rDNA Sequences of *Anopheles* Species from the Iberian Peninsula and an Evaluation of the 18S rRNA Gene as Phylogenetic Marker in Anophelinae

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ABSTRACT The complete 18S rDNA and internal transcribed spacer (ITS)-2 rDNA sequences were obtained from *Anopheles atroparvus* Van Thiel and *Anopheles plumbeus* Stephens from two areas of Spain. The number of nucleotide differences in the 18S rDNA of the two species is high compared with differences in the same gene of other invertebrate vectors. In *Anopheles*, short 18S rDNA sequences are richer in AT than the longer sequences, which are richer in GC and include extremely GC-biased expanded regions. Four small regions in the variable regions V4 and V7 contain the majority of nucleotide differences. The results did not support the use of partial sequences for relationship analyses. Genetic distances and phylogenetic analyses supported the most recent classification of *Anopheles*. The complete 18S rDNA sequence is better for studying anopheline phylogenetics.

KEY WORDS *Anopheles*, 18S and ITS-2 rDNA sequences, phylogenetic analysis, malaria, Iberian Peninsula

Climatic changes have increased the risk for the reintroduction of malaria and other infectious diseases into Spain and Portugal (Muentener et al. 1999). Malaria has occurred in Greece (Kampen et al. 2002a,b), Italy (Baldari et al. 1998, Romi et al. 2001), Spain (Cuadros et al. 2002), and Morocco (Faraj et al. 2003).

In Europe, three species of the Maculipennis Subgroup (Harbach 2004), *Anopheles atroparvus* Van Thiel, *Anopheles sacharovi* Favr, and *Anopheles labranchiae* Falleroni, are known to be efficient current or historical malaria vectors (Ribeiro et al. 1988, Kasap 1990, Jetten and Takken 1994, Romi 1999, Romi et al. 2001). Previous studies indicated that the sibling species are not equally important as vectors for malaria parasites because of their feeding preferences (Fantini 1994) and their differential susceptibility to infection (reviewed in Jetten and Takken 1994).

In both Spain and Portugal, *An. atroparvus* seems to be the most efficient vector. It is halophilous and is found nearly throughout Europe. Details about the biology of this species can be found in Linton et al. (2002) and Rees and Snow (1990). In entomologic surveys conducted in previously malarious areas in Spain, only *An. atroparvus* has been found in high densities similar to those observed during the years malaria was endemic (Blazquez 1974).

Another European mosquito species not in the Maculipennis Subgroup, *Anopheles plumbeus* Stephens, is capable of transmitting *Plasmodium falciparum* (Marchant et al. 1998). Moreover, its capacity to transmit *Plasmodium vivax* was experimentally proven long ago (Blacklock and Carter 1920); it was implicated in malaria transmission in the United Kingdom (Shute 1954) and was recently involved in authochthonous cases in Germany (Krueger et al. 2001). *An. plumbeus* is a species of the western Palaearctic, overwintering as larva, with females biting in the daytime as well as in the evening. Its biology is described in Karch (1997). This species is more locally distributed in Spain (Encinas 1982).

Because of the changes in *Anopheles* distribution in Spain, we are interested in the influence of global warming on anophelines and the risk of reintroduction of malaria in the Iberian Peninsula. We are studying *Anopheles* in Spain by using DNA methods to differentiate cryptic species and analyze species relationships.

The utility of nuclear rDNA for such studies is generally accepted. The nuclear rRNA genes are arranged in tandemly repeated arrays with each unit containing genes for conserved (18S, 5.8S, and 28S) and variable regions (spacers) (Beckingham 1982, Hil-

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lis and Dixon 1991). Because of their ubiquity in all cell types and their functional and structural constancy, sequence analyses from the 18S gene are commonly used (Hillis and Dixon 1991, Olsen and Woese 1993). The 18S ribosomal gene has proven to be informative for analyses of distantly related organisms because it is relatively long (usually ≈1,800–1,900 nucleotides) and highly conserved, although the taxonomic level for which it is useful differs widely among different organisms (Bargues and Mas-Coma 1997).

The efficacy of 18S rDNA for resolving evolutionary relationships among taxa is well documented (Bargues et al. 1997, 2000; Mangold et al. 1998, Wuyts et al. 2000). There have been relatively few 18S rDNA sequence studies on Culicidae (Baldridge and Fallon 1991, Brown and Knudson 1997, Miller et al. 1997, Beebe et al. 2000a, Cooper et al. 2000, Sallum et al. 2002).

The purpose of this study was to genetically characterize anopheline species from Spain by means of the analysis of the complete nucleotide sequences of the 18S rRNA gene. The respective internal transcribed spacer (ITS)-2 sequences were also obtained for the verification of species classification. Mosquito materials were collected in the province of Salamanca and in the Ebro river delta. Malaria was very prevalent in these two areas in the first half part of the 20th century (Blazquez 1974, Encinas 1982). Finally, an evaluation of the 18S rRNA gene as phylogenetic marker in Anophelinae is made by means of a compared analysis with sequences available in the GenBank-European Molecular Biology Laboratory (EMBL).

Materials and Methods

Mosquitoes. Anophelinae adult specimens were initially classified as 1) *Anopheles* (*Anopheles*) *maculipennis* Meigen s.l. captured in Santa Marta de Tormes, Salamanca, Spain; 2) *An.* (*An.*) *plumbeus*, from Santa Marta de Tormes, Salamanca, Spain; and 3) *An.* (*An.*) *atroparvus*, from Poble Nou, Ebro Delta, Tarragona, Spain. The entire 18S rDNA sequence was obtained from two to three adults of each species for nucleotide confirmation, if needed. For *An. atroparvus*, two individuals from the same locality from a colony also were sequenced.

Adults were identified from morphological characters (Schaffner et al. 2001) and descriptions of local populations and keys (Encinas 1982). Voucher specimens are deposited in the laboratories of Salamanca (Santa Marta de Tormes materials) and Valencia (Ebro Delta material). The systematic classification recently proposed for the genus *Anopheles* by Harbach (2004) is used here.

DNA Extraction. Either the entire adult, thorax, or legs of each mosquito were used for DNA extraction with phenol-chloroform (Sambrook et al. 1989) and alternatively by the InstaGene Matrix (Bio-Rad, Hercules, CA) kit. Mosquito extracts were suspended in 400 μl of lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, and 1% sodium dodecyl sulfate) containing 500 μg/ml proteinase K (Promega, Madison, WI). The lysed preparation was gently mixed and then incubated for 2 h at 55°C with alternate shaking each 15 min. Total DNA was extracted using standard extraction techniques (Bargues and Mas-Coma 1997). We also used the InstaGene Matrix kit according to manufacturer's protocol (Bargues et al. 2006). The thorax and legs of the mosquito were suspended in 1 ml of sterilized H_2O, homogenized, and centrifuged for 1 min at 12,000 rpm. The pellet was suspended in 200 μl of InstaGene matrix and incubated at 56°C for 30 min. Tubes were vortexed for 10 s and placed in a 100°C boiling waterbath for 8 min, vortexed for 10 s, and centrifuged at 12,000 rpm for 3 min. The supernatant was stored at −20°C until used. We used 0.4–4 μl of the supernatant per 25 μl of polymerase chain reaction (PCR) reaction.

18S and ITS-2 Sequencing. The complete 18S rRNA gene of each mosquito was amplified by PCR according to methods outlined previously (Bargues and Mas-Coma 1997). To amplify and sequence the complete 18S rRNA gene, two new primers (18SANF 5′-TCATGTGAGAGAATTCTGGTTGAT-3′ and 18SANR 5′-AGGCACTCAAAATGTGTACACC-3′) were designed and used with primers 18SA, 18SB, 18SG, and 18SAP2 of Beebe et al. (2000a). Amplifications were generated in a Peltier thermal cycler (MJ Research, Watertown, MA) by 30 cycles of 30 s at 94°C, 30 s at 50–55°C, and 1 min at 72°C, preceded by 30 s at 94°C and followed by 7 min at 72°C. The ITS-2 rDNA region was amplified by PCR using primers based on conserved sequences of the 5.8S and 28S coding regions (Marinucci et al. 1999). Amplifications were performed by 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, preceded by 5 min at 94°C and followed by 7 min at 72°C after the last cycle. Negative controls (with no template) were always run simultaneously, and reaction mixtures were discarded when DNA occurred in the control. We examined 10 μl of the reaction mixture by 1% agarose gel electrophoresis, followed by ethidium bromide staining.

Primers and nucleotides were removed from PCR products by purification using Ultra Clean PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA) according to the manufacturer's protocol and resuspended in 50 μl of 10 mM TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.6). The final DNA concentration in micrograms per milliliter and the absorbance at 260/280 nm was determined in a spectophotometer UV-VIS (Biophotometer, Eppendorf, Hamburg, Germany).

The sequencing of the 18S rRNA gene and the ITS-2 region was performed on both strands by the dideoxy chain-termination method (Sanger et al. 1977), and was carried with the *Taq* dye-terminator chemistry kit for ABI 3730 and ABI 3700 capillary system (Applied Biosystems, Foster City, CA), by using PCR primers.

Sequence Alignment. Sequences were aligned using CLUSTAL-W version 1.8 (Thompson et al. 1994), and assembly was made with the Staden Package version 1.5. The alignment was made using the anopheline species studied with other known Culicidae se-

quences. The following complete or almost complete (lacking ≈30 bp in the 3′ end) 18S rDNA sequences of Culicidae species present in GenBank-EMBL were used: *Culex tritaeniorhynchus* (incomplete sequence; GeneBank accession no. U48385); *Aedes aegypti* (incomplete sequence; U65375); *Aedes vexans* (AM071382), and *Ochlerotatus caspius* (AM071383); *Anopheles clowi* (AF178683); *Anopheles annulipes* A (AF121053); *Anopheles farauti* 1 (AF121054); *Anopheles farauti* 3 (AF121056); *Anopheles koliensis* (AF121061); *Anopheles punctulatus* (AF121062); and *Anopheles* sp. near *punctulatus* (AF121063); *Anopheles maculatus* (incomplete sequence; AF440198); *Anopheles albimanus* (L78065); *Anopheles pseudopunctipennis* (incomplete sequence; U49735); and *Anopheles gambiae* (AM157179). Among the different *An. farauti* units, only one (species 1) from those presenting a long sequence and another (species 3) species from those with a short sequence (Beebe et al. 2000a) were selected for comparisons. The following dipteran Nematocera (Psychodidae) also were used: *Phlebotomus papatasi*, clone PAPa (AJ244413) and *Sergentomyia minuta*, clone MIN8 (AJ244421).

Concerning ITS-2, the following sequences available in the GenBank were used: *An. atroparvus* (Z50103.2, AF504237–AF504248, AY63453, AF436064, and AY050640); *An. plumbeus* (AJ555483.1); and *An. claviger* (AY129232.1 and incomplete sequence including many indeterminate nucleotides, AJ555157.1).

The DCSE version 2.54 program (De Rijk and De Wachter 1993) was used to examine potential secondary structures. In addition, the program RNAstructure (Mathews et al. 1999) was used to predict parts of the structure. The published secondary structure prediction for eukaryote 18S rRNA (Van De Peer et al. 1999) was used to predict the secondary structure of the 18S rRNA of the mosquito species studied. The RnaViz program was employed for graphic representation (De Rijk and De Wachter 1997).

Phylogenetic Analysis. Phylogenies were inferred using maximum-likelihood (ML) estimates with PAUP version 4.0b10 (Swofford 2001). Maximum-likelihood parameters such as model, base frequencies, transition/transversion ratio (ti/tv), shape of gamma distribution, and proportion of invariant sites were optimized using the hierarchical likelihood ratio test (hLRTs) implemented in Modeltest 3.06 (Posada and Crandall 1998). The ML phylogeny was estimated using the HKY85 (Hasegawa et al. 1985) model, and PAUP was used to obtain the likelihood scores of each phylogeny. Starting branch lengths were obtained using least-squares method with ML distances. To provide an assessment of the reliability of the nodes of the trees, a quartet puzzling analysis was used with 1,000 puzzling steps.

For comparison, distance methods also were used in phylogeny reconstruction. For distance analysis, a neighbor-joining (NJ) tree was generated from a Kimura three-parameter distance matrix by using PAUP version 4.0b10 and the Molecular Evolutionary Genetics Analysis (MEGA) program version 3.0 (Kumar et al. 2004). Minimum evolution (ME) trees were generated and statistical support of each NJ tree was assessed with bootstrap-resampling technique (Felsenstein 1985) over 1,000 replications.

Results

rDNA ITS-2 Sequence Analysis. The ITS-2 of the three mosquito populations studied were sequenced and deposited in the GenBank under the following accession numbers: *An. atroparvus* (=*An. maculipennis* s.l. from Salamanca), AM076979; *An. plumbeus*, AM076978. The length of the ITS-2 sequence was 309 bp for *An. atroparvus* and 337 bp for *An. plumbeus.* The average nucleotide composition was slightly GC biased, being 56.67% for *An. atroparvus* and 54.37% for *An. plumbeus.*

The sequence of the specimens of *An. atroparvus* from Ebro Delta were identical to *An. maculipennis* s.l. mosquitoes from Salamanca. It was almost identical to the following ITS-2 sequences of *An. atroparvus* found in the GenBank: Z50103.2, AF504237–AF504248, and AY63453. Only one mutation (transition) was detected in the position 174 of the ITS-2 alignment with the sequence of accession number AF436064, and only one difference (a deletion) in position 56 of the ITS-2 alignment with the sequence AY050640, both also ascribed to *An. atroparvus.*

The ITS-2 of *An. plumbeus* was compared with sequences of the same spacer of other anopheline species deposited in GenBank. A surprisingly very high degree of nucleotide differences was detected (divergence percentage of 46.17%), compared with the 279-bp ITS-2 sequence for *An. plumbeus* (AJ555483.1; 368 bp deposited including parts of 5.8S and 28S). The sequence of *An. plumbeus* from Salamanca only seemed to be close to that of *Anopheles claviger* (Meigen). The pairwise ITS-2 comparison of *An. plumbeus* with *An. claviger* (AY129232.1) showed in total 33 nucleotide differences in a 350-bp alignment, representing a 9.43% divergence, including 11 (3.14%) transitions, 7 (2.00%) transversions, and 15 (4.28%) indels. Similar results were obtained compared with the other ITS-2 sequence of *An. claviger* available in GenBank (AJ555157.1). In total, 31 nucleotide differences in a 307-bp alignment (10.09% divergence) were observed, which include several indeterminate nucleotides.

18S rDNA Sequence Analysis. The 18S rDNA sequences obtained have been deposited in GenBank-EMBL under the following accession numbers: *An. atroparvus* (=*An. maculipennis* s.l. from Salamanca): AM072973; *An. plumbeus*: AM072974. The length of the sequence was 1,965 bp for the specimens classified as *An. atroparvus* and *An. maculipennis* and 1,981 bp for *An. plumbeus.* The average nucleotide composition was slightly AT biased, being 52.52% for *An. atroparvus* (=*An. maculipennis* s.l. from Salamanca) and 51.27% for *An. plumbeus.*

The alignment of the three 18S rDNA sequences resulted in a total of 1,988 characters, including gaps, showing 202 nucleotide differences, which represents

a genetic divergence of 10.16%. No nucleotide difference was detected between the sequence of *An. atroparvus* from Ebro Delta and that of *An. maculipennis* s.l. from Salamanca. Compared with *An. plumbeus*, a very high number of differences was detected (202), including 174 true mutations (8.75%), of which 91 were transitions (4.57%) and 83 transversions (4.17%), and only a few (28) insertions-deletions (indels) (1.41%).

When the 18S rDNA sequence of *An. atroparvus* was compared with that of *An. pseudopunctipennis* from GenBank, the number of nucleotide differences was only 38, representing a 1.93% genetic divergence. Substitutions seemed to be the most numerous, with 24 transitions (1.22%), 13 transversions (0.66%), and only one indel (0.05%).

A third alignment was performed, including our two different 18S rDNA sequences and the 11 complete or almost complete sequences of other anopheline species available in the GenBank: *An. farauti* 1 (2,046 bp), *An. farauti* 3 (1,983 bp), *An. koliensis* (2,095 bp), *An. punctulatus* (2,102 bp), *An.* sp. near *punctulatus* (2,073 bp), *An. pseudopunctipennis* (1,931 bp), *An. clowi* (2,085 bp), *An. annulipes* (2,019 bp), *An. albimanus* (1,977 bp), *An. maculatus* (1,950 bp), and *An. gambiae* (2,015 bp). The alignment, including these 13 sequences, resulted in a total of 2,231 characters, including gaps, of which 1,573 sites were constant and 433 were parsimony-informative. In this alignment, the mean frequencies of adenine, cytosine, guanine and thymine were 0.248, 0.240, 0.275, and 0.236, respectively ($\chi^2 = 84.76$ df = 36, $P < 0.001$).

In the latter alignment, three regions comprising positions 700–975, positions 1,014–1,050, and positions 1,670–1,791 contained the majority of nucleotide differences. We found that these three regions include differences that enable not only subgenera, series, group, subgroup, complexes, and species distinction, but also distinction of cryptic species as in the two *An. farauti* species 1 and 3 selected. The first of these three regions corresponds to the variable region V4, the second also to V4, and the third to V7. The other nucleotide differences were distributed throughout the rest of the 18S sequence.

Expanded regions were responsible for the long length of the 18S rDNA, as in *An. farauti* 1, *An. koliensis*, *An. punctulatus*, *An.* sp. near *punctulatus*, *An. annulipes*, *An. clowi*, and *An. gambiae*, whose length varied between 2,015 and 2,102 bp (mean 2,062.1 bp). The species *An. atroparvus* (=*An. maculipennis* from Salamanca), *An. plumbeus*, *An. pseudopunctipenis*, *An. albimanus*, and *An. maculatus* do not have these expanded regions; consequently, their 18S rRNA gene is shorter, 1,931–1,983 bp (mean 1,964.5 bp). Interestingly, the longer 18S rDNA sequences are richer in GC than the shorter sequences, which are usually richer in AT. Among the species presenting a longer sequence, only *An. gambiae* shows an almost balanced nucleotide composition.

Six expanded regions were detected and were named A, B, C, D, E, and F. They are located between positions 197 and 222 (A), 756 and 846 (B), 888 and 941 (C), 1,022 and 1,050 (D), 1,670 and 1,791 (E), and 1,906 and 1,920 (F) of the alignment of the 12 sequences analyzed. Region A is located in the variable area V2, B, C, and D in the variable area V4, region E in V7, and region F in V8. When comparing these expanded regions with the regions containing the majority of nucleotide differences detected in the 18S rRNA gene, it seems that expanded regions A and F do not contribute to differentiation of several concrete species of short 18S sequence.

The expanded regions are rich in GC: *An. farauti* 1, 80.69%; *An. koliensis*, 80.11%; *An. punctulatus*, 74.25%; *An.* sp. near *punctulatus*, 76.51%; *An. annulipes*, 77.20%; *An. clowi*, 82.75%; and *An. gambiae*, 71.42%. However, the expanded regions did not explain the biased GC content of the 18S rDNA sequences. The sequences showed GC biased without the expanded regions, i.e., *An. farauti* 1, total GC = 54.11%, GC after discounting expanded regions = 52.08%; *An. farauti* 3, total GC = 53.86%, GC after = 52.81%; *An. koliensis*, total GC = 54.65%, GC after = 52.32%; and *An. annulipes*, total GC = 52.45%, GC after = 50.66%. The only exception is *An. gambiae*, total GC = 50.42%, GC after = 49.35%.

The genetic distances within the 18R rRNA gene were evaluated using Kimura's three-parameter model, in an analysis including all species of Culicidae whose 18S rDNA sequences are available in GenBank-EMBL (Table 1). Within Anophelinae, the most genetically related species are *An. punctulatus* and *An.* sp. near *punctulatus* and also *An. atroparvus* and *An. pseudopunctipennis*. The most distant seem to be *An. atroparvus* versus *An. clowi*.

Phylogenetic Analyses Inferred from 18S rDNA Sequences. The alignment of the 17 18S rDNA sequences representing 13 Anophelinae and 4 Culicinae species was used to infer phylogenies. Two psychodids, also presenting long 18S rDNA sequences (to avoid long branch attraction effects) as *Ph. papatasi* (2,095 bp) and *S. minuta* (2,058 bp), were used as outgroups.

The ML model best fitting the 18S data of mosquitoes was HKY85+I using the ts/tv ratio of 1.213 and the proportion of invariable sites of 0.372, in which kappa was 2.425 and the log likelihood was 12,687.29. Puzzle values obtained were very high in all main branches and quartets were examined using a least-squares method with ML distances (Fig. 1). For comparison, in distance analysis, an ME tree was generated using Kimura's three-parameter, in which negative branch lengths were allowed, but set to zero for tree-score calculation. The minimum evolution score of the tree obtained was 0.8745 (tree not shown).

ML and NJ analyses both yielded trees with identical branch topology and similar high supports (Fig. 1), where Anophelinae species clustered independently of Culicinae species. Within the Anophelinae cluster, three branches were noted. *An. atroparvus* and *An. plumbeus* were in the same branch, together with *An. pseudopunctipennis*. *An. albimanus* was basal to all other anophelines. The rest of the species were in one clade, in which *An. maculatus* was basal (Fig. 1).

Table 1. Pairwise comparisons of nucleotide divergences according to Kimura's three-parameter model for the whole set of 18S sequences from Culicidae species analyzed

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	—	0.02265	0.01043	0.03339	0.14840	0.14211	0.17575	0.17710	0.17869	0.17994	0.17660	0.18408	0.17729	0.12862	0.12422	0.16106	0.15712	0.21495	0.21939
2	44	—	0.02613	0.03821	0.15468	0.14850	0.18041	0.18050	0.18196	0.18428	0.17988	0.18568	0.17899	0.13178	0.12390	0.16441	0.16193	0.21351	0.21794
3	18	45	—	0.03601	0.15385	0.14744	0.17717	0.17749	0.17973	0.18112	0.17741	0.18573	0.17700	0.13554	0.12653	0.15802	0.16102	0.22371	0.22741
4	62	71	60	—	0.14610	0.13899	0.16667	0.16748	0.16919	0.17154	0.16811	0.17253	0.16712	0.12750	0.11310	0.15121	0.14860	0.21425	0.21838
5	287	299	264	270	—	0.01969	0.16738	0.16906	0.17185	0.17089	0.16913	0.17520	0.16826	0.10491	0.13910	0.14970	0.14689	0.23144	0.23679
6	270	282	248	257	38	—	0.16319	0.16328	0.16561	0.16463	0.16338	0.17163	0.16515	0.10729	0.13424	0.14943	0.14423	0.23242	0.23470
7	329	337	298	306	315	305	—	0.02045	0.03087	0.03835	0.06501	0.03108	0.05197	0.14836	0.14316	0.10902	0.09891	0.27217	0.26457
8	331	337	298	307	318	305	40	—	0.02934	0.03494	0.03046	0.05589	0.05225	0.14725	0.14566	0.11094	0.10270	0.25857	0.25352
9	337	343	305	313	326	312	63	58	—	0.04470	0.03657	0.07449	0.05988	0.14990	0.15037	0.11267	0.10181	0.27872	0.26954
10	339	347	307	317	324	310	78	69	92	—	0.00966	0.06104	0.06169	0.15489	0.14797	0.11560	0.10963	0.26817	0.26406
11	332	338	300	310	320	307	63	60	75	20	—	0.05634	0.05536	0.15312	0.14565	0.11059	0.10410	0.26784	0.26182
12	347	350	315	319	332	323	131	110	153	125	115	—	0.06399	0.15356	0.15320	0.11405	0.10992	0.28209	0.26960
13	331	334	297	306	316	308	103	102	120	124	111	129	0.14936	0.14852	—	0.11001	0.10843	0.26778	0.25825
14	249	255	233	236	205	206	281	278	286	296	292	293	281	—	0.10216	0.13333	0.13537	0.23187	0.23587
15	240	239	217	209	270	256	270	275	286	281	276	292	282	199	—	0.12392	0.12663	0.23231	0.22619
16	297	303	262	274	278	276	209	213	217	223	213	220	211	248	230	—	0.08619	0.23362	0.23595
17	299	308	271	270	281	271	190	198	197	214	203	215	211	261	243	166	—	0.23890	0.23975
18	414	411	385	397	449	443	534	498	558	535	533	567	527	454	453	442	468	—	0.09042
19	421	418	390	404	457	445	513	486	531	521	515	533	501	459	437	445	468	185	—

Below diagonal, total character differences; above diagonal, mean character differences. 1, *Ae. vexans;* 2, *Oc. caspius;* 3, *St. aegypti* (=*Ae. aegypti*); 4, *Cx. tritaeniorhynchus;* 5, *An. atroparvus;* 6, *An. pseudopunctipennis;* 7, *An. farauti* 1; 8, *An. farauti* 3; 9, *An. koliensis;* 10, *An. punctulatus;* 11, *An.* sp. near *punctulatus;* 12, *An. clowi;* 13, *An. annulipes;* 14, *An. plumbeus;* 15, *An. albimanus;* 16, *An. maculatus;* 17, *An. gambiae;* 18, *Ph. papatasi;* 19, *Se. minuta.*

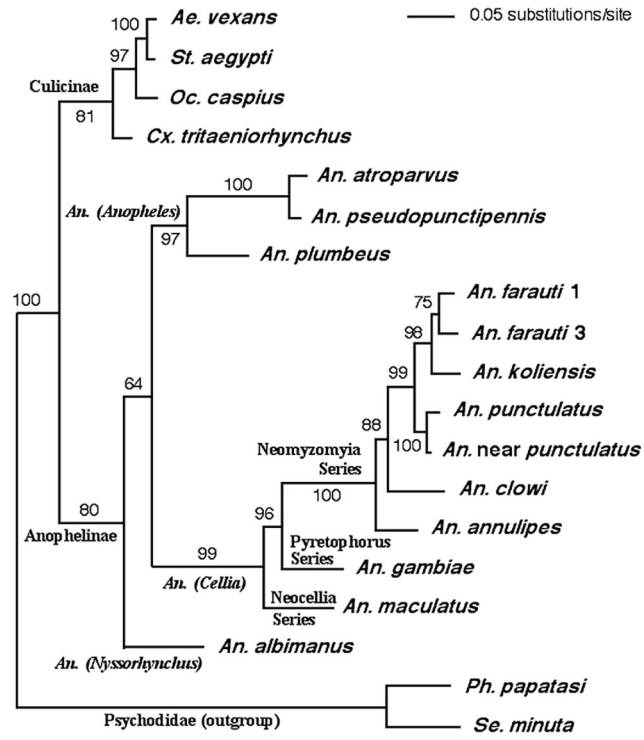

Fig. 1. Phylogenetic tree of Culicidae showing the *Anopheles* cluster, based on 18S rDNA sequences, derived from the maximum likelihood (ML HKY85+I) model, by using the transition/transversion ratio of 1.213 and obtained using the psychodids *Phlebotomus papatasi* and *Sergentomyia minuta* as outgroup. Scale bar indicates the number of substitutions per sequence position. Numbers represent the percentage of 1,000 puzzling replicates.

Discussion

ITS-2 Sequences and Species Classification. The absence of nucleotide differences in the ITS-2 sequence of *An. atroparvus* from Ebro Delta and *An. maculipennis* s.l. mosquitoes from Salamanca proves that both are the same species. This species seems to be the same *An. atroparvus* found in the rest of Europe, according to the identical ITS-2 sequences found in the GenBank from Italy (Marinucci et al. 1999), from the United Kingdom (Linton et al. 2002), and from Romania (Nicolescu et al. 2004). It is also identical to *An. atroparvus* from Portugal, The Netherlands, Italy, and Spain (Catalonia) according to ITS-2 sequence by Proft et al. (1999), suggesting that this species is very homogeneous throughout Europe. Similar ITS-2 sequence uniformity has been observed in other species of the Maculipennis Subgroup (Di Luca et al. 2004). Only one mutation in the population studied by Djadid (2001), erroneously identified as *An. maculipennis* (Linton et al. 2002), and only one deletion detected by Djaid et al. (2001), suggest a slight polymorphism in Iranian populations of *An. atroparvus* in the eastern range of the species.

The difficulty in differentiating adults in the species belonging to the Maculipennis Subgroup (=Palaearctic sibling species of the group known previously as the Maculipennis Complex) (Harbach 2004) is well known. Traditionally, the sibling species of this complex have been classified on the basis of egg morphology (White 1978). A variety of other identification methods have been used, such as cross-breeding experiments, larval chaetotaxy, isoenzyme analysis, cuticular hydrocarbon chromatography, and cytotaxonomy (reviewed in Proft et al. 1999).

The results suggest that the very high degree of nucleotide differences detected between *An. plumbeus* ITS-2 sequence and Asian *An. plumbeus* sequence (Gordeev et al. 2004) may indicate different species. The Asian mosquito material should be reviewed and its classification verified. Differences are too numerous as to include the two sequences in the same species. A high number of nucleotide differences have already been found between morphologically similar species belonging to the same complex (Kampen et al. 2003, Wilkerson et al. 2004).

A low genetic distance of *An. plumbeus* from Salamanca with *An. claviger* from different European countries and Israel (Kampen et al. 2003) was observed. *An. claviger*, subgenus *Anopheles* is known to be a malaria vector (Jetten and Takken 1994), and is present in Spain and Salamanca province (Encinas 1982). *An. claviger* forms the Claviger Complex together with its sibling species *An. petragnani* Del Vecchio, another anopheline also present in the province of Salamanca (Encinas 1982). This complex has recently been included within the Anopheles Series by

Harbach (2004), although it remained as unassigned to a group. The results in the current study suggest that the Claviger Complex is close to and may be included within the Plumbeus Group.

The rDNA ITS-2 sequence is very efficient for mosquito classification. Anopheline species have ITS-2 sequences that do not vary greatly among populations throughout the geographical distribution of each species (Fritz et al. 1994, Onyabe and Conn 1999, Di Luca et al. 2004). Only a reduced intraspecific and intragenomic sequence variability have been found in some *Anopheles* species (Wilkerson et al. 2004, Fairley et al. 2005).

18S rDNA Sequences and Phylogenetic Analyses. The length of the 18S rDNA sequence of the anopheline species studied (1,965 and 1,981 bp) is similar in length range to other *Anopheles* (Miller et al. 1997, Cooper et al. 2000, Beebe et al. 2000a). The range in length is large, from 1,931 to 2,102, and considerably longer than 18S rDNA sequences of other eukaryotic organisms (usually 1,800–1,900 bp). The same gene seems to be somewhat shorter and more uniform (1,948–1,952 bp) in Culicinae (Baldridge and Fallon 1991, Bargues et al. 2006). A long 18S rDNA seems to be a synapomorphic characteristic of Diptera as a result of expanded variable helices.

The nucleotide composition of the 18S rDNA in the anophelines studied is only slightly AT biased. This slight AT bias agrees with results on Culicinae (Baldridge and Fallon 1991, Bargues et al. 2006) and Culicomorpha in general (Miller et al. 1997). A difference in nucleotide composition between core regions and expansion segments has been found in Diptera, where the AT richness derives mostly from the variable domains (Morales-Hojas et al. 2002). This is in accordance with the hypothesis that there has been a mutational bias toward an increase of the AT content during the evolution of Diptera (Friedrich and Tautz 1997). The AT bias is only observed in *Anopheles* species having shorter 18S rDNA sequences (1,931–1,983 bp), such as *An. atroparvus, An. plumbeus, An. pseudopunctipenis, An. albimanus,* and *An. maculatus.* This nucleotide content bias changes to GC in those species presenting longer sequences (2,019–2,102 bp), as *An. farauti* 1, *An. koliensis, An. punctulatus, An.* sp. near *punctulatus, An. annulipes,* and *An. clowi.* Although long 18S rDNA sequence seems related to the GC-rich expanded regions, the rest of the gene is still slightly GC without the expanded regions (50.66–52.81%). In *An. gambiae,* the sequence length is intermediate (2,015 bp), and the nucleotide composition is almost balanced (50, 42% GC and 49, 56% AT), despite presenting GC-rich expanded regions. The nucleotide content bias in Anophelinae is slight, which makes the 18S rDNA sequence an appropriate tool. Balanced DNA regions seem better for taxonomic and phylogenetic studies because substitutions become more detectable when bases occur with approximately similar frequency (25%) (Luton et al. 1992).

The lack of variation detected between distant populations of the same species as *An. atroparvus* in the current study support the usefulness of this gene as a marker for Culicidae. The high number of differences between *An. atroparvus* and *An. plumbeus,* both belonging to the Anopheles Series and Angusticorn Section within the subgenus *Anopheles* (Harbach 2004), is a good example.

The long 18S rDNA sequence includes conserved regions that enable the alignment of sequences of anophelines belonging to evolutionarily distant species. This represents a decisive advantage compared with the ITS-2 marker, in which nucleotide differences prohibit alignment between distant species.

The number of nucleotide differences in the 18S rDNA of mosquitoes is higher compared with differences in this gene in other invertebrates (Bargues and Mas-Coma 1997, Bargues et al. 1997) or other insects (Mangold et al. 1998; Bargues and Mas-Coma 2002). This indicates a higher evolutionary rate and faster molecular clock in mosquitoes compared with other organisms (Bargues et al. 2000, 2002). The high number of informative nucleotide differences provides genetic distances with high puzzle and bootstrap support values in ML and NJ trees, respectively, as in the current study. The 18S rRNA gene is excellent for the reconstruction of Anophelinae phylogenetics and to help systematics and taxonomy toward an evolutionary classification of Culicidae in general.

The 18S rDNA sequence may lack sufficient resolution for distinguishing cryptic or sibling species (Beebe et al. 2000a), compared with ITS-2 (Porter and Collins 1991, Wesson et al. 1992, Collins and Paskewitz 1996, Cornel et al. 1996, Marinucci et al. 1999, Proft et al. 1999, Di Luca et al. 2004, Wilkerson et al. 2004). A study using 18S structural alignment was able to resolve such a group of sibling species of *Anopheles,* whereas a computer generated similarity-based alignment did not (Beebe et al. 2000b). Studies on the three structural domains identified domain two (including nucleotides between positions 643 and 1,313, in a total of 2,169) as the only region informative at the sibling species level and resulted in the same tree as the full structural sequence and helicoidal regions. This finding may be argued to support the phylogenetic results on Anophelinae using only a partial sequence (733 bp, including positions 630–1,425 in the alignment), which includes the same 18S rDNA domain (Sallum et al. 2002). Our study on Anophelinae, including well separated species and not limited to only sibling species, did not support this. Although the variable region V4 containing a high number of nucleotide differences is included in this partial sequence, the highly informative variable region V7 as well as many potentially informative differences throughout the rest of the complete 18S sequence are not contained in this partial sequence. Hence, we recommend using the complete 18S rDNA sequence for analyses of Anophelinae.

A new classification of the genus *Anopheles* has been recently proposed to provide a framework for studies of evolutionary relationships at specific and supraspecific levels of divergence (Harbach 2004). The genetic distances and phylogenetic results obtained in the current study fully support the supraspecific arrangements included in this classification. The three sub-

genera *Anopheles*, *Cellia*, and *Nyssorhynchus* (represented by only *An. albimanus* from Central America, the Caribbean, and northern South America) seem well separated in independent clades. Within the subgenus *Anopheles*, *An. atroparvus* (Maculipennis Group) seems more close to the American *An. pseudopunctipennis* (Pseudopunctipennis Group) than to *An. plumbeus* (Plumbeus Group). This may be understood taking into account that *An. pseudopunctipennis*, a species distributed from southern North America throughout Central America and the northern and Pacific South America up to northern Argentina, is close to the Maculipennis Group, which also comprises several species in the Nearctic region (Porter and Collins 1996).

Among the subgenus *Cellia*, the Neocellia Series (represented by only *An. maculatus* from the Malaysian-Indonesian Subregion), Pyretophorus Series (represented by only *An. gambiae* from Africa), and Neomyzomyia Series seem well differentiated. Within the Neomyzomyia Series, the Australasian *An. annulipes* (not assigned by Harbach 2004) is basal to all species belonging to the southwest Pacific Punctulatus Group, the species of the Farauti Complex included. These results further support the appropriateness of the 18S rRNA gene for the analysis of Anophelinae phylogenetics and to help systematics and taxonomy toward an evolutionary classification of *Anopheles* mosquitoes.

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