IDENTIFICATION OF SPECIES D, A NEW MEMBER OF THE *ANOPHELES QUADRIMACULATUS* SPECIES COMPLEX: A BIOCHEMICAL KEY

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ABSTRACT. Sibling species D, a new member of the *Anopheles quadrimaculatus* species complex was identified in collections from Pickwick Lake, Tishomingo County, Mississippi and Choctawhatchee, Bay County, in West Florida. This species occurred sympatrically with the previously described species, A, B and C. Evidence for identification of species D includes diagnostic allozymes, a lack of polytene chromosomes in the ovarian nurse cells, and inviability of F_1 progeny and lack of sperm transfer in hybridization crosses. An electrophoretic taxonomic key for distinguishing species D from A, B and C is presented.

INTRODUCTION

Until recently, *Anopheles quadrimaculatus* Say, which has a widespread distribution over the eastern part of the United States, was considered to be a single species (Darsie and Ward 1981). However, cytogenetic, hybridization and electrophoretic studies led initially to the identification of sibling species A and B (Lanzaro 1986[1] and Lanzaro et al. 1988) and later species C (Kaiser et al. 1988b; Narang and Seawright 1988; Narang et al. 1989a).

Analysis of polytene chromosomes from the ovarian nurse cells of species A and B revealed diagnostic fixed and floating inversions on the autosomes and on the X chromosome (Kaiser and Seawright 1987, Kaiser et al. 1988c). The ovarian polytene chromosomes of species C had indistinct, diffuse bands which were not suitable for comparison with species A and B (Kaiser et al. 1988b).

Hybridization crosses between species A and B (Lanzaro 1986,[1] Lanzaro et al. 1988, Kaiser et al. 1988a) and between species A, B and C (Kaiser et al. 1988b) have been reported. Some F_1 hybrid adults were produced in all of the crosses, although results varied considerably among crosses because some resulted in all-female families, others produced all-male families, and still others contained fairly normal sex ratios. Results also varied within crosses because some lines produced F_1 males and some did not.

Narang et al. (1989a, 1989b) undertook a combination of electrophoretic and chromosomal studies to identify species-specific diagnostic loci and prepared a dichotomous electrophoretic key for taxonomic identification of species A, B and C.

During further investigation of the distribution of these 3 sibling species and analysis of the genetic structure of natural populations, we collected evidence for a 4th species, D. The initial evidence was the occurrence of a new cytotype, in a collection from the Yellow River, a tributary of Pickwick Lake in the TVA system (PIC), Tishomingo County, Mississippi, and at Choctawhatchee Bay (CHO), Walton County, in West Florida. Electrophoretic data on samples from these localities were analyzed by the electrophoretic taxonomic key of Narang et al. (1989a) suitable for identification of species A, B and C. When some adults from PIC and CHO did not key to either A, B or C, a combination of chromosomal and electrophoretic studies was used to characterize the presumed new species, D. In this paper, we present the combined evidence of electrophoretic, chromosomal, and hybridization studies that indicate the occurrence of sibling species D.

MATERIALS AND METHODS

The primary purpose in this paper is to show the evidence of a 4th sibling species of the *An. quadrimaculatus* complex. In keeping with that objective, we have included electrophoretic and chromosomal analyses of adult mosquitoes from 5 localities that were used to classify species D. The collection sites (with the sibling species present at each site) were: 1) species A from Lake Chickamauga, Hamilton Co., TN (CHI); 2) a mixed population from the Yellow River (near the confluence into Pickwick Lake), Tishomingo Co., MS, (PIC); 3) a mixed population from Choctawhatchee River, Bay Co., West Florida (CHO); 4) species C from Bear Bay Swamp, Dixie Co., FL (BBS); and 5) a mixed A and B population from Lake Octahatchee, Hamilton Co., FL (LOC). The CHI population is

[1] Lanzaro, G. C. 1986. Use of enzyme polymorphism and hybridization crosses to identify sibling species of the mosquito, *Anopheles quadrimaculatus* (Say). Ph.D. dissertation, University of Florida, Gainesville, FL, 92 pp.

typical of species A in the TVA system and was included for direct comparison to PIC. The BBS population is typical of species C throughout its known range. The LOC population was included for comparison to CHO. Wild males and most females were frozen at −70°C until used for electrophoresis. The voucher specimens of species A, B, C and D were deposited with the Florida State Collection of Arthropods, Florida Department of Agriculture and Consumer Services, Gainesville, Florida.

Preparation of ovarian nurse cell polytene chromosomes was done as described by Kaiser and Seawright (1987). To correlate chromosome types with electromorph patterns, females were analyzed electrophoretically either after oviposition or after the preparation of ovarian polytene chromosomes. A coding system was employed that removed any bias in the identification of the mosquitoes by the two methods. Adult F_1 progeny of the species D type were used for chromosomal identification and for hybridization crosses (by means of induced copulation, Baker et al. 1962), to a laboratory strain of species A.

Starch-gel electrophoresis was conducted according to Steiner and Joslyn (1979) with a few modifications (Narang et al. 1989a, 1989b). Homogenates of individual mosquitoes (grinding buffer, pH 7.0:10 mM Tris, 1 mM EDTA, 1 mM 2-mercaptoethanol and 5 mM dithiothreitol) were absorbed onto three 10 × 2.5 mm wicks (Whatman 3 mm paper) and applied to three 10 mm thick starch gels (1 wick per gel). Electrophoresis was terminated when the marker dye (bromophenol blue) moved about 10 cm toward the anode. Each gel was cut into six, 1.5 mm thick slices, stained for specific enzymes and fixed as soon as bands were of desired intensity (30–90 min). When necessary, a single slice was stained for 4 enzyme systems such as malate dehydrogenase (MDH), malic enzyme (ME), glucose phosphate isomerase (PGI) and hydroxy acid dehydrogenase (HAD). These multiple enzyme zymograms were easy to score due to differences in migration of their respective electromorphs in the gel. Thus, electrophoretic data on about 25 loci in each mosquito were routinely obtained. To analyze enzyme systems which showed low activity (faint bands) in the 1-mosquito-3 gels system, the entire homogenate of each mosquito was used to load 1 gel. Data were collected on electromorphs (alleles) at 34 presumptive loci in 16 enzyme systems. The enzyme systems (including the Enzyme Commission number, number of loci in each gene-enzyme system, and electrophoresis buffers) were listed previously (Narang et al. 1989a, 1989b).

A laboratory stock, Q2, of species A, (Lanzaro 1986[1]) homozygous for 34 loci was used as a reference standard. The electromorph coded by each locus of Q2 was given a mobility value of 100. The mobility values of electromorphs of the corresponding locus in natural populations were calculated relative to the reference standard band. In zymograms with allozymes at 2 or more loci, these were designated in order of decreasing anodic mobility from the origin (Figs. 1–3).

Analyses of electrophoretic variability data were performed using a Fortran IV computer program, BIOSYS-1 (Swofford and Selander 1981). The presence of sympatric reproductively isolated mating groups in some collections were identified by significant deviation of observed electromorph frequencies from their expected frequencies under the assumption of Hardy-Weinberg equilibrium. Electrophoretic data on adults from such collections were partitioned into mating groups (2 or more species) based on species-specific allozymes and chromosome patterns [for details on species A, B and C, see Kaiser et al. (1988b) and Narang et al. (1989a)] observed from analysis of individual gravid females. The diagnostic values of allozymes in each species were calculated according to Ayala and Powell (1972). A locus was considered diagnostic, if the probability of correct assignment of an individual to a given sibling species was 99% or higher.

RESULTS AND DISCUSSION

Chromosomal identification of species D: The first evidence of species D was the lack of polytene chromosomes in the ovarian nurse cells of a majority of the mosquitoes from PIC. These observations were highly unusual, in that by using our standard technique on species A and B we had never observed nurse cells without polyteny. The polytene chromosomes of species A and B are not always suitable for detailed analysis of the banding patterns, but they are always present and can be identified on the basis of obvious fixed and polymorphic inversions (Kaiser et al. 1988c). Although the polytene chromosomes of species C have diffuse banding patterns that preclude detailed comparisons to species A and B, their appearance is uniquely different from species A and B, and they are also always present in the nurse cells. In the correlation of chromosome types with electrophoretic data, the 47 females from PIC that were classified as species D lacked polytene chromosomes in the nurse cells. There was also a perfect correlation of the cytotype with the identification by electrophoresis for the other mosquitoes from PIC that were classified as species A ($n = 4$) and B ($n = 6$).

Hybridization crosses: Species D male and female progeny of females collected at Pickwick

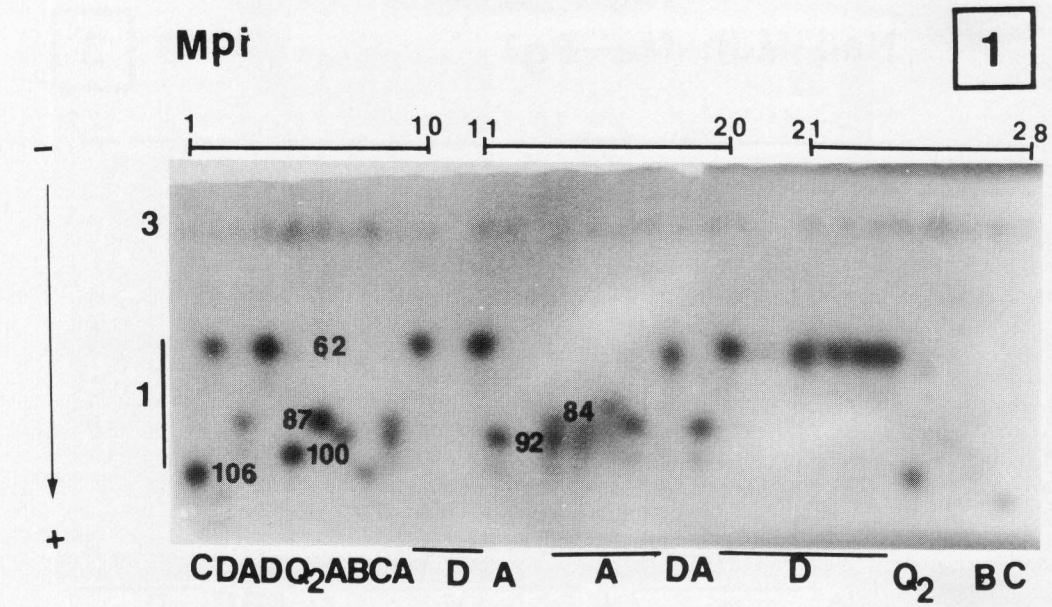

Fig. 1. *Mpi-1* and *Mpi-3*: samples 5 and 25, Q_2 strain; 3, 6, 9, 12, 14–17, 19 and 26, species A; 7 and 27, B; 1, 8, 13 and 28, C; remaining samples, D (samples were all females preidentified by chromosomal patterns). Note the species-specific *Mpi-1* bands (Rf, 62 in species D and 106 in C). *Mpi-3* was monomorphic and identical in the 4 species. Faint bands of *Mpi-2* migrated to about the same distance as *Mpi-1* bands in species D.

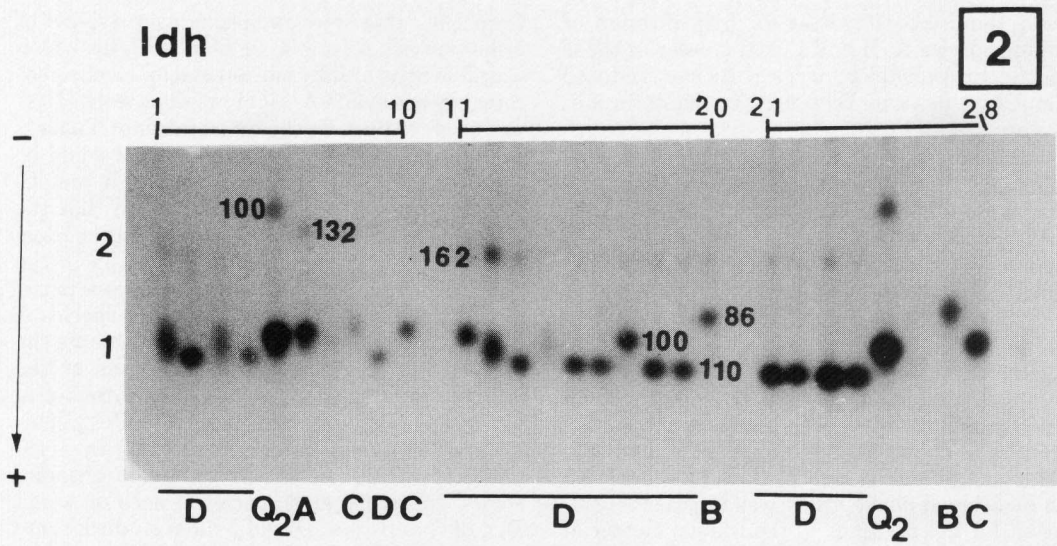

Fig. 2. *Idh-1* and *Idh-2*: samples 5 and 25, Q_2 strain; 6 and 26, species A; 7, 20 and 27, B; 8, 10 and 28, C; remaining samples are of species D.

were mated to species A (ORLANDO strain) by using the standard forced copulation technique employed previously for crosses of species A, B and C. Of the 50 mated D females, only 7 laid egg batches and 5 of these hatched (40.6 ± 21.7% hatch), but all F_1 larvae died as first instars. Such mortality was not observed for a control groups of families of species D (77.3% ± 7.1, n = 11) and species A (87.3% ± 11.9, n = 14). The PIC males that were mated to species A repeatedly grasped the female with the claspers, but they failed to complete copulation, as evidenced by a lack of sperm in the spermathecae of species A females that were dissected. Over the past few years in our studies on the *An. quadrimaculatus* complex, we have used forced copulation to

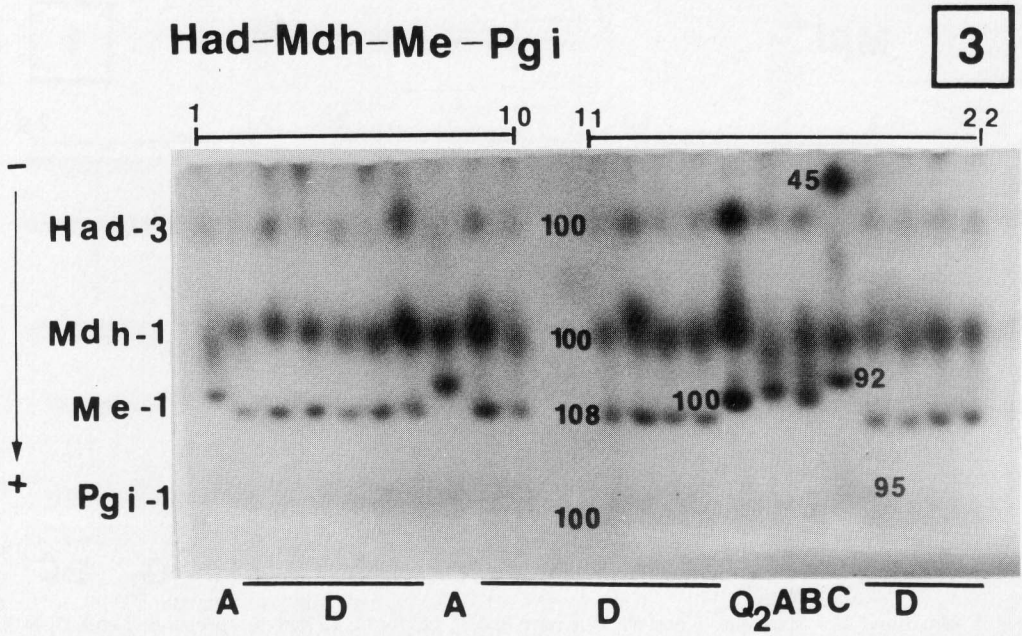

Fig. 3. *Had-3*, *Mdh-1*, *Me-1* and *Pgi-1*: sample 15, Q_2 strain; 1, 8 and 16, species A; 17, B; 18, C; remaining samples, D. Bands of *Had-1* and *Had-2* appear very faint under the electrophoretic condition used.

make hundreds of crosses for hybridization of sibling species A, B and C and crosses of those species to *Anopheles freeborni* Aitken. Induced copulation has also been used to maintain laboratory stocks of the sibling species and to introduce genes from natural populations into those laboratory stocks. In these previous experiments, we did not encounter the degree of difficulty that was experienced in making the crosses between species A and D. We have even had more success in making crosses between species A and B to *An. freeborni* than with A to D.

Diagnostic electrophoretic patterns of species D: In 91% of the adults (of both sexes) from PIC (n = 106), there was a cluster of electromorphs at 10 loci which were different from electromorphs of species A, B and C (Table 1). As mentioned above, there was a perfect correlation for the presence of the unique cluster of allozymes and a lack of polytene chromosomes in the ovarian nurse cells. For the PIC samples, a test of conformance of observed electromorph frequencies to those expected under Hardy-Weinberg equilibrium showed significant deficiencies of heterozygotes at 8 loci (Table 2), viz. peptidase (*Pep-2*), aconitase (*Acon-1*), malic enzyme (*Me-1*), glutamate oxaloacetate transaminase (*Got-1* and *Got-2*), mannosephosphate isomerase (*Mpi-1*), and isocitrate dehydrogenases (*Idh-1* and *Idh-2*). Of the remaining mosquitoes from PIC, their electromorphs were typical of either species A (n = 4) or B (n = 6), for which a sizable body of information and an electrophoretic key are available from previous work (Narang et al. 1989a). Of the data shown in Table 2, *Idh*-1 and *Idh*-2 are the only loci that could be used to distinguish between species A and B. These results were taken as evidence that the deficiencies of heterozygotes for the other 8 loci indicated the occurrence of a 3rd taxon in the collection from PIC. When the data were partitioned into clusters corresponding to species A (n = 4), B (n = 6) and D (n = 96) (Table 2), the frequencies of observed electromorphs at the various loci in species D were in conformance to those expected under the Hardy-Weinberg equilibrium. The small sample from CHO (n = 16) analyzed for both electromorphs and chromosomes, contained all 4 species (1 each of A and B, 4 of C and 10 of D). Of 34 loci studied, there were several diagnostic allozymes that could be used to separate species D from the other sibling species (Tables 3 and 4). Species D can be distinguished by a combination of characteristic electromorphs at *Idh-1* and *Idh-2* (alleles 100 and 110 at *Idh-1* and 162 at *Idh-2*), *Pep-2* (110), *Me* (108), *Got-2* (38), *Acon-1* (118), *Mpi-1* (62) and electromorph 100 at each of *Pgi-1* and *Had-3*. Species D shared with C electromorphs at *Idh-1/Idh-2*, *Pep-2*, *Got-2* and *Acon-1* but differed from the latter in alleles at *Me-1*, *Pgi-1*,

Table 1. Frequency distribution of diagnostic clusters of electromorphs in species D. The species A, B and C are included for comparison. The diagnostic allozymes in clusters in species B, C and D (relative to A) are underlined. P, polymorphic; H, each adult in this category is heterozygous at either one or two of the loci.

	Electromorph clusters in indicated populations							
Locus	PIC D (n = 96)		CHI A (n = 35)		LOC B (n = 60)		BBS C (n = 113)	
Pep-2	110	110	100	H	100	100	110	H
Pgi-1	100	H	100	100	100	H	95	H
Had-3	100	H	100	100	100	H	45	H
Me-1	108	108	100	H	100	100	100	H
Got-1	89	H	100	H	100	100	100	H
Got-2	38	H	100	H	100	H	38	H
Acon-1	118	118	100	H	100	100	118	H
Mpi-1	62	62	P	H	P	P	106	H
Idh-1	P	P	100	H	86	86	P	P
Idh-2	162	H	P	P	162	162	162	162
Freq. (%)	93	7	91	9	75	25	86	14

Table 2. Deviations in number of observed heterozygote electromorphs from those expected under Hardy-Weinberg equilibrium at some loci in a sample from PIC. PIC and PIC-D represent unpartitioned and partitioned electrophoretic data, respectively. (+, significant deviation by chi-square test; −, not significant chi-square value).

			Heterozygotes		Chi-square
Locus	Pop.	n	Exp.	Obs.	test
Pep-2	PIC	106	18	0	+
	PIC-D	96	0	0	−
Me-1	PIC	106	18	0	+
	PIC-D	96	0	0	−
Got-1	PIC	106	24	7	+
	PIC-D	96	9	7	−
Got-2	PIC	106	24	6	+
	PIC-D	96	8	6	−
Acon-1	PIC	106	18	0	+
	PIC-D	96	0	0	−
Mpi-1	PIC	106	19	4	+
	PIC-D	96	0	0	−
Idh-1	PIC	106	56	36	+
	PIC-D	96	44	36	−
Idh-2	PIC	106	10	2	+
	PIC-D	96	2	2	−
Pgi-1	PIC	106	2	3	−
	PIC-D	96	4	3	−
Had-3	PIC	106	4	4	−
	PIC-D	96	4	4	−

Mpi-1 and *Had-3*. Species B showed a characteristic *Idh-1*/*Idh-2* pattern (86/162). Species A was polymorphic for both *Idh* loci with a low frequency of allele 162 of *Idh-2*. Due to small sample sizes of species A and B from PIC and Species A, B, C and D from CHO, these electromorph data were not included in Table 3.

Dichotomous electrophoretic taxonomic key: The diagnostic electromorphs at 15 loci (Table 4 and underlined electromorphs in Table 3) provide wide flexibility in the choice of any 3 or more enzyme systems for taxonomic identification of the 4 species. Table 5 describes a short key, based on 4 enzymes (5 loci), which can be used for taxonomic identification of species A, B, C and D. If the reference strain Q_2 is not available, the relative migration of diagnostic enzymes in the gel can be used. Even a fraction of an adult mosquito is adequate for electrophoretic identification of species. For example, when thoraces and abdomens are needed for other studies, only the heads of individual mosquitoes can be run in one 6-mm-thick gel. After electrophoresis, three 1.5-mm-thick slices can be stained for 5 enzymes (one slice for IDH, a second slice for MPI, and the third slice for HAD, ME and PGI). If more tissue (thorax or abdomen) is available, all diagnostic loci (Tables 1 and 4) can be analyzed in a 10-mm thick gel. Under our standard electrophoretic conditions, the run was terminated after the marker dye-front moved 9–10 cm in the gel. *Had-3* (45) band migrated 6 mm or less in species C and 10 mm or more in A, B and D. *Pgi-1* band in C moved 3–4 mm slower than those of A, B and D, which moved 60–65 mm in most gels. The *Got-1* (89) band in D moved 3–4 mm slower than those of A, B and C. The *Got-2* (38) band moved 6 mm or less in C and D and about 10 mm or more in A and B. The *Pep-2* electromorphs in C and D moved about 4 mm faster than those in A and B (35–40 mm). The most common electromorph at *Pep-4* in A, B and C moved 9–10 mm or less, but in species D it moved 13–14 mm or more. Similarly, the *Me-1* electromorph moved 3–4 mm faster in species D than those in A, B and C, which moved about 50 mm or more. *Mpi-1* in species D moved slower (30–35 mm) than in A,

Table 3. Frequencies of electromorphs in species D, which differ from those in species A, B and C. The diagnostic electromorphs in species B, C and D (relative to species A) are underlined.

Locus	A (CHI)	B (LOC)	C (BBS)	D (PIC)
Acon-1				
N	35	60	113	96
86	0	0.02	0	0
100	0.98	0.98	0.01	0
109	0	0	0.03	0
118	0.02	0	0.96	1.00
Idh-1				
N	35	60	113	96
83	0	0.01	0	0
86	0.18	0.99	0.01	0
100	0.81	0	0.96	0.35
110	0.01	0	0.03	0.65
Idh-2				
N	35	60	113	96
100	0.01	0	0	0
132	0.92	0	0	0.01
162	0.07	1.00	1.00	0.99
Pep-2				
N	35	60	113	96
89	0.01	0	0	0
100	0.97	1.00	0.02	0
110	0.02	0	0.98	1.00
Me-1				
N	35	60	113	96
85	0.03	0	0	0
92	0	0	0.18	0
96	0	0	0.02	0
100	0.97	1.00	0.80	0
108	0	0	0	1.00
Mpi-1				
N	35	60	113	96
62	0	0	0	1.00
78	0.09	0.01	0	0
82	0.06	0	0	0
87	0.11	0.05	0.01	0
92	0.47	0.48	0.05	0
95	0.23	0.45	0	0
100	0.04	0	0.08	0
106	0	0.01	0.86	0
Pep-4				
N	35	60	113	96
50	0.05	0.03	0	0
100	0.95	0.97	0.98	0.18
135	0	0	0.02	0.02
165	0	0	0	0.67
190	0	0	0	0.06
215	0	0	0	0.07
Est-2				
N	17	19	36	46
68	0	0	0.01	0
77	0	0	0.24	0
81	0	0.18	0.11	0
84	0	0.72	0.53	0
88	0	0.05	0.11	0
93	0	0.05	0	0
96	0.18	0	0	0
100	0.47	0	0	0.18
103	0.23	0	0	0.34
105	0.12	0	0	0.20
108	0	0	0	0.22

Locus	A (CHI)	B (LOC)	C (BBS)	D (PIC)
Pgi				
N	35	60	113	96
86	0	0	0	0.02
95	0	0	0.98	0
100	1.00	0.99	0.01	0.98
110	0	0.01	0.01	0
Had-3				
N	35	60	113	96
45	0	0.03	0.96	0
100	1.00	0.93	0.04	0.98
156	0	0.04	0	0.02
Got-1				
N	35	60	113	96
65	0	0	0.01	0.01
89	0	0.05	0.01	0.95
100	0.97	0.90	0.97	0.03
104	0	0.04	0	0
109	0	0	0	0.01
122	0.03	0.01	0.01	0
Got-2				
N	35	60	113	96
25	0	0	0	0.01
38	0.34	0.06	0.92	0.96
100	0.66	0.93	0.08	0.03
170	0	0.01	0	0
Xdh-3				
N	16	26	34	34
94	0.05	0	0	0.03
100	0.82	0.64	0.06	0.12
108	0.05	0	0	0.04
117	0.08	0.31	0.04	0.25
126	0	0.05	0.15	0.38
135	0	0	0.25	0.06
140	0	0	0.41	0.09
150	0	0	0.09	0.03
Pgm-3				
N	35	49	35	96
57	0.02	0	0	0.02
64	0.06	0	0.03	0.13
78	0.01	0.02	0	0.15
83	0	0.01	0.05	0
100	0.87	0.67	0.43	0.65
113	0	0	0.03	0
119	0.01	0.01	0.06	0.03
124	0.02	0.28	0.37	0.01
140	0.01	0	0	0.01
152	0	0.01	0.03	0
Ao-1				
N	11	35	44	41
58	0	0	0	0.01
76	0	0	0	0.12
85	0	0.13	0.13	0.02
88	0	0.53	0.34	0.56
91	0	0.13	0.14	0.01
96	0	0.07	0	0.18
100	0.91	0.11	0.30	0.08
103	0.09	0.03	0.09	0.02

Table 4. Diagnostic loci in species A, B, C and D of the *Anopheles quadrimaculatus* species complex where the probability of correct identification is >99%.

Diagnostic loci for indicated species					
A:B	A:C	A:D	B:C	B:D	C:D
Idh-1	*Acon-1*	*Acon-1*	*Acon-1*	*Acon-1*	*Got-1*
Idh-2	*Idh-2*	*Idh-2*	*Idh-1*	*Idh-1*	*Had-1*
Est-2	*Had-1*	*Got-1*	*Had-1*	*Got-1*	*Had-3*
Est-5	*Had-3*	*Got-2*	*Had-3*	*Got-2*	*Pep-4*
Est-7	*Pep-2*	*Pep-2*	*Got-2*	*Pep-2*	*Pgi-1*
Had-1	*Got-2*	*Pep-4*	*Pep-2*	*Pep-4*	*Me-1*
6Pgd-1	*Pgi-1*	*Me-1*	*Pgi-1*	*Me-1*	*Est-2*
	Est-2	*Mpi-1*	*Est-4*	*Est-2*	*Mpi-1*
	Est-6		*Est-5*	*Est-7*	
	Mpi-1		*Est-6*	*Mpi-1*	
	6Pgd-1		*Est-7*		
	Xdh-3		*Mpi-1*		
	Ao-1		*Xdh-3*		

Table 5. Electrophoretic key for identification of sibling Species A, B, C and D of the *Anopheles quadrimaculatus* complex. (Refer to Figs. 1–3).

1. MPI-1 slow, *Rf* 62 (rarely with 52/62 heterozygotes) (Fig. 1) ..... Species D
   MPI-1 faster, *Rf* 78 or greater ..... (2)
2. IDH-1 slow, *Rf* 86 and IDH-2 fast, *Rf* 162 (Fig. 2) ..... Species B
   IDH-1 faster, *Rf* 100 or greater (sometimes with 86/100 heterozygotes), IDH-2 fast or slower, *Rf* 100, 132, 162 (sometimes heterozygotes for 100, 132, 162 ..... (3)
3. HAD-3 slow, *Rf* 45; PGI slow, *Rf* 95 (Fig. 3) ..... Species C
   HAD-3 faster, *Rf* 100 (sometimes with 45/100 heterozygotes; PGI faster *Rf* 100 (rarely with 95/100 heterozygotes ..... Species A

B and C (45 mm or more). The ratio of migrations of *Mpi-1*: *Mpi-3* electromorphs was 3.10 ± 0.12 or lower in species D, 4.00 ± 0.20 or higher in A and B, and 5.30 ± 0.26 in species C.

Although the short key (Table 5) is adequate for species identification, we routinely analyze samples from different localities in the distribution range of the *An. quadrimaculatus* species complex for all 15 diagnostic loci (Tables 3 and 4) in addition to other gene-enzyme systems for genetic fingerprinting of geographically and ecologically distinct populations including identification of possible new taxa. Similar biochemical keys for identification of cryptic species in *Anopheles*, *Culex* and *Aedes* mosquitoes have been published earlier (see review articles by Munstermann (1988) and Narang and Seawright (1989) for pertinent references).

Our inability to use the technique of nurse cell polytene chromosomes for comparison of all 4 sibling species means that an improved technique for preparation of salivary gland chromosomes must be developed. We originally used nurse cell preparations because of the unsurpassed success of making preparations of species A and B that were suitable for detailed analysis. Although a salivary gland polytene chromosome map is available for species A (Klassen et al. 1965), we have found that it is typically difficult to achieve acceptable preparations of salivary gland chromosomes, especially from field populations.

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ERRATUM

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Table 1 on page 255 should be replaced by the table below.

Table 1. Spearman rank correlations for 13 stations in subplot 1 and 25 stations in subplot 2.

	G. affinis	*L. cyanellus*	Depth
Subplot 1 (n = 13)			
An. freeborni	—	−0.48*	−0.38
L. cyanellus	—	—	0.16
Subplot 2 (n = 25)			
An. freeborni	−0.40**	−0.39**	−0.28
L. cyanellus	0.31	—	0.32
G. affinis	—	0.31	0.67***

* $P < 0.10$
** $P < 0.05$
*** $P < 0.01$