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## The *Anopheles (Anopheles) maculipennis* complex (Diptera: Culicidae) in Greece

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### Abstract

Mosquitoes belonging to the Maculipennis Complex of *Anopheles* subgenus *Anopheles* were collected in 10 prefectures across Greece: Attiki, Evros, Florina, Fthiotida, Ioannina, Lakonia, Magnesia, Rodopi, Serres, and Xanthi. DNA was extracted from 276 specimens and sequences for the nuclear rDNA ITS2 region were obtained from 257 of these (93.1%). Four members of the *An. maculipennis* Meigen complex were identified: *An. maculipennis*, *An. sacharovi* Favre, *An. melanoon* Hackett, and *An. messeae* Falleroni. Species were identified based on correlation of their sequences with those available in GenBank. All four species have been incriminated as primary or secondary vectors of malaria. Species distributions in relation to potential threats of reintroduction of malaria in Greece are discussed. This study comprises the most complete study of the Maculipennis Complex in Greece to date, and contributes substantially to the current knowledge of the genetics and distributions of the four species.

**Keywords:** *Anopheles maculipennis*, *An. melanoon*, *An. messeae*, *An. sacharovi*, *distribution*, *Greece*, *ITS2*, *Maculipennis Complex*

### Introduction

Malaria was prevalent across Europe as late as the mid-20th century. Being abundant and widespread, *Anopheles maculipennis* Meigen was circumstantially incriminated as the primary vector. Following studies into the phenomenon of “anophelism without malaria”, this taxon was the first mosquito sibling species complex to be recognized when two biologically/reproductively distinct species were distinguished by means of egg and wing morphology, correlated with behavioural and ecological differences (Falleroni 1926; van Thiel 1927). Following these early works, extensive efforts were made to elucidate all

Palearctic members of the *Maculipennis* Group, which comprise what is now known as the *Maculipennis* Complex (*sensu* White 1976; Guy et al. 1976; White 1978; Harbach 1994).

Understanding of the species composition of the *Maculipennis* Complex stems from White's (1978) assessment in which he recognized nine Palearctic species: *An. atroparvus* van Thiel, *An. beklemishevi* Stegnii and Kabanova, *An. labranchiae* Falleroni, *An. maculipennis*, *An. martinius* Shingarev, *An. melanoon* Hackett (with its variety *subalpinus* Hackett and Lewis), *An. messeae* Falleroni, *An. sacharovi* Favre, and *An. sicaulti* Roubaud. White also proposed the suppression of *alexandraeschingarevi* Schingarev, *lewisi* Ludlow, and *selengensis* Ludlow, synonymized *An. subalpinus* with *An. melanoon*, and resurrected two nominal species, *An. martinius* and *An. sicaulti*, on the basis of evidence available at the time. Later, field and laboratory investigations using integrated morphological, electrophoretic, cross-mating, and chromosomal methods revealed that *An. sicaulti* was conspecific with *An. labranchiae*, and the former name was synonymized with the latter (de Zulueta et al. 1983). Data from isoenzyme studies by Cianchi et al. (1987) were used by Ribeiro et al. (1988) to re-instate *An. subalpinus* to species status. Using DNA evidence, *An. subalpinus* was formally synonymized with *An. melanoon* based on samples collected in this study by Linton et al. (2002b). Using integrated morphological and molecular data, three new taxa have recently been discovered: *An. persiensis* Linton, Sedaghat, and Harbach from northern Iran (Sedaghat et al. 2003b), *An. daciae* Linton, Nicolescu, and Harbach (Nicolescu et al. 2004), and *An. artemievi* Gordeev, Zvantsov, Goriacheva, Shaikevich, and Ezhov (Gordeev et al. 2004). These works contributed to the current recognition of 11 Palearctic members of the *Maculipennis* Complex: *An. artemievi*, *An. atroparvus*, *An. beklemishevi*, *An. daciae*, *An. labranchiae*, *An. maculipennis*, *An. martinius*, *An. melanoon*, *An. messeae*, *An. persiensis*, and *An. sacharovi*.

Early reports of mosquitoes in Greece recorded the presence of *An. maculipennis* (Nicolot 1917; Joyeux 1918, 1920; Waterston 1918; Russel 1927; Cardamatis 1931; Papadakis 1934; Barber 1935; Hackett and Lewis 1935; Pandazis 1935; Shannon 1935; Rice and Barber 1937; Stephanides 1937; Livadas and Sphangos 1940; Shannon and Hadjinicolaou 1941; Eichler 1944; Peus 1954) and *An. messeae* (Hackett and Missiroli 1935; Pandazis 1935; Shannon 1935). Following a morphological study of eggs from Kavala (Macedonia), Hackett and Lewis (1935) confirmed the presence of *An. messeae*, *An. maculipennis* (as *An. typicus* Hackett and Missiroli), and *An. subalpinus*. In 1936, Bates found *An. messeae* eggs on the Albanian shore of Lake Prespa, and suggested this distribution would also include the Greek shore, which lies in Florina Prefecture (in Livadas and Sphangos 1940). The identity of *An. messeae* in early reports is unclear because prior to the description of the egg of *An. subalpinus* (Hackett and Lewis 1935), eggs of this species were thought to belong to a variety of *An. messeae* (Livadas and Sphangos 1940). Despite earlier suggestions to the contrary, Bates (1940) could not confirm the presence of *An. messeae* in Greece, and thus, Weyer (1942) declared all reports of *An. messeae* in Thrace and Macedonia prior to the recognition of *An. subalpinus* to be unreliable.

Based on the older literature reports, Samanidou-Voyadjoglou and Darsie (1993) and Ramsdale and Snow (2000) suggested that *An. messeae* might still be present in Greece. The presence of this species in Florina Prefecture, NW Greece was established beyond doubt by Linton et al. (2001b, 2002a), who reported sympatric populations of *An. messeae* and *An. maculipennis* based on DNA sequence identification. *Anopheles maculipennis* (as *An. typicus*), *An. melanoon* (as *An. subalpinus*), and *An. sacharovi* (as *An. elutus* Favre) have been reported from Ioannina Prefecture, NW Greece (Livadas and Sphangos 1940) and

Macedonia (Shannon and Hadjinicolaou 1941). *Anopheles maculipennis* and *An. sacharovi* were reported from the island of Corfu (Stephanides 1937, 1938) and the Copias district of mainland Greece (Sakellariou and Lane 1977). In the early literature, *An. sacharovi* was widely reported in Greece (Cardamatis 1931; Barber 1935; Shannon 1935; Rice and Barber 1937; Weyer 1942; Eichler 1944; Peus 1954), although populations were decimated and the species believed to be eliminated following the activities of the Malaria Eradication Programme in the mid-1950s. The reintroduction of *An. sacharovi* was reported in the central region of Lamia (Fthiotida Prefecture) (Hadjinicolaou and Betzios 1973) and, more recently, it was found in sympatry with *An. maculipennis* in the wetlands of northeastern Greece (Ouzounis and Samanidou-Voyadjoglou 1993).

Following his synonymy of *An. subalpinus* with *An. melanoon*, White (1978) suggested that *An. maculipennis*, *An. sacharovi*, and *An. melanoon* were the three members of the Maculipennis Complex present in Greece. The proposed distribution of *An. atroparvus* (Dahl and White 1978; White 1978) suggested that this species may also be present in Greece. For these reasons, *An. atroparvus* was also included in the most recent species list (Samanidou-Voyadjoglou and Darsie 1993) and morphological keys (Darsie and Samanidou-Voyadjoglou 1997; Samanidou-Voyadjoglou and Harbach 2001) of Greek mosquitoes.

Three species of the Maculipennis Complex, *An. atroparvus*, *An. sacharovi*, and *An. labranchiae*, are known to be efficient current or historical malaria vectors in Europe (Jaenson et al. 1986; Ribeiro et al. 1988; Kasap 1990; Jetten and Takken 1994; Romi et al. 1997, 2001, 2002; Romi 1999; Alten et al. 2000). Climatic changes, including global warming and associated increase of precipitation, are expected to extend vector ranges and population sizes of some species, potentially increasing malaria transmission rates (Snow 2000). Indicative of possible increased involvement in malaria transmission, *An. maculipennis* and *An. melanoon* (as *An. subalpinus*) were recently incriminated as secondary vectors in the Biga Plains of Turkey (Alten et al. 2000), and most recently *An. sacharovi* has re-emerged as a vector of malaria in Armenia (Romi et al. 2002). *Anopheles messeae*, a reportedly efficient vector in western Asia but not previously in Europe (Bruce-Chwatt and de Zulueta 1980), has been identified as the principal vector of resurgent malaria in the Ukraine and Russia (Nikolaeva 1996).

Despite the eradication of malaria in Europe following extensive vector control programmes after the Second World War, increasing numbers of malaria cases have been reported in southern European countries over the last decade (Sartori et al. 1989; Nikolaeva 1996; Baldari et al. 1998; Linton et al. 2001b, 2002a; Romi et al. 2001). These cases, along with *Plasmodium* carriers in the form of exotic travellers (Steffen and Brethens 1992) and increasing immigrant labour from endemic areas, have heightened concern for the reintroduction of malaria into southern European countries. This is especially true in Greece and Italy, where malaria was once highly endemic and competent mosquito vectors still exist (Jetten et al. 1996; Romi et al. 1997, 2001; Lindsay and Birley 1996; Romi 1999; Linton et al. 2001b).

Correct vector identification is essential to assess the potential risk of malaria in these border regions, and to devise appropriate control or monitoring strategies. In view of the heightened risk of reintroducing malaria, unreliable historical records, and incomplete knowledge about the ecology and disease relations of the mosquitoes, it is important to characterize the species composition and distributions of members of the Maculipennis Complex in Greece. These studies are essential for the development of effective mosquito control programmes and will serve to identify those areas of highest risk for the

reintroduction of malaria. In this study, we investigated the composition and distribution of members of the *Maculipennis* Complex from 10 prefectures in Greece and determined their identity by correlation of their nuclear ITS2 sequences with those available in GenBank (Marinucci et al. 1999; Proft et al. 1999; Djadid, direct GenBank submissions 2001; Linton et al. 2001b, 2002a, 2002b, 2002c, 2003, 2005; Sedaghat et al. 2003a, 2003b; Nicolescu et al. 2004; Kampen 2005a, 2005b).

## Materials and methods

Mosquitoes were collected in 10 prefectures of Greece, namely Evros, Rodopi, Serres, and Xanthi in the northeast, Ioannina and Florina in the northwest, Attiki, Fthiotida, and Magnesia in the heart of the country, and Lakonia in the south, from June 1996 to August 2001 (Table I). Ioannina borders Albania, Florina borders the Former Yugoslav Republic of Macedonia and Albania, and Rodopi, Serres, and Xanthi border Bulgaria. Evros is unique in that it shares borders with both Bulgaria in the northwest and Turkey in the east. Collection data, along with the numbers of mosquitoes collected and sequenced from each collection site are given in Table I. Larval collections were carried out in all prefectures, except Florina and Attiki; resting adults were collected in Attiki, Florina, Salino (Xanthi), and Monastiraki (Evros), and one specimen was captured on human bait (Table I). Specimens were collected from a light trap in Didymoticho (Evros) and Roditsa (Fthiotida) (Table I).

In the case of resting collections, captured females were held for 2 days before being induced to lay eggs, which were then reared as progeny broods to obtain adults with associated larval and pupal exuviae. At least 10 eggs from each brood were stored in Bouin's solution (BDH, Poole, UK) for future study. Link-reared mosquitoes from progeny broods and larval collections serve as voucher specimens for this work, and are retained in the mosquito collections of The Natural History Museum, London (BMNH), the Benaki Museum, Athens, and The National School of Public Health, Athens. For the purposes of this study, and to reflect the relative species composition in each area, the DNA sequence of only one individual per progeny brood was used in the data analysis.

DNA was extracted from 276 mosquitoes following the phenol–chloroform extraction detailed by Linton et al. (2001a). Amplification of the ITS2 nuclear ribosomal spacer was carried out using the 5.8SF and 28SR primers listed by Collins and Paskewitz (1996), using the PCR amplification conditions outlined by Linton et al. (2001a). Following the manufacturer's instructions, products were cleaned using the QIAgen PCR purification kit (QIAgen, Sussex, UK). Purified products were diluted to 1 ng  $\mu\text{l}^{-1}$  per 200 bp of product prior to undergoing the cycle sequencing reaction, using the Big Dye Terminator Kit (PE Applied Biosystems, Warrington, UK). Sequences were obtained in both directions. An ABI 377 automated sequencer (PE Applied Biosystems) was used to read the sequences, and the data were edited and aligned using Sequencher<sup>TM</sup> version 3.1.1 (Genes Codes Corporation, Ann Arbor, MI, USA) and CLUSTAL X (Thompson et al. 1997). The FASTA search engine (<http://www.ebi.ac.uk/fasta33/>) was used to assess the similarity of sequences with those in GenBank. Sequence analyses were conducted using MEGA version 2.1 (Kumar et al. 2001). Following sequencing, the template DNA was dried and retained at  $-70^{\circ}\text{C}$  in the mosquito DNA bank of the Molecular Systematics Laboratory, Department of Entomology, Natural History Museum for future reference.

Sequences for ITS2 were used for species identification based on comparisons with ITS2 sequences available in GenBank. Restriction enzyme cutting sites were located in the ITS2

Table I. Collection sites in Greece, listing co-ordinates, numbers of *Anopheles* specimens sequenced and their identification based on ITS2 sequence data.

Prefecture	Exact locality	Co-ordinates	Date	No. extracted (sequenced)	<i>An. maculipennis</i> (149)	<i>An. melanoon</i> (23)	<i>An. messeae</i> (2)	<i>An. sacharovi</i> (83)
Attiki (central)	Schinias (near airport)	38°08'N, 24°01'E	3 June 1996 <sup>a</sup>	5 (1)	–	–	–	1
Evros (NE)	Didymoticho	41°24'N, 26°33'E	17 July 1999 <sup>c</sup>	2 (1)	1	–	–	–
	Monastiraki, Alexandroupolis	40°51'N, 25°53'E	9 June 2001 <sup>a</sup>	24 (23)	1	16	–	6
	Pythio (1 km W Pythio)	41°31'N, 26°39'E	6 October 1998 <sup>a</sup>	2 (2)	2	–	–	–
	River Tis Mantheas, Itea	40°58'N, 26°05'E	9 June 2001 <sup>b</sup>	19 (19)	18	1	–	–
	River Erithropotomos, Didymoticho	41°21'N, 26°30'E	10 June 2001 <sup>b</sup>	6 (6)	5	1	–	–
	River Erithropotamos, Polia, Metaxades	41°27'N, 26°07'E	10 June 2001 <sup>b</sup>	2 (2)	2	–	–	–
	River Ardas, Marasia, Kastanies	41°40'N, 26°21'E	11 June 2001 <sup>b</sup>	1 (1)	1	–	–	–
	River Soufliou, Dadia	41°05'N, 26°05'E	12 June 2001 <sup>b</sup>	6 (6)	6	–	–	–
Florina (NW)	Anargyroi (48 km SE Florina)	40°36'N, 21°36'E	23 May 1999 <sup>a</sup>	2 (1)	1	–	–	–
	Kato Kaliniki (11 km NE Florina)	40°53'N, 21°28'E	22 May 1999 <sup>a</sup>	3 (2)	2	–	–	–
	Kato Kleine (9 km N Florina)	40°52'N, 21°28'E	22 May 1999 <sup>a</sup>	2 (2)	2	–	–	–
	Lake Petron (37 km E Florina)	40°45'N, 21°41'E	23 May 1999 <sup>a</sup>	1 (1)	–	–	1	–
	Lake Zazari (45 km SE Florina)	40°37'N, 21°34'E	23 May 1999 <sup>a</sup>	2 (2)	1	–	1	–
Fthiotida (central)	Anthili, Lamia	38°54'N, 22°25'E	July 1997 <sup>b</sup>	8 (3)	–	–	–	3
			25 May 2001 <sup>b</sup>	2 (2)	–	–	–	3
	Roditsa	38°53'N, 22°27'E	18 July 2001 <sup>d</sup>	1 (1)	–	–	–	1
			18 July 2001 <sup>c</sup>	1 (1)	–	–	–	1
Ioannina (NW)	Aoos lake, Metsovo	39°49'N, 21°05'E	18 June 2001 <sup>b</sup>	5 (5)	5	–	–	–
	Louros Springs, Hani, Terovou	39°24'N, 20°53'E	30 May 2001 <sup>b</sup>	11 (11)	11	–	–	–
	Nr Lake Zaravina (47 km NE Ioannina)	39°56'N, 20°29'E	31 May 2001 <sup>b</sup>	20 (20)	20	–	–	–
	N Baltouma Bridge (25 km E Ioannina)	39°42'N, 20°57'E	31 May 2001 <sup>b</sup>	4 (3)	3	–	–	–
	Krya (4 km NE Ioannina)	39°44'N, 20°48'E	5 October 2001 <sup>b</sup>	1 (1)	1	–	–	–
	Kouklesi	39°23'N, 20°52'E	27 July 2001 <sup>b</sup>	5 (5)	5	–	–	–
	Ieromnimi Springs	39°48'N, 20°33'E	16 July 2001 <sup>b</sup>	2 (2)	2	–	–	–
	Ieromnimi Springs	39°47'N, 20°33'E	26 June 2001	2 (2)	2	–	–	–
	Lake Metsovo, Agrapidia	39°51'N, 21°08'E	13 July 2001 <sup>b</sup>	1 (1)	1	–	–	–
	Lake (after pump)	39°41'N, 20°53'E	14 June 2001 <sup>b</sup>	1 (1)	1	–	–	–
	Vella's Springs	39°53'N, 20°36'E	14 July 1999 <sup>b</sup>	2 (1)	–	1	–	–
	Paliouri Springs	39°42'N, 20°37'E	26 June 1999 <sup>b</sup>	14 (13)	13	–	–	–
	Kakavia, River Gyftopotamos	39°55'N, 20°21'E	19 June 1999 <sup>b</sup>	6 (6)	6	–	–	–

Table 1. Continued.

Prefecture	Exact locality	Co-ordinates	Date	No. extracted (sequenced)	<i>An. maculipennis</i> (149)	<i>An. melanoon</i> (23)	<i>An. messeae</i> (2)	<i>An. sacharovi</i> (83)
Lakonia (SW)	Ageranos, Lakonia (14 km SW Gythio)	36°44'N, 22°33'E	30 August 2001 <sup>b</sup>	6 (4)	1	–	–	3
Magnisia (central)	Carla Lake Basin, Volos	39°22'N, 22°57'E	26 May 2001 <sup>b</sup>	1 (1)	–	–	–	1
Rodopi (NE)	Nesti-Krovilli, Maronia	40°54'N, 25°31'E	8 June 2001 <sup>b</sup>	10 (10)	9	1	–	–
	Loutros village	40°35'N, 22°24'E	9 June 2001 <sup>b</sup>	3 (3)	2	1	–	–
Serres (NW)	Limnochori	41°12'N, 23°13'E	9 June 1999 <sup>b</sup>	1 (1)	1	–	–	–
	Strymon River, Koumaria	41°00'N, 23°28'E	10 June 1999 <sup>b</sup>	1 (1)	1	–	–	–
Xanthi (NE)	Selino village	41°01'N, 25°08'E	8 June 2001 <sup>a</sup>	69 (67)	3	2	–	62
	Paralia Mandras, N. Kessani	41°00'N, 25°01'E	8 June 2001 <sup>b</sup>	3 (3)	1	–	–	2
	Vosvodi River, Pagouria	41°02'N, 25°18'E	8 June 2001 <sup>b</sup>	19 (19)	19	–	–	–



sequences to facilitate rapid species diagnosis of members of the Maculipennis Complex in future studies.

Results

DNA was extracted from 276 specimens identified as *An. maculipennis* s.l. on the basis of morphology. Sequences for the nuclear ITS2 region were obtained from 257 of these (93.1%) (Table I). The sequences generated in this study are available in GenBank under the accession numbers listed in Table II.

Bionomics and species distribution

Species identification and exact locality data of specimens collected in Greece are given in Table I, along with numbers of specimens for which DNA was extracted and sequenced. Relative species composition in relation to specimens sequenced per prefecture is shown in Figure 1. In this study, *An. maculipennis* was the most abundant species, followed by *An. sacharovi*, with the latter species having the widest distribution, occurring along the eastern seaboard, from northern to southern Greece.

*Anopheles maculipennis* was identified among specimens from Ioannina (70), and Florina (six) in the northwest, Xanthi (23), Rodopi (11), Serres (two), and Evros (34) in the northeast, and in the southern prefecture of Lakonia (one). *Anopheles sacharovi* was collected in highest densities in the northeastern prefecture of Xanthi (64), where it comprised 71.9% of the samples analysed. It was also found among samples from Evros (seven) and the central and southern prefectures of Attiki (one), Magnesia (one), Fthiotida

Table II. GenBank accession numbers for the 257 ITS2 sequences generated for four members of the *Anopheles maculipennis* complex found in 10 prefectures of Greece.

Species	Prefecture	N	GenBank accession numbers
<i>An. maculipennis</i> (149)	Evros	36	AF455821–AF455853, AF533581, AF533582
	Florina	6	AF342713–AF342715, AF455818–AF455820
	Ioannina	70	AF455854–AF455887, AF469847–AF469852, AF485808–AF485810, AF533552–AF533578
	Lakonia	1	AF485806
	Rodopi	11	AF455888–AF455898
	Serres	2	AF533579, AF533580
	Xanthi	23	AF455899–AF455921
<i>An. melanoon</i> (23)	Evros	18	AF452389–AF452406
	Ioannina	1	AF469853
	Rodopi	2	AF452407, AF452408
	Xanthi	2	AF452409, AF452410
<i>An. messeae</i> (2)	Florina	2	AF342711, AF342712
<i>An. sacharovi</i> (83)	Attiki	1	AF533585
	Evros	7	AY070272–AY070276, AF462110, AF533588
	Fthiotida	7	AF462077–AF462079, AF533583, AF533584, AF533586, AF533587
	Lakonia	3	AF469854, AF485805, AF485807
	Magnesia	1	AF462080
	Xanthi	64	AF462081–AF462109, AF462111–AF462143, AF466747, AF466748

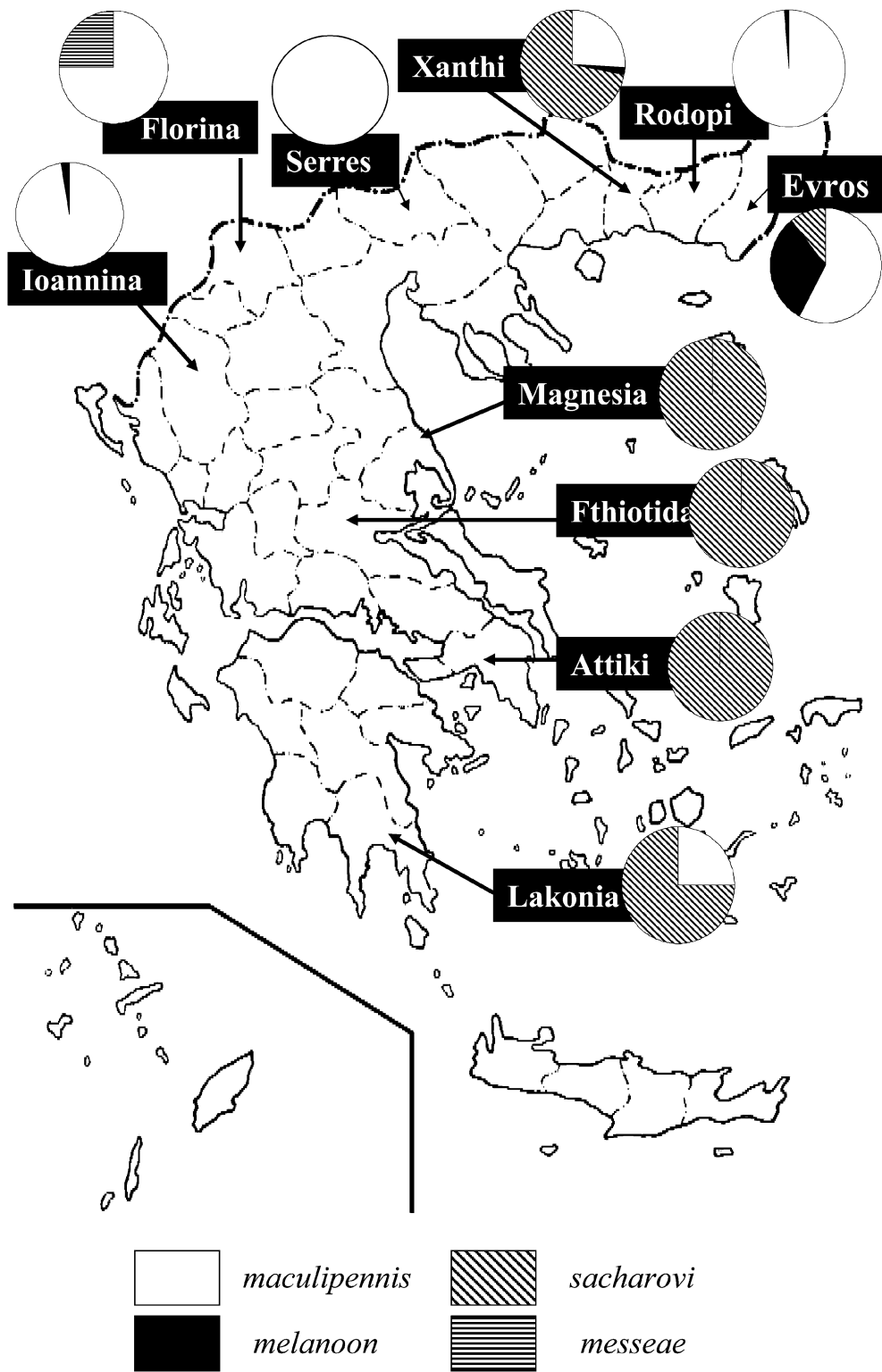


Figure 1. Map of Greece showing the distribution of species of the *Anopheles maculipennis* complex collected in 10 prefectures. Pie charts represent the relative proportions of each species found in each prefecture.

(eight), and Lakonia (three). *Anopheles melanoon* was prominent in the northeastern prefectures, being collected in highest densities in Evros (18), but samples were also found in Rodopi (two) and Xanthi (two). A single specimen of *An. melanoon* was also found in Ioannina, where it represented only 1.4% of *An. maculipennis* s.l. captured there. The presence of *An. messeae* in northwestern Greece was confirmed by the detection of two specimens from Lake Petron and Lake Zazari in the Florina Prefecture, representing a new distribution record for the country (Linton et al. 2001b, 2002a).

Members of the *An. maculipennis* complex were rarely found in biotic sympatry. However, larvae of *An. maculipennis* and *An. melanoon* were found together in Evros (River Tis Mantheas, Itea and River Erithropotomas, Didymoticho) and in Rodopi (Nesti-Krovilli, Maronia and Loutros village). *Anopheles maculipennis* and *An. sacharovi* were found in the same larval habitat at Paralia Mandras, northern Kessani in the Xanthi Prefecture. Highest species diversity was recorded from resting collections made in sheep and goat stables in Monistiraki, Evros Prefecture and Salino, Xanthi Prefecture, where *An. maculipennis*, *An. sacharovi*, and *An. melanoon* were collected together as blood-fed adults. Adults of *An. maculipennis* and *An. messeae* were also found resting together in sheepfolds near Lake Zazari, Florina Prefecture (Linton et al. 2001b).

#### *Identification based on ITS2 sequence data*

Based on comparisons of the ITS2 sequences with those in GenBank, four species of the Maculipennis Complex were identified: *An. maculipennis*, *An. melanoon*, *An. messeae*, and *An. sacharovi* (Table I). One hundred and forty-nine specimens were identified as *An. maculipennis* (98.86% similarity to AF436065, Djadid, direct GenBank submission 2001; 97.56% similarity to Z50104, Marinucci et al. 1999), 83 as *An. sacharovi* (100% identity with AY114204–AY114211, Sedaghat et al. 2003a; 99.38% similarity to AF436062, erroneously identified as *An. maculipennis* by Djadid, direct GenBank submission 2001; 98.38% similarity to Z83198, Marinucci et al. 1999), 23 as *An. melanoon* (99.54% identity with AJ224330 (as *An. subalpinus*), Marinucci et al. 1999) and two as *An. messeae* (99.56% identity with AY050639, Djadid et al., direct GenBank submission 2001; 99.52% identity with AF305556, Yajun and Fengyi, direct GenBank submission 2001) (Table I).

#### *Intraspecific variation in the ITS2 sequences*

Sequences representing 149 *An. maculipennis*, 23 *An. melanoon*, two *An. messeae*, and 83 *An. sacharovi* produced an alignment of 496 bases (Figure 2). No intraspecific variation was found either in the length or composition of the sequences in any of the four species (Figure 2). Inclusive of primers (43 bp), the size of the PCR fragment varied according to species, being 472 bp in *An. maculipennis*, 482 bp in *An. melanoon*, 485 bp in *An. messeae*, and 494 bp in *An. sacharovi*. Interspecific length differences were accounted for in full by length variability in the ITS2 spacer region, not in the 5.8S or 28S flanking genes (Figure 2). Percentage CG content of the entire fragments was 50.8% in *An. maculipennis* (26.3% A, 22.9% T, 26.9% C, 23.9% G), 51.1% in *An. melanoon* (25.9% A, 23.9% T, 26.3% C, 23.9% G), 51.1% in *An. messeae* (26.2% A, 22.7% T, 26.8% C, 24.3% G), and 48.0% in *An. sacharovi* (29.1% A, 22.9% T, 25.1% C, 22.9% G). These values are concordant with 40–50% AT values reported for other mosquitoes of subgenus *Anopheles*, including Nearctic members of the Freeborni (Porter and Collins 1991) and Quadrimaculatus Complexes (Cornel et al. 1996), and the Palaearctic Maculipennis

	1	1111111112	2222222223	3333333334	4444444445	5555555556	6666666667	7777777778
	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
<i>maculipennis</i>	<u>ATCACTCGGC</u>	<u>TCGTGGATCG</u>	<u>ATGAAGACCG</u>	<u>CAGCTAAATG</u>	<u>CGCGTCACAA</u>	<u>TGTGAAGTGC</u>	<u>AGGACACATG</u>	<u>AACACCGATA</u>
<i>messeae</i>	.....	.....	.....	.....	.....	.....	.....	.....
<i>melanoon</i>	.....	.....	.....	.....	.....	.....	.....	.....
<i>sacharovi</i>	.....	.....	.....	.....	.....	.....	.....	.....
		1	1111111111	1111111111	1111111111	1111111111	1111111111	1111111111
	8888888889	9999999990	0000000001	1111111112	2222222223	3333333334	4444444445	5555555556
<i>maculipennis</i>	<u>AGTTGAACGC</u>	<u>ATATTGCGCA</u>	<u>TCGTGCGACA</u>	<u>CAGCTCGATG</u>	<u>TACACATTTT</u>	<u>TGAGTGCCTA</u>	<u>TATTTGACCC</u>	<u>---AGGTCA</u>
<i>messeae</i>	.....	.....	.....	.....	.....	.....C	.....	.....
<i>melanoon</i>	.....	.....	.....	.....	.....	.....	.....T	.....
<i>sacharovi</i>	.....	.....	.....	.....	.....	.....	.....T	.....
	1111111111	1111111111	1111111111	1111111112	2222222222	2222222222	2222222222	2222222222
	6666666667	7777777778	8888888889	9999999990	0000000001	1111111112	2222222223	3333333334
	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
<i>maculipennis</i>	<u>AACACTCGT</u>	<u>ACCTCCGGGT</u>	<u>ACGTGCAT</u>	<u>G</u>	<u>ATGATGAAAG</u>	<u>AGTTTGGAA</u>	<u>---CACCAT</u>	<u>CCTTCTCTTG</u>
<i>messeae</i>	.....	.....	.....	.....	.....	.....	.....	.....
<i>melanoon</i>	.....	.....	.....	.....	.....	.....	.....	.....
<i>sacharovi</i>	.....CG	G.GG.GCC	.....	.....	.....	.....	.....	.....
	2222222222	2222222222	2222222222	2222222222	2222222222	2222222223	3333333333	3333333333
	4444444445	5555555556	6666666667	7777777778	8888888889	9999999990	0000000001	1111111112
<i>maculipennis</i>	<u>CGCAGCGTGT</u>	<u>AGCAACCCCA</u>	<u>GGTTTCAACT</u>	<u>TGCAAAGTGG</u>	<u>CCATGGGGCT</u>	<u>GACACCTCAC</u>	<u>CACCATCAGC</u>	<u>GTGC-TGTGT</u>
<i>messeae</i>	.....	.....	.....	.....	.....	.....	.....	.....
<i>melanoon</i>	.....	.....	.....	.....	.....	.....	.....	.....
<i>sacharovi</i>	.....T	.....	.....	.....	.....	.....	.....	.....
	3333333333	3333333333	3333333333	3333333333	3333333333	3333333333	3333333333	3333333334
	2222222223	3333333334	4444444445	5555555556	6666666667	7777777778	8888888889	9999999990
<i>maculipennis</i>	<u>AGCGTGTTCG</u>	<u>GCCAGTTCG</u>	<u>GTCAATCGTGA</u>	<u>GGCGTTACCT</u>	<u>AACGGGGAGG</u>	<u>CACA-CACTG</u>	<u>TTCGCGGTAT</u>	<u>CTCATGGTT</u>
<i>messeae</i>	.....	.....AA	.....	.....	.....	.....	.....	.....
<i>melanoon</i>	.....	.....	.....	.....	.....	.....	.....	.....
<i>sacharovi</i>	.....	.....	.....	.....	.....	.....	.....	.....
	4444444444	4444444444	4444444444	4444444444	4444444444	4444444444	4444444444	4444444444
	0000000001	1111111112	2222222223	3333333334	4444444445	5555555556	6666666667	7777777778
<i>maculipennis</i>	<u>TA</u>	<u>---ACCAACC</u>	<u>ATAGCAGCAG</u>	<u>AGATACAACA</u>	<u>CGGCTCCTA</u>	<u>GT</u>	<u>-----AG</u>	<u>CCCATGGGCC</u>
<i>messeae</i>	.....	.....	.....	.....	.....	.....	.....	.....
<i>melanoon</i>	.....	.....	.....	.....	.....	.....	.....	.....
<i>sacharovi</i>	.....	.....	.....	.....	.....	.....	.....	.....
	4444444444	4444444444	4444444444	4444444444	4444444444	4444444444	4444444444	4444444444
	8888888889	9999999990	0000000001	1111111112	2222222223	3333333334	4444444445	5555555556
<i>maculipennis</i>	<u>CCCTAAATTT</u>	<u>AAGCAT</u>	.....	.....	.....	.....	.....	.....
<i>messeae</i>	.....	.....	.....	.....	.....	.....	.....	.....
<i>melanoon</i>	.....	.....	.....	.....	.....	.....	.....	.....
<i>sacharovi</i>	.....	.....	.....	.....	.....	.....	.....	.....

Figure 2. A 496bp alignment representing the 257 ITS2 sequences of the four members of the *Anopheles maculipennis* complex in Greece: *An. maculipennis* (472 bp), *An. messeae* (485 bp), *An. melanoon* (482 bp), and *An. sacharovi* (494 bp). Amplification primers are underlined. Figures in parentheses indicate numbers of sequences represented per species. Dashes (–) indicate gaps and dots (·) indicate identity of bases within the alignment.

Complex (Marinucci et al. 1999; Proft et al. 1999; Linton et al. 2001b, 2002a, 2002b, 2002c, 2003, 2005; Sedaghat et al. 2003a, 2003b; Kampen 2005a, 2005b).

Interspecific variability in ITS2 sequences

Levels of sequence divergence for the entire fragments of the four Greek species of the Maculipennis Complex are shown in Table III. Over the whole length of the amplicon, excluding gaps, *An. messeae* and *An. maculipennis* were shown to be most similar with only 3.3% sequence divergence. Sequence divergence between *An. maculipennis* and *An. melanoon*, and *An. messeae* and *An. melanoon* were similar, at 3.7 and 3.8%, respectively (Table III). The sequence of *An. sacharovi* was most distant from all other taxa, showing

Table III. Upper right: pairwise values for p-distance transition/transversion employing pairwise deletion of gaps for *Anopheles maculipennis* (149 sequences), *An. melanoon* (23), *An. messeae* (2) and *An. sacharovi* (83). Lower left: pairwise distance matrix employing Kimura's two-parameter model and pairwise deletion of gaps and missing data (lengths of pairwise alignments are shown in parentheses).

	<i>An. maculipennis</i>	<i>An. messeae</i>	<i>An. melanoon</i>	<i>An. sacharovi</i>
<i>An. maculipennis</i>		2.000	2.400	1.167
<i>An. messeae</i>	0.033 (485)		1.571	1.103
<i>An. melanoon</i>	0.037 (486)	0.038 (486)		1.103
<i>An. sacharovi</i>	0.154 (496)	0.139 (496)	0.140 (496)	

13.9 and 14.0% divergence from *An. messeae* and *An. melanoon*, respectively, and 15.4% from *An. maculipennis* (Table III). This is indicative of the basal position of *An. sacharovi* before the more recent speciation of *An. maculipennis*, *An. messeae*, and *An. melanoon* (White 1978). Indeed, *An. sacharovi* is the only member of the Maculipennis Complex that can reliably be differentiated from other members of the Maculipennis Complex on the basis of adult morphology alone (Shahgudian 1960; White 1978; Sedaghat et al. 2003a).

#### *Species-diagnostic restriction enzyme sites*

The ITS2 sequences generated in this study (Figure 2) were screened for restriction enzyme cutting sites. Resulting patterns following digestion with *Hsp92* II (CATG) were diagnostic for each of the four species of the *An. maculipennis* complex in Greece (Figure 3). Following digestion of the whole ITS2 amplicon (including primers), fragments that are species-diagnostic and at least 50 bp follow: *An. maculipennis* (113/109/89/70/51), *An. melanoon* (206/109/70/53), *An. messeae* (169/89/70), and *An. sacharovi* (214/179/70).

## Discussion

Concerted evolution of multigene families (Zimmer et al. 1980), such as ITS2 rDNA, within species has resulted in the wide utilization of these fast evolving spacer regions to determine the species composition of *Anopheles* mosquito complexes (Porter and Collins 1991; Fritz et al. 1994; Severini et al. 1996; Xu and Qu 1997; Beebe et al. 1999; Marelli et al. 1999; Torres et al. 2000; Hackett et al. 2000; Sharpe et al. 2000; Mukabayire et al. 2001; Linton et al. 2001b, 2002a, 2002b, 2002c, 2003). The low level of intraspecific variation in the ITS2 region has proven useful as the basis for both species-specific PCR assays (Scott et al. 1993; Crabtree et al. 1995; Cornel et al. 1996; Proft et al. 1999) and RFLP assays (Van Bortel et al. 2000; Manonmani et al. 2001) to differentiate members of mosquito species complexes. Previous studies of ITS2 sequences have shown no intraspecific variation in sibling species of *Anopheles*, including species A of the Dirus Complex (Xu and Qu 1997), members of the Maculatus Complex (Torres et al. 2000), and the Sundaicus Complex (Linton et al. 2001a) of *Anopheles (Cellia)*. Intraspecific variation has been reported to be negligible in *An. (C.) funestus* Giles (Mukabayire et al. 1999), species D of the Dirus Complex (Xu and Qu 1997), and in genotype C of the Bancroftii Group (Beebe et al. 2001) of *Anopheles (Anopheles)*. Intraspecific variation in five colony strains of species of the Gambiae Complex was reported to range between 0 and 0.43% (Paskewitz et al. 1993). Contrary to all other studies, Beebe et al. (2001) reported population-specific ITS2 sequences in the Bancroftii Group between Queensland, Australia and the western province of Papua New Guinea, but it remains to be seen

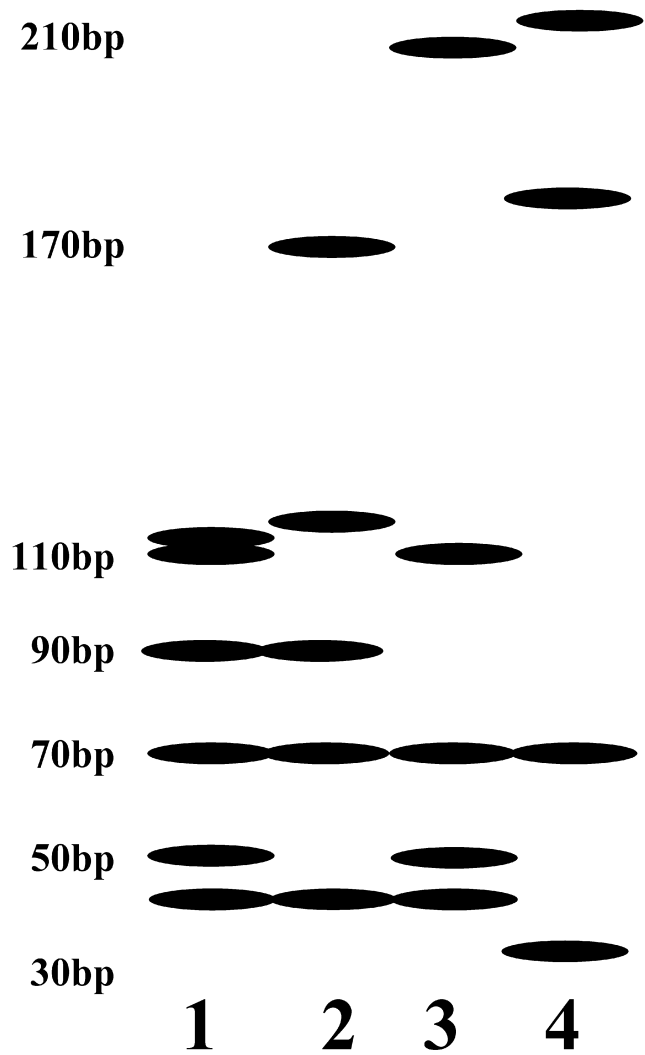


Figure 3. Schematic diagram of the proposed restriction patterns of (1) *Anopheles maculipennis*, (2) *An. messeae*, (3) *An. melanoon*, and (4) *An. sacharovi* after digestion with the enzyme *Hsp92* II.

whether unique haplotypes may indeed represent incipient species. No intraspecific variation was noted in this or previous studies of the ITS2 sequences of *An. atroparvus* (Linton et al. 2002c), *An. maculipennis* (Linton et al. 2001b, 2002a, 2003), *An. melanoon* (Linton et al. 2002b), *An. messeae* (Linton et al. 2001b, 2002a, 2002c), or *An. sacharovi* (Sedaghat et al. 2003a). Analysis of the ITS2 sequences of *An. freeborni* Aitken and *An. hermsi* Barr and Guptavni, two Nearctic members of the Maculipennis Group, showed intraspecific variability to be negligible (Porter and Collins 1991).

The multiple copies per genome and absolute intraspecific homogeneity of the ITS2 region indicate that this region would be well suited to rapid diagnostic assays. Based on the ITS2 sequences generated for the four species in this study, restriction enzyme sites were isolated which yield species-diagnostic patterns. Sequences were screened with 113

commercially available enzymes (Roche Molecular Biochemicals, Sussex, UK) to provide a simple, single enzyme assay that could be used to differentiate *An. maculipennis*, *An. melanoon*, *An. messeae*, and *An. sacharovi*. Enzyme *Hsp92* II (with the cutting site CATG) proved to be the only enzyme capable of distinguishing these four species (Figure 3). Recent studies in our laboratory have shown that ample products can be obtained when a single leg of insects, captured and dried directly over silica gel, is placed directly in the ITS2 PCR (as described for extracted DNA: see "Materials and methods"). The PCR products were found to be sufficiently clean to be used in subsequent RFLP assays. This method provides a cheap, rapid means of identifying the four species in Greece.

In this study, ITS2 sequences generated from 257 wild-caught specimens of *An. maculipennis* s.l. from 10 prefectures across Greece were used to identify the mosquitoes by comparison with those sequences available in GenBank. Samples were identified as *An. maculipennis* (58.0%), *An. sacharovi* (32.3%), *An. melanoon* (8.9%), and *An. messeae* (0.8%). These species distributions, with the addition of the new country record for *An. messeae* (Linton et al. 2001b, 2002a), agreed with the species list of White (1978). The predicted presence of *An. atroparvus* (Dahl and White 1978; White 1978) was not confirmed, despite extensive collections, suggesting that this species is not present in Greece. *Anopheles daciae*, the cryptic sibling species of *An. messeae*, was also not detected.

All four species of the Maculipennis Complex found in Greece, *An. maculipennis*, *An. melanoon*, *An. messeae*, and *An. sacharovi*, have been implicated in malaria transmission, but *An. sacharovi* is the most efficient vector. *An. sacharovi* is the primary vector in endemic zones in Turkey (Kasap 1990; Alten et al. 2000), Syria (Berberian 1946; Abdel-Malek 1958), Armenia (Romi et al. 2002), and elsewhere in the Middle East (Christophers 1920; Manouchehri et al. 1974; Zahar 1974). It is also regarded as the principal potential vector in regions of southern Europe (Romi 1999; Ramsdale and Snow 2000). In this study, *An. sacharovi* was found on the eastern coastal seaboard of Greece, with highest densities in Xanthi and Evros Prefectures in northeastern Greece, near the border of Turkey. The species was also found in the drier central and southern prefectures of Attiki, Fthiotida, Magnisia, and Lakonia (Figure 1). Low densities of the species in this region may reflect the active insecticide-spraying campaigns in these regions or our restricted sampling in these areas.

*Anopheles maculipennis*, *An. melanoon* (as *An. subalpinus*), and *An. messeae* have recently been incriminated as primary or secondary vectors in restricted geographical regions (Bruce-Chwatt and de Zulueta 1980; Nikolaeva 1996; Alten et al. 2000). *Anopheles maculipennis* and *An. melanoon* are found predominantly in the north of Greece along with *An. sacharovi*, and *An. messeae* has only been found in the northwestern prefecture of Florina. Currently, *An. maculipennis* and *An. melanoon* (as *An. subalpinus*) are not regarded as efficient vectors of malaria, and, in the Biga Plains of Turkey (Alten et al. 2000), vectorial competence is only noted where these species are present in high densities. Indeed in Portugal, *An. maculipennis* is known to be predominately zoophilic (Ribeiro et al. 1988), but in certain situations, i.e. at high densities, or where non-human hosts are limited, the species will be anthropophagic and in these cases exhibits malarial vector capacity (*sensu* Garrett-Jones 1964). The distributions of the highest mosquito population densities were found in the northeastern corner of Greece, comprising Xanthi, Rodopi, and Evros Prefectures, where *An. sacharovi*, *An. maculipennis*, and *An. melanoon* are often sympatric. This region encompasses the delta of the Evros and Nestos Rivers, and is surrounded by the borders of Turkey and Bulgaria. With the endemic status of malaria in Turkey (Kasap 1990; Alten et al. 2000) and the economic difficulties experienced in Bulgaria at present, it

seems increasingly important that mosquito control programmes are established and maintained in these northeastern prefectures of Greece. Given the vector competence and population density of the mosquitoes, and the passage of potentially infected persons through this border zone, mosquito control is needed to reduce the risk of malaria being reintroduced and re-established in Greece.

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