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

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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, 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 (Month 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 (Carpen ter 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 Cx. pipiens complex.

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-
tuans, Cx. p. pippiens, 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. p. pippiens 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. pippiens, but were not able to resolve Cx. p. pippiens 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. pippiens collected in Illinois, Cx. quinquefasciatus collected in Florida, Cx. quinquefasciatus collected in Louisiana, and Cx. p. pippiens (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), ISS215f (5'-CTGGGCTGCACGGCG- CGCT-3') and ILS228r (5'-GTTAGTTTCCTTTTT- CCTCC-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, 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 MgCl₂, 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 4 µl of 0.3 M ammonium acetate and 2 µl of ethanol, then 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 Na₃EDTA).

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 Rsal restriction enzymes (BRL, Gaithersburg, MD). Several other 4-base recognition restriction enzymes were tried, but only the above 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
rDNA segments from Cx. p. pipiens, Cx. p. 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 P₁).

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 p. 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, *Culex quinquefasciatus* from Florida; Q1, *Culex 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. P₁, P₂, P₃, Culex pipiens; M₁, M₂, Culex pipiens (variant molestus); Q₁, Q₂, Q₃, Culex quinquefasciatus from Florida; Q₁, Q₂, Q₃, LA, Cx. quinquefasciatus from Louisiana; R₁, R₂, 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. p. pisiens by restriction analysis of amplified rDNA.

The species, subspecies, and infraspecific forms that have been attributed to the Cx. p. p. pisiens 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. p. pisiens 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. p. p. pisiens 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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