IDENTIFICATION OF *ANOPHELES MINIMUS* COMPLEX AND RELATED SPECIES IN VIETNAM

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Abstract. *Anopheles minimus* A and C and several closely related species of mosquitoes have been identified in Vietnam, where some have been implicated in malaria transmission. Morphological variation within and between *Anopheles minimus* A and C makes identification using alpha taxonomy difficult and several molecular techniques have been developed to separate them. However the difficulties of applying these techniques and the benefits of morphological identification in the field have seen morphological characteristics, such as the humeral pale spot on the costa, being used to separate these two species. In this study, the morphological and molecular examinations of 2,206 specimens collected in Vietnam indicate that pale scaling on the proboscis reliably separates *An. aconitus* from *An. minimus* s.l., but hind tarsal banding cannot separate *An. jeyporiensis* from *An. minimus* s.l., and the presence or absence of the humeral pale spot is not a reliable characteristic for differentiating *An. minimus* A from C due to variation of this characteristic in *An. minimus* C.

INTRODUCTION

Anopheles minimus complex consists of three sibling species currently designated A, C, and E (Green *et al*, 1990; Harbach, 2004). *Anopheles minimus* E is restricted to the non-malarious Ryukyu Archipelago, while *Anopheles minimus* A and C have a wide distribution from parts of India through Southeast Asia and into southern China. Throughout this range *An. minimus* A and C are believed to be responsible for malaria transmission. However, accurate information regarding vector competency, distribution and behavioral characters associated with malaria transmission is difficult to elucidate due to difficulties in morphologically separating the two species from each other and from the other closely related species: *Anopheles aconitus*, *Anopheles jeyporiensis*, *Anopheles varuna*, *Anopheles culicifacies* and *Anopheles pampani*. For example, Van Bortel *et al* (2001) recently described the misidentification of the zoophilic species *An. varuna* as the vector species *An. minimus* A in Vietnam.

To resolve the problem of morphological identification, various molecular techniques have been developed for separating *An. minimus* s.l. and closely related species (Green *et al*, 1990; Van Bortel *et al*, 1999, 2000; Phuc *et al*, 2003). While the results from these methods are unequivocal, these are laboratory based techniques requiring preservation and transportation of material from the field and then time consuming processing through sophisticated and expensive equipment. This could be greatly simplified if reliable morphological markers could be found which would allow identification in the field. Unfortunately, this group of mosquitoes appears to be highly variable in regard to morphology (Harrison,

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1980; Jaichapor *et al*, 2005). Despite this, the convenience and expediency of identifying specimens in the field, has promoted the use of possibly unreliable characteristics or at best ones of only regional significance. For *An. minimus* s.l., the presence or absence of the humeral pale spot (HP) and the presector pale spot (PSP) on the costa have been used to separate A and C (Sucharit *et al*, 1988; Sharpe, 1999; Rwegoshora *et al*, 2002; Garros *et al*, 2005). These authors suggest that the presence of a HP spot is a reliable indicator of *An. minimus* C and if absent of *An. minimus* A. But there is some discourse among workers in regard to the reliability of this characteristic (Green *et al*, 1990; Van Bortel *et al*, 1999; Chen *et al*, 2002). Green *et al* (1990) working with specimens in Thailand estimated a 37% error rate in separating *An. minimus* A and C based on the absence or presence of the HP. Jaichapor *et al* (2005) looked at a large series of *An. minimus* A and showed that only 4.11% of 1,715 specimens studied had a HP indicating that this characteristic might reliably separate this species, however a study on similar numbers of *An. minimus* C has not yet been conducted to confirm this.

In this study we examined a series of *An. minimus* s.l., *An. aconitus* and *An. jeyporiensis* from Vietnam to see if the morphological characteristics currently advocated can separate these species.

MATERIALS AND METHODS

Anopheline mosquitoes were collected from villages in Truong Xuan Commune, Quang Binh Province, north-central Vietnam. Collections were made during wet (September - November) and dry (April - May) seasons from 2004 - 2007 using human, cattle and buffalo baits.

Specimens were identified using the national key, - Identification Key for *Anopheles* in Vietnam (National Institute of Malariology, Parasitology and Entomology, 1987). Proboscis morphology (the presence or absence of pale scaling on the distal end of the proboscis) was used to separate *An. aconitus* from *An. minimus* s.l. and hind tarsal bands were used to separate *An. jeyporiensis* from *An. minimus* s.l.. For specimens identified as *An. minimus* s.l., wing morphology was recorded for the presence or absence of: the humeral pale (HP), the presector pale (PSP), the accessory sector pale (ASP) and the median pale spot on the media 1 vein (M_1). Following morphological examination all specimens were identified using molecular characteristics by PCR-RFLP. DNA was extracted from each specimen using the methods of Beebe *et al* (1999). The Internal Transcribed Spacer 2 region of the ribosomal DNA was amplified using the primers and methods of Beebe and Saul (1995) and the product was digested and electrophoresed using the technique of Van Bortel *et al* (2000).

RESULTS

A total of 2,206 specimens of *An. minimus* s.l. and two closely related species, *An. aconitus* and *An. jeyporiensis*, were collected. Of these, 1,034 were identified as *An. minimus* s.l., 1,152 as *An. aconitus* and 20 as *An. jeyporiensis* by morphology. By PCR-RFLP, the 1,034 specimens identified as *An. minimus* s.l. by morphology were identified as 15 specimens of *An. minimus* A, 1,017 of *An. minimus* C, one of *An. aconitus* and one of *An. jeyporiensis*. Of the 1,152 specimens identified as *An. aconitus* by morphology, 1,150 were *An. aconitus*, one was *An. minimus* A and one was *An. minimus* C by PCR-RFLP. Of the 20 specimens indentified as *An. jeyporiensis* by morphology, 19 were found to be *An. minimus* C and one was *An. jeyporiensis* by PCR-RFLP.

Of the 1,017 *An. minimus* C identified by PCR-RFLP, 132 were too damaged to provide

any reliable wing morphological characteristics, of the remaining specimens, 885 provided information on the HP and PSP and 723 and 615 provided information on the ASP and M_1, respectively (Table 1). Of the 15 *An. minimus* A identified by PCR-RFLP, 4 were too damaged to be included in the wing morphology analysis (Table 1).

DISCUSSION

Harrison (1980), in a comprehensive study of *Anopheles* in the Myzomyia Series, stated in his key to the Thailand species that both *An. minimus* and *An. aconitus* have pale scales on the distal half of the proboscis and cited Christopher as concluding that such scales on the proboscis are a reliable characteristic for identifying *An. minimus*. However, Harrison (1980) found that 1.3% and 6.1% of *An. minimus* specimens from Hong Kong and Thailand, respectively, had pale scales on the proboscis. From these observations and the raising of the subspecies *An. minimus flavirostris* to species status, which typically has pale scaling on the proboscis Harrison (1980) believed this characteristic needs to be reassessed and suggests that pale scaling on the proboscis may be uncommon in *An. minimus*. In our study, 99.9% (1,151/1,152) of specimens that had pale scaling on the distal half of the proboscis were *An. aconitus*; this characteristic was found in only 0.1% (1/1,017) of *An. minimus* C specimens and 6.6% (1/15) of *An. minimus* A specimens. Though the numbers of *An. minimus* A examined were few, they indicate that pale scaling on the distal part of the proboscis can reliably separate *An. aconitus* and *An. minimus* s.l. There was difficulty in separating *An. jeyporiensis* and *An. minimus* s.l. using the pale bands on the tarsi as indicated in the national key; of the 20 *An. jeyporiensis* specimens identified by morphology, 19 (95%) were found to be *An. minimus* C by PCR-RFLP.

A number of authors have found the presence or absence of HP can be used to separate *An. minimus* A and C. Jaichapor *et al* (2005) looked at a large number (*n*=1,715) of *An. minimus* A specimens and found that 95.9% lacked the HP; other researchers have also found a similar absence in the HP

Table 1
Wing characteristics present in *An. minimus* C and *An. minimus* A whose identification was confirmed by PCR-RFLP.

Wing characteristic	On at least one wing	
	n	%
An. minimus C		
Humeral pale spot (HP)	592/885	66.9
Presector pale spot (PSP)	788/885	89.0
Accessory sector pale spot (ASP)	72/723	10.0
Medium pale spot on M_1 vein (M_1)	132/615	21.5
An. minimus A		
Humeral pale spot (HP)	1/11	9.1
Presector pale spot (PSP)	10/11	90.9
Accessory sector pale spot (ASP)	5/11	45.5
Medium pale spot on M_1 vein (M_1)	7/11	60.0

in *An*. *minimus* A; 95% lacked the HP in a study by Green *et al* (1990) and 91% lacked the HP in a study by Sharp (1997). In our study 33.1% of *An*. *minimus* C specimens lacked the HP. In north-central Vietnam 33.1% of *An*. *minimus* C specimens could be misidentified as *An*. *minimus* A if we rely on the absence of the HP. This level of misidentification is unacceptably high. Similar error rates for *An*. *minimus* C using this characteristic are cited by Green *et al* (1990) (22%) and Sharp (1997) (37%) for specimens collected in Thailand. Van Bortel *et al* (1999) using isozyme electrophoresis detected *An*. *minimus* A and C species in northern Vietnam and found the absence of the HP was common (99%) in *An*. *minimus* A and common (92%) in *An*. *minimus* C. The variable nature of the HP in *An*. *minimus* C precludes it from being an effective marker in separating *An*. *minimus* A from C.

Other characteristics examined appeared to be no more informative. The presence of the PSP was common in both *An*. *minimus* A and C. Jaichapor *et al* (2005) found that 74.8% of their *An*. *minimus* A specimens had this characteristic, while our study found this characteristic in 89.0% of *An*. *minimus* C specimens. With regards to the presence of the medium pale spot on the M_1, 11.2% of *An*. *minimus* A specimens showed this characteristic in a study by Jaichapor *et al* (2005) while we found it in 21.5% of *An*. *minimus* C specimens.

In Vietnam molecular techniques are now being used to validate the presence and distribution of *An*. *minimus* s.l. and closely related species (Van Bortal *et al*, 2000, 2001; Phuc *et al*, 2003). *An*. *minimus* A is common and wide spread throughout Vietnam, while *An*. *minimus* C is usually found only in the North, above 18ºN. Garros *et al* (2005) recently found it in the southern Vietnamese province of Khanh Hoa (12º14´N) where it appears to be replacing *An*. *minimus* A. *An*. *aconitus* appears to be common and widespread, while *An*. *varuna*, *An*. *jeyporiensis* and *An*. *pampani* are uncommon with patchy distributions. At our field site (17º 15´N) the dominant species during the wet and dry seasons over the past four years were *An*. *minimus* C (1,017/1,034, 98.3%) and *An*. *aconitus* (1,150/1,152, 99.8%) with few *An*. *minimus* A and *An*. *jeyporiensis* specimens.

Our study found that in Vietnam the morphological characteristics used in the identification key for *Anopheles* in Vietnam (National Institute of Malariology, Parasitology and Entomology, 1987) reliably identified *An*. *aconitus* but not *An*. *jeyporiensis*. For *An*. *minimus* s.l. the use of the HP should be used with caution to separate *An*. *minimus* A and C due to the variation of this characteristic in *An*. *minimus* C.

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