Parasitol Res (2005) 97: 118–128
DOI 10.1007/s00436-005-1393-8

ORIGINAL PAPER

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The ITS2 ribosomal DNA of *Anopheles beklemishevi* and further remarks on the phylogenetic relationships within the *Anopheles maculipennis* group of species (Diptera: Culicidae)

Received: 31 January 2005 / Accepted: 15 April 2005 / Published online: 29 June 2005

Abstract *Anopheles beklemishevi* specimens from Russia were analysed by their ITS2 ribosomal DNA sequence to amend and to specify the phylogenetic tree of the *Anopheles maculipennis* species complex. Surprisingly, with 638 base pairs, the ITS2 regions of all the 34 *An beklemishevi* specimens examined were considerably longer than those of all their sibling species. Sequence alignment with GenBank derived sequences of the other siblings was only possible in the beginning (for approx. 335 bp) and at the end (for approx. 150 bp) of the PCR-amplified DNA fragment, whereas in the middle, the *An beklemishevi* DNA sequence found no counterpart in sequences of the other siblings. Closer analysis of this intermediate part suggests a duplicated insertion of about 140 bp that has undergone subsequent mutational changes. Due to this large putative insertion, computerized phylogenetic analysis by the Bayesian inference method locates *An beklemishevi* in a closer relationship to the nearctic than to the palaearctic sibling species. However, when only ITS2 regions are compared, that have corresponding sequences in the other siblings, *An beklemishevi* forms a lineage with the palaearctic species although it is still most remotely related. It is hypothesized that during the evolution *An beklemishevi* separated first from the common ancestor of the palaearctic species, which had presumably made its way from the Nearctic to the Palaearctic.

Keywords *Anopheles beklemishevi* · *Anopheles maculipennis* complex · Phylogeny · ITS2 rDNA · Bayesian analysis

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Introduction

The *Anopheles maculipennis* complex, containing the former and the present Eurasian and North American malaria vectors, was first described in 1927 by van Thiel. According to White (1978) and Barr and Guptavanij (1988), it consists of 11 palaearctic and five nearctic sibling species (Table 1). Seven of the palaearctic species are indigenous in Europe and two in Middle Asia and North Africa, respectively (Jetten and Takken 1994). Among the first, *An messeae* has the largest distribution covering the whole of Central Europe and considerable areas of the former Soviet Union. *An maculipennis* s.s. and *An atroparvus* occur, partly sympatrically, in most of Europe, whereas *An labranchiae*, *An sacharovi* and *An melanoon* are mainly restricted to the coastal regions of the Mediterranean and the Black Sea (Coluzzi 1970). The distribution of *An beklemishevi* ranges from Scandinavia via East Europe to Russia (Lokki et al. 1979; Stegniy 1982; Jaenson et al. 1986), and *An martinius* and *An sicaulti* are non-European species to be found in Middle Asia and North Africa, respectively.

While there is hardly any doubt on the species status of eight of the palaearctic *An maculipennis* sibling species, *An sicaulti* is not unrestrictedly considered a sibling. In fact, evidence predominates that it is rather a local variant of *An atroparvus* than a good species (e.g. de Zulueta et al. 1983). Also, the species status of *An subalpinus*, which is sometimes listed as a further palaearctic member of the *An maculipennis* complex is uncertain. In contrast to various cytogenetic studies (White 1978), it can locally be distinguished from other complex species by allozymes (Cianchi et al. 1987). However, recent data based on nucleotide sequence analysis suggests synonymity of *An subalpinus* with *An melanoon* (Linton et al. 2002, Boccolini et al. 2003; Kampen, unpublished).

At times of endemic malaria in Europe, *An labranchiae* and *An sacharovi* used to be the most dangerous vectors (Jetten and Takken 1994), and even nowadays *An sacharovi* plays an important role in the transmission

Table 1 The sibling species of the *An maculipennis* complex

Palaearctic (Eurasia, North Africa)	Nearctic (North America)
An maculipennis s.s. Meigen 1818	*An occidentalis* Dyar and Knab 1906
An sacharovi Favre 1903	*An aztecus* Hoffmann 1936
An labranchiae Falleroni 1926	*An freeborni* Aitken 1939
An messeae Falleroni 1926	*An earlei* Vargas 1943
An atroparvus van Thiel 1927	*An hermsi* Barr and Guptavanij 1988
An melanoon Hackett 1934	
An beklemishevi Stegnii and Kabanova 1976	
An martinius Shingarev 1926	
An sicaulti Roubaud 1935	

of endemic malaria in southeast Turkey (Çağlar and Alten 2000).

The nearctic species of the *An maculipennis* complex are mainly found in the west of North America and only little overlap in their distributions (White 1978; Barr 1988). *An earlei* occurs in the south of Canada and in the North of USA transcontinentally from the Atlantic to the Pacific Ocean, whereas the other nearctic siblings are restricted to the west of the Rocky Mountains. Except for the close Pacific coast, *An freeborni* populates nearly the whole western North America (Aitken 1945). In contrast, *An occidentalis* can be found only in coastal areas of western North America beginning from the middle of the USA and reaching as far as Alaska. *An aztecus* occurs in the highlands of central Mexico, and *An hermsi*, recognized as a separate sibling species not before 1987 (Barr and Guptavanij 1988), so far has been demonstrated only for California (Cope et al. 1988). Both *An freeborni* und *An hermsi* are considered efficient malaria vectors (Service 2000).

Except for *An freeborni* and *An hermsi* (cf. Fritz et al. 1991), the nearctic *maculipennis*-siblings can be identified by slight but consistent morphological differences in all developmental stages (Pratt 1952) suggesting that evolution has likely been acting for a longer time or more quickly on the nearctic than on the palaearctic mosquitoes. This hypothesis is backed by the fact that the distributions of the various nearctic sibling species hardly overlap (Kitzmiller et al. 1967). Due to their apparently shorter evolution, the palaearctic sibling species are isomorphic or only present minor morphological variations in some developmental stages. Nevertheless, as well as the nearctic species, they may exhibit different behaviour, ecologies and even vector competences for one pathogen or the other (Jetten and Takken, 1994). Hence, reliable species differentiation is essential.

Classically, the palaearctic *An maculipennis* complex species are differentiated by the egg shell morphology (Hackett et al. 1932; Bates and Hackett 1939; Korvenkontio et al. 1979) but various other identification techniques have been employed: larval chaetotaxy (Bates 1939; Suzzoni-Blatger and Sevin 1982; Deruaz et al. 1991), cytotaxonomy (Frizzi 1953; Kitzmiller et al. 1967; Stegnii 1987), isoenzyme analysis (Bullini and Coluzzi 1978; Korvenkontio et al. 1979; Stegniy 1982) and cuticular hydrocarbon chromatography (Phillips et al. 1990). Owing to a lack of reliability and/or other disadvantages of the forementioned techniques in practical routine application, alternate identification methodologies have been searched for.

A few years ago, a PCR based diagnostic assay was established for six of the palaearctic *An maculipennis* sibling species that makes use of interspecific sequence differences in the ITS2 ribosomal mosquito DNA (Proft et al. 1999). Using this test, the sibling species can be distinguished unambiguously by means of the species-specific lengths of their PCR products.

When the assay was developed, however, no *An beklemishevi* specimens were available to comparatively investigate their ITS2 region. As we could get hold of specimens from several Russian *An beklemishevi* populations in the meantime, we were able to analyse the ITS2 region and, based on its nucleotide sequence, the phylogenetic relationships of this species in relation to the other *An maculipennis* siblings.

Materials and methods

Mosquito origin

An beklemishevi specimens from five collections at three different geographical sites in Russia were included. They were identified by egg morphology, larval cytotaxonomy or taxonprinting (Novikov and Shevchenko 2001) by experienced Russian scientists:

1. Krivosheino, Tomsk oblast, Siberia (57N, 83E): seven specimens, collected 1999, identified by egg morphology.
2. Krivosheino, Tomsk oblast, Siberia (57N, 83E): four specimens, collected 2001, identified by egg morphology.
3. Krivosheino, Tomsk oblast, Siberia (57N, 83E): ten specimens, collected 2002, identified by egg morphology.
4. Sredniy Vasyugan, Tomsk oblast, Siberia (59N, 78E): ten specimens, collected 1982, identified by cytotaxonomy.
5. Cherga, Gorno Altay Republic, Siberia (51N, 85E): three specimens, collected 1998, identified by taxonprinting.

Individuals from collections one to four were larvae fixed in ethanol. From the Cherga specimens (collection 5) extracted DNA was provided for further analysis.

DNA extraction

DNA was extracted from two to three abdominal segments or, in some cases, from whole individuals using short protocols described by Walsh et al. (1991) and

Guy and Stanek (1991). The first includes homogenization of mosquito tissue in Chelex resin and the second boiling of homogenized tissue in ammonia.

DNA amplification

The ITS2 ribosomal DNA was amplified using primers 5.8S and 28S that hybridize to conserved flanking regions (Collins and Paskewitz 1996):

5.8S: 5′-TGTGAACTGCAGGACACATG-3′
28S: 5′-ATGCTTAAATTTAGGGGGTA-3′

1–3 µl of DNA solution was added to a PCR mixture (50 µl total volume) composed of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 µM dNTPs, 200 nM of each primer, 1 mM $MgCl_2$, and 1.25 units of Taq DNA-polymerase (Invitrogen). Thermoconditions were 4 min initially at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, and finally 10 additional minutes at 72°C.

DNA sequencing

PCR products were seperated by electrophoresis on 1.5% agarose gels, and bands were excised with a scalpel. DNA was recovered using the 'DNA Gel Extraction Kit' (Qiagen) and prepared for DNA sequencing. Sequencing was done by cycle sequencing on an ABI Prism 310 DNA sequencer (Applied Biosystems) using primers 5.8S and 28S as sequencing primers. All PCR products were sequenced in duplicate in both directions.

Phylogenetic analysis

ITS2 sequences to be analysed in relation to the *An beklemishevi* sequences were derived from the GenBank. 421 ITS2 sequence entries for the palaearctic and nearctic sibling species of the *An maculipennis* complex were found. They were aligned according to species in order to obtain species-specific consensus sequences. Such consensus sequences, based on 129 *An maculipennis* s.s., 96 *An sacharovi*, 47 *An messeae*, 41 *An atroparvus*, 12 *An labranchiae* and 3 *An martinius* entries as well as one entry each for *An hermsi* (GenBank accession no. M64482), *An freeborni* (M64483) and *An occidentalis* (M64484), were further analysed together with the newly obtained *An beklemishevi* data. Phylogenetic trees were rooted using the ITS2 sequences of *An quadrimaculatus* species A (U32503) and of *An dirus* species A (U60410), respectively, as outgroups. *An dirus* was selected as one outgroup because its ITS2 region is of similar length as the *An beklemishevi* one turned out to be, whereas the *An quadrimaculatus* ITS2 region is comparable in length with those of the other palaearctic sibling species.

Sequences were aligned using ClustalX software (Thompson et al. 1997), and phylogenetic trees were built by means of PAUP 4.0 beta 10 (Swofford 1999). Maximum likelihood models were fitted using the Modeltest 3.0 (Posada and Crandall 1998), and relationships between DNA sequences were tested with the Bayesian inference method (MrBayes V3 b4 v2win software; Huelsenbeck 2001). For employing the optimal model complexity, simulations were run with four chains for 2,000,000 steps with a 2,000-step burn-in period, consensus 95% cumulative probability and sampling every 100 steps.

Results

Gel electrophoresis of the amplified DNA-fragments already indicated a considerably larger length of the *An beklemishevi* ITS2 region compared to the other palaearctic *An maculipennis* sibling species (Fig. 1). DNA sequencing showed the amplicons to be invariant for all populations and all individuals examined (Fig. 2). The PCR product of *An beklemishevi* consisted of 782 base pairs (bp), 638 of which represent the presumptive ITS2 region (cf. Porter and Collins 1991; Marinucci et al. 1999). The GC content amounts to 46.6%.

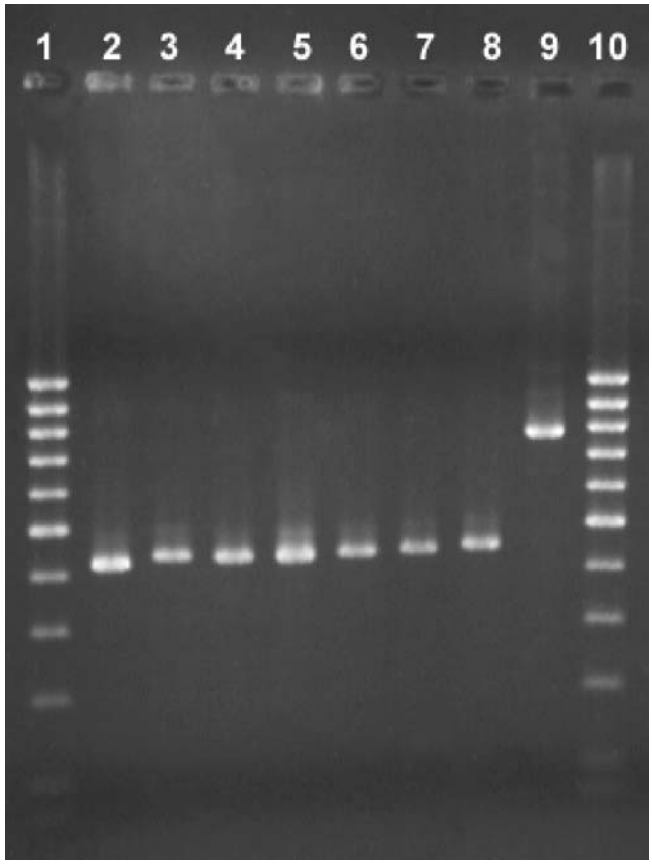

Fig. 1 PCR-amplified ITS2 regions of the European sibling species of the *An maculipennis* complex (lanes *1*, *10*:100 bp ladder; lane *2*: *An maculipennis* s.s., *3*: *An messeae*; *4*: *An melanoon*; *5*: *An subalpinus*; *6*: *An labranchiae*; *7*: *An atroparvus*; *8*: *An sacharovi*; *9*: *An beklemishevi*)

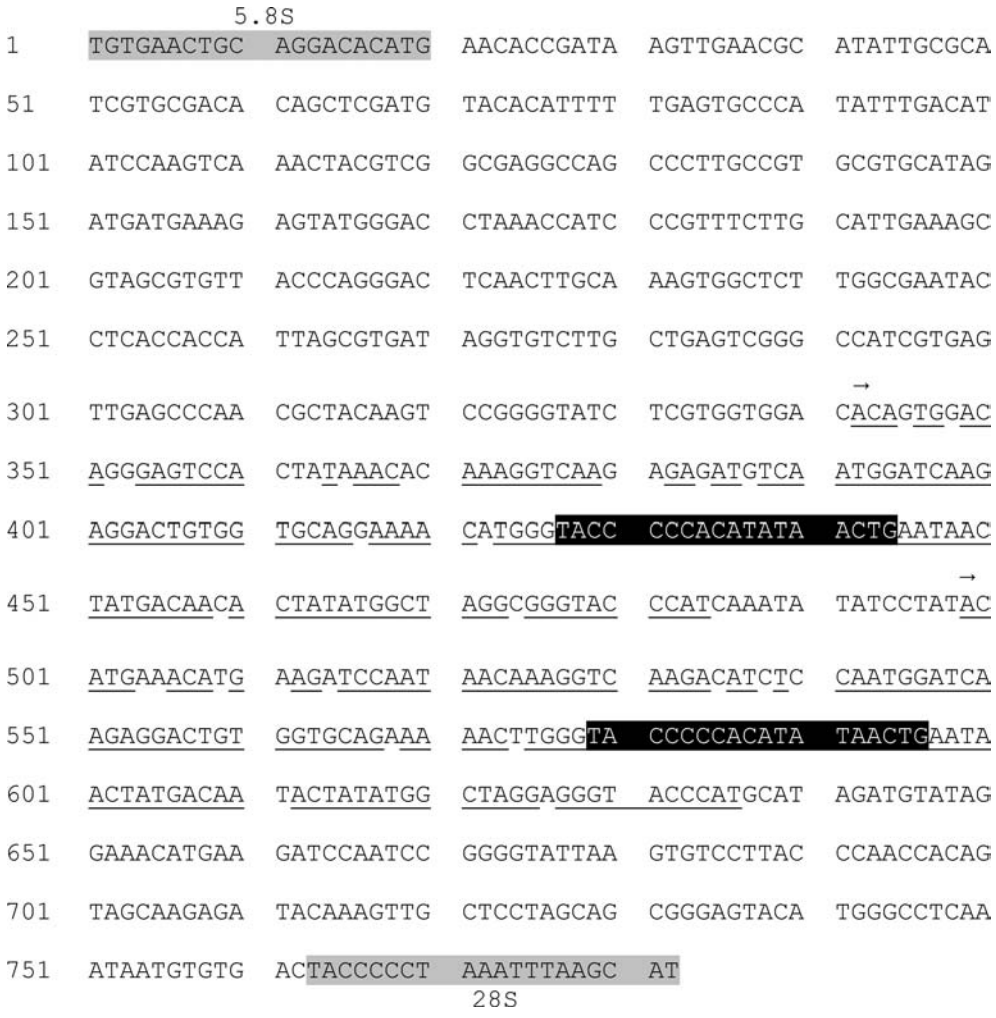

Fig. 2 Nucleotide sequence of *An beklemishevi* ITS2 rDNA (GenBank accession no. AY593958; amplifying primers are marked *grey*; *arrows* denote beginnings of hypothesized duplications, whereby identity between the two regions is underlined; sequences within the duplications similar to the 28S primer are marked *black*)

The alignment of the *An beklemishevi* consensus sequence with GenBank derived ITS2 sequences of the other *An maculipennis* complex species displays nucleotide concordance to a large extent in the beginning (for approx. 335 bp) and at the end (for approx. 150 bp) of the amplified DNA fragment (Fig. 3). However, in the middle of the *An beklemishevi* sequence there is a region of more than 300 nucleotides finding no counterpart in the other siblings. Closer analysis of this intermediate region suggests that an insertion comprising about 140 nucleotides has duplicated and later on underwent further mutational changes (Fig. 2). Interestingly, an 18mer sequence can be found within the hypothesized duplication that is nearly identical to the 28S primer whereby absolute identity exists for the first seven 3′-end nucleotides (Fig. 2).

Surprisingly, according to the Bayesian analysis, *An beklemishevi* is predicted to build a phylogenetic lineage with the nearctic *maculipennis* sibling species (Fig. 4) among which *An freeborni* and *An hermsi* are most closely related. In the same cladogram, *An atroparvus* and *An messeae* seem to be more closely related than the other palaearctic species while *An sacharovi* has an isolated position. However, this phylogenetic tree is generally very weakly supported, most probably due to the *An beklemishevi* putative insertion that cannot phylogenetically be compared with corresponding sequences in the other species. When this insertion is deleted from the *An beklemishevi* ITS2 sequence for comparative purposes, leaving more or less equally long ITS2 sections for all sibling species, the Bayesian analysis results in the expected closer relationship of *An beklemishevi* with the palaearctic siblings (Fig. 5) although it still appears to be phylogenetically most isolated. Moreover, in the case of this second approach much higher posterior probability values are given.

Discussion

An beklemishevi is a true cytospecies of the *An maculipennis* complex and was first described in 1975 (Stegnii and Kabanova 1978). It only occurs in Scandinavia and parts of Russia (Stegniy et al. 1978; Korvenkontio et al. 1979; Jaenson et al. 1986). So far, no published work exists on it being examined on a DNA basis, probably due to a lack of collection material. Except for one GenBank entry (accession no. AJ511867), this is the first

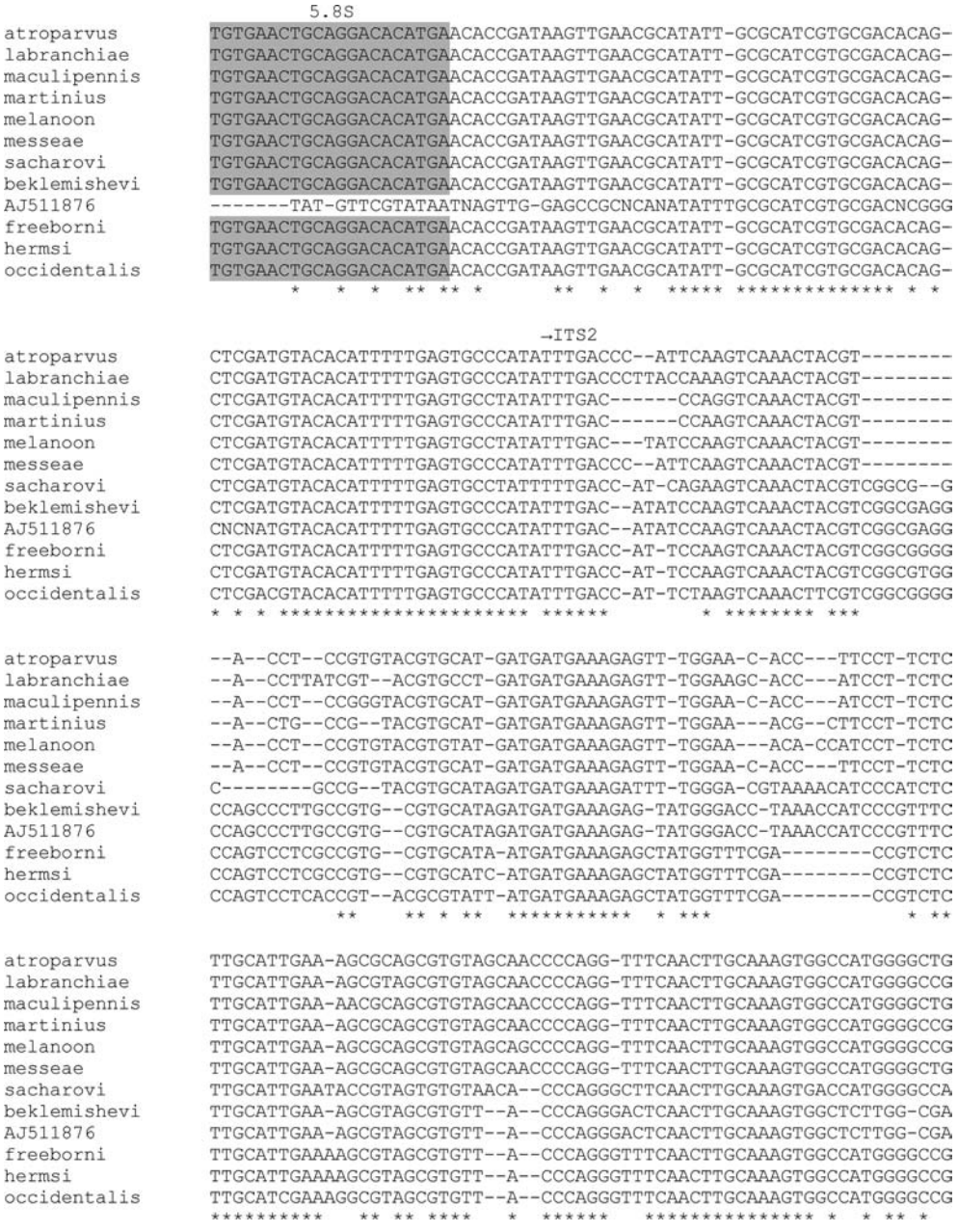

Fig. 3 Alignment of ITS2 of the European sibling species of the *An maculipennis* complex and *An martinius* (AJ511876: *An beklemishevi* sequence from GenBank; *asterisks* denote identical nucleotides)

time that sequence data for *An beklemishevi* are presented. According to the sequence already deposited in the GenBank, the *An beklemishevi* ITS2 region is 335 nucleotides long, but acceptable concordance with the other siblings can only be found for several short sections (data not shown). However, when this *An beklemishevi* GenBank entry is aligned with the sequence obtained within this study, there is a very high sequence similarity beginning from some 30 to 40 nucleotides after the 5.8S-end of the two sequences (Fig. 3). The GenBank sequence ends after approximately 410 nucleotides of the sequence presented here, still matching it very well but at the same time lacking the 28S-end both of this sequence and of that of the other *An maculipennis* sibling species sequences. Unfortunately, there is no information on how the *An beklemishevi* GenBank entry sequence was obtained.

The ITS2 sequence presented here is nearly twice as long. Compared to other anopheline species, it is so far actually identified at the maximum length (Beebe and Cooper 2000). Typical ITS2 lengths are between 350 bp and 500 bp, however, for *An punctulatus* and *An dirus* complex species more than 600 bp and 700 bp, respectively, have been determined (Xu and Qu, 1997; Beebe et al. 1999). Quite unusual is a significant discrepancy in length within the same complex as found between *An beklemishevi* and the other palaearctic *An maculipennis* sibling species whose ITS2 regions are between 290 bp and 312 bp (Proft et al. 1999). Sequence characteristics indicating that the putative insertion responsible for the

Fig. 3 (contd.)

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atroparvus      ACACCTCACCACCAT--CAGCGTGC-TGTGT---AGCGTGTTCGGCCCAGTAAGGTCATCGTGAG
labranchiae     ACACCTCACCACCAT--CAGCGTGC-TGTGC---AGCGTGTTCGGCCCAGTTCGGTCATCGTGAG
maculipennis    ACACCTCACCACCAT--CAGCGTGC-TGTGT---AGCGTGTTCGGCCCAGTTCGGTCATCGTGAG
martinius       ACACCTCACCACCAT--CAGCGTGC-TGTGT---TGCGTGTTCGGCCCAGTTCGGTCATCGTGAG
melanoon        ACACCTCACCACCAT--CAGCGTGC-TGTGT---AGCGTGTTCGGCCCAGTTCGGTCATCGTGAG
messeae         ACACCTCACCACCAT--CAGCGTGC-TGTGT---AGCGTGTTCGGCCCAGTAAGGTCATCGTGAG
sacharovi       ACACTTCACCGCCAT--CT-TGTGCATGTGT---AGTGTGTTCGGCCTAGCTTGGTTAACGTGAG
beklemishevi    ATACCTCACCACCATT--AGCGTG-ATA-G-----GTGTCTT--GCTGAGTCGGGCCATCGTGAG
AJ511876        ATACCTCACCACCATT--AGCGTG-AAA-G-----GTGTCTT--GCTGAGTCGGGCCATCGTGAG
freeborni       ACACCTCACCACCGTCACAGCTTGC-TGTGCTGCAGTGTGTTCGGCCCAGTTCGGTCATCGTGAG
hermsi          ACACCTCACCACCGTCACAGCGTGC-TGTGC---AGTGTGTTCGGCCCAGTTCGGTCATCGTGAG
occidentalis    ACACTTCACCACCATC--AGCGTGC-TGTGC---AGTGTGTTCGGCCTATTTCGGTCATCGTGAG
                * ** ***** ** *       **     *     *******  **  *    **  * ******

atroparvus      GCGTCACCTAACG-------------------------------------------------------
labranchiae     GCGTTATCTAACG-------------------------------------------------------
maculipennis    GCGTTACCTAACG-------------------------------------------------------
martinius       GAGTAACCTAAT--------------------------------------------------------
melanoon        GCGTTACCTATCG-------------------------------------------------------
messeae         GCGTCACCTAACG-------------------------------------------------------
sacharovi       GCGA-ACCCAACG-------------------------------------------------------
beklemishevi    TTGA-GCCCAACGCTACAAGTCCGGGGTATCTCGTGGT-GGACACAGTGGACAGGGAGTCCACTA
AJ511876        TTGA-GCCCAACGCTACAAGTCCGGGGTATCTCGTGGTTGGACACAGTGGACAGGGAGTCNACTA
freeborni       GCGA-GCCCAACC-------------------------------------------------------
hermsi          GCGA-GCCCAACG-------------------------------------------------------
occidentalis    GCGA-GCCCAACG-------------------------------------------------------
                  *    * *

atroparvus      ----------------------------------------------------------------------
labranchiae     ----------------------------------------------------------------------
maculipennis    ----------------------------------------------------------------------
martinius       ----------------------------------------------------------------------
melanoon        ----------------------------------------------------------------------
messeae         ----------------------------------------------------------------------
sacharovi       ----------------------------------------------------------------------
beklemishevi    TAAA-CACAAAGGTCAAGAGAGATGTCAATGGATCAAGAGGACTGTGGTGCAGGAAAACATGGGT
AJ511876        TAAAACACAAAGGTCAAGAGAGATNTCAATGGANCAAGAGGAAT-T----------------------
freeborni       ----------------------------------------------------------------------
hermsi          ----------------------------------------------------------------------
occidentalis    ----------------------------------------------------------------------

atroparvus      ----------------------------------------------------------------------
labranchiae     ----------------------------------------------------------------------
maculipennis    ----------------------------------------------------------------------
martinius       ----------------------------------------------------------------------
melanoon        ----------------------------------------------------------------------
messeae         ----------------------------------------------------------------------
sacharovi       ----------------------------------------------------------------------
beklemishevi    ACCCCCACATATAACTGAATAACTATGACAACACTATATGGCTAGGCGGGTACCCATCAAATATA
AJ511876        ----------------------------------------------------------------------
freeborni       ----------------------------------------------------------------------
hermsi          ----------------------------------------------------------------------
occidentalis    ----------------------------------------------------------------------

atroparvus      ----------------------------------------------------------------------
labranchiae     ----------------------------------------------------------------------
maculipennis    ----------------------------------------------------------------------
martinius       ----------------------------------------------------------------------
melanoon        ----------------------------------------------------------------------
messeae         ----------------------------------------------------------------------
sacharovi       ----------------------------------------------------------------------
beklemishevi    TCCTATACATGAAACATGAAGATCCAATAACAAAGGTCAAGACATCTCCAATGGATCAAGAGGAC
AJ511876        ----------------------------------------------------------------------
freeborni       ----------------------------------------------------------------------
hermsi          ----------------------------------------------------------------------
occidentalis    ----------------------------------------------------------------------
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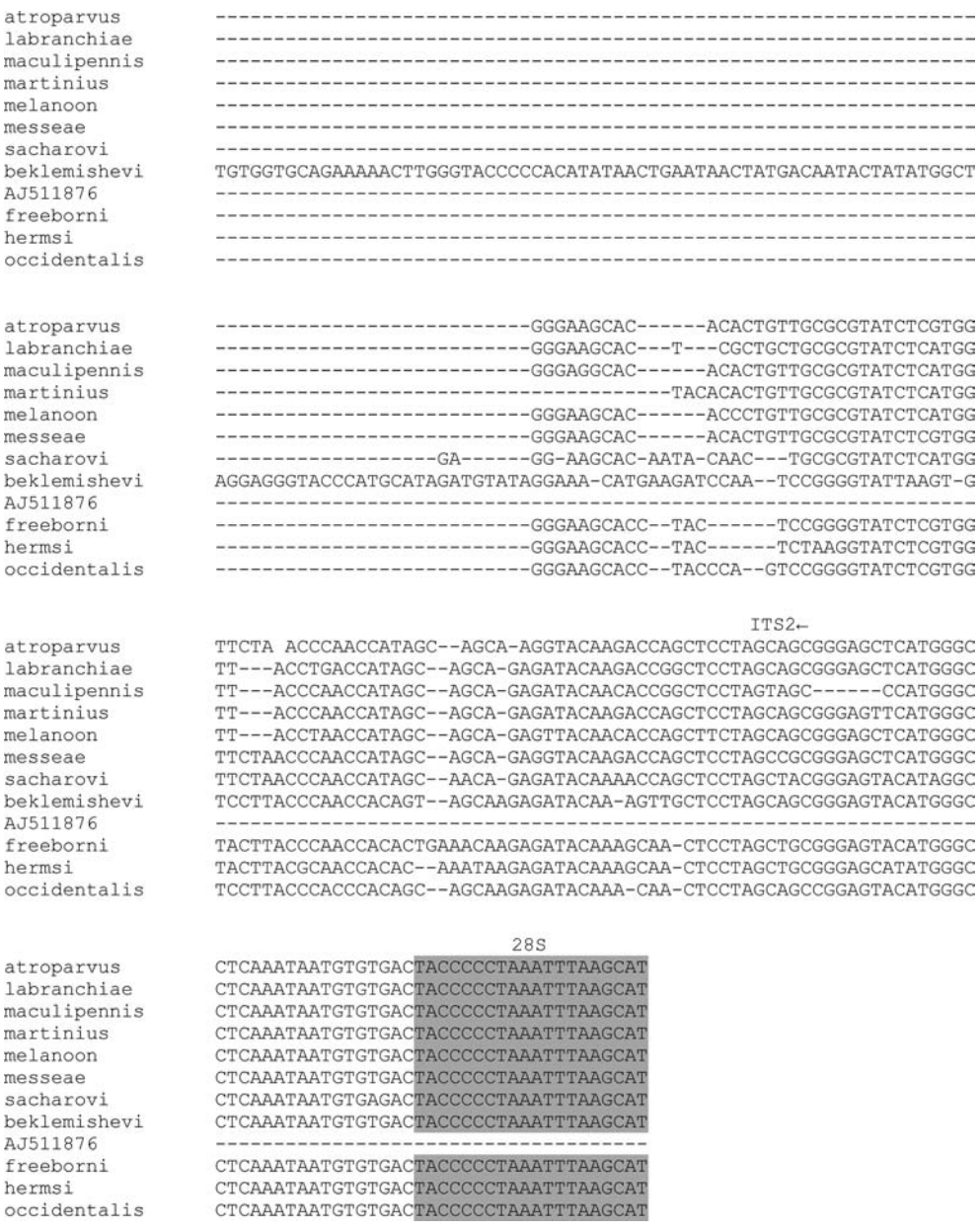
considerable length of the *An beklemishevi* sequence is the result of a former integration of a mobile element could not be recognized when checked by BLAST search.

Beginning with nucleotides 427 and 579 of the *An beklemishevi* PCR product, respectively, there is a repeated motif of 18 nucleotides that finds complementary bases in the 28S primer and exactly matches within the first seven nucleotides. Given a hybridization of the 28S primer to these regions, a 444- and a 596-bp amplicon ought to be expected. However, respective DNA bands were never observed after gel electrophoresis. Furthermore, alignments with sequences corresponding to such hypothetical PCR

Fig. 3 (Contd.)



products would not be possible since considerable parts matching the ITS2 regions of the other siblings follow only past these sites.

Marinucci et al. (1999) were the first to infer phylogenetic trees for the whole *An maculipennis* complex from DNA sequence data. Their results generally correspond with the data presented here, however, their trees do not include *An beklemishevi* and are based on a quite limited number of mosquitoes resulting in weak posterior probabilities. Their ITS2 sequence data and many more are involved in the phylogenetic study presented here. Correspondingly, in both analyses, *An freeborni* and *An hermsi* are more closely related to each other than to *An occidentalis*. Within the palaearctic branch, *An sacharovi* is in both studies somewhat isolated from the other siblings.

With regard to *An beklemishevi*, the computer analysis locates this species closer to the nearctic than to the palaearctic *maculipennis* species although both groups have ITS2 sequences of only around 300 bp. It is to be taken into account that only one ITS2 sequence each was available for the nearctic species, whereas considerable numbers of sequences were used for the palaearctic species.

The hypothesis, that the nearctic and palaearctic *An maculipennis* species build distinct lineages, was already expressed decades ago (Kitzmiller et al. 1967). Contrasting former tacit assumptions that the nearctic *maculipennis* species have been derived from a Beringconnexion immigrant, genetic and cytogenetic as well as morphological, distributional and taxonomic data highly support a descent of the palaearctic from the nearctic species. Therefore, *An beklemishevi* must be

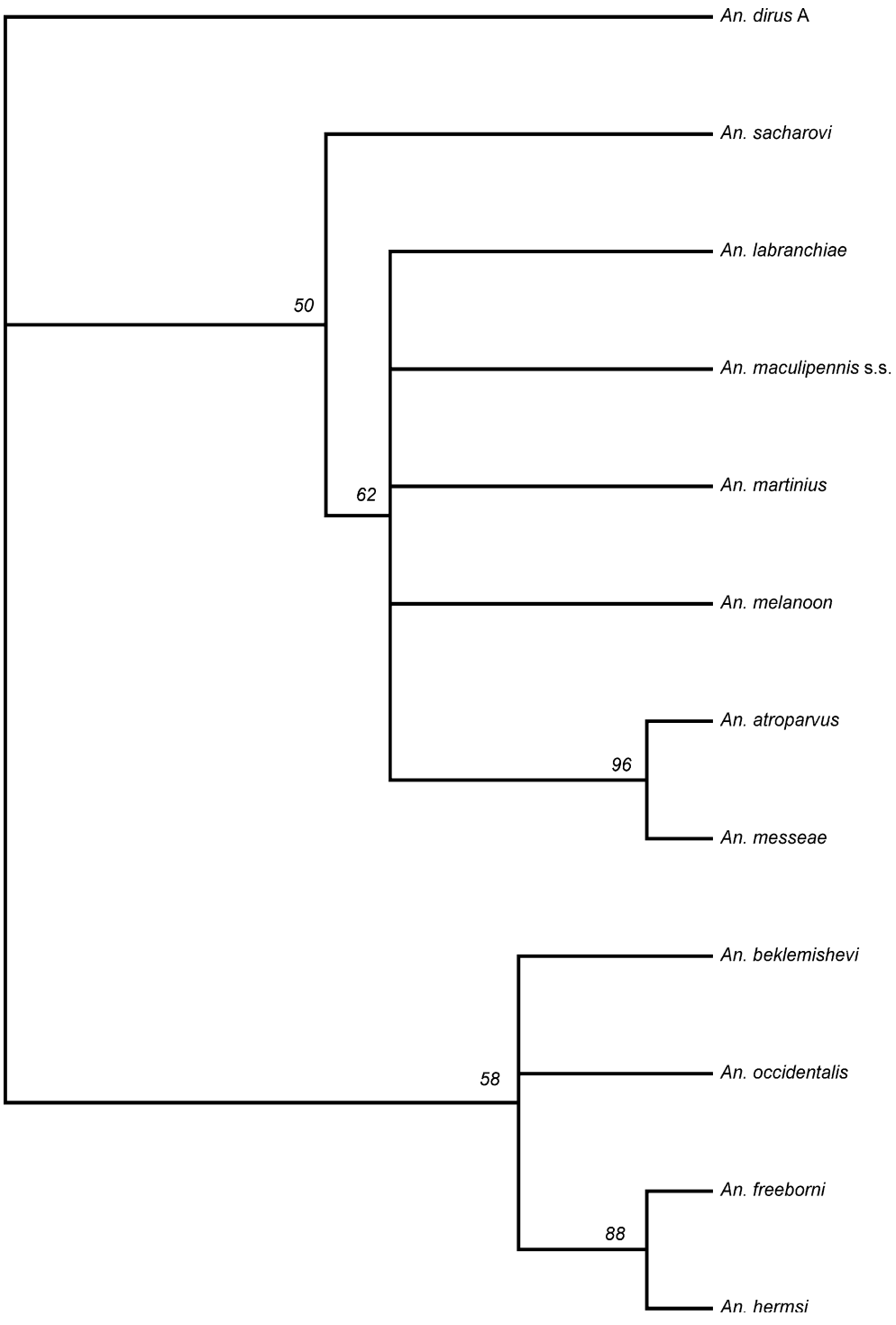


Fig. 4 Phylogenetic tree of the *An maculipennis* sibling species complex (*An dirus* species A (Genbank accession no. U60410) = outgroup)

expected to be more closely related to the palaearctic sibling species and in fact cannot be distinguished from them by morphological features. Based on the cytogenetic and biochemical features, Stegnii (1981, 1991) has constructed a scheme of speciation for the palaearctic *An maculipennis* complex, according to which the ancestral species developed to *An labranchiae* which in its turn was the ancestor of the other palaearctic species except for *An beklemishevi*. *An beklemishevi* is said to have originated from the nearctic evolutionary branch and is not directly related to the other palaearctic species. Later on, when investigating interspecific differences in pericentromeric heterochromatin, Sharakhova et al. (1997) concluded that *An labranchiae* and *An beklemishevi* have a common ancestor originating from the nearctic complex. Only recently, Harbach (2004) presented a revised classification of the genus *Anopheles* including the formal and informal taxa and preliminary hypothetical

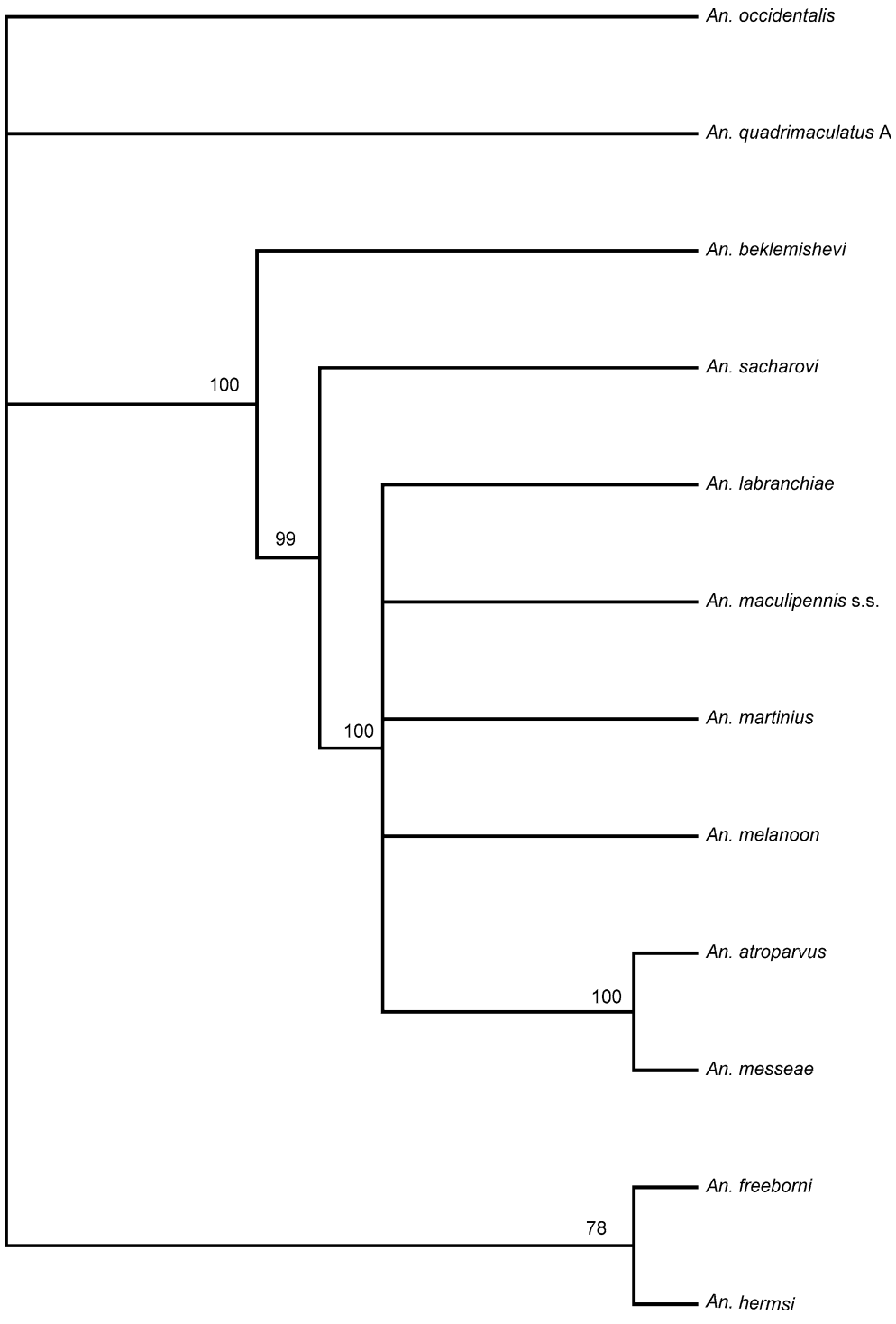


Fig. 5 Phylogenetic tree of the *An maculipennis* sibling species complex after deletion of the putative insertion in the middle of the *An beklemishevi* ITS2 region (*An quadrimaculatus* species A (Genbank accession no. U32503) = outgroup)

phylogenetic relationships. He added two more sibling species to the palaearctic branch of *An maculipennis* group found in Iran and in Romania: *An persiensis* (Sedaghat et al. 2003) and *An daciae* (Nicolescu et al. 2004). Moreover, *An beklemishevi* is not grouped together with the other palaearctic siblings in the '*maculipennis* subgroup' but in the '*quadrimaculatus* subgroup'.

The data presented here demonstrate the exceptional position of *An beklemishevi* and support the theory of an early split of *An beklemishevi* from the ancestor of the other palaearctic species of the *An maculipennis* complex. The differences in the ITS2 nucleotide sequence have been exploited to integrate *An beklemishevi* in a multiplex PCR assay diagnostic for the palaearctic *An maculipennis* sibling species in order to facilitate species identification (Kampen 2005).

Acknowledgments I heartily thank all the Russian scientists for promptly and unselfishly providing *An beklemishevi* mosquito specimens involved in the study: Dr. Alexey V. Katokhin (Institute

of Cytology and Genetics, Russian Academy of Science, Novosibirsk, Russia), Dr. Yuriy M. Novikov (Research Institute for Biology and Biophysics, Tomsk State University, Tomsk, Russia), Dr. Olga P. Braginets (presently Department of Biological Sciences, State University of New York at Buffalo, Buffalo, New York, USA), and particularly Dr. Maria V. Sharakhova (presently Center for Tropical Disease Research and Training, University of Notre Dame, Notre Dame, Indiana, USA) who organized mosquitoes herself and arranged all the other contacts. Furthermore, I am indebted to Carolin Wiersch (Institute for Medical Parasitology, University of Bonn, Germany) and Dr. Bernhard Misof (Zoological Research Institute and Museum Alexander Koenig, Bonn, Germany) for their introduction into the Bayesian analysis and their assistance in generating the phylogenetic trees.

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