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RESTRICTION ANALYSIS OF THE RIBOSOMAL DNA INTERNAL TRANSCRIBED SPACER REGION OF *CULEX RESTUANS* AND MOSQUITOES IN THE *CULEX PIPIENS* COMPLEX

BETTINA A. DEBRUNNER-VOSSBRINCK,[1] CHARLES R. VOSSBRINCK,[2] MICHAEL H. VODKIN[1] AND ROBERT J. NOVAK[2,3]

ABSTRACT. Members of the *Culex pipiens* Linn. complex in the eastern, southern, and central United States are the primary vectors of St. Louis encephalitis virus. Although species and subspecies in the complex can be identified as 4th-instar larvae and by characters on the male genitalia, adult females cannot be identified accurately. In this study a ribosomal DNA (rDNA) segment that includes the internal transcribed spacer region (ITS) was amplified from *Culex pipiens pipiens* Linn., *Culex quinquefasciatus* Say, and *Culex restuans* Theobald. The DNA was amplified from single abdomens or single legs. The amplified rDNA segment from *Cx. restuans* is 90 base pairs smaller than those from members of the *Cx. pipiens* complex. Ribosomal DNA was amplified separately from 3 individuals for each population of *Cx. pipiens* and analyzed by restriction digestion. Intrapopulation variation is seen, because for each population, bands are present that are common to all 3 individuals within the population, but are also unique to that population. These results indicate that this method may provide a means for distinguishing among the mosquitoes in the *Cx. pipiens* complex.

INTRODUCTION

The primary epidemic vectors associated with the transmission cycle of St. Louis encephalitis virus (SLEV) are all members of the subgenus *Culex.* In the United States these mosquitoes include *Culex tarsalis* Coq. in the west, the *Culex pipiens* complex in the eastern, southern, and central States, and *Culex nigripalpus* Theobald in Florida (Hammon and Reeves 1942, Reeves et al. 1942, Chamberlain et al. 1964, Dow et al. 1964). The *Cx. pipiens* complex is composed of 2 species, *Culex pipiens pipiens* Linn. and *Culex quinquefasciatus* Say, and a single subspecies *Culex pipiens pallens* Coquillett (Knight and Stone 1977; Harbach et al. 1984, 1985). Harbach et al. (1984, 1985) synonymized both *Culex pipiens molestus* Forskal and *Culex pipiens calloti* Rioux and Pech as behavioral/physiological variants of *Cx. p. pipiens.* Two other members of the subgenus *Culex* have also been implicated as playing a role in the enzootic transmission among vertebrate animals as well as epidemic transmission of SLEV to humans. These two species are *Culex salinarius* Coq. and *Culex restuans* Theobald (Monath 1980).

Based on morphologic characteristics of adult females, only *Cx. tarsalis* and *Cx. nigripalpus* can be easily identified to species. The only reliable diagnostic characters that can be used to separate *Cx. pipiens* from *Cx. quinquefasciatus* are the phallosome of the male genitalia (Belkin 1962), and the shape of the larval siphon (Carpenter and LaCasse 1955). Although *Cx. salinarius* and *Cx. restuans* have diagnostic characteristics in the adult female (Carpenter and LaCasse 1955), these characters are often lost during sampling, transport, or aging. The problem of female identification is further complicated in areas where *Cx. pipiens* and *Cx. quinquefasciatus* are sympatric. Jacob et al. (1979) used DV/D ratios to determine that 39–50% of the *Culex* males collected in Memphis, TN, were *Cx. pipiens/Cx. quinquefasciatus* hybrids. Sundararaman (1949) defined DV/D as the extension of the ventral arm of the phallosome beyond the intersection with the dorsal arm (DV) relative to the distance between the dorsal arms (D). Collections of the *Cx. pipiens* complex from different sites in North America were analyzed using the DV/D ratio, and a north–south cline was found between the 2 subspecies (Barr 1957). *Culex pipiens* was found north of latitude 39°N, and *Cx. quinquefasciatus* occurred south of latitude 36°N. Both species and hybrids occurred between the latitudes 36°N and 39°N.

Several investigators have used biochemical techniques to characterize and distinguish members of the *Cx. pipiens* complex. Cupp and Ibrahim (1973) used immunoelectrophoresis to detect differences among *Cx. pipiens, Cx. quinquefasciatus,* and their hybrids. Cupp and Ibrahim (1973) were also able to distinguish *Cx. pipiens (molestus)* from *Cx. p. pipiens* and *Cx. quinquefasciatus.* Saul et al. (1977) used polyacrylamide gel electrophoresis and a double staining technique to distinguish among *Cx. res-*

[1] Illinois Natural History Survey, Center for Economic Entomology, Medical Entomology Program, 607 E. Peabody Drive, Champaign, IL 61820.

[2] University of Illinois, Department of Entomology, Urbana, IL 61801.

[3] To whom reprint requests should be addressed.

tuans, Cx. p. pipiens, and *Culex territans* Walker. Preliminary work indicated that *Cx. quinquefasciatus* and *Cx. salinarius* could also be identified using this method. Cheng et al. (1982) determined the biochemical genetics of the *Cx. pipiens* complex through investigations of several isoenzyme loci. All of these biochemical techniques require that the specimen be sacrificed.

Several investigations have utilized the ribosomal DNA (rDNA) locus to distinguish among anopheline species. Two of the studies (McLain and Collins 1989, Collins et al. 1990) demonstrated variability in this locus by Southern blotting of DNA digested with a restriction enzyme. An alternative strategy was developed by Porter and Collins (1991). They sequenced the rDNA from 2 sibling species and designed 2 species-specific primers that yielded products of different size.

In a recent report, Crabtree et al. (1995) used the rDNA internal transcribed spacer (ITS) region to differentiate among *Culex* species. The ITS region was amplified from 2 individuals of each population studied in order to design species-specific or population-specific primers. The amplified DNA was cloned and 2 independent clones from each individual were sequenced. Based on this sequence information the authors were able to design primers to differentiate among *Cx. salinarius, Cx. restuans,* and *Cx. p. pipiens,* but were not able to resolve *Cx. p. pipiens* from *Cx. quinquefasciatus.*

The objective of this study was to distinguish among populations of the subgenus *Culex* by restriction analysis of the amplified rDNA ITS region. We wished to develop a rapid assay that could be done without destruction of the mosquito, leaving it available for genetic crossing, morphologic analysis, or viral detection studies.

MATERIALS AND METHODS

Mosquitoes: Culex restuans was collected in Illinois. Egg rafts were collected in the field and reared to adults in the laboratory. Fourth-instar larvae reared from each egg raft were used for species identification. Laboratory colonies were established from *Cx. p. pipiens* collected in Illinois, *Cx. quinquefasciatus* collected in Florida, *Cx. quinquefasciatus* collected in Louisiana, and *Cx. p. pipiens* (variant *molestus*) collected in Rome, Italy. Mosquitoes were maintained at 18–30°C, a photoperiod of 16:8 (L:D), and an RH of 60%. Adults were provided with a 10–20% honey solution and were fed on quail 1–2 times a week. Eggs were collected and hatched as needed. Larvae were reared on Tetramin (TetraWerke, Melle, Germany) and rabbit chow.

Specimen preparation: Homogenates were prepared from the abdomen or a leg of individual female mosquitoes. Single abdomens and legs were homogenized with a Teflon pestle in a 1.5-ml microcentrifuge tube containing 150 µl or 50 µl, respectively, of STE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Homogenates were incubated for 5 min at 95°C and then centrifuged in a Microfuge-E (Beckman, Fullerton, CA) for 5 min at 16,000 × *g,* 4°C. Supernatants were transferred to new tubes and held at −20°C until frozen. The supernatants were then thawed, centrifuged for 5 min at 16,000 × *g,* 4°C, and transferred to new tubes.

Amplification of the rDNA ITS region: The primers used for the polymerase chain reaction (PCR), ISS2156f (5′-CTGGGCTGCACGCGCGCT-3′) and ILS228r (5′-GTTAGTTTCTTTTCCTCC-3′), were selected to amplify a segment of the rDNA with about 370 bases of the 3′ end of the 18S rDNA, the ITS1 region, the 5.8S rDNA, the ITS2 region, and 75 bases at the 5′ end of the 28S rDNA. Each 100-µl reaction mix contained 4 µl of homogenate, 4.2 pmoles of each primer, 0.1 mM each of dATP, dGTP, dTTP, and dCTP, 1.5 mM $MgCl_2$, 1× buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 0.1% Triton X-100, and 2.5 units of Taq DNA polymerase (Promega Corporation, Madison, WI). Reactions were incubated at 94°C for 3 min, followed by 40 cycles of 94°C for 30 sec, 40°C for 1 min, and 72°C for 1.5 min. For DNA extracted from single abdomens, 3 100-µl PCR reactions were pooled for each sample, and the DNA was precipitated with 0.5 volume of 7.5 M ammonium acetate and 2.5 volumes of ethanol at −20°C. The precipitated DNA was resuspended in 50 µl of sterile water. For single legs, the product of a 100-µl PCR reaction was precipitated with ethanol as above and resuspended in 10 µl of sterile water. Four microliters of each sample was analyzed on a 1% agarose gel in 1× TAE buffer (40 mM Tris-HCl, pH 8.0, 5 mM sodium acetate, and 2 mM Na_2EDTA).

Restriction analysis of PCR products: Amplified DNA (0.35 µg per reaction, as determined by ethidium bromide-stained gels) was digested in separate reactions with Hae III, Hpa II, Alu I, and Rsa I restriction enzymes (BRL, Gaithersburg, MD). Several other 4-base recognition restriction enzymes were tried, but only the above 4 proved informative. Digested DNA was analyzed on 3% or 5% 3:1 NuSieve agarose gels (FMC BioProducts, Rockland, ME) in 1× TAE buffer.

RESULTS

The region of the rDNA that was amplified is shown in Fig. 1. As seen in Fig. 2, the amplified

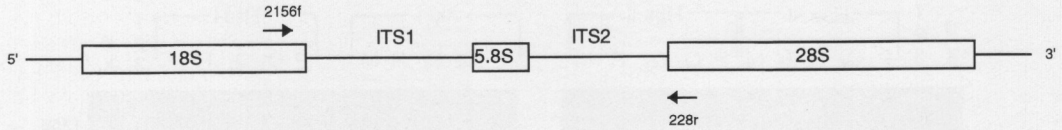

Fig. 1. Diagram of a single rDNA unit, showing the location of primers 2156f and 228r and the region of the rDNA that is amplified by these primers.

rDNA segments from *Cx. p. pipiens, Cx. p. pipiens* (variant *molestus*), and the *Cx. quinquefasciatus* populations from Florida and Louisiana are the same size, approximately 1,415 base pairs (bp). The *Cx. restuans* spacer region, however, is about 90 bp smaller (1,325 bp in size). Figure 2 also shows that DNA can be amplified from a single *Cx. p. pipiens* leg (lane P_L).

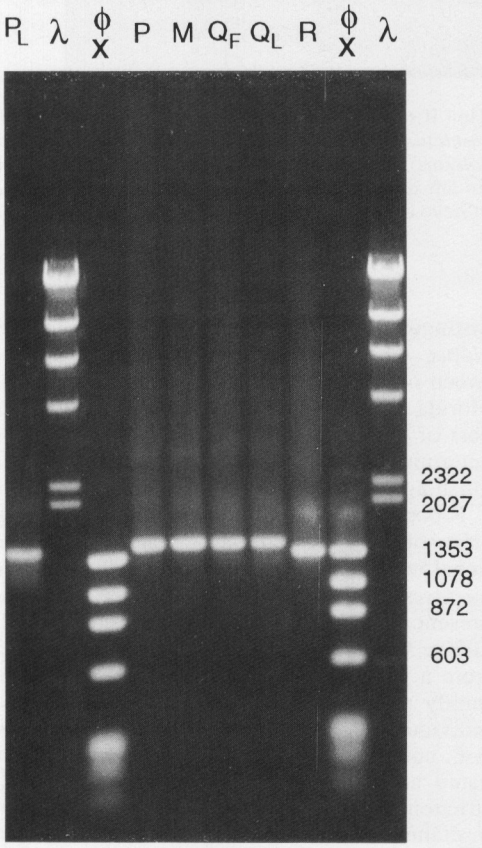

Fig. 2. Amplified rDNA analyzed on a 1% agarose gel. P_L, *Culex pipiens* leg; P, *Cx. pipiens* (single abdomen); M, *Cx. pipiens* (variant *molestus*) (single abdomen); Q_F, *Culex quinquefasciatus* from Florida (single abdomen); Q_L, *Cx. quinquefasciatus* from Louisiana (single abdomen); R, *Culex restuans* (single abdomen); λ, λ DNA Hind III digest; ϕX, ϕX174 DNA Hae III digest. Sizes of standards are shown in base pairs in right margin.

Figure 3 shows Hae III, Hpa II, Alu I, and Rsa I restriction patterns of the rDNA segment amplified from a single individual of each population. The products of the amplified DNA of *Cx. restuans* show banding patterns distinct from those of the members of the *Cx. pipiens* complex. The Hae III, Alu I, and Rsa I restriction digests show a unique banding pattern for each member of the *Culex p. pipiens* complex examined. The 2 *Cx. quinquefasciatus* populations can be distinguished from each other by their Rsa I restriction banding patterns; the Louisiana *Cx. quinquefasciatus* mosquito shows a band at 120 bp, which is absent from that of the Florida *Cx. quinquefasciatus* mosquito.

Figure 4 shows Rsa I restriction digests of the amplified rDNA segment for 3 separate individuals from each *Culex* population included in this study, except for *Cx. pipiens* (variant *molestus*), where the M3 PCR product of one individual was lost during ethanol precipitation. Variability in banding pattern was seen among individuals within a population. However, bands that are common to all 3 individuals within a population and are diagnostic for that population are also seen. *Culex p. pipiens* individuals are distinguished by the presence of a single band at 270 bp; individuals from the other *Cx. p. pipiens* populations all have an additional band of slightly higher molecular weight. *Culex pipiens* (variant *molestus*) individuals have a band at approximately 180 bp, which is absent from *Cx. quinquefasciatus* individuals. Individuals from the Louisiana *Cx. quinquefasciatus* population have a band at approximately 120 bp, which is not present in the Florida *Cx. quinquefasciatus* individuals.

DISCUSSION

The amplified rDNA region of *Cx. restuans* is approximately 90 bp smaller than that of mosquitoes of the *Cx. pipiens* complex examined in this study. This size difference provides a clear, simple, and rapid means for distinguishing between adult females *Cx. restuans* and *Cx. p. pipiens*. The amplified *Cx. restuans* DNA shows banding patterns distinct from those of the *Cx. pipiens* mosquitoes with all of the restriction enzymes used (Fig. 3).

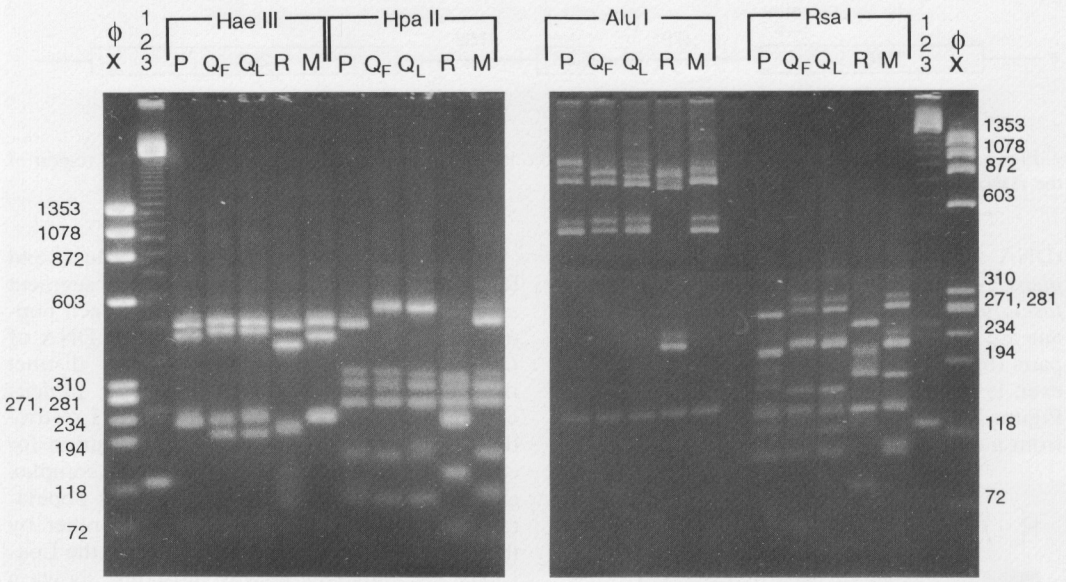

Fig. 3. A. Amplified rDNA digested with Hae III or Hpa II restriction enzyme and analyzed on a 3% 3:1 NuSieve agarose gel. P, *Culex pipiens*; Q_F, *Culex quinquefasciatus* from Florida; Q_L, *Cx. quinquefasciatus* from Louisiana; R, *Culex restuans*; M, *Culex pipiens* (variant *molestus*); 123, 123 bp DNA ladder; ϕX, ϕX174 DNA Hae III digest. Sizes of standards are shown in base pairs in left margin. B. Amplified rDNA digested with Alu I or Rsa I restriction enzyme and analyzed on a 5% 3:1 NuSieve agarose gel. Lanes and size standards as in A. Sizes of standards in base pairs are shown in right margin.

Within the *Culex pipiens* complex, restriction analysis of the rDNA fragment amplified from a single individual from each population shows differences among the populations (Fig. 3). Restriction analysis of rDNA amplified from 3 individuals from each population shows heterogeneity within a population. This observation concurs with the results of Crabtree et al. (1995), who found sequence variation among individuals within a population. They found that within the *Cx. pipiens* complex, sequence variation within an individual and among individuals within the same population was as high as variation between populations, precluding identification of subspecies using diagnostic primers. Their strategy relied on designing primers to distinguish among members of the *Culex* complex. Assay results are scored as positive or negative depending on the presence or absence of a specific amplified product (band). Sequence differences between 2 species, subspecies, or individuals at either or both designed primer sites do not necessarily predict whether those primers will lead to amplification (Gelfand and White 1990). In our study, however, although some intraspecific variability in restriction patterns was seen, bands diagnostic for each population were also apparent. Based on our limited sample size (3 individuals per population), we were able to distinguish among *Cx. pipiens, Cx. quinquefasciatus, Cx. pipiens* (variant *molestus*), and between populations of *Cx. quinquefasciatus* from Florida and Louisiana. Studies with larger numbers of individuals per population are needed to determine the utility of this technique for distinguishing within and between *Culex* populations.

Ribosomal DNA consists of a heterogeneous array of multiple randomly repeated transcriptional units (Kumar and Rai 1993). In *Cx. quinquefasciatus* approximately 87 rDNA copies are present per haploid genome (Kumar and Rai 1990). When sequencing cloned PCR products from a gene family, it is possible that different family members are being sequenced for each individual. As a result, different gene copies (i.e., nonhomologous characters) are being compared among individuals and populations. Restriction analysis of amplified rDNA allows simultaneous analysis of variants abundant enough to be visualized by agarose gel electrophoresis. As a result, heterogeneity at many positions may be seen. The design of primers that can distinguish among closely related sequences often requires more than one base pair change within a short oligonucleotide stretch. In contrast, a restriction site difference involving a single base pair change can be diagnostic for distinguishing closely related sequences. The utility

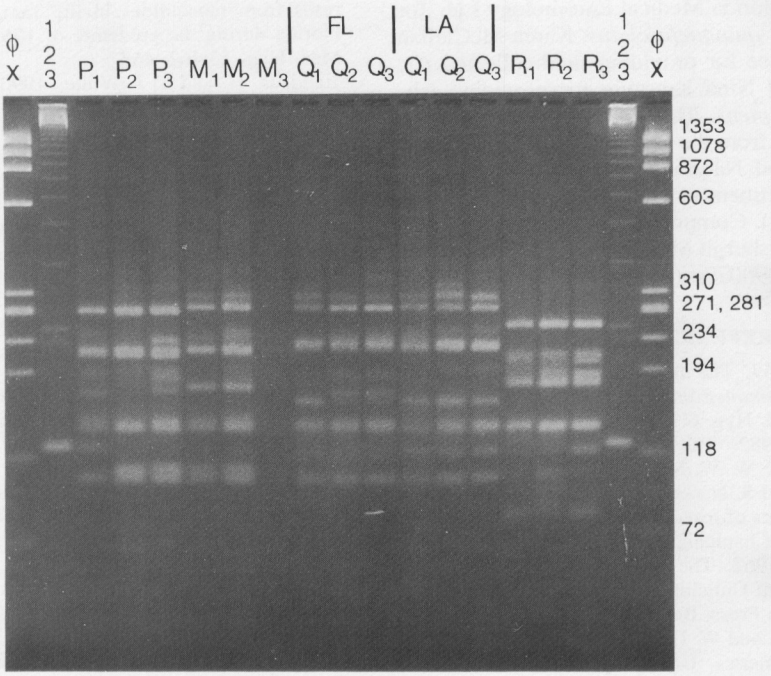

Fig. 4. Rsa I restriction digest of amplified rDNA from 3 separate individuals from each *Culex* population included in the study, analyzed on a 5% 3:1 NuSeive agarose gel. P_{1-3}, *Culex pipiens*; M_{1-3}, *Culex pipiens* (variant *molestus*); Q_{1-3} FL, *Culex quinquefasciatus* from Florida; Q_{1-3} LA, *Cx. quinquefasciatus* from Louisiana; R_{1-3}, *Culex restuans*; ϕX, ϕX174 DNA Hae III digest; 123, 123 bp DNA ladder. Sizes of standards are indicated in base pairs in right margin.

of our approach is manifest in the ability to distinguish *Cx. quinquefasciatus* from *Cx. p. pipiens* by restriction analysis of amplified rDNA.

The species, subspecies, and infraspecific forms that have been attributed to the *Cx. pipiens* complex continue to represent one of the major problems in mosquito taxonomy. The nomenclatural problems associated with this worldwide complex have been addressed to a degree by Harbach et al. (1984, 1985) examining populations from the Middle East, Africa, and southwestern Asia. A comprehensive worldwide study is critically needed not only for systematic purposes but for ecological, vector capability, and mosquito management needs, all of which must depend on a firm taxonomic basis. The use of our technique gives researchers the ability to characterize mosquitoes by assaying only a single leg, thus providing a voucher specimen and allowing for its subsequent use for morphologic analysis. Mosquitoes survive our technique and can then be used to address a myriad of biological questions associated with this complex through genetic studies to determine the heritability of physiologic, morphologic, and behavioral characteristics. It should be pointed out that both in this study and in that of Crabtree et al. (1995), wild-caught individuals may represent a mixture of populations. Gene flow can occur between sibling species in areas of overlap. For example, Sudararaman (1949), Barr (1957, 1982), and others have shown areas of hybridization between *Cx. p. pipiens* and *Cx. quinquefasciatus*. The detection of hybrid forms and the potential consequences of the resulting hybrid genotypes on biology and behavior are questions that continue to challenge researchers.

Our current research goal is to genetically characterize mosquito populations within the *Cx. pipiens* complex relative to their involvement with the transmission of SLEV. We will attempt to determine whether the population genotypes exhibit temporal change and whether certain genotypes predominate during both epidemic and enzootic cycles.

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