

Mosquitoes of *Anopheles hyrcanus* (Diptera, Culicidae) Group: Species Diagnostic and Phylogenetic Relationships

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Abstract. Herein, we report the results of study of *Anopheles* species in Primorsk and Khabarovsk regions of Russia. Three species of the *Anopheles hyrcanus* group: *An. kleini*, *An. pullus*, and *An. lesteri* were identified by molecular taxonomic diagnostics for the first time in Russia. Surprisingly, *An. sinensis*, which earlier was considered the only species of *Anopheles* in Russian Far East, was not observed. We analyzed nucleotide variation in the 610-bp fragment of the 5' end of the cytochrome c oxidase subunit I (COI) region. All species possessed a distinctive set of COI sequences. A maximum likelihood phylogenetic tree was constructed for members of the hyrcanus group. The examined *Anopheles hyrcanus* group members could be divided into two major subgroups: subgroup 1 (*An. hyrcanus* and *An. pullus*) and subgroup 2 (*An. sinensis*, *An. kleini*, and *An. lesteri*), which were found to be monophyletic.

INTRODUCTION

The presence of numerous sibling species displaying high morphological similarity is a common feature of the genus *Anopheles* (Diptera, Culicidae).^{1–3} These species play distinct roles in transmitting malaria pathogens. Identification of potential vectors and a study of their distribution and role in a pathogen transmission are important components of malaria control measures.

The *Anopheles hyrcanus* group of mosquitoes includes about 30 closely related species distributed throughout the Palearctic and Oriental regions.⁴ Some species of this group are vectors of malaria parasites, in particular *Plasmodium vivax*.⁵ Sibling species are difficult or impossible to distinguish morphologically. The method of identification by polymerase chain reaction (PCR) was developed to differentiate five sibling species in the *An. hyrcanus* group (*An. sinensis* Wiedemann, 1828; *An. pullus* Yamada, 1937; *An. lesteri* Baisas and Hu, 1936; *An. belenrae* Rueda, 2005; *An. kleini* Rueda, 2005).⁶

In this study, we have investigated species composition of the *Anopheles hyrcanus* group in the Russian Far East. The results of phylogenetic analysis are provided.

MATERIALS AND METHODS

Mosquito larvae were collected from eight locations in the Primorsk and Khabarovsk regions of Russia from August 22 to September 05, 2011 and from June 22 to June 26, 2013 (Table 1). *An. hyrcanus* larvae were collected in the southern Kazakhstan and Zhambul regions of the Republic of Kazakhstan from April 28 to May 18, 2012. The larvae were preserved in 96% ethyl alcohol for subsequent molecular and genetic analyses. In total, we identified and studied 913 mosquitoes of the *Anopheles hyrcanus* group.

DNA was extracted using DNA Invisorb[®] Spin Tissue Mini Kit (Invitex, Berlin, Germany). Species identification was performed by PCR analysis as described by Li and others.⁶ The phylogenetic analysis of species from the *Anopheles hyrcanus* group was carried out on the basis of the comparison

of cytochrome c oxidase subunit I (COI) nucleotide sequences. The standard LCO1490 and HCO2198 primers⁷ were used to amplify the 5'-region of the COI gene.

The 5'-region of the COI gene was sequenced using the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with an ABI 3130 sequencer in 5–9 specimens of each species. The sequences of 610-bp fragments for 28 specimens of four mosquito species from the *Anopheles hyrcanus* group were obtained (GenBank accession numbers KC855638–KC855665). The 25 COI sequences of five other species from the *Anopheles hyrcanus* group and the COI sequence of *An. messeae* Falleroni, 1926 (an out-group) were retrieved from the GenBank and were also included in the analysis.

The sequences were edited and analyzed using the Sequencing Analysis 5.2 (Applied Biosystems) and SeqMan[™] II (DNASTAR Inc., Madison, WI). For the phylogenetic analysis, the sequences were aligned using ClustalW. A maximum likelihood analysis in MEGA5 was used to examine phylogenetic relationships among taxa.^{8,9} Pairwise nucleotide sequence divergences were calculated using the Kimura 2-parameter (K2P) model.¹⁰

RESULTS AND DISCUSSION

An. hyrcanus, a member of the *Anopheles hyrcanus* group, is believed to be present in the territories of the former Union of Soviet Socialist Republics (USSR).¹¹ Within this species “western” and “eastern” forms were identified on the basis of the morphological features: the coloration of the fourth hindtarsal segment and the wing pattern. In the western specimens the fourth hindtarsal segment is more often pale, wing is more often with sharp pattern, and both costal spots well developed, pattern sometimes diffuse. In the eastern specimens the fourth hindtarsal segment is more often dark with narrow pale apical ring, sometimes with pale basal ring, wing is more often with diffuse pattern, and pale costal spots sometimes partly reduced, one may be nearly or wholly lacking. The western form included *An. hyrcanus* of the European part of Russia, while the eastern form included *An. hyrcanus* mosquitoes from the Russian Far East.¹¹ However, it was impossible to identify the taxonomic status of these forms because of a great variability in the morphological characters.

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TABLE 1
Species composition of malaria mosquitoes (*Anopheles*) in the Primorsk and Khabarovsk regions

No.	Collection localities, coordinates, and dates	n	Frequencies ($f \pm s_r$, %)		
			<i>An. kleini</i>	<i>An. pullus</i>	<i>An. lesteri</i>
1	Slavyanka, Primorsk region; 42°51' N 131°23' E; August 28, 2011	48	75.00 ± 6.25	16.67 ± 5.38	8.33 ± 3.99
2	Vladivostok, Sputnik; 43°14' N 132°02' E; August 29, 2011 (IV stage)	24	100.0	0	0
3	Artem, Primorsk region; 43°22' N 132°09' E; August 27, 2011 (IV stage)	24	79.17 ± 8.29	20.83 ± 8.29	0
4	Artem, Primorsk region; 43°22' N 132° 9' E; August 27, 2011 (II stage)	48	79.17 ± 5.86	18.75 ± 5.63	2.08 ± 2.06
5	Ussuriisk, Primorsk region; 43°49' N 131°57' E; August 31, 2011 (IV stage)	24	87.50 ± 6.75	12.50 ± 6.75	0
6	Ussuriisk, Primorsk region; 43°49' N 131°57' E; August 31, 2011 (II stage)	24	66.67 ± 9.62	33.33 ± 9.62	0
7	Sibirtsevo, Primorsk region; 44°12' N 132°27' E; August 30, 2011	60	100.0	0	0
8	Priamurskii, Khabarovsk region, Amur river floodplain; 48°32' N 134°54' E; September 4, 2011 (IV stage)	30	96.67 ± 3.28	3.33 ± 3.28	0
9	Priamurskii, Khabarovsk region, Amur river floodplain; 48°32' N 134°54' E; September 4, 2011 (II stage)	30	95.74 ± 2.94	4.26 ± 2.94	0
10	Khabarovsk; 48°30' N 135°03' E; September 5, 2011	48	95.83 ± 2.88	4.35 ± 2.88	0
11	Sovetskaya Gavan', Khabarovsk region; 48°56' N 140°15' E; August 22 and 23, 2011	66	100.0	0	0
12	Priamurskii, Khabarovsk region, Amur river floodplain; 48°32' N 134°54' E; June 25, 2013 (IV stage)	117	91.45 ± 2.58	8.55 ± 2.58	0
13	Priamurskii, Khabarovsk region, Amur river floodplain; 48°32' N 134°54' E; June 25, 2013 (II stage)	21	100.0	0	0
14	Khabarovsk; 48°30' N 135°03' E; June 25, 2013 (IV Stage)	71	98.61 ± 1.38	1.39 ± 1.38	0
15	Khabarovsk; 48°30' N 135°03' E; June 25, 2013 (I–III stage)	87	100.0	0	0
16	Vladivostok, Sputnik; 43°14' N 132°02' E; June 23, 2013 (IV stage)	23	100.0	0	0
17	Vladivostok, Sputnik; 43°14' N 132°02' E; June 23, 2013 (II stage)	96	95.83 ± 1.84	0	4.16 ± 1.84
18	Vladivostok, Ugol'naya; 43°16' N 132°02' N; June 24, 2013	54	85.18 ± 3.28	0	14.81 ± 3.28

s_r = standard deviation.

On the basis of the egg exochorion and cytogenetic analysis of larval karyotype, the eastern form of *An. hyrcanus* was identified as *An. sinensis*; it was stated that it was the only species of the *Anopheles* genus in the Russian Far East.¹² However, more species of the *Anopheles hyrcanus* group have been described in the nearby countries of the Oriental zone. In particular, the list of *hyrcanus* species in China comprises 21 species¹³ and in the Republic of Korea comprises six species,⁵ five of which (*An. sinensis*, *An. pullus*, *An. lesteri*, *An. kleini*, and *An. belenrae*) are extremely difficult to identify morphologically.^{3,6} The molecular genetic assay used here allowed five species of “*hyrcanus*” group to be identified: *An. sinensis*, *An. pullus*, *An. lesteri*, *An. belenrae*, and *An. kleini*.⁶ For the first time, we identified three species of the *Anopheles hyrcanus* group in Russia: *An. kleini*, *An. pullus*, and *An. lesteri*.¹⁴ In 2011 and 2013, we found variation in species composition according to different habitats. Table 1 presents the data on the distribution and proportion of species from the *Anopheles hyrcanus* group in the Primorsk and Khabarovsk regions.

Among a total of 913 mosquitoes from the *Anopheles hyrcanus* group were identified by PCR, *An. kleini* was the most commonly collected species; it was found in all samples with a frequency from 66.67% to 100%. *An. pullus* is subdominant and was identified in 7 habitats with the frequency from 1.39% to 33.33%, followed by *An. lesteri* (from 2.08% to 14.81%), which was collected in Vladivostok, Slavyanka, and Artem (Primorsk region) (Table 1). The identified species composition is not constant and is subject to a seasonal dynamics, as has been suggested by the data from the Republic of Korea.^{3,15–17} Of special importance is that *An. sinensis* has not been detected in the examined region, although it has been regarded as the only representative of the genus *Anopheles* in the Russian Far East.¹²

A phylogenetic analysis of the *Anopheles hyrcanus* group species was carried out on the basis of the comparison of the COI nucleotide sequences.

The conspecific K2P divergence averaged 0.73% (range 0.36–1.09%), and it was higher than the conspecific K2P divergence reported earlier for mosquitoes from China (0.39%), Canada mosquitoes (0.55%) and *Aedes* and *Ochlerotatus* genera from Tomsk and Kemerovo regions (0.57%).^{18–20} The sequence divergences between species averaged 3.71% (range 2.34–4.50%), which is higher than the threshold of the intra- and interspecific differences (2%).²¹

Individuals of a single species always occurred on a common clade, regardless of where they were collected (Figure 1). The phylogenetic tree divides the *Anopheles hyrcanus* group into two major subgroups: the first subgroup comprises *An. hyrcanus* and *An. pullus*; the second subgroup consists of *An. sinensis*, *An. kleini*, and *An. lesteri* (Figure 1). These patterns correlate with those found using the ribosomal internal transcribed spacer.^{22,23} The observed low interspecific divergence is characteristic of *An. hyrcanus* and *An. pullus*, the K2P distance (2.34%) only slightly exceeds the interspecific variability. In addition, the lowest intraspecific divergence (K2P, 0.36%) occurred in *An. hyrcanus*. The interspecific divergence was the highest for *An. hyrcanus* and *An. kleini* (K2P, 4.50%). The maximal intraspecific variability (K2P, 1.09%) was observed in *An. lesteri*. At the same time, probably, *An. lesteri* species may consist of three forms.²³

Thus, we obtained new data on species composition and phylogenetic relationships for malaria mosquitoes in the Russian Far East. The list of mosquito species in Russia is now supplemented by three additional species, *An. kleini*, *An. pullus*, and *An. lesteri*. More comprehensive and detailed morphological, cytogenetic, and genetic research studies of the *Anopheles hyrcanus* group species are still required to better understand the malaria vector system in the Russian Far East.

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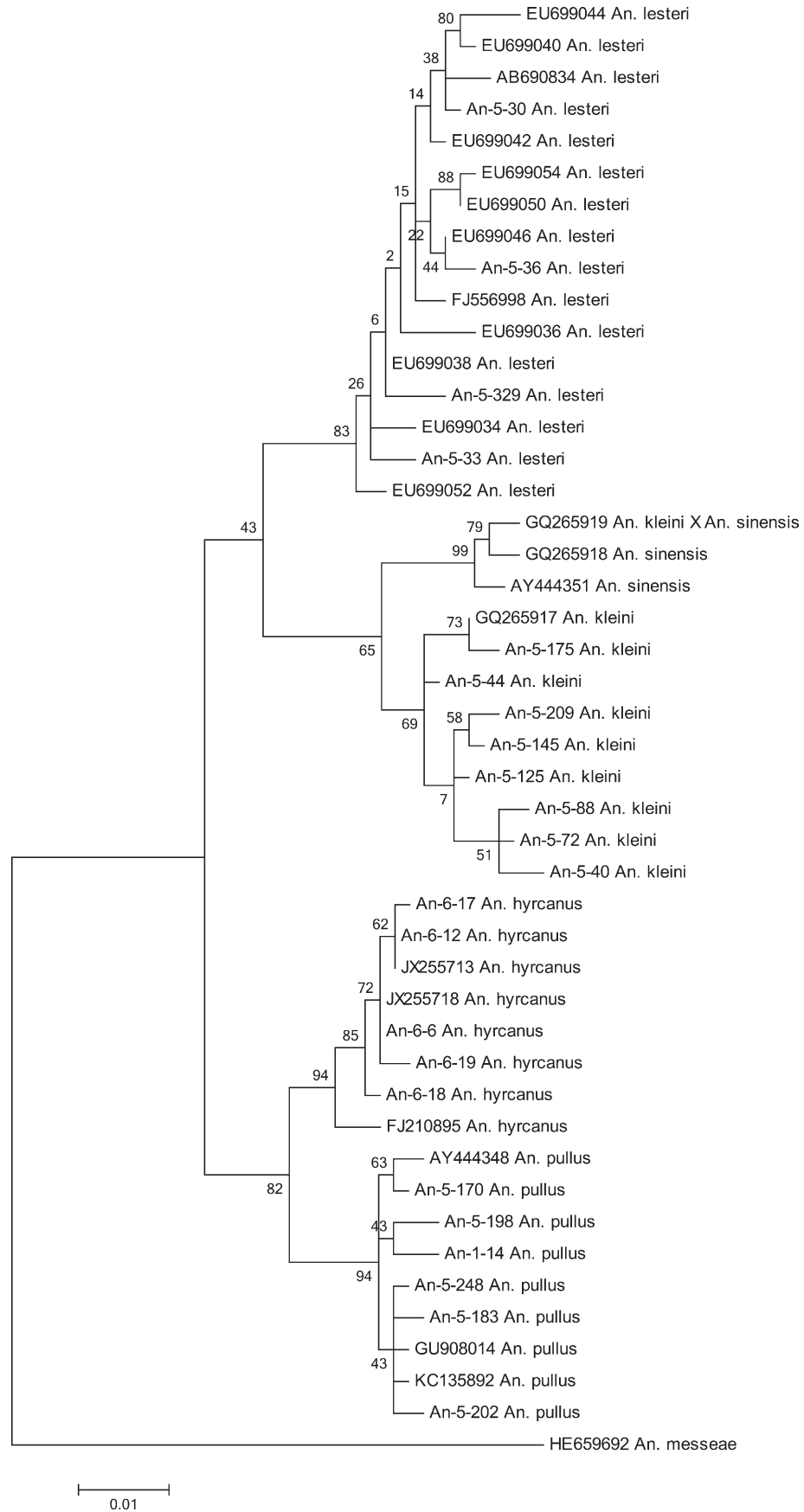


FIGURE 1. Maximum likelihood phylogenetic tree of Kimura 2-parameter (K2P) of cytochrome c oxidase subunit I (COI) *Hyrcanus* group members sequences. The numbers at the branches bases indicate the bootstrapping percentages (1,000 replicates).

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