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# Identification of two species within the *Anopheles minimus* complex in northern Vietnam and their behavioural divergences

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**Summary** Elucidating the complex taxonomic status of the major malaria vector taxa and characterising the individual species within each complex is important for understanding the complexity of the vector system in the south-east Asian region and will allow to estimate the impact of vector control measures. This applies to countries such as Laos, Cambodia and Vietnam that spend about 60% of their malaria control budget on implementing vector control activities. We used isozyme electrophoresis to clarify the *Anopheles minimus s.l.* species composition in northern Vietnam and identify behavioural divergences of individual species. Using different collection methods, adult mosquitoes were caught at monthly intervals from June to November 1995 in four villages. *An. minimus s.l.* could be distinguished from closely related species, *An. aconitus* and *An. jeyporiensis*, at the Octanol dehydrogenase (*Odh*) enzyme locus. Significant positive $F_{is}$ values gave clear evidence of nonrandom mating within the *An. minimus s.l.* population. The highest heterozygote deficiency was observed at locus *Odh*, which was diagnostic for 2 sympatric *An. minimus* species in Vietnam similar to the *An. minimus* A and C species known from Thailand. We found no evidence for restricted gene flow between monthly samples, villages, or collection methods in either of the two *An. minimus* species. They occurred in sympatry, but in different proportions depending on the collection site, and had dissimilar resting and biting behaviours. Thus a vector control strategy will have a nonuniform effect on the various components of this diverse vector system.

**keywords** Myzomia Series, *Anopheles minimus*, species complex, isozyme electrophoresis, population genetics, malaria control, South-east Asia, Vietnam

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## Introduction

The original geographical distribution of the malaria vector *Anopheles minimus sensu lato* (subgenus *Cellia*, Myzomya Series, Minimus Group) extended from Uttar Pradesh in India eastwards to South-east Asia, China, Taiwan and Japan (Harrison 1980). The species seems to have disappeared from Nepal and parts of India (Parajuli *et al*. 1981) and has become rare in Taiwan (Lien 1991). Its disappearance from these regions is primarily attributed to the use of DDT residual spaying. In other countries of Asia, the response of *An. minimus* to insecticide use was heterogeneous. In the plain regions of Thailand, where rice is cultivated, *An. minimus s.l.* was highly endophilic and responded favourably to residual spraying while in forested hilly and cleared forested foothill areas, malaria eradication projects faced difficulties in interrupting transmission. Poor results were attributed to variations in the behaviour of *An. minimus s.l.* and to the important role of *An. dirus* as a malaria vector (Ismail *et al*. 1974). In Vietnam and Burma, DDT pressure induced the selection of exophilic *An. minimus* populations (Myo Paing *et al*. 1988; Vu Thi Phan 1996). Several authors (Ismail *et al*. 1974; Suthas *et al*. 1986) suspected the occurrence of at least two cryptic species within *An. minimus* to explain the heterogeneous responses to insecticides. This was confirmed by genetic studies of *An. minimus s.l.* from Thailand (Sucharit *et al*. 1988; Green *et al*. 1990). Two species within the *An. minimus* complex are commonly accepted,

informally designated *An. minimus* A and C (Harbach 1994), and defined as electromorphs controlled by the enzyme locus Octanol dehydrogenase (Green *et al.* 1990). Apart from the genetic studies from Thailand, and some studies of specimens from Japan and China (Kanda *et al.* 1984; Yu Yuan in Zahar 1996) little is known of this complex in other parts of South-east Asia. The differences between the two species regarding their biting and resting behaviour, degree of anthropophily, longevity and ecological differences have hardly been studied. These factors determine the efficiency of a species as a vector.

Elucidating the nature of malaria vector species complexes and characterising the individual species within each complex will provide insight into the complexity of the vector systems in South-east Asia and assessments of the impact of vector control measures (Coluzzi 1992). This is important for countries such as Laos, Cambodia and Vietnam that spend about 60% of their malaria control budget on vector control.

We studied *An. minimus s.l.* from northern Vietnam using isozyme electrophoresis to clarify its species composition in this part of South-east Asia. Variations in biting and indoor resting behaviour in relation to population genetic findings were examined. Misidentification using morphological characteristics to separate *An. minimus s.l.* from closely related species is common (Harrison 1980). Therefore we searched for diagnostic loci for two other species, *An. aconitus* and *An. jeyporiensis*, which also occur in northern Vietnam.

## Materials and methods

### Study area

Mosquitoes were collected in four villages of Hoa Binh Province in northern Vietnam, located south-west of Hanoi. The area is characterized by plains, narrow valleys (< 100 m), hills and mountains. Annual rainfall, mainly from May to October, is between 1500 and 2500 mm. Average monthly temperatures range from 15 °C in the winter to about 30 °C in the summer. Humidity is very high all year round, rarely lower than 80%. Four collection sites were selected. Rong Vong village is situated on the border between two ecological systems, the Red River Delta plain and a hilly area. The site is located 40 km from Hanoi at an altitude of 30 m. Houses are built directly on the ground or on stilts not higher than 0.5 m, and separated cattle sheds are present. The villages of Co Phay and Xolo are situated at altitudes of 400 m and 380 m, respectively. Houses are built on stilts and cattle are kept under the houses at night. Co Phay and Xolo are surrounded by hills up to 600 m high. The fourth village, Khoi, is situated in a large U-shaped valley at an altitude of 330 m. Houses are comparable with those found in Co Phay and Xolo. Rice is cultivated around all four villages and houses are located near possible *An. minimus* breeding places. Khoi has a complex system of fishponds and rivers.

Malaria reached alarming proportions in Vietnam in 1991 and became a priority for the government (Verlé *et al.* 1998). In Hoa Binh Province transmission is currently low, but malaria epidemics remain a constant threat because people travel between this region and endemic areas, and potential malaria vectors are ubiquitous. During the last three years preceding our collections, no vector control measures had been applied in the study villages.

### Mosquito collections

Adult mosquitoes were caught at monthly intervals from June to November 1995. Night landing collections on humans were done inside and outside three selected houses during three consecutive nights from 1800 h until 0600 h. Morning collections of indoor resting mosquitoes took place on two consecutive days in different houses from those selected for the human night landing collections. Adult mosquitoes were also captured in the vicinity of cattle by two persons for two consecutive nights from 2100 h until 2400 h.

### Morphological identification

Mosquitoes were identified morphologically using the identification key developed by the Institute of Malariology, Parasitology and Entomology (IMPE 1987). Specimens belonging to *An. minimus s.l.* were scored for presence or absence of humeral and presector pale spots on the wings and separated into three morphotypes (Table 1). This morphological variation is known to occur in *An. minimus s.l.* from Vietnam (Trung Ho Dinh, unpublished observation) and in *An. minimus* populations from Thailand (Sucharit *et al.* 1988). We performed tests to determine if any relation exists between the 3 morphotypes and results of the population genetic analysis based on the isozyme electrophoresis. *An. minimus s.l.*, *An. aconitus*, and *An. jeyporiensis* specimens were put into liquid nitrogen immediately after morphological identification.

**Table 1** Definition of each morphotype, based on the presence or absence of the humeral and presector pale spots on the wings, and the percentage of each morphotype found in each of 2 different *Odh* forms of *Anopheles minimus*

| Morphotype | Presector pale spot | Humeral pale spot | *Odh* form I | | *Odh* form II | |
|---|---|---|---|---|---|---|
| | | | % | Number | % | Number |
| 1 | Present | Absent | 89 | 529 | 62 | 197 |
| 2 | Absent | Absent | 10 | 60 | 30 | 96 |
| 3 | Present | Present | 1 | 6 | 8 | 26 |

**Table 2** Enzyme systems and migration conditions for cellulose acetate electrophoresis adapted to three taxa of the Myzomia Series, in northern Vietnam

| Enzyme abbreviation | Enzyme name | E.C. number | Number of loci | Buffer* | Application position | Time† (mins) |
|---|---|---|---|---|---|---|
| AAT | Aspartate aminotransferase | 2.6.1.1 | 2 | III-SP | Cathodal | 30 |
| | | | | III-SP | Central | 36 |
| ACP | Acid phosphatase | 3.1.3.2 | 1 | D | Cathodal | 36 |
| GPI | Glucose-phosphate isomerase | 5.3.1.9 | 1 | B | Cathodal | 24 |
| α-GPD | α-Glycerophosphate dehydrogenase | 1.1.1.8 | 1 | I-SP | Cathodal | 18 |
| HADH | Hydroxy-acid dehydrogenase | 1.1.99.6 | 1 | Morph | Cathodal | 30 |
| IDH | Isocitrate dehydrogenase | 1.1.1.42 | 2 | I-SP | Cathodal | 24 |
| LDH | Lactate dehydrogenase | 1.1.1.27 | 1 | D | Cathodal | 36 |
| MDH | Malate dehydrogenase | 1.1.1.37 | 1 | HR | Central | 30 |
| ME | Malic enzyme | 1.1.1.40 | 1 | III-SP | Cathodal | 18 |
| MPI | Mannose-phosphate isomerase | 5.3.1.8. | 1 | I-SP | Cathodal | 24 |
| ODH | Octanol dehydrogenase | 1.1.1.73 | 1 | I | Cathodal | 36 |
| 6-PGD | 6-Phosphogluconate dehydrogenase | 1.1.1.44 | 1 | I-SP | Cathodal | 30 |
| PGM | Phosphoglucomutase | 2.7.5.1 | 1 | XVIII-SP | Cathodal | 18 |
| SOD | Superoxide dismutase | 1.15.1.1 | 1 | visible on ODH gels | | |

* Buffer B, 0.020 M Phosphate, pH 7.0; D, 0.015 M Tris-EDTA-borate $MgCl_2$, pH 7.8; I, 0.025 Tris-glycine, pH 8.5 (Richardson *et al.* 1986); I-SP, 0.155 M Tris-0.043 M Citric acid, pH 7.0; III-SP, 0.500 M Tris-versene-borate, pH 8; XVIII-SP, 0.100 M Tris-0.100 maleic-0.010 M EDTA, pH 7.4 (Shaw & Prasad 1970); HR, Electra HR buffer (Helena Laboratories); Morph, 0.040 M Citric Acid-morpholine, pH 6.1 (Clayton & Tretiak 1972). †Electrophoretic running time in minutes.

### Isozyme electrophoresis

Isozyme electrophoresis of 14 enzymes on cellulose acetate gels (Titan III, Helena Laboratories, U.K.) followed procedures described by Smits *et al.* (1996). Enzymes and migration conditions were adapted to the *Anopheles* mosquitoes of Vietnam (Table 2). LDH (Lactate dehydrogenase, E.C. number 1.1.1.27) and ODH (Octanole dehydrogenase, E.C. number 1.1.1.73) were tested on concentrated samples because of their low activity (Smits *et al.* 1996). The banding patterns of SOD (Superoxide dismutase, E.C. number 1.15.1.1) were visible on the ODH gels.

### Data analysis

Mosquitoes collected in one village during one round by the different collection methods counted as a sample set (Richardson *et al.* 1986). *An. aconitus* and *An. jeporiensis* can be identified morphologically when the classical characteristics are present. Mosquitoes with these diagnostic characteristics were used as standards for the detection of diagnostic loci separating *An. minimus s.l.*, *An. aconitus* and *An. jeyporiensis*. These loci were used to withdraw misclassified *An. aconitus* and *An. jeyporiensis* from the sample sets of morphologically identified *An. minimus s.l.*

More than 2 species might exist within the *An. minimus* complex (Zahar 1996), therefore no attempt was made to separate *a priori* the *An. minimus s.l.* sample sets by the criteria of Green *et al.* (1990). The null hypothesis that random mating occurs within the morphologically identified *An. minimus s.l.* was tested by means of the *F*-statistics ($F_{is}$, $F_{it}$ and $F_{st}$) (Wright 1951), which enable the measurement of deviations from Hardy–Weinberg expectations at different levels. Any heterozygote deficit is classed into within and among population components. $F_{is}$ is a measure of the 'within population' heterozygote deficit while $F_{st}$ is a measure of the 'among populations' heterozygote deficit. $F_{it}$ measures the global heterozygote deficit. This partition of the heterozygote deficit permits inferences about the levels of inbreeding and gene flow of the populations under investigation (Goudet *et al.* 1994). The computer program FSTAT (Goudet 1995) was used to obtain estimates of $F_{is}$, $F_{it}$ and $F_{st}$, based on the hierarchical analysis of variance developed by Weir and Cockerham (1984), which explicitly accounts for sample size. The 95% confidence intervals (95CI) of the overall *F*-statistics were obtained by bootstrapping over loci (Goudet 1995). Significance of the *F*-statistics was tested with the method of permutations (10000 perms per test) (Goudet 1995).

Deviations from the Hardy–Weinberg expectation of the individual loci in each sample set were tested by an exact test using the program GENEPOP (Raymond & Rousset 1995). This test is not adversely affected by small expected values and appropriate for small sample sets or when rare alleles are present (Lessios 1992; Rousset & Raymond 1995).

Nei's unbiased genetic distance (Nei 1978), which evaluates genetic similarities between populations, was calculated using

BIOSYS (Swofford & Selander 1981). To avoid type-1 errors resulting from multiple simultaneous tests, significance levels were adjusted through sequential Bonferroni procedures (Hochberg 1988; Lessios 1992).

## Results

### The Myzomya Series: species identification

*An. minimus s.l.*, *An. aconitus* and *An. jeyporiensis*, belonging to the Myzomya Series, were collected in northern Vietnam. Two loci were found for MDH by cellulose acetate electrophoresis. Only *Mdh*-2, migrating cathodally on the cellulose acetate gels, displayed clearly interpretable band patterns. It could not separate the 3 taxa unambiguously. However, the *Mdh*-$2^{100}$ allele could be considered typical for *An. minimus s.l.*, whereas the *Mdh*-$2^{135}$ allele was typical for the other two species. Only 1.2% (12/996) of *Mdh*-$2^{100/135}$ heterozygotes were found in samples of the morphological identified *An. minimus s.l.* population. The *Odh* electromorphs were diagnostic for the three taxa. Four different *Odh* alleles were found in the *An. minimus s.l.* population: $Odh^{100, 118, 133, 142}$. The $Odh^{87}$ allele was diagnostic for *An. aconitus* and the $Odh^{56, 73, 80}$ alleles were found in the morphologically identified *An. jeyporiensis*. Using the *Odh* and *Mdh*-2 loci, 3% (31/996) of the specimens were misidentified and withdrawn from the morphologically identified *An. minimus s.l.* sample.

### *An. minimus s.l.*: inter- and intra-specific variability

Eleven enzymes were suitable for further analysis. *Mpi* was excluded from analysis because interpretation of the zymograms was unreliable due to the high number of alleles. The *Sod* and *Me* loci were monomorphic and not used in the analysis of *An. minimus s.l.* Frequencies of the following alleles were pooled because they were difficult to distinguish when not on the same gel: *Aat*-$1^{104}$ with *Aat*-$1^{100}$, *Acp*$^{112}$ with *Acp*$^{100}$ and 6-*Pgd*$^{104}$ with 6-*Pgd*$^{100}$ and 6-*Pgd*$^{96}$.

Table 3 gives the number of morphologically identified *An. minimus s.l.* per sample set. Those smaller than 5 were excluded from the analysis. The overall estimates (over sample sets and loci) of the *F*-statistics of *An. minimus s.l.* showed positive values of $F_{it}$, $F_{st}$, and $F_{is}$ (Table 4, criterion 1) significantly different from zero. The significant positive value of $F_{is}$ denoted that nonrandom mating occurred within the sample sets, whereas the $F_{st}$ value indicated a high heterozygote deficiency between the sample sets of the *An. minimus s.l.* population. Among the 13 enzyme loci $F_{is}$ and $F_{st}$ values were highest for *Odh* (Table 5, criterion 1).

Four different *Odh* alleles were found in the morphologically identified *An. minimus s.l.* Based on the frequencies of heterozygotes, two distinct groups could be identified: (1) *Odh* form I with homozygotes $Odh^{100/100}$ and heterozygotes $Odh^{100/118}$; (2) *Odh* form II including genotypes $Odh^{142/142}$, $Odh^{133/142}$, $Odh^{133/133}$ and $Odh^{118/133}$ with $Odh^{133/133}$ as predominant genotype. Eight heterozygotes between form I and form II, 1 $Odh^{100/142}$ and 7 $Odh^{100/133}$ (0.88% of total collection), were collected during this study. All were collected in Khoi, six of them in October. No specimens with genotypes $Odh^{118/142}$ or $Odh^{118/118}$ were caught. The number of mosquitoes per *Odh* form, and per sample set is shown in Table 3.

Dividing each sample set by *Odh* forms, excluding the heterozygotes $Odh^{100/142}$ and $Odh^{100/133}$, reduced the $F_{is}$ value to 0.034 (Table 4, criterion 2) but it was still significantly different from zero. Analysing the two populations of the *An. minimus Odh* forms separately showed that only form II had significant positive $F_{is}$ and $F_{it}$ values (Table 4, criteria 3 and 4).

**Table 3** Number of mosquitoes per sample set of *Anopheles minimus* collected in northern Vietnam in 1995

| | | Number of *Anopheles minimus* | | |
|---|---|---|---|---|
| Village | Month | *sensu lato** | *Odh* form I† | *Odh* form II† |
| Co Phay | June | 37 | 23 | 9 |
| | July | 10 | 8 | 1 |
| | August | 1 | 1 | 0 |
| | September | 2 | 2 | 0 |
| | October | 19 | 18 | 0 |
| | November | 7 | 6 | 0 |
| Xolo | June | 65 | 50 | 12 |
| | July | 32 | 21 | 9 |
| | August | 6 | 4 | 2 |
| | September | 4 | 1 | 3 |
| | October | 33 | 4 | 29 |
| | November | 9 | 1 | 8 |
| Rong Vong | June | 123 | 118 | 0 |
| | July | 95 | 93 | 1 |
| | August | 36 | 34 | 0 |
| | September | 30 | 29 | 0 |
| | October | 22 | 17 | 1 |
| | November | 47 | 43 | 2 |
| Khoi | June | 57 | 23 | 32 |
| | July | 55 | 23 | 31 |
| | August | 98 | 41 | 52 |
| | September | 25 | 6 | 18 |
| | October | 142 | 27 | 105 |
| | November | 7 | 2 | 4 |
| Number of sample sets > 5 | | 21 | 17 | 10 |

Sample sets smaller than 5 were excluded from the analysis. *Morphologically identified *An. minimus*, not including initially misidentified *An. aconitus* and *An. jeyporiensis*. †Not all morphologically identified *An. minimus* specimens could be scored for the *Odh* locus. Also heterozygotes $Odh^{100/142}$ and $Odh^{100/133}$ were excluded.

**Table 4** Overall estimates of *F*-statistics over 13 gene loci for collections of *Anopheles minimus* from northern Vietnam. Each criterion, except criterion 1, defined an additional partition of each sample set. Tests of significance were performed by permutations, 95% confidence interval (95%CI) by bootstrapping over loci

| Criterion | Number of sample sets | $F_{it}$ | (95%CI) | $F_{st}$ | (95%CI) | $F_{is}$ | (95%CI) |
|---|---|---|---|---|---|---|---|
| *An. minimus sensu lato*[1] | 21 | 0.278** | (0.052–0.572) | 0.115** | (0.005–0.262) | 0.185** | (0.044–0.421) |
| Divided by *Odh* form | 27 | 0.275** | (0.032–0.580) | 0.250** | (0.008–0.565) | 0.034* | (0.005–0.062) |
| *Odh* form I only | 17 | 0.020 | (−0.003–0.038) | 0.002 | (−0.001–0.006) | 0.018 | (−0.006–0.037) |
| *Odh* form II only | 10 | 0.058* | (0.017–0.118) | −0.003 | (−0.006–0.002) | 0.061* | (0.020–0.118) |

[1] Morphologically identified *An. minimus*, excluding misidentified specimens of *An. aconitus* and *An. jeyporiensis*.* $P < 0.01$; ** $P < 0.001$.

**Table 5** $F_{is}$ and $F_{st}$ values per locus for *Anopheles minimus* from northern Vietnam. Each criterion, except criterion 1, defined an additional partition of each sample set (criteria identical to Table 4). Tests of significance were performed by permutations

| | Criterion 1. *sensu* lato† | | 2. divided by *Odh* form | | 3. *Odh* form I | | 4. *Odh* form II | |
|---|---|---|---|---|---|---|---|---|
| Locus | $F_{is}$ | $F_{st}$ | $F_{is}$ | $F_{st}$ | $F_{is}$ | $F_{st}$ | $F_{is}$ | $F_{st}$ |
| *Aat-1* | 0.007 | 0.014* | −0.019 | 0.033* | −0.015 | 0.011 | −0.038 | 0.003 |
| *Aat-2* | 0.033 | −0.004 | 0.042 | −0.003 | 0.032 | 0.000 | 0.056 | −0.007 |
| *Acp* | 0.073 | 0.025* | 0.048 | 0.057* | 0.044 | 0.000 | 0.053 | −0.007 |
| (α– *Gpd* | 0.458* | 0.003 | 0.004 | −0.007 | 0.004 | −0.006 | 0.005 | −0.011 |
| *Gpi* | 0.159* | 0.005 | 0.125* | 0.004 | 0.048 | −0.003 | 0.306* | 0.013 |
| *Hadh* | 0.125* | 0.029* | 0.096 | 0.044* | −0.006 | 0.000 | 0.120 | −0.001 |
| *Idh-1* | 0.236* | 0.009 | 0.064 | 0.009 | −0.008 | 0.001 | 0.144 | 0.018 |
| *Idh-2* | 0.052 | 0.001 | −0.002 | 0.002 | −0.016 | −0.002 | −0.029 | 0.002 |
| *Ldh* | 0.091 | 0.011 | 0.095 | 0.010 | 0.032 | 0.001 | 0.497* | −0.005 |
| *Mdh-2* | 0.083 | 0.009 | −0.011 | 0.004 | −0.011 | 0.002 | −0.012 | 0.011 |
| *Odh* | 0.806* | 0.425* | −0.007 | 0.908* | monomorph | − | −0.007 | −0.011 |
| *6-Pgd* | 0.049 | 0.004 | 0.051 | 0.003 | 0.042 | 0.004 | 0.076 | −0.010 |
| *Pgm* | −0.025 | −0.003 | −0.027 | −0.001 | −0.027 | −0.003 | −0.028 | 0.001 |

†Morphological identified *An. minimus*, excluding misidentified specimens of *An. aconitus* and *An. jeyporiensis*. * Indicates significant values for sequential Bonferroni adjusted significance levels.

No population structuring could be inferred for either *An. minimus* form because of the nonsignificant $F_{st}$ values.

Mean $F_{is}$, $F_{it}$ and $F_{st}$ values of *An. minimus* form I were not significantly different from zero (Tables 4 and 5, criterion 3). In the 17 sample sets of form I, none of the polymorphic loci, defined as a locus whose most common allele has a frequency of 99% or lower, showed significant deviation from Hardy–Weinberg expectations. The per-locus $F_{is}$ of *Odh* form II ranged from − 0.038–0.497, and significant $F_{is}$ values were observed for the *Ldh* and *Gpi* loci (Table 5, criterion 4). These loci deviated significantly from Hardy–Weinberg expectations in sample set Khoi-October. Table 6 shows the frequencies of the most common allele per locus and $F_{is}$ values of 13 enzyme loci for the pooled sample of *An. minimus* forms I and II.

### Genetic distance between the species of the *minimus* group

Based on the 13 enzyme loci, Nei's unbiased genetic distance between the two *An. minimus* forms from Hoa Binh was 0.092. The genetic distance between *An. aconitus* and *An. minimus* forms I and II was 0.649 and 0.671, respectively.

### Characterising the forms of *An. minimus*

All three morphotypes were found in both *An. minimus* forms (Table 1). The relative importance of the morphotypes in the collections of both forms differed significantly between the monthly catches (exact test $P < 0.001$ for both forms). In collections of form I, morphotype 1 decreased significantly from June to November ($\chi^2$ for Trend; $P < 0.001$). Neither the

**Table 6** Number of alleles per loci, frequency of the most common allele per locus and $F_{is}$ per locus for the pooled sample of each *Anopheles minimus Odh* form. Tests of significance were performed by permutations

| | *Odh* form I | | | *Odh* form II | | |
|---|---|---|---|---|---|---|
| Locus | Number of alleles per locus | Frequency of most common allele | $F_{is}$ | Number of alleles per locus | Frequency of most common allele | $F_{is}$ |
| *Aat-1* | 6 | 0.828 | −0.005 | 8 | 0.928 | −0.036 |
| *Aat-2* | 5 | 0.966 | 0.031 | 5 | 0.957 | 0.053 |
| *Acp* | 5 | 0.760 | 0.039 | 5 | 0.516 | 0.048 |
| α–*Gpd* | 3 | 0.997 | −0.002 | 3 | 0.993 | −0.004 |
| *Gpi* | 3 | 0.947 | 0.045 | 4 | 0.958 | 0.320* |
| *Hadh* | 5 | 0.990 | −0.006 | 3 | 0.909 | 0.121 |
| *Idh-1* | 5 | 0.988 | −0.007 | 5 | 0.979 | 0.157 |
| *Idh-2* | 4 | 0.953 | −0.017 | 5 | 0.957 | −0.028 |
| *Ldh* | 4 | 0.948 | 0.032 | 3 | 0.985 | 0.495* |
| *Mdh-2* | 3 | 0.988 | −0.009 | 3 | 0.995 | −0.003 |
| *Odh* | 2 | 0.999 | 0.000 | 3 | 0.931 | −0.016 |
| *6-Pgd* | 8 | 0.892 | 0.046 | 6 | 0.926 | 0.068 |
| *Pgm* | 8 | 0.938 | −0.029 | 6 | 0.930 | −0.028 |

* Indicates significant values for sequential Bonferroni adjusted significance levels.

humeral pale spot nor the presector pale spot could be used to discriminate between the two *An. minimus* forms (Table 1). Based on the polymorphic loci, there was no heterozygote deficit among morphotypes within each *An. minimus* form (form I: $F_{st} = -0.002$, $P = 0.699$; form II: $F_{st} = -0.001$, $P = 0.513$).

Both *An. minimus* forms were found in sympatry in the four study villages of Hoa Binh. Form I was omnipresent

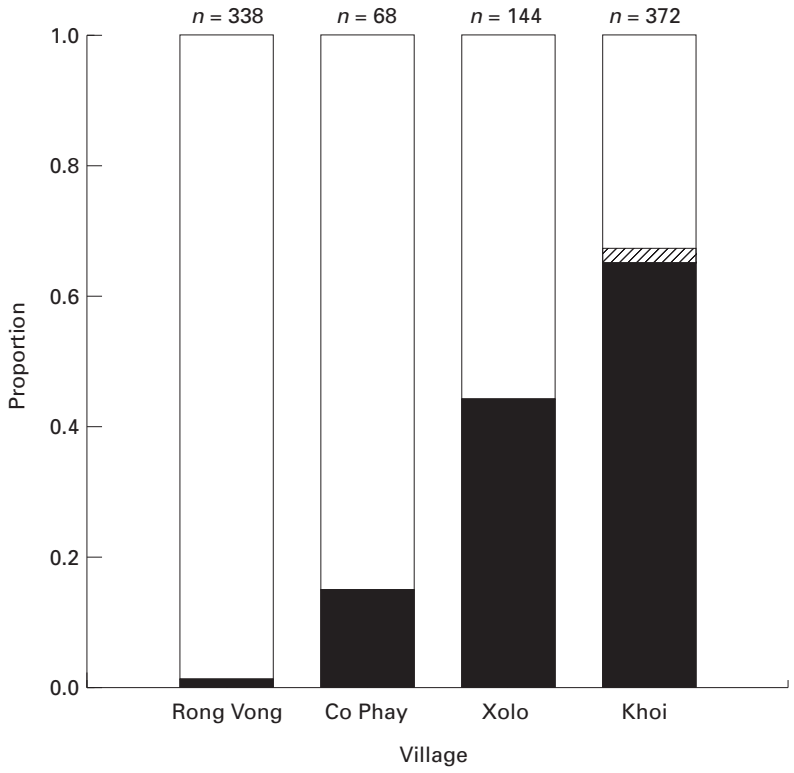


**Figure 1** Geographical distribution of *An. minimus* forms I and II collected during the study period: proportion of the two forms per village. □ Form I; ▨ Form I/II; ■ Form II.

while form II was mainly collected in Xolo and Khoi and almost absent in Rong Vong (Figure 1). The distribution of both forms was not identical across collection months in Xolo (exact test $P < 0.001$) and Khoi (exact test $P = 0.001$). Despite seasonal changes in the proportion of *An. minimus* forms, their distribution between villages was significantly different (Mantel-Haenszel test, stratified by collection month, $P < 0.001$ for all combinations of villages). Based on polymorphic loci, there was no heterozygote deficit among villages within each *An. minimus* form (form I: $F_{st} = 0.001$, $P = 0.212$; form II, Rong Vong excluded because sample $< 5$: $F_{st} = -0.001$, $P = 0.542$).

The distribution of *An. minimus* differed for each collection method across villages (exact $p$-value $< 0.001$) and collection month (exact $p$-value $< 0.05$). Odds ratios of collecting form I by each collection method adjusted for village and collection month were calculated by logistic regression. The effect of each collection method was compared to the overall effect. All collection methods yielded both *An. minimus* forms, but their frequencies in collection methods differed. Twice as many form II were collected by the outdoor human landing and the outdoor cattle collection, and about 5 times as many form I by the indoor resting collection. No difference in distribution was observed for the indoor human landing collection type (Table 7). Based on polymorphic loci, no heterozygote deficit among collection methods was observed within either *An. minimus* form (form I: $F_{st} = 0.000$, $P = 0.428$; form II: $F_{st} = 0.002$, $P = 0.225$).

## Discussion

Misidentification of *Anopheles minimus s.l.* using morphological characters could be avoided by the diagnostic enzyme loci identified during this study. The *Odh* locus was diagnostic for the three taxa of the Myzomya Series, *An. minimus s.l.*, *An. aconitus* and *An. jeyporiensis*, found in northern Vietnam. In contrast to the findings of Green *et al.* (1990), MDH could not separate unambiguously *An. minimus s.l.* from *An. aconitus*.

**Table 7** Odds ratio of collecting *Odh* form I for each collection method obtained by logistic regression

| Collection method | Number | Odds ratio form I/form II | 95% confidence limits | |
|---|---|---|---|---|
| | | | Lower | Upper |
| Indoor human | 133 | 0.9295 | 0.5811 | 1.4867 |
| Outdoor human | 161 | 0.5100** | 0.3497 | 0.7438 |
| Outdoor cattle | 292 | 0.4609** | 0.3384 | 0.6277 |
| Indoor resting | 318 | 4.5771** | 3.0452 | 6.8796 |

** $P < 0.001$.

Significant positive $F_{is}$ values provided clear evidence of nonrandom mating within the *An. minimus s.l.* population from northern Vietnam. The highest heterozygote deficiency within the sample sets was observed at locus *Odh*, which could be identified as a diagnostic locus for 2 sympatric forms of *An. minimus* in Vietnam. In Thailand, Green *et al.* (1990) also recognized the *Odh* locus as diagnostic for two isomorphic species within *An. minimus*, using a different gel system. Comparison of the Vietnamese specimens with Thai *An. minimus* mosquitoes indicated that form I is the same as the Thai species A and that form II can be equated with species C from Thailand (W. Van Bortel, unpublished data; R. Sharpe, personal communication). The two *An. minimus* species recognized by Green *et al.* (1990) were monomorphic for the *Odh* locus, while *An. minimus* species C from Vietnam (form II) was polymorphic for the same locus. The *An. minimus* species from Vietnam are genetically very similar. Nei's unbiased genetic distance was 0.092, which is slightly lower than the distance (0.134–0.172) found between the two isomorphic African malaria vectors *An. gambiae* and *An. arabiensis* (Cianchi *et al.* 1983). The distance between these two *An. minimus* species and *An. aconitus*, a closely related species, was approximately 0.65.

Hybrids in nature are found in all major groups of higher organisms, and taxa that maintain their integrity despite this overlap have been classified as separate species (Barton & Hewitt 1989). In our samples, 8 (0.88%) hybrids between the *An. minimus* species A and C were collected in Khoi. Six of them were collected in the sample set Khoi-October in which an heterozygote deficiency at loci *Ldh* and *Gpi* was observed in species C.

Sucharit *et al.* (1988) proposed the presence of the presector pale spot and humeral pale spot as diagnostic markers for the Thai *An. minimus* species C, while Rattanarithikul *et al.* (1995) used only the presence of the presector pale spot to distinguish species C from species A. We could not correlate these characters with one of the *An. minimus* species from Vietnam. Moreover, a change of the relative importance of the different morphotypes in each of the *An. minimus* species from Vietnam was observed during the study period (wet season to cool dry season). The use of these morphological characteristics to identify the two *An. minimus* species from Thailand led to 37% error (Green *et al.* 1990) and to 33% in the Vietnamese samples.

No population structure could be inferred in either species populations from Hoa Binh; there was no evidence of restricted gene flow between monthly samples nor between the samples from different locations or between the collection methods.

Two closely related species occurring in sympatry are expected to be bionomically different. If these differences are epidemiologically relevant, they can increase the complexity

of disease transmission patterns. Behavioural differences between cryptic species may also influence the effectiveness of vector control measures (Coluzzi 1992). In our study both *An. minimus* species were attracted to man as well as to cattle. However, twice as many species C specimens were captured on humans outdoors and on cattle as species A, while similar proportions of both species were collected on humans indoors. This indicates that species C is likely to be more exophagic and zoophilic than species A. The most important finding was the highly endophilic behaviour of species A, which was 5 times more abundant in indoor resting collections than species C. The difference in resting catches between both species was even more striking considering the fact that cattle were kept under the houses during the night, and that mosquitoes of species C, prone to feed on cattle, could easily enter the houses to rest. Based on the endophilic behaviour, we may expect a lower impact of indoor spraying on species C than on species A.

In different regions of its distribution, *An. minimus s.l.* exhibits a wide range of responses to indoor spray campaigns with insecticides (Harrison 1980; Parajuli *et al.* 1981; Suthas *et al.* 1986; Lien 1991). This can in part be explained by the presence of two cryptic species within *An. minimus s.l.* occurring in sympatry, but in different proportions depending on the location and dissimilarities in resting behaviour.

Introducing impregnated bednets in Assam, where the main vector, *An. minimus s.l.*, is anthropophilic, endophagic and endophilic, reduced considerably the positive slide rate of malaria (Jana-Kara *et al.* 1995). In Thailand, however, the impact of treated bed nets on malaria was variable and the poor results were mainly attributed to the exophagic and exophilic behaviour of the vector (Somboon 1993). At this point it is not clear to what extent these variations could be explained by the presence of different *An. minimus* species. Differential impact of impregnated bednets on cryptic species is complex and extends beyond simply killing the mosquitoes. This was shown for two members of the *An. punctulatus* complex, *An. koliensis* and *An. farauti*, in Papua New Guinea. The survival rate of *An. koliensis*, which is more anthropophilic and endophilic than *An. farauti*, was affected after introduction of impregnated nets but this was not so for *An. farauti*. However, regularity and duration of the oviposition cycle was disturbed in *An. farauti*, which may shift the peak biting activity from postmidnight towards premidnight. This may increase the potential of the mosquito to transmit the parasite (Charlwood & Graves 1987). Such a shift of the biting cycle was also observed in *An. minimus s.l.* from Thailand after indoor spraying (Ismail *et al.* 1975).

In Vietnam impregnation of bednets is largely promoted in the framework of a comprehensive malaria control strategy, including disease management and prevention. This resulted in a decrease of malaria morbidity and mortality, particulary in northern Vietnam, over the past five years. But malaria transmission still occurs in remote areas. Indoor spraying is reserved to control these epidemics (Verlé *et al.* 1998). The relative role of the different cryptic species in the maintenance of these foci and their behavioural changes in relation to vector control should be further analysed.

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