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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. messeae* Falleroni, *An. sacharovi* Favre and *An. sicaulti* Roubaud. White proposed the suppression of *alexandraeschingarevi, lewisi* and *selengensis* and the resurrection of two nominal species (*martinius* and *sicaulti*) 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. sicaulti* was conspecific with *An. labranchiae*, and the former name was synonymised with the latter (de Zulueta *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. messeae, An. sacharovi* and *An. subalpinus*. These species are notoriously difficult to distinguish morphologically in the adult and larval stages and, despite differential chromosome and isoenzyme 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. messeae* (Pandazis, 1935; Shannon, 1935; Hackett & Missiroli, 1935). Following a morphological study of eggs from Kavala (Macedonia), Hackett & Lewis (1935) confirmed the presence of *An. messeae, An. maculipennis* (as *An. typicus* Hackett & Missiroli) and *An. subalpinus*. The status of *An. messeae* 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. messeae* (Livadas & Sphangos, 1940). Despite earlier suggestions to the contrary, Bates (1940) could not confirm the presence of *An. messeae* in Greece, and thus, in his 1942 paper, Weyer 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 & Darsie (1993) and Ramsdale & Snow (2000) suggested that *An. messeae* 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. messeae* 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. melanoon*, White (1978) indicated that *An. maculipennis*, *An. sacharovi* and *An. melanoon* 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* (Cianchi *et al.*, 1987; Ribeiro *et al.*, 1988). Given the recent incrimination of *An. subalpinus* as a secondary vector in the Biga Plains, Turkey (Alten *et al.*, 2000) and documented malaria cases in neighbouring Greece in recent years (Linton *et al.*, 2001b), it is important to determine whether *An. subalpinus* and/or *An. melanoon* 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, Alexandropoulis, 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. melanoon* (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 (AF469853), 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. melanoon* (AJ224330), *An. messeae* (AF305556; 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 bionomics

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

on DNA correlation, larvae were collected in biotic sympatry with *An. maculipennis* in Evros (River Tis Mantheas, Itea and River Erithropotomas, Didymoticho) and in Rodopi (Nesti-Krovilli, Maronia and Loutros village). The species was not found in larval surveys in the prefectures of Florina, Fthiotida, Lakonia or Magnisia.

Intraspecific variation in the ITS2 sequences

No intraspecific variation was found in the sequences obtained from the twenty-three specimens collected in Greece; all exhibited the same ITS2 haplotype (Fig. 1). Previous studies of intraspecific variability in ITS2 sequences for members of the Maculipennis Group have shown it to be negligible for the Nearctic species, *An. freeborni* Aitken and *An. hermsi* Barr & Guptavanij (Porter & Collins, 1991), and three Palaearctic species, *An. atroparvus*, *An. maculipennis* and *An. messeae* (Linton *et al.*, 2001b, 2002a, 2002b). Inclusive of primers (43 bp), the size of the ITS2 fragment was 482 bp and percentage GC content of the whole fragment was 51.1% (25.9% A, 23.9% T, 26.3% C, 23.9% G). This falls within the range of 50-60% previously reported for ITS2 regions in other members of the Maculipennis Group (Porter & Collins, 1991; Marinucci *et al.*, 1999; Proft *et al.*, 1999; Linton *et al.*, 2001b, 2002a, 2002b).

Identification based on ITS2 sequence data

No sequence data for *An. subalpinus* were generated during previous DNA studies of the *An. maculipennis* complex (Marinucci *et al.*, 1999; Proft *et al.*, 1999; Linton *et al.*, 2001b, 2002a, 2002b). However, an *An. melanoon* ITS2 sequence was submitted to GenBank (AJ224330) by Marinucci *et al.* (1999) and a consensus sequence for this species is available in the published alignment of Proft *et al.* (1999) (not entered in Genbank). Proft *et al.* obtained sequences for the ITS2 region from specimens collected in Frosinone, Italy and Evros, Greece, and the ITS2 sequences generated by Marinucci *et al.* were obtained from specimens collected in Lazio, Italy. In these two studies, specimens of *An. melanoon* were identified on the basis of egg morphology using the keys of Weyer (1942) and Angelucci (1955), respectively. These keys differentiate *An. melanoon* from other Palaearctic species of the complex by its uniformly black eggs.

Based on correlation of the ITS2 sequences with those published previously (Marinucci *et al.*, 1999; Proft *et al.*, 1999), the twenty-three specimens identified as *An. subalpinus* on the basis of egg morphology were identified as *An. melanoon* (Fig. 1). A FASTA search revealed highest homology with the sequence for *An. melanoon* submitted to GenBank (AJ224330) by Marinucci *et al.* and the sequence for this species published by Proft *et al.* However, the sequences amplified by these authors were slightly shorter than ours. Whereas we sequenced 482 bases, they sequenced only 432 and 479, respectively. As shown in Fig. 1, the 479 bp sequence of Proft *et al.* amplified from black-egg *An. melanoon* is identical to the homologous sequence we obtained from specimens derived from *subalpinus*-type eggs. When the 432 bp sequence of Marinucci *et al.* is aligned with the 479 bp region, a single A↔T substitution is noted at base 351 (Fig. 1). These alignments show that the ITS2 sequences of specimens derived from *subalpinus*-type eggs and black *melanoon*-type eggs share a minimum of 99.54% identity, clearly indicating that these species are conspecific.

Discussion

The specific status of *An. subalpinus* has always been questionable. The origins of this taxonomic problem stem from the earliest studies of the *An. maculipennis* (reviewed by White, 1978). *Anopheles messeae* was originally described from specimens (now non-extant) collected in the Pontine marshes which previously existed near Rome, Italy (Falleroni, 1926). The species was described as having characteristically dark eggs, with larger floats than *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 oviposit 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 homosequential 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. sicaulti* was deemed conspecific with *An. labranchiae* and synonomised 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. messeae*, 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 purport 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. messeae* 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. messeae* 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. messeae*, not *An. maculipennis*. Although the populations were clearly distinct, it remains unclear whether the specimens they analysed were *An. subalpinus*, *An. messeae* 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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Table 1. Collection sites in Greece, listing site co-ordinates, collection dates and numbers of specimens sequenced from each site. [R]Resting collections, females used to obtain progeny broods; [L]Larval collections, larvae link-reared to adults.

Prefecture	Exact locality	Co-ordinates	Date	n =
Evros (NE)	Monastiraki, Alexandropoulis	40°51'N, 25°53'E	09.VI.01[R]	16
	River Tis Mantheas, Itea	40°58'N, 26°05'E	09.VI.01[L]	1
	River Erithropotomos, Didymoticho	41°21'N, 26°30'E	10.VI.01[L]	1
Ioannina (NW)	Vella's Springs	39°53'N, 20°36'E	14.VII.99[L]	1
Rodopi (NE)	Nesti-Krovilli, Maronia	40°54'N, 25°31'E	08.VI.01[L]	1
	Loutros village	40°35'N, 22°24'E	09.VI.01[L]	1
Xanthi (NE)	Selino village	41°01'N, 25°08'E	08.VI.01[R]	2

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.

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                  1111111111222222222233333333334444444444555555555566666
                  12345678901234567890123456789012345678901234567890123456789012345
subalpinus        ATCACTCGGCTCGTGGATCGATGAAGACCGCAGCTAAATGCGCGTCACAATGTGAACTGCAGGAC
Proftetal         .............................................................
AJ224330          ---------------------------------------------.....------------

                                                  1111111111111111111111111111111
                  66667777777777888888888899999999990000000000111111111122222222223
                  67890123456789012345678901234567890123456789012345678901234567890
subalpinus        ACATGAACACCGATAAGTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGTACACATTTT
Proftetal         .............................................................
AJ224330          .............................................................

                  1111111111111111111111111111111111111111111111111111111111111111
                  3333333334444444444555555555566666666667777777777888888888899999
                  1234567890123456789012345678901234567890123456789012345678901234
subalpinus        TGAGTGCCTATATTTGACTATCCAAGTCAAACTACGTACCTCCGTGTACGTGTATGATGATGAAA
Proftetal         .............................................................
AJ224330          .............................................................

                  1111222222222222222222222222222222222222222222222222222222222222
                  9999000000000011111111112222222222333333333344444444445555555556
                  67890123456789012345678901234567890123456789012345678901234567890
subalpinus        GAGTTTGGAAACACCATCCTTCTCTTGCATTGAAAGCGCAGCGTGTAGCAGCCCCAGGTTTCAAC
Proftetal         .............................................................
AJ224330          .............................................................

                  2222222222222222222222222222222222222233333333333333333333333333
                  6666666667777777777888888888899999999990000000000111111111122222
                  1234567890123456789012345678901234567890123456789012345678901234
subalpinus        TTGCAAAGTGGCCATGGGGCCGACACCTCACCACCATCAGCGTGCTGTGTAGCGTGTTCGGCCCA
Proftetal         .............................................................
AJ224330          .............................................................

                  3333333333333333333333333333333333333333333333333333333333333333
                  2222333333333344444444445555555555666666666677777777778888888889
                  6789012345678901234567890123456789012345678901234567890123456789
subalpinus        GTTCGGTCATCGTGAGGCGTTACCTATCGGGGAAGCACACCCTGTTGCGCGTATCTCATGGTTAC
Proftetal         .............................................................
AJ224330          ........................T....................................

                  3333333334444444444444444444444444444444444444444444444444444444
                  9999999990000000000111111111122222222233333333334444444444555555
                  1234567890123456789012345678901234567890123456789012345678901234
subalpinus        CTAACCATAGCAGCAGAGTTACAACACCAGCTTCTAGCAGCGGGAGCTCATGGGCCTCAAATAAT
Proftetal         .............................................................
AJ224330          .............................................................

                  44444444444444444444444444
                  55556666666666777777777888
                  67890123456789012345678901
subalpinus        GTGTGACTACCCCCTAAATTTAAGCAT
Proftetal         ........................---
AJ224330          ..........................
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