Am. J. Trop. Med. Hyg., 54(1), 1996, pp. 46–48

MISIDENTIFICATION OF A PHILIPPINE MALARIA VECTOR REVEALED BY ALLOZYME AND RIBOSOMAL DNA MARKERS

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Abstract. Morphologically identified *Anopheles flavirostris* (Diptera: Culicidae), the principal malaria vector in the Philippines, comprised two species in collections from the Bataan Province of Luzon based on allozyme and internal transcribed spacer 2 ribosomal DNA analysis. Seven percent of morphologically identified specimens were the closely related nonvector *An. filipinae*. Morphologic variability of *An. filipinae* may account for some of these misidentifications. Genetic identification tools promise to be useful not only for verifying the identification of morphologically defined taxa but also for detecting the presence of morphologically indistinguishable sibling species in the Philippines.

Malaria is endemic in the Philippines in 72 of 75 provinces where it is among the 10 leading causes of morbidity.[1] The principal malaria vector in the Philippines is *Anopheles flavirostris*, with *An. balabacensis*, *An. litoralis*, *An. maculatus sensu lato* (s.l.), and *An. mangyanus* as secondary vectors.[2] The taxonomic status of some Philippine anophelines requires investigation. *Anopheles maculatus* s.l. is thought to be composed of two species in the Philippines based on morphologic differences (i.e., *An. greeni* and *An. dispar*), but assortative mating has yet to be confirmed in the field.[3] The status of *An. flavirostris* has also been brought into question with variation noted in its propensity to bite humans[4] and in its tendency to rest indoors following surface spraying with DDT.[5]

Study of anopheline taxa in other parts of the world has revealed complexes of morphologically indistinguishable species.[6] Species within these complexes differ in vectorial importance or ease of control due to differences in host preference and their tendency to rest indoors, e.g., within the *An. gambiae* complex.[7] Genetic identification techniques using chromosome, allozyme, and DNA markers have aided the elucidation of these complexes, e.g., the *An. punctulatus* complex.[8,9] Recently internal transcribed spacer 2 (ITS-2) ribosomal DNA (rDNA) markers have distinguished closely related species of mosquitoes.[10]

Genetic identification techniques were used by us to investigate variation within morphospecies in the Philippines. In this paper, we describe a technique to obtain correlated data on allozymes and rDNA from morphologically identified samples of *An. flavirostris*. An unexpected finding was that these samples sometimes included a second morphologically defined species.

MATERIALS AND METHODS

Mosquito collections. Mosquitoes were collected in July 1994 from Anahao (120°17′E, 14°42′N) and Cabayo (120°23′E, 14°37′N) within the Bataan province of Luzon by night landing catches from humans or water buffalo, or as larvae that were reared to the adult stage. Adult female mosquito specimens were identified according to the illustrated keys of Cagampang-Ramos and Darsie[11] and either stored frozen at −70°C or blood fed and allowed to lay eggs to provide progeny for morphologic and genetic studies. Alternatively, the forelegs, wings, and palps were mounted on a microscope slide according to the method of Hunt and Coetzee[12] and the remainder of the carcass was frozen.

Identification by morphology. The keys of Cagampang-Ramos and Darsie[11] separate *An. filipinae* from *An. flavirostris* and *An. mangyanus*. The morphologic criteria are 1) number of dark-scaled areas on the anal vein (3 for *An. filipinae* and 2 for the alternative), 2) the color of the wing fringe adjacent to the anal vein (pale for *An. filipinae* and dark for the alternative), and 3) the length of the subapical dark band of the palpi relative to the adjacent subapical pale band (dark band subequal for *An. filipinae* and shorter for the alternative).

Identification by allozymes. Electrophoresis was performed using cellulose acetate plates (Helena Laboratories, Beaumont, TX) and enzyme stains according to the methods of Foley and others.[9,13] Briefly, adult mosquitoes were homogenized with 30 µl of double-distilled water in the wells of a microtiter plate by a hand-operated multiwell grinder.[14] Thirty samples were loaded onto cellulose acetate plates via a modified applicator system.[15] Microtiter plates were numbered, covered, and then stored dry or frozen at or below −20°C. Colony-bred specimens of *An. farauti* s.s. no. 2 and no. 3[9] were included on each plate as standards. Where more than one locus per enzyme was visualized, the locus with the lowest anodic mobility was designated 1 and the slowest allelomorph a. Twenty-four enzymes were screened from each mosquito: aconitate hydratase (ACON), acid phosphatase (ACP), adenylate kinase (AK), amylase (AMY), esterase (EST), fructose-1,6-diphosphatase (FDP), β-galactosidase (βGAL), glutamate-oxaloacetate transaminase (GOT), α-glycerophosphate dehydrogenase (αGPD), 6-glucophosphate dehydrogenase (6GPD), glucose-6-phosphate isomerase (GPI), β-hydroxybutyrate dehydrogenase (HBDH), hexokinase (HK), isocitrate dehydrogenase (IDH), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), malic enzyme (ME), mannose-6-phosphate isomerase (MPI), octanol dehydrogenase (ODH), peptidase-B (PEPB), peptidase-D (PEPD), phosphoglucomutase (PGM), pyruvate kinase (PK), and L-threonine-3-dehydrogenase (THDH).

Identification by rDNA. Following allozyme study, 20

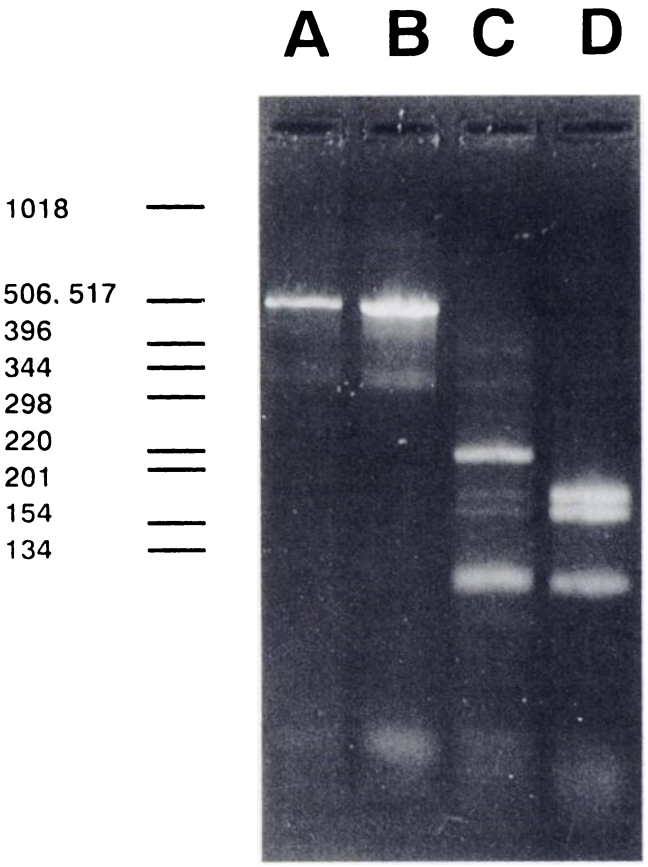

FIGURE 1. Polymerase chain reaction (PCR) products and PCR restriction fragment length polymorphism (RFLP) patterns for the ribosomal DNA internal transcribed spacer 2 fragment of Philippine anophelines using the restriction endonuclease *Msp* I. Lanes A and B are the amplification products for *Anopheles flavirostris* and *An. filipinae*, respectively. Lanes C and D are the restriction patterns for *An. flavirostris* and *An. filipinae*, respectively. Misidentified *An. flavirostris* gave identical RFLP patterns to *An. filipinae*. Values are in basepairs.

μl of TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) was added to mosquito debris in the wells of the microtiter plate and samples further ground to make available DNA template for the polymerase chain reaction (PCR) of the ITS-2 region of rDNA. The primers and cycling conditions were similar to those of Beebe and Saul.[16] The final PCR mixture contained Perkin Elmer-Cetus (Norwalk, CT) *Taq* buffer II, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 50 μM of each primer, 5% dimethylsulfoxide, 2.5 units of *Taq* polymerase, and 1 μl of template. After the initial denaturation step, the PCR conditions consisted of two cycles of 94°C for 1 min, 51°C for 2 min, and 72°C for 2 min, followed by 33 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 1.5 min. The PCR product was digested with the restriction endonuclease *Msp* I according to the protocol of Beebe and Saul.[16]

RESULTS

The allozyme profiles of a number of *An. flavirostris* were unusual, with fixed differences at approximately 50% of the loci in sympatric locations (n = 35 loci). This indicates that they are different species. The allozyme and restriction fragment length polymorphism (RFLP) patterns of the different *An. flavirostris* were identical to *An. filipinae*. Figure 1 shows the 500-basepair (bp) PCR product and the RFLP pattern for *An. flavirostris* and *An. filipinae*. The RFLPs comprised a two-banded pattern for *An. flavirostris* (approximately 220 and 100 bp) and a three-banded pattern for *An. filipinae* (approximately 175, 160, and 100 bp). The presence of faint bands corresponding to the *An. filipinae* pattern in lane C of Figure 1 probably indicates a minor polymorphism in the copies of ITS-2 in *An. flavirostris*. Additional samples of *An. flavirostris* were screened for RFLP and a subsample of allozymes, i.e. ACP, GPI, βGAL-1, βGAL-2, and PGM. The allozyme profiles for *An. flavirostris*, *An. filipinae*, and anomalous specimens are given in Table 1. Except for the anomalous specimens, both the allozyme and RFLP patterns were unique for *An. flavirostris* and *An. filipinae* in the study areas, i.e., different from *An. annularis*, *An. litoralis*, *An. maculatus* s.l., and *An. manyganus* (Foley DH, unpublished data).

TABLE 1

Allele frequencies for allozymes of *Anopheles flavirostris* (Flav), *An. filipinae* (Fil), and anomalous *An. flavirostris* (Anom) collected from Anahao and Cabayo, Bataan Province, Philippines

Locus*	Flav	Fil	Anom
ACP (N)	33	4	6
c	1.00	0.00	0.00
e	0.00	1.00	1.00
βGAL-1 (N)	33	4	5
b	0.00	1.00	1.00
c	1.00	0.00	0.00
βGAL-2 (N)	33	4	5
b	1.00	0.00	0.00
c	0.00	1.00	1.00
GPI (N)	31	4	6
c	1.00	0.00	0.00
d	0.00	1.00	1.00
PGM (N)	32	4	6
a	0.00	1.00	0.75
b	0.00	0.00	0.25
c	1.00	0.00	0.00

*Allelic designations are those of Foley and others.[9,13] ACP = acid phosphatase; N = no. of individuals; βGAL = β-galactosidase; GPI = glucose-6-phosphate isomerase; PGM = phosphoglucomutase.

The morphologic diagnoses of some of the anomalous specimens were rechecked from slides of forelegs, wings, and palps. In one instance, the specimen was identified as *An. flavirostris* a second time. However, progeny from this specimen were identified morphologically as *An. filipinae*. Variability in the number of spots on the anal vein (even between wings on one insect) and in the color of the wing fringe opposite the anal vein was noted in specimens genetically identified as *An. filipinae*. Seven of 107 morphologically identified *An. flavirostris* from the study area were anomalous.

DISCUSSION

The allozyme and RFLP patterns indicate that some morphologically identified *An. flavirostris* were misidentified *An. filipinae*. The number of dark-scaled areas of the anal vein and fringe coloration are unreliable characters, at least for delineating *An. filipinae*. It has not been established whether

these characteristics are also variable for *An. flavirostris* and *An. mangyanus*, although a survey of 25 *An. flavirostris* did not reveal any anomalies except for variation in palpal coloration approaching the *An. filipinae* condition in three individuals.

Anopheles filipinae, *An. flavirostris*, and *An. mangyanus* belong to the *An. minimus* species group and as such are morphologically similar. Unreliable characteristics coupled with the poor condition of some specimens could lead to misidentification of mosquitoes. The level of error would depend on geographic or temporal variability in morphology. However, human error must also play a role in some of these misidentifications. Criteria for separating *An. flavirostris* from *An. mangyanus* include proboscis coloration (ventral gold patch usually confined to the apical half for *An. flavirostris*, without gold patch for *An. mangyanus*). If the proboscis was checked, even after *An. filipinae* was identified, the misidentification rate of *An. filipinae* for *An. flavirostris* would be minimized.

Misidentification of *An. filipinae* for *An. flavirostris* was not confined to larval collections but also occurred in water buffalo–baited trap collections. No misidentifications were found in human bait collections but numbers in this category were low.

It is possible that presently recognized malaria vectors in the Philippines contain cryptic species with differences in biology affecting vectorial importance. This study demonstrates that concurrent typing of morphologically identified mosquitoes with allozyme and ITS-2 rDNA markers is a powerful methodology for addressing questions of identity. However, it must be borne in mind that phenotypic variation and human error can blur the limits of morphologically defined taxonomic species even before these species limits are tested by genetic means.

Acknowledgments: We gratefully acknowledge Ferdinand Salazar and Leonard Bueno for technical assistance, the local aides from Anahao for cooperation and help in collecting mosquitoes, and Joan Bryan for comments on the manuscript.

Financial support: This project was undertaken when Desmond H. Foley was an NIH/Tropical Medicine Research Center–funded visiting scientist at the Research Institute for Tropical Medicine (Manila).

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