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POLYMERASE CHAIN REACTION–BASED DIFFERENTIATION OF THE MOSQUITO SIBLING SPECIES *ANOPHELES CLAVIGER* S.S. AND *ANOPHELES PETRAGNANI* (DIPTERA: CULICIDAE)

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Abstract. A polymerase chain reaction (PCR)–based diagnostic assay was developed that rapidly and reliably differentiates the sibling species of the *Anopheles claviger* complex, *An. claviger* s.s. and *An. petragnani*. The assay makes use of nucleotide differences in the internal transcribed spacer 2 ribosomal DNA sequences to generate PCR products of specific length for each of the two species. In evaluating the test, 580 of 592 field-collected *An. claviger* s.l. specimens were unambiguously identified as one of the two sibling species. Due to poor DNA quality, the remaining 12 specimens yielded no PCR product. Of the 592 mosquitoes, 407 larval specimens had been identified morphologically prior to species-specific DNA amplification, and in all instances PCR identification corroborated with morphologic identification. Mosquitoes identified as *An. claviger* s.s. came from various localities all over Europe and from Israel. Those identified as *An. petragnani* were collected in southern France and Spain. The species-diagnostic PCR assay would facilitate data collection on the temporal and spatial distribution of the two *An. claviger* sibling species because they represent possible vectors of disease in Europe, the Near and Middle East, and north Africa.

INTRODUCTION

Anopheles claviger s.l. is a western palaearctic culicid species complex composed of two siblings, *An. claviger* s.s. and *An. petragnani*. Although they were recognized as two morphologic forms of *An. claviger* more than 60 years ago by Del Vecchio[1] and Lupascu,[2] it was not until 1962 that Coluzzi demonstrated their species status.[3] Up to now, these sibling species could only be distinguished by minor morphologic characters of the immature stages[4–6] and laborious isoenzyme techniques.[7–9] The occurrence of at least one of the *An. claviger* complex species has been observed in nearly all of Europe, the Near East, parts of the Middle East, and north Africa.[10,11] However, there are little reliable data on the respective distribution of each of the two complex members, although *An. claviger* s.s., in contrast to *An. petragnani*, was formerly shown to be a vector of malaria in various regions of Europe.[12–15]

The contemporary discussion on emerging and resurging vector-borne diseases with regard to possible climatic and environmental changes[16,17] has again focused interest on European mosquitoes, especially as possible vectors of malaria[18–21] and of various viral diseases.[22,23] Recent autochthonous, i.e., locally acquired, malaria cases in Italy, Bulgaria, and Greece[20,21,24] demonstrate that decades after malaria eradication from European vector competent *Anopheles* mosquitoes are still indigenous in these regions. Although the *An. maculipennis* complex in this respect deserves the most attention, there are other *Anopheles* species, including *An. claviger* s.s., that were involved in malaria transmission when this disease was rife in Europe. Presently, *An. claviger* is considered a malaria vector in some states of the former Soviet Union where malaria is again on the increase.[25] *An. claviger* s.l. has also been shown to be capable of transmitting Tahyna virus,[26] and it was found to be naturally infected with several other pathogens of medical and veterinary relevance, but without evidence for a vector role.[27–31]

As a result of increasing numbers of autochthonous cases of allegedly tropical vector-borne diseases in countries with moderate climates, concern in Europe is growing to map known and putative vectors of disease and follow their spatial distribution with time. Such studies are made easier by quick yet reliable tools for species identification. To aid this identification, we have developed a diagnostic polymerase chain reaction (PCR)–based assay for the *An. claviger* complex that fulfills these requirements by producing amplicons of different length for each of the two sibling species.

MATERIALS AND METHODS

Mosquito origin. *Anopheles claviger* s.l. specimens, comprising larvae, pupae, and adults, were collected from 1993 to 2003 in France, Spain, Scotland, England, Denmark, Sweden, Czech Republic, Austria, The Netherlands, Germany, and Israel (Table 1). The mosquitoes from The Netherlands, Israel and the Bonn area of Germany (North-Rhine Westfalia), as well as three specimens from France (Tarn), were classified to the complex level by morphologic features using classic determination keys.[32–34] The others were further identified as *An. claviger* s.s. or *An. petragnani* by morphologic characteristics of the larvae[4,6] and partly characterized by isoenzyme polymorphisms.[35]

Extraction of DNA. Two to three abdominal segments of a specimen were used for extraction of DNA following two different extraction protocols. For sequencing purposes, DNA was isolated according to the method of Collins and others[36] with minor modifications described by Proft and others.[37] During this procedure, the mosquito tissue is homogenized in a tube with grind buffer and incubated at 65°C for 30 minutes. Potassium acetate is added to give a final concentration of 1 M, and the solution is incubated on ice for 30 minutes. The debris is removed by centrifugation and the supernatant is transferred to a new tube. Ice-cold 100% ethanol is added to precipitate the DNA. After incubation for at least two hours at -20°C, the DNA is pelleted by centrifugation, washed twice with ethanol, air-dried, and resuspended in sterile water to give 100 μL of DNA solution.

The same protocol was used for PCR identification purposes, as well as a short protocol involving tissue homogenization in a tube containing 100 μL of 1.25% ammonium hy-

TABLE 1

Origin of *Anopheles claviger* s.l. specimens processed, and results of species identification by larval morphology and the polymerase chain reaction (PCR)*

Country	Locality	Number of mosquitoes	Morphologic identification	PCR identification
France	Aisne, Aube, Bas-Rhin, Corrèze, Côte d'Or, Doubs, Haute-Corse, Haute-Marne, Haut-Rhin, Haute-Savoie, Ille-et-Villaine, Indre, Loire, Loire-Atlantique, Maine-et-Loire, Meurthe-et-Moselle, Oise, Puy-de-Dôme, Saône-et-Loire	203	*An. claviger* s.s.†	*An. claviger* s.s.
	Gard, Haute-Corse, Hérault, Tarn, Var	94	*An. petragnani*†	*An. petragnani*
	Tarn	3	*An. claviger* s.l.‡	2 *An. claviger* s.s. 1 *An. petragnani*
Spain	Serrania	6	*An. petragnani*†	*An. petragnani*
Scotland	Highland, Strathclyde	10	*An. claviger* s.s.†	*An. claviger* s.s.
England	North Humberland	1	*An. claviger* s.s.†	*An. claviger* s.s.
The Netherlands	Zuidholland	39	*An. claviger* s.l.‡	*An. claviger* s.s.
Denmark	Himmerland	6	*An. claviger* s.s.†	*An. claviger* s.s.
Sweden	Öland	3	*An. claviger* s.s.†	*An. claviger* s.s.
Czech Republic	Bohemia	5	*An. claviger* s.s.†	*An. claviger* s.s.
Austria	Salzburg	48	*An. claviger* s.s.†	*An. claviger* s.s.
Germany	Bonn	129	*An. claviger* s.l.‡	117 *An. claviger* s.s. 12 NI
	Black Forest, Mecklenburg	31	*An. claviger* s.s.†	*An. claviger* s.s.
Israel	Banias	14	*An. claviger* s.l.‡	*An. claviger* s.s.

* NI = not identifiable.
† Specimens were morphologically identified to species prior to PCR.
‡ Specimens were morphologically pre-identified to complex level only.

droxide and subsequent boiling for approximately 20 minutes.[38] The tube was then opened while in an incubator until half of the solution, mainly the ammonium hydroxide, had evaporated. The remaining volume of approximately 50 μL represents the DNA solution used for PCR amplification.

Amplification and sequencing of DNA. Due to a high degree of interspecific variation compared with intraspecific variation[39,40] the internal transcribed spacer (ITS2) region of mosquito ribosomal DNA (rDNA) was selected for DNA sequence analysis. Following previous studies, the 5.8S and 28S primers were used to amplify the ITS2 region:[41–43] 5.8S: 5′-TGTGAACTGCAGGACACATG-3′ and 28S: 5′-ATGCTTAAATTTAGGGGGTA-3′.

The PCR mixture had a total volume of 50 μL and contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM dNTPs, 200 nM of each primer, 1 mM $MgCl_2$, 2.5 units of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 1–3 μL of DNA solution. The thermoprofile consisted of an initial denaturation step at 94°C for 10 minutes, followed by 35 cycles at 94°C for one minute, 50°C for one minute, and 72°C for one minute, and a final extension step at 72°C for 10 minutes.

Amplicons were subjected to electrophoresis for approximately one hour at 100 V on standard 1.5% agarose gels, excised from the gels, and eluted by means of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. They were then sequenced directly by cycle sequencing in an ABI Prism 310 DNA sequencer (Applied Biosystems, Foster City, CA) using the PCR primers as sequencing primers. The ITS2 regions of seven *An. claviger* s.s. specimens from France and Germany and five *An. petragnani* specimens from France were amplified and sequenced in both directions in duplicate. The sequences were aligned using CLUSTALW software (Intelligenetics, Mountain View, CA).

Design of species-specific primers and the diagnostic PCR. Differences in the ITS2 sequence of the two species were used to design species-specific (reverse) primers that in combination with the 5.8S universal (forward) primer would generate PCR products of species-specific lengths. The primer sequences were selected on the criteria that they had similar lengths and melting temperatures and low propensities to form primer-dimers and intramolecular secondary structures.[43] The compositions of the PCR mixtures were the same as for the amplification of the ITS2 region, except that the 28S primer was replaced with the species-specific primers. The conditions consisted of an initial step at 94°C for three minutes, followed by 35 cycles at 94°C for one minute, 50°C for one minute, and 72°C for two minutes, and a final step at 72°C for 10 minutes. The PCR products were subjected to electrophoresis on 1.5% agarose gels as described earlier in this report.

RESULTS

DNA sequencing showed the ITS2 region lengths of *An. claviger* s.s. and *An. petragnani* to be 341 and 302 basepairs, respectively (Figure 1). The sequencing electropherograms displayed no evidence of multiple peaks, and the consensus sequences from each individual within a species were invariant, suggesting that intra-individual and intraspecific sequence polymorphisms are quite rare or not existent. The GC

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                  5.8S
AP     1 TGTGAACTGC AGGACACATG AACACCGACA AGTTGAACGC ATATTGCGCA TCGTGCGACC
AC     1 TGTGAACTGC AGGACACATG AACACCGACA AGTTGAACGC ATATTGCGCA TCGTGCGACC
          ********** ********** ********** ********** ********** **********

                                                 →ITS2
AP    61 AGCACGGTGT ACACATTTTT GAGTGCCTAT ATTTATCCTT CATTCGACTG CGCGGTGACA
AC    61 AGCACGGTGT ACACATTTTT GAGTGCCTAT ATTTATCCTT CATTCAACTG CGCGCTGCGT
          ********** ********** ********** ********** ***** **** **** **

AP   121 CCCG-TCTCG GAA-CGACGC GGCACCGACG CGACCACTGA TGCGTTGTGC GACCGCCCGC
AC   121 GCCGGTCTAA CAAACCGCGC GACAGCGT-G CGCCCACTGA TGCGTTGTGC AACCGCCCGC
           *** ***    ** *  *** * ** **  * ** ******* **********  *********

AP   179 CGCGGAAGCG CAAACATTGA ATACCGAAGC GTGTCGACCG GCCAC----- -----GTGCG
AC   180 CGCGGTAGCG CAAACATTGA ATACCGAAGC GTGTTTTCCG GCCACATAAC GAGATGTGCT
          ***** **** ********** ********** ****   *** *****          ****

AP   229 TG-CAACCAC ACACAACAGG CGCACTCGCT AGCGTGCCCC GGACCAACAC CTCACCGACG
AC   240 TGTCTTCCAA GGACA-CAGT -GCACTTGCT AGCGTGCCCC GGGCCAACAC CTCACCGACG
          ** *  ***    *** ***   ***** *** ********** ** ******* **********

AP   288 TTAGCGCGTT CGCGTGTGCG TGTGCTCGGC CCGAACTGCC GCCGCGAAGT GCCGCCGTGC
AC   298 TTAGCGCGTT CGCGTGTGCG TGTGCTCGGC CCGAAGTGCC GCCGCGAGGT GCTGTTTTGG
          ********** ********** ********** ***** **** ******* ** ** *   **

AP   348 CGTGGCCACC AAAGTGTT-- ---------G CGCGCAACCG G------GAT CT----ACAC
AC   358 ATAATACACG CAAGTACAAA TAAACTCGGG TGTGTAACCA GATAAACGCT TTCACGACAC
               ***   ****                *  * * ****  *      * *  *    ****

                    ITS2←                                    28S
AP   387 --------AA CCACAGTAGG CCTCAAATAA TGTGTGGGTA CCCCCTATAT TTAAGCAT
AC   418 GAAGAAAAAA CCTCAGTAGG CCTCAAATAA TGTGTGGGTA CCCCCTAAAT TTAAGCAT
                  ** ** ******* ********** ********** ******* ** ********
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FIGURE 1. Internal transcribed spacer 2 (ITS2) ribosomal DNA sequence alignment for the sibling species *Anopheles petragnani* (AP) and *An. claviger* s.s. (AC). Underlined regions represent specific primer hybridization sites, dashes represent gaps introduced to maintain alignment, and asterisks represent identical nucleotides.

content was 56.3% for *An. claviger* s.s. and 64.9% for *An. petragnani*. The ITS2 regions of the two species showed 21% differences when nucleotide substitutions and insertions or deletions are considered; 9% of these differences are insertions and deletions.

The specific primers selected for the species differentiation are 20 (*An. claviger* s.s.) and 19 nucleotides (*An. petragnani*) long and have melting temperatures of approximately 62°C and 60°C, respectively. Their sequences are 5′-CTAGC AAGTGCACTGTGTCC-3′ for *An. claviger* s.s. (AC) and 5′-GCAACACTTTGGTGGCCAC-3′ for *An. petragnani* (AP). Based on the ITS2 sequences, these species-specific primers were expected to produce DNA fragments of 269 basepairs for *An. claviger* s.s. and 367 basepairs for *An. petragnani* when used in combination with the 5.8S primer. This could be verified experimentally as depicted in Figure 2.

The PCR identification assay yielded correct and identical results when the 5.8S PCR primer was used with a single species-specific primer, and when a multiplex PCR including the 5.8S primer and both species-specific primers was conducted. The success of the multiplex PCR demonstrates that no interference of the primers and no cross-hybridization with the heterologous DNA took place.

To show the specificity of the assay, multiplex PCRs were conducted with DNA of several other *Anopheles* species and some species of the genera *Aedes*, *Ochlerotatus*, and *Culex* that are commonly found in sympatry with the *An. claviger* sibling species: *An. maculipennis* s.s., *An. atroparvus*, *An. sacharovi*, *An. labranchiae*, *An. melanoon*, *An. messeae*, *An. subalpinus*, *An. beklemishevi*, *An. stephensi*, *An. plumbeus*, *An. hyrcanus*, *Aedes vexans*, *Ae. albopictus*, *Ochlerotatus caspius*, *Oc. geniculatus*, *Oc. detritus*, *Culex modestus*, *Cx.*

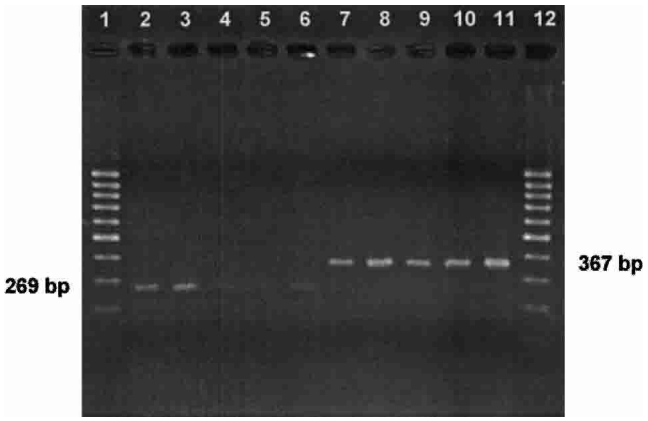

FIGURE 2. Specific polymerase chain reaction products for *Anopheles claviger* s.s. (269 basepairs [bp]) and *An. petragnani* (367 bp). Lanes 1 and 12, 100-basepair DNA ladder; lanes 2–6, *An. claviger* s.s.; lanes 7–11, *An. petragnani*.

pipiens, and *Cx. impudicus.* No PCR product was generated with any of these species except for *An. beklemishevi*, which produced a DNA fragment of approximately the same length as *An. petragnani*. When separate PCRs with only one of the specific primers were performed, the AC primer specific for *An. claviger* s.s. was responsible for the amplification. DNA sequencing of the amplicon produced a 357-basepair segment of the *An. beklemishevi* rDNA starting at the 5.8S end (Kampen H, unpublished data). The first 10 3′-end nucleotides of the AC primer were completely complementary to the *An. beklemishevi* sequence, and this proved sufficient for annealing and priming the *Taq* DNA polymerase activity. Altogether, 16 nucleotides in the 20mer AC primer were identical with the *An. beklemishevi* DNA sequence. Increasing the annealing temperature gradually from 50°C to 60°C did not prevent the generation of amplicons with *An. beklemishevi* DNA.

The diagnostic assay was evaluated in 592 field-collected *An. claviger* s.l. specimens (Table 1). The PCR results obtained were in concordance with the morphologic species identification in all 307 *An. claviger* s.s. and all 100 *An. petragnani* mosquitoes. Of the remaining 185 specimens that had been pre-classified to the complex level only, the PCR identified 172 as *An. claviger* s.s. and 1 as *An. petragnani*. In 12 cases in which the assay produced no amplicons, the entire ITS2 region also failed to be amplified.

DISCUSSION

The ITS2 region lengths of the *An. claviger* sibling species obtained by PCR amplification with primers 5.8S and 28S are within the range of other anopheline mosquito species,[44] although at lower limits. With little more than 300 basepairs, they are of similar lengths as those of the European members of the *An. maculipennis* complex.[37]

The GC content of the two *An. claviger* siblings is quite different, with the *An. claviger* s.s. falling in a range typical for culicid species with shorter ITS2 regions. However, the ITS2 GC content of *An. petragnani* is nearly 9% higher and reaches values usually found in species with longer ITS2 sequences.[44]

The specific primers designed for the two sibling species performed consistently under the PCR conditions tested and yielded specific DNA fragments that could be easily visualized in an agarose gel. This was the case for a simple PCR including two primers (the forward and the reverse), as well as for a multiplex PCR including the universal (forward) 5.8S primer and the two (reverse) species-specific primers in parallel.

When tested against DNA from several European and eastern *Culex*, *Aedes*, *Ochlerotatus*, or *Anopheles* species other than *An. claviger* s.l., the PCR primers unexpectedly were reactive with *An. beklemishevi* DNA. Although *An. beklemishevi* is known to occur only in Scandinavia and Russia,[45–47] and may not be frequently included in batches to be analyzed, this result demonstrates the importance of a morphologic identification of mosquito specimens to complex level prior to PCR examination. Otherwise, *An. beklemishevi* specimens may be incorrectly identified as *An. petragnani* in the PCR because the amplification products are too similar in size for visual separation in an agarose gel. In fact, this observation cannot be excluded for other *Anopheles* species not tested against the *An. claviger*-specific primers.

In contrast, we successfully used the PCR to identify field-collected *An. claviger* complex specimens. A PCR product was generated in all cases as a single and distinct band on an agarose gel that had a fragment length specific either for *An. claviger* s.s. or for *An. petragnani*. Due to the significant difference in length between the specific PCR products, the two species could easily be recognized and distinguished. Since the ITS2 genetic marker should be amplifiable with conserved primers in all mosquito species, the absence of DNA amplification in 12 mosquito specimens from the Bonn area of Germany suggests poor quality DNA. Indeed, the whole batch of mosquitoes had been conserved in a single tube in which conservation of the material may have been insufficient.

It is generally believed that *An. claviger* s.s. occurs throughout the range of the *An. claviger* complex, i.e., mainly Europe, north Africa, and the Near East, whereas *An. petragnani* is confined to the western Mediterranean basin.[9,10] Since the *An. petragnani* specimens in this study were demonstrated only from collection sites in southern France and Spain, the PCR results are concordant with published data[10,11] obtained with other identification techniques. Nevertheless, studies on the distribution of the sibling species of the *An. claviger* complex are required, and the PCR can be an exceedingly useful instrument in these studies.

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