

# Update on the distribution of *Anopheles maculipennis* s.l. members in the Republic of Moldova with the first record of *An. daciae*

Tatiana Șuleșco

[tatiana.sulesco@bnitm.de](mailto:tatiana.sulesco@bnitm.de)

Bernhard-Nocht-Institute for Tropical Medicine

Felix Gregor Sauer

Bernhard-Nocht-Institute for Tropical Medicine

Renke Lühken

Bernhard-Nocht-Institute for Tropical Medicine

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## Short Report

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# Abstract

**Background:** *Anopheles maculipennis* complex members are important vectors of human malaria and potentially also for other pathogens, but there is a substantial gap in the current knowledge on their distribution, particularly in Eastern Europe. Therefore, the aim of the study was to update the distribution data for the individual members of *An. maculipennis* complex in the Republic of Moldova.

**Methods:** New data were collected between 2013 and 2019 using CDC traps and manual aspirators in the Republic of Moldova. Randomly selected 297 *An. maculipennis* s.l. females from 49 sampling sites were subjected to the molecular species identification targeting the ITS2 gene. These data were combined with previously collected data in the 21<sup>st</sup> century and historical data before the 1950s from the literature to present a comprehensive overview on the distribution for the members of *An. maculipennis* complex.

**Results:** The most prevalent species was *An. maculipennis* s.s. (number of specimens = 115, 38.9%), followed by *An. daciae* (72, 24.3%), *An. atroparvus* (56, 18.6%) and *An. messeae* (54, 18.2%). *Anopheles daciae* was firstly reported for the country, while *An. melanoon* was confirmed by sequencing of PCR products of the specimens collected in 2012 and 2013.

**Conclusion:** Five members of the *An. maculipennis* complex are present in the Republic of Moldova: *An. atroparvus*, *An. maculipennis* s.s., *An. messeae*, *An. melanoon*, and *An. daciae*. *Anopheles labranchiae* and *An. sacharovi* were not detected.

## Background

The Palaearctic *Maculipennis* subgroup of the *Anopheles maculipennis* complex comprises 10 sibling species: *Anopheles artemievi*, *An. atroparvus*, *An. daciae*, *An. labranchiae*, *An. maculipennis* sensu stricto (s.s.), *An. martinius*, *An. melanoon*, *An. messeae*, *An. persiensis* and *An. sacharovi* [1, 2]. Due to their different role as vectors of malaria parasites, *Anopheles maculipennis* s.l. was in focus of mosquito research in Europe since the beginning of mosquito surveillance at the beginning of the 20th century [3]. With the disappearance of malaria in the cause of vector control measurements, land-use change and the overall increase in hygiene and health care, the research interest in this mosquito species complex considerably declined [4, 5]. Besides malaria parasites, there is lack of knowledge on the vector competence of the *An. maculipennis* s.l. members, although field-collected specimens have been found to be positive for a variety of other pathogens, e.g. Batai virus [6, 7], West Nile virus [8], Usutu virus [9] or *Dirofilaria repens* / *D. immitis* [10, 11, 12]. However, the species-specific importance of the different members of *An. maculipennis* s.l. in the transmission cycles for most pathogens except malaria parasites is unclear.

The earliest evidence for the presence of *An. maculipennis* s.l. was mentioned in studies conducted between 1932 and 1938 in Bessarabia, a historic region of Romania, which included a huge proportion of the later country borders of the Republic of Moldova [13, 14]. These studies were related to the

distribution and survival rates of diapausing mosquitoes in the basements of human dwellings during the winters and the mosquito human biting activities during the warm season in the villages with malaria outbreaks. However, the members of *An. maculipennis* s.l. were not differentiated [13, 14]. Zotta et al. [15] conducted the first detailed surveillance of the *An. maculipennis* s.l. during an ongoing malaria outbreak in Bessarabia in the 1930s. Based on morphological differences of the egg's chorion, the presence of three species was reported: *An. atroparvus*, *An. maculipennis* s.s. (as *An. typicus* Hackett and Missiroli) and *An. messeae*. At the same time, Zotta et al. [15] highlighted the absence of *An. sacharovi* (as *An. elutus* Favre), *An. melanoon* and *An. labranchiae* in Bessarabia, although *An. sacharovi* was shown to occur on the Black Sea coast and the Danube Delta in neighboring Romania. In the 1940s, further surveys confirmed the presence of three species for the country: *An. atroparvus*, *An. maculipennis* s.s. and *An. messeae* [16, 17].

Until 1950, Moldova experienced huge outbreaks of malaria and favorable climatic conditions allowed the development of 5–6 generations of *An. maculipennis* s.l. per year with peak abundance in July–August [18]. The causative agents of malaria in Moldova were *Plasmodium vivax*, *P. falciparum* and *P. malariae*. *Anopheles messeae* and *An. maculipennis* s.s. were considered the most important vectors, while in contrast to other countries like Germany [19], *An. atroparvus* was considered epidemiologically of little importance due to its sporadic occurrence in the villages with malaria outbreaks. In another work, various water bodies have been inspected on the presence and abundance of *An. maculipennis* s.l. larvae during the national malaria control program [20], but the specimens were not further identified to species level. As observed for other European countries [19], research on *Anopheles* species was mainly neglected after malaria eradication in Moldova and only fragmented data were obtained during the general monitoring of mosquitoes [18, 21, 22, 23].

Only few studies used more reliable identification techniques to analyze the distribution of *An. maculipennis* s.l. in Moldova. Cytogenetic analysis of *An. maculipennis* s.l. larvae collected in Bălți and Fălești during the 1970s confirmed their identity with West-European population of *An. maculipennis* s.s. [24]. Much later, the gel PCR assay elaborated by Proft et al. [25] was applied to identify immature stages of *An. maculipennis* s.l. [26]. These results demonstrated the presence of *An. atroparvus*, *An. maculipennis* s.s., *An. melanoon* and *An. messeae* in the country. *Anopheles maculipennis* s.s. larvae were found in different breeding sites throughout the country. Adults predominated in the resting sites of central hilly forested area, while *An. messeae* has been shown to dominate along river systems. However, the applied PCR assay used by Șuleșco et al. [26] does not separate *An. messeae* from *An. daciae*. The later species was described as a new member of the *An. maculipennis* species complex in 2004 [27]. *Anopheles melanoon* and *An. atroparvus* were sporadically observed in permanent ground pools located in urban and rural areas. Previous records of *An. sacharovi*, based on morphological observations of adults only, were not confirmed [28].

In depth analysis of species-specific ecology or vector competence for members of the *An. maculipennis* complex is hampered due to morphological similarity of immature and adult stages. As summarized above, before the establishment of cytogenetic or molecular identification tools, species

identification in the Republic of Moldova was mostly based on morphological differences of the egg's chorion [29, 15]. However, due to overlapping inter- and intraspecific variations, these results should be interpreted with caution [29, 30, 31]. Thus, data on the distribution of the *An. maculipennis* s.l. members especially in Eastern Europe are outdated and only available for some European countries [5]. In addition, new members of *An. maculipennis* s.l. have been described in the 21st century (*An. artemievi*, *An. daciae* and *An. persiensis*) [32, 27, 33], which were not included in previous applied cytogenetic or molecular screening assays. Thus, the aim of the present study was to summarize historic and current distribution data of the different members of *An. maculipennis* s.l. present in the Republic of Moldova, updating the data by molecular typing of recently collected specimens.

## Methods

Mosquito sampling was conducted between July and August 2016, 2017 and 2019 at 66 rural and semi-urban localities and four natural reserves in the Republic of Moldova. About 2,400 *Anopheles maculipennis* s.l. females were collected at 85 sampling sites in 45 localities and four natural reserves belonging to 14 regions. Sampling was performed using CO<sub>2</sub>-baited CDC traps (Trappola per Monitoraggio Zanzare, IMT Original 2002, Italy) or CDC light traps (model 512, John W. Hock Company, USA) operating overnight and placed inside or outside the animal stables (livestock sheds, hen houses, dog kennels, etc.). In addition, active mosquito sampling with a manual aspirator was applied inside animal stables and natural caves. Mosquitoes collected in 2016 and 2017 were stored in 96% ethanol and specimens sampled in 2019 were kept at -80°C. Adult females were identified by morphology as *An. maculipennis* s.l. using the taxonomic key by Becker et al. [34]. Randomly selected 297 *An. maculipennis* s.l. females from 49 sampling sites (Fig. 1, Additional file 1) were subjected to the molecular species identification. In total, one female collected in 2013, 129 females captured in 2016, six females captured in 2017 and 161 females captured in 2019 were analyzed. These specimens were collected with traps installed indoor (142) and outdoor (40). A further 115 specimens were caught by manual aspirators.

Each mosquito specimen was individually placed into an individual sterile 2 ml tube with 700 µl of Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) with 100 µg/ml streptomycin (PAN-Biotech, Aidenbach, Germany), and 2.5 µg/ml amphotericin B (PAN-Biotech). The samples were homogenized with 5–9 zirconia beads (2 mm, BioSpec Products Inc.) for 3 min at 30 Hz with a TissueLyser II (Qiagen, Hilden, Germany). Total nucleic acid was extracted from 200 µl of supernatant with the 5X MagMAX™ Pathogen RNA/DNA Kit (Thermo Fisher Scientific, Vilnius, Lithuania) using the KingFisher™ Flex Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. DNA eluates were stored at -20°C. The PCR assays by Lühken et al. [19] targeting the internal transcribed spacer 2 (ITS2) ribosomal DNA were used for molecular species identification. The ITS2 gene was subjected to Sanger sequencing (LGC Genomics, Berlin, Germany) to potentially confirm taxa not included in the PCR assay, but previously reported for the Republic of Moldova, e.g. *An. melanoon*. In addition, PCR products of 5 randomly selected specimens (3 larvae, 2 females) previously identified by PCR gel band identification as belonging to *An. atroparvus*, *An.*

*maculipennis* s.s., *An. melanoon* and *An. messeae* [26] were subjected to Sanger sequencing in order to confirm the results of the previously applied gel-based PCR assay by Proft et al. [25] (Additional file 2). The data extracted from Şuleşco et al. [26] and unpublished data of gel PCR assay for additional 86 females are presented in Additional file 2.

Individual, updated distribution maps of the different *An. maculipennis* s.l. members were generated for the Republic of Moldova combining historical data until 1950 [15, 16, 17] with PCR-based results by Şuleşco et al. [26] and with the newly collected data (Fig. 1, Additional file 1–3). Maps were produced with the program R [35]. Following packages were used: geodata [36], ggplot2 [37], ggspatial [38] and sf [39].

## Results

From 2016 to 2019, *An. maculipennis* s.l. were detected in 85 sampling sites. Our molecular screening confirmed the presence of three *An. maculipennis* s.l. species: *An. maculipennis* s.s., *An. atroparvus* and *An. messeae*. In addition, *An. daciae* was firstly reported for the country, while *An. melanoon* was not detected again. The most prevalent species was *An. maculipennis* s.s. (number of specimens = 115, 38.9%; sampling sites = 28, 57.1%), followed by *An. daciae* (72, 24.3%; 20, 40.8%), *An. atroparvus* (56, 18.6%; 15, 30.6%) and *An. messeae* (54, 18.2%; 12, 24.5%) (Fig. 1, Additional file 1).

*Anopheles maculipennis* s.l. was always considered widely distributed and abundant in the Republic of Moldova. Lepşi [14] mentioned large population of the mosquito in Chişinău, which was unknown to be a species complex at that time. Zotta et al. [15] presented detailed data with a map showing the occurrence of different *An. maculipennis* “races” separated by the egg’s chorion morphology in Romania, including the Bessarabia province, covering wide parts of the current territory of the Republic of Moldova. According to Zotta et al. [15], *An. messeae* was the dominant species (602/905 egg batches, 66.5%), followed by *An. atroparvus* (204/905, 22.5%) and *An. maculipennis* s.s. (99/905, 10.9%). Their study mainly focused on the areas located along the Prut and Dniester Rivers and the northern part of the country. In the late 1940’s, Prendel et al. [16] presented the results of the *An. maculipennis* “subspecies” abundance and distribution in the Moldavian Soviet Socialist Republic, corresponding to the territory of the present Republic of Moldova. The authors described the presence of three *An. maculipennis* s.l. species occurring mainly as mixed populations: *An. messeae* (1453/2198 egg batches, 66.1%), *An. maculipennis* s.s. (617/2198, 28.1%) and *An. atroparvus* (128/2198, 5.8%). In the same period, studies conducted in south-western Ukraine located close to the Republic of Moldova showed the following general prevalence among the species: *An. messeae* (426/575, 74.2%), *An. maculipennis* s.s. (60/575, 10.4%) and *An. atroparvus* (89/575, 15.4%) [30]. The short-term field studies conducted between 1949 and 1950 [17] confirmed the findings by Prendel et al. [16]: *An. atroparvus* (7/59, 11.9%), *An. messeae* (40/59, 67.8%), *An. maculipennis* s.s. (12/59, 20.3%).

According to Zotta et al. [15], *An. maculipennis* s.s. (as *An. typicus*) is mainly distributed in hilly areas of Bessarabia, but also present in the lowland areas everywhere along shadowed watercourses in the

vicinity of forests. Prendel [16, 30] confirmed that elevation is an important factor for *An. maculipennis* s.s. distribution and abundance in Moldova. The species has been found predominantly at higher altitudes in hilly, forested areas of the Codri Forest Upland. Recent data demonstrated that *An. maculipennis* s.s. was found in large permanent water bodies (e.g. lakes, water reservoirs) with stagnant water, but also occurs in ditches and low-level small streams distributed in central hilly forested areas of Moldova [26]. In our recent collections, the species was frequently found resting in animal shelters of cows, sheep, goats and poultry. *Anopheles maculipennis* s.s. is a frequent species and occurred at most of our collecting sites (Fig. 1, Table 1, Additional file 1–3).

Table 1  
Sampled regions and species composition of *Anopheles maculipennis* s.l.

| Region       | No. of sampling sites | No. mosquitoes identified | <i>An. maculipennis</i> s.s. | <i>An. atroparvus</i> | <i>An. messeae</i> | <i>An. daciae</i> |
|--------------|-----------------------|---------------------------|------------------------------|-----------------------|--------------------|-------------------|
| Glodeni      | 9                     | 117                       | 58                           | 17                    | 11                 | 23                |
| Cahul        | 16                    | 91                        | 7                            | 1                     | 41                 | 42                |
| Falesti      | 5                     | 28                        | 11                           | 17                    |                    |                   |
| Criuleni     | 6                     | 21                        | 20                           | 1                     | 2                  | 6                 |
| Ceadir-Lunga | 2                     | 19                        |                              | 19                    |                    |                   |
| Straseni     | 2                     | 7                         | 7                            |                       |                    |                   |
| Calaras      | 2                     | 4                         | 4                            |                       |                    |                   |
| Riscani      | 1                     | 3                         | 3                            |                       |                    |                   |
| Grigoriopol  | 1                     | 2                         | 2                            |                       |                    |                   |
| Ialoveni     | 1                     | 1                         | 1                            |                       |                    |                   |
| Bender       | 1                     | 1                         | 1                            |                       |                    |                   |
| Dubasari     | 1                     | 1                         |                              |                       |                    | 1                 |
| Anenii Noi   | 1                     | 1                         |                              | 1                     |                    |                   |
| Slobozia     | 1                     | 1                         | 1                            |                       |                    |                   |
| total        | 49                    | 297                       | 115                          | 56                    | 54                 | 72                |

As shown for other European countries [40, 41], *An. atroparvus* was described to colonize plains and slopes of eroding hills, also being present in brackish waters with dense populations at the Black Sea coast. Due to its ecological plasticity, the species could be regularly found in fresh waters together with *An. maculipennis* s.s. [15]. Previous reports showed the probability of the occurrence of *An. atroparvus* generally increased with increasing salinity of natural breeding sites from North to South of the Black

Sea region [30, 42]. In the 1940s, *An. atroparvus* was found to be sporadic in the villages with malaria outbreaks and considered epidemiologically of little importance [18]. Small number of larvae were also found in brackish waters inside the country, where the soil was more or less rich in the salt in southern and eastern Moldova [26]. In our study the adult females are mainly present as single specimens in the resting sites in rural localities in south-eastern and north-western parts of the country. The historical and present data suggest that the species is widely distributed but not abundant in Moldova, with sporadically higher densities at a few sampling sites (Fig. 1, Table 1, Additional file 1–3).

Zotta et al. [15] described *An. messeae* as a “race” of *An. maculipennis* s.l. predominantly present in large stagnant freshwater bodies such as lakes, swamps, floodplains of the rivers Danube, Prut and Dniester. Studies of Prendel [16, 30] and our data confirmed the association of this species with river systems and dominance of the species in floodplains of the rivers Prut and Dniester and vicinity villages. Other studies conducted in Europe also demonstrated the higher frequency and predominance of *An. messeae* along large river systems [40, 43]. We found that *An. messeae* was mainly present in the villages located near the Prut River. The data suggest a wide distribution of *An. messeae* with a lower prevalence in central Moldova (Fig. 1, Table 1, Additional file 1–3).

*Anopheles daciae* was described as a new member of the *An. maculipennis* species complex in 2004 [27] and is reported here for the first time in Moldova. Our field study showed that *An. daciae* can be found inside the living houses located close to the nature, in the natural shelters like caves and different animal shelters with cows, sheep, goats and poultry present, that may indicate the wide host preferences of the species. Knowledge on the ecology of *An. daciae* is still limited, but the species is considered widely distributed in Europe [5]. Our study demonstrated that the species is frequent and distributed throughout Moldova, predominating in the villages along the Prut River (Fig. 1, Table 1, Additional file 1–3).

*Anopheles melanoon* was detected in 2011 for the first time, using gel PCR assays for specimens from Natural Reserves Padurea Domneasca, Prutul de Jos, Plaiul Fagului and Chisinau municipality [26, 28]. In addition, 13 adult females, collected between 2011 and 2012 by manual aspiration inside the animal shelters and a human dwelling mainly located in the Natural Reserves Plaiul Fagului, Prutul de Jos and Codrii (Additional file 2) were identified as *An. melanoon* by the PCR assay of Proft et al. [25] (Șuleșco, unpublished data). The identity of this species was confirmed here by DNA sequencing of two gel PCR products previously used for PCR gel band identification of *An. melanoon* [26]. However, *An. melanoon* was not detected in the present study among the *An. maculipennis* s.l. adult females collected by CDC traps or manual aspirators inside or outside the animal shelters in the rural localities. The species was also absent in the Prutul de Jos Reserve and Ceadir - Lunga, the only sampling sites where we previously found *An. melanoon* (Șuleșco, unpublished data) and repeatedly sampled the sites in this study. In summary, the species has to be considered very rare in Moldova.

Zotta et al. [15] found *Anopheles sacharovi* (as *An. elutus*) in the brackish and well heated water bodies at the Black Sea coast and Danube Delta, but the species was considered absent in the remaining areas

in Romania and Bessarabia province. Previous records of *An. sacharovi* in Moldova were based on morphology of adult mosquitoes only, which was not confirmed by molecular identification [28]. The presence of *An. sacharovi* in Moldova was not confirmed in this study. Most probably the northern distribution limit of the species is the Danube Delta coast [15]. *Anopheles labranchiae* has probably never been detected in the Republic of Moldova. Two larvae of the species have been detected in a natural pond in Iasi, Romania in 2012 far east from its distribution area [44]. This species is distributed in the Mediterranean basin and have never been detected in Romania before, suggesting the recent introduction of this species from Mediterranean region.

## Conclusion

In conclusion, we confirmed the presence of *An. atroparvus*, *An. maculipennis* s.s., *An. messeae* and *An. melanoon* in Moldova. In addition, we detected for the first time *An. daciae*. This study provides a comprehensive update of the distribution of *An. maculipennis* s.l. members and highlights the importance of mosquito monitoring to address potential health risks in Eastern Europe.

## Declarations

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### Availability of data and materials

All data are available in the manuscript and the supplementary files.

### Authors' contributions

Conceptualization: TS, RL; data collection: TS; data analysis: FGS, TS, RL; first drafting: TS, RL; writing and editing: all authors.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

## Competing interests

The authors declare no competing interests.

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## Figures

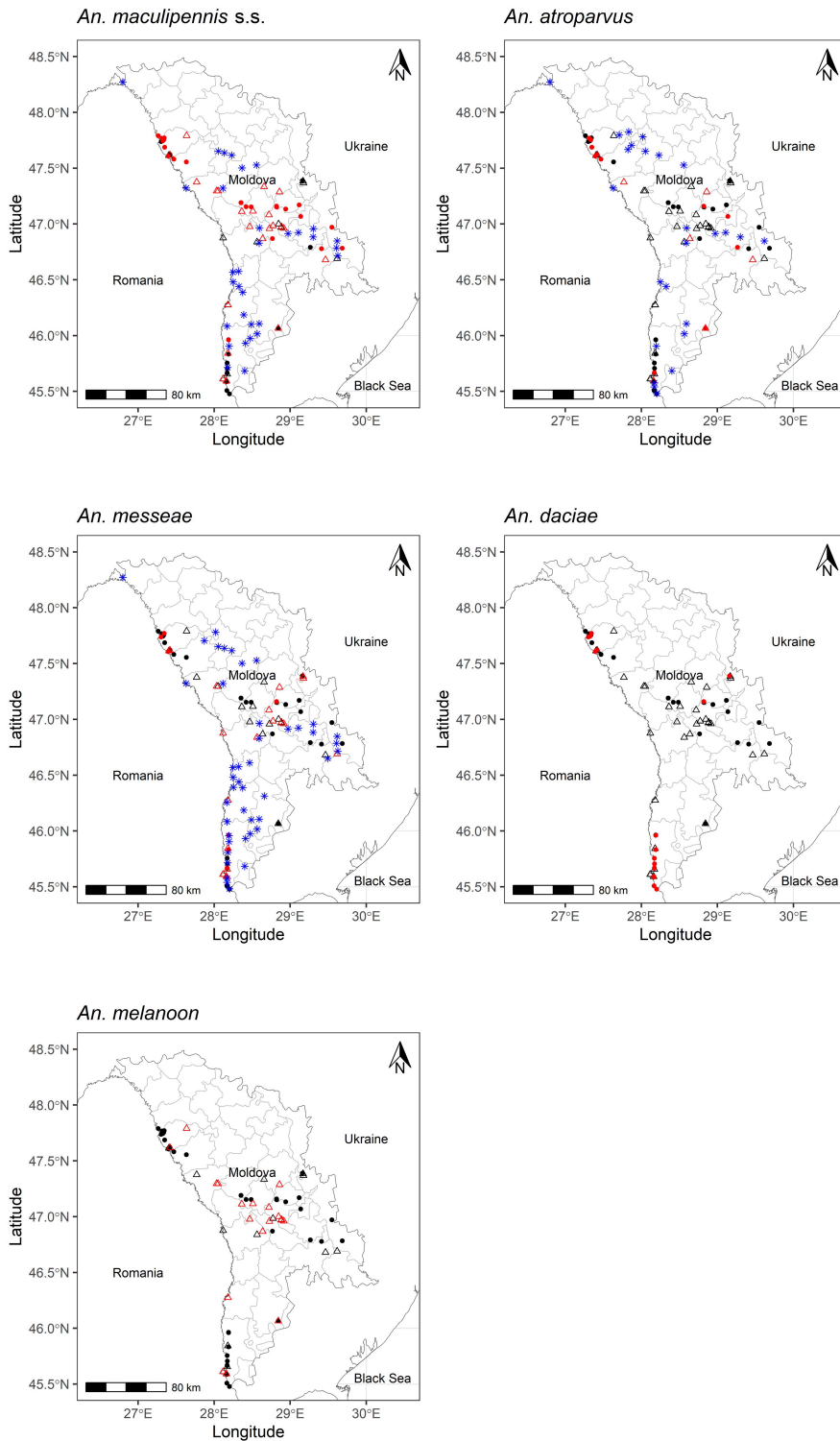


**Figure 1**

Occurrence maps of *Anopheles maculipennis* complex members in the Republic of Moldova. Red dot: current presence (sequenced specimens from 2013 and from 2016 to 2019), black dot: current absence; red triangle: presence (data from 2010 to 2013, obtained using Proft et al. [25] PCR assay); black triangle: absence (data from 2010 to 2013); blue star: historic presence (data from 1938 to 1949)

## Supplementary Files

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