WORLD HEALTH ORGANIZATION
EUROPE

Mosquitoes of the genus *Anopheles* in countries of the WHO European Region having faced a recent resurgence of malaria

Regional research project, 2003–2007

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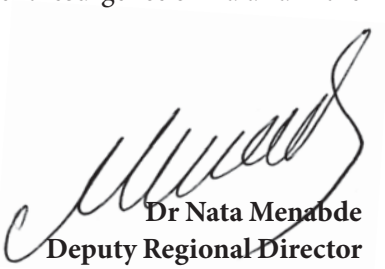
Introduction

From 1999–2007, the reported number of malaria cases within the WHO European Region declined from 90 712 to 1226, and it is likely that only 400–500 autochthonous cases will be reported in the Region in 2008. At present, locally acquired malaria continue to pose a challenge in 6 out of the 53 Member States of the European Region, namely Azerbaijan, Georgia, Kyrgyzstan, Tajikistan, Turkey and Uzbekistan. The incidence of malaria in all affected countries has been brought down to such levels that interruption of transmission of *P. falciparum* and *P. vivax* malaria has become a feasible objective. Interrupting malaria transmission by 2015 and eliminating the disease within all affected countries of the European Region is the ultimate goal of the new WHO regional strategy. Since 2008 all malaria-affected countries of the Region have moved to the elimination phase and their national strategies on malaria have been revised to reflect new elimination realities. It seems very probable that Armenia and Turkmenistan will initiate the process of certification of malaria elimination in 2009–2010. The transmission of autochthonous *P. falciparum* malaria reported in Tajikistan is most likely to be interrupted in 2009, and the WHO European Region as a whole will be free from this type of malaria starting from this year. In areas and countries where malaria had been eliminated, attention is given to maintaining the malaria-free status.

In the framework of the new WHO regional strategy aimed at malaria elimination, special attention is given to operational research. Malaria research capabilities are weak in most of the affected countries of the Region, and poor quality of research may lead to inappropriate changes in policy and practice. In order to update the scientific knowledge on malaria, the WHO Regional Office for Europe has initiated a regional programme on operational research related to malaria entomology and vector control, which is being carried out successfully with the assistance of research institutions and partners in affected countries of Middle Asia and South Caucasus. The objectives of the research are closely tied to the particular situation and problems identified within a single country or a group of neighbouring countries. The identification and geographical distribution of *Anopheles* mosquitoes, prevalence of sibling species and their role in malaria transmission, taxonomy, biology and ecology of malaria vectors are of particular interest in the Region. This research has been neglected, but is presently being reconsidered in order to make vector control more effective in producing the desired result. This paper describes the results of the studies conducted over the past five years in countries facing a recent resurgence of malaria in the WHO European Region.



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1. Problems related to research on the taxonomy of malaria vectors in the middle Asian and south Caucasian countries

It is well known that various species of the genus *Anopheles* play unequal roles in the transmission of human malaria parasites. Some *Anopheles* species are not involved in malaria transmission at all because of their rare occurrence and weak interrelations with humans. Therefore, as far as malariology is concerned, the importance of taxonomic research on malaria vectors is more than justified (*Artemiev, 2001*).

There are major difficulties in species-specific identification of the *Anopheles* mosquitoes due to the existence of numerous sibling species that are virtually identical in their external morphology at the larval and adult stages. Some species of the *An. maculipennis* complex, whose egg exochorion structure has been considered the only reliable differentiation criterion (*Gutsevich et al.,* 1970), serve as an example hereof. However, as shown later, far from all of the species of this complex can be reliably differentiated by this feature.

An important step forward has been made owing to cytogenetic analysis based on chromosome identification; chromosome map development; detection of chromosome rearrangements and chromosome localization in the nucleus. The polytene chromosome photomaps of a number of different Palearctic species of the *An. maculipennis* complex have been compiled to allow cytodiagnosis of malaria mosquitoes and registration of chromosome rearrangements. Yet, comparison of the chromosome sets of the Palearctic *An. maculipennis* complex members revealed that some of the species had identical banding patterns of polytene chromosomes (homosequential species). These species included *An. atroparvus, An. labranchiae* and *An. maculipennis, An. melanoon (An. subalpinus), An. artemievi* (*Frizzi,* 1953; *Stegnii*, 1991; *Gordeev et al.,* 2005). Thus, an analysis of the polytene chromosome banding patterns also has limited capacities for discrimination between the sibling species. In the process of detecting and defining the status of a new species among the *An. maculipennis* complex members it is extremely important to analyse the architectonics of the ovarian nurse cells nuclei and the mechanisms of chromosomes attachment to the nuclear membrane (*Stegnii, 1993*).

Currently, molecular genetic studies based on the polymerase chain reaction (PCR) have become increasingly recognized. The PCR allows precise and efficient identification of the *An. maculipennis* complex species. The main molecular genetic marker for species differentiation is the second internal transcribed spacer of the ribosomal gene cluster (ITS2). The species identification is based on an analysis of this ribosomal deoxyribonucleic acid (DNA) region by amplification with subsequent analysis of the restriction fragment length polymorphism (RFLP) and, if necessary, by sequencing the amplification products.

Owning to the modern methods of taxonomy, the list of the *An. maculipennis* complex mosquito species that inhabit the major part of the former USSR territory has increased and now includes the following

nine species: *An. maculipennis, An. messeae, An. beklemishevi, An. atroparvus, An. melanoon, An. sacharovi, An. martinius, An. artemievi* and *An. persiensis.* However, the list may be extended further.

Taxonomy problems related to the members of the subgenus *Cellia* in countries of the WHO European Region facing a resurgence of malaria have not been fully resolved. This issue is of importance, because two species of the subgenus, *An. superpictus* and *An. pulcherrimus*, play a significant role in malaria transmission in the mountainous, foothill and plain areas of the middle Asian and south Caucasian countries. A discovery of *An. multicolor* in southern and eastern Turkmenistan, where this species may be involved in malaria transmission in the plains, also requires serious attention. Yet, at present, the *An. multicolor* distribution in these countries and its actual contribution to malaria transmission is unknown (though, it is not unlikely that the species occurs in the southern territories of Uzbekistan and Tajikistan). Mamedniyazov's (2005) discovery of *An. Sergenti sergenti* in the fauna of Turkmenistan is also extremely interesting as this species is considered a malaria vector in western Asia and North Africa.

2. Materials and methods

Sample collection

Adult mosquitoes were collected both at their resting habitats, using the human bait technique (the collection technique and geographical location of the collected imagoes were specified during labeling). After the preliminary identification based on external morphological traits, some of the *An. maculipennis* complex females with mature eggs, collected in cattle sheds and in-doors, were placed in cubic containers (10×10×10cm), in which vessels with water were placed for egg collection. The egg batches thus obtained were either identified at once (up to the species or species group) or preserved on strips of filtering paper in small glass vials in 1–2% formalin solution vapors for subsequent identification. The remaining insects were fixed in ethanol (for further molecular genetic research) and kept on cotton wool plugs for morphological identification.

The *Anopheles* larvae were collected from various breeding sites using a standard larval net or a photographic pan (all water reservoirs and their geographical locations were specified during labeling). The larvae were fixed in 96% ethanol, and a part of *An. maculipennis* complex larvae was fixed in ethanol/acetic acid solution for further cytogenetic examination.

Identification of larvae and imago stages by their external morphology was carried out using the standard identification key tables (*Gutsevich et al.,* 1970; *Zvantsov et al*., 2003).

Cytogenetic analysis

For the cytogenetic research, four instar larvae were fixed in a mix of 96% ethanol and glacial acetic acid (3:1). Larval salivary glands were used for staining polytene chromosomes using the lacto-aceto-orsein technique (*Stegnii,* 1991). To detect and identify inversion polymorphism, the polytene chromosomes banding patterns were compared with photomaps of the studied species karyotypes (*Stegnii,* 1991). The

chi-square test (*Gershkovich,* 1968) was used to compare the chromosomal composition of the *An. messeae* populations.

Molecular genetic methods

DNA isolation

For the molecular genetic research, mosquitoes were fixed in 96°C ethanol. DNA isolation was performed using the DIAtom DNA Prep kit (Isogene, Russia), according to the instructions of the manufacturer. For amplification, 0.1 μg of the isolated total DNA was taken each time.

PCR-RFLP

For species identification of *An. maculipennis* complex mosquitoes, the PCR-RFLP method was used. PCR was run in a thermocycler GeneAmp RCR System 2700 (Aplied Biosystems, USA), using primers 5.8S-5'-TGT GAA CTG CAG GAC ACA TG-3' and 28S-5'-ATG CTT AAA TTT AGG GGG TA-3' (*Porter, Collins,* 1991), complementary to the regions of 5,8S and 28S rRNA genes and Universal amplification kits (Isogene, Russia). The PCR temperature regime followed the published protocols (*Proft et al.,* 1999).

Restriction of the amplification products, i.e. of the second transcribed spacer area flanked by 5,8S and 28S rDNA regions, for the *An. maculipennis* complex mosquitoes was carried out in two stages. The first stage was conducted by employing restriction endonuclease *CfoI (Hhal),* (*Nikolescu et al.*, 2004). The restriction of the amplification products revealed in *An. sacharovi* forms DNA fragments of 48, 78, 111 and 207; in *An. messeae* and *An. daciae,* of 48, 111, 135 and 141; in *An. melanoon,* of 48, 108, 135 and 141 bp; in *An. persiensis,* of 48, 108, and 136 bp; in *An. maculipennis,* of 48, 102, and 272 bp; and in *An. atroparvus,* fragments of 48 and 389 bp.

As the restriction fragments patterns of *An. messeae, An. daciae* and *An. melanoon* proved to be similar and indiscernible by 2.5% agarose gel electrophoresis, the second stage involved additional restriction of the PCR products. For this purpose, species-specific restriction endonucleases were used. *MroXI* endonuclease cleaved the *An. messeae* ITS2 nucleotide sequence into 158- and 277-bp fragments; *SacII,* the corresponding sequence of *An. daciae* into 384- and 51-bp fragments; and *BstEII,* the sequence of *An. melanoon* into 335- and 97-bp fragments. In order to examine the individual polymorphism of *An. messeae* and *An. daciae*, *MroXI* and *BseGI* restriction enzymes were used. Restriction endonuclease *Bse*GI cleaves *An. daciae* ITS2 into 158; 277 bp; *Mro*XI cleaves ITS2 of *A. messeae* into 159, 276 bp.

Electrophoresis

For visualization of the restriction analysis and PCR results, 1% and 2.5% agarose gel electrophoresis were performed, respectively.

Elution of the amplification products

For sequencing, the PCR products were purified using a JETQUIK elution kit (Germany) in accordance with the instructions of the manufacturer.

Sequencing

To assess the species-specific differentiation, DNA from some of the mosquito samples was sequenced. Sequencing of the amplification products was carried out in an ABI PRISM 310 sequencer using both primers and reagents provided by Applera (USA), according to the instructions of the manufacturer. The resultant ITS2 nucleotide sequences were compared and aligned with sequences from the GenBank database with the help of the software package CLUSTAL W 1.83. The chromatogramme analysis of the ITS2 sequences was carried out using the Cromas Pro 13.3 procedure.

RAPD assay

The Random Amplified Polymorphic DNA (RAPD) assay involved total DNA of the mosquitoes from the collected samples. Total DNA was isolated by SDS-lysis assay with subsequent phenol-chloroform extraction. After that, the total DNA was precipitated by 96% ethanol or isopropanol. The sediment was dried at 60°C for 10 minutes and re-suspended in 100 µl of sterile de-ionized water. This technique is well suited for total DNA isolation from mosquito (imaginal and larval) tissues.

After DNA isolation and purification, its concentration, purification efficiency and fragmentation rate were determined. The DNA fragmentation rate was established using the 0.8% agarose gel electrophoresis assay. The DNA of the sample was considered suitable for further analysis, if the 10-20-kb fraction constituted at least 20% of the total amount of the isolated DNA. For assessing the concentration and purification rates, the optical absorption of the DNA samples at 260 and 280 nanometer wave lengths was measured using a SP26 spectrophotometer. After all measurements, the DNA was diluted up to the final concentration of the 20 ng/µl. The DNA purification level was measured by the ratio of optical absorption 260/280. If the ratio exceeded 1.8, the DNA sample was considered suitable for further analysis.

The PCR assay was carried out using Biomaster (Russia) reagent kits and commercial RAPD-primers developed by Operon (USA). All the primers were provided by Sintol (Russia). For each sample, the reaction was carried out in the reaction mix of 25 µl that contained a single-use buffer solution of the Biomaster kit, 2 mM of the magnesium chloride, 200 µM of each dNTP (dATP, dTTP, dCTP, dGTP), 62.5 pM of the corresponding RAPD-primer, 0.25 units of *Taq* polymerase, 60 ng of the total DNA, and de-ionized water up to the total volume of 25 µl. The amplification was performed in a PTC-150 thermal cycler (MJ Research, USA), according to the following protocol: 94°C for 3 min (one cycle); 94°C for 1 min, 36°C for 30 seconds, 72°C for 1 min (36 cycles); 72°C for 10 min (one cycle).

The gel electrophoresis assay was carried out using 0.8% agarose gel or 6% polyacrylamide gel (PAAG) in a single-use TBE buffer under the standard conditions (*Maniatis et al.,* 1982). The λ phage DNA, digested by *Pst*1 restriction enzyme and synthetic molecular weight markers of Fermentas (Vilnius, Lithuania), were used as molecular weight markers. After electrophoresis, the PAAG or the agarose gel were stained by the ethidium bromide according to the standard protocol (*Maniatis et al.,* 1982). After the RAPD amplification, the DNA fragments were visualized in transmitted UV light.

3. Results

3.1. Malaria vectors in middle Asia and Kazakhstan

Traditionally, the term Middle Asia refers to an extensive area, stretching from the Caspian Sea in the west to the borders with China in the east, and from the Aral-Irtysh watershed in the north to the borders with Iran and Afghanistan in the south. The area of Middle Asia includes the republics of Kyrgyzstan, Tajikistan, Uzbekistan, Turkmenistan and the major part of Kazakhstan. The northern parts of Kazakhstan (to the north from the Turgay plateau and Kazakh knolls) belong to the West Siberian lowland (*Suslov*, 1954). The territory of Middle Asia is subdivided into three areas: semi-deserts, deserts and mountains.

The area of semi-deserts is a comparatively narrow strip that stretches in latitudinal direction from the upper reaches of the Emba River to the Zaisan Lake, and represents a geographical transition zone between steppes and deserts. The area includes the Mugodzhar mountains, the Turgay plateau and the Kazakh knolls. High summer temperatures and low precipitation, leading to high salinity of both subterranean and superficial waters, and the abundance of salt-lakes are characteristic for this area.

The area of deserts of Middle Asia extends to the south of semi-deserts of Central Kazakhstan, approximately along an imaginary line that begins at the northern precipice of the Ustyurt plateau, passes just north of the Aral and Balkhash lakes, takes direction towards the southern slopes of the Tarbagatay ridge in the east, and ends in the foothills that border the area from the south. The territory comprises self-contained internal-drainage basins with several powerful transit rivers, carrying abundant waters from the mountains, thus having an important irrigational value. For deserts, the oasis agriculture with artificial irrigation is a common feature.

The mountainous area of Middle Asia comprises very elevated highlands, strongly dissected, with great contrasts of altitudes, an extremely complex geological structure and diverse geographical landscapes. The largest mountain systems include Tien Shan with the highest peak of Khan Tengri, from which to the west and to the southwest two mountain arcs branch off: the northern arc – the Tien Shan, and the southern arc – the Alai. To the south of the Trans Alai ridge the great ridges of Pamir stretch out. Between the main ridge systems lie the largest intermountain depressions: Fergan, Naryn, Issyk Kul and Iliy. Turbulent mountainous rivers, flowing down from the ridges, provide irrigation for the fertile foothill plains and fluvial terraces in the mountains. Apart from the above there are two independent mountain systems, the Dzungarian Ala Tau and the Kopet Dag.

Owing to a warm climate and an abundance of water sources, the huge part of the Middle Asian territory is malariogenic, except for the water-lacking deserts and high altitude mountainous areas. Historically, malaria foci emerged in the irrigated agricultural zones and in the settlements located on fluvial terraces in the mountains (at altitudes up to 2000m above sea level). Due to the extreme variety of landscapes and climatic conditions in this vast area, several malaria vectors cause transmission of malaria, and their significance varies according to location.

Abundant literature is dedicated to the species composition of the genus *Anopheles* mosquitoes and their role in malaria transmission, but we will restrict ourselves to mentioning only the main reports.

Traditionally, members of the *An. maculipennis* complex (subgenus *Anopheles*) present difficulties for species identification because they are morphologically similar both at the imago and larval stages. According to the views expressed in the late 20th and early 21st century (*Anufrieva*, 2001*; Zvantsov et al.,* 2003), three species of the complex (*An. maculipennis*, *An. messeae* and *An. martinius*) occur in Middle Asia. However, our examination of the species composition of the *An. maculipennis* complex, conducted in the Fergana valley region in Kyrgyzstan in 2003, has revealed mosquitoes whose egg exochorion structure was similar to that of *An. martinius*, though their polytene chromosomes appeared to be homosequential with those of *An. maculipennis*. Based on this, we have recognized this form as a new species of the *An. maculipennis* complex, which was named *Anopheles artemievi* after the outstanding Russian entomologist, Dr Mikhail Artemiev (1943-2002).

In order to check the status of the newly discovered species, we have conducted a comparative analysis of the genetic structure of the *An. maculipennis* complex mosquitoes from two regions of Middle Asia: the plains of the Amu Darya valley (Karakalpakstan) and the Fergana valley area (Kyrgyzstan).

PCR with primers specific to the 5.8S and 28S rRNA genes produced amplification products (the second transcribed spacer ITS2 region flanked by the 5.8 and 28S rDNA sections) for the DNA of the mosquitoes collected in the Karakalpakstan area. According to cytogenetic evidence, they belonged to the species *An. martinius*.

PCR assays were conducted using the DNA samples prepared from 60 imago and larvae stages produced 447-bp amplification products. Amplification products of the DNA of five mosquitoes from the vicinity of the town of Nukus (Uzbekistan) were sequenced. The consensus nucleotide sequence was deposited in the GenBank under the accession number AJ849885.

The *An. martinius* ITS2 region sequence was compared with the corresponding sequences of *An. artemievi* (AJ849886) and *An. maculipennis* (AY238435). The *An. martinius* and *An. maculipennis* sequences were homologous by 84%; and *An. martinius* and *An. artemievi* sequences - by 87 %. A comparison of *An. martinius* and *An. maculipennis* sequences revealed 7 insertions, 2 deletions and 34 single-base substitutions (point mutations); *An. martinius* and *An. artemievi* differed by 12 insertions, 2 deletions and 40 single-base substitutions (*Fig. 1*).

Thus, the compared species share the specific structure of the ITS2 region. It is worth noting that all investigated mosquitoes from different localities of the Amu Darya River valley were identical by the nucleotide composition of the ribosomal DNA locus under study. Examination of mitochondrial DNA produced similar results.

PCR with the primers, complementary to the cytochrome oxidase I gene (COI), produced characteristic 311-bp amplification products. The COI nucleotide sequences were established for two Karakalpak populations of *An. martinius*, and for two Kyrgyzs populations of *An. artemievi*.

```
artemievi        TGTGAACTGCAGGACACATGAACACCGATAAGTTGAACGCATATTGCGCATCGTGCGACA
maculipennis     TGTGAACTGCAGGACACATGAACACCGATAAGTTGAACGCATATTGCGCATCGTGCGACA
martinius        TGTGAACTGCAGGACACATGAACACCGATAAGTTGAACGCATATTGCGCATCGTGCGACA
                 ************************************************************

artemievi        CAGCTCGATGTACACATTTTTGAGTGCCCATATTTGA------CCCAAGTCAAACTACGT
maculipennis     CAGCTCGATGTACACATTTTTGAGTGCCTATATTTGA------CCCAGGTCAAACTACGT
martinius        CAGCTCGATGTACACATTTTTGAGTGCCCATATTTGATCTTAACCTAAGTCAAACTACGT
                 **************************** ********     ** * ************

artemievi        --ACTGCCG--TACGTGCATG-ATGATGAAAGAGTTTGGAAA--CGCTTCCT----TCTC
maculipennis     --ACCTCCGGGTACGTGCATG-ATGATGAAAGAGTTTGGAAC--ACCATCCT----TCTC
martinius        CGGCGAAGCCGTACGTGCATGGATGATGAAAGAGTTTGGGACTAGACATCCCATCATCTC
                   *      ********** ***************** *     * ***     ****

artemievi        TTGCATTGAAAG-CGCAGCGTGTAGCAACCTCAGGTTTCAACTTGCAAAGTGGCCATGGG
maculipennis     TTGCATTGAAAA-CGCAGCGTGTAGCAACCCCAGGTTTCAACTTGCAAAGTGGCCATGGG
martinius        TTGCATCGAAAATCGTAGCGTGTAACA-CCCAGGGCTTCAACTTGCAAAGTGGCCATGGG
                 ****** ****  ** ******** ** **   ** ************************

artemievi        GCCGACACCTCACCACCATCAGCGTGCTGTGTTGCGTGTTCGGCCCAGTTCGGTCATCGT
maculipennis     GCTGACACCTCACCACCATCAGCGTGCTGTGTAGCGTGTTCGGCCCAGTTCGGTCATCGT
martinius        GCCGACACCTCACCACCATCAGCGTGCTGTGTAGTGTGTTCGGCCCAGTTCGGTCATCGT
                 ** ***************************** * ************************

artemievi        GAGGAGTAACCCCA-----AT-TACACACTGTTGCGCGTATCTCATGGTT---ACCCAAC
maculipennis     GAGGCGTTACCTAACGGGGAGGCACACACTGTTGCGCGTATCTCATGGTT---ACCCAAC
martinius        GAGGCG-AACCCAACGGGGATGCACCTGCAATTGCGCCTATCCCATGGTTCTCACCAAAC
                 **** *  ***  *     *   **   *  ****** **** *******   *** ***

artemievi        CATAGCAGCAGAGATACAAGACCAGCTCCTAGCAGCGGGAG--TTCATGGGCCTCAAATA
maculipennis     CATAGCAGCAGAGATACAACACCGGCTCCTAGTAGC--------CCATGGGCCTCAAATA
martinius        CATAGCAGCAGGGATACAAAACCAGCTCCTAGCTACGGGAGAGTACATGGGCCTCAAATA
                 *********** ******* *** ********   *        ***************

artemievi        -TGTGTGACTACCCCCTAAATTTAAGCAT
maculipennis     ATGTGTGACTACCCCCTAAATTTAAGCAT
martinius        ATGTGAGACTACCCCCTAAATTTAAGCAT
                 **** ************************
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Fig. 1 **Nucleotide composition of the rDNA ITS2 region in the three sibling species of the *An. maculipennis* complex**

The results of comparison of the aligned 228-bp sequences are shown in *Fig. 2*.

The COI sequences of *An. artemievi* from Jalalabad and Osh differed by one insignificant substitution and were identical to sequence AY258166, registered in the GenBank (*Di Luca et al.,* 2004). At the same time, these sequences differed from that of *An. martinius* from the Karakalpak region by 19 nucleotide

```
AY258166           SDFPDSYLAW NIVSSLGSTI SLFAILYFLF IIWESMITQR APAFPMQLSS
artemievi Djelalabad..... ..... ..... ..... .....
artemievi Osh         ..... ..... ..... ..... .....
maculipennis AY258165..... ..... ..... ..... T.....
martinius Nukus      .....R.... ..... ..... ..... T.....
martinius Kungrad    .....R.... ..... ..... ..... T.....

AY258166           SIEWYHPLP AEHTYAELPL LTNNF
artemievi Djelalabad..... ..... .....
artemievi Osh         ..... ..... .....
maculipennis AY258165..... ..... .....
martinius Nukus      ....F..... ..... .....
martinius Kungrad    ....F..... ..... .....
```

Fig. 2 **Variants of the amino acid sequence of the cytochrome oxidase I gene region in three sibling species of the *An. maculipennis* complex**

substitutions. It is worth noting that sequence AY258166 is recorded in the GenBank as a diagnostic character of *An. martinius*, which is an obvious error. Actually, this sequence is characteristic of the new species *An. artemievi.*

The comparison of the COI nucleotide sequences of *An. artemievi* and *An. maculipennis* (AY258165) yielded 14 substitutions. The substitution of the first nucleotide in the triplet GCC (Ala) of *An. maculipennis* by the ACA (Thr) in *An. artemievi* leads to the change of the amino acid structure (*Fig. 4.2*). Other substitutions do not influence the functions of the cytochrome oxidase gene I, due to genetic code degeneration.

COI sequences of *An. martinius* from two areas of the Karakalpak region are identical and differ from *An. maculipennis* (AY258165) by 13 nucleotide substitutions. The substitution of the first nucleotide in triplet AGT (Ser) of *An. maculipennis* by the CGT (Arg) in *An. martinius*, and the second nucleotide in the TAT (Tyr) triplet of *An. maculipennis* by TTC (Phe) in *An. martinius* lead to amino acid substitution (*Fig. 2*).

On the whole, our results show significant differences among species by two genetic markers of *An. martinius*, *An. maculipennis* and *An. artemievi* malarial mosquitoes. This proves the status of these forms as isolated species.

In 1986-2005, we carried out morphological, cytogenetic and molecular genetics analyses of the *An. maculipennis* complex from the Tien Shan area (*Gordeev et al.,* 2006a), the Kyzyl-Orda region of Kazakhstan and the Kashkadarya region of Uzbekistan. The results of the cytogenetic and molecular genetics analysis showed a predominance of *An. messeae* in the northern Tien Shan valleys. In this region, *An. messeae* uses warm backwaters as breeding sites, as well as flood-lands, droves, shallow waters in lakes, ponds and swamps. The Tien Shan ridges form the southern border of this species' natural habitat in the southeast of Kazakhstan and in Kyrgyzstan. According to our findings, the southernmost bound-

ary of the *An. messeae* range is at the southeast foot of the Karatau ridge (South Kazakhstan region) and at the Issyk Kul depression. This species occurs in plains and in mountains at altitudes up to 2000m above sea level, which corresponds to a mountain steppe belt (*Dubitskii*, 1970). In Middle Asia, *An. messeae* is considered an active malaria vector in the north of Kyrgyzstan (*Petrischeva,* 1940, 1940a).

An. Artemievi, which was wrongly identified as *An. martinius* in the past (*Plishkin*, 1989), occurs in the Batken, Osh, Naryn and Jalalabad regions of Kyrgyzstan. *An. artemievi* was described as a new species in the Kyrgyz Fergana valley region (*Gordeev et al.,* 2005). *An. artemievi* predominates in the intermountain depressions of the internal southwestern Tien Shan and the adjacent territories of the Ghissaro-Alay. According to our data, *An. artemievi* prefers stagnant or slowly flowing water reservoirs and well-warmed biotopes, such as swamped areas, filtration reservoirs, stagnant waters in the pebbly riverbeds and rice checks.

However, none of the samples collected in the Tien Shan territory included *An. martinius* (known as *An. sacharovi* in old publications) individuals. Previously, *An. martinius* was thought to be one of the predominating species in the western and northern Tien Shan (*Dubitskii*, 1970). It was believed that its distribution was limited from the west by the Karatau ridge and the Aral Sea (*Beklemishev, Zhelokhovtsev*, 1945). On the other hand, it was presumed that this species was spread along the Tien Shan foothills towards the northeast and up to lake Zaisan, where a few findings of *An. martinius* were masked by abundant *An. messeae* occurrence (*Dubitskii*, 1970). According to our data, *An. martinius* does not occur in the Tien Shan mountains. In our opinion, *An. artemievi* that is recorded to date was previously mistakenly identified as *An. martinius* on the basis of egg exochorion analysis. Note that the egg structure and the exochorion pattern of *An. artemievi* and *An. martinius* are similar and discrimination between these species should involve cytogenetic and molecular genetics markers (*Gordeev et al.*, 2005).

The reliable findings of *An. martinius* took place in the northeast of Turkmenistan (*Stegnii*, 1976), the Kopet Dag (*Mamednyazov*, 1995), Karakalpakstan and the Khorezm area of Uzbekistan (*Gordeev et al.*, 2006), the city of Kyzyl-Orda and its region in Kazakhstan (our unpublished data).

The reports on *An. maculipennis* discovery in the southwest Tien Shan and in the adjacent areas deserve special discussion. In particular, this species has been identified by its egg batches and by the polytene chromosome structure in the Sokh enclave of Uzbekistan (*Stegnii,* 1976; *Tadjieva,* 1979). Note than, from a cytogenetic point of view, it is problematic to differentiate between *An. maculipennis* and *An. artemievi*, because these two species have the same polytene chromosome banding pattern. According to our cytogenetic data, the individuals with *maculipennis-artemievi* karyotypes occur in the Southwest, West and Internal Tien Shan. In all cases the additional research on the ITS2 region nucleotide structure of the mosquitoes with such karyotypes from different localities, has revealed *An. artemievi*. The ITS2 consensus sequence of *An. artemievi* was deposited in the GenBank database under the accession number AJ849886. In 2006, we conducted research on egg batches in the settlement of Karatokoy (Batken region, Kyrgyzstan) at the border with the Uzbek Sokh enclave. The study revealed only the *artemievi*-type eggs. At present, the reliable finds of *An. artemievi*, confirmed by the molecular genetic assay, include those made in the vicinity of Khudjand, which before the discovery and description of *An. artemievi*, was attributable to *An. maculipennis* (*Gordeev et al.,* 2004; 2005). The discovery of *An. maculi-*

pennis in the Kopet Dag (*Mamedniyazov*, 1995), that apparently is the exclusive finding of this species in Middle Asia, is very interesting. Proving the existence of the species in the Kopet Dag, however, will require additional studies using molecular genetic methods.

Thus, evaluating the current data on the *An. maculipennis* complex species distribution in Middle Asia (*Fig. 3*), we conclude that the southern border of *An. messeae* range runs along the southern Kazakhstan and northern Kyrgyzstan territories, and that these territories are the southernmost sites of detection of this mainly European-Siberian species. As for *An. martinius*, its distribution is limited by the western regions of the Middle Asian deserts and semi-deserts. *An. artemievi* predominantly inhabits the Middle Asian mountainous area and the mountain depressions (Naryn and Fergana), occurring at altitudes up to 1600m above sea level (Ugut, Naryn province, Kyrgyzstan). The northern border of this species follows the southern edge of Karatau and runs across the northern slopes of the Talass Ala Too and the Naryn depression. The western border of the species range goes approximately along the line Shymkent-Karshi, while the southern border runs along the line of the Karshi-northern slopes of the Alay Range. The epidemiological significance of *An. artemievi* is unknown and requires future investigation.

The few findings of *An. maculipennis* in the Kopet Dag (*Mamadnyazov O., 1995*) suggest that it is a rare species in Middle Asia, which, most likely, has a disrupted range. Further studies will allow determining the distribution of this species in Middle Asia.

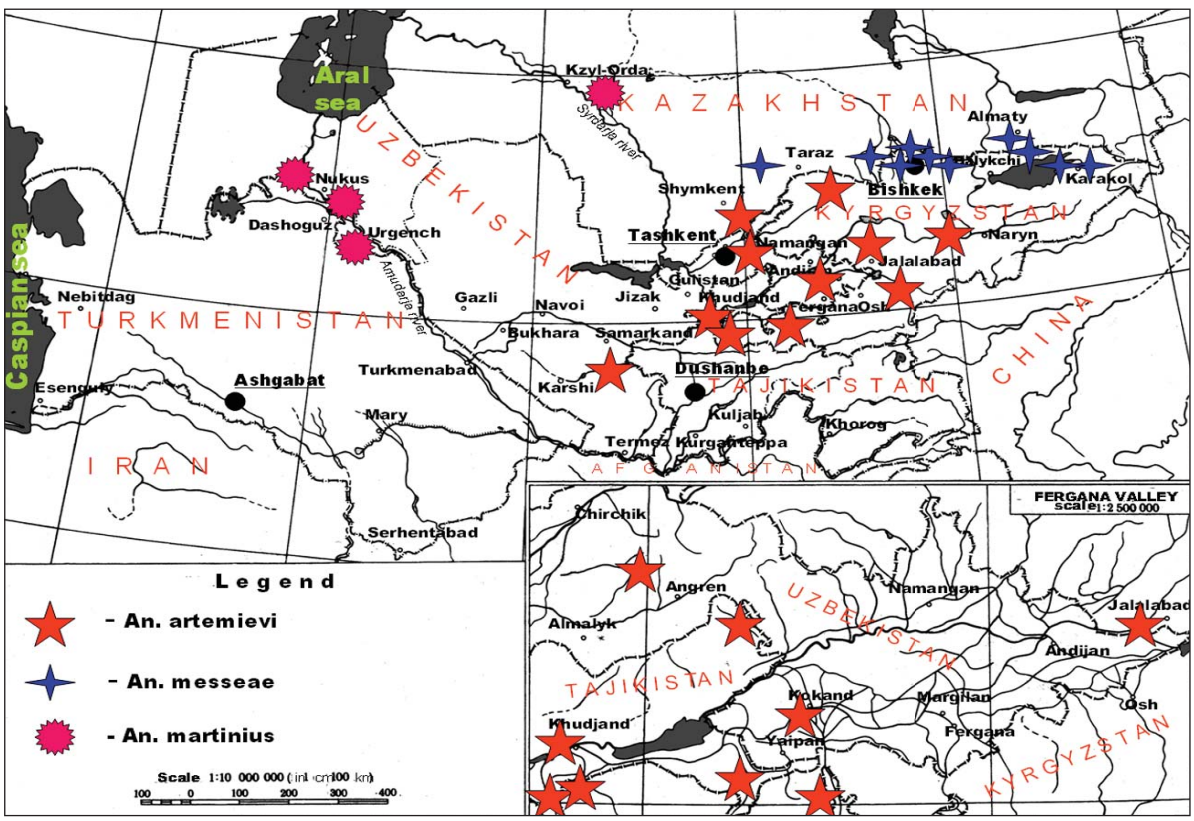


Fig. 3 **Distribution of the *An. maculipennis* complex species in Middle Asia**

Systematics of the subgenus *Cellia* inhabiting Middle Asia requires thorough revision. In his last paper, *O. Mamednijazov* (2005) indicated the existence of an active malaria vector (*An. sergenti sergenti*) in Turkmenistan, but the exact locations of his findings of this species remain unknown. Moreover, this author identified eight new species of this subgenus, however, before he could name and describe them, this remarkable scientist passed away. Further research of the mosquitoes of the subgenus *Cellia* is needed, especially in the south of Turkmenistan, Uzbekistan and Tajikistan.

One of the aims of the operational research is to examine the genetic organization of populations of the main malaria vectors, *An. superpictus* Grassi and *An. pulcherrimus* Theobald, in the malaria foci in Tajikistan and in the neighbouring territories of Uzbekistan (*Abramova et al.*, 2005).

To estimate the genetic structure of mosquito populations, we used the single-primer RAPD, a technique for local amplification of the comparatively short sequences (100 to 2500 bp), flanked by short inverted repeats. This was aimed at obtaining RAPD fingerprints to identify the species and populations of the principal malaria vectors in Middle Asian.

The *An. superpictus* larval samples were collected in the following localities:

- **Tajikistan:** Khatlon region, Dangara settlement, swamps (20 July 2002); Hodzhamaston district, Mekhnat settlement, Novobod-2 plot, rice fields (22 July 2002); Shaartuz district, Berlaish settlement, rice fields (23 July 2002); Sogd region, Khodjent outskirts, Yova outskirts, Golomaydon canal (25 July 2002); Nurek outskirts, Langar settlement, swamps (21 July 2002);
- **Uzbekistan:** Surkhan-Darya region, Uzun district, constant water reservoir in the flood-lands of the Obizarang river (10 August 2002); Termez district, Zhajrankhand sanatorium, the Surkhan river riverbed (11 August 2002).

The samples of *An. pulcherrimus* larvae were collected in the following localities:

- **Tajikistan:** Khatlon region, Bokhtar district, a lake in the flood-lands of the Vakhsh river (22 July 2002);
- **Uzbekistan:** Surkhan-Darya region, Termez district, Zhairankhand sanatorium, the Surkhan riverbed (11 August 2002).

The samples of the *An. superpictus* gonoactive females were collected in the Mekhnat settlement of the Hodzhamaston district and the Berlaish settlement of the Shaartuz district in the Khatlon region, Tajikistan, 22-23 July 2002. The *An. pulcherrimus* imago stages were collected in the Yeruglik settlement of the Shurchik district in the Surkhan Darya region of Uzbekistan on 9 August 2002. The adult mosquitoes were captured at their day resting sites in cattle-sheds and fixed in 96% ethanol.

For seven *An. superpictus* and two *An. pulcherrimus* populations, RAPD-fingerprints were obtained, using decamer primers A09 and B08 (Operon). One of the methods of electrophoretic fractionation in 6% PAAG of the RAPD-amplification products with the B08 primer is presented in *Fig. 4*.

A comparison of *An. superpictus* individuals from different populations has shown that they differ only in frequency of different RAPD variants. *An. superpictus* and *An. pulcherrimus* had species-specific RAPD-fingerprints.

For *An. superpictus* and *An. pulcherrimus*, we compared the DNA-fingerprints of their imago and larvae stages from the same, different, or neighbouring localities. The RAPD-fingerprints of the imago and larvae stages of both species were highly similar.

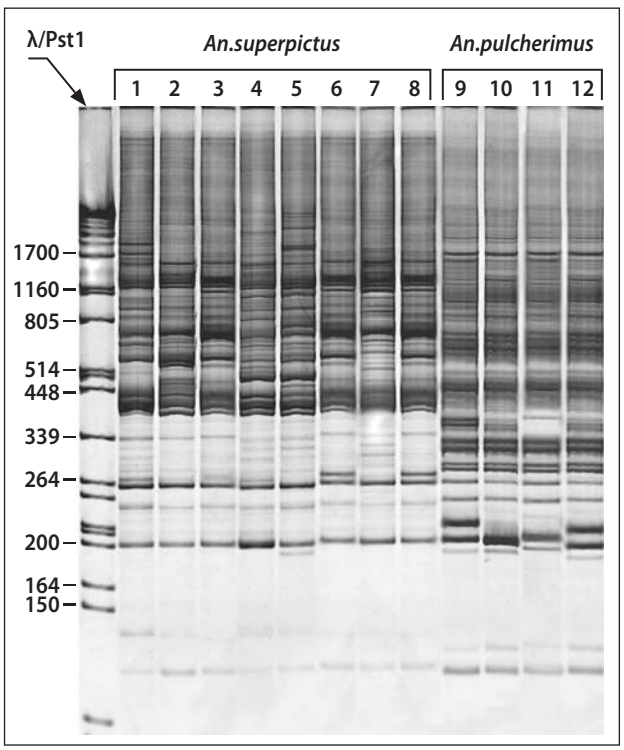


Fig. 4 **RAPD-amplification products with the B08 primer**

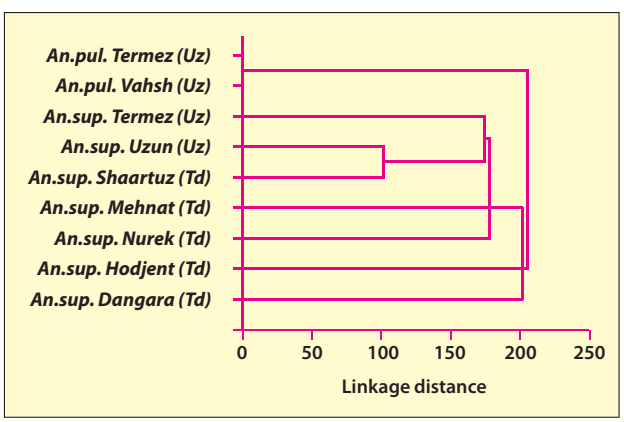


Fig. 5 **The dendrogram for seven** *An. superpictus* **and two** *An. pulcherrimus* **populations using UPGMA**

Based on the frequencies of 15 RAPD variants produced with primers A09 and B08, we constructed an UPGMA (unweighted pair group method with arithmatic mean) dendrogram showing the inter-population differences of the species examined. The dendrogram for seven *An. superpictus* and two *An. pulcherrimus* populations is presented in *Fig. 5*.

The smallest genetic distance has been detected between the *An. superpictus* populations from localities closely located to each other in the Uzun (Uzbekistan) and Shaartuz (Tajikistan) districts. The geographically distant populations of the Dangara settlement and Khodjent are most genetically distant from all other *An. superpictus* populations. As expected, *An. pulcherrimus* clustered separately from all *An. superpictus* populations. The RAPD results conform to the geographical distribution of these two species and allow identification of the genetic composition of the vectors in the malaria foci.

Inter-population differences based on the RAPD-loci frequencies among *An. superpictus* mosquitoes were lowest in the Tajikistan regions with high malaria morbidity (middle part of the dendrogram). Note that by genetic composition the populations from the malaria foci in Tajikistan are close to the *An. superpictus* populations from the adjacent Surkhan-Darya region of Uzbekistan. It is possible to assume that genetically close populations (Shaartuz, Mehnat, Nurec, Termez, Uzun) are

descended from the same population nucleus and form an integrated subpopulation system. Genetic and ecological similarities of the mosquitoes that belong to this system provide an opportunity for the spread of malaria from Tajikistan neighbouring Uzbekistan. Further research with different molecular genetic markers will allow studying in more detail the genetic variability of the main malaria vectors of Middle Asia.

Preliminary PCR-assay of malaria mosquitoes with infected blood was conducted in the Khatlon province of Tajikistan and the adjacent Surkhan-Darya region of Uzbekistan (Zaciepina, Sokolova, Gordeev, 2002; unpublished). *P. falciparum* parasites were detected by nested-PCR in *An. pulcherrimus*; and parasites of *P. vivax* were detected in *An. pulcherrimus* and *An. superpictus* in the borderland of Uzbekistan and southern Tajikistan. The genetic composition of infected mosquitoes is of prime interest for future molecular-genetic investigations.

3.2. Analysis of malaria vectors of the *An. maculipennis* complex in southern Caucasia

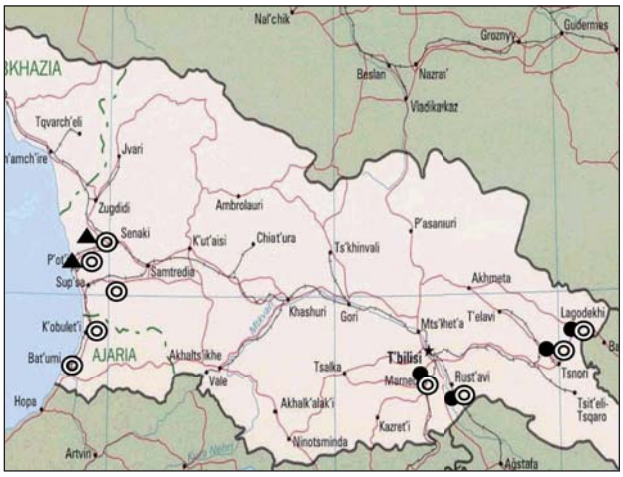
In South Caucasian countries, the species composition of the malaria vectors was investigated most extensively before the 1950s. In the subsequent period, after the eradication of malaria throughout the territory of the former USSR, no large-scale studies of the species composition of malaria mosquitoes and their distribution have been carried out.

A recent deterioration of the malaria situation calls for a revision of the entomological data on malaria. One of the objectives of this research project was to examine closely related species of the *An. maculipennis* complex from the South Caucasian region. The research tasks included determining the composition of vector species by using morphological, cytogenetic and molecular genetic markers, and an analysis of their geographical distribution, including the sympatric species.

Study of the malaria vectors in Georgia

The geographical position of this mountainous country plays an essential role in the formation of malarial mosquitoes' fauna, influenced by the semi-humid Mediterranean climate, arid internal-drainage Aral-Caspian depression and the continental Western Asian upland. In the past territories highly affected by malaria were located in the Kolkhida (western Georgia) and the Iverian (eastern Georgia) plains and hilly depressions, which are separated from each other by the Dzirul Massif (Upper Imeretian Plateau) (*BSE,* 1972).

Conditions favorable for malaria transmission exist in an area covering nearly 52% of the country where 93% of the total population lives. In recent years, the highest risk of resurgence of malaria and its spread is in the areas bordering Azerbaijan and Armenia (Marneuli, Gardabani, Ladokhegi, Signani, Bolnisi, etc.) in eastern Georgia, the Black Sea coastal areas, and the Kolkhida lowlands in the western part of the country, where more than 68% of the total population resides, and where the transmission season may last more than 150 days (*Imnadze*, 2000). The species composition of the *An. maculipennis* complex malaria vectors has been studied mostly in these areas (*Fig. 6*).



◎ *An. maculipennis,* ● *An. sacharovi,* ▲ *An. melanoon*

Fig. 6 **Distribution of the *An. maculipennis* complex mosquitoes in the localities studied in Georgia**

In total, 177 mosquitoes from the above complex have been investigated, 128 of them by the molecular genetic assay (PCR-RFLP), and 49 cytogenetically. The genetic assay allowed identifying three species of malarial mosquitoes that occur in Georgia: *An. maculipennis*, *An. melanoon* and *An. sacharovi*. To confirm the species diagnostics, the ITS2 regions of the *An. maculipennis* (AM269898, AM269738), *An. sacharovi* (AM269899), *An. melanoon* (AM271001) mosquitoes were sequenced. The homology of these sequences with those presented in the GenBank constituted 100%. The ITS2 sequence of *An. melanoon* from Poti was identical to the Balkan form of this species (*Di Luca et al*., 2004). Thus, it was confirmed that *An. maculipennis, An. sacharovi* and *An. melanoon* were monomorphic in the marker in question. The primary structure of the ITS2 sequence is a reliable diagnostic character for identification of these species in different parts of their range, including the territory of Georgia.

The species *An. maculipennis* and *An. Melanoon* were found in western Georgia, and *An. maculipennis* and *An. sacharovi* in eastern Georgia, (*Fig. 6*). Thus, it has been established that the Kolkhida and Iverian depressions differ by the composition of their malaria vectors. *An. melanoon* only occurs in the coastal Black Sea belt with its humid subtropical climate, while *An. sacharovi* inhabits the more arid lowlands of the Iverian depression, where the ecological conditions are more compatible with the continental climate of the Aral-Caspian region.

Among the species studied, *An. sacharovi* is considered the most active malaria vector due to its large-scale contact with man (the species is strictly endophillic), numerous blood-feedings of the gonoactive females during the single gonotrophic cycle and their ability to feed on blood during the diapause (*Zvantsov, Ejov, Artemiev,* 2003). The more ecologically flexible species *An. Maculipennis* has a similar behaviour, including high endophilicity and the ability to blood-suck during the diapause. During the 1930s-1950s, far lower numbers of *An. sacharovi* compared to *An. maculipennis* were recorded (*Kanchaveli,* 1955). At present, the number of *An. sacharovi* in East Georgia is equal to that of *An. maculipennis*. Obviously, the existence of these two active malaria vectors has contributed to the rise of autochthonous malaria in the border areas of East Georgia.

In all, the data on the composition of the *An. maculipennis* complex mosquitoes are compatible with the results of former studies. Previously, four species of malarial mosquitoes of the *An. maculipennis complex* were recorded in Georgia: *An. maculipennis, An. sacharovi, An. melanoon* and *An. messeae* (*Kalandadze, Sagatelova,* 1938; *Kanchaveli,* 1955; *Sichinava,* 1973), though some authors have expressed doubts concerning the finding of *An. messeae* (*Beklemishev,* 1948). The egg batches of the colour char-

acteristic for *An. messeae* were found in Abkhazia, Poti and Kakhetia (*Gakett, Barber*, 1935, *Kalandadze, Sagatelova,* 1938). In the same study, egg batches with eggs, strongly resembling the eggs of *An. messeae*, were also detected. The eggs in these egg batches were darker in colour and had no obvious transversal folds on the intercoastal membranes of the floats (*Fig. 7*). These egg batches were obtained from the mosquito populations from Poti and the vicinity of Khobi. PCR-RFLP analysis showed that the females that had laid these eggs belonged to the species *An. melanoon*.

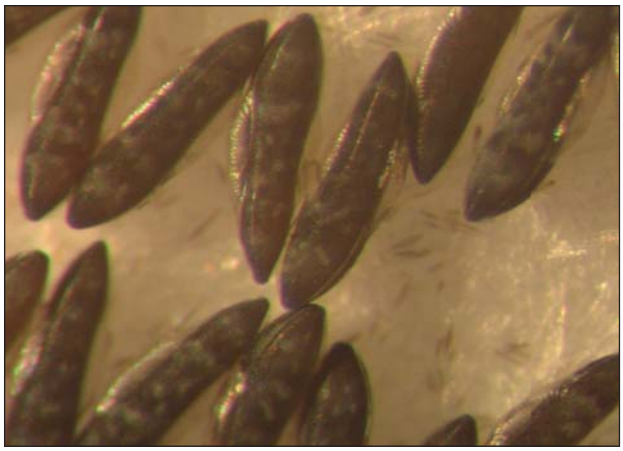


Fig. 7 **Eggs of *An. melanoon* mosquitoes from Poti and Khobi**

Study of malaria vectors in Armenia

At the initial stage of studying the Armenian fauna, two species, *An. maculipennis* and *An. sacharovi*, were discovered (*Chubkova,* 1949). Years later, after the long-term national anti-malaria campaign, only the former of the two was detected (*Manukyan,* 1975). At the end of the 1990s, *An. sacharovi* has again appeared in several settlements of the Ararat Valley (*Romi et al*., 2002).

In total, 116 malarial mosquitoes of the *An. maculipennis complex* were investigated (72 individuals identified by PCR-RFLP, and 44 by the cytogenetic technique). The morphological analysis of the eggs of the females, captured in four different sites, proved the existence in Armenia of two species of the *An. maculipennis complex: An. maculipennis* and *An. sacharovi*. These results were confirmed by PCR-RFLP analysis (*Fig. 8*).

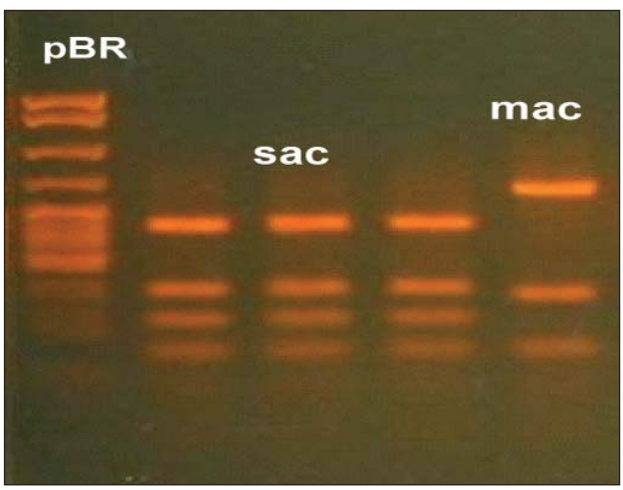


Fig. 8 **Results of the *CfoI (HhaI)*restriction of the ITS2 PCR product**
pBR*322/*Msp*I - molecular weight marker; sac - *An. sacharovi;* mac - *An. maculipennis

An. maculipennis was predominating (75%), while *An. sacharovi* was found only in low-lying areas of the Ararat Valley, where its populations constituted up to 25% (*Fig. 9*).

Our results are in agreement with those found by other authors, according to whom only two malaria mosquito species, *An. maculipennis* and *An. sacharovi*, occur in Armenia: (Chubkova, 1949). Interestingly, in the late 1950s early 1960s, the mosquitoes of these species were totally eradicated in most of Armenia (*Manukyan,* 1975). However, after the use of insecticides was discontinued (in 1966-1970), the numbers of *An. maculipennis* were restored, whereas *An. sacharovi* was not detected at all.

▲ *An. maculipennis* ● *An. sacharovi*

Fig. 9 **Distribution of the *An. maculipennis* complex mosquitoes in the localities examined in Armenia**

✸ *An. persiensis* ● *An. sacharovi* ◎ *An. maculipennis*

Fig. 10 **Distribution of the *An. maculipennis* complex mosquitoes in the localities examined in Azerbaijan**

In the late 1990s, the latter species was again spotted in the Ararat Valley, its numbers being already over 10% (*Romi et al.*, 2003). Our data support these observations.

Study of malaria vectors in Azerbaijan

For several decades, different authors have reported three mosquito species of the *An. maculipennis* complex in Azerbaijan: *An. maculipennis, An. sacharovi* and *An. melanoon* (= *subalpinus*) (*Lemer,* 1948; *Remennikova,* 1953, 1958: *Kiyasov*, 1970, 1973; *Anufrieva et al.*, 1975).

For our study, 179 malarial mosquitoes of the *An. maculipennis* complex were identified (104 by PCR-PFLP, 75 by examination of egg exochorion).

Based on the egg characters, three mosquito species were identified: *An. sacharovi*, *An. maculipennis* and *An. melanoon*. The molecular genetic assay only confirmed identification of the two first-mentioned species. This assay also showed that a female, whose eggs were similar to those of *An. melanoon*, actually represented a newly discovered and recently described species in northern Iran, *An. persiensis* (*Sedaghat et al.*, 2003).

An. sacharovi (90.5% of all detected mosquitoes) predominated in the low-lying areas of the country; *An. maculipennis* (8.9%) was discovered exclusively in the northern parts, in the foothills of the Greater Caucasus and in the Caspian depression. In the south, in the Talysh foothills, *An. persiensis* (0.6%) made a solitary appearance in a coastal zone of Azerbaijan.

The results of the molecular genetic analysis concerning geographical distribution and relative numbers of *An. maculipennis* and

An. sacharovi in Azerbaijan comply with the corresponding data in the literature (*Lemer,* 1948; *Kiyasov,* 1970; *Anufrieva et al*., 1975). *An. sacharovi* prevails in dry and warm plains of the country, while *An. maculipennis* is drawn towards the mountainous areas. The latter also occurs in the north of the country in the Samur-Divichinsk depression.

Using the molecular genetic approach, we detected a new species in the fauna of the South-Caucasian countries, *An. persiensis*. The similarity of the ITS2 sequence structure of *An. persiensis* from Azerbaijan and that from northern Iran, was demonstrated. It is very likely that the early finds of *An. melanoon* (= *subalpinus*) in the Lenkoran district (*Remennikova,* 1953, 1958; *Kiyasov,* 1970, 1973) were in fact members of this new species. The distribution of the *An. maculipennis* complex species in Azerbaijan is shown in *Fig. 10*.

3.3. Cytological and molecular genetic analysis of malaria vectors in the Russian Federation

Examination of the species composition of malaria mosquitoes, conducted in the European part of the Russian Federation, has shown that the most widely distributed species of the *An. maculipennis* complex were *An. atroparvus*, *An. maculipennis* and *An. messeae* (*Gornostaeva, Danilov,* 2002). *An. sacharovi* was observed only in Dagestan (*Shipitsina,* 1936), and *An. melanoon (= subalpinus)* in the south of Krasnodarskii Krai (*Kalita,* 1939), in Kabardino-Balkaria (*Markovitch*, 1936), and Dagestan (*Kalita,* 1939). Only *An. maculipennis* and *An. messeae* occur in the central part of European Russia. The presence of *An. beklemishevi* was recorded in the Moscow, Yaroslavl and Vladimir regions (*Novikov, Alekseev,* 1989), though these solitary findings require confirmation, as they contradict the data by other authors (*Stegnii,* 1978). In many regions, both in southern and central Russia, the species composition of the *An. maculipennis* complex has not been studied. The species composition of the *An. maculipennis* complex in these areas is apparently scarcely investigated, and some of the available data seem outdated.

We have detected *An. atroparvus*, *An. maculipennis* and *An. messeae* in the territory of the Russian Federation (in the Volgograd, Astrakhan, Rostov, Penza, Moscow regions, Krasnodarskii Krai, Adygei Republic, in Kalmykia, Karachaevo-Cherkessia) where in all 557 mosquitoes were examined, (*Fig. 11*).

An. atroparvus occurs more often in the coastal area of the Rostov region and in the northern part of Krasnodarskii Krai; to the east, its range extends as far as the Volgograd region, and to the south, up to Cherkesk, which is in good agreement with the literature published (*Beklemishev, Zhelokhovtsev,* 1937; *Danilova, Lapin,* 1937; *Kalita,* 1937, 1938). *An. maculipennis* was found in all regions studied, except Kalmykia, which confirms the results of the previous studies (*Polikarpova,* 1936; *Pokrovskii, Muratova,* 1936; *Beklemishev, Zhelokhovtsev,* 1937; *Danilova, Lapin,* 1937; *Danilova, Budymko,* 1938; *Kalita,* 1937, 1938; *Zima,* 1964; *Sharkova,* 1964). Note that in Krasnodarskii Krai and the Rostov region, *An. maculipennis* is attracted to mountainous areas, which is a characteristic feature of this mosquito. This species has been detected by many authors in these areas (*Danilova, Lapin,* 1937; *Kalita*, 1937, 1938; *Shipitsina,* 1941).

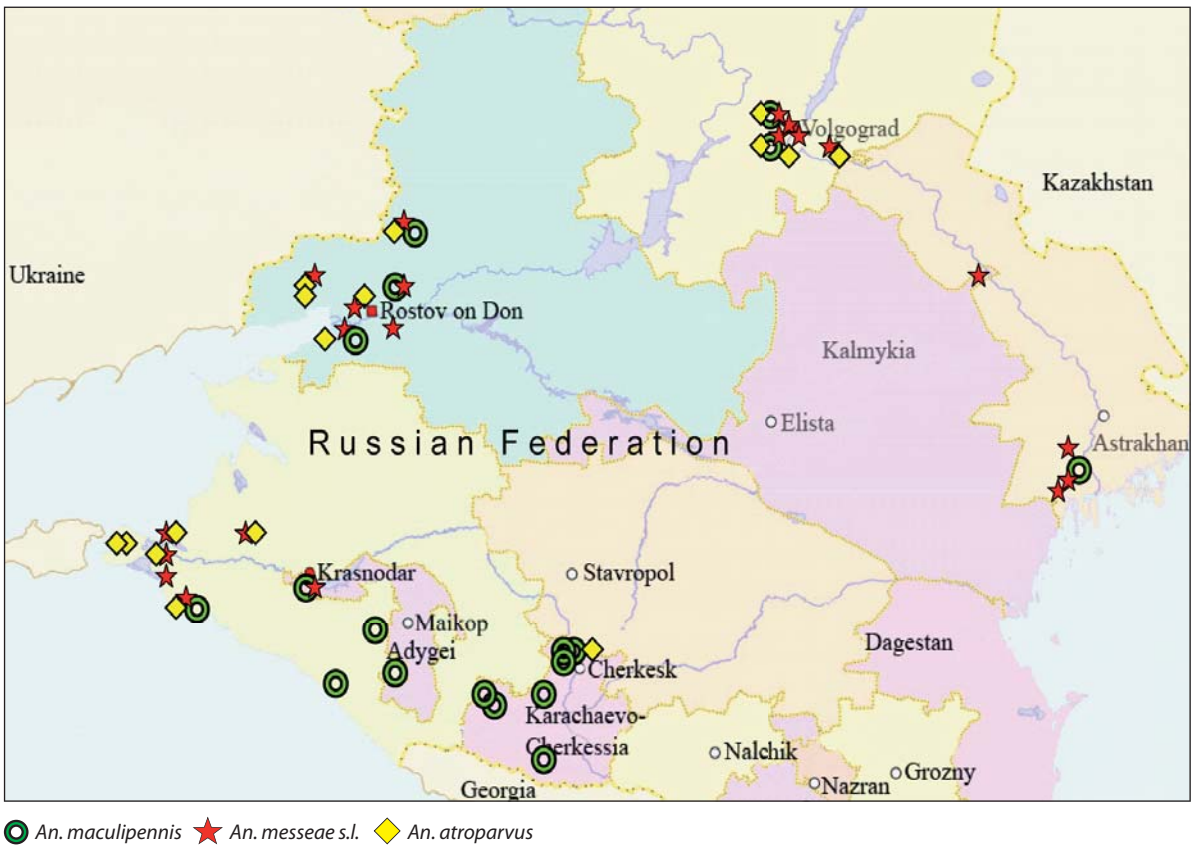


Fig. 11 **Distribution of the *An. maculipennis* complex species in the south of the Russian Federation**

The issue of the *An. melanoon* distribution in the south of the Russian Federation is still unclear. This question is extremely pressing, particularly because of the detection and description of *An. persiensis* in the Near-Caspian lowland. Probably, this species is also distributed in Kabardino-Balkaria and Dagestan, where it was formerly described as *An. subalpinus* (*Markovitch,* 1936; *Kalita,* 1939). More complicated is the situation with *An. messeae*, polymorphic by molecular genetic and cytogenetic markers. To establish the distribution of the earlier described molecular forms of *An. messeae* (*Di Luca et al.,* 2004) and confirm their taxonomic status, *An. messeae* populations in the Russian Federation were examined using molecular genetic and cytogenetic assays. The molecular genetic analysis revealed an intragenomic (individual) polymorphism of the ITS2 region sequences among the mosquitoes collected in Kalmykia, in the Astrakhan, Volgograd, Krasnodar, Penza, Moscow and Rostov regions.

Cytogenetic analysis of the *An. messeae* populations studied revealed the following paracentric inversions (*See Table 1*): XL0, XL1, XL4; 2R0, 2R1; 3R0, 3R1; 3L0, 3L1.

Based on the inversion polymorphism, the studied area could be subdivided into three zones: (1) the western zone: Black Sea region: Rostov, Krasnodar regions and Adygei Republic with a balanced combination of XL_0 and XL_1 variants; (2) the central zone: Moscow and Penza regions, where all inversions occurring in the European part of the Russian Federation exist; and (3) the eastern zone: Near-Caspian

Table 1 **Inversion frequencies in *An. messeae* populations**

Inversion	B.Elan' settlement, Penza region, lake	Frequency ($f \pm s_f$, %)	Timonovo settlement, Moscow region, pond	Frequency ($f \pm s_f$, %)	Aksai of Rostov region, Mukh. hollow	Frequency ($f \pm s_f$, %)	pr. Khurgun, Astrakhan region	Frequency ($f \pm s_f$, %)
XL0	6	19±6,9	4	20±9	28	51±6,7	41	79±5,6
XL1	25	78±7,3	15	75±10	27	49±6,7	11	21±5,6
XL4	1	3±3	1	5±5				
N'	32		20		55		52	
2R0	39	93±3,9	13	43±9	72	100	70	100
2R1	3	7±3,9	17	57±9				
3R0	32	76±6,6	24	80±7,3	65	90±3,5	58	83±4,5
3R1	10	24±6,6	6	20±7,3	7	10±3,5	12	17±4,5
3L0	40	95±3,4	27	90±5	66	92±3	59	84±4,4
3L1	2	5±3,4	3	10±5	6	8±3	11	16±4,4
N'	42		30		72		70	

Inversion	Bir Kosa district, Astrakhan region, flow channel	Frequency ($f \pm s_f$, %)	Zhitno settlement, Astrakhan region, pond	Frequency ($f \pm s_f$, %)	Nijniaya Gostogaevka district, Krasnodar region	Frequency ($f \pm s_f$, %)	Tliustenhabl district, Adygei republic, the Kuban river overflows	Frequency ($f \pm s_f$, %)
XL0	12	80±10	27	57±7,2	23	48±7,2	18	42±7,5
XL1	3	20±10	20	43±7,2	25	52±7,2	25	58±7,5
XL4								
N'	15		47		48		43	
2R0	20	100	50	100	60	100	52	100
2R1								
3R0	13	65±11	36	72±6,3	51	85±4,6	48	92±3,8
3R1	7	35±11	14	28±6,3	9	15±4,6	4	8±3,8
3L0	15	75±10	40	80±5,6	49	82±5		92±3,8
3L1	5	25±10	10	20±5,6	11	18±5		8±3,8
N'	20		50		60			

Lowland: Astrakhan region, where XL_0 inversion predominates. All three zones differ significantly by the ratio of inversions.

The mosquitoes from the central zone differ from those from the two other zones in 2R chromosome polymorphism. The 2R1 inversions were discovered only in the populations of the Bolshaya Elan' and Timonovo settlements. The variability of the inversion composition of chromosome 3 has not been detected.

The difference of the XL and 2R inversion frequencies could be explained on the basis of their adaptive significance in different environments. As confirmed experimentally, inversions affect mosquito fitness parameters at different development stages (*Stegnii,* 1991).

Compared to the Near-Caspian, the high XL_1 inversion frequency in the central and Black Sea populations is most likely connected with the effect of antropogenic factors. In particular, a high frequency of XL_1 inversion was detected in the Moscow population (*Gordeev et al.,* 2005).

The central zone differs from the other two in the 2R chromosome polymorphism. Evidently, the appearance of 2R1 inversion in the Bolshaya Elan' and Timonovo populations was determined by climatic effects. Across the territory of the Russian Federation, by this inversion a distinct latitudinal cline in this inversion frequency was detected (*Stegnii,* 1991).

These molecular genetic and cytogenetic data contradict the results of the Romanian authors (*Nicolescu et al.,* 2004), who recognized the fifth molecular form of *An. messeae* as a distinct species, *An. daciae,* based on the stable substitutions at five positions (161, 165, 167, 362, 382) in the ITS2 region. It is not surprising that *An. messeae,* with its vast range and high plasticity, is characterized by intraspecific and individual ITS2 variability, which may be adaptively significant for this species. Considering the above, the recognition of *An. daciae* as a separate species seems to be erroneous. Clearly, further research is required on the genetic variability of *An. messeae* across the whole habitat of this species.

4. Conclusions and recommendations

The results of the operational research presented in this paper will help national health authorities to re-examine the current vector control strategies, taking into account the updated knowledge of existing and potential malaria vectors.

While malaria still remains widespread in large areas of the world, the re-introduction of malaria may also occur in countries and territories from which malaria has previously been eliminated. Having achieved complete interruption of malaria transmission, the area under consideration might still be receptive to malaria, and receptivity could be increased by development projects or other activities that create favourable conditions for the vectors and increase human-vector contact.

The threat of the re-establishment of malaria transmission in the WHO European Region should not be downgraded, despite the substantial progress achieved. In this connection, further research on the taxonomy, biology, ecology, behaviour and genetics of mosquitoes of the genus of *Anopheles* will lead to a better understanding of the nature of malaria vectors and their role in transmission in the WHO European Region, and to providing advice on the ways to best address the problem.

The priority areas for operational research regarding malaria vectors in the WHO European Region could be as follows:

- To revise the species composition of malaria vectors in countries, primarily in those where autochthonous malaria is reported or a high risk of resurgence of malaria exists;
- To study the present geographical distribution of malaria vectors using the detailed Geographic Information System (GIS) for mapping of malaria vectors;
- To examine the variability of the vector populations using morphological, cytogenetic and molecular genetic techniques;
- To study the prevalence of sibling species in different eco-epidemiological settings and their role in malaria transmission;
- To detect the ecological and behavioural features of malaria vectors, and to determine their relationship with man and their role in malaria transmission;
- To investigate parasite-host relationships in the triad "mosquito-host-malaria parasites", which is particularly relevant in case of recently described vector species;
- To study the response of the vectors to control measures, in particular mosquito resistance to insecticides and excito-repellency effect of insecticides.

Carrying out this comprehensive research agenda constitutes a major step towards the day when malaria in the WHO European Region is completely eliminated.

Annexes

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Within the framework of the new WHO regional strategy aimed at malaria elimination, special attention is given to operational research. In order to update scientific knowledge on malaria, the WHO Regional Office for Europe has initiated a regional programme on operational research related to malaria entomology and vector control, which is being carried out successfully with the assistance of research institutions and partners in affected countries of Middle Asia and South Caucasus. The objectives of the research are closely tied to the particular situation and problems identified within a single country or a group of neighbouring countries. The identification and geographical distribution of *Anopheles* mosquitoes, the prevalence of sibling species and their role in malaria transmission, taxonomy, biology and ecology of malaria vectors are of particular interest in the Region.

The results of the research presented in this paper conducted over the past five years in countries having faced a recent resurgence of malaria in the WHO European Region, will help national health authorities to re-examine the current vector control strategies, taking into account the updated knowledge of existing and potential malaria vectors. The threat of the re-establishment of malaria transmission in the Region should not be downgraded, despite the substantial progress achieved. In this connection, further research on the taxonomy, biology, ecology, behaviour and genetics of mosquitoes of the *Anopheles* genus will lead to a better understanding of the nature of malaria vectors and their role in transmission in the WHO European Region, and to providing advice on the ways to best address the problem.

Carrying out this comprehensive research agenda is a major step towards the day when malaria in the WHO European Region is completely eliminated.

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