Bulletin of Entomological Research (2004) **94**, 525–535 DOI: 10.1079/BER2004330

Mosquitoes of the *Anopheles maculipennis* group (Diptera: Culicidae) in Romania, with the discovery and formal recognition of a new species based on molecular and morphological evidence

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Abstract

Mosquitoes of the *Anopheles maculipennis* group were collected in five districts of Romania (Constanţa, Giurgiu, Ilfov, Mehedinţi and Suceava) between March 2000 and June 2003. Two hundred and ninety-seven specimens were identified by molecular methods. Nuclear rDNA ITS2 sequences of 178 specimens were compared with GenBank sequences for nine known Palaearctic species of the group, and 119 specimens were identified using an ITS2 PCR–RFLP assay developed during the study. Five genetically distinct species of the group were identified: *A. atroparvus* van Thiel, *A. maculipennis* Meigen, *A. melanoon* Hackett and *A. messeae* Falleroni and a previously unrecognized species. The new species, herein formally described and named *A. daciae* **sp. n.**, was collected in the Black Sea coastal region and plains adjacent to the Danube River in southern Romania. *Anopheles daciae* is most similar to and sympatric with *A. messeae*. It is contrasted with *A. messeae* and characterized on the basis of unique nuclear ITS2 and mitochondrial COI DNA sequences and morphological characters of the eggs. The larval, pupal and adults stages of the two species were also compared, but no reliable characters were found to distinguish them. It seems likely that *A. daciae* is more widespread in eastern Europe and the Balkan States, and could be responsible for malaria transmission in these regions that is currently attributed to *A. messeae*. *Anopheles melanoon* is reported from Romania for the first time.

Introduction

DNA studies have proven to be invaluable for resolving taxonomic questions, especially at the species-group level. DNA sequencing of the mitochondrial cytochrome c oxidase gene (COI) (Linton *et al.*, 2001a, 2003; Sedaghat *et al.*, 2003a) and molecular methods using nuclear rDNA ITS2 sequences have been employed previously to identify nine species of the *A. maculipennis* group (Diptera: Culicidae) in the Palaearctic Region (Marinucci *et al.*, 1999; Proft *et al.*, 1999; Linton *et al.*, 2001a, 2002a,b,c, 2003, 2004; Romi *et al.*, 2002; Boccolini *et al.*, 2003; Sedaghat *et al.*, 2003a,b; Gordeev *et al.*, 2004). These species include *A. atroparvus* van Thiel, *A. beklemishevi* Stegnii & Kabanova, *A. labranchiae* Falleroni, *A. maculipennis* Meigen, *A. martinius* Shingarev, *A. melanoon* Hackett, *A. messeae* Falleroni, *A. persiensis* Linton, Sedaghat

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& Harbach and *A. sacharovi* Favre. White (1978) suggested that *A. lewisi* Ludlow, described from Siberia, may be synonymous with *A. messeae* or *A. beklemishevi*, but this nominal form is still regarded as a valid species because its identity has not been resolved. Consequently, ten taxonomic species of the *A. maculipennis* group are currently formally recognized in the Palaearctic Region.

Historical transmission of malaria in Romania (as in other areas of Europe) is attributed principally to members of the *A. maculipennis* group (Martini & Zotta, 1934; Zotta, 1938; Zotta *et al*., 1940). A review of Romanian literature (Nicolescu, 1995) revealed that *A. maculipennis, A. messeae*, *A. sacharovi* and *A. atroparvus* have been identified in Romania based on egg morphology (see Nicolescu, 1995 for numerous references, and Nicolescu, 1996). *Anopheles atroparvus*, *A. labranchiae* and *A. sacharovi* are the most efficient malaria vectors of the *A. maculipennis* group in the Palaearctic Region (Jetten & Takken, 1994), and *A. maculipennis* and *A. melanoon* are believed to play a role when present in high densities (Zotta, 1938; Nicolescu, 1996; Alten *et al*., 2000).

The vector status of *A. messeae* has been the subject of debate for some time. This species is not considered to be a vector in northwestern Europe (Jetten & Takken, 1994), but it was previously considered to be a vector in eastern Europe and western Asia (Martini & Zotta, 1934; Zotta, 1938; Bruce-Chwatt & de Zulueta, 1980) and was recently incriminated as the main vector of resurgent malaria in the Ukraine and Russia (Nikolaeva, 1996).

Anopheles messeae was originally named from specimens collected in the Pontine marshes near Rome, Italy (Falleroni, 1926). It was described as having characteristically dark eggs, with larger floats than *A. labranchiae* and variable amounts of grey barring on the deck (Falleroni, 1926, translation in Missiroli, 1939). At the same time, Dutch workers also applied the name *messeae* to a species of the *A. maculipennis* group with a more northerly distribution and strongly barred eggs ('Dutch *messeae*'). Consequently, the name *A. melanoon* was proposed for the southern species with dark eggs, and it was formally described from Viareggio, Tuscany, Italy (Hackett, 1934). Another barred-egg form was subsequently described from Albania as *A. maculipennis subalpinus* (Hackett & Lewis, 1935). Later studies provided genetic and chromosomal evidence for the specific status of *A. melanoon* and *A. messeae* and indicated that *A. subalpinus* represented an alternative egg phenotype of *A. melanoon* (Frizzi, 1953; Kitzmiller *et al*., 1967; Linton *et al*., 2002c; Boccolini *et al*., 2003).

Enzyme differentiation of members of the Palaearctic *A. maculipennis* group by Cianchi *et al*. (1987) resulted in the recognition of *A. melanoon* from Massarosa, Italy and a questionable species tentatively referred to as *A. subalpinus* from Scutari Lake, Yugoslavia. Cianchi *et al*. stated that the Yugoslav population was similar to Italian populations of *A. subalpinus* [= *A. melanoon*] from Pavia, Rovigo and Ferrara, but also showed similarities to populations of *A. messeae* from central Europe and Italy. Although the populations were distinct, it is not clear whether the specimens were *A. melanoon* or *A. messeae*. Given the genetic similarity of the new species described below to *A. messeae*, it would seem that this species, 'tentatively assigned to *A. subalpinus*', may have been unwittingly included in the enzyme study of Cianchi *et al*. (1987). An inversion polymorphism in the polytene chromosomes reported in *A. messeae* from areas east and west of the Caspian Sea (Stegnii, 1982), and reported differences in the vector status of *A. messeae* between these regions (Martini & Zotta, 1934; Zotta, 1938; Bruce-Chwatt & de Zulueta, 1980; Jetten & Takken, 1994; Nikolaeva, 1996), may be explained by the failure to distinguish the new species (see below) from *A. messeae*. [While this paper was in press, Di Luca *et al*. (2004) reported polymorphic DNA sequences in European populations of *A. messeae*.]

Variation noted between egg batches of *A. messeae* by the first author (G.N.) over a period of several years provided the impetus for the present study. Given the availability of sequences for the second internal transcribed spacer (ITS2) of nuclear ribosomal DNA for all Palaearctic members of the *A. maculipennis* group (except *A. lewisi*, see above) for comparison, this region was sequenced from specimens collected in Romania to assess this variation and to determine the composition and distribution of the group to aid control strategies should they become necessary in future.

Materials and methods

Mosquitoes

Mosquitoes resting in animal shelters were collected between March 2000 and June 2003 at 16 sites in five widely separated districts of Romania, namely Suceava (north-east), Mehedinţi (south-west), Giurgiu (south), Ilfov (south) and Constanţa (east) on the Black Sea coast (fig. 1; table 1). Mosquitoes were identified to group by morphology, and to species by analysis of their nuclear rDNA ITS2 sequences. In addition to specimens listed herein, the progeny of many females unequivocally identified to species during this study are deposited in The Natural History Museum in London and the Cantacuzino Institute in Bucharest.

Molecular studies

Polymerase chain reaction (PCR) amplification was carried out using either 2 µl of template of DNA or by placing a single leg directly in the PCR reaction, following the protocol of Linton *et al*. (2001b). Amplification of the ITS2 was achieved using the 5.8SF and 28SR primers (Collins & Paskewitz, 1996), and the primers C1-J-1718 and C1-N-2191 (Simon *et al*., 1994) were used to amplify a portion of the mitochondrial cytochrome oxidase I gene (COI). Polymerase chain reaction products were amplified using the reaction and thermocycler parameters described by Linton *et al*. (2001b), and cleaned using a commercially available PCR purification kit (QIAgen Ltd, Sussex, England). Sequencing was carried out in both directions using the Big Dye Terminator Kit (PE Applied Biosystems, Warrington, England) and chromatograms read by an ABI 377 automated sequencer (PE Applied Biosystems). Sequences were edited and aligned using Sequencher™ version 3.1.1 (Genes Codes Corporation, Ann Arbor, Michigan) and CLUSTAL X (Thompson *et al*., 1997). Similarity with ITS2 sequences for other members of the *A. maculipennis* group available in GenBank was assessed using FASTA search (http://www.ebi.ac.uk/fasta33/). Conversion of COI nucleotide fragments to amino acids, using the insect mitochondrial code, and calculations of intraspecific sequence variability were carried out using MEGA2 (Kumar *et al*., 2001). After sequencing, the template DNA was dried and retained at –70°C in the Molecular Systematics Laboratory, Department of Entomology, The Natural History Museum, London, for future reference.

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Fig. 1. Map of Romania showing the five districts where mosquitoes were collected in 2000–2003. 1, Suceava District (*Anopheles maculipennis*); 2, Mehedinţi District (*A. daciae*, *A. maculipennis*, *A. messeae*); 3, Giurgiu District (*A. atroparvus*, *A. daciae*, *A. maculipennis*, *A. messeae*); 4, Ilfov District (*A. atroparvus*, *A. daciae*, *A. maculipennis*); 5, Constanţa District (*A. atroparvus*, *A. daciae*, *A. maculipennis*, *A. melanoon*).

For restriction fragment length polymorphisms (RFLP), total reaction volumes were 20 µl. Four µl of the ITS2 PCR product was added to a clean 200-µl PCR tube containing 0.5 U of *Cfo* I (*Hha* I) (CGC↓G) (Roche Molecular Biochemicals Ltd, Sussex, England), 2 µl of the manufacturers incubation buffer (Buffer L) and 13.5 µl ddH_2O. The tubes were incubated at the optimal enzyme activity temperature of 37°C in a thermocycler for a minimum of 2 h to ensure full cutting of the fragments. Restriction fragments were visualized on 3% agarose gels containing 1% ethidium bromide.

Morphological studies

Morphological studies were carried out on eggs and siblings from progeny broods identified from DNA sequence. Progeny of wild-caught females were individually reared to obtain adults with associated fourth-instar larval and pupal exuviae. A number of fourth-instar larvae were also retained for study. At least ten eggs from each brood were stored either in Bouin's solution (BDH, Poole, England), 80% ethanol or 2% formalin. With the exception of samples stored in 80% ethanol, all eggs were first rinsed twice in 20% ethanol to remove any fixative then held in 20% ethanol overnight. The samples were then dehydrated through a graded ethanol series in 5% increments to 100%. The eggs were held for 2–3 h in each concentration of ethanol. Fully dehydrated eggs of individual progeny broods were pipetted into millipore capsules (Agar Scientific Ltd, Stansted, England) and stored in a vial containing a molecular sieve and cold 100% ethanol before being critical-point dried. Eggs were 'tapped' out of capsules onto specimen mounts (one brood per mount) covered with Sticky Tabs (Agar Scientific Ltd, Stansted, England), and individual eggs were re-positioned, if necessary, using a single-hair artist's brush. Specimens were then sputter-coated with palladium and examined and photographed in a Philips XL-30 scanning electron microscope.

Measurements and counts were made from at least ten specimens. The morphological terminology follows Harbach & Knight (1980).

Results and Discussion

Species identification based on ITS2 sequence

One hundred and seventy-eight sequences were generated (table 1) and identified to species based on similarity with the ITS2 sequences for members of the *A. maculipennis* group available in GenBank. Eighty specimens were identified as four currently recognized species, including *A. atroparvus* (30 specimens), *A. maculipennis* (32 specimens), *A. melanoon* (1 specimen) and *A. messeae* (17 specimens). The ITS2 fragments of these species were 487, 472, 482 and 485 bp, respectively, inclusive of primers (43 bp). Differences in the length of the fragments were accounted for in full by the variability in the ITS2 spacer region, not in the 5.8S or 28S flanking genes. The other 98 sequences were identical, but did not correspond to the sequence of any known member of the *A. maculipennis* group. The sequences most resembled those of *A. messeae* in having a fragment length of 485 bp, but the nucleotide sequence differed by five fixed variable sites (1.03%) in all specimens analysed, indicating that they belong to a previously unrecognized member of the group (fig. 2), which is formally described and named *A. daciae* below and hereafter referred to by this name. Sequences generated in this study are available in GenBank under the accession numbers listed in table 2.

Pairwise distances using pairwise deletion are given in table 3. *Anopheles daciae* shares 99.0% sequence identity with *A. messeae*, making these taxa the most closely related members of the *A. maculipennis* group. *Anopheles maculipennis* showed 3.18% sequence divergence from both of these species, and 3.40% dissimilarity with *A. melanoon*. *Anopheles melanoon* showed fractionally higher sequence similarity to *A. messeae* (96.3%) than *A. daciae* (96.0%). *Anopheles atroparvus* is most distant from the other four species, with sequence divergence ranging from 7.22 to 7.87%.

FASTA searches revealed that the ITS2 sequences of *A. maculipennis* share 100% homology with specimens from Greece (AF455818–921, AF342713–5, AF469847–52, AF485808–10, AF533552–82, AF485806; Linton *et al.*, 2003, 2004) and Iran (AY137781–816, AF536332–7; Sedaghat *et al.*, 2003b). The sequences of *A. atroparvus* are identical with those of specimens from England (Linton *et al.*, 2002b), Italy (Z50103; Marinucci *et al.*, 1999) and Italy and Spain (AY365007; Proft *et al.*, 1999). The single specimen of *A. melanoon* exactly matches specimens from Greece (AF452389–406, AF469853, AF452407–10; Linton *et al.*, 2004) and the sequences of *A. messeae* are identical to those of specimens from Greece (AF342711, AF342712; Linton *et al.*, 2001a, 2002a, 2004) and England (AF504197–236; AF452699–700; Linton *et al.*, 2002b). As noted above, the ITS2 sequence of *A. daciae* shares 99.0% identity with *A. messeae*.

Species diagnosis by PCR–RFLP assay

Sequences were screened for 113 restriction enzyme cutting sites to facilitate choice of a simple, single enzyme assay that would produce species-diagnostic banding patterns. Due to the relatively short ITS2 fragment sizes and

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Table 1. Locality data and identification of species of the *Anopheles maculipennis* group collected in Romania. Identifications are based on ITS2 sequence and use of a PCR–RFLP developed during the study.

District	Locality	Co-ordinates	Altitude (m)	Date	No. identified (sequenced)	*A. atroparvus*	*A. maculipennis*	*A. messeae*	*A. melanoon*	*A. daciae*
Constanţa	Agigea	44°06′03′N, 28°37′00′E	13	10.vii.02	7 (1)	5	1	–	–	(1)
	Dulceşti	44°07′55′N, 28°16′51′E	35	10.vii.02	3 (2)	1	–	–	–	(2)
	Herghelia Mangalia	43°49′50′N, 28°35′50′E	5	10.vii.02	6 (4)	1	1	–	(1)	(3)
	Limanu	43°48′00′N, 28°31′58′E	48	10.vii.02	7 (2)	4	1	–	–	(2)
	Mamaia-Sat	44°17′41′N, 28°36′58′E	10	11.vii-02	8 (5)	3	–	–	–	(5)
Giurgiu	Budeni	44°11′55′N, 26°06′43′E	60	2000[a]	(42)	(18)	(11)	(1)	–	(12)
				2001[b]	(9)	–	(4)	–	–	(5)
				08.vii.02	20 (14)	3	3	–	–	(14)
				19.vi.03	(33)	–	(1)	–	–	(32)
	Giurgiu	43°54′02′N, 25°58′33′E	30	15.vii.01	(1)	–	–	–	–	(1)
Ilfov	Săftica	44°37′00′N, 26°04′31′E	85	2000[c]	(47)	(12)	(16)			(19)
Mehedinţi	Dubova	44°37′12′N, 22°15′31′E	200	06.vii.02	27 (1)	–	26			(1)
	Gruia	44°16′03′N, 22°42′17′E	100	07.vii.02	25	25	–	–	–	–
	Ieselnita	44°41′06′N, 22°21′05′E	?	06.vii.02	(1)	–	–	(1)	–	–
	Ostrovu Corbului	44°31′07′N, 22°43′24′E	71	07.vii.02	26 (16)	6	4	(15)	–	(1)
	Pristol	44°13′38′N, 22°42′33′E	70	07.vii.02	11	11	–	–	–	–
Suceava	Băişeşti	47°28′22′N, 26°07′17′E	370	03.vii.02	1	–	1	–	–	–
	Cornu Luncii	47°27′46′N, 26°08′59′E	370	03.vii.02	9	–	9	–	–	–
	Mănăstirea Humorului	47°35′53′N, 25°51′54′E	500	03.vii.02	3	–	3	–	–	–
	Vatra Moldoviţei	47°38′60′N, 25°34′00′E	577	02.vii.02	11	–	11	–	–	–

[a] 21.iii, 28.iii, 8.v and 19.vi.2000.
[b] **28.vi and 12.viii.2001.**
[c] 26.vi and 29.viii.2000.

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                  1111111111222222222233333333334444444444555555555566666666667777777778
                  1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
daciae            atcactcggctcgtggatcgatgaagaccgcagctaaatgcgcgtcacaatgtgaactgcaggacacatgaacaccgata
messeae           ..........................................................................
maculipennis      ..........................................................................
melanoon          ..........................................................................
atroparvus        ..........................................................................

                                 1111111111111111111111111111111111111111111111111111111111111111
                  8888888889999999999000000000011111111112222222222333333333344444444445555555556
                  1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
daciae            agttgaacgcatattgcgcatcgtgcgacacagctcgatgtacacattttgagtgcccatatttgaccc--attcaagt
messeae           ..........................................................................--........
maculipennis      ..................................................t...........--.----g..
melanoon          ..................................................t.........t---..c.....
atroparvus        ..........................................................t.ata.cc....c

                  1111111111111111111111111111111111111111222222222222222222222222222222222222222
                  6666666667777777777888888888899999999990000000000111111111122222222233333333334
                  1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
daciae            caaactacgtacctccgtgtácgtgcatgatgatgaaagagtttggaa-caccatccat-ttcttgcattgaaagcgcag
messeae           ..........................................-....t...t.-c...................
maculipennis      ..................g.....................-........t.-c.............a.....
melanoon          ......................t.....................a........t.-c...................
atroparvus        .....gg........acc.......g.-.t.........g..c....ta.g......t.tc........c...gt..t..

                  2222222222222222222222222222222222222222222222222222222233333333333333333333
                  4444444445555555555666666666677777777778888888888999999999900000000011111111112
                  1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
daciae            cgtgtagcaaccccaggtttcaacttgcaaagtggccatggggctgacacctcaccaccatcagcgtgctgtgtagcgtg
messeae           ..........................................................................
maculipennis      ..........................................................................
melanoon          .........g..................................c.............................
atroparvus        ..........................................................................

                  3333333333333333333333333333333333333333333333333333333333333333333333333334
                  2222222223333333334444444444555555555566666666667777777777888888888999999999900
                  1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
daciae            ttcggcccagtaaggtcatcgtgaggcgtcacctaacggggaagcacacactgttgcgcgtatctcgtggttctaaccca
messeae           ..........................................................................
maculipennis      ..........tc..............t............g.....................a.....---.....
melanoon          ..........tc..............t.....t.............c.................a.....---...t.
atroparvus        ..........tc..............t.........a.......cag...c....t.......a.....ac-c....

                  4444444444444444444444444444444444444444444444444444444444444444444444444444
                  0000000001111111111222222222233333333334444444444555555555566666666667777777778
                  1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
daciae            accatagcagcagagatacaagaccagctcctagccgcgggagctcatgggcctcaaataatgtgtgactacccctaaa
messeae           ..............g..................g...........................................
maculipennis      ....................c...g........t-------...c...............................
melanoon          ..............t.....c.......t.....a...........................................
atroparvus        ..................................a..............t.............a.............

                  444444444
                  888888888
                  123456789
daciae            tttaagcat
messeae           .........
maculipennis      .........
melanoon          .........
atroparvus        .........
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Fig. 2. A 489-bp alignment of 178 ITS2 sequences derived from the five members of the *Anopheles maculipennis* group identified in Romania: *A. atroparvus* (30 specimens), *A. maculipennis* (32 specimens), *A. melanoon* (1 specimen), *A. messeae* (17 specimens) and *A. daciae* (98 specimens). Dashes (-) indicate alignment gaps and dots (.) indicate identity of bases within the alignment.

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Table 2. GenBank accession numbers for ITS2 sequences generated from mosquitoes of the *Anopheles maculipennis* group collected in five districts in Romania.

Species	District	Number	GenBank accession numbers
atroparvus (30)	Giurgiu	18	AY634523-AY634534
	Ilfov	12	AY634523-AY634534
maculipennis (32)	Giurgiu	16	AY634551-AY634566
	Ilfov	16	AY634505-AY634522
melanoon (1)	Constanţa	1	AY634504
messeae (17)	Giurgiu	1	AY648982
	Mehedinţi	16	AY648983-AY648998
daciae (98)	Constanţa	13	AY634470-AY634482
	Giurgiu	64	AY634485-AY634503
	Ilfov	19	AY634406-AY634469
	Mehedinţi	2	AY634483-AY634484

Table 3. Pairwise distance values employing pairwise deletion of gaps between the ITS2 sequences of the five species of the *Anopheles maculipennis* group in Romania.

	A. daciae	*A. messeae*	*A. maculipennis*	*A. melanoon*
A. messeae	0.0103			
A. maculipennis	0.0318	0.0318		
A. melanoon	0.0395	0.0347	0.0340	
A. atroparvus	0.0787	0.0787	0.0722	0.0748

the close genetic relationship of the species involved, finding a single enzyme that could differentiate the five species of the *A. maculipennis* group in Romania proved difficult. Although *A. sacharovi* was not detected in this study, it was included in the assay so that its presence could be quickly detected in future surveys. The optimal species-diagnostic fragments were recovered following digestion with *Cfo I* (*Hha I*), which recognizes the sequence GCGC and cuts before the final C base. All six species produced identical fragments with lengths of 42 and 56 bp, respectively. However, these are not usually visible on agarose gels. Excluding these short fragments, visible fragments include those for *A. atroparvus* (389 bp), *A. maculipennis* (102 bp, 272 bp) and *A. sacharovi* (78 bp, 111 bp, 207 bp). *Anopheles daciae* (111 bp, 135 bp, 141 bp), *A. messeae* (111 bp, 135 bp, 141 bp) and *A. melanoon* (108 bp, 135 bp, 141 bp) were indistinguishable using this one-step assay, and thus all specimens with this shared RFLP pattern were sequenced to determine their identity.

Species distributions in Romania

Based on ITS2 sequences, *A. maculipennis* was collected in all five districts surveyed in this study, and was the only member of the *A. maculipennis* group found in Suceava District in northeastern Romania near the border with the Ukraine (table 1, fig. 1). *Anopheles atroparvus* was common in the Black Sea coastal district of Constanţa, and in the plains of the Danube River running through the southern districts of Giurgiu, Ilfov and Mehedinţi, which border Bulgaria and Serbia. The single specimen of *A. melanoon* was found at Herghelia Mangalia near the Black Sea, and represents a new country record for this species. *Anopheles daciae* was collected in nine of the 17 localities surveyed (table 1), including Budeni and Giurgiu in Giurgiu District, Săftica in Ilfov District, Dubova and Ostrovu Corbului in Mehedinţi District and Dulceşti, Herghelia Mangalia, Limanu and Mamia-Sat in Constanţa District on the Black Sea coast (table 1, fig. 1), which suggests that it is quite common in southern Romania. *Anopheles messeae* and *A. daciae* were found together at Budeni in Giurgiu District and Ostrovu Corbului in Mehedinţi District (table 1, fig. 1). *Anopheles sacharovi* was not found during the surveys, suggesting that this species was effectively eradicated in 1962 and has not re-entered the country.

Anopheles (*Anopheles*) *daciae* Linton, Nicolescu & Harbach, sp. n.

The eggs, fourth-instar larvae, pupae and adults of this species were studied and compared with those of *A. messeae*, as well as with the recently published descriptions of *A. maculipennis* (Linton *et al*., 2003) and *A. sacharovi* (Sedaghat *et al*., 2003). The pupae of *A. melanoon* and *A. persiensis* were also studied. No differential or diagnostic characters were observed in the larvae, pupae or adults. The chaetotaxy of the larval and pupal stages of *A. daciae* and *A. messeae* is compared in tables 4 and 5, respectively. Setae 7 and 8 on abdominal segment VII of pupae, however, distinguish *A. daciae*, *A. melanoon*, *A. messeae* and *A. persiensis* from *A. maculipennis* and *A. sacharovi*. Seta 7 is borne on the intersegmental membrane between terga VII and VIII in *A. daciae*, *A. melanoon*, *A. messeae*, *A. persiensis* and *A. sacharovi*, but *A. sacharovi* differs from the other four species in having seta 8 inserted on the lateral side of the fold line instead of on the mesal side. Seta 7 is borne on tergum VII and seta 8 is inserted on the mesal side of the fold line in *A. maculipennis*.

Anopheles daciae was confused for *A. messeae* in the past because the eggs of these two species are quite similar. Needless to say, the eggs of *A. daciae* are identifiable as those of *A. messeae* in the keys of White (1978) and Jetten & Takken (1994). They differ, however, as shown in fig. 3 and table 6. The eggs of *A. daciae* (fig. 3A) are generally smaller than those of *A. messeae* (fig. 3B) and are distinguished from the latter by the patches of larger deck tubercles that contrast more sharply with the patches of smaller tubercles to impart greater definition to the mottled surface of the deck (fig. 3A,C). In contrast, the deck of *A. messeae* eggs has a more diffuse or weakly mottled appearance (fig. 3B,D).

Nuclear ITS2 sequences for Palaearctic members of the *A. maculipennis* group published to date show no intraspecific variation, thus these sequences represent species-diagnostic characters. Based on these sequences, *A. daciae* is most similar to *A. messeae* (99.0%), with five fixed variable sites. The two species are unique among the Palaearctic members of the group in having ITS2 of the same length (485 bp) (fig. 2, table 3). Diagnostic ITS2 sequences for *A. daciae* are available in GenBank under accession numbers AY634406–AY634503.

The COI gene fragment (522 bp including primers) was sequenced for 33 specimens (29 for which ITS2 sequences were obtained) and identified as *A. daciae*. Twenty-three specimens originated from Budeni in Guirgui District (7 specimens collected 21.iii, 28.iii, 8.v and 19.vi.00; 16 collected 19.vi.03) and the 10 from Săftica in Ilfov District (collected 26.vi and 29.viii.00) (table 1, fig. 1) Due to the structural and functional restraints on this protein-coding region, all fragments were the same length and the alignment was unambiguous. Twenty unique COI sequences were generated from the 33 specimens (fig. 4). Haplotype 1 occurred in 12 individuals (B6.03, B14.03, B48.03, B50.03, B51.03 collected in Budeni in 2003, 173B and 182B collected there in 2000 and 7S, 11S, 12S, 43S, 72S collected in Săftica in 2000) and haplotype 2 in two individuals (1S collected in Săftica in 2000 and B20.03 collected in Budeni in 2003). The 18 other specimens revealed unique COI sequences (fig. 4). Upon conversion to the protein sequence (174 amino acids) using the invertebrate mitochondrial code (after Clary & Wolstenholme, 1985), all 33 sequences shared the same haplotype. All but one base change (first codon position, T⇔C transition at base 28 in specimen 13S from Săftica) occurred in the third position of the codon and all were 'silent' or synonymous changes. Close analysis of the sequences revealed 29 variable sites (5.55% of the 522-bp amplicon). Thirteen (44.8%) of these were parsimony informative and the remainder (16)

https://doi.org/10.1079/BER2004330 Published online by Cambridge University Press

Table 4. Range of numbers of branches for fourth-instar larval setae of *Anopheles daciae* (in bold) and *A. messeae* (below bold entries). Modes in parentheses. A question mark (?) indicates the seta was missing on all specimens examined.

Setae no.	Head	Thorax			Abdominal segments								
	C	P	M	T	I	II	III	IV	V	VI	VII	VIII	X
0	**1**	**1**	**–**	**–**	**–**	**1,2(1)**	**1,2(1)**	**1,2(1)**	**1–3(1)**	**1,2(1)**	**1,2(1)**	**1,2(1)**	**–**
	1	1	–	–	–	1,2(1)	1,2(1)	1,2(1)	1,2(2)	1,2(1)	1,2(1)	1,2(1)	–
1	**1**	**1–6(1)**	**15–21(18)**	**1–3(1)**	**4–31(6)**	**5–21(8)**	**14–24(21)**	**14–25(20)**	**15–25(20)**	**14–20(20)**	**12–20(15)**	**1**	**1**
	1	1,2(2)	15–24(16)	1,2(1)	6–9(6)	6–11(8)	18–28(22)	17–24(22)	17–24(19)	15–23(18)	14–21(21)	1	1
2	**1–8(1)**	**6–10(8)**	**1–4(1)**	**1,2(1)**	**2–9(5)**	**5–10(8)**	**6–10(7)**	**2–5(4)**	**2–4(3)**	**4–6(5)**	**4–8(6)**	**6–7(6)**	**16–34(19)**
	2–6(3)	4–10(8)	1–3(1)	1	3–7(5)	6–13(6)	6–10(7)	3–6(5)	2–5(3)	3–5(5)	4–8(5)	6–8(7)	22–40(32)
3	**21–38(26)**	**1–3(1)**	**1**	**3–12(9)**	**1,2(1)**	**1**	**1**	**2–4(3)**	**1**	**1**	**1–3(3)**	**8–10(8)**	**10–13(11)**
	22–41(29)	1	1	9–15(9)	1,2(1)	1	1	2–4(4)	1	1	1–3(2)	5–11	10–13(11)
4	**1–3(2)**	**14–20(15)**	**2,3(3)**	**1–5(3)**	**4–8(5)**	**3–8(5)**	**3–7(5)**	**3–6(4)**	**3–6(4)**	**1**	**1**	**1**	**14–19(18)**
	1–4(2)	11–18(16)	3,4(3)	3–6(5)	2–9(4)	4–8(5)	4–6(4)	2–5(5)	3–6(3)	1	1	1	14–19(18)
5	**10–18(13)**	**15–23(23)**	**1**	**14–22(21)**	**4–8(7)**	**7–11(9)**	**7–12(9)**	**5–9(7)**	**5–8(6)**	**3–8(8)**	**6–11(8)**	**4–7(5)**	**–**
	13–17(15)	20–32(20)	1	23–33(28)	5–10(6)	7–17(9)	10–14(10)	5–11(7)	5–11(7)	7–10(10)	7–12(7)	5–7	–
6	**11–19(14)**	**1**	**1–3(3)**	**1,2(2)**	**14–17(17)**	**17**	**9–13(12)**	**2,3(3)**	**1–3(2)**	**1–3(1)**	**2–4(2)**	**–**	**–**
	13–22(15)	1	3,4(3)	1,2(2)	16–26(22)	18–30(21)	9–22(17)	2–10(3)	1–3(2)	2–4(3)	3,4(4)	–	–
7	**15–22(20)**	**?**	**1,2(2)**	**16–21**	**11–14(14)**	**12–21**	**3–5(4)**	**3–5(5)**	**3–5(3)**	**2–4(2)**	**3–6(6)**	1–S,	**5–7(6)**
	14–24(20)	16–25(23)	1–5(2)	20–28(24)	14–26(17)	16–28(21)	2–5(4)	2–6(5)	3,4(3)	2,3	5–9(7)		5–7(5)
8	**5–10(6)**	**15**	**15**	**1**	–	**2–4(3)**	**2,3(3)**	**1–3(2)**	**2,3(2)**	**2,3(2)**	**2–4(4)**	2–S,	**2–6(3)**
	6–9(7)	17–24(24)	12–23(14)	10–27(27)	–	2–5(4)	2–4(3)	2–4(3)	1–4(3)	1–4(3)	3–5(5)		3–5(5)
9	**5–7(5)**	**2**	**1**	**1**	**4–6(4)**	**5–7(7)**	**3–9(7)**	**6–13(7)**	**6–10(9)**	**6–11(8)**	**2–4(3)**	6–S,	**1–3(2)**
	6–11(7)	2,3(3)	1	1	4–7(5)	7–11(10)	5–14(6)	9–12(10)	6–11(9)	8–11(8)	2–5(3)		2–4(3)
10	**1–4(3)**	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1,2(1)**	**2**	**2–4(3)**	7–S,	**1,2(1)**
	2–4(3)	1	1	1	1	2–5(4)	1	1	1	1,2(1)	2–5(3)		1,2(1)
11	**27–35(30)**	**1**	**1**	**1**	**3,4**	**1**	**2**	**3**	**2**	**2**	**1,2(1)**	8–S,	**2–5(3)**
	30–36(30)	1	1	1	4,5(5)	1	2–4(3)	2–5(3)	2–5(3)	2,3(2)	2,3(2)		3–6(3)
12	**2–4(3)**	**1**	**1**	**1–3(1)**	**2**	**1**	**2,3**	**2**	**2,3**	**1**	**1**	9–S,	**3–6(5)**
	3–6(5)	1	1	1–3(1)	2	1	2–4(3)	2,3(3)	1–5(3)	1	1,2(1)		4,5(4)
13	**1–5(4)**	**3–9(6)**	**5**	**1**	4	**6–8(6)**	**7–10(9)**	**5**	**5**	**7**	**3,4**	**–**	**–**
	3–7(5)	6–12(9)	4–9(6)	3–5(3)	4–7(5)	6–12(10)	5–12(9)	4–6(5)	2–6(5)	6–9(7)	3–5(4)	–	–
14	**3,4**	**10,11**	**21**	**–**	**–**	**–**	**1**	**1**	**1**	**1**	**1**	**1**	**–**
	?	8–15(11)	7–9(9)	–	–	–	1	1	1	1	1	1	–
15	**5**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**
	?	–	–	–	–	–	–	–	–	–	–	–	–

Table 5. Range (mode) of branches for pupal setae of *Anopheles daciae* (in bold) and *A. messeae* (below bold entries) from Romania. Modes in parentheses.

Setae no.	Cephalothorax CT	Abdominal segments I	II	III	IV	V	VI	VII	VIII	Paddle Pa
0	**–**	**–**	**1,2(1)**	**1–3(1)**	**1–3(2)**	**1–3(2)**	**1–3(1)**	**1–3(1)**	**1**	**–**
	–	–	1,2(1)	1,2(1)	1,2(1)	1,2(1)	1,2(1)	1,2(1)	1,2(1)	–
1	**2,3(2)**	**23–67(34)**	**4–8(6)**	**6–11(6)**	**4–9(6)**	**3–5(3)**	**1,2(1)**	**1,2(1)**	**–**	**1–3(1)**
	2–4(3)	16–32(27)	5–7(6)	6–10(8)	5–10(7)	3–6(4)	1–3(2)	1,2(1)	–	1
2	**2–4(3)**	**2–5(2)**	**4–7(7)**	**4–9(6)**	**3–5(4)**	**3–6(3)**	**2–5(3)**	**2–5(3)**	**–**	**1,2(1)**
	1–3(2)	2,3(2)	2–7(5)	6–10(6)	3,4(3)	3–5(4)	2–4(4)	3,4(4)	–	1
3	**2–4(3)**	**2–5(2)**	**1**	**1–5(3)**	**3–6(5)**	**2–4(3)**	**1–3(2)**	**2–4(3)**	**–**	**–**
	2–5(3)	3–6(4)	1	2–4(3)	3–6(5)	2,3(2)	1–3(2)	1–5(2)	–	–
4	**2–8(3)**	**3–8(4)**	**1–5(2)**	**1–4(4)**	**1–4(2)**	**2–4(3)**	**1,2(2)**	**1–4(1)**	**1–4(2)**	**–**
	2–5(4)	3–5(5)	2–5(4)	1–4(2)	1–3(2)	1–3(2)	1,2(2)	1,2(1)	2,3(2)	–
5	**3–6(5)**	**2–5(2)**	**3–6(4)**	**6–10(9)**	**5–8(7)**	**4–7(5)**	**2–5(3)**	**1–4(3)**	**–**	**–**
	3–6(4)	2–4(3)	3–6(4)	6–11(8)	5–7(6)	3–6(5)	3,4(3)	2–5(3)	–	–
6	**2–5(3)**	**1–4(2)**	**2–4(3)**	**3–6(4)**	**2–5(3)**	**1–3(2)**	**1**	**1,2(1)**	**–**	**–**
	1–3(2)	2–4(3)	2–4(3)	2–6(5)	2–5(4)	1–3(2)	1	1,2(1)	–	–
7	**1**	**1–5(3)**	**1–5(3)**	**2–5(4)**	**1–4(4)**	**2–4(3)**	**1,2(1)**	**1**	**–**	**–**
	1,2(1)	1–5(3)	2–5(4)	1–5(1)	1–4(1)	1–4(2)	1	1	–	–
8	**1–3(1)**	**–**	**–**	**2–4(2)**	**1–4(2)**	**1,2(1)**	**1–3(2)**	**2–6(3)**	**–**	**–**
	1–3(1)	–	–	1–3(3)	1–3(2)	1–3(1)	1–3(1)	2–4(3)	–	–
9	**1–4(1)**	**1–3(2)**	**1**	**1**	**1**	**1**	**1**	**1**	**8–15(10)**	**–**
	2–4(3)	1–3(2)	1	1	1	1	1	1	7–15(8)	–
10	**1–4(1)**	**–**	**–**	**2,3(2)**	**1,2(1)**	**1,2(1)**	**1,2(1)**	**1,2(1)**	**–**	**–**
	1,2(1)	–	–	2,3(2)	1,2(1)	1,2(1)	1	1	–	–
11	**1–4(3)**	**–**	**–**	**1,2(1)**	**1,2(1)**	**1**	**1**	**1,2(1)**	**–**	**–**
	3,4(3)	–	–	1,2(1)	1	1	1	1	–	–
12	**1–5(1)**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**
	1–3(1)	–	–	–	–	–	–	–	–	–
14	**–**	**–**	**–**	**1**	**1**	**1**	**1**	**1**	**1**	**–**
	–	–	–	1	1	1	1	1	1	–

Table 6. Egg characteristics of *Anopheles daciae* and *A. messeae* (ranges of lengths and counts with means and modes in parentheses, respectively).

Character	*A. daciae*	*A. messeae*
Egg length	547–680 µm (611 µm)	589–667 µm (634 µm)
Float length	200–307 µm (246 µm)	211–300 µm (257 µm)
Float ridges	18–21 (19)	19–22 (20)
Anterior lobed tubercles	7–12 (9)	7–11 (8)
Number of convolutions	4–10 (8)	6–11 (8)
Posterior lobed tubercles	6–11 (7)	5–10 (7)
Number of convolutions	5–11 (7)	5–10 (8)

comprised singleton polymorphic changes (fig. 4). All base changes are accounted for by uniform single-base substitutions. Overall, the average pairwise distance in the data set was 1.02%, and ranged from 0.19% (between haplotypes 1 and 2) to 1.34% (Budeni specimens B49.03 and B45.03). This level of genetic diversity and the fact that shared mtDNA haplotypes exist between these two geographically separate populations re-enforces the specific status of *A. daciae* and suggests it is present in southern Romania as a stable, breeding population. A FASTA search of sequences in GenBank revealed that haplotype 1, the most common one in the data set, shared 100% identity with a taxon identified as *A. messeae* from Pavlodar, Kazakhstan (AY258181; Di Luca *et al*., 2004), suggesting that *A. daciae* is widely distributed in Eurasia. COI sequences generated in this study are available in GenBank under accession numbers AY757922–AY757954.

To satisfy Articles 16.4 and 72.3 of the *International Code of Zoological Nomenclature* (International Commission on Zoological Nomenclature, 1999), a name-bearing type is fixed for *A. daciae* from the type series listed below. The series consists of 139 specimens from eight progeny broods identified on the basis of ITS2 sequence: 25 males (♂), 23 females (♀), 28 larval exuviae (Le), 49 pupal exuviae (Pe) and 14 fourth-instar larvae (L). The series is deposited in The Natural History Museum (BMNH), London. Many additional specimens and progeny broods of *A. daciae* collected and reared in parallel with this study are housed in the BMNH and the Cantacuzino Institute in Bucharest, Romania.

Holotype ♀LePe (RO207-1), offspring of female number RO52-34 (GenBank accession AY634463) collected ROMANIA: Muntenia, Giurgiu, Budeni (44°11′55′N, 26°06′43′E), 8.vii.2002, resting in cow shed (Ciulacu-Purcărea). The holotype bears the following label: 'RO207-1' // 'Progeny of mother RO52-34' // 'ROMANIA:' // 'Muntenia, Giurgiu,' // 'Comana, Budeni.' // ~80m.' // '8.vii.2002 // 'Ciulacu-Purcărea' // 'resting in cow shed'.

Paratypes, 2♀LePe (RO196-1, -3), 1♂Pe (RO196-2), 1Le (RO196-4), offspring of female number RO52-12 (GenBank accession AY634461), same data as holotype; 1♂Pe (RO197-1), 1♀LePe

https://doi.org/10.1079/BER2004330 Published online by Cambridge University Press

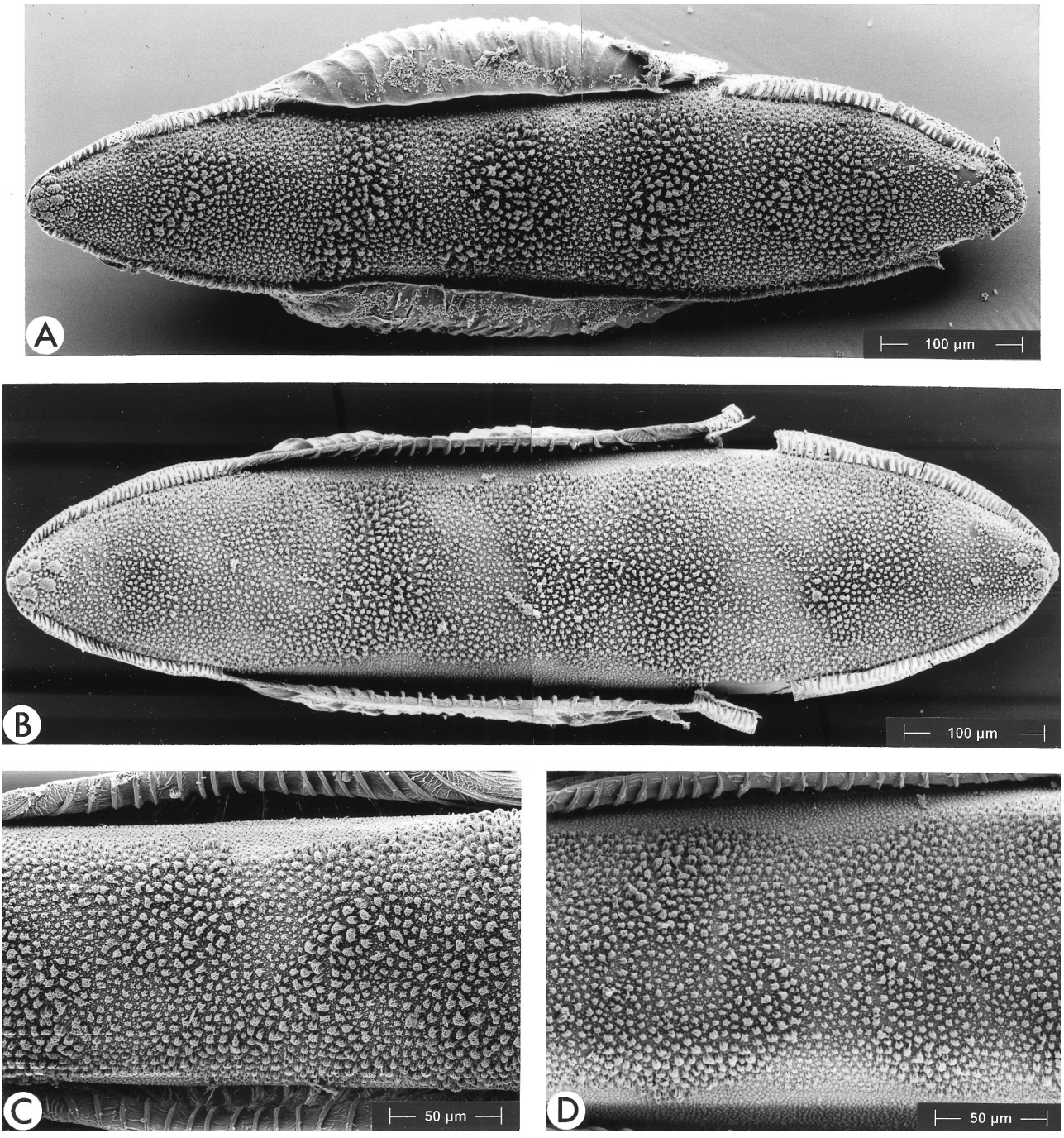

Fig. 3. Eggs of *Anopheles daciae* (A,C) and *A. messeae* (B,D). Ventral (upper) surface of whole eggs (A,B, anterior to right) and middle of deck (C,D). Scales in µm.

(RO197-2), 1Le (RO197-3), offspring of female RO52-15 (GenBank accession AY634462), same data as holotype; 2♀LePe (RO207-2, -3), siblings of holotype; 1♀LePe (RO214-1), offspring of female RO52-66 (GenBank accession AY634465), same data as holotype; 4♂ LePe (RO222-1, -2, -3, -5), 1♂Pe (RO222-4), offspring of female RO52-101 (GenBank accession AY634456), same data as holotype; 2♀LePe (RO224-1, -6), 5♂LePe (RO 224-2, -4, -8, -9, -10), 1♂Pe (RO224-3), 2♀Pe (RO224-5, -7), offspring of female RO52-103 (GenBank accession AY634457), same data as holotype; 2♂Pe (RO249-1, -2), 2♂LePe (RO2494-4, -7), 1♀Pe (RO249-5), 2♀LePe (RO249-6, -8), 1LePe (RO249-3), offspring of female RO58-36 (GenBank accession AY634464) collected ROMANIA: Dobrogea, Constanţa, Herghelia

https://doi.org/10.1079/BER2004330 Published online by Cambridge University Press

```
                    1111111222222333444 4
           2345559934568892578918893468 8
           8921473681020955598484798480 9
Haplotype1 TACGTCTGACGTACGAAAATATAATATTT
Haplotype2 .............T..............
180B       ..T....A..A...A.GT.C....C...C
187B       ........................C..
197B       .......A..............G......
213B       .GTAAT.A..A.G.AG.......GC....
190B       ...........C..A..............
8S         ...............G..G..........
13S        C............................
47S        ...........C.............G...
B10.03     .......AG............C....C..
B12.03     ..T....A......A....CG........
B18.03     .........T...TA..............
B19.03     ......C......T...............
B21.03     ......C...A..................
B22.03     ..........A..T...............
B23.03     ..........A..................
B45.03     .......A......AG..G........C.
B47.03     ...........C.................
B49.03     ...........C....G............
```

Fig. 4. Alignment of 20 unique COI sequences obtained from 33 specimens of *Anopheles daciae* collected at Budeni in 2000 (e.g. 180B) and 2003 (e.g. B21.03), and at Săftica (e.g. 8S) in 2000, in southern Romania. Haplotype 1 represents 12 specimens (B6.03, B14.03, B48.03, B50.03, B51.03 from Budeni, 2003; 173B and 182B from Budeni, 2000; 7S, 11S, 12S, 43S, 72S from Săftica, 2000) and haplotype 2 represents two specimens (1S from Săftica, 2000 and B20.03 from Budeni, 2003). Only those variable bases in the 522-bp COI fragment are shown.

Mangalia (43°49′50′N, 28°35′50′E), 10.vii.2002, resting in horse stable (Nicolescu/Harbach); 1♂ LePe (RO271-1), 8♀ Pe RO271-2, -4, -6, -8, -9, -13, -14, -15), 7♂ Pe (RO271-3, -5, -7, -10, -11, -16, -17), 1♀ LePe (RO271-12), 14L (RO271, on separate microscope slides), offspring of female RO61-98 (GenBank accession AY634481) collected ROMANIA: Dobrogea, Constanţa, Mamaia-Sat (44°17′41′N, 28°36′58′E), 11.vii.2002, resting in cow shed (Nicolescu).

The specific name *daciae* is taken from the Latin name (*Dacia*, *-iae*, feminine) for the country corresponding principally to modern Romania.

Acknowledgements

The authors are grateful to The Natural History Museum, London for funding the field collections, and to the EU-supported Sys-resource programme for supporting training visits for G. Nicolescu and A. Vladimirescu. They also gratefully acknowledge Valeria Ciulacu-Purcărea and Elena Claudia Coipan, Cantacuzino Institute, Bucharest, and Lisa Smith, The Natural History Museum, for technical assistance in both the field and the laboratory.

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(Accepted 5 August 2004)