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Anopheles maculipennis Complex in The Netherlands: First Record of *Anopheles daciae* (Diptera: Culicidae)

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Abstract: Despite their past importance as vectors of indigenous malaria, the species composition and spatial distribution of the members of the *Anopheles maculipennis* complex have been studied to a limited extent in the Netherlands. Therefore, this investigation focuses on the distribution of the members of this complex in the Netherlands, including *Anopheles daciae*, which has recently been found in countries bordering the Netherlands. In the framework of a national mosquito surveillance between 2010 and 2021, a total of 541 specimens of *An. maculipennis* s.l. were analyzed from 161 locations covering the entire territory. In addition, 89 specimens were analyzed from overwintering sites during the winter of 2020/2021. All individual mosquitoes were identified to species-level using Sanger sequencing of the ribosomal internal transcribed spacer 2. To characterize the habitat of *An. maculipennis* s.l. in the Netherlands, land cover use data was extracted in a 1 km buffer area around each finding location. For populations collected in summers between 2010 and 2021, the most frequent species was *An. messeae*, present in 88.19% of the locations, followed by *An. maculipennis* s.s. (11.80%), *An. atroparvus* (3.72%) and *An. daciae* (3.72%). *Anopheles daciae* was found in the southern inland areas of the country. Furthermore, *An. messeae* and *An. daciae* occurred in sympatry at overwintering sites. This study provides relevant information on the occurrence of species of the *Anopheles maculipennis* complex in the Netherlands, contributing to a better estimation of the risk of mosquito-borne disease in the country.

Keywords: mosquitoes; DNA-based species identification; ribosomal internal transcribed spacer 2 (ITS2); malaria vector

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1. Introduction

In the Netherlands, the last published checklist of mosquitoes (Diptera: Culicidae) included 35 indigenous species [1]. This list is not static and was recently updated with a new indigenous species, *Culiseta longiareolata* [2]. Four members of the *Anopheles maculipennis* complex (= s.l.) are reported as present in the published checklist [1], some of which are capable of carrying pathogens of medical importance, including malaria: *Anopheles atroparvus* van Thiel, 1927, *An. messeae* Falleroni, 1926, *An. maculipennis* sensu stricto Meigen, 1818, and *An. melanoon* Hackett, 1934. However, this latter species is not considered to

occur in the Netherlands by [3], and *An. maculipennis* s.s. is considered uncommon in the country [4]. *Anopheles atroparvus* is reported as the only malaria vector (*Plasmodium vivax* and *Plasmodium malariae*) in the coastal areas of the Netherlands. Experimentally, *An. atroparvus* can also be an effective host and capable of transmitting *Plasmodium ovale* to humans [5]. A comparison of the anopheline species composition between 1935 and 1999 showed a prevalence shift from *An. atroparvus* to *An. messeae* in the Delta of the Rivers Rhine and Meuse, coinciding with the disappearance of indigenous malaria [6]. In a study of overwintering mosquitoes in several farms in the Netherlands, *An. messeae* individuals were more frequently found and *An. atroparvus* was less common [4]. A decline of *An. atroparvus* over the 20th century was recorded in multiple European countries and was assumed to be linked to major ecological changes, such as drainage practices, surface water pollution, loss of suitable resting sites for hibernation, etc. [6–8].

Despite their past importance as vectors of indigenous malaria and their potential role in the transmission of imported tropical malaria, leading to the reappearance of autochthonous malaria cases in Europe [9], the species composition and spatial distribution of the members of *An. maculipennis* s.l. in the Netherlands has been poorly studied. Individuals of *An. maculipennis* s.l. were found at 144 sampling sites during a nationwide inventory of indigenous mosquitoes, involving natural, rural and urban habitats [10]. However, this nationwide inventory did not include DNA-based species identifications to distinguish the members of the complex. Yet, the nuclear ribosomal internal transcribed spacer 2 (ITS2) flanked by portions of the conserved 5.8S and 28S rDNA is useful in this respect [11–13]. Since the members of the *An. maculipennis* complex are difficult to discriminate by morphological characteristics, their identification needs to be verified by ITS2 sequencing.

Anopheles daciae Linton, Nicolescu & Harbach, 2004, is a recently described species of the *An. maculipennis* complex that is distributed throughout continental Europe [14] and has been found over the past years in the countries bordering the Netherlands, including Germany [15,16], the United Kingdom [11], and Belgium [17]. Nevertheless, *An. daciae* has not yet been reported in the Netherlands. Therefore, the aim of this study was 1) to investigate the distribution of *An. maculipennis* s.l. members present in the Netherlands by applying ITS2 sequencing and 2) to find evidence of the presence of *An. daciae* in the Netherlands, where it is expected to occur, given its presence in neighboring countries.

2. Materials and Methods

2.1. Sampling

Mosquito specimens for the study were collected from different surveys. Most of the specimens (n = 531) were collected between the months of May and September using Mosquito Magnet Liberty Plus traps (WoodstreamTM Co., Lititz, PA, USA) using octenol, in the framework of the National Mosquito Survey [10]. This included 145 specimens collected in 2011, 74 in 2012, 146 in 2013, 72 in 2014, 88 in 2015, 3 in 2016, 12 in 2017 and one in 2021. Additional specimens were collected during Exotic Mosquito Surveys [18] using a variety of sampling methods such as BG-Sentinel traps (n = 4) or BG-Mosquitaire traps (n = 5) (Biogents AG, Regensburg, Germany) both using BG-Sweetscent, and larval sampling using aquarium nets (n = 1). In total, 541 specimens (540 adults and 1 larva) of *An. maculipennis* s.l. were collected at 161 locations covering the entire territory of the Netherlands.

In addition to these 541 specimens, a total of 89 *An. maculipennis* s.l. specimens were collected in February (n = 65) and March (n = 24) 2021 from six bunkers of the New Dutch Waterline, located in the municipality of West-Betuwe, the Netherlands. These bunkers are well-known overwintering sites for several mosquito species [19].

All specimens were transported to the laboratory and were morphologically identified to the *Anopheles maculipennis* complex level using the key of Becker et al. [20]. After identification, specimens were placed in sterile vials and kept frozen at –20 °C until further processing.

2.2. DNA-Based Species Identification

Individual DNA was extracted from a leg or a part of abdomen using the NucleoSpin® Tissue DNA extraction kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocols, with an elution volume of 70 µL. For the mosquitoes collected from overwintering sites in February and March 2021, DNA was extracted following the ammoniumhydroxide-protocol as described by [21]. The ITS2 fragment was amplified using the primers of [16], with thermal cycling conditions, PCR reactions and purification for sequencing following [17], except for the mosquitoes collected in overwintering sites in February and March 2021. For these latter, ITS2 fragment were amplified using MyTaq® HS Red mix (Bioline, UK) using the primers as described in [16] and the following thermal cycling conditions: 1 min at 95 °C, followed by 35 cycles of 95 °C for 15 s, 53 °C for 15 s, and 72 °C for 10 s. Forward and reverse strands were assembled and corrected with Geneious® Prime v.2019.2.3 (Biomatters Ltd., Auckland, New Zealand), after which consensus sequences were generated and trimmed to remove the primers and low-quality ends.

ITS2 consensus sequences were used as queries to search for most similar sequences in GenBank (NCBI, National Centre for Biotechnology), using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 15 June 2022). To discriminate between *An. daciae* and *An. messeae*, aligned consensus sequences were visually checked for the presence of the five species-specific diagnostic sites [12]. Species-assigned consensus sequences were then aligned together with sequences of all other *Anopheles* species occurring in the Netherlands, namely *An. algeriensis* Theobald, 1903, *An. claviger* (Meigen, 1804), *An. plumbeus* Stephens, 1828 [22], and with outgroup sequences (namely *An. funestus* sensu stricto Giles, 1900, and *An. minimus* Theobald, 1901), using ClustalW in Geneious® Prime v.2019.2.3. We also included ITS2 sequences of *An. melanoon* Hackett, 1934, which is part of *An. maculipennis* s.l. and was reported to occur in the Netherlands in the past [22] but was not identified in previous reports [3]. Conspecific identical sequences were removed from the database to retain unique ITS2 sequences in the final alignment. Using the web application FindModel (http://hiv.lanl.gov/content/sequence/findmodel/findmodel.html, accessed on 15 June 2022), the Kimura 2-parameter was identified as the best evolution model describing our data [23,24]. A rooted maximum likelihood tree (ML) was constructed using MEGA X v.10.0.5 (Kumar et al., 2018), with branch support assessed by 1000 bootstrap replicates. Average interspecific K2P distances were calculated with the R v.3.6.1 package Spider v3.6.2 [25,26].

2.3. Habitat Characterization

To characterize the habitat of *An. maculipennis* s.l. in the Netherlands, a 1 km buffer area was created around each finding location (excluding overwintering locations). Using this pre-defined buffer zone in ArcGIS v.10.7.1 [27] and the 2018 version of the raster file of the Corine Land Cover [28], the areas covered by the five main Land Cover Classes were extracted: artificial or urban areas, agricultural areas, forest and seminatural areas, wetlands, and water bodies. The expected number of specimens per Land Cover Class was calculated using the following formula: (total number of specimens per species × percentage Land Cover in the Netherlands)/100. Habitat association per species was verified by comparing observed number of specimens per species per Land Cover Class with expected number of specimens per species per Land Cover Class using a Fisher's exact test. All statistical analyses were conducted in RStudio v1.4.1717 [25].

3. Results

The ITS2 fragment was scored in the 541 specimens collected between May and September. Of these, 496 specimens were assigned as *An. messeae*, 25 as *An. maculipennis* s.s., 11 as *An. atroparvus*, and nine as *An. daciae*. Of the 89 mosquitoes collected from the six bunkers, 82 specimens were identified as *An. messeae* (92.13%), and seven were identified as *An. daciae* (8.99%).

No ITS2 sequences were shared between the four species of *An. maculipennis* s.l. collected in the Netherlands, with each of the four species involving one unique species-specific haplotype (Figure 1). Average interspecific K2P distances ranged from 0.687 to 8.258% (Table 1). Double peaks at two of the five supposedly diagnostic sites discriminating *An. messeae* from *An. daciae* were observed in three ITS2 sequences of *An. daciae*, namely position 218 (A/T) (n = 2) and 220 (C/T) (n = 1) (site numbering following [12]). Such ambiguities were not recorded in the 578 *An. messeae* ITS2 sequences (214 (T), 218 (T), 220 (C), 416 (G), and 436 (G)).

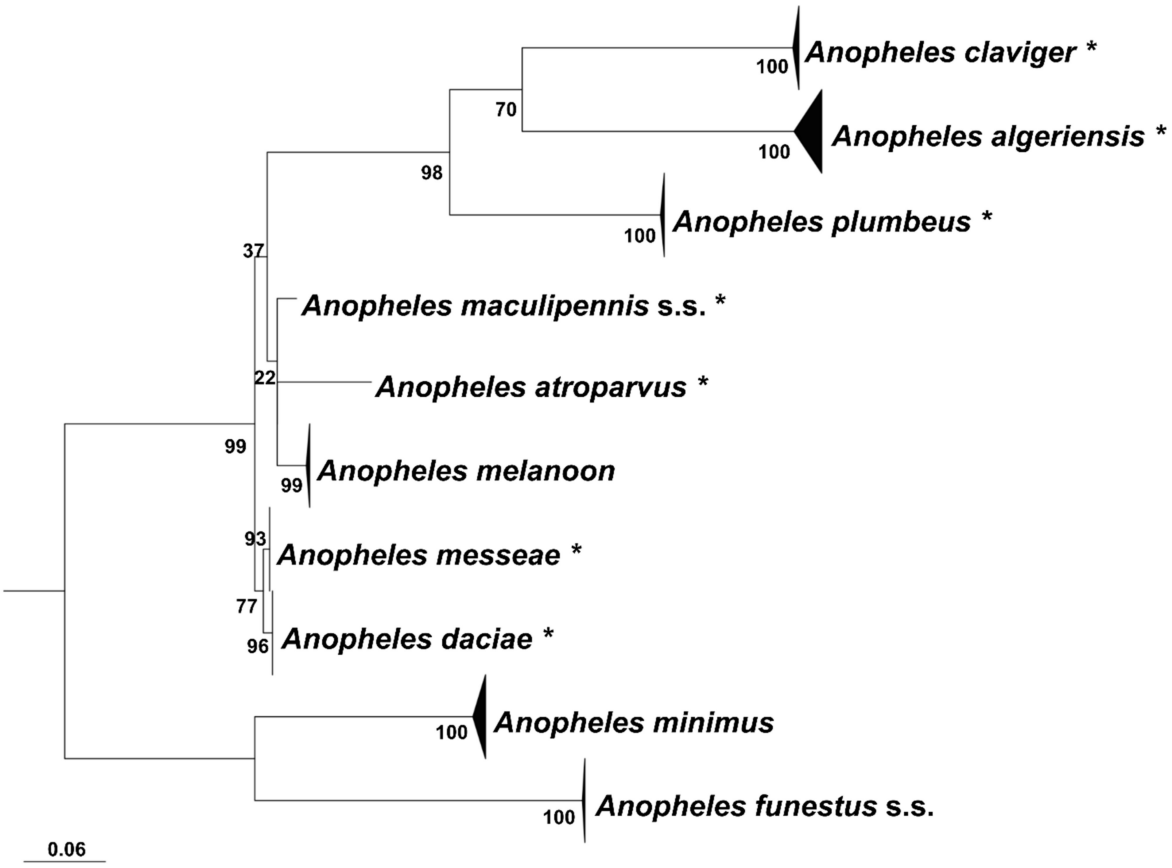

Figure 1. Condensed ITS2 ML-tree (K2P model) of five members of *Anopheles maculipennis* s.l. (*An. maculipennis* s.s.; *An. messeae*; *An. atroparvus*; *An. daciae*; *An. melanoon*), four of which were collected in the Netherlands in the present study, including *An. plumbeus*, *An. claviger* and *An. algeriensis* occurring in the Netherlands, and *An. funestus* sensu stricto and *An. minimus* as outgroups (GenBank accession numbers: KP298399, KP298400, OK570292, OK570315). Duplicate sequences per species were excluded, with the ITS2 databases for *An. messeae* and *An. daciae* including a few sequences displaying ambiguous sites. Numbers at nodes are bootstrap support values. * species reported in the Netherlands.

Excluding the sampled overwintering sites (i.e., the six bunkers), the most frequent species captured was *An. messeae*, being present in 88.19% of the locations, followed by *An. maculipennis* s.s. (11.80%), *An. atroparvus* (3.72%) and *An. daciae* (3.72%) (Figure 2, Table S1). *Anopheles messeae* was found in a total of 142 locations, being found in sympatry with *An. maculipennis* s.s. at four locations and with *An. atroparvus* at five locations. *Anopheles daciae* was found in sympatry with *An. maculipennis* s.s. at three out of the six locations where it was identified (Figure 2). Using ITS2, the present investigation provides the first solid evidence of the occurrence of *An. daciae* in the Netherlands. The 16 identified specimens were captured only in 2011, 2012, 2013 and 2021, in the southern part of the country (Figure 2). At overwintering collection sites, the seven *An. daciae* were collected in

February from five out of six bunkers; no *An. daciae* were identified in March. *Anopheles maculipennis* s.s. and *An. atroparvus* were not found at the overwintering sites.

Table 1. Descriptive statistics of the genetic diversity of ITS2 within *Anopheles maculipennis* s.l. in the Netherlands, including the average interspecific K2P distances among sequences (excluding conspecific identical sequences).

	n	N_H	N_P	Average Interspecific K2P (%) ± Range (%)
An. atroparvus	11	1	0	8.258 ± 0.000
An. daciae	9 + **7**	1	0 *	0.881 ± 0.327
An. maculipennis s.s.	25	1	0	3.114 ± 0.000
An. messeae	496 + **82**	1	0	0.687 ± 0.003

n: sample size. N_H: number of haplotypes. N_P: number of polymorphic nucleotide sites. * ambiguities recorded at two of the five species-diagnostic sites. Numbers in bold are specimens collected at overwintering sites (2020/2021).

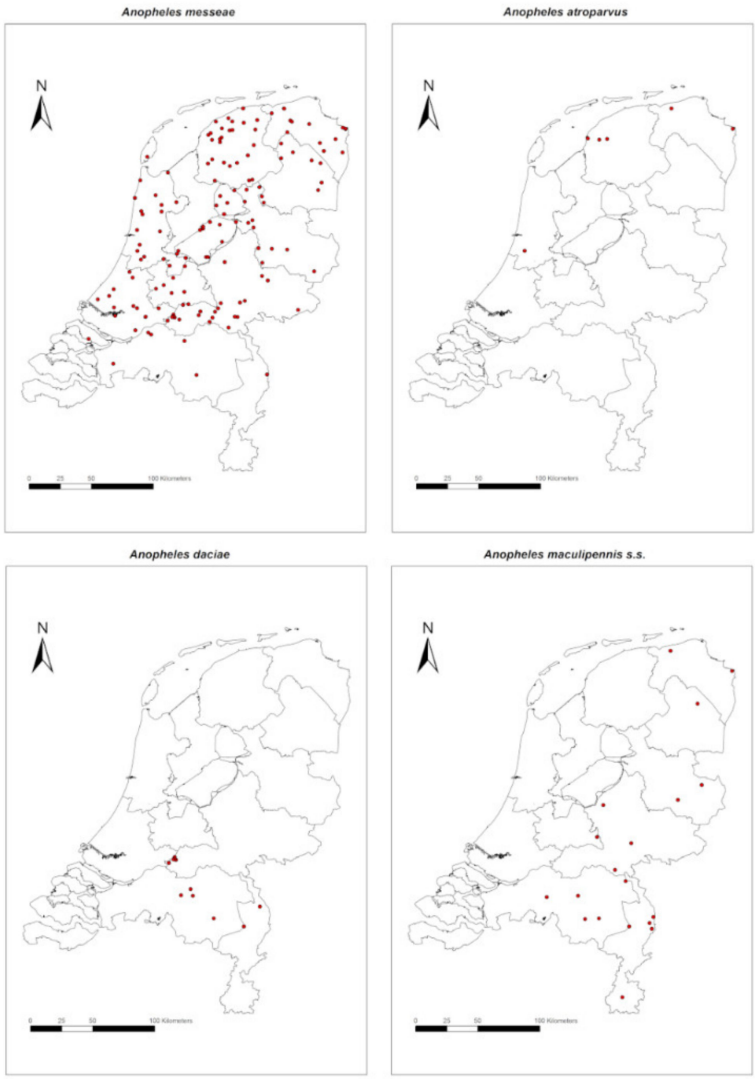

Figure 2. Distribution of *Anopheles maculipennis* s.l. in the Netherlands (details in Table S1) identified using DNA-based techniques: *Anopheles messeae*, *Anopheles atroparvus*, *Anopheles daciae* and *Anopheles maculipennis* sensu stricto.

Except for *An. maculipennis* s.s., preferred Land Cover Class for *An. messeae*, *An. daciae* and *An. atroparvus* are areas with predominant agricultural use (Figure 3). *Anopheles maculipennis* s.s. was found in Land Cover Classes artificial habitats (e.g., industrial or

residential areas) and agricultural areas in similar proportions. *Anopheles atroparvus* was not found in Land Cover Classes forests or seminatural areas. *Anopheles atroparvus* was collected in the north of the country, at six locations near the coast, where it is more probable to encounter mixing seawater and fresh water. A significant difference between expected and observed distributions per Land Cover Class was observed for *An. messeae* ($p < 0.001$), indicating a significantly higher occurrence at artificial Land Cover Classes than expected. For *An. maculipennis* s.s. ($p = 0.164$), *An. daciae* ($p = 1$) and *An. atroparvus* ($p = 1$), no significant differences between expected and observed distributions were found (Figure 3).

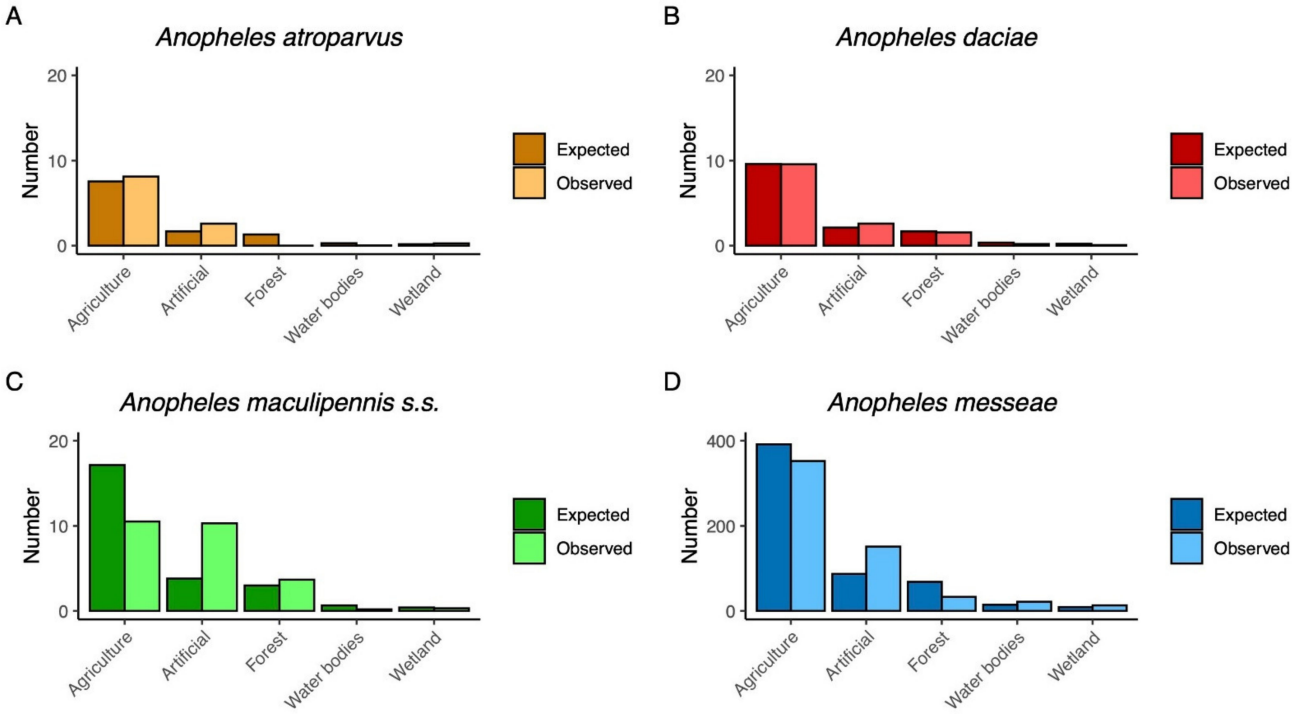

Figure 3. Expected distribution versus observed distribution per Land Cover Class of (**A**). *An. atroparvus* ($n = 11$), (**B**). *An. daciae* ($n = 16$), (**C**). *An. maculipennis* s.s. ($n = 25$) and (**D**). *An. messeae* ($n = 478$). Significant differences between observed and expected distributions were observed for *An. messeae* only (Fisher's exact test, $p < 0.001$).

4. Discussion and Conclusions

In the Netherlands, four species of *Anopheles maculipennis* s.l. were identified: *An. messeae*, *An. maculipennis* s.s., *An. atroparvus* and *An. daciae*. Our study presents the first report of *An. daciae* in the Netherlands using ITS2 sequencing.

Each identified species of the complex involved a single ITS2 haplotype, with smallest average interspecific K2P distances between *An. daciae* and *An. messeae* (0.7%). However, ambiguous sites at some of the species-diagnostic positions were observed [17,29–31]. Double peaks in chromatograms can result from slight differences among ITS2 copies (heterozygosity) and their regular occurrence in specimens from different countries, surveys and years, suggests that only two sites are diagnostic between *An. daciae* and *An. messeae*, namely positions 416 (A/G) and 436 (C/G). For the remainder, phylogenetic DNA sequence analyses of ND4, ND5, COI and Hunchback gene fragments provide no support for the distinction of *An. daciae* and *An. messeae* [17,31], while other taxonomically diagnostic features between these nominal species are still poorly investigated (e.g., hybrid incompatibility, morphology, ecology, cytotaxonomy, etc.). Therefore, it has been proposed to regard *An. daciae* as a species inquirenda (i.e., a species of doubtful identity [32]) [17,33].

Our study shows that *An. messeae* is the species with the widest distribution in the Netherlands compared to any other species of *An. maculipennis* s.l. Similarly to a study

in Germany [14], *An. messeae* was the most frequent species in the analyzed samples. In the Netherlands, the species was most commonly found in agricultural areas. Important and ecologically relevant features of the Dutch agricultural landscape are the drainage ditches, which are an important refuge for biodiversity in agricultural landscapes [34] and represent a preferred breeding site for *Anopheles* mosquitoes. However, when comparing the distribution of *An. messeae* with the overall distribution of Land Cover Classes in the Netherlands, the species seems to be associated with artificial Land Cover Classes, more than we would expect based on the land cover distribution of the entire country. Interestingly, the prevalence of *An. messeae* observed in the present study is comparable to that reported by [4], which focused on overwintering mosquitoes. In the latter study, *An. maculipennis* s.s. was not detected, and the presence of *An. daciae* was not investigated by ITS2 sequence data. *Anopheles atroparvus*, a common species breeding in brackish waters, was found at three sites in the coastal areas of the Netherlands during the study of [4].

Anopheles maculipennis s.s. is also widely distributed in the Netherlands, covering large areas from North to South, but it was not found near the coastal areas. Our data show that this species occurs in similar proportions in both urban and agricultural Land Cover Classes. In our study, no preference of *An. maculipennis* s.s. towards a specific class was identified. However, similar to *An. atroparvus* and *An. daciae*, the collected number of *An. maculipennis* s.s. specimens was too low for adequate analyses. As such, further research is needed to identify habitat preferences. In Belgium, *An. maculipennis* s.s. appears to be the most frequent and widespread species of the complex [17]. However, this observation was based on a survey of artificial breeding sites. Therefore, the higher prevalence of *An. maculipennis* s.s. is not surprising, since this species seems better adapted to artificial habitats compared to other species of *An. maculipennis* s.l. [35–37]. In the present study, most of the specimens were collected using adult traps at randomly generated locations across the country, including urban, rural and natural Land Cover Classes, thus preventing a sampling bias [10]. The occurrence of *An. maculipennis* s.s. in urban areas in our study (>40% of the sampling sites) seems to corroborate its preference for man-made habitats.

Except for *An. atroparvus* [6], the potential role of *An. messeae, An. maculipennis* s.s. and *An. daciae* in the historical transmission of malaria in the Netherlands is unknown. For *An. daciae*, the present highlighted species distribution does not fit with the historical areas where the malaria parasite occurred [6]. The main vector of malaria was *An. atroparvus* [6], a species dependent on brackish water. Nowadays, in comparison with the other members of the species complex, the species distribution of *An. atroparvus* indicates the presence of the species in scarce locations nearby coastal areas.

This study also shows that *An. messeae* and *An. daciae* live in sympatry during winter. It remains unclear, however, how the species within the *An. maculipennis* complex differ in overwintering strategies. Earlier studies have shown that *An. atroparvus* enters diapause in early winter, but occasionally continues its blood-feeding behaviour to maintain fat reserves, in contrast to *An. messeae*, which remains inactive throughout winter [38,39]. To our knowledge, this is the first study to find *An. daciae* overwintering in artificial shelters together with other mosquito species that are in diapause [19]. Furthermore, *An. daciae* was only found in the southern inland areas and occurring in areas with Land Cover Classes associated with agricultural activities.

This study provides accurate and unbiased information on the occurrence of the species of the *Anopheles maculipennis* complex in the Netherlands and shows that *An. messeae* is the most frequent species in the country during summers and can occur in sympatry with *An. daciae* in winters. Unbiased occurrence data are needed to develop mosquito species distribution models that can contribute to a better estimation of the risk of mosquito-borne diseases in the country. In addition, while the ITS2 gene fragment is an adequate tool for the identification of the species of the *Anopheles maculipennis* complex, exploring the species' whole genomes will further help elucidate the phylogenetic relationships between the complex members and support their taxonomic status.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14080636/s1, Table S1: Sampling locations (latitude, longitude) and methods (BGS: BG-Sentinel trap; BGM: BG-Mosquitaire trap; MMLP: Mosquito Magnet Liberty Plus traps), collection year, and DNA-based identification results of specimens collected in the Netherlands.

Author Contributions: Conceptualization, A.I.-J., A.S. and N.S.; methodology, A.I.-J., N.S., A.V., F.J., R.B., K.M. and S.G.; analyses, A.I.-J., N.S., A.V., R.B. and C.J.M.K.; data curation, A.I.-J., N.S., A.V., F.J., R.B., C.J.M.K., K.M. and S.G.; writing—original draft preparation, A.I.-J. and N.S.; writing—review and editing, A.V., F.J., R.B., K.M., S.G., T.B., C.J.M.K., J.S.G., M.D.M. and A.S.; visualization, A.I.-J., N.S., C.J.M.K. and R.B.; supervision, T.B., M.D.M. and A.S.; project administration and funding acquisition, T.B., M.D.M. and A.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/, accessed on 15 June 2022. Accession numbers: *An. atroparvus*: ON033651-ON033660 and ON033662; *An. daciae*: ON053456-ON053464 and ON407975-ON407981; *An. maculipennis* s.s.: ON033742-ON033766; *An. messeae*: ON053466-ON053930, ON053932-ON053962 and ON407982-ON408063.

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

References

1. Verdonschot, P.F.M.; Beuk, P.L.T. Family Culicidae. In *Checklist of the Diptera of the Netherlands*; Beuk, P.L.T., Ed.; KNNV Uitgeverij: Utrecht, The Netherlands, 2019. Available online: https://diptera-info.nl/news.php?fam=Culicidae (accessed on 13 June 2022).
2. Deblauwe, I.; Ibanez-Justicia, A.; De Wolf, K.; Smitz, N.; Schneider, A.; Stroo, A.; Jacobs, F.; Vanslembrouck, A.; Gombeer, S.; Dekoninck, W.; et al. First detections of *Culiseta longiareolata* (Diptera: Culicidae) in Belgium and the Netherlands. *J. Med. Entomol.* **2021**, *58*, 2524–2532. [CrossRef] [PubMed]
3. Takken, W.; Kager, P.A.; Verhave, J.P. Will malaria return to North-West Europe? In *Emerging Pests and Vector-Borne Diseases in Europe*; Takken, W., Knols, B.G.J., Eds.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2007; pp. 23–24.
4. Scholte, E.J.; Mars, M.H.; Braks, M.; Den Hartog, W.; Ibañez-Justicia, A.; Koopmans, M.; Koenraadt, C.J.; De Vries, A.; Reusken, C. No evidence for the persistence of Schmallenberg virus in overwintering mosquitoes. *Med. Vet. Entomol.* **2013**, *28*, 110–115. [CrossRef] [PubMed]
5. Collins, W.E.; Jeffery, G.M. Plasmodium ovale: Parasite and disease. *Clin. Microbiol. Rev.* **2005**, *18*, 570–581. [CrossRef] [PubMed]
6. Takken, W.; Geene, R.; Adam, W.; Jetten, T.H.; van der Velden, J.A. Distribution and dynamics of larval populations of *Anopheles messeae* and *A. atroparvus* in the delta of the rivers Rhine and Meuse, The Netherlands. *Ambio* **2002**, *31*, 212–218. [CrossRef]
7. Rodhain, J.; Van Hoof, M.T. Recherches sur l'anophélisme en Belgique. *Ann. De La Société Belg. De Médecine Trop.* **1942**, *21*, 19–43.
8. Van Seventer, H.A. The disappearance of malaria in the Netherlands. *Ned. Tijdschr. Voor Geneeskd.* **1969**, *113*, 2055–2056.
9. ECDC. Multiple Reports of Locally-Acquired Malaria Infections in the EU. Available online: https://www.ecdc.europa.eu/sites/portal/files/documents/RRA-Malaria-EU-revised-September-2017_0.pdf (accessed on 20 May 2022).
10. Ibañez-Justicia, A.; Stroo, A.; Dik, M.; Beeuwkes, J.; Scholte, E.J. National mosquito (Diptera: Culicidae) survey in The Netherlands 2010-2013. *J. Med. Entomol.* **2015**, *52*, 185–198. [CrossRef]
11. Danabalan, R.; Monaghan, M.T.; Ponsonby, D.J.; Linton, Y.M. Occurrence and host preferences of *Anopheles maculipennis* group mosquitoes in England and Wales. *Med. Vet. Entomol.* **2014**, *28*, 169–178. [CrossRef]
12. Nicolescu, G.; Linton, Y.M.; Vladimirescu, A.; Howard, T.M.; Harbach, R.E. Mosquitoes of the *Anopheles maculipennis* group (Diptera: Culicidae) in Romania, with the discovery and formal recognition of a new species based on molecular and morphological evidence. *Bull. Entomol. Res.* **2004**, *94*, 525–535. [CrossRef]
13. Proft, J.; Maier, W.A.; Kampen, H. Identification of six sibling species of the *Anopheles maculipennis* complex (Diptera: Culicidae) by a polymerase chain reaction assay. *Parasitol. Res.* **1999**, *85*, 837–843. [CrossRef]

14. Bertola, M.; Mazzucato, M.; Pombi, M.; Montarsi, F. Updated occurrence and bionomics of potential malaria vectors in Europe: A systematic review (2000-2021). *Parasit Vectors* **2022**, *15*, 88. [CrossRef] [PubMed]
15. Luhken, R.; Czajka, C.; Steinke, S.; Jost, H.; Schmidt-Chanasit, J.; Pfitzner, W.; Becker, N.; Kiel, E.; Kruger, A.; Tannich, E. Distribution of individual members of the mosquito *Anopheles maculipennis* complex in Germany identified by newly developed real-time PCR assays. *Med. Vet. Entomol.* **2016**, *30*, 144–154. [CrossRef]
16. Weitzel, T.; Gauch, C.; Becker, N. Identification of *Anopheles daciae* in Germany through ITS2 sequencing. *Parasitol. Res.* **2012**, *111*, 2431–2438. [CrossRef] [PubMed]
17. Smitz, N.; De Wolf, K.; Gheysen, A.; Deblauwe, I.; Vanslembrouck, A.; Meganck, K.; De Witte, J.; Schneider, A.; Verle, I.; Dekoninck, W.; et al. DNA identification of species of the *Anopheles maculipennis* complex and first record of *An. daciae* in Belgium. *Med. Vet. Entomol.* **2021**, *35*, 442–450. [CrossRef]
18. Ibañez-Justicia, A.; Koenraadt, C.J.M.; Stroo, A.; van Lammeren, R.; Takken, W. Risk-based and adaptive invasive mosquito surveillance at Lucky bamboo and used tire importers in the Netherlands. *J. Am. Mosq. Control. Assoc.* **2020**, *36*, 89–98. [CrossRef] [PubMed]
19. Koenraadt, C.J.M.; Mohlmann, T.W.R.; Verhulst, N.O.; Spitzen, J.; Vogels, C.B.F. Effect of overwintering on survival and vector competence of the West Nile virus vector *Culex pipiens*. *Parasit Vectors* **2019**, *12*, 147. [CrossRef] [PubMed]
20. Becker, N.; Petric, D.; Zgomba, M.; Boase, C.; Madon, M.; Dahl, C.; Kaiser, A. *Mosquitoes and Their Control*; Kluwer Academic/Plenum Publishers: Heidelberg, Germany; Dordrecht, The Nertherlands; London, UK; New York, NY, USA, 2010.
21. Vogels, C.B.; van de Peppel, L.J.; van Vliet, A.J.; Westenberg, M.; Ibanez-Justicia, A.; Stroo, A.; Buijs, J.A.; Visser, T.M.; Koenraadt, C.J. Winter Activity and Aboveground Hybridization Between the Two Biotypes of the West Nile Virus Vector Culex pipiens. *Vector Borne Zoonotic Dis.* **2015**, *15*, 619–626. [CrossRef]
22. Robert, V.; Günay, F.; Le Goff, G.; Boussès, P.; Sulesco, T.; Khalin, A.; Medlock, J.M.; Kampen, H.; Petrić, D.; Schaffner, F. Distribution chart for Euro-Mediterranean mosquitoes (western Palaearctic region). *J. Eur. Mosq. Control Assoc.* **2019**, *37*, 1–28.
23. Posada, D.; Crandall, K.A. MODELTEST: Testing the model of DNA substitution. *Bioinformatics* **1998**, *14*, 817–818. [CrossRef]
24. Tao, N.; Richardson, R.; Bruno, W.; Kuiken, C. FindModel. 2016. Available online: http://hiv.lanl.gov/content/sequence/findmodel/findmodel.html (accessed on 18 February 2021).
25. RStudioTeam. RStudio: Integrated Development for R. RStudio. 2020 Boston, MA: PBC. Available online: https://rstudio.com/ (accessed on 26 February 2021).
26. Brown, S.D.; Collins, R.A.; Boyer, S.; Lefort, M.C.; Malumbres-Olarte, J.; Vink, C.J.; Cruickshank, R.H. Spider: An R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. *Mol. Ecol. Resour.* **2012**, *12*, 562–565. [CrossRef]
27. ESRI. *ArcGIS Desktop: Release 10*; Environmental Systems Research Institute: Redlands, CA, USA, 2011.
28. Copernicus. CORINE Land Cover. Available online: https://land.copernicus.eu/pan-european/corine-land-cover (accessed on 17 April 2022).
29. Bezzhonova, O.V.; Goryacheva, I.I. Intragenomic heterogeneity of rDNA internal transcribed spacer 2 in *Anopheles messeae* (Diptera: Culicidae). *J. Med. Entomol.* **2008**, *45*, 337–341. [CrossRef] [PubMed]
30. Culverwell, C.L.; Vapalahti, O.P.; Harbach, R.E. *Anopheles daciae*, a new country record for Finland. *Med. Vet. Entomol.* **2020**, *34*, 145–150. [CrossRef] [PubMed]
31. Lilja, T.; Eklof, D.; Jaenson, T.G.T.; Lindstrom, A.; Terenius, O. Single nucleotide polymorphism analysis of the ITS2 region of two sympatric malaria mosquito species in Sweden: *Anopheles daciae* and *Anopheles messeae*. *Med. Vet. Entomol.* **2020**, *34*, 364–368. [CrossRef] [PubMed]
32. ICZN. *International Code of Zoological Nomenclature*, 4th ed.; International Trust for Zoological Nomenclature: London, UK, 1999; 306p.
33. Calzolari, M.; Desiato, R.; Albieri, A.; Bellavia, V.; Bertola, M.; Bonilauri, P.; Callegari, E.; Canziani, S.; Lelli, D.; Mosca, A.; et al. Mosquitoes of the Maculipennis complex in Northern Italy. *Sci. Rep.* **2021**, *11*, 6421. [CrossRef] [PubMed]
34. Mukete, B.; Vermaat, J.E.; van Straalen, N.M. Variability in microhabitats prevalence in low lying peat polder ditches of the Netherlands. *Appl. Ecol. Environ. Res.* **2013**, *11*, 629–643. [CrossRef]
35. Becker, N.; Petrić, D.; Zgomba, M.; Boase, C.; Madon, M.B.; Dahl, C.; Kaiser, A. *Mosquitoes: Identification, Ecology and Control*; Springer Nature Switzerland AG: Cham, Switzerland, 2020; 570p.
36. Dakić, Z.; Zoran, K.; Novica, S.; Mijomir, P.; Miloje, C.; Stanimirovic, Z.; Nikola, I.; Poluga, J.; Pavlovic, M. Ecology of *Anopheles* mosquitoes in Belgrade area. Estimating vector potential for malaria retransmission. *Acta Veterinaria* **2008**, *58*, 603–614.
37. Weyer, F. Die geographische Verbreitung der Rassen von *Anopheles maculipennis* in Deutschland. *Z. Für Parasitenkd.* **1938**, *10*, 437–463. [CrossRef]
38. Ramsdale, C.D.; Wilkes, T.J. Some aspects of overwintering in southern England of the mosquitoes *Anopheles atroparvus* and *Culiseta annulata* (Diptera: Culicidae). *Ecol. Entomol.* **1985**, *10*, 449–454. [CrossRef]
39. Weyer, F. Versuche zur Übertragung der affen-malaria durch stechmucken. *Arch. Schiff. f. Tropenhyg.* **1937**, *41*, 167–172.