

CONFIRMATION OF *ANOPHELES VARUNA* IN VIETNAM, PREVIOUSLY MISIDENTIFIED AND MISTARGETED AS THE MALARIA VECTOR *ANOPHELES MINIMUS*

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Abstract. Malaria control programs in Southeast Asia are faced with several questions concerning vector behavior and species identification, which need to be answered to consolidate and further improve the results of control practices. The vector system in Southeast Asia is complex because of the number of species potentially involved in malaria transmission. Additionally, the follow-up and evaluation of preventive control measures are hampered by the misidentification of vectors due to overlapping morphological characters of the female mosquitoes. In central Vietnam, control practices are aimed at 2 main species, *Anopheles dirus* s.l. and *Anopheles minimus* s.l. These reputed vectors were studied in an area of Binh Thuan Province of south-central Vietnam. Different collection methods were used to capture mosquitoes quarterly during a 1-year period. Mosquitoes were identified in the field and later subjected to detailed morphological examination and polymerase chain reaction-restriction fragment length polymorphism analysis. What was thought to be an unusual morphotype of *An. minimus* was shown to be *Anopheles varuna*, and most specimens identified as the former species in the field proved to be the latter species. Very few *An. minimus* individuals were found during the study period. The population of *An. varuna* was found to be highly zoophilic, and based on this behavior, it cannot be considered a vector in Vietnam. Because this species was previously being misidentified as *An. minimus*, a nonvector was mistargeted as a malaria vector in Binh Thuan Province. *Anopheles dirus*, which was found positive for *Plasmodium falciparum* circumsporozoite via enzyme-linked immunosorbent assay, is clearly the main vector in this area. Despite the fact that several potential secondary vectors were found during the study, the primary target for vector control in the region should be *An. dirus*.

INTRODUCTION

The important economic and social implications caused by malaria in Southeast Asia have prompted governments to make this disease a public health priority and to implement integrated national malaria control programs adapted to the specific needs of their individual countries. In Vietnam, a comprehensive malaria control strategy, including disease management and prevention, has resulted in a significant reduction of malaria cases, especially in the northern part of the country. In central Vietnam, however, malaria remains a major public health problem. Although a similar decrease in the number of malaria cases in this part of the country can be expected as a result of national and international financial input, several questions concerning vector behavior and species identification need to be resolved to further rationalize the available resources.¹

In central Vietnam, mosquito control practices are aimed at 2 principal vector taxa, *Anopheles dirus* s.l. and *Anopheles minimus* s.l. (henceforth referred to simply as *An. dirus* and *An. minimus*, respectively). Bed nets are impregnated twice a year, once before the presumed transmission by *An. minimus* and a second time to precede transmission by *An. dirus*. *Anopheles minimus* belongs to a group of related species that on mainland Southeast Asia includes *Anopheles aconitus*, *Anopheles culicifacies*, *Anopheles fluviatilis*, *Anopheles jeyporiensis*, *Anopheles pampanai*, and *Anopheles varuna*. The identification of these species, including vectors and non-vectors, is problematic as a result of overlapping morphological characters in the blood-sucking females, and identification requires confirmation from associated larval and pupal exuviae.²

In a recent study, Van Bortel and others³ showed that most

discrepancies between morphological and molecular methods of identification were caused by specimens that were incorrectly identified as *An. minimus* on the basis of adult morphology. Obviously, failure to identify mosquito species hampers the study and monitoring of vectors and hence complicates the follow-up and reassessment of preventive control measures. Ultimately, this will impede progress toward the consolidation and further improvement of the malaria situation in Vietnam.¹ In this article, we report the use of morphological and molecular methods of identification for studying the malaria vectors in central Vietnam and the implications of correct identification for vector control.

MATERIALS AND METHODS

Description of study site. Mosquitoes were collected in a village of Suoi Kiet commune (10°50' N, 107°40' E), Binh Thuan Province, south-central Vietnam. The village is situated in a hilly, forested area 50 km from the provincial capital. The village has a thousand inhabitants who grow manioc, maize, and cashew nut trees. People also work in the forest that directly surrounds the village. The nearest health center is located 7 km from the village. The annual average temperature at the district town is 26.7°C. The coldest months are December through February; the hottest are from April through August. The dry season runs from December through April, with almost no rain during the first 3 months of the season. During the rainy season from May through November, monthly average precipitation is 250 mm.

Mosquito collections and species identification. Mosquitoes were captured quarterly from May 1998 to April 1999 during 10 nights and mornings each time. Human land-

ing collections were made by one collector inside and 2 collectors outside each of 2 fixed houses. One person collected resting mosquitoes during the night both inside and outside of a third house. The night collections were made at 1800–0600 hr. Two collectors made collections on cattle at 2100–2400 hr. Morning collections of indoor resting mosquitoes took place in 10 different houses at 0600–0830 hr. After capture, mosquitoes were identified morphologically in the field by use of a standardized key for the medically important anophelines of Southeast Asia (modified from the Institute of Malariology, Parasitology and Entomology).⁴ Specimens of *An. minimus* and 2 closely related species, *An. aconitus* and *An. pampanai*, were stored to verify the morphological identifications using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays developed by Van Bortel and others.³

Progeny broods were obtained from blood-fed females. Progeny were individually reared to obtain adults with associated larval and pupal exuviae for identification, which was based on correlated morphological characters of the 3 life stages.²

The entire internal transcribed spacer 2 (ITS2) of rDNA of one specimen of *An. varuna* originating from Thailand and of one mosquito from a progeny brood were sequenced by means of the procedure described by Van Bortel and others.³ The *An. varuna* from Thailand corresponds to strain number 146 described by Sharpe and others.⁵

Specimens identified morphologically as *An. minimus* and *An. dirus* were subjected to enzyme-linked immunosorbent assay (ELISA) to detect *Plasmodium falciparum*, *Plasmodium vivax* 210, and *P. vivax* 247 circumsporozoite proteins in the head-thoracic portion of individual mosquitoes.^{6–8}

RESULTS

On the basis of correlated differential and diagnostic anatomical characters of the adult and immature stages of individual mosquitoes,² a single progeny brood was identified as *An. varuna*. This came as a surprise to field workers, who had identified adults of this brood as an unusual morphotype of *An. minimus* on the basis of previous practice. The identification of the brood as *An. varuna* was confirmed by comparison of the ITS2 rDNA sequence of siblings with the sequence of *An. varuna* from Thailand. A sequence of 517 bp was produced that includes the entire ITS2 rDNA fragment and a partial sequence of the 5.8S and 28S genes. The sequences of the progeny and the specimen from Thailand were in complete agreement. Sequence obtained for the progeny brood was deposited in GenBank under accession number AF230465. The ITS2 rDNA sequences obtained during this study and by Sharpe and others⁵ for the same Thai strain of *An. varuna* differed, however, at 2 positions. This was probably because of the reading errors produced by the *Taq* polymerase.

Subsequently, all specimens identified as *An. minimus* in the field on the basis of morphology, and at least 30% of specimens similarly identified as *An. aconitus* and *An. pampanai*, were analyzed by PCR-RFLP. The results are shown in Table 1. Most of the specimens identified as *An. minimus* (69 of 75) proved to be *An. varuna*, one was actually *An. aconitus*, and 2 were *An. pampanai*. Only 3 of the specimens

TABLE 1
Morphological versus molecular identification of *Anopheles* species

Morphological identification*	Molecular identification†			
	<i>An. aconitus</i>	<i>An. minimus</i> A	<i>An. pampanai</i>	<i>An. varuna</i>
<i>An. aconitus</i>	67	—	—	4
<i>An. minimus</i>	1	3	2	69
<i>An. pampanai</i>	—	—	135	—

* Morphological identification of adult females in the field.

† On the basis of the method developed by Van Bortel and others.³

were identified correctly as *An. minimus* (species A of the *An. minimus* complex). Of 71 specimens identified in the field as *An. aconitus*, 4 proved to be *An. varuna* on the basis of PCR-RFLP.

During the 1-year study in Suoi Kiet, 18 *Anopheles* species were collected in the vicinity of human dwellings. The vector *An. minimus* was found infrequently during the collection period: one specimen was collected on human outdoors and 2 were captured on cattle. *Anopheles varuna* were almost all captured on cattle (66 of 69). Not one of 59 *An. varuna* initially identified as *An. minimus* on the basis of morphology was positive for *P. falciparum*, *P. vivax* 210, or *P. vivax* 247 circumsporozoite proteins. In addition to the main vector *An. dirus*, of which 1.3% (2 of 151) were positive for *P. falciparum* circumsporozoite protein, a number of potential secondary vectors were collected landing on humans (Table 2). However, most specimens of these species—for example, *An. aconitus*, *Anopheles maculatus*, and *Anopheles vagus*—were captured on cattle, indicating a stronger preference for nonhuman hosts. Proportionately more specimens of *Anopheles barbirostris*, *Anopheles sinensis*, and *Anopheles tessellatus* were collected landing on humans, but few females of these species were captured overall (Table 2).

DISCUSSION

The geographic distribution of *An. varuna* is uncertain because the adult females are so morphologically similar to those of *An. aconitus* and *An. minimus*.² On the basis of published records and specimens examined, Harrison² gave the following distribution for *An. varuna*: Bangladesh, Burma, India, Nepal, Sri Lanka, and Thailand. He did not include the only record of *An. varuna* from Vietnam (Cam Ranh Bay, Khanh Hoa Province)⁹ because the identification was based on specimens collected in light traps and could not be confirmed. On the basis of morphological and molecular evidence, the present study definitely establishes the presence of *An. varuna* in Vietnam. Since Binh Thuan is located at 10°50' N latitude, this locality is much farther south than the sites where *An. varuna* is recorded from Thailand.²

Published accounts of the adult behavior of *An. varuna* are contradictory and confusing. In the plains of India, this species reportedly feeds primarily on cattle and is found more commonly resting in animal sheds than in human habitation.¹⁰ However, in the hilly areas of east-central India, adults are often captured in human habitation and are decidedly anthropophilic.¹¹ In Sri Lanka, *An. varuna* has been found infected with *P. falciparum* and *P. vivax* by circum-

TABLE 2
Adult *Anopheles* mosquitoes collected quarterly during a 1-year study (May 1998–April 1999) in Suoi Kiet commune, south-central Vietnam

Species	Collected on humans (no./person/10 nights)		Collected resting during the night (no./house/10 nights)		Collected resting in the morning (no./house/10 nights)	Collected on cattle (no./collector/10 nights)
	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor
<i>An. aconitus</i> *	0.88	1.56	0	0	0	23.75
<i>An. argyropus</i>	0	0.06	0	0	0	0
<i>An. barbirostris</i>	0.25	0.38	0	0	0	1.13
<i>An. barbumbrosus</i>	0	0.13	0	0	0	0
<i>An. culicifacies</i> s.l.	0	0	0	0	0	0.13
<i>An. dirus</i>	0.88	4.06	12.25	13.00	0	0.13
<i>An. karwari</i>	0	0.44	0	0	0	4.13
<i>An. maculatus</i> s.l.	2.00	9.94	1.00	0.25	0	44.50
<i>An. minimus</i> A†	0	0.06	0	0	0	0.25
<i>An. nivipes</i>	0.75	0.75	0.25	0	0	9.13
<i>An. pampanai</i> †	1.13	3.88	0	0.25	0	25.00
<i>An. peditaeniatus</i>	1.50	3.50	0	0	0	2.63
<i>An. philippinensis</i>	0.25	0.44	0	0	0	4.88
<i>An. sinensis</i>	0.25	0.63	0.50	0	0	0.25
<i>An. splendidus</i>	1.25	2.19	0.25	0	0	12.50
<i>An. tessellatus</i>	0.75	0.50	0.75	0	0	1.00
<i>An. vagus</i>	4.38	4.50	20.00	0.75	12.95	38.25
<i>An. varuna</i> †	0.13	0.13	0	0	0	8.25

* Mixture of *An. aconitus* and *An. varuna*: 4 *An. varuna* were found in a sample of 71 morphologically identified *An. aconitus*.

† Identification made on the basis of polymerase chain reaction–restriction fragment length polymorphism.

sporozoite ELISA, and it was implicated in a relatively low level of malaria transmission in a traditional tank-irrigation-based rice-producing village.^{12,13} The population in Binh Thuan Province of Vietnam, however, is highly zoophilic, and on the basis of this behavior, it cannot be considered as a malaria vector in that area of the country. Moreover, the circumsporozoite ELISA assay on *An. varuna* mosquitoes supports this, despite the small number of samples tested.

These contradictory observations could be explained by the misidentification of adult females, but the vector system in Southeast Asia is also very complex. In addition to the main vectors, *An. dirus* and *An. minimus*, a number of secondary vectors also occur in the region. The involvement of each of these secondary vector species in malaria transmission can differ from region to region.^{14–16}

Malaria is one of the major health problems in Binh Thuan Province, especially in the villages located in mountainous areas, which also includes the site where the mosquitoes were collected during the present study. Vector control is focused on 2 species, *An. dirus* and *An. minimus*. However, what was thought to be an unusual morphotype of *An. minimus* is now known to be *An. varuna*. Hence, a non-vector was considered to be a malaria vector. *Anopheles dirus*, which was found positive for *P. falciparum* circumsporozoite by ELISA, is clearly the main vector in this area. During the study, a number of species were encountered that are known to be secondary vectors in various areas of Southeast Asia. Of these, *An. aconitus*, *An. maculatus*, *An. sinensis*, and *An. vagus* are suspected secondary vectors in Vietnam, but none of these species has been implicated with certainty in malaria transmission in the country.¹⁷ Hence, the primary target for vector control in this region should be *An. dirus*.

The presence of *An. minimus*, although in small numbers, and the importance of the secondary vectors should not be neglected. In Sri Lanka, evidence exists for multispecies in-

volvement in transmission, where secondary vectors enhance malaria transmission that is mainly attributable to *An. culicifacies* species B.^{12,13} Furthermore, secondary vectors often gain in importance after environmental change or changes in human practices, and may contribute substantially to malaria transmission.^{12,18} It is questionable, however, whether these species can maintain malaria endemicity in the absence of principal vector species. Consequently, it can be hypothesized that one round of bed net impregnation, preceding the transmission season of *An. dirus*, should be enough to control malaria in the study area. But the exophagic behavior of this vector and the possibility of malaria transmission in the forest will complicate control practices, and other interventions outside the villages may be required to control transmission by *An. dirus*.

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