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Discovery of a third member of the Maculipennis Group in SW England

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Abstract

Currently the *Anopheles maculipennis* complex in UK is believed to comprise two species - *An. atroparvus* van Thiel and *An. messeae* Falleroni. Following the recent description of a new species *An. daciae* Linton, Nicolescu & Harbach from Romania, which closely resembles *An. messeae*, we herein report its presence in the Somerset Levels of SW England. Specimens of *An. daciae* in SW England were identified by comparison of their nuclear ITS2 and mitochondrial COI gene sequences generated from the type series. *Anopheles daciae* and *An. messeae* cannot be differentiated using the current *messeae*-specific primers designed by Proft *et al.* (1999).

Introduction

The Maculipennis Group of *Anopheles* mosquitoes in the UK is currently understood to include two species – *An. atroparvus* van Thiel and *An. messeae* Falleroni. *Anopheles atroparvus* has been reported from Berkshire, Cheshire, Dartford, Devon, Dorset, Essex, Hayling Island, Kent, Pevensey Levels, Romney Marsh, Surrey, the Thames Estuary and in the lower reaches of Sussex rivers in England (Ramsdale & Snow, 2000; Linton *et al.*, 2002b) and from Anglesey, Llanfaglan, the southern coast of the Menai Straits and at Gwyrfai on the River Afon estuary in Wales (Wright, 1924; Evans, 1934; Ramsdale & Snow, 2000). *Anopheles messeae* has been reported from Cambridgeshire, Cheshire, Devon, Kent, Norfolk, Northumberland, Suffolk, Surrey and Sussex in England (Lewis in Evans, 1934; Evans, 1934; Ramsdale & Snow, 2000; Linton *et al.*, 2002b). Genetic identification of the sympatric presence of *An. atroparvus* and *An. messeae* in Kent, SE England (Linton *et al.*, 2002b) and *An. messeae* in Yorkshire, England (Linton *et al.*, unpublished data) were confirmed by ITS2 sequencing. Although members of the Maculipennis Group are also present in both Scotland (Ashworth, 1927) and Ireland (Ashe *et al.*, 1991), their species composition was not documented. Recently ITS2 sequencing of two specimens from Galway in western Ireland confirmed the presence of *An. messeae* (Y.-M. Linton & F. Geraghty, unpublished data).

Recent molecular studies on the Maculipennis Group in Romania revealed a new species, *Anopheles daciae* Linton, Nicolescu & Harbach, on the Black Sea coast and in the plains adjacent to the Danube River in southern Romania (Nicolescu *et al.*, 2004). The new species is most similar to *An. messeae* Falleroni, and was often found in sympatry with the latter species in Romania (Nicolescu *et al.*, 2004). Although diagnostic morphological characters could not be assigned in the larval, pupal or adult stages, the species are separated by unique nuclear ITS2 and mitochondrial COI DNA sequences and differences in egg morphology. As well as being generally smaller than those of *An. messeae*, the eggs of *An. daciae* are further distinguished by their more distinctly mottled appearance caused by 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. As well as their close morphological similarity, the nuclear ITS2 sequences of *An. daciae* and *An. messeae* are the same length, differing by only five bases (1.03%) showing that these taxa are also the most genetically similar in the Palaearctic Maculipennis Group (Nicolescu *et al.*, 2004).

While the paper describing *An. daciae* (Nicolescu *et al.*, 2004) was in press, Di Luca *et al.* (2004) published the most comprehensive study of intraspecific polymorphism in populations of *An. messeae* (n=79) across Eurasia (Italy, The Netherlands, Former Yugoslavia, Kazakhstan and England) to date. The paper described highly variable mitochondrial COI sequences and variability of up to 1.15% in nuclear ITS2 sequences of *An. messeae*, with their most extreme sequence types originating from specimens from Garslade Farm, Somerset Levels in SW England. These findings raised our suspicion that *An. daciae* may account for some of this reported variability and thus additional specimens from the same locality were sequenced to determine whether *An. daciae* is indeed present in SW England.

Materials and Methods

Mosquitoes belonging to the Maculipennis Group were collected resting in horse stables in the Somerset Levels, SW England between June and July 2001 as part of an MSc project (Lee, 2001). Specimens were individually identified by the removal of two legs and placement directly into the PCR reaction, following the species-diagnostic PCR assay of Proft *et al.* (1999). Five specimens identified as *An. messeae* using the above assay were subsequently used for comparative molecular analysis. DNA was individually extracted from the remainder of the mosquito and PCR amplification of the nuclear ITS2 region was carried out using the 5.8SF and 28SR primers of Collins & Paskewitz (1996), following the conditions listed in Linton *et al.* (2001a). PCR products were directly sequenced in both directions on a ABI 3730 automated sequencer using the Big Dye Terminator Kit (PE Applied Biosystems, Warrington, England). Sequences were edited using Sequencher™ version 3.1.1 (Genes Codes Corporation, Ann Arbor, Michigan) and similarity with previously published ITS2 sequences from members of the Maculipennis Group available in GenBank was assessed using FASTA search (http://www.ebi.ac.uk/fasta33/). Sequence alignments were carried out in Clustal X (Thompson *et al.*, 1997) and calculations of inter- and intraspecific sequence variability were carried out using MEGA2 (Kumar *et al.*, 2001). Template DNA is stored at -70°C in the Molecular Systematics Laboratory, Department of Entomology, The Natural History Museum, London, for future reference.

Nuclear ITS2 sequences generated or used in this study are available under the following GenBank accession numbers: *An. daciae* (n=98) [Romania; AY634406-AY634503 Nicolescu *et al.*, 2004)] and *An. messeae* (n=61) [Florina, Greece (n=2; AF342711-12 Linton *et al.*, 2001b, 2002a, 2005); Yorkshire, central England (n=2; AF452699-700 Linton *et al.*, 2002, direct submission); Kent, southeast England (n=40; AF504197-236 Linton *et al.*, 2002b); Romania (n=17; AY648982-98 Nicolescu *et al.*, 2004)]. The ITS2 sequences from the Somerset Levels (n=5) are available under GenBank accession numbers (AY822585-9), and AY238412 represents 10 individuals from the same site (Di Luca *et al.*, 2004).

Results and Discussion

Nuclear ITS2 sequences were generated for five specimens identified as *An. messeae* using the species diagnostic PCR assay of Proft *et al.* (1999). The five sequences obtained from the Somerset population were 435 bp in length and identical. An alignment was created of ITS2 sequences for *An. messeae* from Greece, England and Romania (n= 61), *An. daciae* from Romania (n=98) and those specimens from Somerset amplified in this study (n=5) and by Di Luca *et al.* (n=10) (Fig. 1). *Anopheles daciae* and *An. messeae* sequences are of equal length at 435 bp and vary by 5 bases (1.15%) (Fig. 1). Figure 1 clearly shows that the sequences from the Somerset Levels, SW England generated in this study are identical to those of *An. daciae* from Romania (n=98, AY634406-AY634503) and to those generated by Di Luca *et al.* (2004)(n=10, AY238412). The combined data of Di Luca *et al.* (2004), obtained from specimens collected in the Somerset Levels in 2000, combined with ours (collected in 2001), confirms that *An. daciae* is present in England. That it was collected from the same farm (Garslade Farm, Godney) over two consecutive years, indicates that *An. daciae* is present as a stable population in the Somerset Levels, and that these results are not likely to be an artefact of mislabelled specimens. No *An. daciae* were detected among specimens sequenced from Kent, SE England (Linton *et al.*, 2001b)

It is clear from these results that the presence of *An. daciae* can be obscured when using the purported *messeae*-specific primer of Proft *et al.* (1999) as occurred in the study of Lee (2001) and Lee *et al.* (2002). Given the similarities of the ITS2 region in the two species, the *messeae*-specific primer (indicated in Fig. 1) unfortunately also amplifies *An. daciae* (Fig. 1). As the fragment lengths are identical (435 bp), there would be no indication by gel electrophoresis that any species other than *An. messeae* was being detected. However the cluster of three variable bases between bases 161-167 of the alignment (Fig. 1) could be exploited in the search for a species-diagnostic probe to differentiate *An. messeae* and *An. daciae*. Further efforts are currently underway in our laboratories to provide species diagnostic tools to separate the two species.

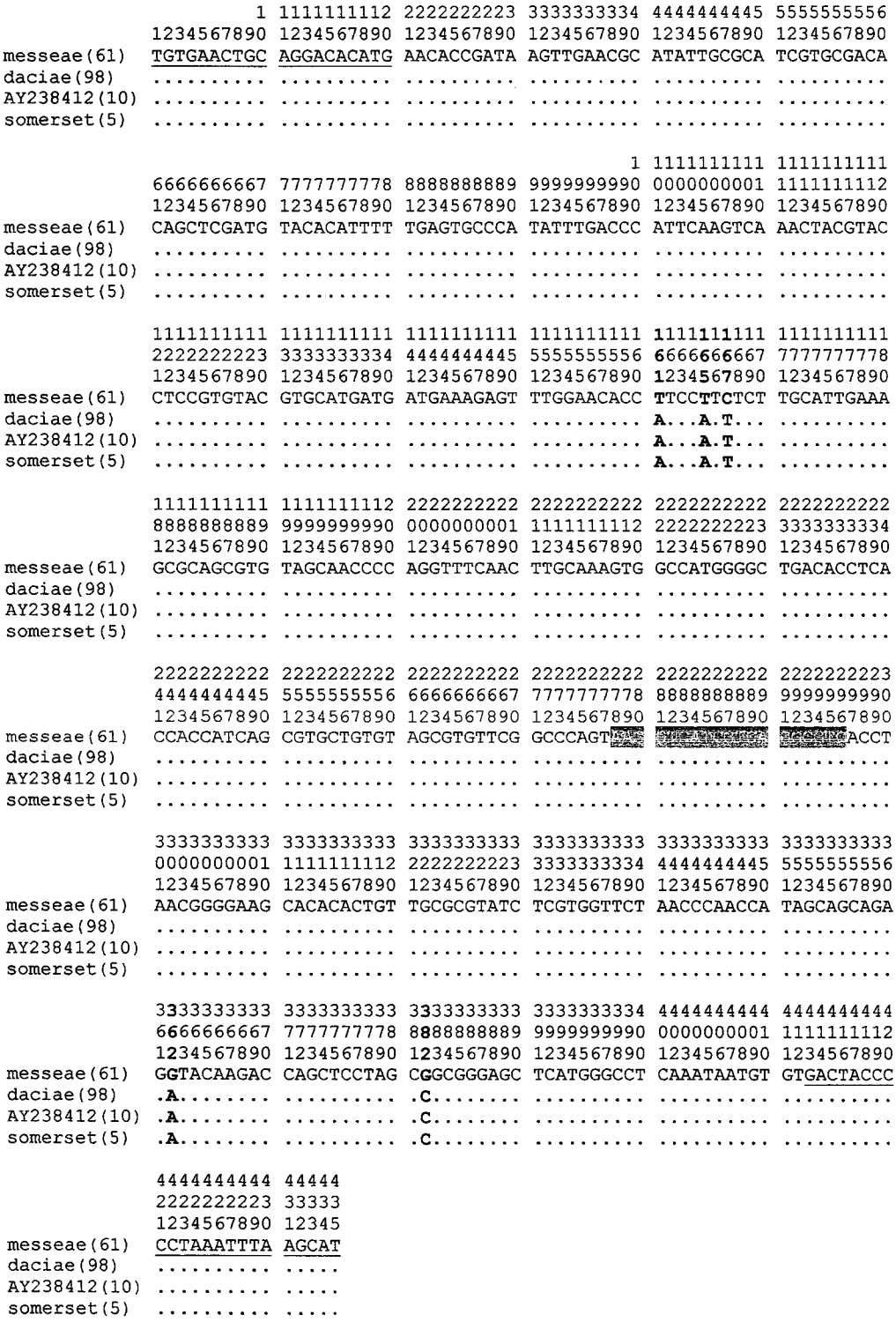

Figure 1: A 435 bp ungapped alignment of nuclear ITS2 rDNA sequences of *An. messeae* from Greece, Romania and England (n=61), *An. daciae* from Romania (n=98), and the ITS2 sequences generated from specimens collected in the Somerset Levels in our study (n=5) and those reported as *An. messeae* in Di Luca *et al.* (2004) (n=10). Primer sequences are underlined. The grey shaded box indicates the purported *messeae*-specific primer of Proft *et al.* (1999).

Additional evidence to support the presence of *An. daciae* in Somerset comes from comparing partial sequences of the mtDNA cytochrome C oxidae I gene in the studies of Di Luca et al. (2004) with those from specimens of the *An. daciae* type series (Nicolescu *et al.*, 2004). A FASTA search of sequences in GenBank revealed that the most common COI haplotype for *An. daciae* (n=12, Nicolescu *et al.*, 2004) shares 100% identity with specimen KZ2 from Pavlodar, Kazakhstan (AY258181; Di Luca *et al.*, 2004), and specimen GB6 from the Somerset Levels, England (AY258176, Di Luca *et al.*, 2004). Other specimens amplified in the Di Luca *et al.* study (2004), including KZ3 (AY258182; Pavlodar, Kazakhstan), GB5 (AY258175; Somerset Levels, England), AL6 (AY258171; Alessandria, Italy) and BG5 (AY258173; Bergamo, Italy), varied from those above by a single point mutation, suggesting these also represent *An. daciae*. These combined data sets reveal that *An. daciae*, present in England, Italy, Romania and Khazastan, is substantially more widely distributed in Eurasia than at first realised (Nicolescu *et al.*, 2004).

Anopheles messeae and *An. daciae* are morphologically and genetically the most similar species in the Holarctic Maculipennis Group. It seems that the presence of *An. daciae* has been obscured by *An. messeae* across their extensive and seemingly sympatric ranges. This may account for differential chromosomal inversions within *An. messeae* east and west of the Caspian Sea (Stegnii, 1982) and the conflicting reports on biting behaviours (Hackett & Missiroli, 1935; Zahar 1990a,b). Currently and historically, *Anopheles messeae* is believed to be a malaria vector in Eastern Europe, including Romania, the Ukraine and Russia, and western Asia (Martini & Zotta, 1934; Zotta, 1938; Bruce-Chwatt & de Zulueta, 1980; Nikolaeva, 1996), but is not considered to be a vector in north-western Europe, where it is reported to be predominantly zoophilic (Jetten & Takken, 1994). Given that *An. daciae* is more widespread in Eurasia than originally anticipated, it seems likely that this species could be responsible for the malaria transmission currently attributed to *An. messeae*.

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