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Source: Florida Entomologist, 87(1): 1-5

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/0015-

4040(2004)087[0001:IOFCCC]2.0.CO;2

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

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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 [Idh-1], could be used in sequence to identify the four Culex species. Culex salinarius and Cx. p. quinquefasciatus could be separated from Cx. p. quinquefasciatus by Idh locus and Idh-1 and/or Idh-2 loci. Randomly combined specimens of these four Idh-2 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

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. Fieldcollected 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 speciesdiagnostic 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-vertielectrophoretic 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-ul 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 BIO-SYS-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 <math>CN = CX. NIGRAIPALPUS). Twenty-four specimens, each from a separate family, were analyzed from each species.

	Species			
Locus & Rf values ^a	CS	CR	CQ	CN
\overline{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

"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.

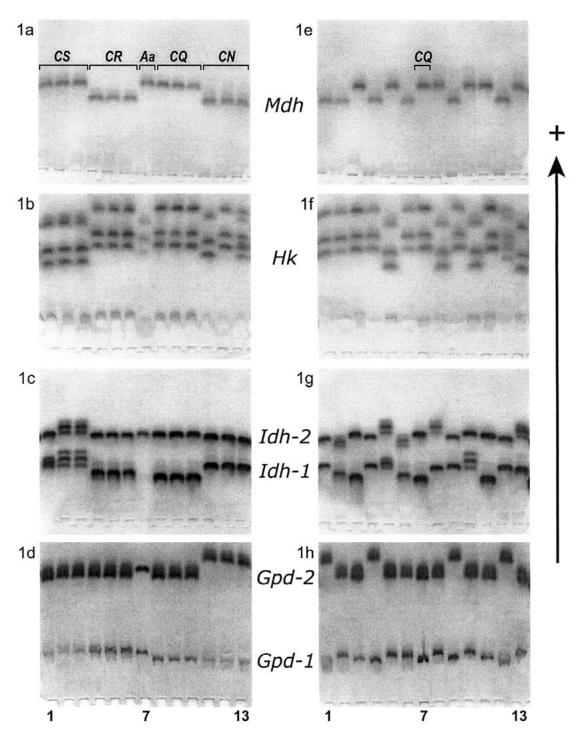


Fig. 1. Isoenzyme profiles of four enzymes (six loci, Mdh, Hk, Idh-1 and Idh-2, and Gpd-1 and Gpd-2). In Figs. 1a-1d, individuals numbered 1-3, 4-6, 8-10 and 11-13 represent known Culex salinarius (CS), Cx. restuans (CR), Cx. p. quinquefasciatus (CQ) and Cx. nigripalpus (CN), respectively. Individual numbered (CR) is (CR) and (CR) are used to identify unknown individuals as described in the text, except that individual numbered (CQ) (CR) 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 Culexspecies (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-1133, 133/147,133/153 but homozygous in the other three species (Cx)restuans Idh-1107, Cx. p. quinquefasciatus Idh-1100 and Cx. nigripalpus Idh-1¹³³) (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-2¹⁰⁰, Cx. salinarius Gpd-2¹⁰⁰, and Cx. p. quinquefasciatus Gpd-2100) (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 $(Cx.\ nigripal-pus, 1.6 \pm 0.2, 46.2\%$ and 1.95 ± 0.07 ; $Cx.\ restuans, 1.3 \pm 0.1, 30.8\%$ and 0.091 ± 0.05 ; and $Cx.\ salinarius, 1.8 \pm 0.2, 69.2\%$ 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 iden-

tify 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.		
2.		
	*	Cx. p. quinquejasciaius
3.	<i>Idh-</i> 1, faster, monomorphic;	
	Gpd-2, faster, usually monomorphic	
	Both <i>Idh-</i> 1 and <i>Gpd-</i> 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.

ACKNOWLEDGMENTS

This article is Florida Agricultural Experimental Station Journal Series No. R-09582.

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