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Further molecular and morphological support for the formal synonymy of *Anopheles subalpinus* Hackett & Lewis with *An. melanoon* Hackett

Daniela Boccolini, Marco Di Luca, Marino Marinucci and Roberto Romi
Laboratorio di Parassitologia, Istituto Superiore di Sanità Viale Regina Elena, 299
00161, Roma E-mail: romi@iss.it

Abstract

The taxonomic status of *Anopheles subalpinus* Hackett & Lewis, a member of the Palaearctic *Anopheles maculipennis* complex, was investigated by re-examining specimens from a study conducted in 1986. The presence of two different taxa was detected in the sample by sequencing ITS2 and a mitochondrial fragment (310 bp) of the COI gene.

The re-examination of 71 larval specimens for the biometric character of the branches of setae 2-IV, V also indicated the presence of two species within the sample. These species were identified as *An. messeae* and *An. melanoon*. Our results confirm the findings of Linton *et al.* (2002) that *An. subalpinus* is simply an alternative egg phenotype of *An. melanoon*.

Introduction

In White's (1978) review of the *Anopheles maculipennis* complex, nine sibling species were recognised in the Palaearctic Region: *An. atroparvus* Van Thiel, *An. beklemishevi* Stegnii & Kabanova, *An. labranchiae* Falleroni, *An. maculipennis* Meigen, *An. martinius* Shingarev, *An. melanoon* Hackett, *An. messeae* Falleroni, *An. sacharovi* Favre, *An. sicaulti* Roubaud and, in addition, a variety of *An. melanoon*, i.e. *An. subalpinus* Hackett & Lewis. However, later studies revealed that *An. sicaulti* is a geographical variety of *An. labranchiae* (de Zulueta *et al.*, 1983), and *An. subalpinus* is a synonym of *An. melanoon* (Linton *et al.*, 2002).

On the basis of the structural characters of its eggs, *An. subalpinus* was first described as a "new variety" of *An. maculipennis* from Albania (Hackett & Lewis, 1935). The *An. subalpinus* eggs were described as "black barred with a pale background and smooth floats", distinguishing them from the eggs of *An. messeae*, which are darker and have rough floats, and from the completely dark *An. melanoon* eggs. The same authors reported *An. subalpinus* in Spain, France and Italy.

Although the egg morphology of this taxon is more similar to that of *An. messeae* than that of *An. melanoon*, the results of successive morphological studies on larvae and genetic analysis, i.e. homosequentiality of polytenic chromosomes and fertility of hybrids, suggested that *An. subalpinus* represents "an alternative egg phenotype of *An. melanoon*, the two forms of eggs apparently being intergrading conspecific varieties that occur as pure populations in limited geographical areas" (White, 1978). Nonetheless, isoenzymatic studies carried out in the 1980s on Italian and Yugoslavian populations, which were morphologically referred to as *An. subalpinus*, elevated this taxon to the species level (Bullini *et al.*, 1980; Bianchi Bullini *et al.*, 1980; Cianchi *et al.*, 1981, 1987).

Recently, Linton *et al.* (2002), on the basis of evidence provided by ITS2 sequences, formally placed this taxon in synonymy with *An. melanoon*. To re-enforce the findings of Linton *et al.* we analysed, using both molecular and morphological approaches, some of the remaining adult specimens and all of the larval specimens of a sample from the former Yugoslavia (Scutari) investigated by Cianchi *et al.* (1987). As a molecular marker for adult identification, we used the internal transcribed spacer 2 (ITS2), which is a reliable tool for species identification within the *An. maculipennis* complex (Marinucci *et al.*, 1999). A 310 bp fragment of the mitochondrial cytochrome oxidase I gene (COI), never studied before in this complex, was also used to confirm a possible heterogeneity in species composition of the Scutari sample (Zhang & Hewitt, 1996). The larval sample was also re-examined for the biometric character of the branches of setae 2-IV,V (antepalmate hairs of Bates, 1939).

Materials and Methods

The sample analysed by Cianchi *et al.* (1987) was collected in 1986 along the northern side of Lake Scutari in Montenegro (Serbia and Montenegro, former Yugoslavia), which borders on Albania, the type locality of *An. subalpinus*. Gravid and half-gravid females were induced to lay eggs for identification; the larvae obtained from single batches of eggs were reared and 71 fourth-instar larvae mounted on microscope slides, and stored in suitable boxes at room temperature. Females were maintained frozen at -20°C in our laboratory.

In 2002, 18 adult females of that sample were processed for ITS2 sequencing. Genomic DNA was extracted from single specimens according to the procedure of Coen *et al.* (1982). PCR was performed as in Marinucci *et al.* (1999), and the PCR products were directly sequenced at MWG Biotech AG (Ebersberg, Germany). The ITS2 sequences were deposited in GenBank: accession numbers AY238411 (*An. melanoon)* and AY238421 (*An. messeae*). Six of the females were also processed for a mtDNA 310 bp fragment, which is the intergenic region between COI and the $tRNA^{leu}$ genes and corresponding to positions 1910-2219 of *An. gambiae* mtDNA (Beard *et al.*, 1993). This fragment was amplified using a pair of primers, UEA9 (5'-GTA AAC CTA ACA TTT TTT CCT CAA CA-3') and UEA10 (5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3'), described by Zhang & Hewitt (1996). The initial denaturation at 94°C for 4 min was followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 1 min, and extension at 72°C for 40 s, with a final elongation time of 7 min at 72°C. Sequences have been submitted to the GenBank, accession numbers AY258206-09 and AY258167-68.

All amplicons were analysed by electrophoresis in 1.5% agarose gel containing ethidium bromide. The products were then purified using Microcon YM 100 (Millipore Corp., Bedford, MA, USA) and sequenced at MWG Biotech AG (Ebersberg, Germany) using the same primers as those used for PCR in both forward and reverse direction.

The original microscope slides of fourth-instar larvae were re-examined. The morphological study was performed considering the sum of the branches of seta 2 on abdominal segments IV and V. The cut-off value for distinguishing the taxa was calculated as the median of the distribution of the number of branches of the entire sample.

Results and Discussion

Sequencing of 18 adult females from the sample collected at Lake Scutari in 1986 yielded two different ITS2 fragments, one of 432 bp shared by 8 specimens and the other of 435 bp shared by 10 specimens, both with 100% nucleotide identity. The sequences showed a high degree of homology with respect to the sequences considered as a reference: the 432 bp fragment shared 99.77% identity with the sequence from specimens of *An. melanoon* collected in the province of Lucca, Toscana (AY238408), which is near the type locality of Viareggio, Tuscany, Italy. The 435 bp fragment shared 99.54% identity with that of *An. messeae* collected in central Italy (province of Rieti, Lazio, AY238419). The 432 bp fragment showed a single A→T transversion at position 301 with respect to *An. melanoon* from Lucca; the 435 bp fragment showed a G→A transition at position 362 and a G→C transversion at position 382 with respect to *An. messeae* from Rieti.

Alignment of the 310 bp mt DNA fragments (Table 1) obtained from 6 females showed 8 variable sites, all substitutions, at position 30, 36, 42, 51, 60, 66, 186 and 228. The variable sites clearly defined two main haplotypes (named 1 and 2, respectively), the second of which presented a polymorphic site at position 228. With respect to the amino acid code, all of the nucleotide substitutions were synonymous, thus generating the same amino acid haplotype. The six specimens were identified by comparison of their ITS2 sequences with the reference sequences AY238408 and AY238419: the two sharing haplotype 1 as *An. melanoon* and the four sharing haplotype 2 as *An. messeae*, respectively.

The analysis of 71 fourth-instar larvae revealed a marked heterogeneity in the distribution of the number of branches of seta 2-IV,V. As illustrated in Figure 1, the distribution showed two distinct peaks, one at 16 branches and the other at 20 branches, suggesting the presence of at least two different taxa. We arbitrarily considered the median number of branches of the entire sample (n=18) as a cut off for distinguishing the two taxa. Of the 71specimens, 40 had 18 or fewer branches, with a mean of 15.90 ± 1.82 SD, and 31 had more than 18 branches, with a mean of 20.87

± 1.58. These mean values are consistent with those reported in the literature for *An. melanoon/An. subalpinus* and *An. messeae,* respectively (Bates, 1939).

Cianchi *et al.*, (1987) reported that the sample from Scutari was homogeneous in terms of the morphology of both the eggs and the larvae, yet based on the isoenzymatic analysis the authors concluded that there were two different species, *An. subalpinus* and *An. melanoon.* Our analysis confirmed the presence of two sympatric taxa in the same sample, even though the biometric character cannot be used to confidently distinguish *An. messeae* from *An. melanoon/An. subalpinus* because of the overlapping range of branching (Boccolini *et al.*, 1986).

The analysis of the mtDNA 310 bp fragment strongly supported the presence of two species, and ITS2 sequences unambiguously identified them as *An. messeae* and *An. melanoon.* On the basis of these results, it is apparent that egg morphology is not reliable for distinguishing *An. messeae* and *An. melanoon* and that screening enzyme patterns may not always allow for the correct identification of *An. messeae.*

In the studies conducted by Bullini *et al.* (1980) and Cianchi *et al.* (1981), the enzymatic pattern attributed to *An. subalpinus* may have reflected the presence of intraspecific genetic polymorphism in *An. messeae.* Significant differences in the frequency of chromosomal inversions among populations of the latter species were detected by Stegnii (1982) and, more recently, we found a noteworthy polymorphism in ITS2 sequences of *An. messeae* populations from different geographic areas (data not shown).

In conclusion, the finding that the Lake Scutari sample did not contain a third taxon beyond *An. messeae* and *An. melanoon* confirms that *An. subalpinus* is simply an alternative egg phenotype of *An. melanoon,* as previously proposed by White (1978), and more recently demonstrated by Linton *et al.* (2002) by analysing samples from Greece.

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AY258167 GTAAACCTAA CATTTTTTCC TCAACATTTT TTAGGATTAG CGGGAATACC ACGACGATAT TCTGACTTTC CTGATAGTTA
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AY258207 .......... .......... .........C .....G.... .A........ T........C .....T.... ..........
AY258208 .......... .......... .........C .....G.... .A........ T........C .....T.... ..........
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AY258168 .......... .......... .......... .......... .......... .......... .......... ..........
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AY258168 .......... .......... .......... .......... .......... .......... ..........
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Table 1. A 310 bp alignment of the mtDNA COI gene fragment sequences of two *Anopheles melanoon* (AY258167-8) and of four *An. messeae* (AY258206-9) derived from adult females collected at Lake Scutari (Montenegro) in 1986, and at that time identified as *An. subalpinus* by egg morphology. Primer regions are in bold and underlined. Dots (.) indicate identical bases and bases in bold the variability between the sequences.

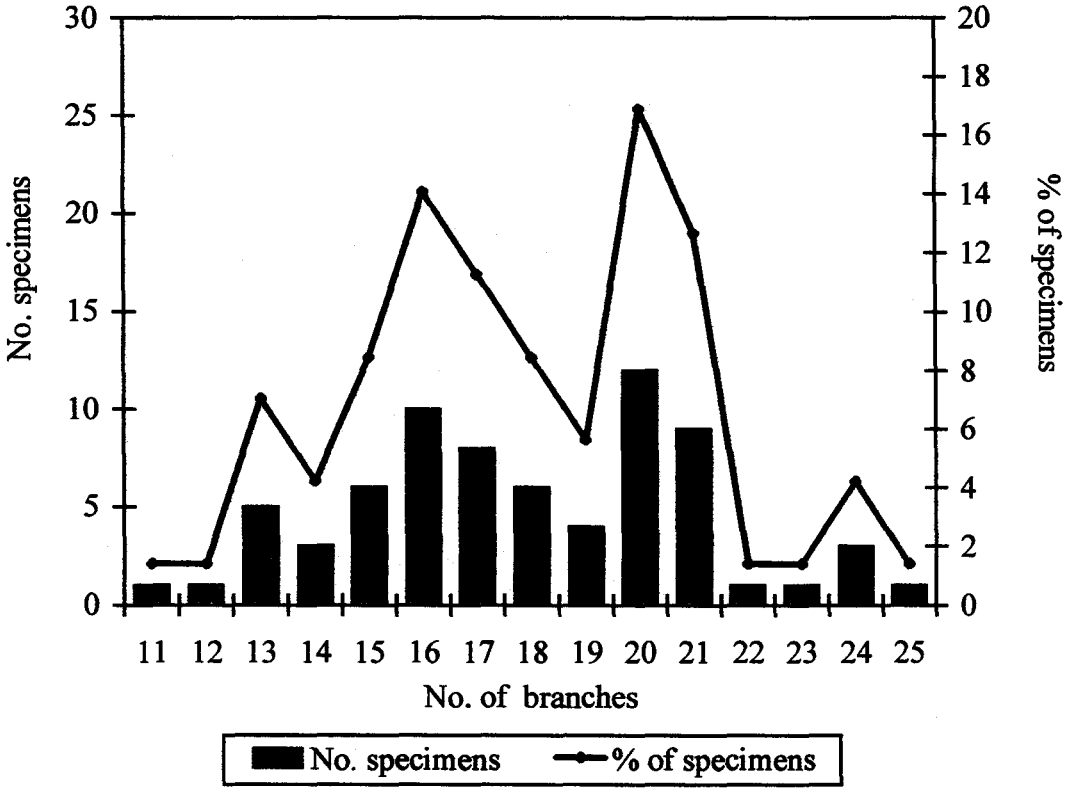

Figure 1. Distribution of the number of branches of the seta 2-IV, V (antepalmate hairs of IV and V abdominal segments) in a sample of 71 fourth-instar larvae collected at Lake Scutari (Montenegro) in 1986.