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Molecular confirmation of sympatric populations of *Anopheles messeae* and *Anopheles atroparvus* overwintering in Kent, southeast England

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Abstract

Mosquitoes of the *Anopheles maculipennis* complex were collected as overwintering adults in a disused war fortification near the Cliffe marshes in the county of Kent, southeast England in January 2002. Fifty-three adult females were collected and fifty-two of these were identified as either *An. atroparvus* van Thiel or *An. messeae* Falleroni on the basis of similarity to rDNA internal transcribed spacer (ITS2) sequences in GenBank. Forty specimens (76.9%) were identified as *An. messeae* and 12 (23.1%) as *An. atroparvus*. DNA sequences for these species from England are provided for the first time, which enhance knowledge of the species composition and distribution of the *An. maculipennis* complex in England.

Introduction

In the past, tertian malaria, which was locally called "ague" or "marsh fever" and caused by *Plasmodium vivax*, was indigenous to Britain. Malaria occurred in the valleys, coastal marshes and estuaries in East Anglia, Essex, Kent and other counties on the south coast of England where competent mosquito vectors were present. Sporadic cases of malaria were reported from as far north as Inverness in northern Scotland (Snow, 2000). Five species of *Anopheles* are known to occur in Britain: *An. atroparvus* van Thiel, *An. algeriensis* Theobald, *An. claviger* (Meigen), *An. messeae* Falleroni and *An. plumbeus* Stephens (Ramsdale & Snow, 2000). Of these, only two are thought to have been historically involved in malaria transmission in Britain. The primary historical vector of *Plasmodium vivax* was reported to be *An. atroparvus*, which breeds in brackish water and can occur in high densities in coastal marshes (Rees & Snow, 1990). In laboratory infection studies, Marchant *et al.* (1998) demonstrated that *P. falciparum* transmission by this species is extremely unlikely. The tree hole breeder *An. plumbeus* was implicated by association in the transmission of two *P. vivax* malaria cases in 1953 in Lambeth, south London (Shute, 1954). Blacklock & Carter (1920) reported the successful infection of *An. plumbeus* with *P. vivax*, leading to sporozoites in the salivary glands at 28°C, but the formation of oocysts only between 17-26°C. Consequently, it would appear unlikely that the parasite could complete its lifecycle in this species in England, except during an unusually prolonged spell of warm weather. A study by Marchant *et al.* (1998) showed the development of *P. falciparum* oocysts in *An. plumbeus* under laboratory conditions, but whether the parasite can complete its whole life cycle in this species remains unknown.

Availability of anti-malarial drugs, large-scale drainage of marshlands for agriculture, and improvements in sanitation and health care all contributed to the decline of malaria in Britain in the Nineteenth Century (Dobson & Fantini, 1994). Malaria transmission occurred again briefly in areas of southeast England following the return of infected soldiers from the Mediterranean and tropical or subtropical theatres, during both the First and Second World Wars. An outbreak of *vivax* malaria began in 1917 on the Isles of Grain and Sheppey, caused by the repatriation of sick and injured soldiers fighting the First World War on the continent. The disease soon spread to the local population, especially children, and 491 cases were confirmed between 1917-1921 (Shute, 1949; Shute & Maryon, 1974). Special measures, including making malaria a notifiable disease, evacuating all cases to special hospitals and undertaking mosquito control measures had to be implemented before the outbreak was curtailed (Snow, 2000). Lessons were learned from this outbreak and during the Second World War, infected personnel were excluded from the marshlands, and only 34 malaria cases were reported between 1941-1948 (Shute, 1949). A total of 1291 imported cases were reported in Britain between 1952-54, related to the return of military units returning from Malaya (Malaysia), Korea and other parts of the Far East (Bruce-Chwatt & de Zulueta, 1980). The last case of malaria attributed to local transmission in Britain occurred in 1957 (C.D. Ramsdale, pers. comm.). Today, there is no endemic malaria in Britain, but localised cases of "airport malaria", whereby an infected mosquito is imported on board an aircraft originating in a malarious zone, have been reported (Curtis & White, 1984; White, 1985). Two cases of *P. falciparum* malaria were reported near around Gatwick airport, south of London, in 1983 (Whitfield *et al.*, 1984). Although risk at present seems minimal, the large populations of competent vectors that are present in Britain combined with the influx of parasites heightens the risk of reintroducing endemic malaria (Snow, 2000).

The most efficient malaria vectors in Europe belong to the *An. maculipennis* complex. Numerous morphological, ecological and molecular studies have contributed to the recognition of the following eight Palaeartic species. These include: *An. atroparvus*, *An. beklemishevi* Stegnii & Kabanova, *An. labranchiae* Falleroni, *An. maculipennis* Meigen, *An. martinius* Shingarev, *An. melanoon* Hackett, *An. messeae* and *An. sacharovi* Favre (White, 1978; de Zulueta *et al.*, 1983; Cianchi *et al.*, 1987; Ribeiro *et al.*, 1988; Linton *et al.*, 2002b).

Three species of the complex, *An. atroparvus*, *An. sacharovi* and *An. labranchiae*, are known to be efficient current or historical malaria vectors (Jaenson *et al.*, 1986; Ribeiro *et al.*, 1988; Kasap, 1990; Jetten & Takken, 1994; Romi, 1999; Romi *et al.*, 2001). More recently, *An. messeae* was identified as the principal vector of resurgent malaria in the Ukraine and Russia (Nikolaeva, 1996), and *An. maculipennis* and *An. melanoon* (as *An. subalpinus*) were incriminated as secondary vectors in the Biga Plains of Turkey (Alten *et al.*, 2000). Because *An. messeae* and *An. atroparvus* have both been incriminated as malaria vectors, it is important to characterise the populations of these species in coastal regions of southeast England where the impact of global warming is predicted to be highest. Correct species identification will also be useful to devise appropriate control strategies for the mosquito nuisance problem already reported in this region (Ramsdale & Snow, 1995; Snow, 1997). This paper provides the molecular identification of the species of the *An. maculipennis* complex collected in the Cliffe marshes on the Isle of Grain, Kent.

Materials and Methods

Diapausing adult mosquitoes were collected on 19 January 2002 resting in a disused war fortification bordering the Cliffe marshes on the Isle of Grain, Kent, England. The exact location of the shelter can be found using an Ordinance Survey map at grid reference 718 758. A total of 53 mosquitoes were collected, all female. DNA was individually extracted using the phenol-chloroform extraction protocol of Linton *et al.* (2001a). Amplification of the ITS2 nuclear ribosomal spacer was carried out with the 5.8SF and 28SR primers of Collins & Paskewitz (1996) using the reaction and thermocycler parameters described in Linton *et al.* (2001a). Products were cleaned using a commercially available PCR purification kit (QIAgen Ltd, Sussex, England). Cycle sequencing reactions were prepared using the Big Dye Terminator Kit (PE Applied Biosystems, Warrington, England) and read by an automated sequencer (ABI 377, PE Applied Biosystems). Following sequencing, the template DNA was dried and retained at -70°C in the Molecular Systematics Laboratory, Department of Entomology, The Natural History Museum, London, as voucher material. Sequences were edited and aligned using Sequencher™ version 3.1.1 (Genes Codes Corporation, Ann Arbor, Michigan) and CLUSTAL X (Thompson *et al.*, 1997) software packages. Similarity with other sequences in GenBank was assessed using FASTA search (http://www.ebi.ac.uk/fasta33/). Inter- and intraspecific variability was carried out using MEGA2 (Kumar *et al.*, 2001).

Results

ITS2 sequence data

Sequence data for the ITS2 region was generated for all but one of the 53 individuals collected. Results of FASTA searches of the 52 sequences revealed that 40 samples (76.9%) were *An. messeae* and the remaining 12 (23.1%) were *An. atroparvus*. Sequences are available under GenBank accession numbers AF504197-AF504236 for *An. messeae* and AF504237-AF504248 for *An. atroparvus*.

Percentage AT content was 48.5% in *An. atroparvus* (26.1% A, 22.4% T, 27.1% C, 24.4% G) and 46.8% in *An. messeae* (24.4% A, 22.4% T, 25.4% C, 27.8% G). These values are concordant with 40-50% AT values reported for other Palaearctic members of the *An. maculipennis* complex (Marinucci *et al.*, 1999; Proft *et al.*, 1999; Linton *et al.*, 2001b; Linton *et al.*, 2002a).

The ITS2 sequence alignment of the two species was 489 bases long (Fig. 1). Excluding primers (43 bp), the PCR products were 442 and 444 bases in *An. messeae* and *An. atroparvus*, respectively. Total sequence divergence between the species, including six indels (based on 44 individual changes), was 9.0%. The indels were present at bases 150-151 (AT), 188 (T), 208 (T), 214 (A) and 396 (A) (Fig. 1). In addition to the indels, 38 species-specific base substitutions were noted: 24 transitions (TS) and 14 transversions (TV) with TS:TV ratio = 1.7. No intraspecific variation was detected in the ITS2 sequences of either species (Fig. 1). Previous studies of intraspecific variability in the ITS2 sequences of members of the Holarctic *An. maculipennis* group have shown it to be negligible, e.g. in *An. freeborni* Aitken and *An. hermsi* Barr & Guptavanij (Porter & Collins, 1991), and in *An. messeae* and *An. maculipennis* (Linton *et al.*, 2001b; Linton *et al.*, 2002a).

Comparison with published ITS2 sequences

Despite a number of earlier DNA studies (Marinucci *et al.*, 1999; Proft *et al.*, 1999; Linton *et al.*, 2001b; Linton *et al.*, 2002a), there is a paucity of sequence data available for *An. messeae* and *An. atroparvus*. Problems with the accessibility and reliability of sequence data for *An. maculipennis* and *An. messeae* resulting from earlier studies of the *An. maculipennis* complex (Marinucci *et al.*, 1999; Proft *et al.*, 1999; Djadid, unpublished; Djadid *et al.*, unpublished) was discussed by Linton *et al.* (2001b, 2002a).

Anopheles messeae is currently represented in GenBank by seven ITS2 sequences (Marinucci *et al.*, 1999; Yajun & Fengyi, direct submission 2001; Linton *et al.*, 2001b; Linton *et al.*, 2002a; Djadid, direct submission 2001), and two COI sequences (Linton *et al.*, 2001b). Sequences generated from 40 individuals in this study show 100% identity to four *An. messeae* in GenBank, including AF342711 and AF342712 from Florina, Greece (Linton *et al.*, 2001b; Linton *et al.*, 2002a) and AF452699 from Bishopthorpe and AF452700 from Borobridge, both Yorkshire, England (Linton *et al.*, direct submission 2002). Absolute homology was also noted with the *An. messeae* sequence published in the alignment of Proft *et al.* (1999) (the sequence was not submitted to GenBank). High levels of homology were shown with the three other *An. messeae* sequences entered in Genbank, including 99.6 % identity with AY050639 from Iran (Djadid *et al.*, direct submission 2001), 99.5% identity with AF305556 from China (Yajun & Fengyi, direct submission 2001) and 97.7% identity with Z50105 (Marinucci *et al.*, 1999).

Excepting those generated in the present study, only two ITS2 sequences are present in GenBank for *An. atroparvus*: AY050640 from Iran (Djadid *et al.*, unpublished) and Z50103 from Italy (Marinucci *et al.*, 1999). Sequences generated from our 12 remaining individuals were shown to have 99.56% and 99.38% identity with AY050640 and Z50103, respectively. Our studies revealed that another GenBank entry, AF436064, from a specimen originating in northern Iran (Djadid, direct submission 2001), was erroneously identified as *An. maculipennis* (Linton *et al.*, 2002a). A FASTA search showed that it shares 98.66% similarity with Z50103, and 99.33% identity with our *An. atroparvus* sequences. The authors were notified of these errors (YML, November 2001) but as yet, the GenBank entries have not been corrected.

Variability between the ITS2 sequences generated for *An. atroparvus* in this study and those already available in GenBank (Z50103, Marinucci *et al.*, 1999; AY050640, Djadid *et al.*, direct submission 2001; AF436064, Djadid, direct submission 2001) are shown in Fig. 2. As highlighted in Linton *et al.* (2002a), problems with base discrepancies were noted between the GenBank entries and those in the published alignment of Marinucci *et al.* (1999) for *An. maculipennis* and *An. messeae*, and sequences generated by Proft *et al.* (1999) were never entered into GenBank. The *An. atroparvus* sequences from the alignments published in Marinucci *et al.* (1999) and Proft *et al.*, (1999) are included in Fig. 2, with those available in GenBank.

Absolute homology was noted in the 435 bp overlap between our English *An. atroparvus* sequences and those generated in the publication of Proft *et al.* (1999) from Portugal, The Netherlands, Italy and Spain (Fig. 2). Three variable bases exist between our sequences and Z50103 (Marinucci *et al.*, 1999), comprising an A↔G transition at base 325 and two indels at bases 353 and 393 of the alignment (Fig. 2). However, the variation at base 393 is recorded as an adenosine in the published alignment by the same authors, which is echoed in all other studies to date (Fig. 2). Of the unpublished GenBank entries of Djadid *et al.* and Djadid, AY050640 varied by one indel at base 198 of the alignment (Fig. 2) and AF436064 (erroneously identified as *An. maculipennis*) revealed variation involving two indels at bases 47 and 73 and a G↔T transversion at base 316 of the alignment (Fig. 2). In addition, variable bases were noted in the reverse primer sequence of the Iranian sequences, featured as a G at base 468 of AY050640, and two indels at bases 482 and 484 as well as two A↔T transitions at bases 487 and 488 in AF436064 (Fig. 2). Polymorphism in the primer sequence is not possible unless the primer utilised has an ambiguous base, and thus it seems that these are indeed errors on the part of the author(s). Although some of these discrepancies may represent valid sequence polymorphisms, the increasing number of invariant sequences presented, and the level of errors and inconsistencies in single entry studies, make the truly variable bases difficult to determine with certainty.

As for *An. atroparvus* (Fig. 2), a similar alignment was created for all known *An. messeae* sequences and published in Linton *et al.* (2002a). As the *An. messeae* sequences in this study were identical to the sequences of *An. messeae* from Florina, northern Greece, it is unnecessary to repeat the figure in this paper. Five bases varied between GenBank entry Z50105 and the ITS2 sequence in the published alignment of Marinucci *et al.* (1999). These comprise an error within the primer sequence of an additional thymine (T) at base 5 of the forward primer, two omitted bases and two base alterations (Linton *et al.*, 2002a). Genbank accession AF305556 by Yajun & Fengyi (unpublished) showed an additional T base, unsupported by the data of other workers. However, the intraspecific variability shown between our data for specimens from England and Greece and that of Yajun & Fengyi from a

specimen from China, did not affect the specificity of the species-specific primer for *An. messeae* designed by Proft *et al.* (1999) (see Fig. 1).

Discussion

There are many previous reports concerning members of the *An. maculipennis* complex (as *An. maculipennis s.l.*) in Britain. Although Ashworth (1927) and Ashe *et al.* (1991) reported *An. maculipennis s.l.* in Scotland and Ireland, respectively, no further studies have been carried out to confirm which of the member species are present (Rees & Snow, 1990). Despite its widespread distribution in surrounding European countries, including northwest France, Germany, The Netherlands and Belgium, *An. maculipennis*, the nominotypical member of the complex, has not been recorded in Britain (Cranston *et al.*, 1987; Snow & Ramsdale, 1992; Ramsdale & Snow, 2000). Ramsdale (1991) suggested it may be present in the Channel Islands, but this has not been confirmed.

In Wales, *An. atroparvus* has been recorded from Anglesey, Llanfaglan, the southern coast of the Menai Straits and at Gwyrfai on the River Afon estuary (Wright, 1924; Evans, 1934; Ramsdale & Snow, 2000). In addition, the species has been recorded from Berkshire, Cheshire, Dartford, Devon, Dorset, Essex rivers, Hayling Island, Pevensey Levels, Romney Marsh, Surrey, Thames Estuary and in the lower reaches of Sussex rivers in England (Harold, 1923; James, 1929; Shute, 1933; Marshall & Staley, 1933; Roubaud & Gaschen, 1933; Ramsdale & Snow, 2000). Records of hibernating *Anopheles* from the Isle of Man (Blacklock & Carter, 1921) suggest that *An. atroparvus* is present there. *Anopheles messeae* has been reported from Cambridgeshire, Cheshire, Devon, Norfolk, Northumberland, Suffolk, Surrey and Sussex in England (Lewis in Evans, 1934; Evans, 1934; Ramsdale & Snow, 2000).

This study revealed the presence of *An. atroparvus* and *An. messeae* overwintering in sympatry in the county of Kent, southeast England. Traditionally, members of the *An. maculipennis* complex have been differentiated on the basis of egg morphology (for keys see Weyer, 1942; Angelucci, 1955; White, 1978; Korvenkontio *et al.*, 1979; Jaenson *et al.*, 1986). Other studies have revealed ecological and biological differences that could also be used to differentiate the species found in England. Preferred breeding sites of *An. messeae* are inland fresh waters that are either stagnant or slow moving, whereas *An. atroparvus* is found in brackish-water pools and ditches in coastal regions. Other reported differences include different hibernation conditions. Only nulliparous females of both species hibernate. *Anopheles atroparvus* is normally found hibernating in warm animal shelters, or sometimes in houses, where it will periodically feed on the inhabitants. In contrast, *An. messeae* seeks cold shelters and undergoes complete hiberation, surviving on its food reserves (Rees & Snow, 1990). The discovery of sympatric populations of *An. atroparvus* and *An. messeae* overwintering in a secluded double-walled war fortification may therefore be indicative of the lack of animal shelters near this collection site. We are unaware of any previous report of these species overwintering in sympatry.

Given the importance of these two species, and the *An. maculipennis* complex as a whole, there is a distinct paucity of sequence data available in GenBank. Therefore, the sequences generated in this

study comprise not only the first DNA sequences to be published for English members of the complex, but also represent the most comprehensive molecular study of these species to date. Correct species identification also provides a valuable addition to the knowledge of the species composition and distribution of the English *Anopheles* mosquito fauna. DNA sequences generated herein comprise part of a larger integrated morphological and molecular investigation in our laboratory, the purpose of which is to fully characterise the Palaearctic members of the complex and provide reliable diagnostic characters to differentiate them.

Several authors, including Snow (2000), Pearce (1992) and Lindsay & Birley (1996), consider that there is always a risk of reintroduction of malaria into Britain due to the presence of capable mosquito vectors, and that this risk is increased due to the effects of global warming. The distribution of malaria is determined by the occurrence and biology of the mosquito vectors, and the temperature requirements of the malarial plasmodia for sporogony within the vector species (WHO, 1990). Climatic changes, including global warming and associated increase of precipitation, are expected to extend vector ranges and population sizes of some species, potentially increasing malaria transmission rates (Lindsay & Birley, 1996; Snow, 2000). This may be evidenced by the altered malarial transmission capacity exhibited recently by *An. messeae* (Nikolaeva, 1996), *An. maculipennis* and *An. subalpinus* (Alten *et al.*, 2000).

Anopheles atroparvus is the most efficient vector in Britain, and if, as predicted, sea levels rise in response to global warming, its breeding places will be significantly expanded leading to higher population densities, especially in the south. Given the predicted ambient temperature increase of 1.2-1.6% by 2050 and 2.5-3.0°C by 2100 (Raper *et al.*, 1997), we could expect an increase in the number of mosquito generations per year and possible optimal

temperatures for sporogeny of the parasite within the mosquito host. If we combine these factors with an increased parasite pool resulting from an increase in recent arrivals from countries with endemic malaria, the risk of reintroduction of malaria to Britain becomes a realistic possibility. However, as Snow (2000) concluded, imminent reintroduction of malaria to Britain is unlikely, given that other countries in southern Europe, whilst they do experience the occasional malaria outbreak, are still free from endemic malaria due to mosquito control measures and improved sanitation. That said, the effects of global warming will increase vector ranges, and it will become important to monitor the mosquito fauna and the appearance or spread of mosquito species which may be vectors of other important human or animal diseases, including filariasis and arboviruses such as West Nile virus.

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re 1. A 489 bp alignment showing the interspecific variability between ITS2 sequences for 12 *An. atroparvus* 40 *An. messeae* collected on the Isle of Grain, Kent, England. Primer regions are indicated in bold and derlined. Bases that are underlined only indicate the position of the corresponding species-specific primer signed by Proft *et al.* (1999).

```
                    1111111111222222222233333333334444444444555555555566666
                    12345678901234567890123456789012345678901234567890123456789012345678901234
atroparvus(12)      ATCACTCGGCTCGTGGATCGATGAAGACCGCAGCTAAATGCGCGTCACAATGTGAACTGCAGGA
messeae(40)         ........................................................................


                                                        11111111111111111111111111111
                    66666777777777788888888889999999990000000000111111111222222222
                    56789012345678901234567890123456789012345678901234567890123456789012345678
atroparvus(12)      CACATGAACACCGATAAGTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGTACACATT
messeae(40)         ........................................................................


                    11111111111111111111111111111111111111111111111111111111111111111111
                    23333333333444444444455555555556666666667777777777888888888899 9
                    90123456789012345678901234567890123456789012345678901234567890123456789012
atroparvus(12)      TTTGAGTGCCCATATTTGATCATAACCCAAGCCAAACGGCGTACCTCACCGTACGTGGA-GTTG
messeae(40)         ...................C.--C.TT....T.....TA........CGT.......C.T.A..


                    11111122222222222222222222222222222222222222222222222222222222
                    99999990000000001111111111222222222233333333334444444444555555 5
                    34567890123456789012345678901234567890123456789012345678901234567890123456
atroparvus(12)      ATGAAAGGGTCTGGATACGCCATCCTTTCTCTTGCATCGAAGTCGTAGCGTGTAGCAACCCCAG
messeae(40)         .......A..T....-..A..-.T.C...........T...AG..C.................


                    22222222222222222222222222222222222222222222333333333333333333333
                    55566666666667777777777888888888999999999900000000011111111112
                    78901234567890123456789012345678901234567890123456789012345678901234567890
atroparvus(12)      GTTTCAACTTGCAAAGTGGCCATGGGGCTGACACCTCACCACCATCAGCGTGCTGTGTAGCGTG
messeae(40)         ........................................................................


                    33333333333333333333333333333333333333333333333333333333333333333333
                    22222222233333333344444444445555555555666666666677777777778888 8
                    12345678901234567890123456789012345678901234567890123456789012345678901234
atroparvus(12)      TTCGGCCCAGTTCGGTCATCGTGAGGCGTTACCTAACGGAGAAGCACCAGCTGCTGCGTGTATC
messeae(40)         ..........AA.............C.........G.......ACA...T....C.....


                    33333333333333344444444444444444444444444444444444444444444444444444
                    88888999999999900000000011111111112222222222333333333344444444 4
                    56789012345678901234567890123456789012345678901234567890123456789012345678
atroparvus(12)      TCATGGTTACC-CCCAACCATAGCAGCAGAGATACAAGACCAGCTCCTAGCAGCGGGAGCTCAT
messeae(40)         ..G.....CTAA.................G.................G.............


                    44444444444444444444444444444444444444444
                    45555555555666666666677777777778888888888
                    90123456789012345678901234567890123456789
atroparvus(12)      GGGTCTCAAATAATGTGAGACTACCCCCTAAATTTAAGCAT
messeae(40)         ...C............T.....................
```

Figure 2. A 488 bp alignment showing discrepancies between the 12 ITS2 sequences generated in this study for *An. atroparvus*, those recorded in GenBank by Marinucci *et al.* (Z50103), Djadid (AF436064, direct submission in 2001), Djadid *et al.* (AY050640, direct submission in 2001), and those in the published alignments of Marinucci *et al.* (1999) and Proft *et al.* (1999). Primer regions used in this study are indicated in bold and underlined. Bold bases indicate the variability shown between the sequences and shaded bases indicate a discrepancy between that published in the alignment of Marinucci *et al.* and the purported same sequence (Z50103) in GenBank.

```
                             1111111111222222222233333333334444444444555555555566
                    1234567890123456789012345678901234567890123456789012345678901
atroparvus(12)      ATCACTCGGCTCGTGGATCGATGAAGACCGCAGCTAAATGCGCGTCACAATGTGAACTGCA
Proft et al.        --------------------------------------------------...........
Z50103              .............................................................
Marinucci et al.    .............................................................
AY050640            --------------------------------.............................
AF436064            --------------------------------..............-..............

                                                          11111111111111111111111
                    6666666677777777778888888888999999999900000000001111111111222
                    2345678901234567890123456789012345678901234567890123456789012
atroparvus(12)      GGACACATGAA-CACCGATAAGTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGT
Proft et al.        .............................................................
Z50103              .............................................................
Marinucci et al.    .............................................................
AY050640            .............................................................
AF436064            ...........A.................................................

                    1111111111111111111111111111111111111111111111111111111111111
                    2222222333333333344444444445555555555666666666677777777778888
                    3456789012345678901234567890123456789012345678901234567890123
atroparvus(12)      ACACATTTTTGAGTGCCCATATTTGATCATAACCCAAGCCAAACGGCGTACCTCACCGTAC
Proft et al.        .............................................................
Z50103              .............................................................
Marinucci et al.    .............................................................
AY050640            .............................................................
AF436064            .............................................................

                    1111111111111111222222222222222222222222222222222222222222222
                    8888889999999999000000000011111111112222222222333333333344444
                    4567890123456789012345678901234567890123456789012345678901234
atroparvus(12)      GTGGAGTTGATGAAAGGGTCTGGATACGCCATCCTTTCTCTTGCATCGAAGTCGTAGCGTG
Proft et al.        .............................................................
Z50103              .............................................................
Marinucci et al.    .............................................................
AY050640            ..............-..............................................
AF436064            .............................................................

                    2222222222222222222222222222222222222222222222222222222333333
                    4444455555555556666666666777777777788888888889999999999000000
                    5678901234567890123456789012345678901234567890123456789012345
atroparvus(12)      TAGCAACCCCAGGTTTCAACTTGCAAAGTGGCCATGGGGCTGACACCTCACCACCATCAGC
Proft et al.        .............................................................
Z50103              .............................................................
Marinucci et al.    .............................................................
AY050640            .............................................................
AF436064            .............................................................

                    3333333333333333333333333333333333333333333333333333333333333
                    0000111111111122222222223333333333444444444455555555556666666
                    6789012345678901234567890123456789012345678901234567890123456
atroparvus(12)      GTGCTGTGTAGCGTGTTCGGCCCAGTTCGGTCATCGTGAGGCGTTACCTAACGGAGAAGCA
Proft et al.        .............................................................
Z50103              ...................A...........................-.............
Marinucci et al.    ...................A...........................-.............
AY050640            .............................................................
AF436064            ..........T..................................................
```

Figure 2 (continued)

```
                    333333333333333333333333333333344444444444444444444444444444
                    666777777777788888888889999999990000000000111111111122222222
                    789012345678901234567890123456789012345678901234567890123456
atroparvus(12)      CCAGCTGCTGCGTGTATCTCATGGTTACCCCCAACCATAGCAGCAGAGATACAAGACCAGC
Proft et al.        ............................................................
Z50103              ........................-...................................
Marinucci et al.    ............................................................
AY050640            ............................................................
AF436064            ............................................................

                    444444444444444444444444444444444444444444444444444444444444
                    223333333333444444444455555555556666666666777777777788888888
                    890123456789012345678901234567890123456789012345678901234567
atroparvus(12)      TCCTAGCAGCGGGAGCTCATGGGTCTCAAATAATGTGAGACTACCCCCTAAATTTAAGCAT
Proft et al.        ............................................................---
Z50103              ............................................................
Marinucci et al.    ............................................................
AY050640            ..........................................G.................
AF436064            ............................................................-.-..TA
```