and Tropical Medicine named after E. Martsinovskii, Sechenov First Moscow State Medical University, Moscow, Russia
[3] Moscow State University, Moscow, Russia

The area of invasive species *Aedes albopictus* and *Aedes aegypti* is expanding. Precise identification and understanding of the genetic diversity of invasive mosquito populations allows us to develop appropriate control methods. Endosymbiotic bacterium *Wolbachia pipientis* has different effects on their arthropod hosts and can influence the transmission and spread of the pathogens. The objective of the presented study was molecular-genetic identification of the *Aedes* mosquitoes collected in sampling sites on the Black Sea coast from 2007 to 2017; determination of genetic variability of *Ae. aegypti*, *Ae. albopictus* and their symbiotic bacteria *Wolbachia*; assessment of mosquitoes ability to be infected and to spread parasitic *Dirofilaria*. Another objective was obtaining the genetic characteristic of laboratory strain *Ae. aegypti* IMPITM. We investigated two markers of nuclear and mitochondrial DNA from *Ae. albopictus* and *Ae. aegypti* and compared them to DNA from *Ae. cretinus* and *Ae. koreicus* sympatrically inhabiting the territory, as well as to one of *Ae. aegypti* from a laboratory line. The study of nuclear and mitochondrial DNA revealed a low level of variability in the invasive mosquitoes *Ae. albopictus* and *Ae. aegypti* collected at different collection sites and in different years. More than a half of *Ae. albopictus* were infected with *Wolbachia*, two strains of bacteria, *w*AlbA and *w*AlbB, occur in the *Ae. albopictus* population on the Black Sea coast. Total infection of *Ae. aegypti* and *Ae. albopictus* with dirofilariae was 1.8 %. *Dirofilaria immitis* was found only in mosquito abdomen, larvae of infective stage L3 were not found. *D. repens* larvae developed to the infective stage in the mosquitoes of both species.

Key words: blood-sucking mosquitoes; *Aedes aegypti*; *Aedes albopictus*; invasion; population; Black Sea coast; ITS2; *COI*; *Wolbachia*; *Dirofilaria*.

Инвазивные виды *Aedes albopictus* и *Aedes aegypti* на Черноморском побережье Краснодарского края: генетика (*COI*, ITS2), зараженность *Wolbachia* и *Dirofilaria*

Е.В. Шайкевич[1] ✉, И.В. Патраман[2], А.С. Богачева[3], В.М. Ракова[2], О.П. Зеля[2], Л.А. Ганушкина[2]

[1] Институт общей генетики им. Н.И. Вавилова РАН, Москва, Россия
[2] Институт медицинской паразитологии, тропических и трансмиссивных заболеваний им. Е.И. Марциновского Первого Московского государственного медицинского университета им. И.М. Сеченова (Сеченовский университет) Министерства здравоохранения РФ, Москва, Россия
[3] Московский государственный университет им. М.В. Ломоносова, Москва, Россия

Ареал инвазивных видов *Aedes aegypti* и *Aedes albopictus*, переносчиков ряда трансмиссивных инфекций, расширяется. Идентификация видов-переносчиков и понимание генетического разнообразия инвазивных популяций позволяют разработать соответствующие профилактические мероприятия. Эндосимбиотическая бактерия *Wolbachia pipientis* оказывает различные эффекты на своих хозяев-артропод и может влиять на процесс передачи и распространения возбудителей. Основной целью работы была молекулярно-генетическая идентификация видов комаров рода *Aedes*, собранных в населенных пунктах Черноморского побережья с 2007 по 2017 г.; определение генетической изменчивости *Ae. aegypti*, *Ae. albopictus* и их симбиотической бактерии *Wolbachia*; оценка способности *Ae. aegypti* и *Ae. albopictus* к заражению и распространению паразитических *Dirofilaria*. Отдельной задачей являлась генетическая характеристика лабораторной линии *Ae. aegypti* ИМПиТМ, которая поддерживается в лаборатории в течение 50 лет. Исследованы маркеры ядерной и митохондриальной ДНК у *Ae. albopictus* и *Ae. aegypti* и проведено их сравнение с *Ae. cretinus* и *Ae. koreicus*, симпатрически обитающими на данной территории, а также с *Ae. aegypti* лабораторной линии. Обнаружен низкий уровень изменчивости *Ae. albopictus* и *Ae. aegypti*, собранных в природе в разных точках сбора и в разное время. У *Ae. albopictus* выявлены четыре гаплотипа на основе сравнения вариабельной области внутреннего транскрибируемого спейсера (ITS2) кластера генов рРНК и два митохондриальных гаплотипа при сравнении последователь-

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✉ e-mail: elenashaikevich@mail.ru

ностей гена первой субъединицы цитохромоксидазы (*COI*). У *Ae. aegypti*, собранных в природе, обнаружены четыре гаплотипа ядерной ДНК и три митохондриальных гаплотипа. Более половины *Ae. albopictus* заражены *Wolbachia*. В популяции на Черноморском побережье Краснодарского края встречаются два штамма бактерии: *w*AlbA и *w*AlbB. Общая зараженность комаров *Ae. aegypti* и *Ae. albopictus* дирофиляриями составила 1.8 %. *Dirofilaria immitis* обнаружены только в брюшках комаров, развития личинок до инфекционной стадии L3 не выявлено. Личинки *D. repens* развились до инфекционной стадии в комарах обоих видов.

Ключевые слова: кровососущие комары; *Aedes aegypti*; *Aedes albopictus;* инвазия; популяция; Черноморское побережье Кавказа; ITS2; *COI*; *Wolbachia*; *Dirofilaria*.

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At the beggining of the 21st century, expansion of two mosquito species – *Aedes* (Stegomiya) *aegypti* (Linnaeus, 1796) and *Aedes* (Stegomiya) *albopictus* (Skuse, 1895) – was registered in Krasnodarskiy Region. These are associated with numerous epidemic outbreaks of denge, chikungunya, zika viral infections, etc. *Ae. aegypti* also acts as a primary carrier for yellow fever (Jeffries, Walker, 2016). Beside arboviruses, both mosquito species are able to transmit the threadworm larvae of *Dirofilaria* family responsible for dirofilariasis in humans and animals (Ganushkina et al., 2014a). This transmissible helmintosis demonstrates an expansive trend in the territory of Russia (Bogacheva et al., 2017).

Ae. aegypti had been absent in Russia for 50 years, until 2001 (Ryabova et al., 2005; Yunicheva et al., 2008) due to the special measures taken in the USSR in the 1920s and 1930s aimed at eradication of the aforementioned species (Martsinovsky, 1929). The measures were taken considering the extreme danger of this carrier and occurrence of substantial high-mortality dengue outbreaks in the countries of South Europe.

Later in the 20th century, greater concerns aroused worldwide about the expansion of *Ae. albopictus*, another effective carrier of dangerous arboviruses, which started to spread beyond its natural habitat in Southeast Asia. This mosquito species expanded into areas, previously inhabited by other endemic species, like morphologically similar *Ae. cretinus* Edwards (Patsoula et al., 2006). For the first time, this species was transferred to Albania from China in the middle of the 1970s (Adhami, Reiter, 1998). Currently this species of mosquito is found in more than 15 countries, and its habitat is growing persistently (Medlock et al., 2015). *Ae. albopictus* are able to cause outbreaks of dengue and chicagunya infections even in absence of *Ae. aegypti* (Delatte et al., 2008; Delisle et al., 2015; Calba et al., 2017; Chuchuy et al., 2018). In the Russian Federation these species were first found in 2011 (Ganushkina et al., 2012). Currently, the Caucasus coast of the Black Sea is the only territory in the WHO European region that geographically includes the Asian region of the Caucasus where the both mosquito species have been registered as active vectors for arbovirus infectious agents (http://www.who.int/about/regions/euro/).

Hematophagous mosquito species may vary in pathogen transmission capability. Their morphological markers used for authentication could be either unclear, or missed or erased during storage of adult specimens, which results in identification errors. For example, cytochrome gene sequence MF148262 annotated in Gene Bank as being derived from *Ae. albopictus* originated in Malaysia, was actually associated with *Ae. aegypti*. For that reason, DNA analysis for verification of the population's structure and diversity had to be performed to devise necessary preventive measures.

Dirofilariasis caused by *Dirofilaria immitis* and *D. repens* is endemic to the Southern parts of Russia (Sergiev et al., 2014; Kartashev et al., 2018). In assessment of epidemiological situation one uses xenomonitoring for entomological control over filariasis infections (Ganushkina et al., 2014a). Dirofilaries are identified by means of total DNA amplification of vector mosquito with specific primers. This method shows fillaries at all stages of development (L1, L2, L3), while not every filaria reaches the pathogenic L3 stage, which can be transmitted through salivary glands of a mosquito to human and animal hosts. Microfilaria development to stage L3 requires certain thermal conditions, therefore the disease is primarily limited to southern regions. *Dirofilaria* can be found in mosquitoes of the following genera: *Aedes*, *Anopheles*, *Ochlerotatus* and *Culex* (Bochková et al., 2013; Kronefeld et al., 2014; Bogacheva et al., 2017). During monitoring, taxonomical study of mosquitoes can be frequently limited to the genus level. *Dirofilaria* identification, specialization towards mosquito species and determining the invasive stage of *Dirofilaria* inside the mosquito are aimed towards discovering the actual epidemiological significance of various mosquito species (Ganushkina et al., 2014a). Major attention in latest research has been paid to discovering the role of endosymbiotic bacteria in the mosquito and other arthropods in the host survivability, as well as in processes of transmission

and spread of infectious diseases (Bourtzis et al., 2014; Jeffries, Walker, 2016).

The main objectives of the presented research were molecular genetics identification of *Ae. albopictus* and *Ae. aegypti* collected in the populated areas of the Black Sea coast from 2007 to 2017; determination of their gene diversity; characterization of sympatric species *Ae. cretinus* and *Ae. koreicus*; measuring of symbiotic bacteria *Wolbachia* appearence in investigated mosquito samples and of *Wolbachia* strains diversity within the population of *Ae. albopictus*; evaluation of the capability of *Ae. albopictus* and *Ae. aegypti* to vector and spread parasitic *Dirofilaria*. Within the framework of the study one also performed genetic sampling of the laboratory line of *Ae. aegypti* IMPITM cultivated for 50 years.

Materials and methods

Collecting the mosquitoes. The mosquitoes were collected on the North Caucasus shore in years 2007, 2011–2013 and 2015–2017 (Table 1, Fig. 1). *Ae. albopictus* and *Ae. aegypti* larvae were picked in temporal water basins using a scoop-net. A part of the larvae was preserved in alcohol, while the others were raised to adults. The adult mosquitoes were caught either with an Electrofrog trap (LMD-Komplekt plus, Russia), or "on self" with an exhauster and preserved dry. More details on the mosquito collection protocol can be found in (Ganushkina et al., 2013, 2016). The total collection consisted of 3 005 specimens: 1 430 *Ae. aegypti* and 1 575 *Ae. albopictus*.

Molecular genetic analysis included mosquitoes from the IMPITM lab line, four dried adult *Ae. cretinus* from the IMPITM museum and five *Ae. koreicus* collected in Sochi in 2013. The *Ae. cretinus* and *Ae. koreicus* were used to determine the genetic differences between morphologically similar species of the *Aedes* genus, which could potentially present in the collections, collected in the territory in question. *Ae. cretinus* is endemic towards the Black Sea coast of the Caucasus. *Ae. koreicus* originating from Southeast Asia, have been found in the area since 2013 (Ganushkina et al., 2016).

Determining species of mosquitoes. Species identification of all the mosquito samples was conducted with account for the morphology data (Gutsevich et al., 1970) and using molecular genetic technologies. Up to 30 specimens from each place and year were used for PCR identification of second inner transcribable spacer of pRNA gene cluster (ITS2). Characteristic to *Ae. albopictus* is PCR sequence of 500 bp in size, for *Ae. koreicus* – 450 bp, for *Ae. cretinus* – 390 bp, and for *Ae. aegypti* – 340 bp.

Mosquito identification by gene sampling. DNA extraction from the mosquitoes was accomplished using DIAtom DNA Prep (Izogen, Moscow). PCR identification was conducted with the Evrogen Encyclo PCR kit (Evrogen, Moscow). For the ITS2 amplification primers 5,8S and 28S were used (Porter, Collins, 1991). A cytochrom oxidase I sequence (*COI*) of nearly 750 bp in length was built up using primers TY-J-1460 (Simon et al., 1994) and COIR (Shaikevich, 2007). Amplificates

Table 1. Years and points of *Ae. aegypti* and *Ae. albopictus* sampling

Year	Sampling site	Geographical coordinates	Number of individuals	
			Ae. aegypti	*Ae. albopictus*
2007	Adler	43°25′44″ N, 39°55′26″ E	52	0
	Sochi	43°35′07″ N, 39°43′13″ E	89	0
	Lazarevskoye	43°54′31″ N, 39°19′52″ E	25	0
	Tuapse	44°06′19″ N, 39°04′48″ E	23	0
2011	Hosta	43°30′53″ N, 39°52′05″ E	1	16
2012	Adler	43°25′44″ N, 39°55′26″ E	3	24
	Hosta	43°30′53″ N, 39°52′05″ E	0	47
	Sochi	43°35′07″ N, 39°43′13″ E	6	116
	Mamaika	43°38′35″ N, 39°42′34″ E	48	406
	Dagomys	43°40′11″ N, 39°40′07″ E	0	24
	Lazarevskoye	43°54′31″ N, 39°19′52″ E	31	48
	Tuapse	44°06′19″ N, 39°04′48″ E	566	28
	New Afon	43°04′50″ N, 40°50′17″ E	45	6
	Pizunda	43°09′43″ N, 40°20′27″ E	7	58
2013	Adler	43°25′44″ N, 39°55′26″ E	6	164
	Hosta	43°30′53″ N, 39°52′05″ E	0	23
	Sochi	43°35′07″ N, 39°43′13″ E	3	46
	Mamaika	43°38′35″ N, 39°42′34″ E	3	34
	Lazarevskoye	43°54′31″ N, 39°19′52″ E	17	19
	Tuapse	44°06′19″ N, 39°04′48″ E	394	21
2015	Adler	43°25′44″ N, 39°55′26″ E	0	120
	Tuapse	44°06′19″ N, 39°04′48″ E	30	20
2016	Dagomys	43°40′11″ N, 39°40′07″ E	0	256
2017	Adler	43°25′44″ N, 39°55′26″ E	81	32
	Sochi	43°35′07″ N, 39°43′13″ E	0	67
	Total		1430	1575

were visualized in 1–2 % agarous gel and purified using a clean-up extraction kit (Evrogen, Moscow) followed by sequencing with the BigDye Termination kit 3.1 (Applied Biosystems, USA). Thirteen ITS2 amplificates of *Ae. aegypti* и *Ae. albopictus* were sequenced (1–2 samples from 10 collection sites for various years), including *Ae. cretinus* and *Ae. aegypti* taken in the amount of one sample from each line. Mitochondrial DNA variability was studied using 28 sequenced sequences of the *COI* gene, 634 bp in length, from 1–4 specimens collected at 1–4 sites and four specimens from the *Ae. aegypti* lab line. The sequences were registered in Gene Bank. The *COI* gene: *Ae. aegypti* MG198586–MG198594, MH251909–MH251911; *Ae. albopictus* MG198595–MG198606; *Ae. aegypti* IMPITM MH023409 и ITS2: *Ae. aegypti* MH142316–MH142320; *Ae. albopictus* MH142321–MH142326; *Ae. aegypti* IMPITM MH142327; *Ae. cretinus* MH142328.

Data analysis. Gene sequence analysis was performed using the following software: ChromasPro, BLASTN, ClustalW, MAFFT v.6, MEGA v.6. Phylogenetic trees were built using the Neighbor-Joining technique, the evolution distances were calculated by maximum composite likelihood method using MEGA v.6 program (Tamura et al., 2013). The DNA sequences of *Ae. albopictus* and *Ae. aegypti* closest to the extracted ones, as well as ones characteristic for certain regions were selected in Gene Bank (https://www.ncbi.nlm.nih.gov/) for the purpose of comparative analysis. Their registry numbers can be found on the diagrams. Sample collection time periods are indicated in the annotations, where it is possible. The statistical reliability of filogenetic tree branches was analyzed using the bootstrap method (1 000 iterations). Evolutionary divergence level between the sequences was evaluated using the MEGA v.6 software (Tamura et al., 2013).

***Wolbachia* contamination.** Identification of the *Wolbachia* symbiotic bacteria was carried out using specific primers for bacterial surface protein gene *wsp* (81F and 691R, see Braig et al., 1998). In order to separate the two strains, multiprimer PCR was used (Zhou et al., 1998): primers 383F and 183F were paired with wsp-691R to separate the *w*AlbA and *w*AlbB strains of *Wolbachia* in *Ae. albopictus.* The PCR fragment corresponding to the *w*AlbA strain was one of 379 bp, and to the *w*AlbB strain – of 501 bp. Validity evaluation for the received data on bacterial contamination in the samples was carried out using the Fischer accuracy test with the error margin set for $N > 10$ (Tokarev et al., 2017).

Susceptibility to *Dirofilaria*. Only hemotrophic gonoactive female mosquitoes collected in the wild were used for analysis. In order to determine dirofilaria contamination in mosquito pools, the mosquito imagos were dissected, their abdomen and head-thorax parts separated, and 2 to 7 sample mosquitoes were grouped into pools based on their collection time and place. In the head-thorax parts, L3 larvae were registered. Contamination of mosquitoes with *Dirofilaria* larvae was revealed by the amplification of the ITS2 area using the DIDR-F1 and DIDR-R1 primers (Rishniw et al., 2006). PCR sequence size, specific

Fig. 1. Sampling points on the Caucasian coast of the Black Sea.

to *D. immitis* was 542 bp, for *D. repens* – 484 bp. For *Dirofilaria* DNA screening was conducted in pools and not individually, contamination was evaluated using the common MIR (minimum infection rate) method. The value was calculated based on the assumption that at least one mosquito specimen in the pool was infected with *Dirofilaria* with the minimum infection rate calculated as the number of positive pools divided by the total specimen quantity and expressed as a percentage (Cancrini et al., 2003).

Results

Geography of sampling

Mosquitoes *Ae. aegypti* and *Ae. albopictus* were collected on the coast of the Black Sea of Krasnodarskiy Region from Adler to Tuapse in years 2007, 2011–2013 and 2015–2017 (see Table 1). In 2012, the specimens of these species were additionally gathered in the Republic of Abkhazia near Pizunda and New Afon (see Fig. 1). In 2007 *Ae. albopictus* were absent in the region, while in four sampling sites from Adler to Tuapse *Ae. aegypti* were common (see Table 1). Starting 2011, *Ae. albopictus* were present in each pool from every populated area. Moreover, the quantity of collected *Ae. albopictus* was vastly superior to the one of *Ae. aegypti* in the area from Adler to Dagomys. In years 2012, 2013 and 2016 *Ae. aegypti* were not registered in Hosta and Dagomys. However, *Ae. aegypti* prevailed numerically in Tuapse, the northern part of the region, in years 2012 and 2013.

In year 2017 collection was conducted only in Sochi and Adler, and the larvae and imagos were attributed solely to *Ae. albopictus*. In the pools of adult mosquitoes no *Ae. aegypti* specimen was present, as no *Ae. aegypti* larvae were found in typical breeding grounds of this species (various small artificial basins filed with water: barrels, cans, decorative pools, old dishware, tires). A sample from a dried car tire found in Adler in August 2017 contained preserved viable eggs of *Ae. aegypti* and *Ae. albopictus* with the prevalence of *Ae. aegypti,* which later developed in the lab into larvae and imagos.

Table 2. Variable sites in the ITS2 region of *Ae. aegypti*

GenBank annotation	Origin	Variable nucleotide sites*													
		2 5 3	2 5 4	2 5 5	3 4 9	3 5 1	3 5 2	3 5 3	3 5 4	3 5 5	3 5 6	3 5 7	3 5 8	4 9 2	4 9 4
MH142327	IMPITM	G	T	G	A	C	T	A	A	C	T	A	G	T	T
MH142320	Sochi, 2007	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MH142318	Mamaika, 2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MH142317	Tuapse, 2012	.	.	A	.	.	.	.	.	.	.	.	.	.	.
MH142326	Adler, 2017	–	–	.	C	.	.	.	.	.	.	.	A	G	.
MH142316	New Afon, 2012	–	–	.	.	–	–	–	–	–	–	–	–	.	C
MH142319	Adler, 2013	–	–	.	.	–	–	–	–	–	–	–	–	.	C

Note: * The nucleotide positions are indicated relative to the MH142327 sequence. In Tables 2 and 3, the points denote the nucleotides identical to those indicated in the first line, dashes – the absence of nucleotides in the sequence (deletions).

Table 3. Variable sites in the ITS2 region of *Ae. albopictus*

Gene Bank annotation	Origin	Variable nucleotide sites*			
		3 0 8	3 3 1	3 6 3	3 6 4
MH142321	Hosta, 2011	G	T	G	C
MH142322	Pizunda, 2012	.	.	.	.
MH142323	Pizunda, 2012	A	C	–	–
MH142324	Tuapse, 2013	A	C	.	.
MH142325	Adler, 2017	.	C	.	.

* The nucleotide positions are indicated relative to the MH142321 sequence.

Table 4. Estimates of average evolutionary divergence over ITS2/*COI* sequence pairs between and within the studied *Aedes*

Species	*Ae. aegypti*	*Ae. albopictus*	*Ae. cretinus*
Ae. aegypti	0.005* 0.0028*		
Ae. albopictus	0.14 0.37	0.004* 0.0127*	
Ae. cretinus	n. d. 0.30	n. d. 0.38	
Ae. koreicus	n. d. 0.28	n. d. 0.46	n. d. 0.39

Note: The number of base substitutions per site from averaging over all sequence pairs within and between the *Aedes* species is shown. Below the diagonal are differences in ITS2 sequences; above the diagonal – in *COI*.

* Intraspecific differences; n. d. – not defined.

PCR analysis

***Ae. aegypti* lab line.** The *Ae. aegypti* mosquito lab line has been constantly sustained for more than five decades in the laboratory of the Martsinovskiy institute. The line was named "IMPITM" after the abbreviated name of the institute. Parts of the *COI* gene were sequenced for two mosquitoes from two different genres. DNA sequences from all four specimens were identical, one sequence was registered in Gene Bank under number MH023409. Two variable sites G148A and T624a (the site numbers correspond to MH023409) in *COI* separated IMPITM-line from lab cultures *Ae. aegypti* Liverpool (AY432648) and RED (AF390098). As a nuclear marker, ITS2 area from *Ae. aegypti* IMPITM (GenBank annotation MH142327) was sequenced. ITS2 DNA in *Ae. aegypti* IMPITM was identical to DNA of the Rockfeller strain (KF471588).

Nuclear DNA variability in *Ae. aegypti* and *Ae. albopictus*. In *Ae. aegypti* four haplotypes with different single-nucleotide substitutions and two deletions in their ITS2 areas were found (Table 2). The first type was *Ae. aegypti* collected in Sochi (2007) and Big Sochi – Mamaika (2012). The second type was from Tuapse (2012) and differed from the first by one G255A replacement. The third haplotype was discovered in the specimens from New Afon and Adler (2013). This haplotype differed by two deletions (two and eight nucleotides) and one T494C replacement. In the ITS2 area of *Ae. aegypti* collected in Adler (2017) two deleted nucleotides were found, which was similar to the first haplotype, and three nucleotide replacements – A349C, G358A and T492G.

Four variable haplotypes were found in *Ae. albopictus*, different by single-nucleotide mutations and one deletion (Table 3). The earliest in this respect is the Hosta collection (2011). We found an identical haplotype in *Ae. albopictus* from Pizunda (2012). The second similar haplotype was found in another specimen from the same collection (Pizunda, 2012), different by deletions of two nucleotides and replacements in G308A and T331C. The third haplotype of *Ae. albopictus* (Tuapse, 2013), unlike the second, had no deletion. The fourth one was found in *Ae. albopictus* (Adler, 2017) and had one T331C replacement.

Intraspecific ITS2 variability in wild *Ae. aegypti* was 0.3 %, while the variability in wild *Ae. albopictus* was 1.3 % (Table 4). The genetic differences between species

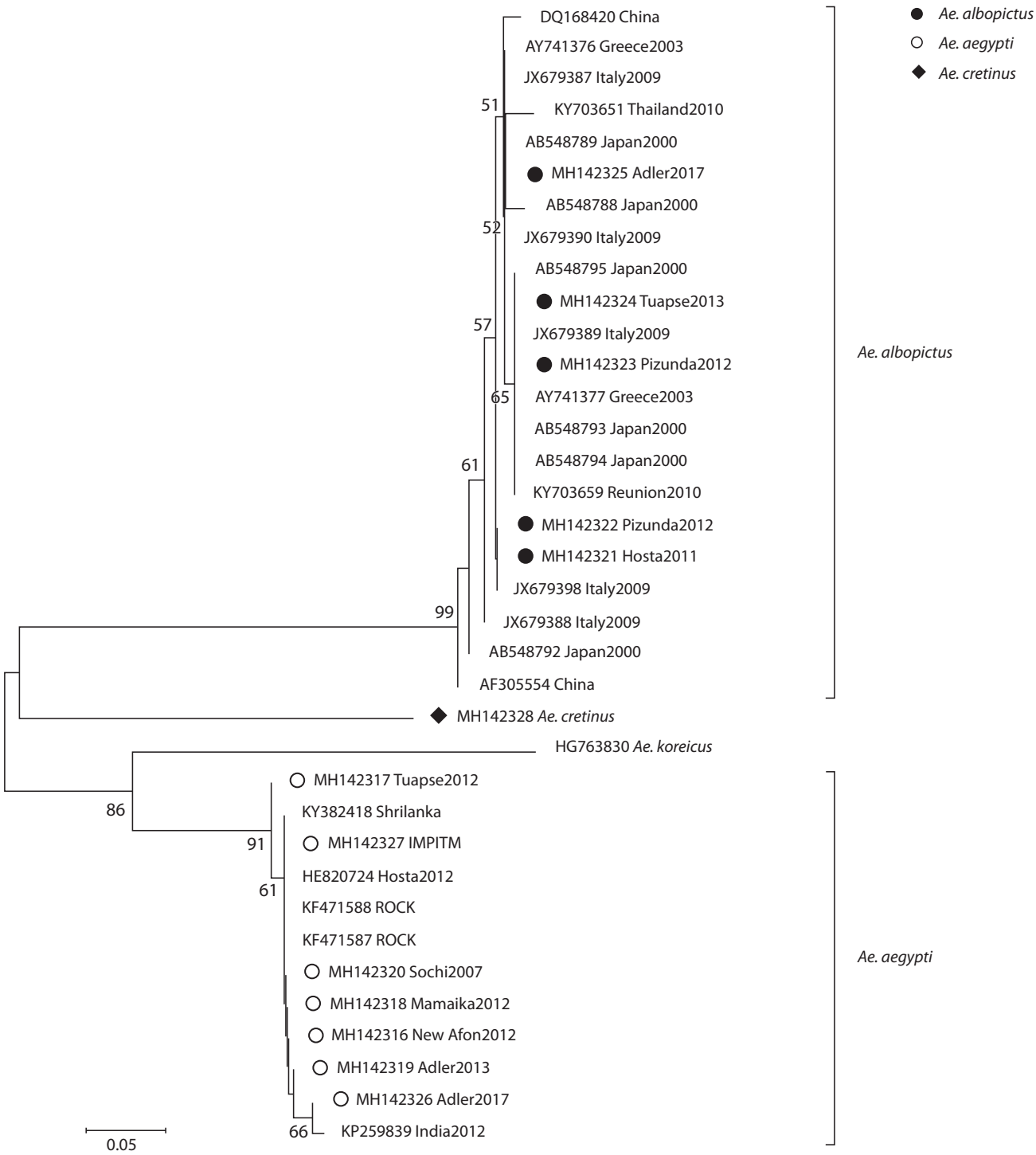

Fig. 2. Similarity dendrogram derived from comparative analysis of the ITS2 areas.
All the deletions were excluded from the analysis.

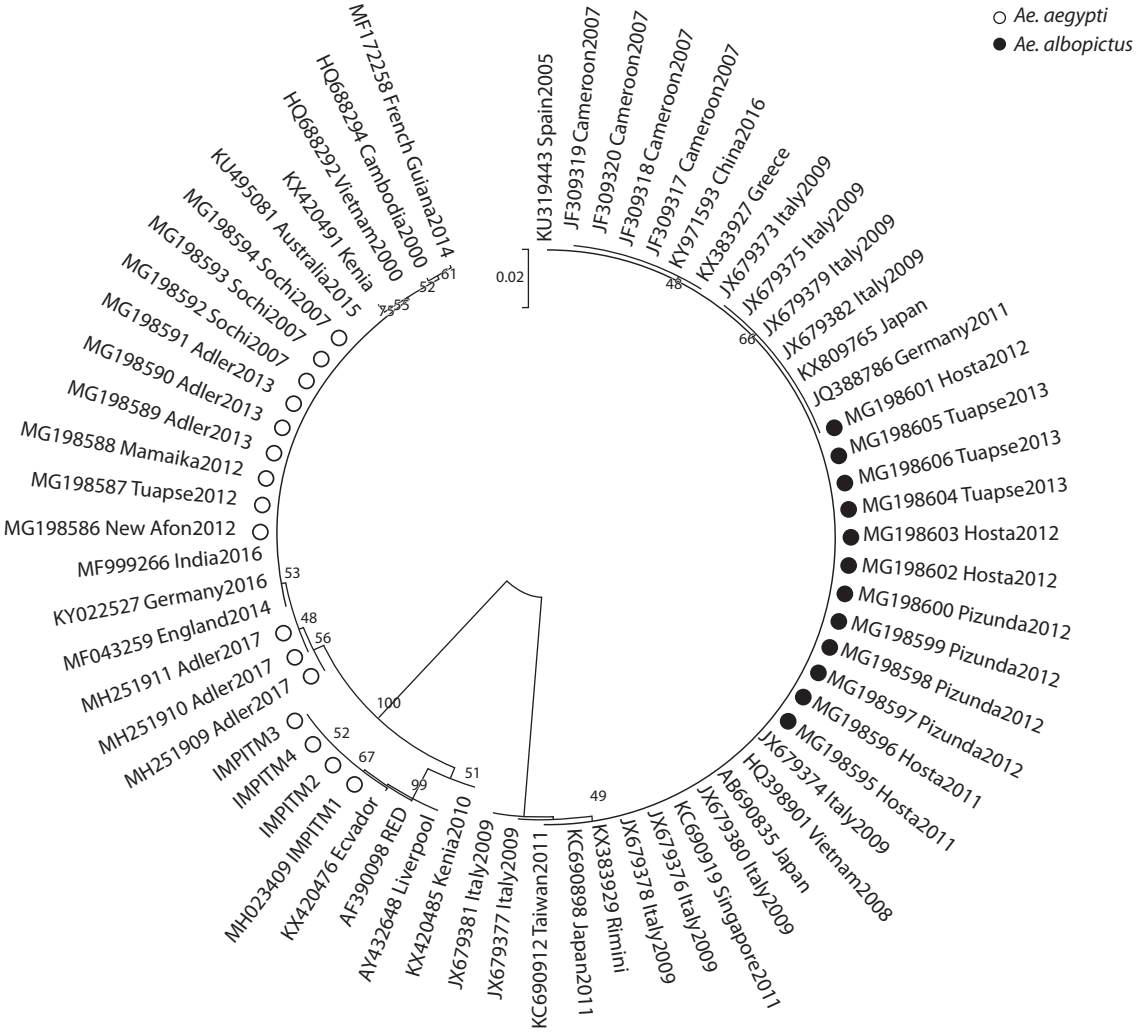

Fig. 3. Similarity dendrogram of the *COI* gene sequences in *Ae. aegypti* and *Ae. albopictus*.

after ITS2 comparison indicated that *Ae. aegypti* was 1.3 times closer to *Ae. koreicus* and *Ae. cretinus* than to *Ae. albopictus* (Table 4).

Comparison of the identified sequences between themselves and with their analogues from Gene Bank showed that, while the ITS2 region was variable in one mosquito species, its identical variants were present in specimens from geographically separated regions (Fig. 2). The ITS2 sequences of each species form different dendrogram clusters, their differences supported by the high values in bootstrap analyses.

Mitochondrial DNA variability in *Ae. aegypti* and *Ae. albopictus*. Nine *COI* sequences from wild *Ae. aegypty* (2007, 2011–2015) were identical. Same variant is present in Gene Bank – annotated *Ae. aegypty* from Cambodia (2000), India, England (2014), French Guyana (2014), Australia (2015), Germany (2016) (Fig. 3). *Ae. aegypti* (Adler, 2017) had two other different mitochondrial haplotypes (MH251909–MH251911) with synonymous nucleotide replacements C48T and additional T189C in MH251909. The genetic diversity between *Ae. aegypti* specimens from the Black sea coast for the *COI* gene was determined to be 0.5 %. The differences between the wild specimens and the lab line included 11 nucleotide substitutes, one of which (G148A) was non-synonymous (see Fig. 3).

Among *Ae. albopictus* species two mitochondrial haplotypes were found. The first one consisted of 11 identical sequences in the specimens (2011–2017) which were also registered in *Ae. albopictus* from Spain (2005), Italy (2009), China, Taiwan (2011), and Japan (2011) (see Fig. 3). The second mitochondrial haplotype that differed from the others by synonymic replacement A79G was aslo found in one mosquito from Hosta (2012) (MG198601). The haplotype identical to second one (79G) had been previously discovered in *Ae. albopictis* from northern Italy (2009), Japan and Germany (2011). The *COI* gene variability among *Ae. albopictus* was 0.4 % (see Table 4).

Endosymbiotic bacteria Wolbachia

The frequency *of Wolbachia* contamination was investigated in 411 specimens of *Ae. albopictus,* 50 specimens of *Ae. aegypti*, 4 – of *Ae. cretinus* and 5 – of *Ae. koreicus*.

Table 5. Prevalence of *w*AlbA and *w*AlbB strains of *Wolbachia* in *Ae. albopictus* from different sampling sites

Sampling site, year	*N*	*w*AlbA	*w*AlbB	*w*AlbA + *w*AlbB	Infection±SD (%)*
Pizunda, 2012	16	0	10	4	87.5 ± 8.3
Adler, 2013	30	0	18	0	60 ± 8.9
Hosta, 2011	6	1	0	5	100
Hosta, 2012	3	0	0	1	33.3
Sochi, 2012	35	0	34	0	97.1 ± 2.8
Mamaika, 2012	63	0	51	12	100
Dagomys, 2012	27	0	24	3	100
Dagomys, 2016	190	1	22	8	16.3 ± 2.7
Lazarevskoye, 2012	30	0	24	6	100
Tuapse, 2013	11	2	1	7	90.9 ± 8.7
Total	411	4	184	46	56.9

* Standard deviation (SD) was considered for samples of more than 10 individuals.

Table 6. Infection of *Ae. albopictus* and *Ae. aegypti* with *D. immitis* and *D. repens*

Species	Number of specimens (pools)	Number of pools, infected with *D. repens*			Number of pools, infected with *D. immitis*		
		Head-thorax	Abdomen	MIR, %	Head-thorax	Abdomen	MIR,%
Ae. albopictus	366 (74)	1	0	0.3	0	5	1.4
Ae. aegypti	21 (4)	1	0	4.8	0	0	0

All screened *Ae. aegypti*, *Ae. cretinus, Ae. koreicus* were not infected with *Wolbachia.* In *Ae. aibopictus* symbiotic bacteria *Wolbachia* were found in all the pools, the percentage of infected insects varied between 16.3 and 100 % (Table 5). On the Caucasus coast all possible variants of infected *Ae. albopictus* were found. Between 234 positive specimens 3 variants of infection were found: rare strain *w*AlbA (1.7 %), common strain *w*AlbB (78.6 %) and superinfection with both strains *w*AlbA and *w*AlbB (19.7 %); 177 specimens were not infected. What is especially interesting is the Dagomys population where in 2016 only 31 (16.3 %) out of the 190 mosquitoes were tested positively for *Wolbachia*. The infection rate of the Dagomys *Ae. albopictus* in 2016 was statistically different from the grand total (Fisher test, $p < 0.0001$). If the Dagomys 2016 pool were removed from analysis, the total contamination of *Ae. albopictus* would be 91.8 %.

The variations in total infected specimens in the pools can be explained by their small count, like in the Hosta case (2012) or, possibly, by the poor condition of bacterial DNA in the preserved mosquitoes. The low total of infected mosquitoes was mainly due to Dagomys (2016). In this case, 190 specimens were checked and considering good PCR results for other genes, poor DNA condition was hardly the reason. Most probably, the low rate of *Wolbachia* infection was consistent and the *Ae. albopictus* population requires further investigation (see Table 5).

Dirofilaria contamination

Using PCR with specific primers for the DNA of two species of *Dirofilaria* 74 pools (366 specimens) of *Ae. albopictus,* and 4 pools (21 specimens) of *Ae. aegypti* were investigated (Table 6). Among 74 pools of *Ae. albopictus* one was infected with *D. epens* (MIR = 0.3 %), five pools were infected with *D. immitis* (MIR = 1.4 %). Only one pool of *Ae. aegypti* out of four was infected with *D. repens* (MIR = 4.8 %). *D. immitis* was found only in the abdomen pools of *Ae. albopictus*. Infective larvae L3 of *D. repens* were found in pools of head-thorax parts of the both species (see Table 6).

Discussion

Stable, replenishing population of *Ae. albopictus* inhabits the territory of Krasnodarsky Region's Black Sea coast. Mosquitoes *Ae. albopictus,* first registered in 2011, have been expanding with the great speed presenting serious competition to *Ae. aegypti*.

Survey of the territory in years 2012–2013 showed presence in this area of consistent, replenishing populations of two dangerous mosquito species – *Ae. aegypti* and *Ae. albopictus* (Ganushkina et al., 2013). In 2012, the areas surrounding New Afon and Pizunda were additionlly investigated, with the same mosquito species discovered. In Russia *Ae. albopictus* were found only recently, in 2011, but they actively, as it is common for invasive species in a

new place, have taken their niche around the Big Sochi in the wet subtropical climate zone, pressing *Ae. aegypti* from Adler to Lazarevskoe. In typical semidry Mediterranean climate zone, Tuapse area, *Ae. aegypti* were predominant in 2011–2013, while the quantity of *Ae. albopictus* in this territory was negligible. Further to the north to Anapa, where semidry Mediterranean climate is also present, no mosquitoes of both species were found during July, September and October of 2013 (Ganushkina et al., 2013). In 2014–2015 the population of *Ae. albopictus* and *Ae. aegypti* on the Caucasus coast of the Black Sea developed in the way similar to the 2012–2013 trends (Ganushkina et al., 2016). In populated areas to the south of Tuapse, mainly *Ae. albopictus* were registered. In Tuapse *Ae. aegypti* were predominant (*Ae. aegypti* share 70 %, *Ae. albopictus* 30 %), however, the count of *Ae. albopictus* began to rise. No further advances of *Ae. albopictus* in 2012–2014 to the northwest of Jubga (57 km from Tuapse, the last locality, where *Ae. albopictus* were registered) were observed. However, as we had predicted (Ganushkina et al., 2014b). *Ae. albopictus* mosquitoes were able to expand in the northwest direction making it essential to investigate the Gelendjik area, where M.V. Zabshata (2016) had found this species in 2015.

Our collections from July and August 2016, as well as the data by Fedotova M.V. et al. (2017a, b) demonstrated that in Adler, Hosta and Sochi no *Ae. aegypti* had been found, and only *Ae. albopictus* had occupied the coast. However, during August 2017 in Adler the viable eggs of both *Ae. aegypti* and *Ae. albopictus* were found, which was confirmed by DNA tests. The *Ae. aegypti* eggs are able to withstand prolonged dehydration, but below-zero temperatures are lethal to them. Consequently, despite the drastic decrease in population, in positive conditions *Ae. aegypti* are able to regain abundance. The population decrease may be linked to the competition between *Ae. aegypti* and *Ae. albopictus* larvae for food resources and the fertility loss in *Ae. aegypti* due to possible interspecies copulation (Bargielowski et al., 2015; Carrasquilla, Lounibos, 2015). A major role in preserving the viability of diapausing eggs of *Ae. aegypti* may be attributed to winter temperatures. Theoretically, *Ae. aegypti* areal may correlate with the lowest rate of night temperature at ground surface (Tsai et al., 2018). Median borderline temperature of 13.8 degrees may play a critical role in limiting of *Ae. aegypti* expansion on the Caucasus Black Sea coast, where during some years it drops down from –3 to –13 centigrade.

Amplification of the ITS2 region enables for identification of *Ae. albopictus*, *Ae. aegypti*, *Ae. aretinus* and *Ae. koreicus* based on the size of the PCR product. This method can be used along with morphological criteria for the accurate species identification. Genetic divergence in the variable non-coding ITS2 area among *Ae. albopictus*, *Ae. cretinus*, *Ae. koreicus* and *Ae. aegypti* constitutes between 28 and 46 %. *Ae. albopictus* is genetically closer to *Ae. cretinus* and *Ae. koreicus* than to *Ae. aegypti*. The divergence between *Ae. albopictus* and *Ae. aegypti* is 37 %, and in the coding sequence of *COI* gene it reaches 14 %.

The methods of molecular genetics were first used to characterize the mosquitoes from the lab line of *Ae. aegypti* cultivated in a laboratory for more than 50 years. The origin of first mosquitoes of the IMPITM line is unknown. According to the nuclear marker, these mosquitoes are identical to the specimens in the Rockfeller line. The *COI* gene analysis has shown that mosquitoes from the *Ae. aegypti* IMPITM lab line contain DNA close to the one of African mosquitoes and the specimens from the Liverpool and RED lab lines. The databases contain neither *COI* sequences for the Rockfeller strain, nor ITS2 for Liverpool and RED. Therefore, no possibility exists to compare *Ae. aegypti* IMPITM with any of the known laboratory cultures of *Ae. aegypti* using the both markers. The origin of the laboratory strains of *Ae. aegypti*, bred in the laboratories of USA, England, France and other countries since 1940–1950s are often unknown (Kuno, 2010). The *Ae. aegypti* Rockfeller line originated from Cuba and the *Ae. aegypti* Liverpool line – possibly from West Africa (Kuno, 2010). The RED line heritage was undetermined, but we know that it is a variant of the Rex-D strain of *Ae. aegypti (*Costa-da-Silva et al., 2017).

Ae. aegypti originated from Africa, from where they expanded firstly to South and North Americas, and then towards Asia (Bennett et al., 2016). *Ae. aegypti* found on the Caucasus coast of the Black Sea have the *COI* gene identical to the one of invasive mosquitoes from Southeast Asia (India, Cambodia), America (French Guyana), Australia and single specimens carried to Europe (Kampen et al., 2016; Dallimore et al., 2017). These invasive *Ae. aegypti* are likely to have adaptive properties to subtropical and even moderate climates.

Southeast Asia is considered to be the place of *Ae. albopictus* origin, from where these mosquitoes expanded worldwide. Previous research of *Ae. albopictus* had shown a low level of diversity in mitochondrial DNA, but found differences in the *COI* gene between the populations that are present in countries with tropical or subtropical climate (Mousson et al., 2005; Patsoula et al., 2006; Kamgang et al., 2011; Zitko et al., 2011), where tropical populations carry 363C, and subtropical – 363T (relative to MG198595). No tropical *COI* gene haplotype was found on the Black Sea coast. One of the haplotypes found in *Ae. albopictus* during our research was typical to *Ae. albopictus* not only from Taiwan and Japan but also from Italy and Spain (see Fig. 3). The second haplotype found in *Ae. albopictus* from Hosta (2012) also presents in *Ae. albopictus* from Japan, Italy and Germany.

Investigation of *Ae. albopictus* and *Ae. aegypti* from the Black Sea coast using the markers of nuclear and mitochondrial DNA and comparison with existing databases has shown a low level of diversity among the mosquitoes of these species collected in various sites and at different times. It confirms that the worldwide expansion of invasive *Ae. aegypti,* and especially *Ae. albopictus,* has been happening very fast and no evolutionary changes have occurred so far.

Our findings confirmed the absence of symbiotic bacteria *Wolbachia* in wild *Ae. aegypti*. *Wolbachia* was not found in the specimens of *Ae. cretinus* и *Ae. koreicus*, but wider screening is required to make conclusive statements regarding symbiont presence in these species. We have revealed the circulation of two *Wolbachia* strains, *w*AlbA and *w*AlbB, in the *Ae. albopictus* population from the Caucasus Black Sea coast. The *w*AlbB strain is prevalent in our findings, which is similar for *Ae. albopictus* from different regions worldwide (Calvitti et al., 2015). It is known that the infection rate in the *Ae. albopictus* species is close to 100 %. Our values are lower, compared to what has usually been registered in *Ae. albopictus*. In the Dagomys collection (2016) less than 16 % of specimens were infected. An *Ae. albopictus* population totally free from *Wolbachia* was discovered in Vietnam in 2012 (Minard et al., 2017). Thorough investigation into *Wolbachia* infection and the genetic structure of *Ae. albopictus* should be continued in Dagomys using the markers of nuclear and mitochondrial DNA in order to determine the nature of infected and non-infected mosquitoes.

One of the goals of this study was revealing of invasive stages of microfilaria in *Ae. albopictus* and *Ae. aegypti* in order to determine their role as dirofilaria vectors. In *Ae. albopictus* total dirofilaria infestation with both species consisted of 6 infected pools out of 74 (MIR = 1.6 %). In *Ae. aegypti* one mosquito infected with *D. repens* was found in a pool of four (MIR = 4.8 %). Such high count should be attributed to low amount of *Ae. aegypti* specimens in the test. Discovery of *D. repens* DNA in the thorax part of mosquitoes points to microfilaria development at larvae stage L3 and that both *Ae. albopictus* and *Ae. aegypti* can infect humans or animals while sucking blood. Spread of dirofilariasis along the Black Sea coast is facilitated by optimal climate conditions for the development of infective agents, as well as intensive migration of people and dogs. Obligate carriers of *D. repens* and *D. immitis* are known to be carnivorous animals of feline and canine family. (Sergiev et al., 2014; Bogacheva et al., 2017). Southern Russia, due to its climate, is the region where consistent dirofilaria transmission has been taking place. In recent years, rising trend of dirofilariasis has been observed not only in animals, but also in humans (Ermakova et al., 2017; Kartashev et al., 2018). Considering the growth of invasive mosquito population (*Aedes* gene) on the Caucasus Black Sea coast, the presence of this suitable carriers may be the cause of dirofilariasis spreading.

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Conflict of interest

The authors declare no conflict of interest.

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