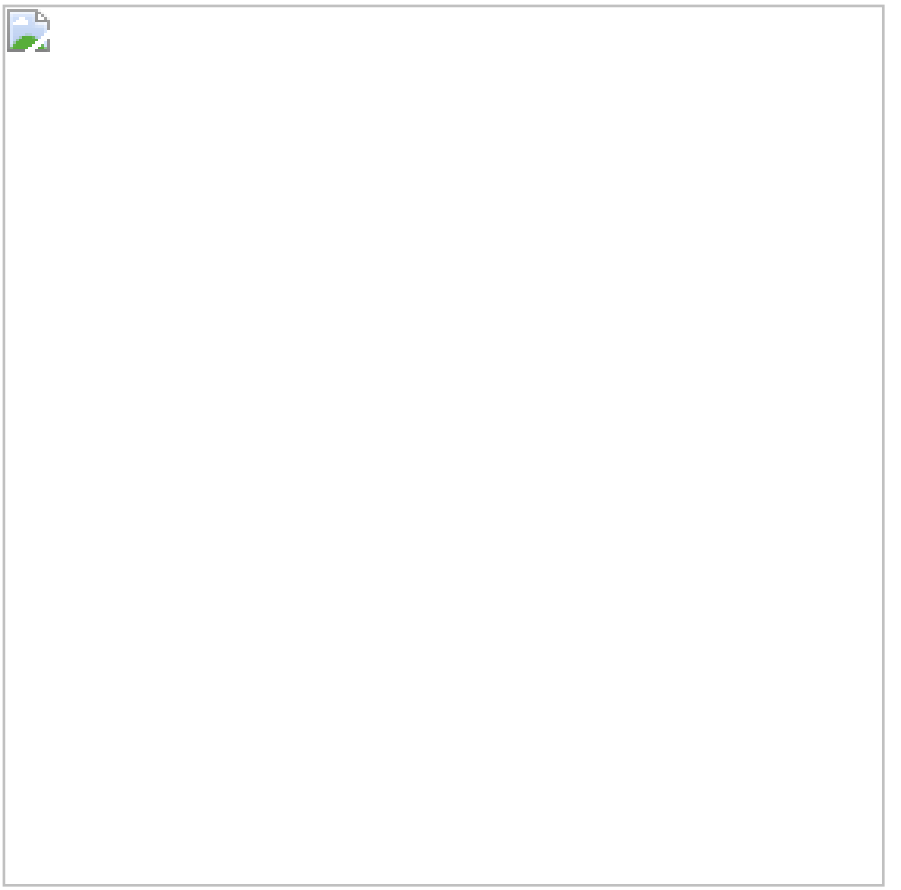

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Authors: Knight, J. W., and Nayar, J. K.

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IDENTIFICATION OF FOUR COMMON *CULEX* (*CULEX*) (DIPTERA: CULICIDAE) SPECIES FROM FLORIDA WITH ISOENZYME ANALYSIS

J. W. KNIGHT AND J. K. NAYAR
Florida Medical Entomology Laboratory, and Department of Entomology and Nematology
IFAS/University of Florida, 200 9th Street, S.E., Vero Beach, FL 32962

ABSTRACT

Females of four common *Culex* (*Culex*) species from Florida were analyzed for isoenzymes using polyacryamide gel electrophoresis. Ten enzymes that yielded 11 putative loci were studied. Most of the loci showed diagnostic characteristics in the four species, but four of the loci (glycerol-3-phosphate dehydrogenase [*Gpd*-2], hexokinase [*Hk*], isocitrate dehydrogenase [*Idh*-1], and malate dehydrogenase [*Mdh*]) could be used in sequence to identify the four *Culex* species. *Culex salinarius* and *Cx. p. quinquefasciatus* could be separated from *Cx. restuans* and *Cx. nigripalpus* by *Mdh* locus. *Culex salinarius* could be distinguished from *Cx. p. quinquefasciatus* by *Hk* locus and *Cx. nigripalpus* could be distinguished from *Cx. restuans*, by *Idh*-1 and/or *Gpd*-2 loci. Randomly combined specimens of these four *Culex* species were identified accurately by using these enzyme loci.

Key Words: Mosquito indentification, *Culex* species, *Culex nigripalpus*, *Culex pipiens quinquefasciatus*, *Culex restuans*, *Culex salinarius*, isoenzyme analysis, Florida

RESUMEN

Las hembras de cuatro especies comunes de *Culex* (*Culex*) de Florida fueron analizadas para isoenzimas usando un gel poliacrilamida de electroforesis. Diez enzimas que produjeron 11 loci (lugares) putativas fueron estudiados. La mayoría de los loci mostraron caracteristicas diagnosticadas en las cuatro especies, pero cuatro de los loci (glicerol-3-fosfato-deshidrogenasa [*Gpd*-2], hexocinasa [*Hk*], isocitrato-deshidrogenasa [*Idh*-1], y el malato-deshidrogenasa [*Mdh*]) pudieron ser utilizados en secuencia para identificar las cuatro especies de *Culex*. *Culex salinarius* y *Cx. p. quinquefasciatus* pudieron ser separadas de *Cx. restuans* y *Cx. nigripalpus* por el locus de *Mdh*. *Culex salinarius* pudieron ser distinguidas de *Cx. p. quinquefasciatus* por el loci de *Hk* y *Cx. nigripalpus* pudieron ser distinguidas de *Cx. restuans*, por los loci *Idh*-1 y/o *Gpd*-2i. Especímenes de las cuatro especies de *Culex*, combinados al azar fueron identificados correctamente utilizando estos loci de enzimas.

Mosquitoes belonging to the *Culex* (*Culex*) species have been shown to be among the important epizootic or epidemic vectors of arboviruses including St. Louis encephalitis (SLE) virus and West Nile Virus (WNV) in the United States (Tsai & Mitchell 1989, CDC 2002). Accurate identification of field-collected *Culex* mosquitoes is essential for epidemiological and control efforts. Field-collected specimens of females of *Culex* (*Culex*) species are often difficult to identify, because adult collections are commonly made with various trapping methods and, unfortunately, the characteristic patterns of scales used to identify *Culex* adult females are frequently rubbed off by the devices or simply lost as the mosquito ages with the result that unidentified *Culex* species are lumped together as *Culex* spp. for identification and for virus analysis. During the last 30 years, several attempts have been made to identify field-collected *Culex* mosquitoes by methods other than the morphological methods. These include identification of *Culex* species by isoenzyme electrophoresis in Indiana (Saul et al. 1977; Corsaro & Munstermann 1984) and by a species-diagnostic polymerase chain reaction assay (Crabtree et al. 1995; Miller et al. 1996; Crabtree et al. 1997). Since some *Culex* species present in Florida are different from species found in other parts of the United States, the objective of this study was to identify females of Florida's four common *Culex* (*Culex*) species (*Cx. nigripalpus* Theobald, *Cx. pipiens quinquefasciatus* Say, *Cx. restuans* Theobald and *Cx. salinarius* Coquillett) by using isoenzyme electrophoresis.

MATERIALS AND METHODS

Mosquito Collection

Egg rafts of the four *Culex* species were collected in oviposition pans containing oak leaf and/or hay infusion from the field at the Florida Medical Entomology Laboratory (Knight & Nayar 1999) from January through April 2003 when all four species are present (O'Meara & Evans, 1983; Provost 1969). Individual egg rafts were allowed to hatch in the laboratory in vials and the first instars of each species were identified (Dodge 1966;

Haeger & O'Meara 1983). Larvae from 16 to 20 egg rafts from each species were reared, one raft per tray, to the adult stage. The identification of newly emerged adults was reconfirmed by morphological characters before samples of females were frozen to be used later in polyacrylamide gel electrophoresis.

In order to confirm our results, 6 individuals/gel of each of the four *Culex* species, each individual representing a different family, were randomly processed for the previously determined four diagnostic enzyme loci as described in the Results section below. A total of 24 individuals of each *Culex* species, each individual representing a different family, were processed.

Electrophoretic Methods

Preparation of individual mosquitoes, buffer systems and electrophoretic protocols were the same as were described by Black and Munstermann (1996). Mini-Protean II Cell® (Mini-vertical electrophoretic system from Bio-Rad Laboratories, Hercules, CA) was used for these studies. Each female was homogenized in 30 µl of loading buffer (20% sucrose, Triton X-100 [0.5%], Tris-citrate pH 7.0 electrode buffer and trace amount of bromophenol blue tracking dye), and centrifuged for 10 min at 2,000 g. The supernatant (24 µl) was dispensed equally (3 µl) into 8, 0.5-ml Eppendorf tubes and frozen at -80°C until used for electrophoresis. At the time of electrophoresis, a 1.0-µl sample was loaded into each lane of the gel. Using this method we could analyze up to 16 enzyme loci from each mosquito (Nayar et al. 2002).

Ten enzyme systems were analyzed and are listed by name, abbreviation and Enzyme Commission number: aconitase hydratase (*Acoh*, EC 4.2.1.3); adenylate kinase (*Ak*-2, EC 2.7.4.3); glycerol-3-phosphate dehydrogenase (*Gpd*-2, EC 1.1.1.8); glucose-6-phosphate isomerase (*Gpi*, EC 5.3.1.9); hexokinase (Hk-2-4, EC 2.7.1.1 scored as one enzyme); isocitrate dehydrogenase (*Idh*-1 and *Idh*-2, EC 1.1.1.42); malate dehydrogenase (*Mdh*, EC 1.1.1.37), malate dehydrogenase (NADP+)/malic enzyme *(Mdhp*-2/*Me*, EC 1.1.1.40); phosphogluconate dehydrogenase (*Pgd*, EC 1.1.1.44), and phosphoglucomutase (*Pgm*, EC 5.4.2.2.). Three females, each from a separate family, were analyzed on each gel, and eight gels were assayed for each group of four species plus controls. Reference females of *Aedes aegypti* L. (ROCK strain) were also included in each run.

Statistical Analysis

Genetic variation was analyzed with a BIOSYS-2 Program for desktop computer (Black 1997). This program is a modification of BIOSYS-1 (Swofford & Selander 1981).

TABLE 1. ALLELE FREQUENCIES IN FOUR *CULEX* SPECIES (*CS* = *CX. SALINARIUS*, *CR* = *CX. RESTUANS*, *CQ* = *CX. P. QUINQUEFASCIATUS* AND *CN* = *CX. NIGRAIPALPUS*). TWENTY-FOUR SPECIMENS, EACH FROM A SEPARATE FAMILY, WERE ANALYZED FROM EACH SPECIES.

Locus & *Rf* values[a]	Species			
	CS	*CR*	*CQ*	*CN*
Acoh				
95	0.000	0.000	0.083	1.000
100	0.875	0.208	0.917	0.000
105	0.125	0.792	0.000	0.000
Ak-2				
90	1.000	1.000	0.000	0.000
95	0.000	0.000	0.000	1.000
100	0.000	0.000	1.000	0.000
Gpd-2				
100	1.000	1.000	1.000	0.083
120	0.000	0.000	0.000	0.917
Gpi				
84	0.000	0.000	0.000	0.042
95	1.000	0.000	0.000	0.000
100	0.000	0.083	1.000	0.833
105	0.000	0.917	0.000	0.125
Hk				
86	0.917	0.000	0.000	0.000
93	0.083	0.000	0.000	0.375
100	0.000	1.000	1.000	0.625
Idh-1				
100	0.000	0.000	1.000	0.000
107	0.000	1.000	0.000	0.000
133	0.625	0.000	0.000	1.000
147	0.292	0.000	0.000	0.000
153	0.083	0.000	0.000	0.000
Idh-2				
94	0.667	0.000	1.000	0.000
97	0.000	1.000	0.000	1.000
100	0.167	0.000	0.000	0.000
111	0.167	0.000	0.000	0.000
Mdh				
83	0.000	1.000	0.000	1.000
100	1.000	0.000	1.000	0.000
Mdhp-2				
95	0.042	1.000	0.000	1.000
100	0.333	0.000	0.875	0.000
103	0.000	0.000	0.125	0.000
108	0.625	0.000	0.000	0.000
Pgd				
67	0.792	0.083	0.125	0.000
100	0.208	0.917	0.875	1.000
Pgm				
87	0.167	0.458	0.000	0.333
100	0.833	0.542	0.958	0.542
109	0.000	0.000	0.042	0.125

[a]The eleven variable enzymes are *Acoh* = aconitase hydratase; *Ak*-2 = adenylate kinase; *Gpd*-2 = glycerol-3-phosphate dehydrogenase; *Gpi* = glucose-6-phosphate isomerase; *Idh*-1 and *Idh*-2 = isocitrate dehydrogenase; *Hk* = hexokinase; *Mdh* = malate dehydrogenase; *Mdhp*-2 = malate dehydrogenase (NADP+); *Pgd* = phosphogluconate dehydrogenase; and *Pgm* = phosphoglucomutase.

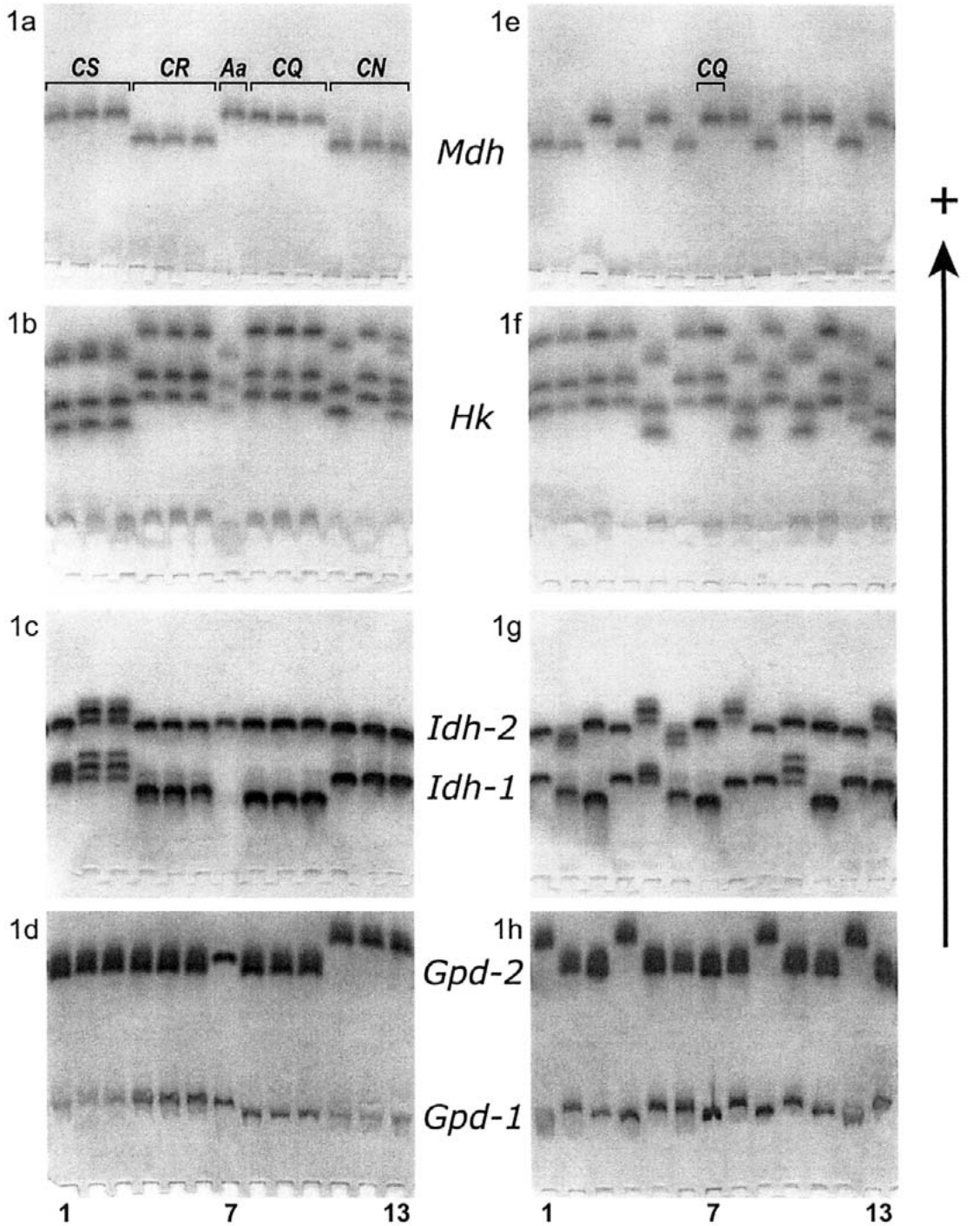

Fig. 1. Isoenzyme profiles of four enzymes (six loci, *Mdh*, *Hk*, *Idh*-1 and *Idh*-2, and *Gpd*-1 and *Gpd*-2). In Figs. 1a-1d, individuals numbered 1-3, 4-6, 8-10 and 11-13 represent known *Culex salinarius* (*CS*), *Cx. restuans* (*CR*), *Cx. p. quinquefasciatus* (*CQ*) and *Cx. nigripalpus* (*CN*), respectively. Individual numbered 7 (*Aa*) is *Aedes aegypti* control. Figs. 1e-1h, are used to identify unknown individuals as described in the text, except that individual numbered 7 (*CQ*) *Cx. p. quinquefasciatus* was used as a control.

RESULTS

Allele frequency data for four *Culex* species from Florida are presented in Table 1. Comparison of the frequency values of enzyme loci showed that even though most of the enzyme loci have differences in *Rf* values that could separate different species from each other, the *Rf* values in only four of the loci (*Gpd*-2, *Hk*, *Idh*-1 and *Mdh*) were distinctive enough to be used to separate the four species (Table 1; Fig. 1). These four loci are as follows: malate dehydrogenase (*Mdh*) is monomorphic in *Cx. salinarius* and *Cx. p. quinquefasciatus* at Mdh^{100}, and in *Cx. nigripalpus* and *Cx. restuans* at Mdh^{83} (Table 1; Fig. 1a). Hexokinase (*Hk*), that is represented by three-banded pattern and sometimes by a six-banded polymorphic pattern (Tabachnick & Howard 1982), is slower in *Cx. salinarius* ($Hk^{86,86/93}$) than in the other three *Culex* species (*Cx. restuans* Hk^{100}, *Cx. p. quinquefasciatus* Hk^{100} and *Cx. nigripalpus* $Hk^{93,100,93/100}$) (Table 1; Fig. 1b). Isocitrate dehydrogenase-1 (*Idh*-1) is polymorphic in *Cx. salinarius* *Idh*-$1^{133,\ 133/147,133/153}$ but homozygous in the other three species (*Cx. restuans* *Idh*-1^{107}, *Cx. p. quinquefasciatus* *Idh*-1^{100} and *Cx. nigripalpus* *Idh*-1^{133}) (Table 1; Fig. 1c). Glycerol-3-phosphate dehydogenase (*Gpd*-$2^{120,\ 100/120}$) is moving faster in *Cx. nigripalpus* in one allele than the other three species (*Cx. restuans* *Gpd*-2^{100}, *Cx. salinarius* *Gpd*-2^{100}, and *Cx. p. quinquefasciatus* *Gpd*-2^{100}) (Table 1; Fig. 1d). Since *Gpd*-2 in *Cx. nigripalpus* is sometimes heterozygous, caution is needed in using it as a distinguishing character. From this information we developed a key to separate the four *Culex* species (Table 2).

Further analysis of the data in Table 1 showed that *Cx. p. quinquefasciatus* exhibited a low number of alleles per locus (1.3 ± 0.1), the lowest percentage of polymporphic loci (23.1%) and the lowest Hardy-Weinberg heterozygosity (0.054 ± 0.03) from the other three species (*Cx. nigripalpus*, 1.6 ± 0.2, 46.2% and 1.95 ± 0.07; *Cx. restuans*, 1.3 ± 0.1, 30.8% and 0.091 ± 0.05; and *Cx. salinarius*, 1.8 ± 0.2, 69.2% and 0.207 ± 0.05, respectively). Since *Cx. p. quinquefasciatus* was monomorphic for the four enzyme loci chosen to be used in the key (Table 2), we used it as a control instead of *Ae. aegypti* (ROCK strain) to identify other *Culex* species. Thus, using *Cx. p. quinquefasciatus* as a control (#7 in Figs. 1e-1h) and the key (Table 2), we were able to identify correctly 24 randomly selected individuals of all four *Culex* species (Figs. 1e-1h, only 12 individuals are shown in these Figs.). Individuals numbered 3, 5, 8, 10, 11 and 13 (Fig. 1e) had a faster moving *Mdh* allele and represented either *Cx. salinarius* or *Cx. p. quinquefasciatus*, whereas individuals numbered 1, 2, 4, 6, 9 and 12 had a slower *Mdh* allele representing either *Cx. restuans* or *Cx. nigripalpus*. Individuals numbered 5, 8, 10 and 13 (Fig. 1f) had a slower moving *Hk* allele that identified it as *Cx. salinarius*, and distinguished it from the other two faster moving individuals numbered 3 and 11 that were identified as *Cx. p. quinquefasciatus*. Individuals that represented either *Cx. restuans* or *Cx. nigripalpus* and were numbered 1, 4, 9 and 12 (Fig. 1g) had a faster moving *Idh*-1 allele that identified it as *Cx. nigripalpus*, and distinguished it from a slower moving *Idh*-1 allele in individuals numbered 2 and 6 that were identified as *Cx. restuans*. *Culex nigripalpus* individuals numbered 1, 4, 9 and 12 were identified by using *Gpd*-2 enzyme loci. The most common *Gpd*-2 in *Cx. nigripalpus* was faster than *Gpd*-2 in the other three *Culex* species (Fig. 1h).

CONCLUSION

Our results show that *Culex* (*Culex*) species from Florida can be unambiguously distinguished from each other by using four isozymes (*Mdh*, *Hk*, *Idh*-1 and *Gpd*-2) in sequence. These studies suggest that from various types of trapping collections for *Culex* species, those individuals that cannot be identified to separate species with standard morphological characters can be identified by isoenzyme analysis, instead of pooling them together as *Culex* spp. It is worth pointing out here that the four species of mosquitoes used in this study were collected from January through April, when all four species were present in Florida. It is possible that some of the isoenzyme systems may show some degree of polymorphism when these species of mosquitoes are collected at different times of the year or from different locations as observed in *Cx. nigripalpus* (Nayar et al. 2002) and *Cx. p. quinquefasciatus* (Nayar et al. 2003).

TABLE 2. ELECTROPHORETICE KEY FOR IDENTIFICATION OF OUR COMMON *CULEX* (*CULEX*) SPECIES IN FLORIDA.

1.	Mdh, faster, monomorphic ..... *Cx. salinarius* or *Cx. p. quinquefasciatus*	(2)
	Slower, monomorphic ..... *Cx. restuans* or *Cx. nigripalpus*	(3)
2.	*Hk*, slower ..... *Cx. salinarius*	
	Faster, monomorphic. ..... *Cx. p. quinquefasciatus*	
3.	*Idh*-1, faster, monomorphic; *Gpd*-2, faster, usually monomorphic ..... *Cx. nigripalpus*	
	Both *Idh*-1 and *Gpd*-2 slower, monomorphic. ..... *Cx. restuans*	

Therefore, a word of caution may be appropriate. A broader application of this technique to identify *Culex* species from other areas must be confirmed with samples from different localities before this technique should be used outside Florida.

Isoenzyme analysis by electrophoresis technique is reliable, accurate and simple to perform once the electrophoretic equipment is set-up in the laboratory (Black & Munstermann 1996) and a person is trained to run the equipment. This technique is especially useful when freshly collected or frozen *Culex* mosquitoes are to be used for virus analysis or surveillance during different seasons of the year; however, this technique cannot be used for dead or dried specimens. Isoenzyme analysis is less expensive and faster than the PCR technique for DNA identification of different *Culex* species (Miller et al. 1996; Crabtree et al. 1995, 1997), but DNA analyses can be used for dead or dried specimens.

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