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 Anoph- eles 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 spe-

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1980; J aichapor 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. J aichapor 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 (M1). 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 M₁, 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>
<thead>
<tr>
<th>Wing characteristic</th>
<th>An. minimus C</th>
<th>An. minimus A</th>
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<tr>
<td>Humeral pale spot (HP)</td>
<td>592/885 66.9%</td>
<td>1/1 9.1%</td>
</tr>
<tr>
<td>Presector pale spot (PSP)</td>
<td>788/885 89.0%</td>
<td>10/11 90.9%</td>
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<tr>
<td>Accessory sector pale spot (ASP)</td>
<td>72/723 10.0%</td>
<td>5/11 45.5%</td>
</tr>
<tr>
<td>Medium pale spot on M₁ vein (M₁)</td>
<td>132/615 21.5%</td>
<td>7/11 60.0%</td>
</tr>
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</table>

**Table 1**

Wing characteristics present in An. minimus C and An. minimus A whose identification was confirmed by PCR-RFLP.
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. J aichapor 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 M1, 11.2% of An. minimus A specimens showed this characteristic in a study by J aichapor 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 widespread throughout Vietnam, while An. minimus C is usually found only in the North, above 18°N. Garros et al (2005) recently found it in the southern Vietnamese province of Khanh Hoa (12°14’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 (17°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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