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A multiplex PCR-based method derived from random amplified polymorphic DNA (RAPD) markers for the identification of species of the *Anopheles minimus* group in Southeast Asia

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Abstract

Effective control of *Anopheles minimus* s.l., an important malaria vector in Southeast Asia, is based on the accurate identification of species within *An. minimus* complex, which cannot be distinguished using morphological characters. Derived from individual random amplified polymorphic DNA markers, sequence characterized amplified regions were analysed for the design of species-specific paired-primers. Combination of these primers resulted in the development of a simple, robust multiplex PCR able to identify both species *An. minimus* A and C belonging to the complex, hybrids AC, and three sympatric and closely related species, *An. aconitus*, *An. pampanai* and *An. varuna*. Hybrids AC do not possess alleles of both parents but exhibit novel adaptive potentials resulting from recombination among parental genes leading to hybrizyme.

Keywords: *Anopheles minimus*, hybrizymes, multiplex PCR, malaria vector, Southeast Asia.

Introduction

Anopheles (*Cellia*) *minimus* s.l. is a major malaria vector that occurs in foothills from northern India (Uttar Pradesh) eastward to southern Japan (Ishigaki Island) and from southern China (Yunnan and Guangxi Provinces, Hainan Island) to northern Malaysia, including Cambodia, Laos, Myanmar, Thailand and Vietnam (Baimai *et al*., 1996; Das & Baruah, 1985; Dev, 1996; Dutta & Baruah, 1987; Khan *et al*., 1998; Kobayashi *et al*., 2000; Sawabe *et al*., 1996; Tsuda *et al*., 1999; Tun-Lin *et al*., 1995; Van Bortel *et al*., 1999) (Fig. 1). This vector taxon belongs to the *An. minimus* group, which includes nine species in southern Asia and one in Africa, previously classified on geographical criteria within the *An. funestus* group (Green, 1982; Harbach, 1994; Pape, 1992). Seven of these species, *An. aconitus*, *An. filipinae, An. flavirostris*, *An. minimus* species A and C, *An. pampanai* and *An. varuna*, occur on mainland Southeast Asia. These seven species are difficult to distinguish in the adult stage because of overlapping characteristics, and therefore misidentification using morphological characters is common (Harrison, 1980).

After indoor spraying with DDT, the occurrence of two biological variants was already suspected in Thailand (Ismail *et al*., 1974, 1975, 1978; Sucharit & Komalamisra, 1997; Suthas *et al*., 1986), and southern China (Yu, 1987; Yu & Li, 1984). We know now that the domestic *An. minimus* species A will be more exposed to residual insecticides than the more zoophilic and exophilic species C (Van Bortel *et al*., 1999). Form B, described from southern China (Sawabe *et al*., 1996; Yu & Li, 1984) has not been ranked as a species yet, because a more thorough study comparing the three putative members of the *An. minimus* complex should be made.

Isoenzyme analysis is the golden standard for identification of the species of the *An. minimus* group (Green *et al*., 1990; Van Bortel *et al*., 1999). Morphological identification of adults on the basis of associated immature skins can also be used for all species except the species of the *An. minimus* complex. However the use of molecular markers is more field-friendly for identifying adult mosquitoes because specimens can be dried and very little material (e.g. one mosquito leg) is needed. RAPD-PCR markers were defined to differentiate both species A and C (Sucharit & Komalamisra, 1997), but the results were not validated. Two additional PCR-based techniques, an allele-specific

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Figure 1. Distribution of *An. minimus* s.l. (shaded area) and localities (dark spot) where studied populations of species A and C, *An. aconitus*, *An. pampanai* and *An. varuna* originated. 1, Kanchanaburi (Thailand); 2, Hoa Binh (Vietnam); 3, Hanoi suburbs (Vietnam); 4, Khanh Hoa (Vietnam); 5, Binh Thuan (Vietnam); 6, Rattanakiry (Cambodia); 7, Vientiane (Laos).

Table 1. Location of the sample sites of the five studied species. In parentheses, the site number as defined on the map (Fig. 1)

Taxon	Country	Localities (Province)
An. minimus A	Thailand	Kanchanaburi (1)
	Vietnam	Hoa Binh (2), Hanoi (3), Khanh Hoa (4), Binh Thuan (5)
	Cambodia	Rattanakiry (6)
	Laos	Vientiane (7)
An. minimus C	Thailand	Kanchanaburi (1)
	Vietnam	Hoa Binh (2)
An. aconitus	Vietnam	Hoa Binh (2), Khanh Hoa (4), Binh Thuan (5)
	Cambodia	Rattanakiry (6)
	Laos	Vientiane (7)
An. pampanai	Vietnam	Khanh Hoa (4), Binh Thuan (5)
	Cambodia	Rattanakiry (6)
An. varuna	Vietnam	Khanh Hoa (4), Binh Thuan (5)

amplification (ASA) and single-strand conformation polymorphism (SSCP), were developed for distinguishing, respectively, *An. minimus* A and C; and both species A and C, as well as *An. aconitus* and *An. varuna* (Sharpe *et al.*, 1999). Recently, a RFLP-PCR procedure was designed (Van Bortel *et al.*, 2000) for the identification of these four species as well as *An. pampanai* and *An. jeyporiensis*. Despite the availability of these identification procedures, as noted by Van Bortel *et al.* (2000), a simpler, more robust technique is needed for epidemiological studies.

This study presents a multiplex PCR, using species-specific paired-primers capable of identifying specifically *An. minimus* species A and C, and the hybrids AC, as well as three sympatric and closely related species, *An. aconitus*, *An. pampanai* and *An. varuna* (Fig. 1, Table 1). These primers were designed from sequence characterized amplified regions (SCAR), obtained from individual RAPD markers.

Results

Screening and identification of RAPD polymorphism

RAPD-PCR generates, from the entire genome, random amplification of numerous DNA fragments using a single decamer primer. A total of sixty-six primers (list available upon request to S.M.) were tested, including forty-seven RAPD, eight microsatellites, and eleven consensus primers based on intergenic repeated sequences. The initial screening of the sixty-six primers was done on four specimens of each of the five species, *An. minimus* A and C, *An. aconitus*, *An. pampanai* and *An. varuna*. Laboratory identification of these species was based on morphological characters of immature stages and/or isozyme analysis or RFLP-PCR.

Of the sixty-six primers, twelve RAPD markers (18%) showed polymorphism with reproducible banding patterns discriminant for species A and C; three of them (5%) did not amplify. Among the other fifty-one primers (77%), eight (12%) showed faint or non-reproducible banding patterns, and thirty-nine (59%) produced non-scorable banding patterns due to the amplification of either too many bands or weak bands. Poor results were obtained with seven of the eight microsatellite primers and nine of the eleven consensus primers tested.

The twelve RAPD primers that discriminate species A and C were further studied with a larger sample (twenty specimens) of each of the five species in order to select the ones presenting reproducible polymorphic fragments

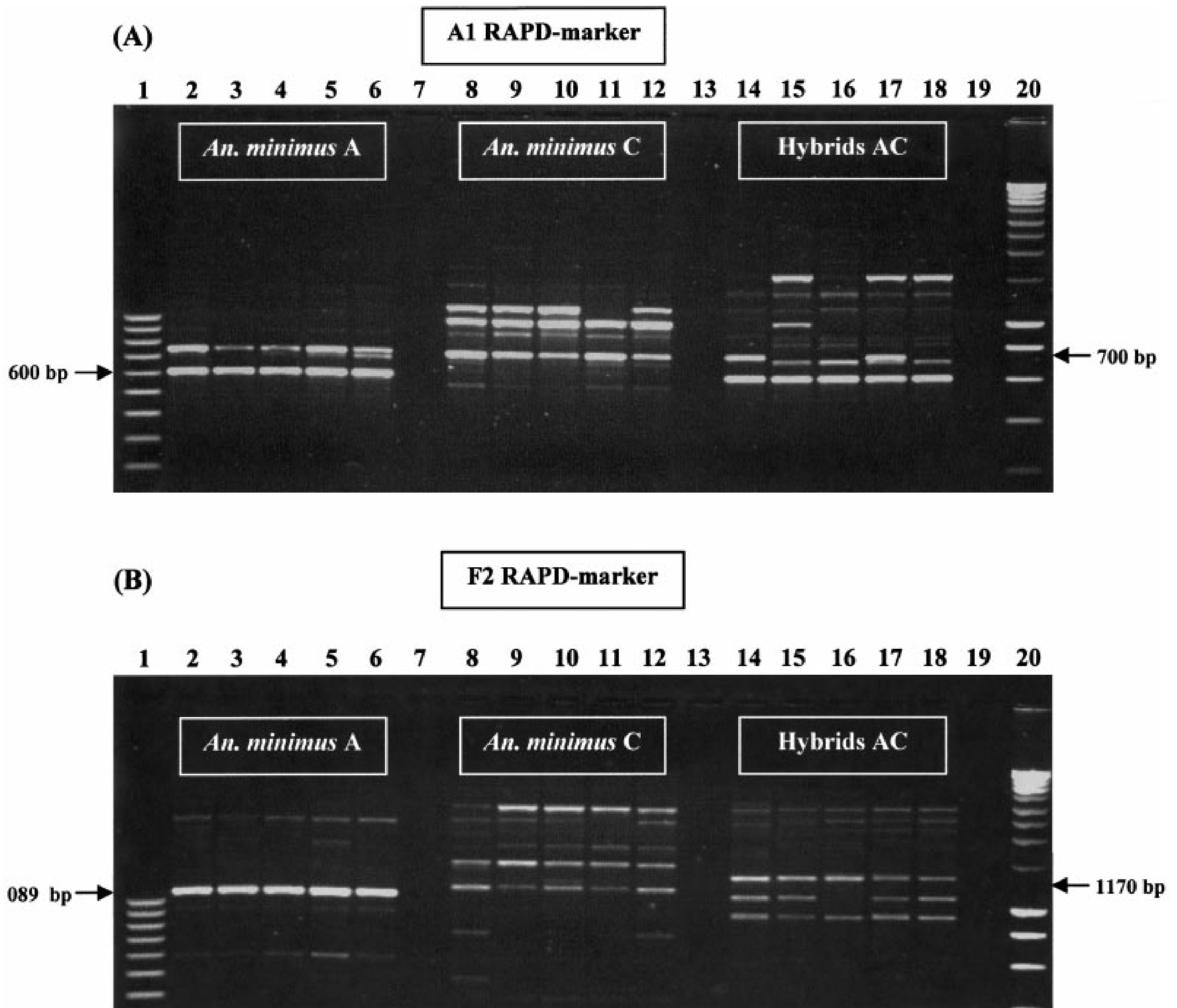

Figure 2. RAPD-PCR profiles of *An. minimus* A and C using two markers: (A) primer A1 and (B) primer F2. Lane 1, 100 bp ladder; Lanes 2–6, *An. minimus* A; Lanes 8–12, *An. minimus* C; Lanes 14–18, hybrids AC; Lane 19, negative control; Lane 20, Smart ladder.

and able to unambiguously differentiate all species. Four primers (A1, A5, F2, F4) were able to do this (Fig. 2). Two primers showed one constant specific band for both species A or C, respectively, at 600 bp (along with another band at 740 bp for species A) and 700 bp for A1 (Fig. 2A), and 1089 and 1170 bp for F2 (Fig. 2B); the other two primers, A5 and F4, presented one specific band for either species A or C, and both bands for the hybrids, respectively, at 850 bp for species C, and 750 for species A. Primers A1 and F2 amplified different bands for the hybrids AC than for either *An. minimus* A or C, respectively, at 580 bp (Fig. 2A), and at 870 and 1300 bp (Fig. 2B). Primer F2 also allowed the amplification of specific fragments for *An. aconitus*, *An. pampanai* and *An. varuna* at 1243 bp, 1515 bp and 1030 bp, respectively (data not shown).

Conversion of RAPD markers into sequence characterized amplified regions (SCARs)

The six fragments specific for *An. minimus* A and C, using RAPD markers A1, A5, F2 and F4, were cloned and sequenced. From these sequences obtained from ten specimens of each species A or C, as well as six hybrids, specific 20–24 mer SCAR primers were designed. Using specific primers from A5 and F4, decamer primer included in the SCAR was amplified in both species A and C, indicating that the original RAPD polymorphism was caused by mismatches in one or a few nucleotides in the priming sites. SCARs obtained from the RAPD primer A1 showed a polymorphism on agarose gel that was nearly the size expected for both *An. minimus* A and C, through hybridization

F2p

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C   AGGTAATGATGTTCATTGTGACGATATATCCATTGAATTAATACACAAATCATAATTAAT
A   AGGTAATGATGTTCAATGTGACGATATATCCATTGAATTAATACACAAATCATAATTAAA
H   AGGTAATGATGTTCATTGTGACGATATATCCATTGAATTAAT--------CATAATTAAT
    *************** **************************        *********
C   TATATAGACTTTAAATCGTACCATAAATCG-AACAAACAACAGAAATTTCGAAAAAGTTT
A   TCTACAGACTTTGAATCGTACCATAAATCG-AACAAACAACAGAAATTTCGAAAAAGTTT
H   TATATAGAGTTTAAATCGTACCATGAATCGTAACAAACAACAGAAATTTCCAACG---TT
    * ** *** *** *********** ***** *******************  **     **
C   GAATACGATACATAAGTTATTCGTTTTAATTCAAAATCATAACACTAAAATTGACTAATC
A   GAATACGATACATACATTATTGGTTGTAATTCAAAACCATTACACTAAAATTGATTAATC
H   GAAAACGATACATAAATTATTCGTAGCAATTCAACACCATAACGTTAAAATTTACTAATC
    *** **********  ***** **   ******* * *** **  ******* * *****
C   TCACTTACCTTCAAGTGGCGAAATGAAATCTGTAACGAAACAAGAAAATAAATTAATTTC
A   TCAC--------------------------------------------------------
H   TCACTTACCTTCAAGTGGCGAAATGAAATCTGTAACGAAACAAGAAAATGAATTAATTTC
    ****
C   GGTGAAATGACACATGTAAACATAAGTAATTGCCACATAT--------------------
A   -------------ATATATACATAAGTAATAGCCACATAT--------------------
H   GGCGAAATGACACATATATGAATAAATAATAGCCACATATGGGCGGTCCGGTGGCCGAGG
                 ** **   **** **** *********
C   ------------------------------------------------------------
A   ------------------------------------------------------------
H   AGATAAGCGGCGCCGGTCTCAAACGGCAGGACCGGGGTTCAAATCCCATCCGGACCGTTC

C   ------------------------------------------------------------
A   ------------------------------------------------------------
H   CCCCGTAGTGAGGACTGACTATCCAACTACGTGGTATTAATAAGTCTCGTAAGCCAGAAA

C   ------------------------------------------------------------
A   ------------------------------------------------------------
H   TGGCAGGCATGACCGAGTAGGTCGTTAGGCCAAAGAAGAAGAAGAAGAAGAAGAATAGCC

C   ------ATGTAGATTTTCATGGTTAATTTTTGTTTAAATATCNATGTTGATCATAAGAAA
A   ------ATGTAGATTTTCATGGTTAATTGTTGTTTAAATATCGTTGTGGATCATAAGAAA
H   ACATATATGTAGATTTTCATGGTTAATTGTTGTTTAAATATCGTTGTTGATCATAAGAAA
          ********************** *************  *** ************
C   CCAAAACCCAAAATTTTTATGGTAGTACATTATTATTCTCACGCTATTTGCATAAAAAAC
A   CAAGAACCTAAAATTTTTATGGTAGTACATTAATATTC--ACGCTATTTGCATAAAAAAC
H   CCAAAACCCAAAATTTTTATGGTAGTACATTATTATTCTCACGCTATTTGCATAAAAAAC
    * * **** *********************** *****  ********************
C   GACACAAGGCGTCCCTTTTTGAACCACT
A   GACACAAG-CGTCCCTTTTTGAATCACT
H   GACACAAGGCGTCCCTTTTTGAACCACT
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F2q

Insert 1: common to species C and hybrids AC (69 bp)

Insert 2: specific of hybrids AC (206 bp)

*, identical nucleotide

Figure 3. Sequence alignment of F2-RAPD fragments specific of *An. minimus* species A (A), species C (C), and hybrids AC (H), with primers F2p and F2q in shaded area. In bold, insert 1 (69 bp) common to *An. minimus* C and hybrids AC; insert 2 (206 bp) specific to hybrids AC. Underlined, 21 bp microsatellite $(GAA)_7$.

with homologous probes. SCARs obtained from the primer F2 were amplified for *An. minimus* A and C at 1089 and 1170 bp, respectively. To improve the efficiency of the PCR, two primers (F2p and F2q) were chosen within the sequence of the SCAR fragment (Fig. 3, Table 2). PCR using these two SCAR primers resulted in the amplification of smaller fragments of 348 for species A and 420 bp for species C (Fig. 4, Table 2). These SCAR markers were tested on hybrids AC from Hoa Binh Province (village Khoi), located in northern Vietnam, initially identified by isozyme analysis (Van Bortel *et al.*, 1999). The amplification showed that both fragments of species A and C were not simultaneously present as expected. Instead, one fragment at 616 bp was amplified in either males or females (Fig. 4, Table 2). This 616 bp fragment was sequenced and compared to both fragments at 348 (for species A) and 420 bp (for species C). Sequences of the three fragments revealed three different alleles that were specific for either *An. minimus* A, species C, or the hybrids AC (Fig. 3). Sequence of species A had a 69 bp deletion compared

Table 2. Primers developed from sequence characterized amplified regions (SCAR) of F2-RAPD marker

Primer	Sequence	Size	Species A	Specific C	Fragment Hybrid AC
F2p	5′-GGTAATGATGTTCAATGTGACG-3′	22	348 bp	420 bp	616 bp
F2q	5′-AGTGATTCAAAAAGGGACGC-3′	20			
		Size	Species *An. aconitus*	Specific *An. pampanai*	Fragment *An. varuna*
Aco3	5′-ATCGTCATCCTTTGCTCTGC-3′	20	252 bp	–	–
Aco4	5′-CCATCCAACAGTGAACAACG-3′	20			
Pam3	5′-TGCAAACCCATACAAATTTCA-3′	21	–	540 bp	–
Pam4	5′-AAAACGCATCCAAAAGTTCG-3′	20			
Var3	5′-TTCTTTAACCAACACTACGGTTCTC-3′	25	–	–	647 bp
Var4	5′-AGGTGTTTATGGTTGTTGTTGTTTT-3′	25			

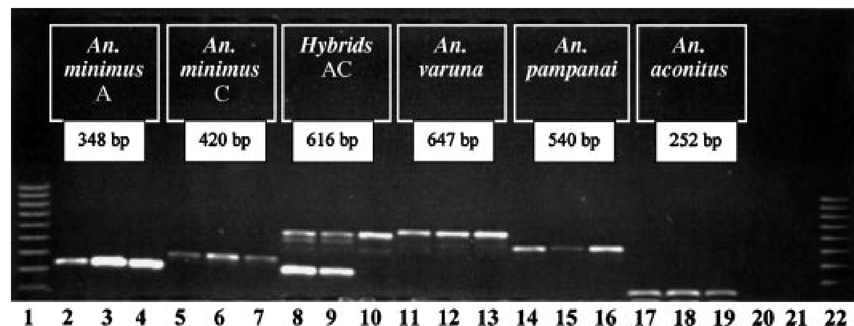

Figure 4. Multiplex PCR for the identification of *An. minimus* species A (lanes 2–4) and species C (lanes 5–7), hybrids AC (lanes 8–10), and *An. varuna* (lanes 11–13), *An. pampanai* (lanes 14–16), and *An. aconitus* (lanes 17–19). Lanes 1 and 22, 100 bp ladder; lanes 20 and 21, negative controls.

to species C and hybrids (Fig. 3, insert 1). In addition, the latter had one major insertion of 206 bp (Fig. 3, insert 2), including a 21 bp microsatellite $(GAA)_7$, compared to species A and C. Except the 69 bp difference between species A and C, the rest of both sequences were similar at 94%. In the case of the hybrid sequence, the similarity was of 88% and 91%, with species A and C, respectively, without considering the differences of 69 and 206 bp evoked above.

Three specific F2-RAPD fragments were cloned and sequenced for *An. aconitus*, *An. pampanai* and *An. varuna*. One paired primer (20–24 mer) for each species was designed (Table 2), with the concern of keeping the annealing temperature close to 55 °C. The PCR amplification with the designed paired-primers, Aco3/Aco4, Pam3/Pam4 and Var3/Var4, produced the expected fragment for each species: 252 bp for *An. aconitus*, 540 bp for *An. pampanai*, and 647 bp for *An. varuna*. No amplification was obtained, using these primers, with either *An. minimus* A and C, or other unrelated anopheline species, including *An. dirus*, *An. funestus*, *An. jeyporiensis*, *An. maculatus*, *An. subpictus* and *An. sundaicus*.

Combination of paired-primers into multiplex PCR amplification

In order to develop a quick, simple, inexpensive, robust molecular technique, using little DNA material and reagents, for species-specific identification, we combined the four species-specific primers into a multiplex PCR assay (Fig. 4). This multiplex PCR was developed for the identification of *An. minimus* A and C, *An. aconitus*, *An. pampanai* and *An. varuna* using the paired-primers F2p/F2q, Aco3/Aco4, Pam3/Pam4 and Var3/Var4, respectively. The results of this multiplex PCR showed the same amplification profiles as those from the individual use of paired-primers. Multiplex PCR combining up to four couples of primers was able to differentiate as many as five sympatric species of the *An. minimus* group within one PCR process.

This method was tested and validated on a large number (956) of specimens of each species (Fig. 1, Table 1), including 657 *An. minimus* A from Vietnam (491), Thailand (23), Cambodia (70), Laos (73); 169 *An. minimus* C from northern Vietnam (135) and Thailand (34); sixty-five *An. aconitus* from Vietnam (26), Cambodia (15) and Laos (24); forty-five *An. pampanai* from Vietnam (30), Cambodia (15); and twenty *An. varuna* from Vietnam.

Sympatry in study sites of Vietnam, Laos and Cambodia

The results confirmed the presence of *An. minimus* C and hybrids AC in Hoa Binh Province (village Khoi) in northern Vietnam, which occurred in sympatry with *An. minimus* A and *An. aconitus* (Table 1). Hybrids AC (< 1%) were identified using isozymes (Van Bortel *et al.*, 1999), RFLP-PCR (Van Bortel *et al.*, 2000), and our multiplex PCR. No

species C or heterozygotes were found in our other study sites in central Vietnam, Cambodia or Laos (Table 1). In central Vietnam, the results showed that *An. minimus* A, *An. aconitus*, *An. pampanai* and *An. varuna* are sympatric in Khanh Hoa and Binh Thuan Provinces. In Cambodia, *An. minimus* A, *An. aconitus* and *An. pampanai* were found to be sympatric in Rattanakiry Province; and in Laos, *An. minimus* A and *An. aconitus* were found together in Vientiane Province (Table 1).

Discussion

Precise identification of anopheline mosquitoes is essential for a better understanding of their potential role in malaria transmission, as well as for improving the effectiveness of vector control strategies. Isozyme and molecular techniques are available for identifying morphologically similar species of the *An. minimus* group. Isozyme electrophoresis is still considered the 'gold standard' method (Green *et al.*, 1990), however it requires frozen material to avoid protein breakdown and this preservation technique, which requires the use of dry ice or liquid nitrogen, is difficult to employ under field conditions. Molecular tools are more field-friendly because specimens can be dried, and because they require very little material, such as one mosquito leg, specimens can be kept alive for crossing experiments or used for more than one type of analysis (isozymes, ELISA tests, PCR, etc.). However, some molecular techniques can be lengthy, expensive or lack reproducibility, and therefore are not always fully adapted to all laboratories or the identification of large number of specimens. Single-strand conformation polymorphism-PCR (SSCP-PCR) (Sharpe *et al.*, 1999) allows the identification of four species of the *An. minimus* group, however, it requires the running (16–20 h) and staining of an acrylamide gel (expensive), which is unrealistic for field studies. Restriction fragment length polymorphism-PCR (RFLP-PCR) (Van Bortel *et al.*, 2000) has the advantage of identifying five species of the *An. minimus* group, as well as *An. jeyporiensis*. However, this technique requires an additional step, which is the digestion with one appropriate restriction enzyme (BsiZI), being processed between the DNA amplification by PCR using two specific primers and the visualization of the digested DNA by electrophoresis. RAPD-PCR is fast, easy and inexpensive, however this method seems too sensitive to some changes, especially DNA concentration, which give rise to unrepeatable and unreliable results (Hoy, 1994). We compared the six RAPD markers identified by Sucharit & Komalamisra (1997) for the differentiation of *An. minimus* species A and C, and the results showed discrepancies. Only the M20-fragment at 400 bp, specific for species C, was shared in both studies. This comparison demonstrated that RAPD markers have to be carefully selected by repeating reactions and validation with a large sample size in order to determine which discriminant bands are scorable and reproducible.

To palliate drawbacks of RAPD-PCR, we developed an allele-specific PCR based on sequence characterized amplified regions (SCAR) obtained from individual RAPD markers (Karp *et al.*, 1998). After cloning and sequencing of desired SCARs, primers were designed (usually 20–25 mers) that allowed the amplification of species-specific fragments. This procedure resulted in markers, either dominant or co-dominant, which were able to distinguish species and their potential heterozygotes. When no species occurrence records are available for a particular locality, combinations of up to four paired-primers in a multiplex PCR are required, otherwise the combination of specific paired-primers will depend on those species of the *An. minimus* group known to be present. In addition, these markers combined in a multiplex PCR made this technique quite reliable, simple, robust and doable under any laboratory conditions.

Even though RAPD markers are dominant, heterozygotes were strongly suspected on particular RAPD profiles showing *An. minimus* specimens collected in village Khoi of Hoa Binh Province in northern Vietnam. After confirmation by isozyme analysis and RFLP-PCR, hybrids AC could clearly be recognized because they exhibited a different banding pattern than their parents (Fig. 2A,B). In the multiplex PCR (Fig. 4), hybrids exhibited one long fragment (616 bp), including a microsatellite $(GAA)_7$, occasionally associated with the specific fragment of species A (348 bp). The presence of novel alleles, also called 'hybrizymes' (Woodruff, 1989), is the consequence of hybridization or introgression, which has been reported in mammals, birds, reptiles, amphibians and insects (reviewed in Woodruff, 1989). These heterozygotes do not exhibit alleles of both parents but present novel adaptive potentials resulting from recombination among parental genes leading to hybrizymes. Several explanations for the occurrence of hybrizymes have been proposed, such as increased mutation rates in hybrids (confirmed by the presence of a microsatellite known to be hypervariable), reduced selection, and intragenic recombination between different alleles of the parent species (Golding & Strobeck, 1983; Woodruff, 1989). In any case, the new alleles resulting from genetic combinations can be viewed as the 'raw material for evolution' (Anderson, 1949) or 'a significant source of genetic novelty upon which selection can act' (Rieseberg, 1991). The presence of heterozygotes in sympatric populations of *An. minimus* A and C in northern Vietnam and north-western Thailand is evidence of introgression between the two species, which can lead to an increase of genetic diversity, a transfer of adaptations, origin of new adaptations, breakdown or reinforcement of isolating barriers, and the origin of new ecotypes or species that could promote dispersal (Potts & Reid, 1988). It would be interesting to study how populations

of *An. minimus* A and C, as well as hybrids AC, might evolve. Considering the fact that barriers, which limit genetic exchange between hybridizing taxa, are often semipermeable, there is considerable variance in the extent to which alleles at different loci introgress. Thus, species can occasionally or extensively hybridize and yet remain distinct. In this sense, hybrid zones pose serious problems for the recognition concept of species (Paterson, 1985). However, they provide a wealth of information about possible states and degrees of divergence between populations that may be in the process of speciation (Harrison, 1993).

For a more complete understanding and a better knowledge of the *An. minimus* complex, it would be necessary to extend the comparison to a larger number of populations throughout its geographical range in order to evaluate the species-specific distribution. Our results show that *An. minimus* A and C occur together in northern Vietnam (Hoa Binh Province), but only species A was found in the suburbs of Hanoi (northern Vietnam), Khanh Hoa and Binh Thuan Provinces (central Vietnam), Rattanakiry Province of Cambodia, and Vientiane Province in Laos.

Current records show a wide distribution of *An. minimus* A in Southeast Asia, while species C was only found in northern Vietnam, in north-western (Kanchanaburi Province) and northern (Chieng Mai Province) Thailand (Sharpe *et al.*, 1999). Even in the hilly regions of northern Vietnam the proportion of the sympatric species A and C may vary between villages of the same district (Van Bortel *et al.*, 1999). This spot distribution of species C is probably linked to environmental conditions, which should be studied over a much larger geographical range than currently prospected.

It is important to note that populations of *An. minimus* from the suburbs of Hanoi (Gian Bien, Hanoi Province) are peculiar because their larvae breed in stagnant water in cement basins instead of the margins of flowing streams. This zoophilic population is not involved in malaria transmission. After the comparison of different populations using isozymes, RFLP-PCR, RAPD-PCR and multiplex PCR, these specimens were unambiguously identified as *An. minimus* A.

The robust and simple allele-specific PCR for identifying *An. minimus* A and C, as well as three closely related *An. aconitus*, *An. pampanai* and *An. varuna*, was developed for easy and reliable use in any properly equipped laboratory. These sympatric species have different behaviours and roles in malaria transmission, and it is quite important to identify the species accurately and reliably to better target the vectors for the improvement of vector-specific control measures.

Experimental procedures

Mosquito populations

Specimens of *An. minimus* s.l., *An. aconitus*, *An. pampanai* and *An. varuna* (Fig. 1, Table 1) were collected during extensive surveys, which included landing catches on humans and bovines, resting captures, and larval collections. These surveys were made in Vietnam during May, August, November 1998, and April 1999, and in Cambodia and Laos in March, June and October 1999. In Cambodia, collections were made in the village of Cha Ong Chan (village K of O Chum District in Rattanakiry Province); and in Laos, in the village of Na Ang (village L of Fuang District in Vientiane Province). In Vietnam, the three concerned sites were located in Khoi (village A of Phu Cuong Commune, Tan Lac District in Hoa Binh Province), Lang Nhot (village B of Khanh Phu Commune, Khanh Vinh District in Khanh Hoa Province), and village C of Suoi Kiet Commune (Tanh Linh District in Binh Thuan Province). Specimens of *An. minimus* A were also obtained from larval collections made from cement basins in Giau Bien Commune near the Duong River on the outskirts of Hanoi. In Thailand, *An. minimus* A and C were collected in village no. 8 of Tasao, Saiyok District, Kanchanaburi Province. No specimens outside these four countries were examined, in particular specimens from India, China or Japan which represent the extreme range of the *An. minimus* distribution.

Standard procedures for identification of all the specimens were done in laboratories using isozyme analysis (Van Bortel *et al.*, 1999) or PCR-RFLP (Van Bortel *et al.*, 2000) and/or morphological identification of immature skins (Harrison, 1980). Our analyses were conducted on either wild-caught adults (Thailand) or adults reared from larval collections and progeny broods (Vietnam, Laos, Cambodia). Adult specimens were stored either at −80 °C (material from Thailand, Vietnam) or dry-preserved (Cambodia and Laos).

RAPD analysis

Genomic DNA from individual mosquitoes was purified following a slightly modified protocol provided by Paskewitz & Collins (1990). Mosquito DNA from different localities (Fig. 1, Table 1) was screened using forty-seven RADP primers (Operon kits A, B, F, M, P), eight microsatellites and eleven consensus primers to identify species-specific markers (Williams *et al.*, 1990). Ten to 25 ng of genomic DNA were combined in a 25 μl total volume with 6 pM of each primer, 3 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates (dNTPs), and one unit of Taq DNA polymerase. The PCR conditions started with a denaturation step at 94 °C for 3 min, then 1 min at 94 °C, 1 min at 35 °C and 2 min at 72 °C for forty-five cycles with a final extension step at 72 °C for 15 min. Amplified fragments were analysed by electrophoresis on a 1.5% agarose gel. PCR fingerprinting patterns were assigned for each primer presenting intense, reproducible and specific bands. Reproducibility was tested by surveying three times all primers revealing polymorphism on sixty specimens. Four RAPD markers (A1, A5, F2, F4) were identified for revealing sequence characterized amplified regions (SCAR) for the differentiation of *An. minimus* A and C. Their specific sequence is as follows: 5′-CAG GCC CTT C-3′ for A1; 5′-AGG GGT CTT G-3′ for A5; 5′-GAG GAT CCC T-3′ for F2; and 5′-GGT GAT CAG G-3′ for F4.

Cloning and sequencing of specific RAPD fragments

Specific RAPD-PCR products were excised from low melting point agarose gel and purified using the PCR purification kit (Qiagen). Purified DNA was reamplified with the same primer in order to evaluate the presence of additional fragments, not revealed in the first amplification. Expected RAPD fragments were cloned using the Zero Blunt TOPO PCR cloning kit. Recombinant plasmids containing mosquito DNA were purified using a miniprep kit (Qiagen). Four to six clones per single RAPD fragment were sequenced from

5′ and 3′ ends by the dideoxy chain termination method using the T3 and T7 universal primers. RAPD sequences were deposited in GenBank with the following accession numbers: AJ299388 for *An. minimus* C, AJ299389 for *An. minimus* A, AJ299390 for *An. minimus* heterozygotes AC, AJ299391 for *An. pampanai*, AJ299392 for *An. varuna*, and AJ299393 for *An. aconitus*.

Primer design and SCAR-PCR

Eight SCAR-primers (including or excluding the RAPD-primer) were designed from the sequences of the species-specific fragments (Table 2) using Pick Primer Program (Primer 3 web site). Each paired-primer was tested in a SCAR-PCR for the amplification of sequence characterized DNA regions of each species. Ten to 20 ng of template mosquito DNA were combined in a 25 µl total volume with 20 pM of each primer, 1X reaction buffer, 1.5 µM $MgCl_2$, 200 µM (each) dNTP, and 0.625 units of Taq DNA polymerase. The PCR conditions started with a denaturation step at 94 °C for 3 min, then 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C for thirty-five cycles with a final extension step at 72 °C for 10 min. After amplification, PCR products were electrophoresed as described above.

Multiplex PCR

Equimolar concentration of up to four pairs of species-specific primers was used in a single multiplex PCR. PCR conditions and analysis were done as described above for SCAR-PCR. Each species was identified by the size of its specific amplified product.

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