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Susan T. Ratcliffe,[1] *Ph.D.; Donald W. Webb,*[2] *Ph.D.; Richard A. Weinzievr,*[1] *Ph.D.; and Hugh M. Robertson,*[3] *Ph.D.*

PCR-RFLP Identification of Diptera (Calliphoridae, Muscidae and Sarcophagidae)—A Generally Applicable Method

ABSTRACT: A simple, rapid method using restriction fragment length polymorphisms (RFLPs) within the internal transcribed spacer (ITS) regions of the ribosomal DNA gene repeat allows identification of insects and other organisms. We used the method to identify the morphologically similar Diptera larvae that are important in forensic entomology for estimating the time and location of death. Polymerase chain reaction (PCR) was used to amplify a region from the 18S to the 28S rRNA genes. The ITS1 and ITS2 regions provided variation between species and homogeneity within species, with the exception of *Cochliomya macellaria.* Combinations of the restriction enzymes *Dde*I, *Hin*fI and *Sau*3AI provided diagnostic bands for identification of the ten species from three families of Diptera (Calliphoridae, Muscidae and Sarcophagidae).

KEYWORDS: forensic science, rDNA, ITS regions, Diptera, forensic entomology

Identification of immature Diptera of forensic importance has relied on slight variations in spiracle patterns and the cephalopharyngeal skeleton (1). These structures differ in each larval stage for each species and as a result, this method requires an expert in the field to assure correct identification (2). Haskell (3) recommends rearing larvae to adulthood to confirm species identification, and this step may add several weeks to the identification process.

A variety of DNA-based methods, each with its own advantages and disadvantages, have been developed recently to identify species and sub-species of insects. Random amplified polymorphic DNA-PCR (RAPD) procedures use a single 10-nucleotide primer to amplify regions of DNA flanked by the random primer sequences. This method may produce multiple DNA fragments that can be used as markers for identifying individuals, populations, or species and genome mapping. Edwards and Hoy (4) used RAPD-PCR to identify microhymenoptera, and Roehrdanz et al. (5) used this method to distinguish laboratory-reared colonies of parasitic Hymenoptera. RAPD-PCR can be extremely sensitive, but replication of results has been difficult, and hundreds of primers may be tested before identifying one that can be used to discriminate between species or strains. Garner and Slavicek (6) tested 222 RAPD primers before locating one that discriminated between Asian and North American gypsy moths, and Schnell et al. (7) tested 63 primers to locate RAPD markers for Caribbean fruit flies. Restriction fragment length polymorphism-PCR (RFLP-PCR) uses paired primers of 18 to 30 nucleotides in length to amplify nuclear or mitochondrial DNA (mtDNA) by PCR. The PCR product is then cut with restriction enzymes to produce multiple fragments used as markers to identify species. Sperling et al. (8,9) used RFLP-PCR of mtDNA to separate species of economically important ermine moths and forensically important Diptera. Taylor et al. (10) unsuccessfully attempted to separate four species of *Muscidifurax* using similar techniques.

An alternative approach that should be applicable to all insect groups is RFLP-PCR of rDNA. The internal transcribed spacer (ITS) regions of the genome can be expected to be highly variable, yet diagnostic for each species. In the past decade, the ITS regions of ribosomal RNA gene repeats have become recognized for species diagnostic sequence characteristics and this region is present in all organisms (11–13). There are two spacers, the ITS 1, located between the 18S (Svedberg coefficient) and the 5.8S rRNA genes, and the ITS 2, located between the 5.8S and the 28S rRNA genes. The ITS 1 region varies in length from approximately 200 bp in Odonata (H.M. Robertson, Personal Communication) to 500 bp in grasshoppers (14) to about 1000 bp in several flies and parasitoids (11,12,15,16). The ITS regions undergo concerted evolution along with tandemly repeated rRNA genes (11), and generally are homogeneous in both length and sequence within a species (12,13,14,17).

In the study reported here, we used primers designed to anneal to the 3′ end of 18S rRNA and the 5′ end of the 28S rRNA genes to amplify both ITS regions and the 5.8S RNA gene. Restriction enzymes were screened to determine those that would produce RFLPs that allow identification of ten forensically important dipteran species in three families (Calliphoridae, Muscidae and Sarcophagidae), including two pairs of congeneric species. This is a generally applicable method for species identification that does not require prior determination of the sequence, extensive screening of RAPD primers, or isolation of novel microsatellites for each group of interest.

[1] Department of Crop Sciences, University of Illinois, Urbana, IL.
[2] Center for Economic Entomology, Illinois Natural History Survey, Champaign, IL.
[3] Department of Entomology, University of Illinois, Urbana, IL.
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Materials and Methods

Live specimens were obtained from laboratory colonies or field sites and then preserved in alcohol or held at −20°C until DNA was extracted. The field specimens, *Musca domestica* (L.) (Muscidae), *Cochliomya macellaria* (F.) (Calliphoridae), *Protophormia terraenovae* Robinaeu-Desvoidy (Calliphoridae), *Cynomyopis cadaverina* Robinaeu-Desvoidy (Calliphoridae) and *Bufolcilia silvarum* Meigen (Calliphoridae), were verified by D. Webb, Illinois Natural History Survey. Laboratory colony specimens sources were from: M. Dickinson, University of Chicago (*Calliphora vicina* Robineau-Desvoidy (Calliphoridae)), D. Denlinger, Ohio State University (*Saracophaga crassipalpis* Macquart (Sarcophagidae)), B. Greenberg, University of Illinois, Chicago (*Cochliomya macellaria*), A. Broce, Kansas State University (*Musca autumnalis* DeGeer (Muscidae)), and Department of Entomology, University of Illinois (*Sarcophaga bullata* Parker (Sarcophagidae), *Phormia regina* Meigen (Calliphoridae) and *M. domestica*).

The thorax of adult flies was used for extraction of DNA. Specimens were held at −20°C prior to extractions. DNA was extracted using the phenol/chloroform method (15). Universal primers, 1975F (TAACAAGGTTTCCGTAGGTG) designed to anneal to the 3′ end of the 18S gene and 52R (GTTAGTTTCTTTTCCTCC CCT) designed to anneal to the 5′ end of the 28S gene, were used for amplification by polymerase chain reaction (PCR). Methods for PCR amplification are described by Ratcliffe (15). Using restriction enzymes, *Alu*I, *Apo*I, *Dde*I, *Hinf*I, *Sau*3AI, *Ssp*I, PCR products were digested as described by Ratcliffe (15) and resolved on a 1.25% agarose gel, stained with ethidium bromide, and visualized with UV light. Blind assays and field assays were conducted using methods described above for extraction, amplification, digestion, and visualization.

Results

Universal primers (108F, 1406F, 1975F, 25R, 35R, and 52R) were tested to determine which pair of primers allowed amplification of the largest fragment. The universal primers, 1975F (TAA CAAGGTTTCCGTAGGTG) for the 3′ end of the 18S gene and 52R (GTTAGTTTCTTTTCCTCCCCT) for the 5′ end of the 28S gene amplified the entire region of interest, including both ITS regions and the 5.8S gene in all ten species included in this study (Fig. 1). PCR products were produced ranging in size from 1.2 to 1.5 kbp. In an initial test, PCR products from two species (*S. bullata* and *P. regina*) were digested using eight restriction enzymes (*Alu*I, *Apo*I, *Dde*I, *Eco*RI, *Hinf*I, *Sau*3AI, *Ssp*I, and *Taq*I) to determine which produced RFLPs allowing species differentiation. *Dde*I, *Hinf*I, *Sau*3AI and *Ssp*I were selected for further testing on all ten species.

The restriction enzyme *Dde*I yielded RFLPs in eight species, however *Phormia* and *Protophormia* species were indistinguishable (Table 1). *Dde*I was the only enzyme that produced RFLPs that distinguished the two *Musca* species. Variation in RFLP pattern was seen between field and laboratory samples of *Cochliomya macellaria*. *Hinf*I allowed identification of four species (*S. bullata,*

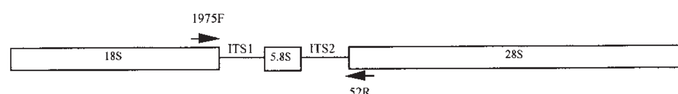

FIG. 1—*Schematic diagram of a single repeat unit of rDNA which consists of the 18S, 5.8S, and 28S rRNA genes and two internal transcribed spacers (ITS) with the primers used in the polymerase chain reaction shown by arrows.*

TABLE 1—*Species identification was evaluated in ten species with four restriction enzymes. RFLP in a column (*DdeI, HinfI, Sau3AI, SspI*) indicates the restriction fragment length polymorphism (RFLP) differentiated species.*

	*Dde*I	*Hinf*I	*Sau*3AI	*Ssp*I
Sarcophaga bullata	RFLP	RFLP	RFLP	RFLP
Sarcophaga crassiplapis	RFLP	RFLP	RFLP	RFLP
Phormia regina		RFLP	RFLP	RFLP
Protophormia terraenovae			RFLP	RFLP
Cochliomya macellaria	RFLP	RFLP		RFLP
Calliphora vicina	RFLP		RFLP	RFLP
Cynomyopsis cadaverina	RFLP			RFLP
Bufolucilia silvarum	RFLP		RFLP	RFLP
Musca autumnalis	RFLP			
Musca domestica	RFLP			

S. crassipalpis, Phormia regina and *Cochliomya macellaria*), but did not cut the PCR products from *Protophormia terranovea, Calliphora vicina, Cynomyopsis cadavarina,* or *B. silvarum* (Table 1). Field samples of *Cochliomya macellaria* produced RFLPs that varied slightly from laboratory samples when PCR products were cut by *Hinf*I. Results indicated *Sau*3AI was useful in identifying six species (*S. bullata, S. crassipalpis, Phorima regina, Protophormia terraenovae, Calliphora vicina,* and *B. silvarum*) (Table 1). The RFLPs produced for *Cochliomya macellaria* and *Cynomyopsis cadaverina* were identical and variation in the banding patterns produced for *M. autumnalis* and *M. domestica* was so slight that species identification was not possible. Eight of the ten species could be identified using *Ssp*I. The two Musca species could not be separated by RFLPs (Table 1). No variation was detected within species from multiple field samples in this study. Blind laboratory and field assays confirmed PCR-RFLP identification of species examined.

Discussion

The ten species of Diptera used in this study are some of the primary indicator species used in central Illinois to assist criminal investigators in determining time and location of death. The presence or absence of these and other forensically important species has been used in criminal investigations and as evidence in trials. However, the timely and correct identification of larvae is necessary in order for the entomological evidence to be of value in these situations. We described a method that provides rapid, inexpensive, and accurate identification of forensically important species. The primers were designed to anneal to the highly conserved regions of the 18S and 28S rRNA genes, so this method is applicable for the identification of any insect group and most other animals.

The primers consistently produced amplification products varying from 1.2–1.5 kb. Since both ITS1 and ITS2 were contained in the fragment, the number of potentially variable sites between closely related species and sub-species are increased. Identifying enzymes that produce informative RFLPs requires some screening if sequence data are not available. In this study, eight enzymes were initially screened and three were selected for further testing. To distinguish all ten species examined in this study, it was necessary to use all three enzymes to determine species identification. However, sequence data from the ten species may identify a single restriction enzyme that would produce informative RFLPs for all the species in this study. Variation within species was detected in *Cochliomya macellaria* when field samples were compared with samples from an isolated laboratory colony. Vogler and DeSalle (18) found

geographical sequence variation in the ITS1 region of *Cicindela dorsalis* Say. *Cochliomya macellaria* sequence data would indicate location of nucleotide variation found in the field and laboratory samples. After the initial identification of restriction enzymes, this method allows differentiation of species in approximately 6 h when quick prep DNA extraction methods are used, followed by PCR amplification, digestions of the PCR products, and visualization of RFLPs on an agarose gel. In the case of criminal investigations, this quick turn around may greatly enhance the chances of identifying a suspect. This method reduces the need for dipteran taxonomists to identify larval samples and allows species identification in any laboratory that is currently capable of PCR-RFLP. As a result, law enforcement agencies that do not have access to a forensic entomologist would have an alternative method for quickly processing entomological evidence.

Due to regional complex variation future research is needed to determine RFLP patterns for additional dipteran species. In order to develop an RFLP identification method, amplification by PCR and sequencing of the region from the 18S to the 28S genes of additional species encountered on or near a corpse is necessary. Examination of these sequence data would allow identification of restriction enzymes that produce unique RFLP patterns for each species. These data combined with the temperature-based larval development data will provide investigators with valuable information regarding time and location of death within six hours of sample collection without the need of forensically trained entomologists.

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Additional information and reprint requests:
Susan T. Ratcliffe
Assistant Professor
University of Illinois
Department of Crop Sciences
S-316 Turner Hall
1102 S. Goodwin Avenue
Urbana, IL 61801
Tel: (217) 333-9656 (Office)
Fax: (217) 333-5245
E-mail: sratclif@uiuc.edu