Observations on the taxonomic status of *Anopheles subalpinus* Hackett & Lewis and *An. melanoon* Hackett

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Abstract

Progeny broods of *An. maculipennis* s.l. from Greece were identified as *An. subalpinus* Hackett & Lewis based on egg morphology and the original species descriptions. DNA sequence data from members of the progeny broods were compared to previously published sequences for members of the *An. maculipennis* complex. The sequences were 99.54-100% identical to those of *An. melanoon* Hackett, typified by its uniformly black egg. These results are discussed in relation to those of other workers, and *An. subalpinus* is formally synonymised with *An. melanoon*.

Introduction

*Anopheles maculipennis* Meigen, the historical malaria vector in Europe, was exposed as a complex of at least two species on the basis of egg morphology in the early 1920s (Falleroni, 1922; van Thiel, 1923). Following these early works, extensive efforts were made to elucidate all members of the *An. maculipennis* complex (for reviews see Kitzmiller et al., 1967; White, 1978). Current understanding of the composition of the *An. maculipennis* complex stems from White (1978), who recognised nine taxa: *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. meseae* Falleroni, *An. sacharovi* Favre and *An. sicuati* Roubaud. White proposed the suppression of *alexandraeschingarevi*, *lewisi* and *selengensis* and the resurrection of two nominal species (*martinius* and *sicuati*) on the basis of evidence available at that time.

Field and laboratory investigations, utilising integrated morphological, enzyme electrophoresis, crossing-mating and chromosome studies, revealed that *An. sicuati* was conspecific with *An. labranchiae*, and the former name was synonymised with the latter (de Zaldueta et al., 1983). The nominal form of *subalpinus* was regarded as a variety of *An. melanoon* until Cianchi et al. (1987) showed enzyme evidence for the reproductive isolation of two forms in sympatric populations. Based on this, Ribiero et al. (1988) treated *An. subalpinus* “as a separate species” and its re-elevation to species status is attributed to these authors (Ward, 1992). 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. meseae*, *An. sacharovi* and *An. subalpinus*. These species are notoriously difficult to distinguish morphologically in the adult and larval stages and, despite differential chromosome and isozyme differences, egg morphology remains the golden standard by which the members of the complex are routinely identified. Several authors have provided keys for the differentiation of members of the *An. maculipennis* complex based on egg characters (Weyer, 1942; Angelucci, 1955; White, 1978; Korvenkontio et al., 1979; Jaenson et al., 1986).

Early reports of mosquitoes in Greece recorded the presence of *An. maculipennis* (Hackett & Lewis, 1935; Shannon, 1935; Shannon & Hadjinicolaou, 1941) and *An. meseae* (Pandazis, 1935; Shannon, 1935; Hackett & Missirolli, 1935). Following a morphological study of eggs from Kavala (Macedonia), Hackett & Lewis (1935) confirmed the presence of *An. meseae*, *An. maculipennis* (as *An. typicus* Hackett & Missirolli) and *An. subalpinus*. The status of *An. meseae* in early reports is unclear because prior to the description of the egg of *An. subalpinus* (Hackett & Lewis, 1935), eggs of this species were thought to belong to a variety of *An. meseae* (Livadas & Sphangos, 1940). Despite earlier suggestions to the contrary, Bates (1940) could not confirm the presence of *An. meseae* in Greece, and thus, in his 1942 paper, Weyer declared all reports of *An. meseae* in Thrace and Macedonia prior to the recognition of *An. subalpinus* to be unreliable. Based on the older literature reports, Samanidou-Voyadogioglou & Duris (1993) and Ramsdole & Snow (2000) suggested that *An. meseae* might also be present in Greece. The presence of this species in Florina Prefecture of Greece was established beyond doubt by Linton et al. (2001b), who reported sympatric populations of *An. meseae* and *An. maculipennis* based on DNA sequence identification. *Anopheles maculipennis* (as *An. typicus*), *An. subalpinus* and *An. sacharovi* (as *An. elutus* Favre) have been reported from Ioannina Prefecture, NW Greece (Livadas & Sphangos, 1940) and Macedonia (Shannon & Hadjinicolaou, 1941). Despite many years of mosquito survey by the Rockefeller Foundation and the Greek National Malaria Control Organisation, there is only a single record of *An. melanoon* in neighbouring Albania (Livadas & Sphangos, 1940), which is the type locality of *An. subalpinus* (Hackett & Lewis, 1935).
As a consequence of synonymising An. subalpinus with An. melanon, White (1978) indicated that An. maculipennis, An. sacharovi and An. melanon were the members of the complex present in Greece. Except for the recent studies of Linton et al. (2001b, 2002a), no studies have been carried out to confirm the identity of the taxa of the An. maculipennis complex present in Greece following the re-elevation of An. subalpinus (Ciunci et al., 1987; Ribeiro et al., 1988). Given the recent incrimination of An. subalpinus as a secondary vector in the Biga Plains, Turkey (Allen et al., 2000) and documented malaria cases in neighbouring Greece in recent years (Linton et al., 2001b), it is important to determine whether An. subalbinus and/or An. melanon are present in Greece. In studies leading to this report, we used an integrated molecular and morphological approach to investigate the status and distribution of these nominal forms in Greece.

Materials and Methods

Mosquitoes belonging to the An. maculipennis complex were collected in eight prefectures of Greece, namely Evros, Rodopi and Xanthi in the north-east, Ioannina and Florina in the north-west, Fthiotida and Magnesia in the heart of the country and Lakonia in the south, from July 1997 to August 2001. Larval collections were carried out in all eight prefectures, and resting adults were collected at two sites; in Selino village, Xanthi and in Monastiraki, Alexandroupolis, Evros (Table 1). Females were held for two days before being induced to lay eggs. The progeny broods were then individually link-reared to obtain adults with associated larval and pupal exuviae for integrated molecular and morphological studies. At least ten eggs from each brood were stored in Bouin’s solution (BDH, Poole, England) for light and scanning electron microscope studies of the eggs. Mosquitoes belonging to progeny broods were identified on the basis of egg morphology and DNA sequences obtained for the nuclear internal transcribed spacer (ITS2) region. Eggs were identified using the keys of Weyer (1942), Angelucci (1955) and the original descriptions of An. melanon (Hackett, 1934) and An. subalpinus (Hackett & Lewis, 1935). DNA sequences obtained from wild-caught larvae were identified on the basis of correlation of their ITS2 sequences with those of progeny broods and ITS2 sequences available in GenBank. Similarities with GenBank entries were assessed using FASTA search (http://www.ebi.ac.uk/fasta33/).

DNA was extracted from individual mosquitoes following a phenol-chloroform extraction (Linton et al., 2001a). Amplification of ITS2 was carried out using 5.8SF and 28SR primers (Collins & Paskewitz, 1996) and the PCR conditions outlined by Linton et al. (2001a). Products were cleaned using the QIAgen PCR purification kit (QIAgen Ltd, Sussex, England) and diluted to 1 ng/μl per 200 bp prior to cycle sequencing using the Big Dye Terminator Kit (PE Applied Biosystems, Warrington, England). An ABI 377 automated sequencer (PE Applied Biosystems) was used to read the sequences, and the data were edited and aligned using Sequencher™ version 3.1.1 (Genes Codes Corporation, Ann Arbor, Michigan) and CLUSTAL X (Thompson et al., 1997). Sequence statistics were computed using MEGA version 2.1 (Kumar et al., 2001). Sequences generated in this study are available in GenBank under the following accession numbers: Evros (AF452389-AF452406), Ioannina (AF469855), Rodopi (AF452407-AF452408) and Xanthi (AF452409-AF452410). The DNA sequence of only one individual per progeny brood was submitted. GenBank ITS2 sequences for members of the An. maculipennis complex include: An. atroparvus (Z50103; AF504237-AF504248), An. labranchiae (Z50102), An. maculipennis (Z50104; AF455818-AF455820; AF342713-AF342715; AF436065), An. martinius (AJ224329), An. melanon (AJ224330), An. meseae (AF205556; Z50105; AY050639; AF452699-AF452700; AF504197-AF504236) and An. sacharovi (Z83198). At present there are no publicly available DNA sequences for An. beklemishevi or An. subalpinus.

Link-reared mosquitoes from progeny broods and larval collections serve as voucher specimens for this work, and are retained in the mosquito collection of The Natural History Museum (NHM), London. Template DNA is also preserved at -70°C in the mosquito DNA bank of the Molecular Systematics Laboratory, Department of Entomology, NHM.

Results

Morphological identification, distribution and bionnomics

On the basis of egg morphology, eighteen progeny broods were identified as An. subalpinus. Eggs were grey, mottled and barred, typical of An. subalpinus, and no melanic eggs were recorded. Mothers of the progeny broods were captured resting in goat and sheep stables in Evros (16) and Xanthi (2), where they comprised 69.5% and 3.0% of the total catch of An. maculipennis s.l., respectively. In both locations, the species was found resting together with An. maculipennis and An. sacharovi (Linton et al., unpublished). DNA sequences were obtained for the eighteen mothers, and five additional specimens, reared from wild-caught larvae, which were identified as An. subalpinus by correlation of their DNA sequences with those of the progeny. The species was prominent in the north-eastern prefectures, being collected in Evros (18), Rodopi (2) and Xanthi (2). A single specimen was also collected in the north-western prefecture of Ioannina, where it comprised only 2.3% of the An. maculipennis complex captured there (Table 1). Based
DNA disregard with A problem al. of 479 specimens these collected the variable species In name from Marinucci prefectures an Frosinone, Group names. status Magnisia. derived of were ("Dutch (1981, specimens pure Proft form typical marshes the On Lintoo of porter two clearly and of eggs melanic that subalpinus that based support identified As the morphology generated (25.9%1 Whereas ITS2 in for mosquitoes limited 50-6%O/O by the ITS2 and of messeae The messeae the Italy. keys Weyer and freeborni of previously species of the An. messeae larvae are of bp Erithropotomas, former the 2002a, translation variation (Rackett, eggs, dark (Fig. I). taxonomic ITS2 the egg 2002b). 23.9«'/0 fragment in found barred-egg correIatioo, larvae were collected in biotic sympatry with An. mocuJipennis in Evros (River Tis Mantheas, ... identical. Melanic eggs have been reported in species of the An. macuJipennis complex other than An.
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as identifiable al. amounts data ITS2 published in A+-+T of (AJ224330) 1978). of and A, I, a on a sequence a in shorter eggs reported of of An. labranchiae and variable amounts of grey barring on the deck (Falleroni, 1926, translation in Missiroli, 1939). However, at the same time Dutch workers were also applying the name An. messeae to a species of the complex with a more northerly distribution and strongly barred eggs ("Dutch messeae"). Consequently, the name An. melanoon was proposed for the southern species with dark eggs, and it was described from Viareggio, Tuscany, Italy (Hackett, 1934). Another barred-egg form of An. messeae was subsequently described from Albania as An. maculipennis subalpinus (Hackett & Lewis, 1935). Later studies provided genetic and chromosomal evidence for the specific status of An. melanoon and An. messeae and indicated that An. subalpinus represented an alternative egg phenotype of An. melanoon (Frizzi, 1953; Kitzmiller et al., 1967). On the basis of these studies, White (1978) suggested that An. melanoon and An. subalpinus represented varieties of the same species that occurred as pure populations in limited areas, and listed An. subalpinus as a junior synonym of An. melanoon. Stegnii (1981, 1982) noted that there was no evidence to support separate species status for An. melanoon and An. subalpinus, but contrary to White (1978) he considered the former to be a melanic egg form of An. subalpinus, with apparent disregard for the priority of names.

Our data clearly show that mosquitoes reared from barred and mottled eggs identifiable as those of An. subalpinus, and those derived from typical black melanic eggs typical of An. melanoon (Proft et al., 1999; Marinucci et al., 1999) are genetically identical. Melanic eggs have been reported in species of the An. maculipennis complex other than An.
melanoon. Recent studies by M. Coluzzi (mentioned in Ramsdale & Snow, 2000) revealed that An. subalpinus occasionally oviposited batches of dark melanoon-type eggs. Additionally, correlated study of ITS2 sequences and egg morphology carried out on Romanian members of the An. maculipennis complex showed that specimens originating from melanic egg batches were An. atroparvus (G. Nicolescu, R. E. Harbach & Y.-M. Linton, unpublished). Melanic eggs of An. atroparvus were also reported from Britain by Evans (1934). From the DNA data and the reports of melanic egg batches in other species of the An. maculipennis complex, it is apparent that An. subalpinus and An. melanoon represent a single species that has polymorphic eggs.

That An. melanoon and An. subalpinus are conspecific is further supported by the reports of homossexual polytene chromosomes in these taxa (Frizzi, 1947; Stegnii, 1981) and cross-matings that result in viable, fertile offspring (in Bullini et al., 1980). It is interesting to note that An. sicaulii was deemed conspecific with An. labranchiae and synonymised with the latter on the basis of the same sort of evidence (de Zulueta et al., 1983). Extensive hybridisation studies were carried out on both Nearctic and Palaearctic members of the Maculipennis Group by Kitzmiller et al. (1967), who stated "there is not enough evidence to consider melanoon as a separate species". Curiously, of all the attempts at phylogenetic reconstruction, using DNA (Marinucci et al., 1999), chromosomes (Kitzmiller et al., 1967; White, 1978; Stegnii, 1981, 1982), cuticular hydrocarbons (Phillips et al., 1990) and hybridisation experiments (Kitzmiller et al., 1967), none have included both An. melanoon and An. subalpinus in the same study. Irrespective of the method used, or whether the taxon was identified as An. melanoon or An. subalpinus, the extremely close relationship with An. maculipennis, and the relationship of these two taxa with An. meseae, is constant (Kitzmiller et al., 1967; Stegnii, 1981, 1982; White 1987; Phillips et al., 1990; Marinucci et al., 1999).

Phylogenetic relationships based on electrophoretic enzyme differentiation of the An. maculipennis complex were reported by Cianchi et al. (1987) and later by Bullini et al. (1980) (incorporating original data of Cianchi et al.). These are the only studies known to us that purported to include both An. melanoon and An. subalpinus in the same study, but the identity of An. subalpinus (denoted as "An. sp = subalpinus") appeared to be uncertain and no indication was given of how the specimens were identified. This is significant, as mentioned earlier, it seems that the results of Cianchi et al. (1987) served as the basis for separate species recognition by Ribeiro et al. (1988). In their study, populations of An. melanoon from Massarosa, Italy and the taxa denoted An. sp = subalpinus? from Scutari Lake, Yugoslavia (close to the type locality of subalpinus in Albania) were shown to have distinct enzyme profiles. They stated that the Yugoslav population was similar to Italian populations of An. sp = subalpinus? from Pavia, Rovigo and Ferrara, but also showed similarities to populations of An. meseae from central Europe and Italy (Cianchi et al., 1987). The close genetic relationship of An. melanoon with An. maculipennis is echoed in the results of Cianchi et al. (1987) (shown again in Bullini et al., 1980), as is the basal relationship of An. meseae to this melanoon+maculipennis clade. However, contrary to the studies of other authors using An. subalpinus specimens (Kitzmiller et al., 1967; Stegnii, 1982), these authors showed that the taxon "An. sp = subalpinus?" was most closely related to An. meseae, not An. maculipennis. Although the populations were clearly distinct, it remains unclear whether the specimens they analysed were An. subalpinus, An. meseae or an undiscovered member of the complex.

On the whole of the aforementioned evidence, it is apparent that An. subalpinus and An. melanoon represent a single species that has polymorphic eggs; therefore, An. subalpinus Hackett & Lewis, 1935 is hereby formally placed in synonymy with An. melanoon Hackett, 1934. A fully integrated morphological and molecular study is underway in our laboratory to fully characterise An. melanoon, and provide reliable diagnostic characters to differentiate this species from other members of the An. maculipennis complex.

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References


**Table 1.** Collection sites in Greece, listing site co-ordinates, collection dates and numbers of specimens sequenced from each site. *Resting collections, females used to obtain progeny broods; Larval collections, larvae link-reared to adults.*

<table>
<thead>
<tr>
<th>Prefecture</th>
<th>Exact locality</th>
<th>Co-ordinates</th>
<th>Date</th>
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<tr>
<td>Evros (NE)</td>
<td>Monastiraki, Alexandroupolis</td>
<td>40°51'N, 25°53'E</td>
<td>09.VI.OIL</td>
<td>16</td>
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<td></td>
<td>River Tis Mantheas, Itea</td>
<td>40°58'N, 26°05'E</td>
<td>09.VI.OIL</td>
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<td>River Erithropotomos, Didymoticho</td>
<td>41°21'N, 26°30'E</td>
<td>10.VI.OIL</td>
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<tr>
<td>Ioannina (NW)</td>
<td>Vella's Springs</td>
<td>39°53'N, 20°36'E</td>
<td>14.VII.OIL</td>
<td>1</td>
</tr>
<tr>
<td>Rodopi (NE)</td>
<td>Nesti-Krovilli, Maronia</td>
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<tr>
<td>Xanthi (NE)</td>
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<td>41°01'N, 25°08'E</td>
<td>08.VI.OIL</td>
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</table>
**Fig. 1.** A 482 bp alignment of the ITS2 sequences of twenty-three mosquitoes derived from *An. subalpinus*-type egg (labelled *subalpinus*) and two sequences of *An. melanoon* from melanic eggs, i.e. Proft et al. (1999) (not entered in GenBank) and the GenBank entry AJ224330 of Marinucci et al. (1999). Note that the sequence Proft et al. is 3 bases shorter in the reverse primer sequence, and the sequence of Marinucci et al. sequence is 50 bases shorter than ours as a result of different primers being used. Amplification primers used in the present study are underlined. Dashes (-) indicate missing data and dots (.) indicate identical bases within the alignment.