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Identification and distribution of sibling species of *Anopheles maculipennis* complex (Diptera: Culicidae) in north-eastern Poland[1]

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Abstract: A previously established species-diagnostic PCR assay was used to determine the species composition and distribution of sibling species of the *Anopheles maculipennis* complex in north-eastern Poland, which was historically affected by malaria. Of all the 1120 mosquitoes of *An. maculipennis* complex (1002 adults and 118 larvae) identified by molecular means, all were either *An. messeae/daciae* or *An. maculipennis* s. s. with the former dominant in all collection localities. Their halophilic sister taxon *An. atroparvus*, previously collected along the Baltic Sea coast was not found. New records of *An. maculipennis* s. s. and *An. messeae* were located in 9 UTM squares respectively and these species were new for Tuchola Forests (Bory Tucholskie) and for the district of Iława Lakeland (Pojezierze Iławskie). *An. messeae* is a new species also for Masurian Lakeland (Pojezierze Mazurskie).

Key words: *Anopheles maculipennis* complex, sibling species, species-diagnostic PCR, Poland

INTRODUCTION

Cryptic species complexes comprise groups of closely related species that are difficult sometimes impossible to distinguish by morphological alone. Many of the major Anopheline malaria vectors are members of such cryptic species complexes, which include both vector and non-vector species. Moreover, a few member species are often found sympatrically (Collins & Paskewitz 1996).

Anopheles maculipennis Meigen 1818, the historical malaria vector in Europe, was first exposed as a species complex in the 1920's by Falleroni (1926) and van Thiel (1927) who described the sibling taxa *An. messeae* and *An. atroparvus*, respectively. The larval, pupal and adult stages of the component taxa in the Maculipennis Complex are morphologically similar to each other. However, most can be on the basis of egg shell patterns, although this is only useful when gravid females are captured. More recently, larval chaetotaxy, cytotaxonomic methods, cross breeding experiments, the use of enzyme electrophoresis and analysis of cuticular hydrocarbons, PCR methods and DNA sequencing have provided evidence for the existence of the different species in the complex (Kitzmiller et al. 1967, Munstermann & Conn 1997).

Currently 11 species of the *An. maculipennis* s. l. are recognized in the Palearctic Region: *An. maculipennis* s. s. Meigen, *An. atroparvus* van Thiel and *An. messeae* Falleroni, which occur also in Poland (Kubica-Biernat 1997, Okróy-Rysop 1991), *An. artemievi* Gordeyev, Zvantsov, Goryacheva, Shaikevich & Yezhov. in Kyrgizstan (Gordeyev et al. 2005).

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An. melanoon Hackett in the southern Europe, *An. labranchiae* Falleroni, and *An. sacharovi* Favr in coastal areas along the Mediterranean Sea and the Black Sea, and *An. beklemishevi* Stegnii & Kabanova, are distributed sympatrically with *An. messeae* in Sweden, Finland and European part of Russia (Ramsdale & Snow 2000). *An. persiensis* Linton, Sedaghat & Harbach has been described from Iran (Sedaghat et al. 2003), *An. martinius* Shingarev, 1928 occur in northern Africa and middle Asia. White (1978) suggested that *An. lewisi* Ludlow may be synonymous with *An. messeae* or *An. beklemishevi*, but this nominal form is still regarded as a valid species because its identity has not been resolved. Recent molecular studies on the *An. maculipennis* s. l. in Romania revealed a new species, *Anopheles daciae* Linton, Nicolescu & Harbach, (Nicolescu et al. 2004) which occurs also in England (Linton et al. 2005) and Poland (Wegner, personal communication in 2009) and probably in Italy, the Netherlands, former Yugoslavia and Kazakhstan (di Luca 2004). *An. messeae* and *An. daciae* are morphologically and genetically the most similar species in the Palearctic Maculipennis Complex. The nuclear ITS2 sequences of both species are the same length, differing by only five bases. These species cannot be differentiated using the current *messeae*-specific primers designed by Proft et al. (1999). It seems that the presence of *An. daciae* has been obscured by *An. messeae* across their extensive and seemingly sympatric ranges and could be responsible for malaria transmission (Nicolescu et al. 2004).

The distribution of malaria is determined by the occurrence of competent mosquito vectors, and the temperature requirements of the *Plasmodium* sp. for sporogony within the vector itself (Knap & Myjak 2009). In Poland, as in many areas of central Europe, malaria was due to *Plasmodium vivax* was observed in the past. This parasite was transmitted almost exclusively by *An. atroparvus* and *An. messeae*. The latter, was involved in malaria transmission in eastern Europe and western Asia, while *An. atroparvus* played the most important role as a malaria vector in north-western Europe (Jetten & Takken 1994).

In Poland, malaria was endemic in some areas during the years following World War I, especially in swampy lowlands. The disease reappeared at the end of World War II. Thus, more attention was paid again to the *An. maculipennis* complex, particularly to *An. atroparvus* and *An. messeae,* which were the main vectors in the country (Kubica-Biernat 2005). Although, the indigenous malaria has been eradicated, the number of infections in Poland is likely to increase with the growth of travel to endemic areas.

Material and methods

Mosquito collections

Anopheline mosquitoes were collected from 1999 to 2002 in the voivodeships of Pomorskie, Warmińsko-Mazurskie and Podlaskie (north and north-eastern Poland). The 54 collection sites covered 22 of the 3.000 UTM (Universal Transverse Mercator) 10 km^2 Poland.

Resting adult were collected from the walls and ceilings of human houses, farm buildings and cellars an Nabokov-Zeifert aspirator. Further specimens, were collected using CO_2 traps and entomological nets. Moreover, the catches on human bait were done by means of an aspirator. Preimaginal stages were captured in water bodies using standard dippers. Then, the larvae of the 1^{st}, 2^{nd} and 3^{rd} instars and pupae were reared in the laboratory in breeders in order to obtain larvae of the 4^{th} stage and adults – forms with fully developed taxonomic features. Mosquitoes were sorted to *An. maculipennis* s. l. based on larval or adult morphological characteristics according to the identification keys of Skierska (1971, 1977) and stored individually in 70% ethanol for later molecular identification.

DNA extraction, amplification and sequencing

DNA was individually extracted, either larval portions or single legs of adults, by lysis in ammonium hydroxide (NH_4OH) (Rijkpema et al. 1996). Lysates were stored at –20°C for further investigation.

Specimens were individually identified using the previously developed species diagnostic PCR assay of Proft et al. (1999). All specimens were tested using individual PCRs for the identification of species previously detected in Poland.

Reactions containing the universal primer UN and one of the three species specific primers: AMA (*An. maculipennis* s. s.), AMS (*An. messeae/daciae*) and AAT (*An. atroparvus*) were used to amplify fragments of different length of the ITS2 region of the ribosomal DNA (410 bp, 305 bp and 117 bp, respectively) (Proft et al. 1999). PCR reactions were carried out in a reaction mixture that contained: 2.5 µl of DNA template, 5 U (0.125 µl) *Taq* polymerase (Gibco), 2.5 µl of 10 x PCR reaction buffer (Gibco), 0.5 µl of 50 mM $MgCl_2$ (Gibco), 1.25 µl of 2.5 mM dNTPs mixture (MBI Fermentas), 1 µl of 10 µM universal primer (UN), 1 µl of 10 µM species specific primer (AMA or AMS or ATT) and sterile double distilled water (DDW) was added to a total volume of 25 µl. Samples were incubated for 3 min in 94°C and then thermally cycled 35 times with DNA denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 72°C for 1 min, with a final extension of 7 min at 72°C.

PCR amplification of the nuclear ITS2 region was carried out using primers 5.8F and 28R (Collins & Paskewitz 1996). The concentrations of the reactants were: 5 µl DNA template, 1.25 U RUN *Taq* polymerase (A&A Biotechnology), 5 µl 10 x PCR buffer containing $MgCl_2$, (A&A Biotechnology), 5 µl dNTPs mixture (MBI Fermentas), 1 µl primers 10 µM (5.8F, 28R) and DDW to a total volume of 50 µl. The samples were heated at 94 °C for 2 min before 35 cycles of amplification at 94 °C for 30 s min, 53 °C for 30 s and 72 °C for 30 s followed by a final extension step of 10 min.

All PCR reactions were carried out in Perkin Elmer GeneAmp PCR System 2400 and 9700 thermocyclers. Amplification products were visualised in 2% agarose gels stained with ethidium bromide. Both positive and negative (sterile DDW) controls were run with each PCR reaction.

Amplicons of 5.8F and 28R primers were purified by using the Clean-up purification kit (A&A Biotechnology) and sequenced in both directions. DNA sequencing reactions were performed with the ABI PRISM 310 Genetic Analyser (Applied Biosystem). Sequences were edited and compared with representative gene sequences deposited in GenBank database using NCBI BLAST program (U.S. National Institute of Health, Bethesda, Maryland).

Positive controls

The DNA from morphologically and molecularly (ITS2 region sequence) verified samples of *An. maculipennis* s. s *An. messeae* and *An. atroparvus* served as positive controls. Bloodfed females of *An. maculipennis* s. s. and *An. messeae* were caught in the field and induced to lay eggs, which were then identified according to patterning of the eggs (Skierska 1971). Control of *An. atroparvus* (insectary strain) originated from Spain (courtesy Dr. Carlos Aranda Pallero, Consell Comarcal del Baix Llobregat).

Results

Thirty six of the 54 prospected sites yielded samples of Anophelinae. In 18 of them only *An. maculipennis* s. l. was noted, while in 11 captures stations it occurred together with *An. claviger* s. l. Moreover, the latter one was recorded in 7 locations where *An. maculipennis* s. l. was absent.

Species identification based on ITS2 sequence

Sixteen sequences were generated and identified to species based on similarity with the ITS2 sequences for members of *An. maculipennis* s. l. available in GenBank. One sequence originated from Spain – it shows 100 % homology with *An. atroparvus* (Z501102) from Italy, AM409779 from Russia, AF504248 from England and AY634530 from Romania.

Six sequences from Masurian (UTM: EF 41), Iława (CE 97) and Kashubian Lakeland (CF 23), Tucholskie Forests (CE 25), Białowieża Primeval Forest (FD 75) and Gdańsk (CF 42). They showed 100% homology with sequences from GenBank: *An. maculipennis* s. s. AF342715 from Greece, Z50104 from Italy, FN665792 from Azerbaijan and from Germany (Proft et al. 1999) (not entered in GenBank).

Nine sequences from Masurian (UTM: EF 41), Iława (CE 97) and Kashubian Lakeland (CF 23), Tucholskie Forests (CE 25), Białowieża Primeval Forest (FD 73, 75), Żuławy (CF 41) and Gdańsk and vicinity (CF 42, 92), they showed 100% homology with sequences *An. messeae* AF452699 from England, AF342711 from Greece, AF305556 from China, EF090191 from Romania and from Germany (Proft et al. 1999) (not entered in GenBank).

Species diagnosis by PCR

In total, 1.120 *An. maculipennis* s. l. specimens were collected and identified by PCR, including 118 larvae (10.53%) (Table 1). Two species were identified: *An. messeae/daciae* (n=912 specimens; 81.43%) and *An. maculipennis* s. s. (n= 208; 18.57%) (Table 1). No *An. atroparvus* were detected in these northerly collections sites. The majority of *An. maculipennis* s. l. specimens found (n=974) were collected in Pomorskie Voivodeship (Table 1). Amongst those, 81.52% were found to be *An. messeae/daciae* and 18.48% *An. maculipennis* s. s.

Both species of the complex occurred in 19 and 15 collection sites, respectively, out of total 44 standings studied in this voivodeship. *An. messeae/daciae* was the dominant both among adults collected (84.35%) and larvae (61.01%). Moreover, it was the dominant species at all capture sites in the Podlaskie and Warmińsko-Mazurskie Voivodeships, where only adults were captured (Table 1).

Anopheles maculipennis s. l. was noted in 26 localities, located in 17 squares of the UTM net. Eleven collection sites (in 6 UTM squares) were situated in urban areas (Table 1). *Anopheles maculipennis* s. l. was reported in 107 UTM squares in Poland prior to this research, and herein *An. maculipennis* s. l. is reported in an additional 10 UTM squares for the first time (CF 13, 23, 41, 42, 92 & CE 25, 97& FD 73, 75 & EF 41) (Fig. 1; Table 1).

In case of *An. maculipennis* s. s., during the present study its occurrence of was confirmed 12 UTM net squares of which 9 were new for this species (CF 13, 23, 41, 42, 92 & CE 25, 97 & FD 75 & EF 41) (Fig. 2, Table 1).

The occurrence of *Anopheles messeae/daciae* was reconfirmed in 13 UTM squares in the present investigations, and new country records were established in a further 9 squares –(CF 23, 41, 42, 92 & CE 25, 97 & EF 41 & FD 73, 75) (Fig. 3, Table 1).

Discussion

Despite numerous publications concerning Culicidae in Poland, detailed information on the specific distribution of taxa within *Anopheles* sibling species complexes are still relatively poorly understood. Since the first record of *Anopheles maculipennis* complex in Poland at the end of 19th century (as *An. maculipennis*, Nowicki 1873), only a few short reports and faunistic notes concerning its distribution have been published. Extensive study on malaria vectors

carried out in the 1920-s showed that *An. maculipennis* s. l. is very common in Poland (Tarwid 1934). Thus some 130 years later, although, *An. maculipennis* s. l. has been reported 107 squares of UTM net (Kubica-Biernat 1999), knowledge of the distribution of component taxa was less clear. The result of our investigations added new distribution records of *An. maculipennis* s. l. in 10 new UTM squares and reconfirmed another three UTM squares where it had been previously recorded. In the present study, specific identification of *An. maculipennis* s. s., *An. messeae/daciae* and *An. messeae* have been confirmed in Poland, using PCR and sequencing of ITS2 region of nuclear DNA. *Anopheles maculipennis* s. s. is widely distributed in Europe and Asia, apart from Great Britain, Ireland, and the southern part of the Iberian peninsula. The distribution range of *An. messeae* covers the almost whole Europe and Asia, except Portugal and Spain, and this species occurs more frequently than *An. maculipennis* s. s. (Ramsdale & Snow 2000).

Table 1. Occurrence of *Anopheles maculipennis* s. s. and *An. messeae/daciae* in the study areas

UTM net square and collection site	*An. maculipennis* s. s.		*An. messeae/daciae*		Total
	adults	larvae	adults	larvae	
Pomorskie Voivodeship					
CE 25 Kasparus	6	0	41	0	47
CE 97 Dzierzgoń	26	-	56	-	82
CF 07 Brzyno	76	-	207	-	283
CF 13 Pomieczyńska Huta	8	-	19	-	27
CF 23 Kielno	-	-	3	-	3
CF 32 Gdańsk-Klukowo	-	13	-	9	22
Gdańsk-Rębiechowo	-	3	1	6	10
CF 33 Banino	-	17	-	17	34
Gdańsk-Owczarnia	1	2	-	4	7
CF 34 Gdynia-Wiczlino	-	1	-	2	3
CF 35 Gdynia-Pierwoszyno	-	2	-	1	3
CF 41 Radunica	-	6	2	25	31
CF 42 Gdańsk-Migowo	-	0	-	2	2
Gdańsk-Wrzeszcz	-	0	-	2	2
Gdańsk-Siedlce	0	0	1	1	2
Gdańsk-Orunia Dolna	1	-	2	-	3
CF 43 Gdańsk-Oliwa	-	1	-	2	3
Sopot-Karlikowo	-	1	-	-	1
CF 92 Krynica Morska	0	0	6	1	7
Przebrno	16	0	384	0	400
In sum [n/%]	134/13.76	46/4.72	722/74.13	72/7.39	974/100.0
Podlaskie Voivodeship					
FD 73 Piaski		0		1	1
FD 74 Poryjewo		2		7	9
Orzeszkowo		9		30	39
FD 75 Dubiny		3		4	7
Postołowo		12		20	32
In sum [n/%]		26/29.54		62/70.46	88/100.0
Warmińsko-Mazurskie Voivodeship					
EF 41 Wesołowo		2/3.44		56/96.56	58/100
In total [n/%]		208/18.57		912/81.43	1120/100.0

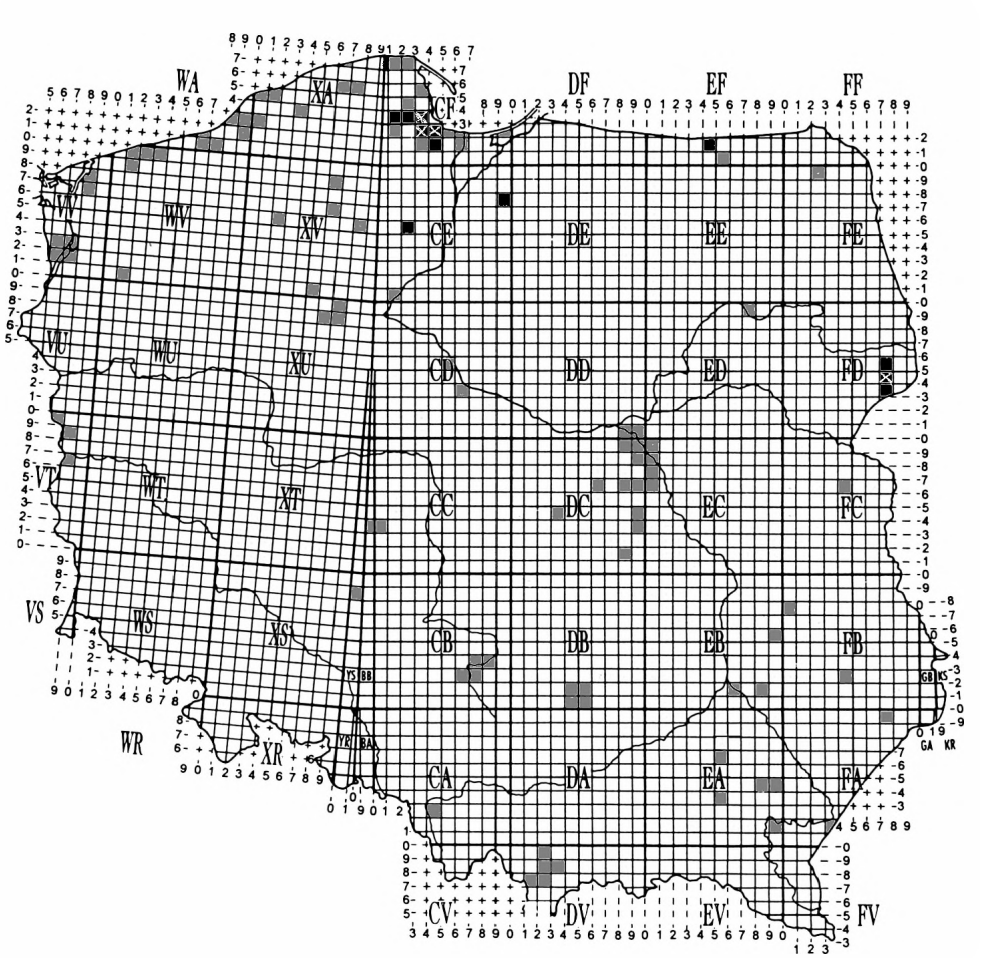

Fig. 1. Recorded distribution of *Anopheles maculipennis* s. l. in Poland by UTM squares; grey squares – recorded distribution before present investigations (Kubica-Biernat 1999), black squares – recorded distribution in present investigations, black squares with white cross – recorded distribution in present and past investigations.

Although *An. atroparvus* has been reported in Poland in previous studies, it was not found in this survey. Although predominantly coastal, the first record of *An. atroparvus* was reported from Rokitnica village near the city of Bytom (CA48) (Weyer 1938), which remains the southernmost record of this species in Poland. *Anopheles atroparvus* was also captured in urban areas in the city of Gdańsk and its vicinity in many subsequent years (Lachmajer 1950, 1952, 1966, 1971, 1972, 1975, 1982, Skierska & Szadziewska 1978). Maculipennis Complex specimens collected in the coastal regions of the in the 1940-s (Lachmajer 1948) showed high percentages of *An. atroparvus* (from 20% to 75%). However, surveys on the western Baltic coast in 1963–1965 showed a significant reduction in prevalence, with *An. atroparvus* accounting for only 2.6% of the total "Maculipennis" Complex mosquitoes (Lachmajer & Skierska 1968). Its presence was first reported in the mid-1980s in the coastal zone between Wejherowo & Darłowo (Pomerania) (Okróy-Rysop et al. 1991). Similar studies carried out about the same time using the same methods revealed only *An. maculipennis* s. s. and *An.*

messeae (Szadziewska & Okróy-Rysop 1988, Wegner et al. 1993). The disappearance of *An. atroparvus* for Poland could be explained by the reduction of the brackish habitats favoured by *An. atroparvus* due to human intervention e.g., land reclamation, such as drainage of small reservoirs in urban or agriculture areas. However, as in other European countries, the targeted larvicide applications of the widespread malaria control measures in the 1940-s and 1950-s could have resulted in the decimation of *An. atroparvus* populations, whose niche was perhaps subsequently filled by *An. messeae*. A similar phenomenon was observed in The Netherlands, where in 1999, most anophelines collected were *An. messeae* (Takken et al. 2002). The authors concluded that the study area has undergone dramatic ecological changes since the earlier *Anopheles* surveys in 1935, causing the near extinction of *An. atroparvus*. Molecular identification of material collected by Lachmajer & Skierska (1968) could be very informative in determining whether *An. atroparvus* was indeed still present at this time present, and in what proportion, relative to its sibling taxa.

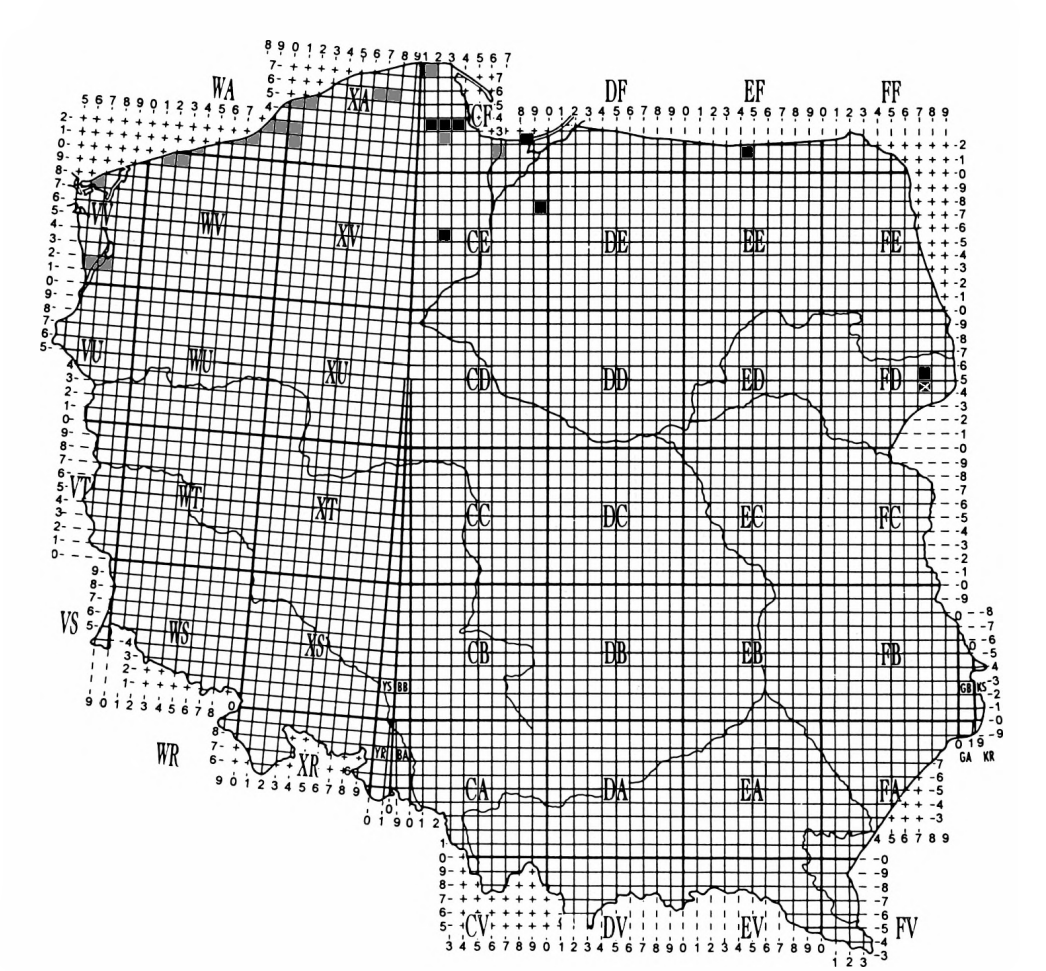

Fig. 2. Recorded distribution of *Anopheles maculipennis* s. s. in Poland by UTM squares. Legend: see Fig 1.

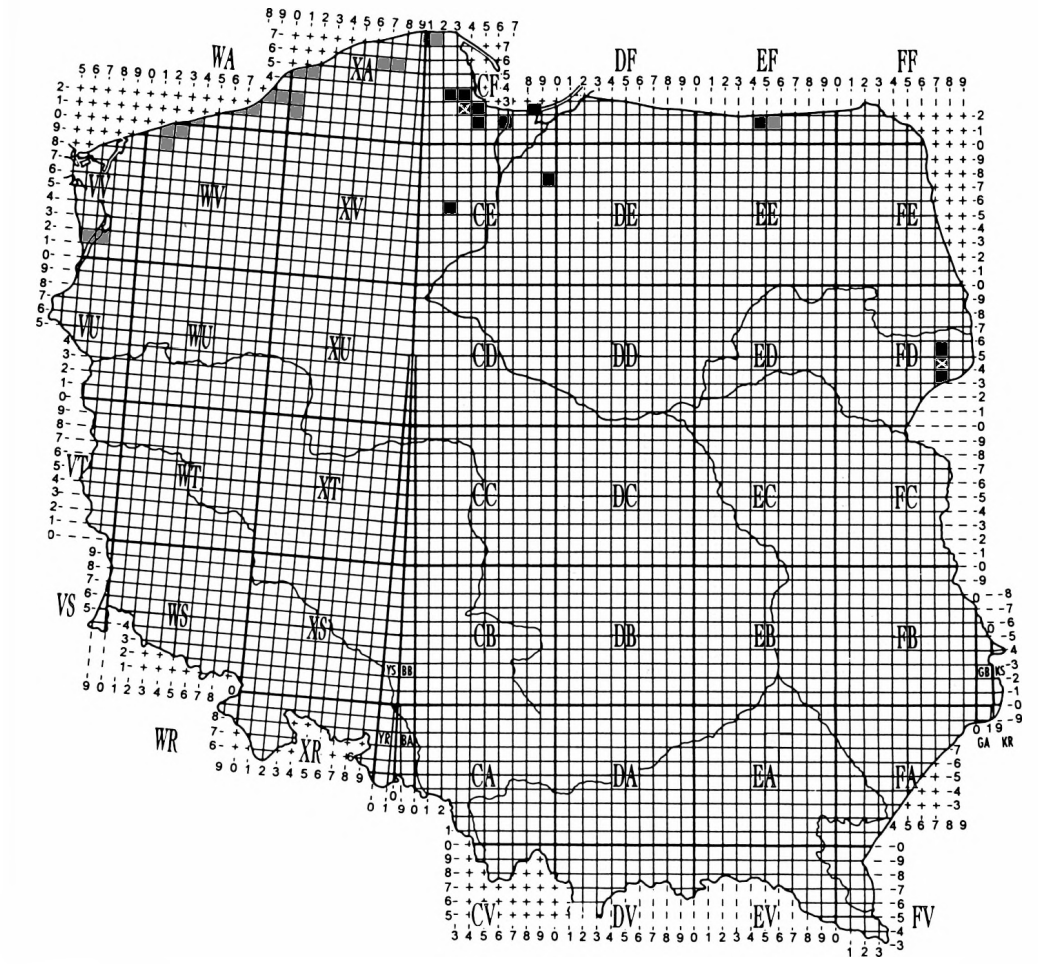

Fig. 3. Recorded distribution of *Anopheles messeae/daciae* in Poland by UTM squares.. Legend: see Fig. 1. UTM squares: CE 97, CF 23, 41, 42, 92, EF 41, FD 73, 75 – *An. messeae* identified using sequencing ITS2

An. messeae/daciae was almost 4 times as prevalent than *An. maculipennis* s. s. Domination of *An. messeae/daciae* was observed both in the Pomorskie Voivodeship (76.60%), and in Podlaskie Voivodeship (70.46%), in agreement with the results previously reported by Lachmajer & Skierska (1968). The area of the Hel Peninsula is the exception, as in the end of the 1940-s *An. atroparvus* was documented as the most numerous species, comprising more than 75% of Maculipennis Complex taxa collected in the region (Lachmajer 1948). The results of investigations from the 1980-s and early 1990s showed that in the area of the western Baltic coast, the occurrence of *An. messeae* (49.3%) was comparable to *An. maculipennis* s. s. (48.5%), whilst that of *An. atroparvus* was significantly lower (2.2%) (Okróy-Rysop et al. 1991). On the other hand, in the Żarnowieckie Lake environs, *An. messeae* was significantly more dominant (72.33%) than *An. maculipennis* s. s. (27.67%), and *An. atroparvus* was not reported at all (Wegner et al. 1993).

In the Pomerania Voivodeship, present study showed new records of *Anopheles maculipennis* s. s. and *An. messeae* in 7 squares of the UTM net, which covers Żuławy Wiślane (CF 41), Kashubian Lakeland (CF 13, 23) and in the urban areas of the city of Gdańsk (CF 42). In addition, the presence of both *An. maculipennis* s. s. and *An. messeae* were showed on Vistula Spit (CF 92), where only *An. maculipennis* complex was reported 50 years ago (Łukasiak 1959). Moreover, both species were also recorded, in the Tuchola Forests and Iława Lakeland for the first time (CE 25, CF 97).

Sack (1925) first reported *An. maculipennis* s. l. from the Podlaskie Voivodeship, but was unable to distinguish between the sibling species. Sampling conducted in the Białowieża Primeval Forest and its vicinity in the Podlaskie Voivodeship, showed a dominance of *An. messeae* (69.32%) over *An. maculipennis* s. s. as reported in the 1950s there by Lachmajer & Skierska (1958). New country records are herein shown for both species: *An. maculipennis* s. s. (UTM square FD 75) (Fig. 2) and *An. messeae* (FD73 & FD75) (Fig. 3). Here we support the absence of *An. atroparvus* reported in Lachmajer & Skierska (1958). In the Masurian Lakeland, in Mikołajki vicinity (EE 36), only *An. maculipennis* s. l. was previously reported (Wojnarowicz 1960), and recent studies reported *An. messeae* only (Kowalska-Ulczyńska & Giłka 2003). Herein we report *An. maculipennis* s. s. from Masurian Lakeland for the first time.

Following WWI, 15 mosquito species, including *An. maculipennis* s. l. (Martini 1920) were reported the TriCity agglomeration, including Gdańsk, Gdynia & Sopot. It is worth to stress the repeated records of occurrence of *Anopheles maculipennis* s. l. in Gdynia, Sopot and Gdańsk, especially *An. messeae/daciae* – the malaria vector in the typically urban areas – as their larvae were found in 11 different collection sites within towns, suggesting favourable habitats and suitable hosts still exist in these regions. Moreover, in a water reservoir close to the Gdańsk-Rębiechowo international airport, the appearance of larvae of both *An. maculipennis* s. s. and *An. messeae/daciae* was noted, with the latter again dominating. As this airport sometime serves countries where malaria is endemic, it is possible for the reintroduction of malaria to occur in Poland through. Malaria-infected mosquitoes entering our country on board incoming international flights. In summer months, mosquitoes may survive long enough to take a blood meal and transmit the disease. The surrounding environs is predominantly farmland where these exotic mosquitoes can find hosts, and suitable resting and breeding places, creating perfect conditions to establish a new foci of malaria in Poland. Furthermore, 37% of 95 cases of imported malaria presented in the clinic of Institute of Maritime and Tropical Medicine in Gdynia, between 1984–1993, were due to *Plasmodium vivax*, the historical malarial parasite in Poland (Jaremin et al. 1993/1994).

The permanent presence of historical vectors of disease in connection with possibility importing pathogens like in The TriCity area, creates potential epidemiological problems. Although endemic malaria has disappeared in Poland, every year more than 20 cases of malaria are imported by tourists and other travelers from malaria endemic areas. This has increased the concern that reintroduction of malaria into Poland is a real danger. Climatic changes such as global warming and associated increased precipitation are expected to extend vector ranges and population sizes of some species, potentially increasing malaria transmission rates (Kuhn et al. 2002).

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STRESZCZENIE

[Identyfikacja i rozprzestrzenienie kryptogatunków *Anopheles maculipennis* s. l. (Diptera: Culicidae) w północno-wschodniej Polsce]

Celem niniejszej pracy była identyfikacja kryptogatunków *Anopheles maculipennis* s. l. na wybranych terenach północno-wschodniej Polski – niegdyś endemicznych terenach występowania malarii. Ogółem odłowiono przy użyciu standardowych dla rodziny Culicidae metod połowów i przebadano metodą PCR 1120 komarów z kompleksu *Anopheles maculipennis*: 1002 imagines i 118 larw. Stanowiska odłowu (n = 54) (obory, zabudowania mieszkalne, „wolna przyroda" oraz zbiorniki wodne) były rozlokowane na terenie północnej Polski, w województwach: Pomorskim, Podlaskim i Warmińsko-Mazurskim i zlokalizowane w obrębie 22 kwadratów siatki UTM. Nie stwierdzono w niniejszych badaniach *An. atroparvus* – halofilnego gatunku charakterystycznego dla wybrzeża Bałtyku, notowanego przez szereg lat głównie na Wybrzeżu Gdańskim. Na całym badanym terenie gatunkiem dominującym był *An. messeae/daciae* – główny wektor *Plasmodium vivax* w Europie środkowo-wschodniej. Stwierdzono nowe stanowiska występowania *An. maculipennis* s. s. i *An. messeae* zlokalizowane w obrębie 9 kwadratów UTM, przy czym w Borach Tucholskich i na Pojezierzu Iławskim wykazano te dwa gatunki po raz pierwszy, jak również pierwszy raz wykazano *An. maculipennis* s. s. na Pojezierzu Mazurskim.

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