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New occurrence records for *Anopheles maculipennis* and *An. messeae* in northern Greece based on DNA sequence data

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Abstract

Bloodfed *Anopheles maculipennis s.l.* females were collected resting in and around sheepfolds in the northwestern prefecture of Florina, Greece. The specimens were identified as *An. maculipennis* and *An. messeae* on the basis of similarity to published DNA sequence data for the rDNA internal transcribed spacer (ITS2) region. Novel DNA sequences were obtained for the mitochondrial cytochrome c oxidase I gene (COI) for both species. Correlations of COI and ITS2 sequences were used to identify specimens, with respect to ITS2 sequences in Genbank. These results constitute new distribution records for these two species in northwestern Greece.

Introduction

Epidemiological studies of malaria in Europe in the 1920s and 1930s revealed the presence of the vector *Anopheles maculipennis* Meigen in regions without the disease, a phenomenon referred to as "anophelism without malaria". Further studies at that time revealed that *An. maculipennis* was not one but several closely related species, thereby exposing the first mosquito species complex (Hackett & Missiroli, 1935). This discovery accounted for the failure of early malaria control programmes in Europe (Hackett & Missiroli, 1935; Bates, 1949) and has revolutionised vector biology studies today.

Species complexes comprise two or more essentially isomorphic sibling species that may exhibit very different ecological, behavioural and physiological characteristics, which ultimately determine vector status. Modern systematic studies have employed ecological, morphological, physiological and biochemical data to characterise members of the *An. maculipennis* complex. These include studies of egg morphology (Falleroni, 1926; Corradetti, 1934; Korvenkontio *et al.*, 1979), biting preferences (Hackett & Missiroli, 1935), larval chaetotaxy (Bates, 1939; Deruaz *et al.*, 1991), hybridization experiments (de Buck & Swellengrebel, 1934), ovarian polytene chromosome banding patterns (Kitzmiller *et al.*, 1967), zymotaxonomy (Korvenkontio *et al.*, 1979; Bullini & Coluzzi, 1982), cuticular hydrocarbons (Phillips *et al.*, 1990) and, most recently, DNA sequences (Marinucci *et al.*, 1999; Proft *et al.*, 1999).

Current understanding of the composition of the *An. maculipennis* complex stems from White's (1978) revision of the *An. maculipennis* complex, where nine Palaeartic members were proposed. These were *An. atroparvus* van Thiel, *An. beklemishevi* Stegnii & Kabanova, *An. labranchiae* Falleroni, *An. maculipennis* Meigen, *An. martinius* Shingarev, *An. melanoon* Hackett (with its variety *subalpinus*), *An. messeae* Falleroni, *An. sacharovi* Favre and *An. sicaulti* Roubaud. White also proposed suppression of the names *alexandraeschingarevi*, *lewisi* and *selengensis* and resurrected two nominal species (*martinus* and *sicaulti*) on the basis of evidence available at that time. Field and laboratory investigations, utilising integrated morphological, electrophoresis, crossing-mating and chromosomal studies revealed that *An. sicaulti* was conspecific with *An. labranchiae*, and the former name was suppressed (de Zulueta *et al.*, 1983). The nominal form of *subalpinus* was regarded as a variety of *An. melanoon* until Cianchi *et al.* (1987) elevated it to species level on the basis of reproductive isolation in sympatric populations. Hence; it follows that the *An. maculipennis* complex currently comprises the following nine species: *An. atroparvus*, *An. beklemishevi*, *An. labranchiae*, *An. maculipennis*, *An. martinius*, *An. melanoon*, *An. messeae*, *An. sacharovi* and *An. subalpinus*.

The distribution of malaria is determined by the occurrence and biology of the mosquito vectors, and the temperature requirements of the malarial plasmodia for sporogony within the vector species (WHO, 1990). Three species of the *An. maculipennis* complex (*An. atroparvus*, *An. sacharovi* and *An. labranchiae*) are known to be efficient current or historical malaria vectors across Europe (Ribeiro *et al.*, 1988; Kasap, 1990;

1994). *Anopheles maculipennis* and *An. subalpinus* were recently incriminated as secondary Biga Plains, Turkey (Alten *et al.*, 2000). Climatic changes, including global warming and increased precipitation, are expected to extend vector ranges and population sizes of some species, increasing malaria transmission rates (Snow, 2000). *Anopheles messeae*, previously involved in malaria transmission in eastern Europe and western Asia (Bruce-Chwatt & de Zulueta, 1980), but not considered to be a vector in north-western Europe (Jetten & Takken, 1994), has now been incriminated as the main vector of resurgent malaria in the Ukraine and Russia (Nikolaeva, 1996).

Recent increased trends for exotic travel and immigrant labour have resulted in the importation of malarial parasites into areas of Europe where competent local vectors are present. This has heightened concern for the reintroduction of malaria into European countries, especially Greece and Italy, where malaria was once highly endemic (Jetten *et al.*, 1996; Lindsay & Birley, 1996). In 1999, two indigenous cases of malaria were reported from Evros Prefecture in northeastern Greece, and in summer 2000, two unconfirmed cases were reported from the Kassandra Peninsula in Chalkidiki Prefecture (G. Koliopoulos, personal communication).

Early records of mosquitoes in Greece reported the presence of *An. maculipennis* (Hackett & Lewis, 1935; Shannon, 1935; Shannon & Hadjinicolaou, 1941) and *An. messeae* (Pandazis, 1935; Shannon, 1935; Hackett & Missiroli, 1935). Following a study of morphology of eggs from Kavala (Macedonia), Hackett & Lewis (1935) confirmed the presence of *An. messeae*, *An. maculipennis* (as *An. typicus*) and *An. subalpinus*. In 1936, Bates reported finding *An. messeae* eggs on the Albanian shore of Lake Prespa, and it was suggested that the distribution would include the Greek shore of this Lake, which is in the Prefecture of Florina (in Livadas & Sphangos, 1940-1). However, Bates (1940) stated that *An. messeae* was not present in Greece. Weyer (1942) declared all existing reports of *An. messeae* in Thrace and Macedonia to be unreliable as they were made prior to the recognition of *An. subalpinus* as a separate entity. The status of *An. messeae* in early reports is unclear because prior to the description of the egg of *An. subalpinus*, eggs of this species were thought to belong to a variety of *An. messeae* (reported in Livadas & Sphangos, 1940-1). In a later study of eggs, *An. maculipennis* (as *An. typicus*), *An. sacharovi* and *An. subalpinus* were reported from Macedonia (Shannon & Hadjinicolaou, 1941). White (1978) suggested that at least three members of the *An. maculipennis* complex, *An. maculipennis*, *An. sacharovi* and *An. melanoon*, were present in Greece. Based on earlier works (Shannon, 1935; Hackett & Missiroli, 1935; Hackett & Lewis, 1935), Samanidou-Voyadjoglou & Darsie (1993) suggested that *An. messeae* may be present in Greece, but this has not been confirmed in subsequent studies. Distribution patterns of *An. atroparvus* by Dahl & White (1978) indicate that *An. atroparvus* may be present in Greece, but again this is unconfirmed.

Neither the historical status nor current distribution of the component members of the *An. maculipennis* complex in Greece are clear. Given the geographical proximity of Greece to areas of resurgent malaria transmission and the potential presence of capable vectors, it is important both to survey and characterise members of the *An. maculipennis* complex. Correct vector identification is essential to assess the potential risk of malaria in these border regions, and to devise appropriate control or monitoring strategies. This paper is the result of preliminary collections and molecular analyses to identify which species of the *An. maculipennis* complex are present in Florina, Greece.

Material and methods

Eleven bloodfed *An. maculipennis s.l.* were captured resting in and around sheep pens in Florina, the most northwesterly prefecture of Greece, in May 1999. Females were held for two days before being induced to lay eggs, which were subsequently preserved in 70% ethanol. Eggs, which can also be used to confirm species identity, have been retained as voucher specimens at The Natural History Museum (NHM), London. Following oviposition, mothers were stored in 95% ethanol for DNA studies. DNA was extracted from each specimen, but sequence data were obtained from only nine individuals.

DNA was extracted from individual mosquitoes following a phenol-chloroform extraction protocol (Linton *et al.*, 2001). Amplification of the ITS2 nuclear ribosomal spacer and mitochondrial cytochrome oxidase I gene (COI) region were carried out using 5.8SF and 28SR primers (Collins & Paskewitz, 1996), and the universal insect COI primers C1-J-1718 and C1-N-2191 (Simon *et al.*, 1994). PCR products were amplified using the reaction and thermocycler parameters described in Linton *et al.* (2001), and were cleaned using a commercially available PCR purification kit (QIAgen Ltd, Sussex, England). Cycle sequencing reactions were

prepared using the Big Dye Terminator Kit (PE Applied Biosystems, Warrington, England) and read by an automated sequencer (ABI 377, PE Applied Biosystems). Following sequencing, the template DNA was dried and retained at minus 70°C in the Molecular Systematics Laboratory, Department of Entomology, NHM for future reference.

Sequence data were edited and aligned using Sequencher™ version 3.1.1 (Genes Codes Corporation, Ann Arbor, Michigan) and CLUSTAL W (Pearson & Lipman, 1988) software packages. Similarity with sequences in GenBank was assessed using FASTA search (http://www2.ebi.ac.uk/fasta3/). Inter- and intra-specific variability was calculated using MEGA version 1.01 (Kumar *et al.*, 1993).

Results

DNA sequences were obtained from the nine females listed in Table 1. Sequence data for ITS2 were generated for five individuals, and these sequences were used for species identification based on comparison with ITS2 sequences available in GenBank. Identification of the samples, based on the ITS2 DNA sequences, was further confirmed on the basis of egg morphology. Two specimens shared closest identity (97.6%) with *An. messeae* (Z50105; Marinucci *et al.*, 1999), and the other three were most similar (97.7%) to *An. maculipennis* (Z50104; Marinucci *et al.*, 1999). Another *An. messeae* ITS2 sequence (AF305556) recently submitted from Yining, Xinjiang, China (Yajun & Fengyi, GenBank direct submission, January 2001) is identical to ours except for an additional T base, which is not supported by this study or Marinucci's sequences in GenBank.

Table 1. GenBank accession numbers of DNA sequences obtained from specimens of *An. messeae* and *An. maculipennis*.

Specimen number	Trapping locality in Florina	Species identity	GenBank numbers COI	ITS2
69	Lake Petron	*An. messeae*	AF342723	AF342711
72	Lake Zazari	*An. messeae*	AF342724	AF342712
67	Kato Kleine	*An. maculipennis*	AF342716	–
68	Kato Kaliniki	*An. maculipennis*	AF342717	–
71	Kato Kleine	*An. maculipennis*	AF342718	AF342713
73	Lake Zazari	*An. maculipennis*	AF342719	–
74	Anargyri	*An. maculipennis*	AF342720	AF342714
76	Kato Kaliniki	*An. maculipennis*	AF342721	AF342715
77	Kato Kaliniki	*An. maculipennis*	AF342722	–

Excluding primers (43 bp), the PCR products were 442 and 429 bases in length for *An. messeae* (ITS2=303 bp) and *An. maculipennis* (ITS2=290 bp), respectively. Interspecifically, the 13 bp length difference is accounted for by three indel events within the ITS2 region. No intraspecific variation was detected in the ITS2 region of either species.

Partial sequences for the COI gene were obtained from the nine specimens. Seven of these were shown to be *An. maculipennis*: three identified by comparison with ITS2 sequences in GenBank and the others by correlation of their COI sequences with those of the former. The COI sequence alignment for both *An. maculipennis* and *An. messeae* was 472 bases long (excluding primers, which totalled 50 bp), and unambiguous. Intraspecific variation within the COI haplotypes of *An. maculipennis* comprise a maximum of two bases (0.4%) in a total of five variable sites, and resulted in five unique haplotypes. No intraspecific variation was detected in the two COI sequences of *An. messeae*. Interspecific similarity of the COI sequences ranged between 96.2 –96.6%.

Discussion

The mosquito fauna of Florina Prefecture in Greece has not been extensively studied, and as such this study represents the first attempt to characterise the *An. maculipennis* complex in this region. Sequencing of five ITS2 and nine COI gene regions for nine individuals identified seven *An. maculipennis* and two *An. messeae* on the basis of their ITS2 similarity to GenBank sequences (Z50104, and Z50105 and AF305556, respectively), and correlation of their COI sequences. *Anopheles maculipennis* was present at Kato Kleine, Kato Kaliniki and Anargyri, *An. messeae* was present at Lake Petron, and sympatric populations of both species were found at Lake Zazari. This small dataset represents new distribution records for these species, which are both capable malaria vectors (Nikolaeva, 1996; Alten *et al.*, 2000).

Despite the recent studies on the ITS2 of the *An. maculipennis* complex by Marinucci *et al.* (1999) and Proft *et al.* (1999), there is a paucity of sequence data available in Genbank for *An. messeae* and *An. maculipennis*. Currently, *An. maculipennis* is represented by the ITS2 sequence Z50104 (Marinucci *et al.*, 1999) and one 28S sequence (X89643) generated for a phylogenetic study of Culicomorpha (Pawlowski *et al.*, 1996). GenBank submissions for *An. messeae* include two ITS2 sequences, Z50105 by Marinucci *et al.* (1999) and AF305556 by Yajun & Fengyi (see above), from Yining, Xinjiang, China.

It is interesting to note the high level of ITS2 sequence homology between the Chinese (AF305556) and our Greek specimens, indicating a high degree of genetic homogeneity in *An. messeae* across its range. Sequence data generated for the two DNA regions in this small study constitute the most comprehensive intraspecific datasets to date for both *An. maculipennis* and *An. messeae*. As such, this study serves as a platform for further intra- and interspecific DNA studies on members of the *An. maculipennis* complex.

The mtDNA COI gene, which has not been studied previously in this group, revealed higher variability than the ITS2 sequences. The levels of variability observed, and the unambiguous alignment of homologous characters, suggest that COI may be useful for phylogenetic reconstruction of the *An. maculipennis* complex, and the *An. maculipennis* group as a whole.

Despite the historical importance and the number of studies carried out on members of the *An. maculipennis* complex so far, there is a distinct lack of fully integrated systematics studies. With funding trends leaning towards molecular systematics at the expense of classical taxonomy, we find ourselves in a position of ageing experienced taxonomists. It is thus increasingly important to obtain molecular sequence data from samples that have been properly identified while this gap is being bridged. In the case of mosquitoes, rearing progeny broods from wild-caught females allows morphological characters in all life stages (eggs, larvae, pupae and adults of both sexes), chromosome banding patterns and molecular data to be studied among specimens that unquestionably belong to the same species. Progeny broods are not only invaluable for defining members of species complexes, they also provide excess material that can be retained as 'voucher specimens' for reference and future study. We advocate that the characterisation of any species, particularly new taxa, should include high quality sequence data for at least two gene regions, from more than one individual, to facilitate subsequent molecular identification (e.g. Nguyen *et al.*, 2000; Linton *et al.*, 2001). In addition to the retention of intact specimens as vouchers and a source for classical taxonomic study, we advocate, where possible, preservation of some of the template DNA. In view of the value of integrated systematics studies incorporating both morphology and molecular data, we have recently undertaken field collections to fully characterise the Greek members of the *An. maculipennis* complex following this methodology.

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