Journal of the American Mosquito Control Association, 14(4):385–389, 1998
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A DIAGNOSTIC POLYMERASE CHAIN REACTION ASSAY FOR SPECIES A AND D OF THE *ANOPHELES DIRUS* (DIPTERA: CULICIDAE) SPECIES COMPLEX BASED ON RIBOSOMAL DNA SECOND INTERNAL TRANSCRIBED SPACER SEQUENCE

XIAOCHUN XU, JIANNONG XU[1,2] AND FENGYI QU

Department of Parasitology, Second Military Medical University, Shanghai 200433, People's Republic of China

ABSTRACT. A polymerase chain reaction assay based on differences in the internal transcribed spacer regions of ribosomal DNA was developed for distinguishing 2 members of the *Anopheles dirus* sibling species complex. This assay distinguished *An. dirus* species A from species D by producing diagnostic bands, 374 base pairs (bp) in length for species A and 663 bp in length for species D. Both laboratory colonies and field collections from Hainan and Yunnan provinces of China were identified with 100% accuracy.

KEY WORDS *Anopheles dirus*, species diagnostic assay, polymerase chain reaction, ribosomal DNA, species complex, second internal transcribed spacer

INTRODUCTION

Anopheles dirus Peyton and Harrison (Peyton and Harrison 1979) is considered a highly efficient malaria vector in forested areas of Southeast Asia (Meek 1995). The range of this species has thus far been found to include Thailand, Myanmar, Cambodia, Laos, Vietnam, China, Bangladesh, India, and Malaysia. Morphologic and cytogenetic studies suggest that *An. dirus*, which until 1979 was considered a subspecies of *Anopheles balabacensis* Baisas, is itself a species complex comprising at least 7 distinct species designated as *An. dirus* species A, B, C, D, E; *Anopheles takasagoensis* Morishita; and *Anopheles nemophilous* Peyton and Ramalingam (Baimai 1988). According to the data available at the present, 5 members found in Thailand (species A, B, C, D, and *An. nemophilous*) exhibit distinct geographic distributions (Baimai 1988, Baimai et al. 1988b). Two species are found in China; species A occurs on Hainan Island and species D is found in Yunnan Province (Xu and Qu 1991). Species A, C, and D are recorded in Myanmar (May 1987, Baimai et al. 1988c). Species D is widespread in Bangladesh (Baimai et al. 1988c). Species E is known only from southwestern India (Baimai 1988, Sawadipanich et al. 1990). *Anopheles nemophilous* is common in Peninsular Malaysia (Baimai 1988). A number of studies on *An. dirus* have addressed seasonal prevalence, feeding behavior, larval habitats, vector capacity, and the role of this species in the transmission of malaria (Rosenberg 1982; Rosenberg and Maheswary 1982; Dutta et al. 1989, 1991, 1996; Gingrich et al. 1990; Rosenberg et al. 1990, Biswas et al. 1992; Rattanarithikul 1995, 1996a, 1996b, 1996c; Somboon et al. 1995, Tun-Lin et al. 1995; Prakash et al. 1997a, 1997b; Rahman et al. 1997). These studies showed that the species is a forest inhabitant and is highly anthropophilic and exophilic in nature, and the larvae breed in either small temporary groundwater bodies or slow-moving perennial streams in shade. A careful study in Thailand provided evidence that species A, B, C, and D differ in geographic distribution. Each species appears to have distinct nocturnal biting times. The species also appear to have differential seasonal abundance (Baimai et al. 1988b). The larvae of species A and D exhibited differences in habitat preference (Rattanarithikul et al. 1995). In an area along the Myanmar border in southern Thailand, the role of *An. dirus* s.l. in malaria transmission is secondary during the wet season, and insignificant during the dry season. This conclusion is opposite to what has been reported for species A of *An. dirus* in southeastern and northern Thailand (Ismail et al. 1975, Rosenberg et al. 1990) and it may be that the *An. dirus* they studied is not species A, as species C and D were also found in the area (Rattanarithikul et al. 1996a). This report stresses the importance of species recognition as a basic requirement for epidemiologic investigations and control programs. Unfortunately, little information exists about the taxonomic status of *An. dirus* s.l. in field studies. This is partially because of the lack of a simple and reliable species identification method that is practical for field studies.

The identification of individual species of the *An. dirus* complex has relied upon techniques such as chromosomal analysis (Baimai et al. 1987) or allozyme typing (Green et al. 1992). Cytologic identification of sibling species is based on distinct banding patterns of salivary gland polytene chromosomes as well as heterochromatin differences in mitotic karyotypes. The method is applicable only to

[1] Author to whom correspondence and reprint requests should be addressed.

[2] Present address: Department of Medical and Molecular Parasitology, New York University Medical Center, 341 E 25th Street, New York, NY 10010.

F_1 progeny larva, which are not always available for field specimens. Allozyme analysis can only be performed on living or freshly frozen material, and difficulties in storage arise when working in the field. Egg characters are also useful in separating the different sibling species, but some differences can only be revealed by scanning electron microscopic observation (Damrongphol and Baimai 1989).

Direct analysis of DNA is an alternative approach for distinguishing among the members of a species complex (Hill and Crampton 1994, Munstermann and Conn 1997). The advantages of DNA-based methods are their applicability to all life stages and both sexes and the stability of the DNA molecules, which make sample storage and transfer more practical under field conditions. A nonradioactive DNA hybridization method has been developed to differentiate among species A, B, C, and D in field collections (Audtho et al. 1995). Ribosomal DNA (rDNA) is an attractive target for molecular taxonomy studies and has been widely used for the identification of cryptic species of mosquito complexes (Collins and Paskewitz 1996). We found sequence differences in the rDNA 2nd internal transcribed spacer (ITS2) between *An. dirus* species A and D from China (Xu and Qu 1997). Here we report a polymerase chain reaction (PCR) method that is capable of distinguishing these 2 member species based on sequence differences in the rDNA ITS2.

MATERIALS AND METHODS

Mosquitoes: Specimens of species A (Armed Forces Research Institution of Medical Sciences [AFRIMS], Bangkok, Thailand, colony) were provided by R. Rattanarithikul of AFRIMS. The Hainan (HN) colony of *An. dirus* was colonized in the mid 1970s, and was identified chromosomally as species A (Xu and Qu 1991). Field specimens of species A were collected in 1995 from 4 sites in Hainan Province, China. Fifty-three specimens were from Daan (the northern part of the *An. dirus* s.l. distribution region on Hainan Island), 29 specimens were from Heping (the central part of the *An. dirus* distribution region), 45 specimens were from Maoyang (the eastern part of the *An. dirus* distribution region), and 21 specimens were from Luokui (the southern part of the *An. dirus* distribution region). Thirty specimens of species D adults were collected in 1994 and 1995 from Mengla County, Yunnan Province, China (near the China–Laos and China–Myanmar border). Six of these specimens were used as sources for ITS2 sequence determination of species D (Xu and Qu 1997). The mosquitoes collected from the same site in 1990 were identified chromosomally as species D (Xu and Qu 1991). Cytogenetic confirmation was not done on field specimens because of our inability to obtain progeny from them. All field specimens were preserved in 75% ethanol and stored at −20°C until being used for PCR.

Polymerase chain reaction method: The DNA was extracted from individual mosquitoes according to the protocol described in Collins et al. (1987), and precipitated DNA was resuspended in 100 µl of sterile water. The DNA from single legs was prepared by triturating one leg in 10 µl of sterile water, then boiling for 5 min.

A universal primer (UP) (5′-TGTGAACTGCAGGACACATGAAC-3′) was selected within the 5.8S rDNA coding region; the species A-specific primer (AP) (5′-GGCGGTCCCCTTGTGCGT-3′) and the species D-specific primer (DP) (5′-GCGACTGAGGTCGGTCAG-3′) were selected within ITS2. Primer AP differs from the homologous sequence in species D by the last 6 most-3′ nucleotides, with a 4-base insertion at position 363–358; primer DP differs from species A by 3 single base mismatches, one in the 3′ position (Xu and Qu 1997). For optimal conditions the PCR reaction was conducted as a 25-µl volume in a Perkin-Elmer-Cetus DNA thermal cycler 480 (Perkin-Elmer, Norwalk, CT). Each sample contained 10 mM Tris buffer (pH 8.3) containing 50 mM KCl; 1.9 mM $MgCl_2$; 0.1 mg/ml bovine serum albumin, 0.1 mM each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate; 0.2 µM each of 3 primers; 1 µl of template DNA (10–50 ng); and 0.625 U Taq DNA polymerase. The amplification conditions included a total of 30 cycles at 94°C for 1 min, 50°C for 30 sec, and 70°C for 45 sec. The PCR products were analyzed by electrophoresis on 2% agarose gels containing ethidium bromide, and were visualized on an ultraviolet transilluminator.

RESULTS

The diagnostic PCR amplified species-specific fragments of 374 base pairs (bp) for *An. dirus* species A and 667 bp for species D. The 2 species can be separated unambiguously by the size of the product band on an ethidium bromide-stained gel (Fig. 1). Specimens of AFRIMS and HN colonies produced the fragments of species A. For the 148 field-collected specimens from the 4 sites in Hainan Province, the PCR yielded the 374-bp fragments of species A. When testing 30 specimens collected in 2 years from Mengla County, Yunnan Province, including 6 specimens used for sequence determination of ITS2 of species D, the PCR produced the 667-bp fragment corresponding to species D. When using extracted DNA, as little as 1/1,600 or less of the DNA extracted from a single specimen gave good results. For specimens dried for 14 months, 1/100 of the extracted DNA produced a well-amplified band. The PCR also worked successfully when 1/5 of the DNA from a single leg triturated in water is used. Figure 1 shows a typical PCR assay that unambiguously identified all specimens.

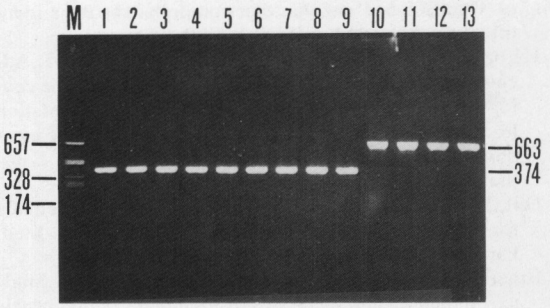

Fig. 1. A 2% agarose gel showing fragments amplified by polymerase chain reaction M: pGEM 7(+) DNA/Hae III marker. Lanes 1 and 2: species A, Armed Forces Research Institution of Medical Sciences colony; lanes 3 and 4: species A, Hainan (HN) colony; lane 5: dried HN specimen; lanes 6–9: field specimens from each of 4 sites in Hainan Province; lanes 10 and 11: field specimens from Yunnan Province; lanes 12 and 13: field specimens from Yunnan Province, 1/5 of the DNA from one leg triturated in water.

DISCUSSION

Diagnostic rDNA PCR assays have been developed for identification of member species of the *Anopheles gambiae* complex (Porter and Collins 1990, Scott et al. 1993, Taylor et al. 1993, Paskewitz et al. 1993; van Rensburg et al. 1996) and the *Anopheles quadrimaculatus* complex (Cornel et al. 1996, Rutledge et al. 1996). These studies showed that PCR methods are in agreement with chromosome or isoenzyme electrophoresis in identification of wild-caught specimens for both species complexes. In our study, PCR identified all specimens from Hainan Province as species A and all specimens from Yunnan Province as species D. This result is consistent with our previous interpretation of the species status for *An. dirus* s.l. in both localities according to chromosomal banding pattern and egg characters determined by scanning electron microscopy (Xu and Qu 1991, Xu et al. 1991). In addition, the Hainan specimens are representatives of 4 distinct *An. dirus* populations sampled from throughout their geographic distribution except for a small western region (see Materials and Methods). Thus, the present results suggest that species A may be the only member of *An. dirus* on Hainan Island. The specimens of species D used in this study are all from Mengla of Yunnan, a region near the border with Myanmar and Laos. Baimai and others (1988a, 1988c) have shown that species D is widespread, and that geographically distinct populations show intraspecies chromosomal polymorphisms. Therefore, our method should be tested for its applicability to species D samples from a wider geographic range or to different chromosomal forms. Because the rDNA ITS2 sequence has shown little intraspecific variation (Xu and Qu 1997), the primer DP may not be able to separate the different chromosomal forms. Fortunately, the rDNA target may be informative enough to deal with the problem, because 3 chromosomal forms of *An. gambiae* s.s. have been separated by polymerase chain reaction–restriction fragment length polymorphism analysis (Favia et al. 1997).

Although several methods are now available for the identification of sibling species of the *An. dirus*, only DNA probes are ideal for large-scale identification of single specimens (Audtho et al. 1995). However, a separate hybridization must be done for each species and each requires an overnight incubation. Problems arise when the number of species in a complex increases, or when one must make duplicate blots or reprobe the same blot for extra species. However, for a PCR diagnostic assay, multiple members of a complex would not cause a problem because primers for all of the species can be tested in one tube, and only one reaction may be required to give an individual species identification. In addition, because the PCR assay works well on a small mount of DNA from one leg (Fig. 1, lane 12, 13), species determination can be done without killing the mosquitoes. This technique will be valuable for epidemiologic investigations to identify blood meal origin, for parity determination, and for parasite detection. We found that DNA isolated from 2nd to 4th instar larvae and pupae can also be amplified, so the method can be used for field studies on larval biology (Harbach et al. 1997).

This study only examined *An. dirus* species A and D because other members of the complex are not available in mainland China. This approach should now be developed for all members in the *An. dirus* complex.

ACKNOWLEDGMENTS

We are grateful to Rampa Rattanarithikul of AFRIMS, Thailand, for providing the specimens. We also thank all the field personnel, especially Shanqing Wang and Xianzheng Cai at Hainan Research Institution of Tropical Disease, and Xueshu Dong at Yunnan Research Institution of Malaria Control, for their help in specimen collection. This work was jointly supported by National Foundation of Natural Science of China and the United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases.

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