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RESEARCH ARTICLE

Molecular Identification of *Anopheles maculipennis* Complex (Diptera: Culicidae) Species in the Northeastern Anatolia Region of Türkiye and Research of *Plasmodium* Presence in These Species

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Abstract

Accurate identification of malaria vectors and detection of pathogens are essential for understanding local transmission dynamics and supporting vector control strategies. In this study a total of 737 adult mosquitoes belonging to the *Anopheles maculipennis* complex (Diptera: Culicidae) were collected from eight ecologically diverse localities in the Aras Valley, northeastern Anatolia, Türkiye, during 2019-2020. Collections were performed using New Jersey light traps and mouth aspirators. Molecular identification was performed using a PCR assay based on ribosomal DNA internal transcribed spacer 2 (ITS2) sequence variation. Electrophoretic profiles and sequence analyses showed that all examined specimens belonged to *Anopheles maculipennis* sensu stricto. This finding indicates that the species is the dominant and widely distributed member of the complex in the study area. To assess the potential for malaria transmission, individual DNA samples were screened for *Plasmodium vivax* and *Plasmodium falciparum* using nested PCR. No Plasmodium DNA was detected in any individual analyzed. Although the present findings suggest a low level of malaria transmission risk in the Aras Valley, the extensive distribution of a competent vector species, combined with favorable environmental conditions, highlights the necessity of sustained entomological and molecular surveillance. This study updates molecular data on the *Anopheles maculipennis* complex in northeastern Türkiye and contributes to regional malaria risk assessment.

Keywords: *Anopheles maculipennis* complex, Aras Valley, Malaria, Plasmodium

Introduction

Malaria has been recognized for centuries as one of the most important vector-borne diseases affecting human populations and continues to represent a major global public health challenge, with an estimated 282 million cases and 610,000 deaths reported worldwide in 2024 [1]. Historically, malaria also represented an important public health problem in Türkiye. However, effective control strategies implemented by the Ministry of Health have substantially reduced indigenous transmission, and no locally acquired malaria cases have been reported since 2013. Despite this achievement, Türkiye remains vulnerable to malaria reintroduction because of increasing human mobility, climate change, and the continued presence of competent vector populations [2]. Previous reports have suggested that individuals returning from malaria-endemic regions, particularly African countries, as well as international travelers and students, may contribute to the importation of malaria parasites into the country [3].

Malaria is caused by protozoan parasites belonging to the genus *Plasmodium*, several species of which infect humans. Among these, *Plasmodium falciparum* (Welch 1897), *Plasmodium vivax* (Grassi & Feletti, 1890), *Plasmodium ovale* (Stephens, 1922), *Plasmodium malariae* (Feletti& Grassi, 1889) and *Plasmodium knowlesi* (Knowles and Gupta, 1932) are recognized as the principal causative agents of human malaria. In Türkiye, *P. vivax* has historically been the predominant malaria parasite, whereas *P. falciparum* infections have generally been associated with imported cases. However, recent reports describing indigenous *P. falciparum* infections and mixed *P. falciparum/P. vivax* infections suggest a potential influence of increasing international travel and population mobility on malaria epidemiology in the country [4,5]. Humans act as intermediate hosts for these parasites, while female *Anopheles* mosquitoes serve as both definitive hosts and vectors.

Among *Anopheles* mosquitoes, the *Anopheles maculipennis* species complex represents one of the

earliest recognized sibling species complexes and includes several epidemiologically important malaria vectors distributed across Europe and the Middle East [6,7]. Accurate identification of members within this complex has historically been challenging because of substantial morphological similarity among sibling species. Consequently, molecular methods have become increasingly important for reliable species discrimination, whereas traditional approaches such as egg morphology and cytogenetic analyses are now mainly used as complementary tools [8-12]. Studies focusing on the molecular characterization of the *An. maculipennis* complex in Türkiye have increased particularly since the 2000s [12-15].

Despite limited data on the vectorial capacities of individual species within the *An. maculipe*nnis complex, *Anopheles* mosquitoes are recognized as vectors of a range of viral pathogens and filarial parasites, in addition to *Plasmodium* spp. Within the Palearctic region, the *An. maculipennis* complex consists of several closely related sibling species exhibiting considerable ecological diversity and varying vector capacities. To date, only a limited number of members of the complex -*Anopheles maculipennis* s.s. Meigen 1818., *Anopheles sacharovi* Favre 1903, and *Anopheles melanoon* Hacket- have been molecularly confirmed in Türkiye, whereas historical records have also reported *Anopheles messae* Falleroni, 1926, whose taxonomic status remains controversial. These differences among sibling species, particularly regarding ecology, distribution, and vector competence, highlight the importance of accurate species-level identification for disease surveillance and vector control programs.

Understanding the biological and ecological characteristics of vector species is essential for evaluating the transmission dynamics of vector-borne diseases. Furthermore, investigating pathogen presence in mosquito populations contributes to a more accurate assessment of their epidemiological importance and potential role in disease transmission. Although previous studies have investigated mosquito fauna in Türkiye, molecular information regarding the species composition of the *An. maculipennis* complex and the occurrence of *Plasmodium* spp. in northeastern Anatolia remains limited. Therefore, the present study aimed to identify species within the *An. maculipennis* complex collected from the Aras Valley in northeastern Türkiye using molecular methods and to investigate the presence of *Plasmodium* spp. in these mosquitoes. The findings of this study may provide baseline information for future vector surveillance studies and contribute to understanding the potential role of *Anopheles* species in malaria transmission in northeastern Türkiye.

Material and Methods

Ethical Approval

This study did not involve any procedures requiring ethical approval.

Field Studies

In this study, members of the *An. maculipennis* complex were collected from eight localities in the Aras Valley, a region characterized by high potential for vector-borne diseases due to its geographic features, climatic conditions, agricultural and human activities, and availability of mosquito breeding sites. The Aras Valley, which includes the Iğdır Plain, is an important ecological corridor with microclimatic climate features where desert fauna enters Anatolia. Iğdır Province is situated along the southern margin of the Iğdır Plain, at the northwestern foothills of Mount Ağrı. The northern and northeastern boundaries of province are defined by the Aras River, which also forms the border with Armenia, while the eastern and southeastern boundaries are bordered by Nakhchivan and Iran. The study area is characterized by saline soils formed from volcanic materials originating from Mount Ağrı and alluvial deposits transported by the Aras River. The presence of agricultural landscapes, extensive water bodies (including irrigation systems, drainage canals, ponds, and temporary floodwaters), and suitable climatic conditions creates favorable habitats that support high mosquito population densities. Furthermore, the region sustains large populations of livestock, including cattle, sheep, and poultry, which provide abundant blood-meal sources. Collectively, these factors enhance vector–host interactions and may increase the potential for vector-borne disease transmission in the region. According to the 1981-2010 data from the General Directorate of Meteorology, the annual average temperature of the area has been measured at 12.1°C.

The aim was to search for *Plasmodium* parasites in the mosquitoes separated at the species level. Eight different localities (Mürşitali, Zülfikarköy, Kozlu, Kuloğlu, Yukarı Çıyrıklı, Kötek, Sürmeli, and Günindi) *(Fig. 1)* and their coordinates, as well as the number of mosquitoes collected from these localities are detailed *(Table 1)*.

DNA Isolation

The DNA isolation of the collected samples was performed using the Quick-DNA Tissue/Insect Miniprep Kit (Zymo Research), following the appropriate protocol. The obtained DNA was measured using a spectrometer, and extracted DNA was preserved at −20°C until PCR analysis was performed.

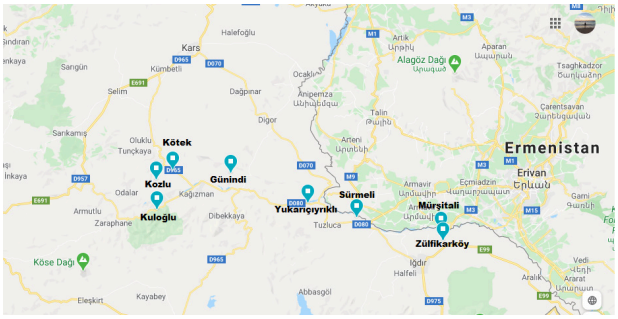

Fig 1. Sampling localities of the evaluated samples

Table 1. *Localities where the samples were collected, with their coordinates, elevations, and numbers of samples*

Localities	Coordinates	Altitude	Sample Number
Zülfikarköy	39°59'33.3"N 44°08'47.3"E	853 m	133
Mürşitali	40°01'36.7"N 44°08'16.5"E	858 m	90
Kuloğlu	40°11'13.3"N 42°56'59.2"E	880 m	2
Yukarı Çıyırklı	40°07'31.1"N 43°36'26.4"E	1020 m	276
Sürmeli	40°03'56.9"N 43°47'07.7"E	1020 m	155
Günindi	40°12'24.4"N 43°15'39.6"E	1521 m	3
Kötek	40°13'06.3"N 43°01'07.7"E	1532 m	53
Kozlu	40°11'13.0"N 42°56'59.4"E	2044 m	25
Total			737

Molecular Identification of the *Anopheles maculipennis* Species Complex

In this study, the identification of the *An. maculipennis* species complex was carried out using a method based on differences in ITS2 sequences, which is the most common method for species differentiation. Differentiation based on ITS2 sequence variation was performed following the protocol described by Proft et al.[16], with additional reference to Akıner and Çağlar[14]. The following molecular protocol was used to distinguish the *An. maculipennis* complex species.

For the PCR protocol, both general (forward) and species-specific (reverse) primers were used for the species differentiation of the *An. maculipennis* complex. The primers are given in *Table 2*.

PCR was performed in a 50 µL reaction mixture with 10 pmol of each primer from the seven listed primers, 25 µL of BioMix Red, and DNase/RNase free water. The PCR was conducted under the following conditions: 30 sec at 94°C for denaturation, 30 sec at 55°C for primer annealing, and 30 sec at 72°C for elongation, over 39 cycles.

The PCR products were run on a 1.5% agarose gel at 100 volts for 30 min. After electrophoresis, the species were classified based on band sizes. A 100 base pair marker was used during the electrophoresis process. The band sizes for the species were given in *Table 3*.

The PCR products were prepared for sequencing and sent for sequencing to obtain raw sequences. After alignment, the sequences were compared to reference sequences in the GenBank database, and species-level matches were identified

Plasmodium Detection

The previously isolated DNA samples of species complex members, which were identified at the species level used for the detection of *Plasmodium* presence by indivudally. For this purpose, a two-step nested PCR method was used [17].

For the initial amplification, a primer pair targeting all four *Plasmodium* species (*P. falciparum, P. vivax, P. malariae,* and *P. ovale*) was used to amplify a region approximately 1200 base pairs long. The primers used for this amplification were rPLU5 (5'-CCT GTT GTT GCC TTA AAC TTC-3') and rPLU6 (5'-TTA AAA TTG TTG CAG TTA AAA CG-3'). Positive samples for *P. falciparum* and *P. vivax* were obtained from the Parasitology Bank of the Faculty of Medicine, Celal Bayar University, Manisa.

Table 2. *Primers used for species differentiation within the Anopheles maculipennis complex*

Parameter	Species	Primer
Forward primer	*An. maculipennis* s.l.	5.8S rDNA (5'-TGT GAA CTG CAG GAC ACA TG-3'
Reverse primers	*An. maculipennis* s.s.	5'-CGT TTG GCT TGG GTT ATG A-3'
	An. atroparvus	5'-CGT TTG GCT TGG GTT ATG A-3'
	An. messae	5'-GAC GCC TCA CGA TGA CCT T-3'
	An. melanoon	5'-TGC AAG TTG AAA CCT GGG GC-3'
	An. labranchiae	5'-GTA TCT CTG CTG CTA TGG TC-3'
	An. sacharovi	5'-CAA GAG ATG GAT GTT TTA CG-3'

Table 3. *The band size for the An. maculipennis complex species*	
Species	**Band Size**
An. maculipennis s.s.	410 base pairs
An. atroparvus	117 base pairs
An. messae	305 base pairs
An. melanoon	224 base pairs
An. labranchiae	374 base pairs
An. sacharovi	180 base pairs

The PCR products obtained from the first step were run on a 1.5% agarose gel, and the presence of bands was checked under UV light.

For the second reaction, PCR was planned to be performed with two primer pairs using the positive samples from the first step. For *P. falciparum*, primers rFAL1 (5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3') and rFAL2 (5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3') were used to amplify a region of approximately 205 base pairs. For *P. vivax,* primers (5'-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3') and rVIV2 (5'-AAG GAA AGA AAG TCC TTA-3') were used to amplify a region of approximately 120 base pairs.

For the first amplification, the PCR-mixture was prepared as follows: 2.5 µL template DNA, 25 µL BioMix Red, and DNase/RNase-free water with 250 nM of each primer (rPLU5 and rPLU6). PCR conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min, with a final elongation at 72°C.

For the second amplification, a total mixture of 20 µL was going to be prepared, and 2 µL of the PCR product from the first amplification was going to used. However, since no positivity for Plasmodium presence was obtained in the first PCR step, this stage was not conducted.

Results

Molecular Identification of the *Anopheles maculipennis* Species Complex

A total of 737 adult mosquitoes belonging to the *An. maculipennis* complex were collected and the identification method based on the differences in ITS2 sequences of adult individuals carried out using the method described by Proft et al.[16] as the main reference and the protocol provided by Akıner and Çağlar [14]. Electrophoresis showed 410 bp bands in all the samples. Sequencing of five randomly selected individuals confirmed that all collected specimens belonged to *An. maculipennis* s.s. Representative gel images showing PCR products of 410 bp are presented *(Fig. 2)*. The forward and reverse sequences obtained from the samples were deposited in the NCBI GenBank database under accession numbers MZ666139–MZ666148.

Plasmodium Detection

For the initial amplification, the first-step PCR was performed using the rPLU5 and rPLU6 primer pairs, which target all four *Plasmodium* species and amplify a region of approximately 1200 base pairs (bp). No *Plasmodium* presence was detected in the first-stage PCR.

Since no positivity was found at this stage, the second-stage PCR was not applied to the samples. The gel image of the nested PCR performed using *P. falciparum* and *P. vivax* positive samples is shown *(Fig. 3)*.

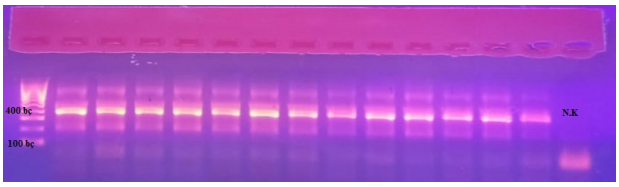

Fig 2. Electrophoresis image of 410 bp agarose gel for *Anopheles maculipennis* s.s

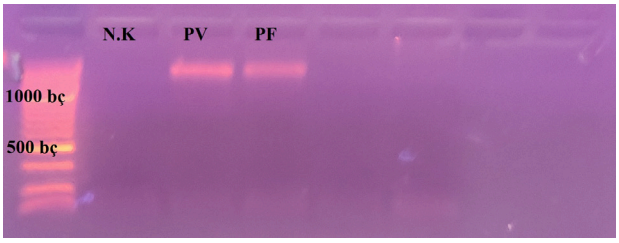

Fig 3. PCR control study performed with *Plasmodium falciparum* and *Plasmodium vivax* positive samples. N.K.: negative control

Discussion

Effective vector control requires accurate identification of vector species and detailed characterization of their spatial distribution. The Aras Valley was selected as the study area because of its ecological characteristics, including extensive agricultural habitats, irrigation systems, and wetland-associated environments that support mosquito populations. Previous studies have documented medically important mosquito genera, including *Anopheles*, *Aedes*, and *Culex*, in the region [15,18-23].

Several *Anopheles* species with epidemiological importance in malaria transmission have been reported in Türkiye. Among these, *Anopheles superpictus* Grassi, 1899 and members of the *An. maculipennis* complex, particularly *An. sacharovi* and *An. maculipennis* s.s., are considered epidemiologically important malaria vectors in the Eastern Mediterranean region [13,24,25].

The *An. maculipennis* complex includes several closely related sibling species that are widely distributed throughout the Palearctic region and recognized as important malaria vectors in Europe and the Middle East [6,26]. Differences among members of the complex in ecology, geographical distribution, and vector competence have increased the importance of accurate species-level identification. Although the epidemiological roles of individual species are not fully understood, members of the complex have been associated with the transmission of *Plasmodium* spp., arboviruses, and filarial parasites [27]. Previous studies lacking precise species discrimination have reported infections with pathogens such as Batai, Tahyna, West Nile, Usutu, and Sindbis viruses, as well as filarial nematodes [27]. In Türkiye, molecular evidence has confirmed the presence of *An. maculipennis* s.s., *An. melanoon*, and *An. sacharovi*, while *An. messeae* has been reported only in historical records [13,15].

Since its first description, the *An. maculipennis* complex has posed persistent taxonomic challenges because of the high morphological similarity among sibling species. The systematic issues, which started with egg morphology in 1926, are still being addressed with the widespread use of molecular tools. The primary identification method used in our study is the rDNA ITS2 region analysis, which is one of the most commonly used methods in both Türkiye and the Palearctic region. But it is recognized that this method does not provide reliable discrimination between *An. messeae* and *Anopheles daciae* Linton, Nicolescu & Harbach, 2004 [16,28]. In our study, based on the method applied by Proft et al. [16], PCR results yielded bands of approximately 400 bp, and these bands were initially identified as *An. maculipennis* s.s. [16]. Subsequent sequence analyses confirmed that the predominant species detected in northeastern Anatolia belonged to the *An. maculipennis* complex, specifically *An. maculipennis* s.s.

An. maculipennis s.s. is the nominotypical taxon of the species complex and was widely distributed throughout most of Europe, with the exception of its northernmost areas [29]. Recent evidence indicates that the range of this species has extended toward northeastern Europe as well as northwestern Asia and is expanding to the northeast of Europe at a speed of approximately 30 km per year [30]. The establishment of this species in the Southern Urals has been associated with shifts in the composition of sympatric *Anopheles* populations, indicating ongoing changes in species distribution within the Palearctic region [30]. Within the complex, *An. maculipennis* s.s. is distinguished by its ability to occur at elevations exceeding 1,000 m, where larval development generally takes place in small, temporary water bodies associated with agricultural environments [31]. This is consistent with our study, as most of the sampling sites were located above 1.000 m. Despite the detection of *Plasmodium* sporozoites in the salivary glands of this species, its strong preference for animal hosts indicates a relatively limited role in malaria transmission [32,33]. In a previous study conducted in the Aras Valley, *Dirofilaria immitis* (Leidy, 1856) and *Dirofilaria repens* Railliet & Henry, 1911 were detected in these complex mosquitoes identified only at the genus level [34]. Considering the predominance of *An. maculipennis* s.s. observed in the present study, it is possible that this species was responsible for the previously detected *Dirofilaria* infections.

A large-scale mosquito survey conducted in Armenia between 2016 and 2018 identified *An. maculipennis* s.l./s.s. as the predominant *Anopheles* species in the country, whereas *An. sacharovi* was detected only in low numbers in the Ararat Valley [35]. Similarly, larval sampling studies from northern Iran reported *An. maculipennis* s.l. as the most abundant *Anopheles* species in the Mazandaran region [36]. Consistent with these findings, Günay [15] detected only *An. maculipennis* s.s. in the Iğdır and Kars regions using COI gene sequences and reported sympatric distribution of genetically distinct groups temporarily designated as *Anopheles* MBI-36. Although Kuçlu [22] identified *An. sacharovi* in the Aras Valley based on a limited number of specimens, this species was not detected in the present study, which may reflect differences in sampling methods, seasonal variation, or habitat preference. Likewise, Öztürk [37] reported *An. maculipennis* s.s. as the only representative of the *An. maculipennis* complex in the Central and Eastern Black Sea regions. Together with previous studies, the present findings suggest that *An. maculipennis* s.s. is a dominant and widely distributed member of the complex across diverse ecological regions of Türkiye, likely reflecting its ecological adaptability.

Entomological studies employ a variety of sampling techniques, broadly categorized into collections of adult mosquitoes (flying or resting) and immature stages (larvae and eggs). The choice of sampling method depends on the study objectives, target species, and environmental conditions of the study area. Attractants such as carbon dioxide, odor cues, hosts, and light are effective for capturing mosquitoes at different gonotrophic stages or those actively seeking hosts, whereas mouth aspirators are particularly useful for collecting certain *Culex* and *Anopheles* species that do not readily respond to traps [38,39]. Larval sampling plays a critical role in control and surveillance studies, as it enables the identification of breeding sites and facilitates the detection of species that are either trap-insensitive or present only for short periods. Similarly, egg traps (ovitraps), which mimic oviposition sites, are effective for sampling species that do not respond to conventional trapping methods. In the present study,

only adult mosquito samples were collected using New Jersey light traps and mouth aspirators. Although the results are consistent with findings from previous studies conducted in the region and nearby areas, some members of the complex may not have been detected due to the sampling methods employed. This limitation highlights the potential for sampling bias and suggests that the inclusion of larval and egg sampling could provide a more comprehensive assessment of species diversity in the study area.

Although indigenous malaria transmission has been successfully controlled in Türkiye and no local cases have been reported since 2013, imported malaria cases continue to occur annually [1,40]. Recent reports describing *P. falciparum*, *P. vivax*, and mixed infections indicate that international travel, cross-border movement, and increasing human mobility may contribute to changes in malaria epidemiology and the potential reintroduction of malaria in the country [2,3,41,42]. Despite *P. vivax* remaining the predominant malaria parasite in Türkiye, the occurrence of imported cases together with the widespread presence of competent vector populations suggests that transmission risk persists under suitable ecological conditions. Furthermore, the Aras Valley, with its suitable microclimate, has been identified as a region in Türkiye favorable for *P. falciparum* transmission, in addition to the lowland areas of the Marmara, Mediterranean, and Southeastern Anatolia regions [10]. Given the location of the study area near international borders, monitoring multiple *Plasmodium* species may be important for assessing the risk of imported infections and potential local transmission in the presence of competent vector populations.

Although no malaria parasites were detected in the sampled mosquitoes, the widespread and sustained presence of competent vectors across Türkiye indicates that the potential for malaria transmission persists. Furthermore, climate-driven environmental changes are expected to alter the distribution and biology of both vectors and parasites, potentially increasing the likelihood of malaria re-emergence under favorable conditions. In addition, pathogen surveillance and detection studies require sufficiently large sample sizes to ensure adequate statistical power and reliable detection of low-prevalence infections. Overall, these findings highlight the need for continuous surveillance and expanded sampling efforts to better assess transmission risk and to support early detection of potential malaria resurgence in the region.

Declarations

Availability of Data and Materials: The authors declare that the data and materials are available on request from the corresponding author (B.D.).

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Ethical Approval: This study did not involve any procedures requiring ethical approval.

Conflict of Interest: The authors declare no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article, tables and figures were not written/created by AI and AI-assisted Technologies.

Author Contributions: Field studies: B.D and H.B.U, Performed the experiments: B.D and H.B.U. Analyzed the data: B.D, Wrote the paper: B.D.

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