**Dirofilaria and Wolbachia in mosquitoes (Diptera: Culicidae) in central European Russia and on the Black Sea coast**

Elena Shaikevich1,*, Anna Bogacheva2, and Ludmila Ganushkina3

1 Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow 119991, Russia
2 Moscow State University, Moscow 119234, Russia
3 Martinovsky Institute of Medical Parasitology, Tropical and Vector-Borne Diseases, Sechenov First Moscow State Medical University, Moscow 119435, Russia

Received 5 October 2018, Accepted 4 January 2019, Published online 15 January 2019

**Abstract** – Dirofilariosis is endemic in Russia, as well as in many other European countries. The aim of this study was to assess the ability of mosquitoes to transfer *Dirofilaria immitis* and *Dirofilaria repens* in regions with temperate and subtropical climates. The possible impact of the symbiotic bacterium *Wolbachia* on *Dirofilaria* transmission was also investigated. 5333 female mosquitoes were collected at 11 points in central European Russia and on the Black Sea coast during the period 2013–2017. Out of 20 mosquito species examined, 14 were infected with *D. repens* and 13 with *D. immitis*. Both species of *Dirofilaria* were found in different climatic regions. The total *Dirofilaria* spp. estimated infection rate (EIR) in the central part of Russia varied from 3.1% to 3.7% and, in the southern region, from 1.1% to 3.0%. The highest estimated infection rate was found in Anopheles messeae, the lowest in Culex pipiens. The greatest epidemiological danger was represented by Aedes aegypti, Ae. geniculatus, An. messeae and Ae. communis. Six out of 20 mosquito species were infected with *Wolbachia*. Pools of *Aedes albopictus*, *Cx. pipiens* and Coquillettidia richiardii were simultaneously infected with *Dirofilaria* and *Wolbachia*. After checking mosquitoes individually, it was found that there was no development of *Dirofilaria* to the infective larval stage in specimens infected with *Wolbachia*. Twenty-two *Dirofilaria*-infected pools were *Wolbachia*-free and only two mosquito pools were *Wolbachia*-infected. The potential for transmission of *Dirofilaria* in mosquito species naturally uninfected with the symbiotic bacterium *Wolbachia* is higher than in species infected with the bacterium.

**Key words:** mosquitoes, *Dirofilaria repens*, *Dirofilaria immitis*, *Wolbachia* pipientis.

**Résumé** – *Dirofilaria et Wolbachia chez les moustiques (Diptera: Culicidae) en Russie centrale et sur la côte de la Mer Noire*. La dirofilariose est endémique en Russie, ainsi que dans de nombreux autres pays européens. L’objectif de ce travail était d’étudier la capacité des moustiques à transmettre *Dirofilaria immitis* et *Dirofilaria repens* dans les régions à climat tempéré et subtropical. L’impact possible de la bactérie symbiotique *Wolbachia* sur la transmission de *Dirofilaria* a également été étudié. 5333 moustiques femelles ont été collectés en 11 points en Russie centrale et sur la côte de la mer Noire au cours de la période 2013–2017. Sur les 20 espèces de moustiques examinées, 14 étaient infectées par *D. repens* et 13 par *D. immitis*. Les deux espèces de *Dirofilaria* ont été trouvées dans différentes régions climatiques. Le taux total d’infection estimé des *Dirofilaria* spp. dans la partie centrale de la Russie variait de 3,1 à 3,7 % et de 1,1 à 3,0 % dans le sud. Le taux d’infection estimé le plus élevé a été observé chez *Anopheles messeae* et le plus faible chez *Culex pipiens*. Le plus grand danger épidémiologique était représenté par *Aedes aegypti*, *Ae. geniculatus*, *An. messeae* et *Ae. communis*. Six espèces de moustiques sur 20 étaient infectées par *Wolbachia*. Des pools d’*Aedes albopictus*, *Cx. pipiens* et *Coquillettidia richiardii* étaient infectés simultanément par *Dirofilaria* et *Wolbachia*. Après avoir examiné les moustiques individuellement, il a été trouvé que les *Dirofilaria* ne se sont pas développées au stade larvaire infectant chez les spécimens infectés par *Wolbachia*. Vingt-deux pools infectés par *Dirofilaria* étaient indemnes de *Wolbachia* et seulement deux pools de moustiques étaient infectées par *Wolbachia*. Le potentiel de transmission de *Dirofilaria* chez les espèces de moustiques naturellement non infectées par la bactérie symbiotique *Wolbachia* est plus élevé que chez les espèces infectées par la bactérie.

*Corresponding author: elenashaivech@mail.ru*
Introduction

Dirofilaria is a vector-borne disease common in many countries on various continents [27, 42, 44, 60]. Sources of infection for mosquitoes are infected dogs, less often cats and wild canines (wolves, foxes, etc.). Dirofilaria immitis and Dirofilaria repens are transmitted by culicid mosquito species belonging to the Culex, Aedes, Ochlerotatus, Anopheles, Coquillettidia, Armigeres and Psorophora genera [42, 58, 69]. Vectors ingest microfilariae during a blood meal on an infected host. In mosquito Malpighian tubules, microfilariae develop to the third stage larvae (L3) [34]. The season for Dirofilaria transmission in the central part of Russia begins in late May to early June [26]. In order for the larvae to develop to L3, a sum of temperatures of 130 degrees-day [27] is necessary. L3 reach the salivary glands and proboscis from where they are transferred while feeding to another host [34, 43]. However, development of larvae to the infective stage does not always occur; Dirofilaria remain in the Malpighian tubules and do not undergo further development or are encapsulated by the immune system of mosquitoes, and may also die within a few hours of entering the intestine of a mosquito [18, 34]. Thus, only mosquitoes in which development has progressed to the third stage larvae (L3) can be considered epidemiologically competent vectors, and the larvae, infective.

Dirofilaria infection is endemic in Russia. Two species of Dirofilaria (D. immitis and D. repens) have been identified in humans [54, 63]. Prior to 2014, D. repens infection was detected in 850 people living in 42 regions of the Russian Federation [54]. The first case of D. immitis was detected in 2015 in the Moscow region; an immature female was removed from a 14-month-old child [63]. The dirofilariasis zone in the north of the European part of Russia has advanced to 58° N [4, 10].

In Russia, mosquitoes infected with Dirofilaria have previously been investigated in the southern regions (Astrakhan, Rostov, Krasnodar Krai and Republic of Adygeya) and the estimated infection rate (EIR) was 1.0%–14.0% [2, 24, 35]. Even though dirofilariasis is a concern in Russia, many areas have not been sufficiently studied. Also, there are no data on the species of mosquito that are potential vectors of dirofilarial worms. Identification of mosquitoes in all cases was conducted only to the genera level: D. immitis and D. repens have been detected in the Culex, Aedes and Anopheles genera and the EIR was established as 1.9%–7.0%, 2.3%–6.7%, and 0.6%–3.4%, respectively [2, 24, 35].

An endosymbiotic, maternally inherited bacterium, Wolbachia pipiensis (Rickettsiales: Rickettsiaceae), hereafter Wolbachia, infects filarial nematodes and many insects, including some mosquito species. Wolbachia is required for the development and survival of filarial nematodes [61], whereas its symbiotic relationship with mosquitoes is largely parasitic [65]. Among the culicid mosquito species, Culex pipiens, Cx. quinquefasciatus and Ae. albopictus are known to be infected with Wolbachia [32, 67] and considered as vectors for Dirofilaria [13–15, 28, 45, 50, 69]. However, it was found that Culex pipiens f. molestus from Madeira, Portugal was unable to support the full development of D. immitis, both in nature and after experimental infection with D. immitis [29]. In continental Portugal, Cx. p. pipiens were found to be infected with D. immitis, but were not potentially infective; filarial DNA was detected only in the abdomen and not in thorax-head samples [25]. However, D. immitis microfilariae development to the L3 stage has recently been found in the thorax-head of one Cx. p. pipiens f. pipiens from Spain [9]. The hypothesis concerning the influence of Wolbachia on the transmission of Dirofilaria by Cx. p. pipiens mosquitoes in nature requires further confirmation, particularly in view of the limited number of infected specimens [9] and the absence of 100% Wolbachia infection of Cx. p. pipiens in nature [22, 56]. There are only three studies that have focused on investigating simultaneous infection with native Wolbachia and Dirofilaria in mosquitoes from natural populations [22, 23, 51]. Therefore, the effect of co-infection with native Wolbachia on mosquito vector competence for Dirofilaria remains unclear.

Prior to clarifying whether naturally occurring Wolbachia has any influence on filarial susceptibility or the development of Dirofilaria to the infective stage in the vectors, it is necessary to understand Wolbachia-mosquito interactions, which mosquito species are infected with the bacterium, the variability of bacterial strains, and the frequency with which Wolbachia occurs in mosquito populations.

The objectives of the current study were to examine mosquito fauna and to identify mosquito species that can potentially transmit filarial worms in rural and urban localities in the central part of European Russia compared with the Black Sea resorts, and to evaluate epidemiologically dangerous mosquito species in which larvae develop to the infective (L3) stage. All mosquito species were screened to determine their Wolbachia infection status.

Materials and methods

Mosquito sampling and taxa discrimination

Mosquitoes were captured in the Tula region, Nizhniy Novgorod region, Moscow region and on the Black Sea coast (Fig. 1, SM1). The climate in the studied regions in the central part of the country is moderately continental with clear seasonality; the average temperature in July is +19 °C, and in January –10 °C. At the resorts of the Black Sea coast, the climate is mild Mediterranean and subtropical with average temperatures in July of +24 °C and in January +3 °C. Collection of mosquitoes in the central part of Russia was conducted throughout the warm season in 2013–2017, and in the southern part for one month at each point in 2012–2013 and 2016. Exact locations and months of gathering are presented in SM1.

Sampling locations in the Tula, Nizhniy Novgorod and Moscow regions were typical areas for a large number of dogs to be found (gardens of private houses, forests and parks) and natural forests as far as 6–8 km from rural and urban areas. At one of the sampling points in the Moscow region (#5 Fig. 1), there was a kennel for stray dogs; this was located in the immediate vicinity of the forest where mosquito collections took place. To compare the infection rate of mosquitoes in urban and rural areas, we collected mosquitoes near human habitations and in forests.
Mosquito collection sites in the south were located in human settlements in a resort area. At the recreation centre “Pribol” (#11 Fig. 1), lakes and ponds are located at a distance of 200–500 m from the collection site and flying mosquito imagoes were observed here. On the Black Sea coast of the Caucasus (#8, 9, 10 Fig. 1) in Anapa, Tuapse and in Sochi, mosquitoes were collected in both urban and rural areas.

At all collection sites, the mosquitoes were captured using a suck tube by human landing during the most active attacking period from 6 pm to 9 pm several times during each month. After trapping, the mosquitoes were frozen at −19 °C for 20–30 min and, afterwards, were identified using taxonomic keys [31]. The specific name of the tribe Aedini is presented according to the studies of Wilkerson et al. [66]. Identification of the molestus and pipiens forms of Cx. pipiens and Cx. torrentium was conducted genetically using a PCR-RFLP assay, based on the DNA variability of the COI gene, as described previously [55, 56]. Representatives of Anopheles maculipennis complex were identified using an ITS2 PCR-RFLP [47].

Molecular Dirofilaria spp. screening

The collected mosquitoes were grouped according to species, collection site and year; there were up to six specimens/pool, usually five. The thorax-head and abdomen of each mosquito in the group were dissected and formed the pool. In some cases, individual thorax-heads were analysed. DNA extraction was performed using the DIAtom™ DNA Prep kit (Isogen, Russia). Extraction was conducted separately for the abdomens and thorax-heads in order to determine infected and infective mosquito specimens, respectively. For the PCR analysis, we used 5333 female mosquitoes which were divided into 1095 pools. Each pool was tested separately to identify D. immitis and D. repens using the following primers: DIR-3: F–5’–CCGGTA-GACCATGGCATTAT–3’ and DIR-4: R–5’–CGGTCTTGGACGTGGTGTTA–3’ for the D. repens DNA repeat region [64] and COIntF – 5’–TGATTGGGTTTTGTTA–3’ and COIntR – 5’–ATAAGTACGATCAATATC–3’ for detection of the COI gene in the mtDNA of D. immitis [46]. The PCR was run on a GeneAmp® PCR System 2700 thermal cycler (Applied Biosystems, USA) with a GenPak PCR MasterMix Core PCR kit (Isogen, Russia) for amplification, according to the manufacturer’s instructions. PCR was performed in 25-ll reaction mixtures, containing 1.5 mM of MgCl2, 10 pmol of each primer and 20–50 ng of mosquito genomic DNA. PCR protocols were as follows: primary denaturing at 94 °C for 5 min and then 48 cycles of 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min for D. repens; primary denaturing at 94 °C for 5 min and then 30 cycles of 94 °C for 1 min, 50 °C for 2 min and 72 °C for 3 min, and final extension for 5 min at 72 °C for D. immitis. Negative and positive controls were used in each PCR analysis to avoid false-positive results. The positive control used in the study was obtained from adult D. repens isolated from a dog. The presence of filarial DNA was confirmed using 1.5% agarose gel electrophoresis. Resulting amplicon sizes were 656 bp for D. immitis and 246 bp for D. repens.

Calculation of the infection rates

Minimum infection rates (MIRs) were calculated using the following standard formula: (number of positive mosquito pools)/(total number of mosquitoes tested) × 100 [13]. Estimated infection rates (EIRs) were calculated using the following formula: 1 – (1 – x/m) 1/k [20], where x is the number of positive pools, m is the number of pools tested, and k is the pool size. In the text, all EIRs are given per 100 specimens. Corresponding 95% confidence intervals (95% CIs) were calculated by the modified Wald method, using GraphPad Scientific software. EIR values with corresponding 95% CIs were calculated for all analysed pools per mosquito and Dirofilaria species. Host effectiveness was determined as the number of infectious mosquito pools with L3 larvae as a proportion of the total number of mosquitoes studied × 100.

Molecular Wolbachia screening

Wolbachia infection was detected in a sub-sample of 2926 individuals (633 pools) of 20 mosquito species by PCR with an Encyclo PCR kit (Evrogen, Russia), using the wsp-specific primers wsp-81F and wsp-691R [8]. In cases where Dirofilaria DNA was detected in pooled abdomens from the mosquitoes, pools of abdomen and individual thorax-heads were tested for Wolbachia infection. In other cases, pooled mosquito thorax-heads were analysed. The PCR fragments were purified from agarose gel with a Clean-Up Extraction Kit (Evrogen, Russia) and were sequenced using the BigDye Termination kit 3.1
(Applied Biosystems, USA) in order to distinguish Wolbachia of mosquito and of filarial nematode origins. Sequences of the Wolbachia wsp locus were deposited in GenBank under numbers MF989984–MF989989.

To distinguish between two strains of Wolbachia in Ae. albopictus, multi-primer PCR was used [70]; primers 383F and 183F were paired with wsp-691R to allow the separation of \textit{wAlbA} and \textit{wAlbB} Wolbachia strains from \textit{Ae. albopictus}. For the \textit{wAlbA} strain, a fragment size of 379 bp was found and, for the \textit{wAlbB} strain, an amplicon length of 501 bp was found.

The \textit{wPip} infections in \textit{Cx. pipiens} were genotyped in a subsample of 24 individuals representative of \textit{Dirofilaria}-positive pools and assigned to the \textit{wPip-II} and \textit{wPip-IV} groups, using PCR-RFLP assays based on two \textit{wPip} markers, \textit{ank2} and \textit{pki}, as previously described [3, 56].

\section*{Results}

\textbf{Dirofilaria spp. infection in mosquitoes}

The collected mosquitoes included 20 species; 16 species were in the central part of Russia and seven species on the Black Sea coast. The most abundant mosquito species in the temperate climate region was determined to be \textit{Ae. cantans}. In the subtropical climate on the Black Sea coast, the most abundant sampled mosquito species was \textit{Ae. albopictus}, followed by \textit{Cq. pipiens} and \textit{Cx. modestus} (Table 1). Filarial DNA was found in 15 species belonging to four genera with \textit{D. immitis} found in 4.66% of tested abdomen pools and in 1.74% of tested head pools and assigned to the Nizhny Novgorod region (Fig. 1 #3), EIRs for both \textit{D. immitis} and \textit{D. repens} were 1.51 and 1.82, respectively. In the Moscow region (Fig. 1 #6, 7), the EIR for \textit{D. repens} was 1.63 and for \textit{D. immitis} 1.97, in a dog kennel in the forest near Moscow (Fig. 1 #5) infected mosquitoes were not found. The first infected mosquitoes were recorded in May and the last in August–September. In the forest zones of the Nizhny Novgorod and Tula regions, with a sample of 525 specimens, no infected mosquitoes were found (SM2).

At the resorts of the Caucasian Black Sea coast, in Anapa, Tuapse and Sochi (Fig. 1 #8, 9, 10), the rates of mosquito infection were much lower; EIR values were 0.32 for \textit{D. repens} and 0.81 for \textit{D. immitis}. However, at the recreation center “Pribor” (Fig. 1 #11), \textit{D. repens} had an EIR of 0.99 and \textit{D. immitis} 2.04; this is comparable to values in the central regions of Russia (Fig. 1).

\textbf{Wolbachia infection in mosquitoes}

The presence of Wolbachia was found in six out of 20 studied mosquito species. 93% of all tested \textit{Cx. pipiens} were infected with Wolbachia, followed by \textit{Cq. richiardii} (68%), \textit{Ae. albopictus} (56%), \textit{Ae. cinereus} (37%), \textit{Cx. modestus} (7%) and \textit{Ae. cantans} (3%). Specific sample sites and screening results are presented in an additional file (SM3). Sequences of \textit{Wolbachia wsp} genes from all six mosquito species demonstrated that all bacteria belonged to supergroups A or B, which were shared between arthropods (Table 3). No filarial bacteria were amplified. Two \textit{Wolbachia} strains were present in studied \textit{Ae. albopictus} (\textit{wAlbA} and \textit{wAlbB}), \textit{wPip-II} in \textit{Cx. pipiens} \textit{f. pipiens} and \textit{wPip-IV} in \textit{Cx. pipiens f. molestus}. Based on the \textit{wsp} gene sequence, \textit{Wolbachia} strains in \textit{Cq. richiardii}, \textit{Ae. cinereus} and \textit{Ae. cantans} differ from \textit{wAlb} and \textit{wPip}, so we named these \textit{wCrich}, \textit{wAcin} and \textit{wOcan}, respectively (pubmlst.org/wolbachia).

A total of 90 \textit{Dirofilaria} positive abdomen and thorax-heads pools were analyzed for simultaneous infection with \textit{Wolbachia} (Table 3). Seventy five of the \textit{Dirofilaria} positive pools (83%), including \textit{Ae. cinereus}, \textit{Ae. cantans} and \textit{Cx. modestus}, were free from \textit{Wolbachia}. Fifteen pools of \textit{Ae. albopictus}, \textit{Cx. pipiens} and \textit{Cq. richiardii} (17%) were positive for both \textit{Wolbachia} and at least one \textit{Dirofilaria} species. \textit{Dirofilaria} was found in 22 thorax-head pools of mosquitoes uninfected with \textit{Wolbachia} and in two thorax-head pools which were positive for \textit{Wolbachia}.

In order to investigate a possible association between the occurrence of \textit{Wolbachia} and the development of \textit{Dirofilaria} to the infective third larval stage (L3) within mosquitoes, we tested the thorax-heads of individual specimens from 12 pools: 25 individuals (five pools) of \textit{Ae. albopictus}, 23 individuals (five pools) of \textit{Cx. pipiens} (21 \textit{f. pipiens} and two \textit{f. molestus}), and 11 individuals (two pools) of \textit{Cq. richiardii} (SM4). There was no possibility to study one pool of \textit{Ae. albopictus} (collected in Sochi 2012) infected with \textit{D. repens} and two pools of \textit{Cx. pipiens} (collected in Tula 2014) – one infected with \textit{D. repens} and one infected with \textit{D. immitis} individually. The development of \textit{D. immitis} to the infective stage (L3) was successful only in one thorax-head of \textit{Wolbachia}-free \textit{Cq. richiardii} (No. 11′-1, SM4), although a pool of five mosquito abdomens gave a
toes were studied in Europe for the presence of
Dirofilaria. A positive signal for both
D. repens and D. immitis. Neither D.
immitis nor D. repens were found in other individual thorax-heads; parasites were found only in pooled abdomens.

Discussion

Mosquito species and Dirofilaria infection

The detection of infection with D. repens and D. immitis was tested in 5333 mosquitoes comprising 1095 pools and representing 20 species collected in geographically remote locations in a temperate and sub-tropical climate. This is the first large-scale study of the infection of mosquitoes in the European part of Russia involving identification of the mosquito species. The published results on mosquito infestation in Europe, including Turkey, in comparison with our data are presented in Table 4. Previously, Ae. cataphylla, Ae. cinereus, Ae. excrucians, Ae. leucomelas, Ae. punctor and Ae. diantaeus mosquitoes were studied in Europe for the presence of Dirofilaria, but no positive samples were detected. In our study, infection with Dirofilaria was newly detected in the first four of these mosquito species. However, development of the larvae did not reach the infectious stage.

Ae. intrudens and Ae. communis were firstly studied here as vectors of Dirofilaria; their EIR values were 3.08 and 3.11, respectively. It should be noted that their epidemiological significance is confirmed by the presence of third-stage larvae in the thorax-heads. Ae. intrudens and Ae. communis host effectiveness was 0.41 and 1.3, respectively.

Special attention should be paid to Ae. aegypti mosquitoes, which were found in Russia on the Black Sea coast of the Caucasus in 2000 [53]. As far as we know, the infection of natural populations of Ae. aegypti has not been studied. Based on a small sample (21 females), the EIR of this species of mosquitoes was reported as 5.33.

In our study, the most abundant species of mosquito was Ae. cantans (1776 specimens out of 5333) with an infection rate that was not high; the EIR for D. repens was 1.63 and for

![Table 1. Mosquito species composition and their collected numbers in studied regions.](image-url)
## Table 2. Mosquito species and infection with *D. immitis* and *D. repens*.

| Mosquito species | Number of indiv. mosquitoes | Number of pools | Average number of specimens per pool | Pools positive for *D. repens* | EIR (95% CI) | Number of abdomen pools | EIR (95% CI) | Number of head-thorax pools | EIR (95% CI) | MIR (%) | EIR (95% CI) | Host effectiveness** |
|------------------|----------------------------|----------------|-------------------------------------|--------------------------------|-------------|------------------------|-------------|--------------------------|-------------|---------|-------------|----------------------|
| *An. messeae*    | 67                         | 15             | 4.47                                | 1                              | 1           | 3.15 (0.21–10.86)      | 3           | 0                        | 0.37 (0.00–1.27) | 0.74    | 8.87 (2.86–16.86) | 1.49                  |
| *Ae. aegypti*    | 21                         | 4              | 5.25                                | 0                              | 1           | 5.33 (0.01–24.42)      | 0           | 0                        | 0.37 (0.00–1.27) | 0.74    | 8.87 (2.86–16.86) | 1.49                  |
| *Ae. geniculatus*| 203                        | 43             | 4.72                                | 2                              | 2           | 2.05 (0.59–5.13)       | 2           | 3                        | 2.59 (0.9–5.8)   | 4.43    | 4.85 (2.23–8.33) | 2.46                  |
| *Ae. cataphylla* | 236                        | 49             | 4.82                                | 6                              | 0           | 2.67 (1.04–5.56)       | 3           | 0                        | 1.3 (0.26–3.85)  | 3.81    | 4.12 (1.91–7.19) | 0                     |
| *Cq. richiardii* | 184                        | 40             | 4.6                                 | 2                              | 0           | 1.11 (0.04–4.13)       | 3           | 1                        | 2.27 (0.65–5.65) | 3.26    | 3.47 (1.34–7.09) | 0.54                  |
| *Cx. modestus*   | 223                        | 45             | 4.96                                | 3                              | 0           | 1.38 (0.27–4.06)       | 2           | 2                        | 1.86 (0.54–4.68) | 3.14    | 3.35 (1.40–6.46) | 0.89                  |
| *Ae. cantans*    | 1776                       | 356            | 4.99                                | 25(8*)                         | 11(8*)      | 1.63 (1.08–2.28)       | 21(1*)      | 4(1*)                    | 1.9 (0.9–2.01)   | 2.93    | 3.11 (2.23–3.83) | 0.84                  |
| *Ae. communis*   | 307                        | 64             | 4.79                                | 3(1*)                          | 1*          | 0.99 (0.12–2.97)       | 3           | 3                        | 2.03 (0.8–4.3)   | 2.93    | 3.11 (1.47–5.56) | 1.3                   |
| *Ae. intrudens*  | 482                        | 98             | 4.92                                | 4                              | 1           | 1.06 (0.37–2.48)       | 8           | 1                        | 1.94 (0.93–3.57) | 2.9     | 3.08 (1.69–4.86) | 0.41                  |
| *Ae. vexans*     | 179                        | 37             | 4.84                                | 2                              | 0           | 1.14 (0.04–4.24)       | 3(1*)      | 1*                       | 1.73 (0.35–5.04) | 2.79    | 2.96 (1.02–5.55) | 0.56                  |
| *Ae. cinereus*   | 259                        | 54             | 4.79                                | 1                              | 0           | 0.39 (<0.01–2.38)      | 4           | 0                        | 1.59 (0.46–4.05) | 1.93    | 2.01 (0.70–4.57) | 0                     |
| *Ae. albopictus* | 366                        | 74             | 4.95                                | 0                              | 1           | 0.27 (<0.01–1.69)      | 5           | 0                        | 1.4 (0.49–3.25)  | 1.64    | 1.69 (0.67–3.62) | 0.27                  |
| *Ae. leucellos*  | 62                         | 13             | 4.77                                | 0                              | 0           | 0                     | 1           | 0                        | 1.66 (<0.01–9.41) | 1.61    | 1.66 (<0.01–9.41) | 0                     |
| *Ae. excrucians* | 68                         | 15             | 4.53                                | 1                              | 0           | 1.51 (<0.01–8.63)      | 0           | 0                        | 0.47 (0.00–1.83) | 0.93    | 1.46 (<0.01–8.63) | 0                     |
| *Cx. pipiens*   | 516                        | 104            | 4.96                                | 1                              | 1           | 0.39 (0.01–1.5)        | 5           | 0                        | 0.99 (0.35–2.32) | 1.36    | 1.39 (0.60–2.83) | 0.19                  |
| *Ae. sticticus*  | 57                         | 13             | 4.38                                | 0                              | 0           | 0                     | 0           | 0                        | 0.00 (0.00–3.3)  | 0       | 0.00 (0.00–3.3)  | 0                     |
| *Ae. caspius*    | 146                        | 30             | 4.87                                | 0                              | 0           | 0                     | 0           | 0                        | 0.00 (0.00–3.3)  | 0       | 0.00 (0.00–3.3)  | 0                     |
| *Ae. punctor*    | 50                         | 11             | 4.55                                | 0                              | 0           | 0                     | 0           | 0                        | 0.00 (0.00–3.3)  | 0       | 0.00 (0.00–3.3)  | 0                     |
| *Ae. diantaeus*  | 128                        | 27             | 4.74                                | 0                              | 0           | 0                     | 0           | 0                        | 0.00 (0.00–3.3)  | 0       | 0.00 (0.00–3.3)  | 0                     |
| *Cx. torrentium*| 3                          | 3              | 4.74                                | 0                              | 0           | 0                     | 0           | 0                        | 0.00 (0.00–3.3)  | 0       | 0.00 (0.00–3.3)  | 0                     |
| Total            | 5333                       | 1095           | 4.87                                | 51(9*)                         | 19(9*)      | 1.17 (0.89–1.47)       | 63(2*)     | 15(2*)                   | 1.47 (1.14–1.78) | 2.57    | 2.71 (2.18–3.03) | 0.64                  |

*MIR = 1.14 MIR = 1.43

* Inclusive pools, in which infection was detected in both abdomens and head-thorax pools;
** Host effectiveness – proportion of infectious mosquitoes with L3 larvae in total number of studied mosquitoes (%).
of these three species were infected with *Wolbachia* s.l. (inclusive heads) 26 (9) 9 (3) 0. A *Ae. cantans* and *Ae. geniculatus* with an EIR of 0.81 and, respectively, EIR 2.52. In the Moscow region, one point was studied in the immediate vicinity of the dog kennel (Fig. 1 #2, 4). However, in the settlements (Fig. 1 #1, 3, 6, 7), mosquito infection was high >3%. Possible reasons for this are that circulation of the pathogen in the two woodlands does not occur, or wild canines are not affected by *Dirofilaria* or are affected to such a small extent that we could not discern infection by examining the mosquito vectors. In the Moscow region, one point was studied in the immediate vicinity of the dog kennel (Fig. 1 #5), located in a woodland 2 km away from the nearest settlement. Infected mosquitoes were not found. In this kennel, the dogs were treated for different infections, including *Dirofilaria*, and the infection from wild animals did not occur or was extremely low.

**Host effectiveness**

According to our findings, under similar conditions (temperature and the presence of definitive hosts), the effectiveness factors, such as season, climate and geographical features, which are specific for each region [27], but also, perhaps to an even greater extent as shown by our results with *Ae. aegypti*, *Ae. cantans* and *Ae. vexans*, connected with the sample size.

**Infection in specific collection regions**

When comparing the total infection of mosquitoes with *D. repens* and *D. immitis* by region (SM2), almost identical EIR results in the settlements of the Central region were noted, with some prevalence of mosquitoes infected with *D. immitis*. On the Black Sea coast of the Caucasus (Fig. 1 #8, 9, 10), mosquitoes were collected in a resort area where the number of dogs near our sample sites was negligible and the infection of mosquitoes was lower. Temperature is an important factor for the maintenance of *Dirofilaria* foci. However, the presence of definitive hosts (mainly domestic, office and stray dogs) basically determines one or another level of mosquito infection with *Dirofilaria*. This is confirmed by the absence of infection in mosquitoes collected in the forest at a distance of 8–10 km from settlements in the Nizhny Novgorod and Tula regions (Fig. 1, “forests” #2, 4). However, in the settlements (Fig. 1 #1, 3, 6, 7), mosquito infection was high >3%. Possible reasons for this are that circulation of the pathogen in the two woodlands does not occur, or wild canines are not affected by *Dirofilaria* or are affected to such a small extent that we could not discern infection by examining the mosquito vectors. In the Moscow region, one point was studied in the immediate vicinity of the dog kennel (Fig. 1 #5), located in a woodland 2 km away from the nearest settlement. Infected mosquitoes were not found. In this kennel, the dogs were treated for different infections, including *Dirofilaria*, and the infection from wild animals did not occur or was extremely low.

**Host effectiveness**

According to our findings, under similar conditions (temperature and the presence of definitive hosts), the effectiveness
of mosquitoes as vectors of *Dirofilaria* was not the same. There were five species of mosquitoes, *Ae. punctor*, *Ae. diantaeus*, *Ae. sticticus*, *Ae. caspius* and *Cx. torrentium*, in which no infected samples were found. Absence of infection in *Ae. sticticus* and *Ae. punctor* was probably associated with a small sample size (57 and 50 mosquitoes, respectively). *Cx. torrentium* rarely attack people, and with our collection method, the sample size was only three mosquitoes. However, of particular interest is the reason for the absence of infection in *Ae. diantaeus* and *Ae. caspius*, which were collected in sufficient numbers (117 and 146 mosquitoes) and not in the natural forests. Another interesting finding was the absence of infection in *Ae. diantaeus*, which were mainly collected in the Nizhny Novgorod region, where other mosquito species of the same biotope were infected (SM2). In contrast to our results, it was reported that *Ae. caspius* was infected with *D. repens* in Italy [38] and Moldova [58], with *D. immitis* in Serbia [37], Portugal [25] and Hungary, based on one positive sample of *D. repens* and *D. immitis* out of 267 collected mosquitoes from four species [71]. The absence of infection in *Ae. caspius* in our

### Table 4. Published results about *Dirofilaria* in mosquito species in Europe, including Turkey, in comparison with data obtained in this study.

| Species                     | N indiv./pools | *D. repens* | *D. immitis* | Host effectiveness | Country, references |
|-----------------------------|----------------|-------------|---------------|--------------------|---------------------|
| *Cx. pipiens*               | 516/104        | EIR = 0.39  | EIR = 0.99    | 0.19               | This study          |
| *Cx. pipiens*               | 1108/412       | MIR = 0.27  | MIR = 0.27    | 0.27*              | Italy 2002–2003 [12] |
| *Cx. pipiens (s.l.)/torrentium* | 2663/132     | EIR = 0.88  | EIR = 0.47    |                    | Moldova 2010–2016 [58] |
| *Cx. pipiens*               | 1595/1123      | EIR = 0.50  |               |                    | Continental Portugal 2011–2013 [25] |
| *Ae. sticticus*             | 2589           | MIR = 0.12  |               | 0.12*              | Turkey 2008–2009 [69] |
| *Cx. pipiens*               | 37,865/835     | MIR = 0.01* | MIR = 0.04*   |                    | Italy 2010 [38]      |
| *Ae. sticticus*             | 5568/115       | MIR = 0.02* | MIR = 0.18*   |                    | Serbia 2013 [37]     |
| *Cx. pipiens complex*       | 2539/187       | MIR = 0.28* |               |                    | Slovakia 2015–2017 [11] |
| *Cx. pipiens/Cx. torrentium*| 12,292/554     | MIR = 0.02* |               |                    | Germany 2011–2013 [36] |
| *Ae. pipiens*               | 136/11         | EIR = 0.58  |               |                    | Belarus 2015 [59]    |
| *Ae. pipiens*               | 666            | MIR = 0.3*  |               |                    | Spain 2004–2006 [46] |
| *Ae. pipiens*               | 604            | MIR = 0.17* |               | 0.17*              | Spain 2012–2013 [9]  |
| *An. messeae*               | 67/15          | EIR = 3.15  |               |                    |                    |
| *An. maculipennis s.l.*     | 400/114        | EIR = 3.12  |               | 1.25*              |                    |
| *An. maculipennis*          | 136/28         | MIR = 1.47* |               |                    |                    |
| *An. maculipennis s.l.*     | 947/62         | EIR = 4.91  |               |                    |                    |
| *Ae. vexans*                | 179/37         | EIR = 1.14  |               | 0.56               |                    |
| *Ae. vexans*                | 3179           | MIR = 0.41  |               | 0.35*              |                    |
| *Ae. vexans*                | 720/25         | MIR = 0.14* |               |                    |                    |
| *Ae. vexans*                | 405/19         | MIR = 0.25* |               |                    |                    |
| *Ae. caspius*               | 146/30         | EIR = 0     |               |                    |                    |
| *Ae. caspius*               | 26/13          | EIR = 22.64 |               |                    |                    |
| *Ae. caspius*               | 270/193        | EIR = 3.73  |               | 1.48*              |                    |
| *Ae. caspius*               | 2264/92        | MIR = 0.18* |               |                    |                    |
| *Ae. caspius*               | 195/13         | MIR = 0.5*  |               |                    |                    |
| *Cq. richiardii*            | 184/40         | EIR = 1.11  |               | 0.54               |                    |
| *Cq. richiardii*            | 34/7           | MIR = 2.94* |               |                    |                    |
| *Cq. richiardii*            | 48/26          | MIR = 2.08* |               |                    |                    |
| *Cq. richardi*              | 19/11          | EIR = 16.25 |               |                    |                    |
| *Ae. cantans*               | 1776/356       | EIR = 1.63  |               | 0.84               |                    |
| *Ae. cantans*               | 15/5           | EIR = 14.84 |               |                    |                    |
| *Ae. sticticus*             | 57/13          | EIR = 0     |               | 0.84               |                    |
| *Ae. sticticus*             | 24/7           | EIR = 4.43  |               |                    |                    |
| *Ae. sticticus*             | 120/7          | MIR = 0.83* |               |                    |                    |
| *Ae. sticticus*             | 414/41         | MIR = 0.24* |               | 0.24*              |                    |
| *Cx. modestus*              | 223/45         | EIR = 1.38  |               | 0.89               |                    |
| *Cx. modestus*              | 203/25         | EIR = 3.26  |               |                    |                    |
| *Ae. geniculatus*           | 203/43         | EIR = 2.05  |               | 2.46               |                    |
| *Ae. geniculatus*           | 26/10          | EIR = 7.45  |               |                    |                    |
| *Ae. albopictus*            | 366/74         | EIR = 0.27  |               | 0.27               |                    |
| *Ae. albopictus*            | 2534/336       | 0           | MIR = 3.19*   | 0.87*              | Italy 2000–2002 [12, 13] |
| *Ae. albopictus*            | 436/436        | MIR = 0.92* |               | 1.15*              | Italy 2002–2003 [16] |
| *Ae. albopictus*            | 528/98         | MIR = 0.19* |               |                    | Italy 2005 [41]     |
| *Ae. albopictus*            | 175/35         | MIR = 1.14  |               | 0.51*              | Italy 2011 [28]     |

*Number calculated based on the results published by the authors.*
collections may be explained by there being no infection or only slight infection at that particular collection point, since specimens of other species were also negative.

In four species (Ae. leucomelas, Ae. cataphylla, Ae. cinereus and Ae. excrucians), Dirofilaria were found only in the abdomens, indicating that its development did not reach an infective L3 stage (Table 2). Also, it should be highlighted that in three species of mosquitoes (Ae. cantans, Ae. communis, and Ae. vexans), there were positive signals for Dirofilaria simultaneously in the thorax-head and abdomen pools. This fact may indicate that mosquitoes could ingest the filariae at different times and repeatedly, and not all nematodes managed to complete the development cycle to become infective larvae and migrate to the front of the body. Similarly, it cannot be excluded that not all filariae reach the infectious stage due to possible defense mechanisms activated by host cells, such as encapsulation, melanization, and coagulation [12, 21, 34]. However, in all mosquito species, except Ae. aegypti where only one pool was infected, the percentage of positive thorax-head pools was lower compared to abdomen pools.

According to published research, the development of Dirofilaria to the infective stage (L3) was recorded in Europe in the mosquito species Ae. caspius, An. maculipennis, Ae. vexans, Ae. geniculatus, Ae. albopictus, and Cx. pipi on Table 4). On the basis of our results, eleven mosquito species are epidemiologically dangerous, when Dirofilaria undergo development to L3 (Table 2). Of particular interest are the species Ae. geniculatus, Ae. communis, Ae. intrudens, Ae. cantans and Cx. modestus, in which L3 were found more than once. Ae. Aegypti, Ae. geniculatus, An. messeae and Ae. communis have host effectiveness values ranging from 1.3 to 4.76. It should be noted that the efficacy of Ae. aegypti as a vector of Dirofilaria has been studied many times in laboratory conditions [34, 57, 62], where the microfilariae developed to third-stage larvae, but not in field-collected Ae. aegypti. In seven mosquito species, the host effectiveness was less than 1 (Table 2).

Wolbachia and Dirofilaria infection in individual mosquitoes

Most mosquito species uninfected with Wolbachia showed higher epidemiological potential for Dirofilaria transmission in all studied regions (host effectiveness 0.41–4.76; EIR = 2.96–8.67, average EIR = 4.2). Ae. cinereus and Cx. modestus pools that had Dirofilaria were free from Wolbachia (Table 3).

Dirofilaria DNA was detected in abdomen pools of both Wolbachia-infected and uninfected mosquitoes. This result shows that Wolbachia does not prevent the acquisition of Dirofilaria by mosquitoes in nature. However, in eight thorax-head pools, D. immitis DNA was only detected in Wolbachia-uninfected mosquitoes. Moreover, after individual study of 11 thorax-heads from two Wolbachia-positive Cq. richiardti abdomen pools, the D. immitis development was successful only in Wolbachia-free sample (Table SM4).

D. repens DNA in thorax-heads was found in 14 Wolbachia-uninfected and in only two Wolbachia-infected pools, in one of Ae. albopictus and in one of Cx. papi on. We could not study mosquitoes from these two pools individually, so it is impossible to determine whether all the individuals in pool were infected with the bacterium, and to what ecological form (f. pifi or f. molestus) of Cx. pipo s they belonged. Therefore, our findings do not prove a clear influence of bacteria on the development of Dirofilaria.

Nevertheless, the ratio of Dirofilaria-infective mosquitoes is much higher in Wolbachia-free mosquito specimens than in Wolbachia-infected, 22:2. Differences in the effects of different strains of Wolbachia were not recorded. However, given the small sample size of Dirofilaria-infected mosquitoes, further investigation into whether Wolbachia is present in individual Cx. pipi on and Ae. albopictus mosquitoes carrying infective L3 stage larvae is required.

It is known that artificial bacterial transfer significantly increases the expression of immune genes, including those involved in the Toll and IMD immune pathways, enhances the mosquito’s resistance to pathogens [5, 48, 49], and inhibits the development of filarial nematodes [1, 33]. In contrast, it has been shown that native Wolbachia does not affect the induction of host immune pathways [17, 39]. As a hypothesis, it could be proposed that there is a resource competition in the host for metabolites, because both Dirofilaria [19, 30] and Wolbachia [68] require them for their development. It should be noted that any Wolbachia anti-pathogen effect is dependent on bacterial density [40], so the development of microfilaria to the infective stage may differ in each mosquito. The study of simultaneous infection of individual mosquitoes with Dirofilaria spp. and a bacterial symbiont, taking into account Wolbachia density, will help us understand the mechanism of Wolbachia interference in the transmission of Dirofilaria by mosquitoes.

In conclusion, Dirofilaria were found in 15 mosquito species. This is the first study conducted in Russia examining the mosquito species as potential vectors of D. immitis and D. repens. Out of 1095 pools studied, there were 114 positive abdomen pools and 34 positive thorax-head pools. The ratio of infected pools to infective pools was 3.35:1. Mosquitoes in central temperate regions are able to spread Dirofilaria no less than mosquitoes in the southern regions. This indicates that the presence of infected dogs has a greater effect on the maintenance of dirofilariosis than temperature. In the forests, the circulation of pathogens occurs with less intensity than in human settlements in rural and urban areas. For the first time in Europe, Ae. aegypti, Ae. intrudens and Ae. communis mosquitoes have been studied as Dirofilaria vectors, in which EIR values ranged from 3.08 to 5.33. Our data showed that Ae. albopictus and Culex pipiens s.l. are not the most important vectors of Dirofilaria. The greatest epidemiological danger was represented by An. messeae, Ae. aegypti, Ae. geniculatus, and Ae. communis. Ae. cantans might be added to this list given the considerable host effectiveness and the very high density.

Acknowledgements. We thank Vera Rakova and Ivan Patraman for the assistance in mosquito sampling and in carrying out certain molecular assays.

Conflict of interest

There is no conflict of interests.
Funding

This work was supported by the Russian Foundation of Fundamental Research [grant N 16-04-00091].

Supporting information

SM1. Mosquitoes collected between 2013 and 2017. Information on the specificities and the coordinates of the sampling site, sampling date, total mosquito number and Dirofilaria screening results.

SM2. Mosquito infection with Dirofilaria spp. in specific sample sites. Information on the sampling date, mosquito species, pool size and Dirofilaria screening results.

SM3. Occurrence of Wolbachia in mosquito species. Information on the sampling region, mosquito number, pool size and Wolbachia screening results.

SM4. Comparison of simultaneous infection with Wolbachia and Dirofilaria individually.

Supplementary materials are available at https://www.parasite-journal.org/10.1051/parasite/2019002/olm.

References

1. Andrews ES, Crain PR, Fu Y, Howe DK, Dobson SL. 2012. Reactive oxygen species production and Brugia pahangi survivability in Aedes polynesiensis with artificial Wolbachia infection types. PLoS Pathogens, 8(12), e1003075.

2. Arakeljan R, Kovtunov A, Bikov V, Shatalin V, Arakeljan E. 2008. Epidemiologic-episentologetic features of three-member system of dirofilariasis (dog-mosquito-people) on the territory of Astrakhan region. Siberian Medical Journal, 7, 13–18 (in Russian).

3. Atyame CM, Delsuc F, Pasteur N, Weill M, Duron O. 2011. Diversification of Wolbachia endosymbiont in the Culex pipiens mosquito. Molecular Biology and Evolution, 28, 2761–2772.

4. Barashkova SV. 2011. Case of dirofilariasis in adolescent in Saint-Petersburg: Clinical and morphological characteristic. Journal Infection Ecology, 3, 108–110 (in Russian).

5. Bian G, Joshi D, Dong Y, Lu P, Zhou G, Pan X, Xu Y, Dimopoulos G, Xi Z. 2013. Wolbachia invades Anopheles stephensi populations and induces refractoriness to Plasmodium infection. Science, 340(6133), 748–751.

6. Biskin Z, Duziu O, Yildirim A, Inci A. 2010. The molecular diagnosis of Dirofilaria immitis in vector mosquitoes in Felahiy district of Kayseri. Turkey Parazitiologii Dergisi, 34(3), 200–205.

7. Bockova E, Rudolf I, Kocisova A, Betasova L, Venclikova K, Mendel J, Hubalek Z. 2013. Dirofilaria repens microfilariae in Aedes vexans mosquitoes in Slovakia. Parasitology Research, 112(10), 3465–3470.

8. Braig HR, Zhou W, Dobson SL, O’Neill SL. 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont Wolbachia pipientis. Journal of Bacteriology, 180(9), 2373–2378.

9. Bravo-Barriga D, Parreira R, Almeida APG, Calado M, Blancho-Ciudad J, Serrano-Aguilera FJ, Perez-Martín JE, Sanchez-Peinado J, Pinto J, Reina D, Frontera E. 2016. Culex pipientis as a potential vector for transmission of Dirofilaria immitis and other unclassified Filarioidea in Southwest Spain. Veterinary Parasitology, 223, 173–180.

10. Byakova OV, Maslemnikova OV, Ermolina SA. 2014. Dirofilariosis dog in the Kirov region. Basic Research, 11, 1297–1300 (in Russian).

11. Cabanova V, Miterpakova M, Valentova D, Blazejova H, Rudolf I, Sloukal E, Hurmikova Z, Dzidova M. 2018. Urbanization impact on mosquito community and the transmission potential of filarial infection in Central Europe. Parasites & Vectors, 11(1), 261.

12. Cancrini G, Frangipane di Regalbono A, Ricci I, Tessarin C, Gabrielli S, Pietrobelli M. 2003a. Aedes albopictus is a natural vector of Dirofilaria immitis in Italy. Veterinary Parasitology, 118(3–4), 195–202.

13. Cancrini G, Romi R, Gabrielli S, Toma L, Di Paolo M, Scaramozzino P. 2003b. First finding of Dirofilaria repens in a natural population of Aedes albopictus. Medical and Veterinary Entomology, 17(4), 448–451.

14. Cancrini G, Magi M, Gabrielli S, Arispici M, Tola F, Dell’Omodarme M, Prati MC. 2006. Natural vectors of dirofilariasis in rural and urban areas of the Tuscan region, central Italy. Journal of Medical Entomology, 43(3), 574–579.

15. Cancrini G, Gabrielli S. 2007. Vectors of Wolbachia nematodes: biology, behaviour and host-parasite relationships, in Dirofilaria immitis and D. repens in dog and cat and human infections, Genchi C, Rinaldi L, Cringoli G, Editors. Veterinary Parasitology and Parasitic Diseases, Department of Pathology and Animal Health, Faculty of Veterinary Medicine, University of Naples Federico II: Napoli, NA, Italy. p. 48–58. ISBN 9788889132142.

16. Cancrini G, Scaramozzino P, Gabrielli S, Di Paolo M, Toma L, Romi R. 2007. Aedes albopictus and Culex pipiens implicated as natural vectors of Dirofilaria repens in central Italy. Journal of Medical Entomology, 44(6), 1064–1066.

17. Caragata EP, Pais FS, Batón LA, Silva JBL, Sorgine MHF, Moreira LA. 2017. The transcriptome of the mosquito Aedes fluviatilis (Diptera: Culicidae), and transcriptional changes associated with its native Wolbachia infection. BMC Genomics, 18, 6.

18. Castillo JC, Reynolds SA, Eleftheriano I. 2011. Insect immune responses to nematode parasites. Trends in Parasitology, 27(12), 537–547.

19. Cotton JA, Benzuru S, Grote A, Harsha B, Tracey A, Beecrook D, Dovie SR, Dunn M, Hotopp JC, Holroyd N, Kikuchi T, Lambert O, Mhashilkar A, Mutowoo P, Nurmisimar Nu, Ribeiro JM, Rogers MB, Stanley E, Swapna LS, Tsa J, Unnasch TR, Voronin D, Parkinson J, Nutman TB, Gheiden E, Berriman M, Lustigman S. 2016. The genome of Onchocerca volvulus, agent of river blindness. Nature Microbiology, 2, 16216.

20. Cowling DW, Gardner IA, Johnson WO. 1999. Comparison of methods for estimation of individual-level prevalence based on pooled samples. Preventive Veterinary Medicine, 39(3), 211–225.

21. De Carvalho GA, Ramos RAN, Trinidad Maia R, de Andrade CPS, Alves CL. 2018. Melanization of Dirofilaria immitis larvae in different culicid species. Journal of Arthropod-Borne Diseases, 12(1), 94–99.

22. de Pinho Mixao V, Mendes AM, Mauricio IL, Calado MM, Novo MT, Belo S, Almeida AP. 2016. Molecular detection of Wolbachia pipientis in natural populations of mosquito vectors of Dirofilaria immitis from continental Portugal: first detection in Culex theileri. Medical and Veterinary Entomology, 30, 301–309.

23. Dyab AK, Galal LA, Mahmoud AE, Mokhtar Y. 2016. Finding Wolbachia in Filarial larvae and Culicidae mosquitoes in Upper Egypt governorate. Korean Journal of Parasitology, 54 (3). 265–272.

24. Ermakova L, Nagorny S, Krivorotova E, Peschenchnaya N, Matina O. 2014. Dirofilaria repens in the Russian Federation:
current epidemiology, diagnosis, and treatment from a federal reference center perspective. International Journal of Infectious Diseases, 23, 47–52.
25. Ferreira CA, de Pinho MV, Novo MT, Calado MM, Gonçalves LA, Belo SM, de Almeida AP. 2015. First molecular identification of mosquito vectors of Dirofilaria immitis in continental Portugal. Parasites & Vectors, 8, 139. DOI: 10.1186/s13071-015-0760-2.
26. Ganushkina LA, Rakova VM, Ivanova IB, Supriaga VG, Sergiev VP. 2014. Entomological monitoring of an area to assess Dirofilaria transmission risk. Meditsinskaiα Parazitologiiα i Parazitarnye Boleznii (Msk), 3, 9–12.
27. Genchi C, Rinaldi L, Mortarino M, Genchi M, Cringoli G. 2009. Climate and Dirofilaria infection in Europe. Veterinary Parasitology, 163(4), 286–292.
28. Giangaspero A, Marangi M, Latrofa MS, Martellini D, Traversa D, Otranto D, Genchi C. 2013. Evidences of increasing risk of dirofilariosis in southern Italy. Parasitology Research, 112(3), 1357–1361.
29. Gouveia M. 2007. Susceptibility of Mosquito Vectors to Dirofilaria immitis on Madeira Island, Portugal. Tese Doutoramento Universidade da Madeira. Funchal, Portugal: Universidade da Madeira. 113 p. hdl.handle.net/10400.13/27.
30. Grote A, Voronin D, Ding T, Twaddle A, Unnasch TR, Lu P, Bian G, Pan X, Xi Z. 2012. Development of Dirofilaria immitis within the mosquito Aedes (Finlaya) koreicus, a new invasive species for Europe. Parasites & Vectors, 8(1), 177.
31. Gutsevich AV, Monchadskii AS, Shtakeberg AA. 1970. Fauna of the USSR. Diptera. Mosquitoes. Nauka: Leningrad (in Russian).
32. Hertig M, Wolbach SB. 1924. Studies on rickettsia-like micro-organisms in insects. Journal of Medical Research, 44(3), 329–374.
33. Kambris Z, Cook PE, Phuc HK, Sinkins SP. 2009. Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes. Science (New York, NY), 326 (5949), 134–136.
34. Kartman L. 1953. Factors influencing infection of the mosquito with Dirofilaria immitis (Leidy, 1856). Experimental Parasitology, 2(1), 27–78.
35. Krivorotova EY. 2016. Xenomonitoring of dirofilaria in the south and north-west of the Russian Federation. Parazitologiia i Parazitarnye Bolezni (Mosk), 3, 9 (in Russian).
36. Kronefeld M, Kampen H, Sassnau R, Werner D. 2014. Prevalence of Dirofilaria immitis (Nematoda: Filarioidea) in mosquitoes from Northeast Arkansas, the United States. Journal of Medical Entomology, 50(4), 871–878.
37. Montarsi F, Cicocchetta S, Devine G, Ravagnan S, Mutinelli F, Frangipane di Regalbano A, Otranto D, Capelli G. 2015. Development of Dirofilaria immitis within the mosquito Aedes (Finlaya) koreicus, a new invasive species for Europe. Parasites & Vectors, 8(1), 177.
38. Moodley K, Govin CN, Peer AKC, Westhuizen MVD, Parbhoo D, Ming Sun L, du Plessis DC, Frenai JA. 2015. First detection of human dirofilarial infection in South Africa. Infectious Disease Reports, 7(1), 5726.
39. Morchón R, Bargues MD, Latorre JM, Melero-Alcivar R, Pou- Barreto C, Mas-Coma S, Simon F. 2007. Haplotyp H1 of Culex pipiens implicated as a natural vector of Dirofilaria immitis in an endemic area of western Spain. Vector Borne and Zoonotic Disease, 7(4), 653–658.
40. Murata K, Yanai T, Agatsuma T, Uni S. 2003. Dirofilaria immitis Infection of a Snow Leopard (Uncia uncia) in a Japanese Zoo with mitochondrial DNA analysis. Journal of Veterinary Medical Science, 65(8), 945–947.
41. Nicolescu G, Linton YM, Vladimiracu A, Howard TM, Harbach RE. 2004. Mosquitoes of the Anopheles maculipennis group (Diptera: Culicidae) in Romania, with the discovery and formal recognition of new species based on molecular and morphological evidence. Bulletin of Entomological Research, 94(6), 525–535.
42. Pan X, Zhou G, Bian G, Lu P, Raikhel AS, Xi Z. 2012. Wolbachia induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito Aedes aegypti. Proceedings of the National Academy of Sciences, 109(1), E23–E31.
43. Pan X, Pike A, Joshi D, Bian G, McFadden MJ, Lu P, Liang X, Zhang F, Raikhel AS, Xi Z. 2018. The bacterium Wolbachia exploits host innate immunity to establish a symbiotic relationship with the dengue vector mosquito Aedes aegypti. ISME Journal, 12(1), 277–288.
44. Paras KL, O’Brien VA, Reiskind MH. 2014. Comparison of the vector potential of different mosquito species for the transmission of heartworm, Dirofilaria immitis, in rural and urban areas in and surrounding Stillwater, Oklahoma, U.S.A. Medical and Veterinary Entomology, 28(Suppl 1), 60–67.
45. Ricci I, Cancrini G, Gabrielli S, Damelio S, Favia G. 2002. Searching for Wolbachia (Rickettsiales: Rickettsiaceae) in mosquitoes (Diptera: Culicidae): Large polymerase chain reaction survey and new identifications. Journal of Medical Entomology, 39, 562–567.
46. Rudolf Ľ, Sebesta O, Mendel J, Betášová L, Bocková E, Jedličková P, Venclová K, Blázková H, Sikutová S, Hubálek Z. 2014. Zoonotic Dirofilaria repens (Nematoda: Filarioidea) in Aedes vexans mosquitoes, Czech Republic. Parasitology Research, 113, 4663–4667.
47. Ryabova T, Yuniachev Y, Markovich N, Ganushkina L, Orabei V, Sergiev V. 2005. Detection of Aedes (Steigomyia) aegypti L. mosquitoes in Sochi. Meditsinskaiα parazitologiia i parazitarnye bolezni, 3, 3–5 (in Russian).
48. Sergiev VP, Supriaga VG, Bronštejn AM, Ganushkina L, Rakova VM, Morozov EN, Fedinanina LV, Frolova AA, Morozova LF, Ivanova IB, Darchenková NN, Zhukova LA. 2014. Results of studies of human dirofilariasi in Russia. Meditsinskaiα parazitologiia i parazitarnye bolezni, 3, 3–9 (in Russian).
55. Shaikevich E. 2007. PCR-RFLP of the COI gene reliably differentiates Cx. pipiens, Cx. p. molestus and Cx. torrentium of the Pipiens Complex. European Mosquito Bulletin, 23, 25–30.

56. Shaikevich E, Vinogradova E, Bouattour A, Almeida APG. 2016. Genetic diversity of Culex p. mosquitoes in distinct populations from Europe. Contribution of Cx. quinquefasciatus in Mediterranean populations. Parasites & Vectors, 9(1), 47.

57. Sulaiman I, Towson H. 1980. The genetic basis of susceptibility of infection with Dirofilaria immitis in Aedes aegypti. Annals of Tropical Medicine and Parasitology, 74, 635–646.

58. Sulesco T, von Thien H, Toderas L, Toderas I, Lühken R, Shaikevich E, Vinogradova E, Bouattour A, Almeida APG. 2019. PCR-RFLP of the COI gene reliably differentiates Cx. p. molestus and Dirofilaria immitis DNA in mosquitoes from Belarus. Parasitology Research, 115, 3535–3541.

59. Terekhova IA, Shaikevich E. 2019. Dirofilaria and Wolbachia: master manipulators of invertebrate biology. Nature Reviews. Microbiology, 6, 741–751.

60. Wilkerson RC, Linton Y-M, Fonseca DM, Schultz TR, Price DC, Strickman DA. 2015. Making mosquito taxonomy useful: a stable classification of Tribe Aedini that balances utility with current knowledge of evolutionary relationships. PLoS One, 10(7), e0133602.

61. Wright JD, Barr AR. 1980. The ultrastructure and symbiotic relationships of Wolbachia of mosquitoes of the Aedes scutellaris group. Journal of Ultrastructure Research, 72, 52–64.

62. Wu M, Sun LV, Vamathavan J, Riegler M, Deboy R, Brownlie JC, McGraw EA, Martin W, Esser C, Ahmadinejad N, Wiegand C, Madupu R, Beanan MJ, Brinkac LM, Daugherty SC, Durkin AS, Kolonay JF, Nelson WC, Mohammed Y, Lee P, Berry K, Young MB, Utterback T, Weidman J, Nieman WC, Paulsen IT, Nelson KE, Tettelin H, O’Neill SL, Eisen JA. 2004. Phylogenomics of the 235-reproductive parasite Wolbachia pipientis wMel: a streamlined genome overrun by mobile genetic elements. PLoS Biology, 2(3), e69.

63. Yıldırım A, İnci A, Duzlu O, Biskin Z, Ica A, Sahin I. 2011. Aedes vexans and Culex pipiens as the potential vectors of Dirofilaria immitis in Central Turkey. Veterinary Parasitology, 178(1–2), 143–147.

64. Zhou W, Rousset F, O’Neill S. 1998. Phylogeny and PCR-based classification of Wolbachia strains using wsp gene sequences. Proceedings of the Royal Society B: Biological Sciences, 265(1395), 509–515.

65. Zittra C, Kocziha Z, Pinnyei S, Harl J, Kieser K, Lacies A, Eigner B, Silbernayr K, Duscher GG, Fok E, Fuehrer HP. 2015. Screening blood-fed mosquitoes for the diagnosis of filarial helminthes and avian malaria. Parasitistes & Vectors, 8, 16.