European Mosquito Bulletin, 17 (2004), 14-19.
Journal of the European Mosquito Control Association
ISSN1460-6127

Variability of the second internal transcribed spacer of the ribosomal DNA among five Palaearctic species of anopheline mosquitoes

M. Gordeev [1], I. Goriacheva [1], E. Shaikevich [1] and M. Ejov [2]
[1]The N.I.Vavilov Institute of General Genetics of the Academy of Sciences of the Russian Federation, Moscow, Russian Federation; [2]The WHO Regional Office for Europe, Copenhagen, Denmark
Email: mgordeev@mtu-net.ru

Abstract

The ITS2 rDNA of five *Anopheles* species (Diptera: Culicidae) from a range of CIS ecotopes were studied. The ITS2 sequences of *An. beklemishevi, An. claviger* and *An. plumbeus* were determined, the malaria mosquitoes of Sogdiiskaya oblast in Tadjikistan (Hodjent) were identified as *An. maculipennis* and the ITS2 sequence of *An. messeae* from eastern Siberia was studied. By means of PCR, diagnostic sequences of five *Anopheles* species were identified.

Introduction

One of the fundamental issues in contemporary molecular biology concerns the creation of a new taxonomy that would combine traditional species identification methods with molecular diagnostic techniques. Of foremost importance here is the elaboration of molecular diagnostics for closely related malarial mosquito species of the genus *Anopheles* (Diptera: Culicidae). The recent evolution and adaptive specialization processes have scarcely affected morphological features, giving rise to cryptic sibling species among *Anopheles*. Despite morphological similarities, these species are ecologically isolated and differ in their capabilities to transmit malaria.

The polymerase chain reaction (PCR) represents an effective diagnostic method. At present, the detection of intraspecific differences is carried out using ribosomal DNA. The ribosomal DNA in mosquitoes, as in other eukaryotes, is expressed in the genome by multiple copies of tandemly repeated transcription units (Bekingdale, 1982). Each transcription unit comprises segments encoding three ribosomal subunits – 18S, 5.8S and 28S. Two internal transcribed spacers, ITS1 and ITS2, divide these areas. The structure and length of ITS2 varies among different mosquito species (Porter & Collins, 1991).

The present study concerns the analysis of ITS2 structure in five anopheline species that inhabit the CIS territories: *An. beklemishevi* Stegnii & Kabanova, *An. claviger* Meigen, *An. maculipennis* Meigen, *An. messeae* Falleroni and *An. plumbeus* Stephens. The operational tasks included determination of the ITS2 nucleotide structure and elucidation of specific primers for identifying these species.

Materials and methods

The research was carried out using alcohol-preserved larvae of *An. maculipennis* (Tadjikistan, Sogdiiskaya oblast, Hodjent, 25.07.02), *An. plumbeus* (Russian Federation, Krasnodar Territory, Hosta, 28.08.00), *An. messeae* (Russian Federation, Irkutskaya oblast, Meget, 10.08.00), *An. claviger* (Tadjikistan, Hatlonskaya oblast, Bohtarskii District, Navobod, 19.07.02) and *An. beklemishevi* (Russian Federation, Tomskaya oblast, Krivosheino, 15.07.01). DNA extraction was carried out using the DNA isolation kit DIAtomTM DNA Prep (Isogene, Moscow), which is based on use of lysic reagent with guanidintiocyanate. One microgram of DNA was used for amplification. PCR was carried out in a GeneAmpR PCR System 2700 (Applied Biosystems, USA) using previously described primers (Porter & Collins, 1991; Collins & Paskewitz, 1996) and the conditions described by Proft *et al.* (1999). The forward primer sequence was complementary to the conserved area of 5.8S rDNA (5'- TGTGAACTGCAGGACACATG-3'), the reverse primer annealed with the conserved area of 28S rDNA (5'- ATGCTTAAATTTAGGGGGTA-3'). The PCR results were checked by electrophoresis in 1-2% gel (Agarose Type I, Sigma). The amplification products were purified from the gel using the JETQUICK Gel Spin Kit (Genomed). Sequencing was carried out with an ABI 373 automated sequencer (Applied Biosystems), using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit. The alignment of sequences with those in the GeneBank database and phylogenetic analysis was carried out using the CLUSTAL X 1.8 phylogenetic software package (Thompson *et al.*, 1997) and Genebee (MSU).

PCR was carried out as follows: preliminary denaturation for 2 min at 94°C, thereafter 25 cycles; denaturation for 15 s at 94°C; primers annealing for 15 s at 59°C; synthesis for 20 s at 72°C; closing synthesis for 6 min at 72°C. For the specific amplification, 20 ng of the DNA, 44 pM of the universal primer and 20 pM of the specific primer were used. The concentrations of the specific primers used were calculated from the primer nucleotide

structure using the Oligo calculator program (Kubarenko A.V.). The total volume of the mix was 25 µl. As a forward primer, the universal primer complimentary to 5.8S rDNA was used. When performing the PCR with a mix of specific primers in combination with the direct universal 5.8S primer, 0.1 µl of the DNA of one of the anopheline species, 44 pM of the non-specific complimentary to the 5.8S rDNA primer, and 20 pM each of all the mixed species-specific primers were used.

Results

After the PCR with primers specific to the 5.8S and 28S rDNA genes, the amplification products (the second transcribed spacer region, flanked by 5.8S and 28S rDNA) were obtained for *An. maculipennis*, *An. plumbeus*, *An. messeae*, *An. claviger* and *An. beklemishevi*. The sizes of the amplification products are presented in Table 1.

Table 1. Amplification products obtained following PCR with primers specific to the 5.8S and 28S rDNA genes.

Species	Product size, bp
An. maculipennis	~ 390
An. messeae	~ 410
An. beklemishevi	~ 750
An. claviger	~ 500
An. plumbeus	~ 400

The ITS2 sequences for the five species are registered in GeneBank as follows: *An. maculipennis* - AJ555481, *An. messeae* - AJ555482, *An. beklemishevi* - AJ511876, *An. claviger* - AJ555157, *An. plumbeus* - AJ555483. The ITS2 regions of *An. beklemishevi* and *An. plumbeus* were sequenced for the first time. The ITS2 region of *An. claviger* was recently sequenced by Kampen *et al.* (2003).

The ITS2 sequences of *An. maculipennis* share 100% identity with *An. maculipennis* sequences AJ342713, AJ342714, AJ342715 and AJ342720 from Greece and with Z50104 from Italy. The ITS2 sequence of *An. messeae* is 99% similar to the sequences AJ452700 and AJ452699 of the same species in Great Britain, AJ3055556 from China and AJ436064 and AJ436065 from Iran.

The length of ITS2 sequence in *An. beklemishevi,* exclusive of the 5.8S and 28S segments, is 650 nt, with a GC percentage of 46.6%. This sequence is 68-70% homologous with those of the Nearctic *An. hermsi, An. freeborni, An. occidentalis* and *An. quadrimaculatus* and the Palaearctic *An. maculipennis, An. messeae, An. martinius* and *An. melanoon* of the Maculipennis Group.

Only 54% of the ITS2 sequences of *An. claviger* and *An. plumbeus* are homologous with those of *An. maculipennis*. The GC percentage of *An. claviger* reaches 51.6% and that of *An. plumbeus* reaches 53.6%.

Significant interspecific differences in the ITS2 allowed us, for the first time, to select a set of species-specific primers for *An. claviger, An. plumbeus* and *An. beklemishevi* (Table 2). The sequences listed in Table 2 were used as reverse primers. The reverse primers AMA and AMS of Proft *et al.* (1999), can be used for *An. maculipennis* and *An. messeae*. The direct universal primer has been used as forward primer in all the cases. The similar annealing temperature permitted use of the same PCR conditions for all the species. As a result of amplification with the species-specific primers, 430 bp and 580 bp fragments were obtained for *An. beklemishevi*, an approximately 250 bp fragment for *An. claviger*, a 226 bp fragment for *An. plumbeus*, a 410 bp fragment for *An. maculipennis* and a 305 bp fragment for *An. messeae*.

Table 2. Specific primers for recognizing three species.

Species	Primer	Nucleotide sequence	Annealing temperature	Product length, bp
An. claviger	Acl	5'-cacatctcgtttatgtgtggccg-3'	58	250
An. plumbeus	Apl	5'-cgaagccacccagatgcgagaacat-3'	59	226
An. beklemishevi	Abe	5'-tacccaagtttttctgcaccaca-3'	58	420, 580

The expected amplification products were obtained in all trials with the species-specific primers (11 *An. beklemishevi*, 10 *An. claviger*, 6 *An. plumbeus*, 10 *An. maculipennis* and 10 *An. messeae* larvae). The DNA of the studied *Anopheles* species has not undergone amplification by PCR with primers that were selected for ITS2 sequence of different species. There was no amplification when the primer for each species was applied to the other four species.

The analysis of the ITS2 sequence for *An. beklemishevi* revealed a 100 bp duplication with insignificant nucleotide replacements. The Abe primer that we selected is complementary to two rDNA segments of *An. beklemishevi*, as shown in figure 1.

Figure 1. ITS2 sequence of *An. beklemishevi*, limited by 5.8S and 28S rDNA
Duplicate regions are printed in italics. Complementary to 28S rDNA primers is underlined.

ATATTGCGCATCGTGCGACACAGGCTCGATGTACACATTTTTGAGTGCCCATATTTGACA
TATCCAAGTCAAACTACGTCGGCGAGGCCAGCCCTTGCCGTGCGTGCATAGATGATGAAAGAGTAT
GGGACCTAAACCATCCCGTTTCTTGCATTGAAAGCGTAGCGTGTTACCCAGGGACTCAACTTGCAA
AGTGGCTCTTGGCGAATACCTCACCACCATTAGCGTGAAAGGTGTCTTGCTGAGTCGGGCCATCGT
GAGTTGAGCCCAACGCTACAAGTCCGGGGTATCTCGTGGTTGGACACAGTGGACAGGGAGTCCACT
ATAAACACAAAGGTCAAGAGAGATGT***CAATGGATCAAGGGGACTGTGGTGCAGGAAAAACTTGGGT***
ACCCCCACATATAACTGAATAACTATGACAACACTATATGGCTAGGCGGGTACCCATCAAATATATCC
TATACATGAAACATGAAGATCCAATAACAAAGGTCAAGACATCTC***CAATGGATCAAGAGGACTGTG***
GTGCAGAAAAACTTGGGTACCCCCACATATAACTGAATAACTATGACAATACTATATGGCTAGGAGGG
TACCCATGCATAGATGTATAGGAAACATGAAGATCCAATCCGGGGTATTAAGTGTCCTTACCCAAC
CACAGTAGCAAGAGATACAAAGTTGCTCCTAGCAGCGGGAGTA

PCR with a mixture of specific primers in combination with the direct universal 5.8S primer and the DNA of the five species was carried out. As after PCR performed individually with Abe, Acl, Apl, Ama and Ams primers, the expected amplification products were obtained for all DNA samples.

Discussion

The DNA diagnostic method opens new horizons in vector biology research. Promising trends in molecular genetic studies of malaria vector mosquitoes include the following: identification of the species that occur together in different geographical regions, detection of cryptic species and assessment of phylogenetic relationships between them, analysis of the variability of genetic markers used for vector diagnostics and determination of genetic processes in a population. For the above purposes, for the first time the ITS2 size and structure of *An. claviger,* abundant in the spring, and the tree-breeding *An. plumbeus* were investigated. Both species are distinguished by clear ecological specialization, are exophilic and are considered secondary vectors of malaria. The epidemiological significance of these species depends on their living conditions. In the foothills of central Asia, *An. claviger* reaches high numbers by the end of the breeding season and may therefore take part in malaria transmission. *Anopheles petragnani*, a sibling species of *An. claviger* (Ramsdale & Snow, 2000), occurs in Europe. The comparative analysis of rDNA of these species, as well as comparison of individuals from European and Asiatic *An. claviger* habitats, is thought to be essential.

An important task is the detection of intraspecific groupings with different ecological specialization in *An. plumbeus*. The present study reveals the data on ITS2 structure among the individuals of a natural tree hole-breeding malaria mosquito population. In future, it is highly desirable to study ITS2 structure of newly formed urban *An. plumbeus* races, as they may well be a potential vector of tropical strains of malaria introduced into western and central Europe (Marchant *et al.*, 1998).

To assess the phylogenetic relationships of *An. claviger* and *An. plumbeus* to the Maculipennis Complex, the ITS2 structure of three members of this complex were analyzed. The sibling species *An. maculipennis*, *An. messeae* and *An. beklemishevi* are sympatric in the Palaearctic taiga zone. *Anopheles messeae* and *An. beklemishevi* occur in sympatry in a zone across Siberia and the north of eastern Europe (Stegnii, 1991). In western, central and eastern Europe, the distributions of *An. messeae* and *An. maculipennis* partly overlap. In Siberian and European ecotopes the larvae of these sibling species develop in the same habitats. In central Asia, *An. maculipennis* and *An. messeae* are ecologically isolated, and the limits of their distribution in Kyrgyzstan and in the south of Kazakhstan are poorly known. The morphological identification of these species is undeveloped and cyto-diagnostic methods require special preparation of material and specially trained staff. The elaboration of molecular diagnostic methodology may help to define the current ecology, distribution and epidemiological significance of these species.

For molecular diagnostic purposes, the ITS2 sequence of *An. beklemishevi* was determined for the first time. The GC percentage of the studied segment reaches 46.5%. In allied species the GC composition is slightly higher and varies within 50-60% (Porter & Collins, 1991; Marinucci *et al.*, 1999; Proft *et al.*, 1999; Linton *et al.*, 2001, 2002). The length of ITS2 in *An. beklemishevi* is twice as long as the other members of the Holarctic Maculipennis Group (Cornel *et al.*, 1996; Porter & Collins, 1991; Proft *et al.*, 1999). Thus the ITS2 structure of *An. beklemishevi* is significantly different from the other species. This fact is probably related to a long period of isolation and specific conditions of the species' evolution.

The vast sympatric zone of *An. beklemishevi* and *An. messeae* lies in the north of Palaearctic taiga. Both species are characterized by high chromosome variability (Stegnii, 1991). Based on inversion polymorphism, the stable population-specific systems have been formed. The populations in the center and at the periphery of specific areas are genetically well defined. The dynamic stability of the genetic population system is maintained owing to variability of its structural components (Altukhov, 2003). One of the aims of the present study was the comparison of ITS2 sequences in *An. messeae* populations from different geographic habitats. To this end, ITS2 sequences of *An. messeae* from the Irkutsk suburbs were studied for the first time. Following comparison of these sequences with those in GeneBank, we concluded that there were no significant intraspecific differences among the species as far as nucleotide structure was concerned. The data illustrate homogeneity of *An. messeae* populations in western and eastern areas within the specific region. It is necessary to point out that all *An. messeae* populations studied comprise individuals with "southern" chromosome inversions. In the northern part of the taiga zone, the "southern" chromosome sequences are replaced by "northern" inversion variants. The issue as to what extend the studied ribosomal loci have been involved in the formation of specific populations remains open. To clarify this, the ITS2 structure of *An. messeae* populations in the north of the taiga zone needs to be investigated.

This research included the integrated study of malarial mosquitoes occurring in the vicinity of Hodjent (Sogdiiskaya oblast, Tadjikistan). Comparison of the ITS2 structure of these mosquitoes and the sequences in GeneBank has shown that the predominant vector in the north of Tadjikistan is *An. maculipennis*. This species was found in malaria foci in Tadjikistan for the first time. Mosquitoes from Hodjent were previously identified as *An. martinius* (Informational and Analytical Bulletin, 2001). Further research on the species composition of malarial mosquitoes may contribute to the assessment of vector populations and their impact on malaria resurgence in the north of Tadjikistan.

The results of ITS2 analysis and data from GeneBank are shown in a dendrogram (figure 2) that reflects the phylogenetic relationships among species of the Maculipennis Group. *Anopheles beklemishevi* falls within a clade that includes the Holarctic species *An. quadrimaculatus, An. occidentalis, An. hermsi* and *An. freeborni*. The data correspond to the results of cytogenetic research and confirm the hypothesis of a Northern American origin for *An. beklemishevi* (Stegnii, 1991). Among Palaearctic species, a close relation was detected between *An. maculipennis* and *An. labranchiae* (Marinucci *et al.*, 1999). These species have identical polytene chromosomes and are considered the phylogenetic basal species of the Maculipennis Group (Stegnii, 1991). Correspondence with cytogenetic analysis is noted for other Palaearctic species, with the exception of *An. sacharovi* and *An. martinius*. The latter are separated on the phylogenetic tree, although, according to polytene chromosome banding pattern, *An. martinius* is sister to *An. sacharovi*. These species differ in the inverted regions of XL and 3L chromosomes (Stegnii, 1991, 1993). In our opinion, it is essential to determine more precisely the phylogenetic relationships within the Palaearctic Maculipennis Complex with the aid of additional markers.

Acknowledgements

We thank Dr V. Perevozkin and Mr A. Panfilov for their assistance in collecting mosquito samples from the field. This study was carried out with the financial aid of the WHO Regional Office for Europe, Programme "Biodiversity" of the Russian Academy of Science, the Russian President's Programme (Science Schools; 827.2003.4), Programme "Integracia" (E0371) and the Russian Fund for Basic Research (02-04-48869).

References

Altukhov, Yu. P. (2003) Genetic processes in populations: manual. 3rd edition, revised. Akademkniga PTC, Moscow, pp 1-431.

Anon (2001) Malaria incidence in Tadjikistan Republic. *Information and Analytical Bulletin, Ministry of Health of Tadjikistan Republic* 1-12 (in Russian).

Beckingdale, K. (1982) Insect rDNA. *The Cell Nucleus* (Ed. Bush, H. & Rothblum, L.). Vol. X, pp.205-263. Academic Press. New York.

Collins, F.H. & Paskewitz, S.M. (1996) A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. *Insect Molecular Biology* **5**, 1-9.

Cornel, A.J., Porter, C.H. & Collins, F.H. (1996) Polymerase chain reaction species diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera: Culicidae) based on ribosomal DNA spacers. *American Journal of Tropical Medicine and Hygiene* **33**, 109-116.

Kampen, H., Sternberg, A., Proft, J., Bastian, S., Schaffner, F., Maier, W.A. & Seitz, H.M. (2003) Polymerase chain reaction-based differentiation of the mosquito sibling species *Anopheles claviger* s.s. and *Anopheles petragnani* (Diptera: Culicidae). *American Journal of Tropical Medicine and Hygiene* **69**, 195-199.

Linton, Y.M., Samanidou-Voyadjoglou, A. & Harbach, R.E. (2002) Ribosomal ITS2 sequence data for *Anopheles maculipennis* and *An. messeae* in northern Greece, with a critical assessment of previously published sequences. *Insect Molecular Biology* **11**, 379-383.

Linton, Y.M., Samanidou-Voyadjoglou, A., Smith, L. & Harbach, R.E. (2001) New occurrence records for *Anopheles maculipennis* and *An. messeae* in northern Greece based on DNA sequence date. *European Mosquito Bulletin* **11**, 31-36.

Marchant, P., Eling, W., van Gemert, G.-J., Leake, C.J. & Curtis, C.F. (1998) Could British mosquitoes transmit *falciparum* malaria? *Parasitology Today* **14**, 344-345.

Marinucci, M., Romi, R., Mancini, P., Di Luca, M. & Severini, C. (1999) Phylogenetic relationships of seven Palaearctic members of the *maculipennis* complex inferred from ITS2 sequence data. *Insect Molecular Biology* **8**, 469-480.

Porter, C.H. & Collins, F.H. (1991) Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). *American Journal of Tropical Medicine and Hygiene* **45**, 271-279.

Proft, J., Maier, W.A. & Kampen, H. (1999) Identification of six sibling species of the *Anopheles maculipennis* complex (Diptera: Culicidae) by a polymerase chain reaction assay. *Parasitology Research* **85**, 837-843.

Ramsdale, C. & Snow, K. (2000) Distribution of the genus *Anopheles* in Europe. *European Mosquito Bulletin* **7**, 1-26.

Stegnii, V.N. (1991) Population genetics and evolution of malaria mosquitoes. Tomsk: Tomsk University Publication. 1-136 (in Russian).

Stegnii, V.N. (1993) Genome architectonics, system-formatted mutations and evolution. Novosibirsk: Novosibirsk University Publication. 1-111 (in Russian).

Thompson, J.D., Gibson, T.G., Plewniak, F., Jeanmougin, F. & Jiggins, D.G. (1997) The Clustal X windows interface flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**, 4876-4882.

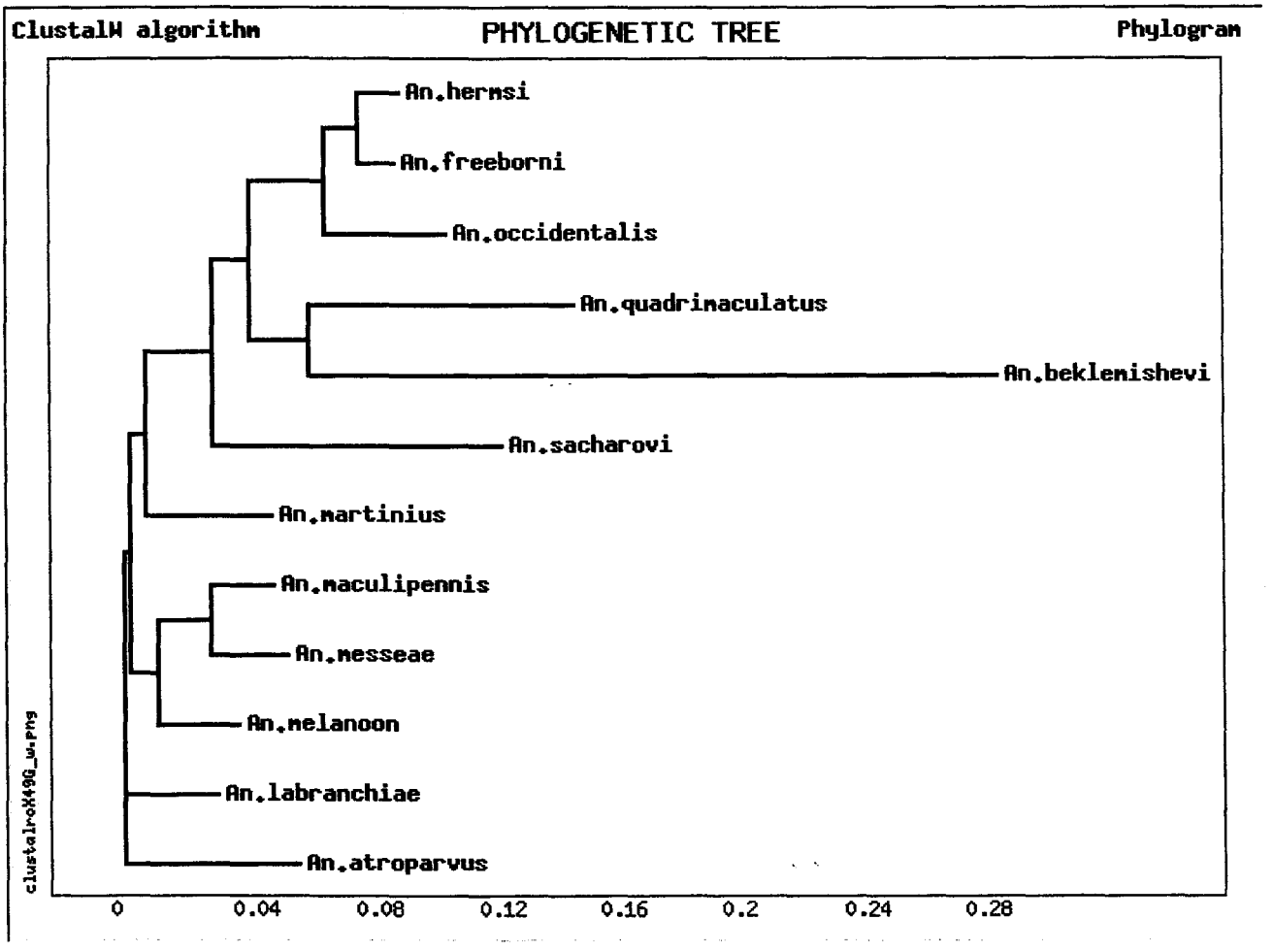

Figure 2. Maximum likelihood tree of members of the Maculipennis Group based on ITS2 sequence data in GenBank: *An. atroparvus* - AY050640, *An. beklemishevi* – AJ511876, *An. freeborni* – M64484.1, *An. hersii* – M64483.1, *An. labranchiae* – Z50102.2, *An. maculipennis* – AJ555481, *An. martinius* –AJ224329.2, *An. melanoon* – AF452410.1, *An. messeae* – AJ555482, *An. occidentalis* – M64482, *An. quadrimaculatus* – U32550.1, *An. sacharovi* – AF462143.