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Bionomics, taxonomy, and distribution of the major malaria vector taxa of *Anopheles* subgenus *Cellia* in Southeast Asia: An updated review

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Abstract

There is high diversity of *Anopheles* mosquitoes in Southeast Asia and the main vectors of malaria belong to complexes or groups of species that are difficult or impossible to distinguish due to overlapping morphological characteristics. Recent advances in molecular systematics have provided simple and reliable methods for unambiguous species identification. This review summarizes the latest information on the seven taxonomic groups that include principal malaria vectors in Southeast Asia, i.e. the Minimus, Fluviatilis, Culicifacies, Dirus, Leucosphyrus, and Sundaicus Complexes, and the Maculatus Group. Main issues still to be resolved are highlighted. The growing knowledge on malaria vectors in Southeast Asia has implications for vector control programs, the success of which is highly dependant on precise information about the biology and behavior of the vector species. Acquisition of this information, and consequently the application of appropriate, sustainable control measures, depends on our ability to accurately identify the specific vectors.

Keywords: Malaria; Vectors; Minimus Complex; Fluviatilis Complex; Culicifacies Complex; Dirus Complex; Leucosphyrus Complex; Sundaicus Complex; Maculatus Group; Asia

1. Introduction

Southeast Asia, including the Mekong area, encompasses 15 countries[3] that experience a high burden of vector-borne diseases, among which malaria remains the most important. Some 2.5 million cases of malaria are reported annually, but it is estimated that as many as 100 million cases may actually occur in the region each year (WHO, 2007a). This region accounts for 30% of the global malaria morbidity and about 8% of the global mortality, with approximately 26,000 deaths per year (WHO, 2007b).

Major progress in malaria control was achieved during the last decade, especially in Bhutan, Cambodia, Laos, Sri Lanka, Thailand, and Vietnam. However, a high rate of malaria still occurs in hilly forested areas and some coastal foci where it is a fatal disease that is endemic in poor rural areas (Trung et al., 2004).

The epidemiology of vector-borne diseases is strongly linked to the biodiversity of known or potential insect vectors such as *Anopheles* mosquitoes that may transmit malarial pathogens. Nowadays one must consider the whole anopheline community present in an area, instead of focusing on just one vector species. Ecological, demographic, and climatic changes influence the composition of anopheline communities and consequently have an impact on malaria transmission. This is quite true in Asia where the biodiversity and specific richness of *Anopheles* species is high compared to the other regions

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(Foley et al., 2007), and where important environmental changes, such as deforestation (Walsh et al., 1993) and irrigation system (Klinkenberg et al., 2004), occur at a quick pace.

The main malaria vectors in Southeast Asia belong to species complexes and groups (Harbach, 2004), which include closely related species that are difficult to distinguish morphologically yet often differ in their bionomics. The sympatric occurrence of the vector species complicates our understanding of malaria transmission and epidemiology. A species may play a primary role in one area and a secondary role elsewhere, and the vector status of an individual species may vary in relation to environmental or seasonal changes. Vector control in the region is therefore potentially hampered by the number and complexity of the primary and secondary vector species. This situation dictates that scientists must come together to study anopheline communities as a whole and to integrate the diverse information about vector systems to define appropriate vector and effective control programs.

The purpose of this review is to synthesize the most recent information on the principal malaria vector taxa of genus *Anopheles* subgenus *Cellia* in Southeast Asia and to highlight the main issues still to be resolved.

2. The Minimus Complex (Funestus Group, Myzomyia Series)

The Minimus Complex comprises two formally named species, *An. minimus* (species A) and *An. harrisoni* (species C), and the informally designated *An. minimus* E. Several putative forms of *An. minimus* are mentioned in published literature that are either morphological or chromosomal variants of the genetic species (see the review of Chen et al. (2002)).

Today, the taxonomy of the Minimus Complex is nearly complete. Harbach et al. (2006) designated a neotype to fix the identity of *An. minimus* s.s., *An. harrisoni* was recently described and named by Harbach et al. (2007), and the description and naming of *An. minimus* E is underway (Harbach, personnel communication). Despite with the formal taxonomy, the three species cannot be distinguished based on morphology (Jaichapor et al., 2005; Sungvornyothin et al., 2006a) and their separation from closely related sympatric species is problematic due to overlapping characters. The situation is complicated by the morphological variability of *An. minimus* (Jaichapor et al., 2005).

The application of molecular techniques has made it possible to reliably identify species in entomological surveys (Table 1). A number of molecular identification assays are now

Table 1
Type and references on the PCR assays developed for each complex and associated species

Method[a] (reference)	*An. culicifacies* A, D/B, C/E	*An. fluviatilis* S, T, U	*An. dirus*, *An. cracens*, *An. scanloni*, *An. baimaii*	*An. minimus*, *An. harrisoni*	*An. aconitus*, *An. pampanai*, *An. varuna*[b]	*An. jeyporiensis*[1]	*An. maculatus* group[c,d]	*An. sundaicus*, species E, *An. epiroticus*
AS (Singh et al., 2004b)	A, D/B, C, E							
RFLP (Goswani et al., 2005)	A, D/B, C/E							
AS (Manonmani et al., 2001; Singh et al., 2004b)		X						
AS (Walton et al., 1999a) AS-SCAR (Manguin et al., 2002)			X					
SSCP (Sharpe et al., 2000)				X	X			
RFLP (Garros et al., 2004b; Van Bortel et al., 1999)				X	X	X		
AS-SCAR (Kengne et al., 2001)				X	X			
AS (Garros et al., 2004a; Phuc et al., 2003)				X	X			
RFLP (Torres et al., 2000)							X[c]	
AS (Ma et al., 2006; Walton et al., 2007)							X[d]	
AS (Dusfour et al., 2007b)								X

[a] AS: Allele-specific; RFLP: restriction fragment length polymorphism; SSCP: single-strand conformation polymorphism; SCAR: sequence characterized amplified region.

[b] These four species are closely related to the Minimus Complex and often sympatric with members of this complex.

[c] Two species identified by this RFLP, *An. dispar* and *An. greeni* (Torres et al., 2000).

[d] Five species identified by these two AS-PCR, *An. maculatus*, *An. dravidicus*, *An. pseudowillmori*, *An. sawadwongporni* and either *An. willmori* for Ma et al. (2006) or chromosomal form K for Walton et al. (2007).

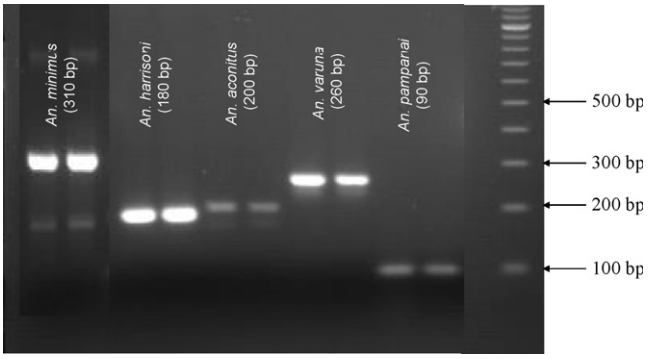

Fig. 1. AS-PCR gel of *An. minimus* and related species (Garros et al., 2004a).

available to distinguish the two sympatric sibling species, *An. minimus* and *An. harrisoni*, as well as related, sympatric species. The RFLP-PCR[4] assay (Garros et al., 2004b; Van Bortel et al., 2000) is very useful for large-scale screening of anopheline fauna, but is technically more time-consuming (two-step PCR assay) and expensive than other assays. The AS-PCR[5] assay is more frequently used to distinguish *An. minimus* and *An. harrisoni*, and also related species such as *An. aconitus*, *An. pampanai*, and *An. varuna* (Table 1, Fig. 1), as it is a quick, reliable, and easy one-step PCR application (Garros et al., 2004a; Phuc et al., 2003).

Despite the availability of molecular assays, important data on the bionomics and distribution of *An. harrisoni* are still unavailable. Studies have shown that *An. minimus* and *An. harrisoni* are considered as main malaria vectors in hilly regions in the Oriental Region. They are commonly found at elevations ranging from 200 to 900 m; they also occur at higher elevation but become quite rare at altitudes above 1,500 m (Duc and Huu, 1973; Harrison, 1980; Oo et al., 2004). *Anopheles minimus* species E is restricted to Ishigaki Island in the Ryukyu Archipelago of Japan, a malaria-free region (Fig. 2) (Green et al., 1990; Harbach et al., 2006; Somboon et al., 2001).

Anopheles minimus extends from northern India eastwards through Vietnam and northward across southern China (up to 24.5°N latitude), including Taiwan (Figs. 2 and 3) (Chen et al., 2002; Garros et al., 2005b; Jambulingam et al., 2005; Phuc et al., 2003; Van Bortel et al., 2000). *Anopheles harrisoni* has been collected in Vietnam, Laos, Thailand, Myanmar, and southern China (up to 32.5°N latitude) (Fig. 2) (Chen et al., 2002; Garros et al., 2005b; Kengne et al., 2001; Phuc et al., 2003; Sharpe et al., 2000; Singh et al., 2006; Trung et al., 2004). *Anopheles minimus* and *An. harrisoni* have been found in sympatry over a large area that includes northern and central Vietnam, southern China, northern Laos, and western Thailand (Fig. 2) (Garros et al., 2006). Whether the two smaller areas in central Vietnam and western Thailand are contiguous with the large areas of sympatry is unknown. Data from Cambodia are scarce and so far no specimens of *An. harrisoni* have been found there (Coosemans et al., 2006). Recently, Singh et al. (2006) recorded the presence of *An. harrisoni* (as *An. minimus* species C) from central Myanmar (Mandalay).

Specific trophic behavior of *An. minimus* and *An. harrisoni* has been studied in four countries, Cambodia, Laos, Vietnam (Garros et al., 2006; Trung et al., 2005; Van Bortel et al., 1999), and Thailand (Sungvornyothin et al., 2006b), but no information is available for species E from Japan. These studies showed that adult females of both species are opportunist feeders as they show a high degree of behavioral plasticity (Trung et al., 2005).

Anopheles minimus is one of the main malaria vectors throughout Southeast Asia (Trung et al., 2004). Nowadays, effective control programs make it difficult to estimate the potential role of *An. harrisoni* as a vector, but its higher exophagic and zoophilic behavior compared to *An. minimus* suggests a lower vectorial capacity in some areas of northern Vietnam (Van Bortel et al., 1999). However, the presence of *An. harrisoni*, without *An. minimus*, in central China where malaria is prevalent suggests that this species, along with three species of the Hyrcanus Group, plays an important role in malaria transmission (Chen et al., 2003, 2006, 2002).

Members of the Minimus Complex occur in the forested foothills of India, Southeast Asia, and southern China where the larvae mainly inhabit clear-water canals and streams with grassy margins and slow moving current (Harrison, 1980). However, larvae of *An. minimus* are also found in water tanks in the suburbs of Hanoi (Van Bortel et al., 1999, 2003). Unpublished data from field observations in northern Vietnam showed that *An. harrisoni* occurs in hilly open areas associated with deforested agro-ecosystems such as maize cultivation, whereas *An. minimus* occurs in more undisturbed closed environments with little anthropogenic change (Garros et al., unpublished data). In western Thailand, *An. harrisoni* was found in fewer types of habitats than *An. minimus*, which occurs in a variety of habitats ranging from agricultural fields to forests with a closed canopy (Rongnoparut et al., 2005). This difference between the two regions could be due to the high behavioral plasticity of both species. Future studies need to focus on the landscape associations of each species for the development of malaria risk maps.

3. The Fluviatilis Complex (Funestus Group, Myzomyia Series)

Cytotaxonomic studies of fixed inversions in polytene chromosomes have identified three chromosomal forms within the Fluviatilis Complex, *An. fluviatilis* S, T, and U, informally recognized as sibling species (Subbarao et al., 1994).

The taxonomic status of the Fluviatilis Complex is unresolved and complicated by the recent publication of molecular variants (mostly based on differences in ITS2 sequence), including species X in Orissa State, India (Manonmani et al., 2003; Naddaf et al., 2003, 2002) and form V recorded only in Iran (Hormozgan Province) (Djadid et al., unpublished data). So far no taxonomic study of the complex has been published and no morphological characters are known to differentiate the different forms. Crossing experiments are required to unequivocally determine whether these chromoso-

[4] RFLP-PCR: Restriction fragment length polymorphism-polymerase chain reaction.
[5] AS-PCR: Allele specific-polymerase chain reaction.

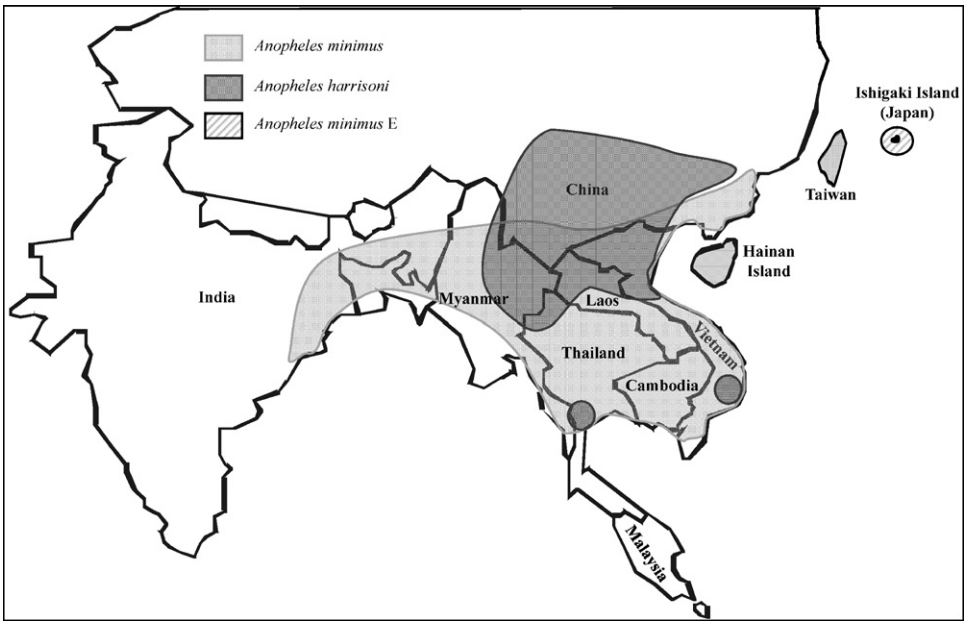

Fig. 2. Distribution of the Minimus Complex based on molecular identifications.

mal forms are definitely distinct species. A complete AS-PCR assay based on sequence differences of the Domain 3 (D3) of 28S rDNA is available for distinguishing members of the complex (Singh et al., 2004b) (Table 1). This assay needs to be applied on a broad scale to determine the precise distribution of each species of the complex.

The Minimus and Fluviatilis Complexes are phylogenetically closely related (Garros et al., 2005a). Based on morphological data and a recent comparison of the D3 sequences of *An. fluviatilis* S and *An. harrisoni*, these two species were deemed to be conspecific (Chen et al., 2003, 2006; Garros et al., 2005a). However, this conclusion was refuted by Singh et al. (2006) who found pair-wise distances of 3.6% and 0.7% for the ITS2 and 28S-D2/D3 loci, respectively, between the species. Chen et al. (2006), in a thorough review, concluded that the Fluviatilis Complex consists of two species, T (with intraspecific variations, including the putative species Y) and U, and two forms, X (different from species S) and V. Singh et al. (2006) removed *An. harrisoni* (as *An. minimus* C) from synonymy with *An. fluviatilis* S, and reported that *An. fluviatilis* X is synonymous with the latter species. Therefore, for Singh et al. (2006) the Fluviatilis Complex includes *An. fluviatilis* species S, T, U, and form V. As currently interpreted, *An. fluviatilis* S is distinct from *An. harrisoni*, which does not occur in India. Further research, however, is needed to clarify the situation.

Little information is available on the bionomics, ecology, and distribution of the taxa outside of India. The Fluviatilis Complex is widely distributed in hilly forested regions of southwestern Asia (Fig. 3) (Bhatt and Kohli, 1996; Malakar et al., 1995; Nanda et al., 2000; Nandi et al., 2000; Subbarao

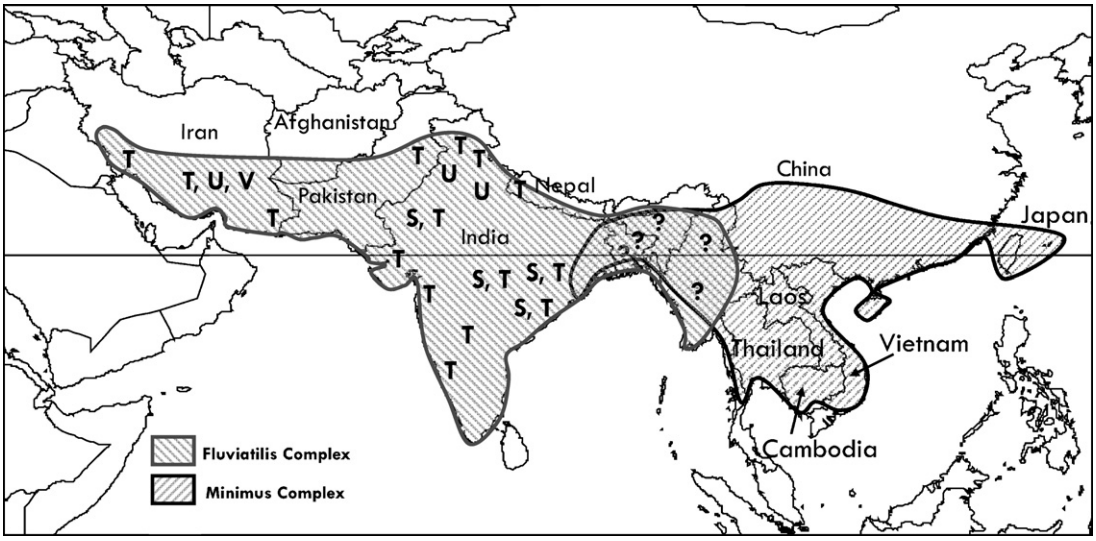

Fig. 3. Distribution of the Fluviatilis and Minimus Complexes (see Fig. 2 for distribution details of the Minimus Complex).

et al., 1994; Vatandoost et al., 2004). However, the distribution of the chromosomal forms has only been well studied in India and Iran. Species T has the largest distribution, which includes India, Nepal, Pakistan, and Iran (Chen et al., 2006). Species U has been recorded in northern India and Iran (Chen et al., 2006; Raeisi et al., 2005). The Fluviatilis and Minimus Complexes overlap in India to Myanmar, but the limits of the overlap are not precisely known (Fig. 3).

Anopheles fluviatilis S is mainly anthropophilic (90%) and endophilic (Nanda et al., 2000), and is known to be a highly competent malaria vector (Nanda et al., 2005), ranking second to *An. culicifacies* species A for total malaria cases transmitted in India (Singh et al., 2004a). *Anopheles fluviatilis* T and U are primarily zoophilic (99%), exophagic, and exophilic, and are regarded as poor or non-vectors, even though species T is known to play a role in the maintenance of malaria in mountainous and hilly regions of India, Pakistan, Iran, and Nepal (Naddaf et al., 2003; Rao, 1984). Members of the Fluviatilis Complex are restricted to forest, especially in mountainous, hilly, and foothill regions of southwestern Asia (Iran, Pakistan, Afghanistan, India, Nepal, Bangladesh, and Myanmar). A study comparing forested and deforested areas of Orissa State in India showed that *An. fluviatilis* S is predominant in forested areas (98% for S; 2% for T), whereas members of the complex are nearly absent in deforested areas where only one specimen of species T was collected (Nanda et al., 2000).

4. The Culicifacies Complex (Funestus Group, Myzomyia Series)

As for the Fluviatilis Complex, the taxonomy of the Culicifacies Complex is unresolved. The complex includes five chromosomal forms, denoted as species A, B, C, D, and E. The members of the complex are cytogenetically defined by fixed paracentric inversions of polytene chromosomes, except for species B and E which are homosequential. However, it is possible to distinguish species B and C based on mitotic chromosomes in semi-gravid females: the Y-chromosome is acrocentric in species B and submetacentric in species E (Kar et al., 1999). Thus, so far no comparative morphological study of the complex has been undertaken.

As cytogenetic analyses can only be done on semi-gravid females, routine field identification is limited during disease control programs. Isozyme analyses based on *Ldh* (Lactate dehydrogenase) distinguish species A and D from species B and C with 95% confidence (Adak et al., 1994), but the main vector, species E, cannot be distinguished from B and C (Kar et al., 1999). Recently developed molecular assays include an AS-PCR based on the D3 domain (Singh et al., 2004c) and a RFLP-PCR based on cytochrome oxidase II (COII) and ITS2 using two restriction enzymes (Goswani et al., 2005). However, these two applications only distinguish A and D from B, C and E (Table 1). In the latter group, an additional RFLP-PCR can distinguish species E from B and C (Goswani et al., 2005), which is useful where species A and B (India) or species B and E (India and Sri Lanka) are sympatric. No currently available application can directly identify all five species, which raises some doubt about their validity.

The Culicifacies Complex is widely distributed from southern China, Vietnam, Laos, Cambodia, Thailand, and Myanmar to India, Pakistan, and Iran, with a western extension into the Arabian Peninsula and Ethiopia (Fig. 4) (Amerasinghe et al., 1999; Kobayashi et al., 1997; Rowland et al., 2002; Van Bortel et al., 2002; Vatandoost et al., 2004; Zhang and Yang, 1996). In parallel with the Fluviatilis Complex, the bionomics and ecology of the species have been largely studied in India, and data are missing for other regions.

Four species of the complex, i.e. species A, C, D, and E, are malaria vectors in India; however, species E is the most efficient vector. Species B is a poor or non-vector (Subbarao et al., 1988). Its distribution is the widest of all the species—it occurs from Iran to China and is the only species of the complex in eastern areas,

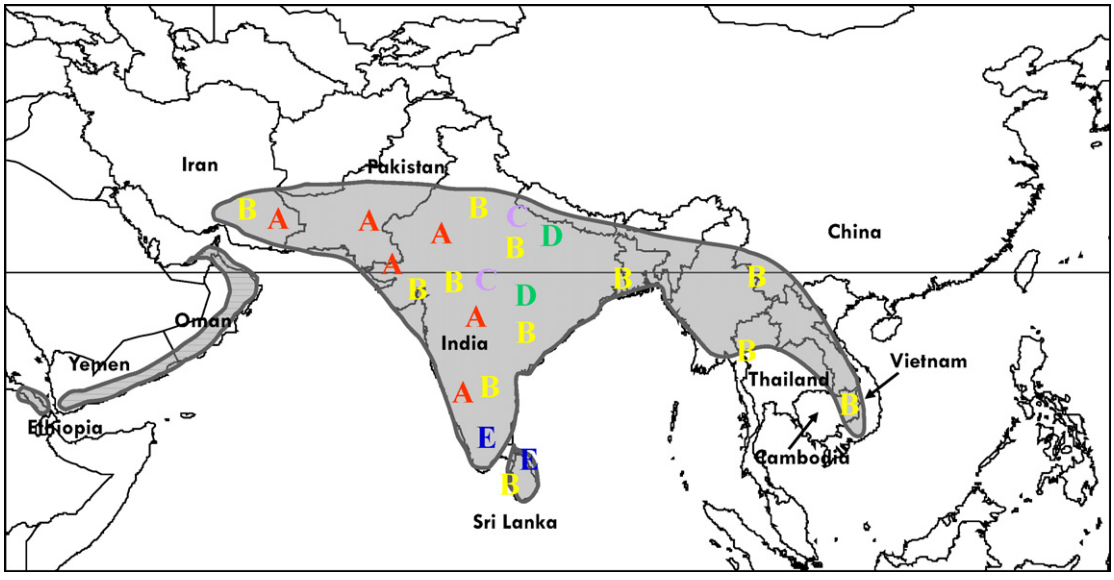

Fig. 4. Distribution of the Culicifacies Complex (grey) and each of the five species (A to E).

including southern China (Sichuan), Vietnam, Laos, Cambodia, and northwestern Thailand (Harrison, 1980; Harrison et al., 1990; Kobayashi et al., 1997; Van Bortel et al., 2002; Vatandoost et al., 2004; Zhang and Yang, 1996). Westwards, it occurs in sympatry with other species of the complex, especially species E, the most important malaria vector in southern India and Sri Lanka (Fig. 4). No data are available for the species that occur in the Arabian Peninsula and Ethiopia.

Anopheles culicifacies s.l. is responsible for the transmission of 60–70% of malaria cases in India, mainly due to species E, which is highly anthropophilic (90%) and endophilic (Subbarao, 1988). Species A, C, and D are mainly zoophilic, with a low anthropophilic index that does not exceed 3–4% (Subbarao and Sharma, 1997). Therefore, these three species play a minor role in malaria transmission (Sharma et al., 1995), although species C was found responsible for local malaria transmission in deforested riverine areas of central India (Nanda et al., 2000). Species B is highly zoophilic but it sometimes plays a role in sporadic epidemics in Myanmar, Laos, and Vietnam (Oo et al., 2004; Sucharit et al., 1988; Trung et al., 2004). *Anopheles culicifacies* s.l. occurs in different ecotypes, such as forests with perennial streams and deforested riverine ecosystems (hills, plains) or irrigated areas. Larval habitats include irrigated canals, rock pools, and sandy pools near paddies or quarries. A study in Sri Lanka showed that species E exploits a wide range of habitats, which reflects a greater environmental adaptability of this malaria vector than species B (Surendran and Ramasamy, 2005). The study by Nanda et al. (2000) in Orissa (India) that compared forested and deforested ecosystems showed that specimens of *An. culicifacies* s.l., unlike those of the Fluviatilis Complex (see above), are present in both ecosystems. In forested areas, *An. culicifacies* C (71%) outnumbered species B; in deforested areas species C (78%), B (21%), and species A (1%) were present. This also shows the ability of *An. culicifacies* C to adapt to environmental changes.

Even though the Fluviatilis and Culicifacies Complexes include some major malaria vectors, further studies are needed to resolve the taxonomic status of the individual species. Other complexes, especially the Dirus and Leucosphyrus Complexes and the Maculatus Group (see below) have been thoroughly studied and serve as models for the delineation of species and the development of molecular identification methods that provide important tools for improving our knowledge of the distribution and bionomics of the individual species.

5. The Dirus Complex (Leucosphyrus Group, Neomyzomyia Series)

The Dirus Complex includes seven species that vary from highly competent malaria vectors to non-vectors of human malaria in tropical evergreen rainforests, cultivated forests, and forest fringes throughout Southeast Asia (Baimai, 1998; Oo et al., 2004) (Fig. 5). The taxonomy of the complex was recently resolved and all the species now have morphological descriptions and formal Latin names (Sallum et al., 2005), and their distributions in Southeast Asia have been mapped (Baimai, 1998; Obsomer et al., 2007).

Anopheles dirus (=*An. dirus* species A) has a wide distribution in eastern Asia—it is known to occur in Myanmar, Thailand, Cambodia, Laos, Vietnam, and Hainan Island (China) (Fig. 6). *Anopheles cracens* (=*An. dirus* B) is known from southern (peninsular) Thailand, peninsular Malaysia, and

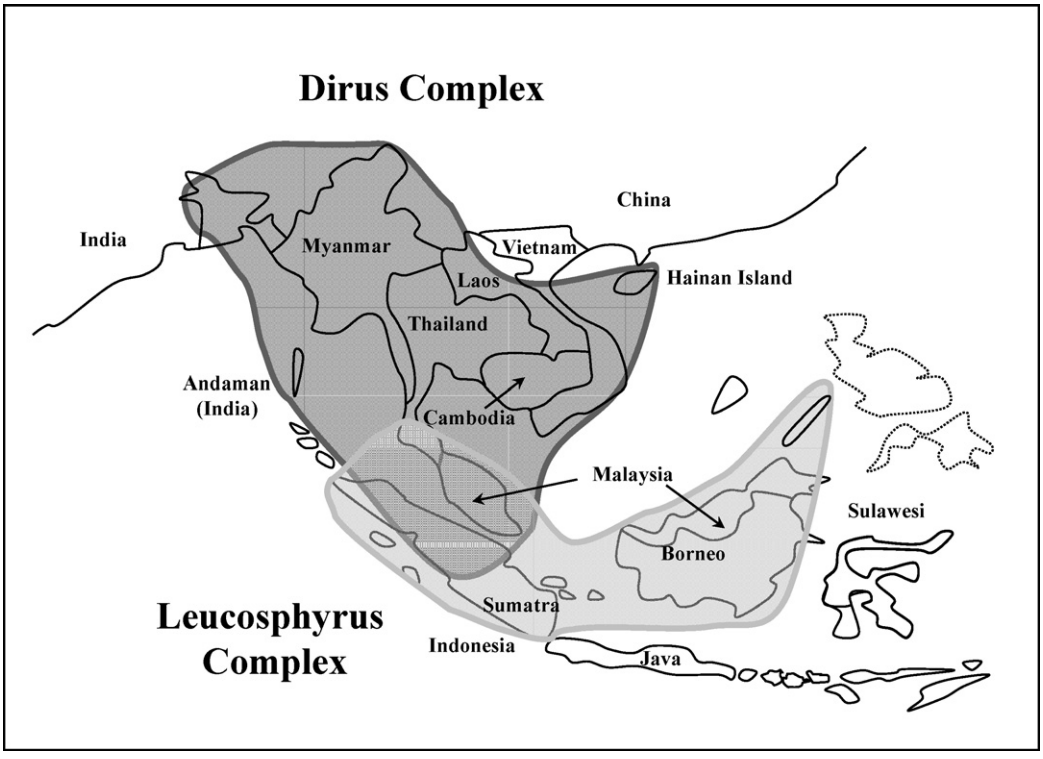

Fig. 5. Distribution of the Dirus (dark grey) and Leucosphyrus (light grey) Complexes showing the zone of overlap in the Malay Peninsula and Sumatra (Indonesia).

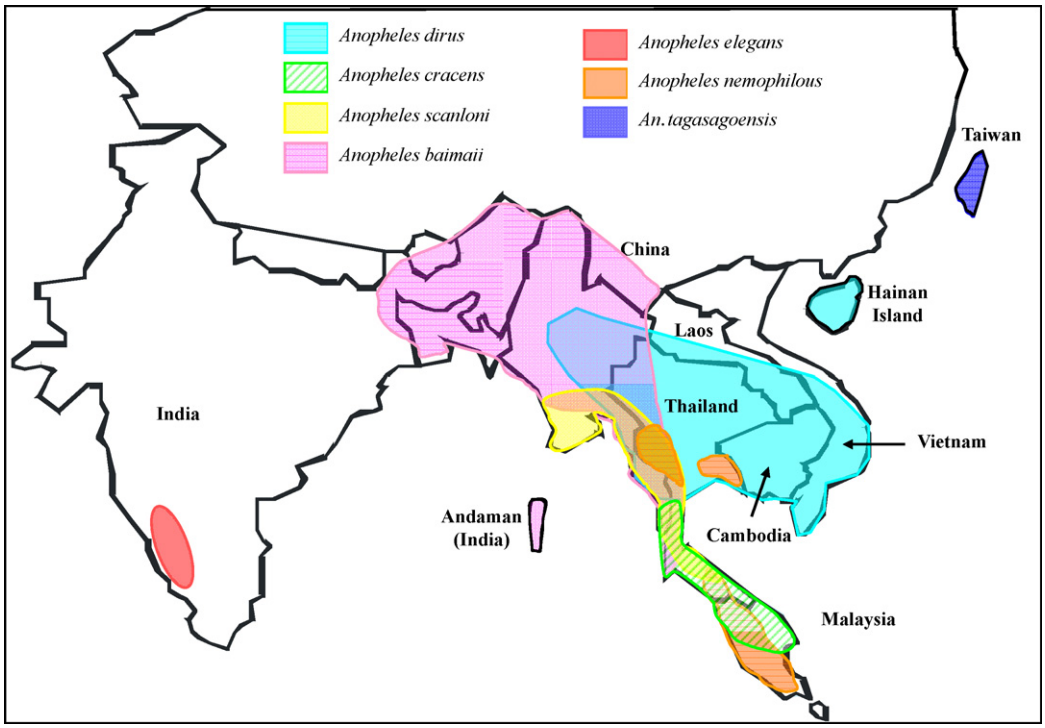

Fig. 6. Distribution of the seven members of the Dirus Complex (records of *An. cracens* in Sumatra are not shown).

Sumatra (Indonesia). *Anopheles scanloni* (=*An. dirus* C) occurs in a relatively narrow area along the borders of southern Myanmar and western and southern Thailand, and appears to be intimately linked to limestone environment. In Thailand, *An. scanloni* populations are restricted to ''islands'' of limestone karst habitat that support high levels of population structure (O'Loughlin et al., 2007). *Anopheles baimaii* (= *An. dirus* D) occurs in areas from southwestern China (Yunnan Province), western Thailand, Myanmar, and Bangladesh to northeastern India and the Andaman Islands (India) (Sallum et al., 2005). *Anopheles elegans* (=*An. dirus* E) is restricted to hilly forests of southwestern India. *Anopheles nemophilous* (=*An. dirus* F) has a patchy distribution along the Thai–Malay peninsula and Thai border areas with Myanmar and Cambodia. Finally, *An. takasagoensis* is restricted to Taiwan.

Initial recognition of the species was achieved primarily by cross-mating experiments and studies of polytene chromosome banding patterns, and subsequently by electrophoresis analyses of allozyme variation (Baimai et al., 1987; Green et al., 1992a; Hii, 1984). Since several species of the complex occur in sympatry, it was important to clearly identify the specimens. Therefore, two AS-PCR assays were developed (Table 1). Walton et al. (1999b) designed an AS-PCR based on ITS2 sequence to distinguish and unambiguously identify *An. dirus*, *An. cracens*, *An. scanloni*, *An. baimaii*, and *An. nemophilous*, and Manguin et al. (2002) developed a SCAR[6]-based PCR to identify the same five species. The recent revisionary study of the complex by Sallum et al. (2005) revealed that morphological characters are present in all life stages that distinguish the species, but the authors stated that ''Due to the variability of their elaborate ornamentations, separating the many species of this group [Leucosphyrus Group] will always be morphologically challenging.''

The Dirus Complex includes primary vectors of forest malaria, principally *An. dirus* and *An. baimaii*, which transmit both *Plasmodium falciparum* and *P. vivax*. Records show that these species are anthropophilic, mainly exophagic, and highly competent vectors (Baimai et al., 1988). *Anopheles cracens* is an anthropophilic species that may play a role in malaria transmission, and also *An. scanloni* or potentially *An. elegans*. The availability of morphological and molecular identification methods will allow researchers to investigate the degree to which these species may be involved in malaria transmission. The two other species of the complex, *An. nemophilous* and *An. takasagoensis*, appear to be non-vectors of human malaria due to their zoophilic behavior (Baimai, 1988; Peyton and Harrison, 1980).

Species of the Dirus Complex are forest mosquitoes (forested foothills, deep forests, cultivated forests), but are occasionally collected in open areas adjacent to forest (forest fringes). Larvae of the species typically inhabit small, usually temporary, mostly shaded bodies of fresh, stagnant water, such as pools, puddles, animal footprints, streams, and even wells in hilly or mountainous regions with primary, secondary evergreen or deciduous forests, bamboo forests, and fruit and rubber plantations (Baimai et al., 1988; Oo et al., 2002; Prakash et al., 2002).

Species of the Dirus Complex are closely related to members of the Leucosphyrus Complex, and this has been the cause of considerable confusion in published literature. Numerous studies, mainly based on crossing experiments, cytogenetics,

[6] SCAR: Sequence characterized amplified region.

allozyme data, and more recently molecular methods, have been necessary to recognize the individual species and to confirm their taxonomic status (Baimai, 1988, 1989; Baimai et al., 1987; Green et al., 1992a; Hii, 1984; Sallum et al., 2005).

6. The Leucosphyrus Complex (Leucosphyrus Group, Neomyzomyia Series)

The Leucosphyrus Complex of four species, recently revised by Sallum et al. (2005), includes *An. balabacensis*, *An. introlatus*, *An. latens* (= *An. leucosphyrus* A), and *An. leucosphyrus* (= *An. leucosphyrus* B). *Anopheles leucosphyrus* and *An. latens* are morphologically indistinguishable, but they can be differentiated from *An. balabacensis* and *An. introlatus* (Sallum et al., 2005). Various members of the complex occur in southern Thailand, Malaysia (Sabah, Sarawak, mainland), Indonesia (Java, Kalimantan, Sumatra), and Balabac Island of the Philippines. *Anopheles latens* and *An. introlatus* are sympatric with members of the closely related Dirus Complex in the Malay Peninsula, including southern Thailand (Fig. 5). *Anopheles latens* is also widely distributed in Borneo (Kalimantan, Sarawak, Sabah), together with *An. balabacensis* in the forested areas of eastern Borneo (Fig. 7) (Rattanarithikul and Harrison, 1973; Rattanarithikul et al., 2006). *Anopheles leucosphyrus* has only been found in Sumatra.

Two species, *An. balabacensis* and *An. latens*, are recognized as malaria vectors with sporozoite infection rates of 1.3% and 1%, respectively, and both species are reported to be exophagic and exophilic (Harbach et al., 1987). No information exists on the vectorial status of *An. leucosphyrus*. *Anopheles introlatus* is known to transmit simian malaria in Malaysia (Eyles et al., 1963). Overall, the importance of the species as vectors of human malarial parasites is not well established because the species have been largely misidentified.

Species of this complex are forest mosquitoes and share the same types of habitats as members of the Dirus Complex. Typical larval habitats are freshwater ground pools along stream margins, flood pools, seepage pools, sandy pools, wallows, small shallow streams, elephant footprints, and even large swamps (Leicester, 1903; Sallum et al., 2005). Water in the habitats may be stagnant or slow running, turbid or clear, and partially or heavily shaded. The species occur at elevation ranging from 70 to 500 m (Sallum et al., 2005).

7. The Maculatus Group (Neocellia Series)

The Maculatus Group includes eight formally named species (Harbach, 2004): *An. pseudowillmori* and *An. willmori* and six species assigned to subgroups, the Maculatus Subgroup, which includes *An. dispar*, *An. greeni*, *An. dravidicus*, and *An. maculatus*, and the Sawadwongporni Subgroup, which includes *An. notanandai* and *An. sawadwongporni* (Ma et al., 2006). Members of the group are variously distributed in areas from India to Indonesia and the Philippines. Two species, *An. dispar* and *An. greeni* (Fig. 8), are found exclusively in the Philippines (Rattanarithikul and Harbach, 1990; Torres et al., 1997). In addition, a recent genetic study of the Maculatus Group found that chromosomal form K in eastern Thailand has an unique ITS2 sequence that is 3.7% divergent from the closest taxon (*An. sawadwongporni*), which indicates it is a distinct species (Walton et al., 2007). Hence, the group would appear to include nine species with form K falling into the Sawadwongporni Subgroup (Ma et al., 2006).

Adults of the complex are difficult to identify to species using morphology because of overlapping characters. In fact, members of the group were first recognized using cytogenetics (Baimai et al., 1993; Green and Baimai, 1984; Green et al., 1985, 1992b). Eleven cytogenetic forms were described that represent eight genetic species (Green et al., 1991; Rattanarithikul and Green, 1986). The correspondence between the formally named species and the 11 chromosomal forms is given by Walton et al. (2007).

A reliable and easy RFLP-PCR assay (Table 1) was developed to distinguish *An. dispar* and *An. greeni* (Torres

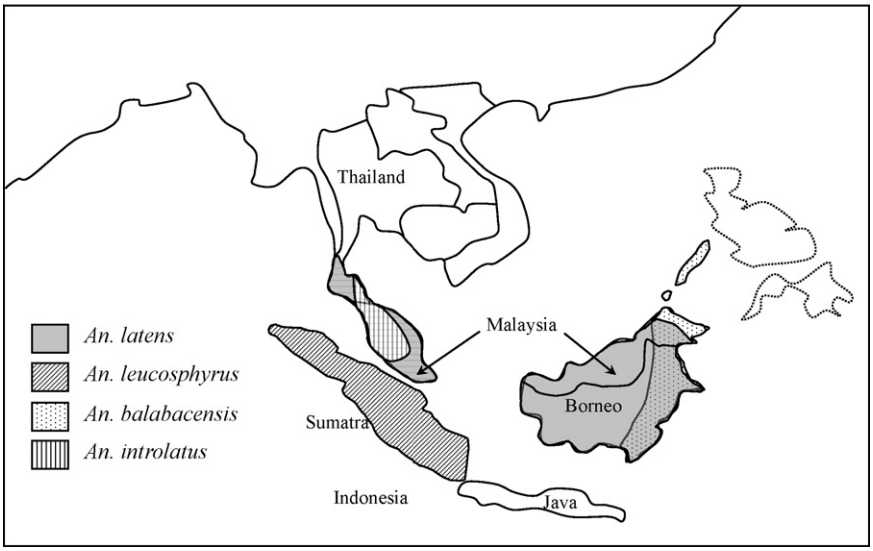

Fig. 7. Distribution of the four members of the Leucosphyrus Complex.

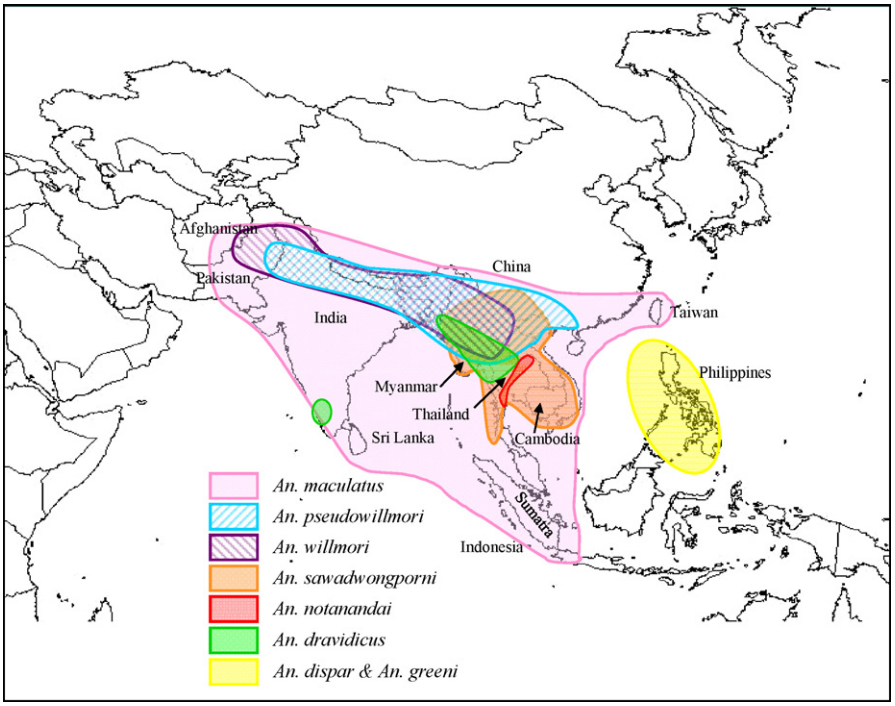

Fig. 8. Distribution of the eight members of the Maculatus Group.

et al., 2000). Use of this method should shed light on the vector status of these two species as previous data collected on *An. maculatus* s.l. in the Philippines is unreliable. Two allele-specific-PCR (AS-PCR) assays (Table 1) have also been developed to distinguish *An. dravidicus*, *An. maculatus*, *An. pseudowillmori*, *An. sawadwongporni*, and either *An. willmori* (Ma et al., 2006) or chromosomal form K (Walton et al., 2007).

Anopheles pseudowillmori occurs from northern India (Punjab, Assam, Kasauli) through northwestern Thailand and southern China (Yunnan), and *An. willmori* is found at higher altitudes in Afghanistan, Pakistan (Kashmir), northern India (Punjab to Assam), Nepal, northern Thailand (Chiang Mai), and southern China (Yunnan) (Green et al., 1992b; Li et al., 2003; Pradhan et al., 1970; Rao, 1984). *Anopheles maculatus* has the widest distribution, ranging from Afghanistan, Pakistan, India, and Sri Lanka eastward through Southeast Asia to Taiwan and Indonesia. *Anopheles dravidicus* has a very peculiar distribution as it is found in two separate areas, one in southwestern India (Nilgiri Hills) and the other in northwestern Myanmar (Kale Valley) and northern Thailand (Kanchanaburi, Tak, Chiang Mai, Chiang Rai, Mae Hong Son, Loei, Phrae) (Rattanarithikul and Green, 1986; Rattanarithikul et al., 2006). This disjunctive distribution is surprising and further investigation must be carried out to determine whether it is actually the same species in both areas and/or to find new populations in the intervening areas. The two members of the Sawadwongporni Subgroup are distributed in the Mekong Region: *An. sawadwongporni* occurs from Myanmar to China and Vietnam and *An. notanandai* is only known from west-central Thailand (Baimai et al., 1993; Green et al., 1992b; Oo et al., 2004; Rattanarithikul and Green, 1986).

Members of the Maculatus Group are variously involved in malaria transmission in the Oriental Region (Rahman et al., 1993; Rongnoparut et al., 1996; Upatham et al., 1988), but individual species may have quite different vectorial capacities. The precise role of each species is unknown due to misidentifications based on morphological characters. In addition, the vectorial capacity of an individual species seems to vary depending on location. *Anopheles maculatus* has a wide distribution (Fig. 8), but it is considered to be a major malaria vector only in eastern India, southern Thailand, peninsular Malaysia, and Java (Barcus et al., 2002; Green et al., 1991; Hodgkin, 1956; Rahman et al., 1993; Rattanarithikul et al., 1996b; Reid, 1968). Whereas *An. willmori* is one of the primary vectors in Nepal (Pradhan et al., 1970), it is rare in Thailand and not involved in malaria transmission. *Anopheles pseudowillmori* is a secondary vector in northwestern Thailand along the Myanmar border (Green et al., 1991, 1992b). *Anopheles sawadwongporni* has been found with sporozoite rates of 1–2% in Thailand where it is an important malaria vector along with *An. maculatus* (Rattanarithikul et al., 1996a; Somboon et al., 1998). The two species that occur in the Philippines, *An. dispar* and *An. greeni*, are regarded as secondary vectors but their specific involvement in malaria transmission has not been determined. These two species exhibit strong exophagic and zoophilic behavior, with a biting rate on water buffalo that is 50 times the human landing rate (Torres et al., 1997). *Anopheles notanandai* and *An. dravidicus* are not known to be involved in malaria transmission (Mouchet et al., 2004).

Members of the Maculatus Group are found in or near hilly and mountainous areas. Larvae of *An. maculatus* s.l. have been collected in a diversity of habitats, including ponds, lakes, swamps, ditches, wells, different pools (grassy, sandy, ground, flood, stream), stream margins, seepages springs, rice fields, foot or wheel prints, and occasionally tree holes or bamboo

stumps (Rattanarithikul et al., 1995, 2006, 1994). More specific studies have shown that the species have preferred habitats. For instance, larvae of *An. willmori* are found only along the margins of streams at altitudes between 990 and 1450 m in northern Thailand, and larvae of *An. pseudowillmori* have been collected primarily in rice fields, stream margins, ponds, pits, and wells (Rattanarithikul et al., 1995, 2006).

8. The Sundaicus Complex (Pyretophorus Series)

Behavioral and ecological differences, notably the occurrence of immature stages in brackish and freshwater, led Reid (1970) to suspect that *Anopheles sundaicus* was a species complex. Subsequently, the presence of three cytogenetic forms (A, B, and C) were detected in Sumatra, Java, and Thailand, and confirmed by allozyme analysis (Sukowati et al., 1996, 1999). *Anopheles sundaicus* species A was found in both Indonesia and Thailand; species B, was strongly linked to freshwater in northern Sumatra and central Java; and species C was only found at a single locality (Asahan) in northern Sumatra where all three cytotypes were collected in sympatry. A fourth cytotype D (Nanda et al., 2004) was later identified from the Nicobar and Andaman Islands of India (Fig. 9) and recently confirmed by molecular analysis of ITS2 and D3 sequences of rDNA (Alam et al., 2006). The identity of *An. sundaicus* s.s. was fixed with the designation of a neotype from the Lundu District of Sarawak (Fig. 9) in northern Borneo (Malaysia) based on morphology and sequences for the ITS2 rDNA and COI mtDNA loci (Linton et al., 2005). Finally, two allopatric species were verified based on two mitochondrial markers, COI and Cytochrome *b* (Cyt-b), and the ribosomal marker, ITS2 (Dusfour et al., 2004b). One of these two species, *An. epiroticus* (=*An. sundaicus* A), occurs in coastal brackish water sites from southern Vietnam to peninsular Malaysia (Fig. 9). The other species, *An. sundaicus* species E (Fig. 9), occurs in Sumatra and Java (Dusfour et al., 2007b). Neither ITS2 nor COI revealed a distinction between cytogenetic forms B and C from Asahan (Sumatra), rather the molecular data indicated the existence of only one species, which was informally designated *An. sundaicus* species E (Dusfour et al., 2007b). An allele-specific PCR was developed for the identification of three of the four species: *An. sundaicus*, species E, and *An. epiroticus* (Table 1) (Dusfour et al., 2007a).

Anopheles sundaicus s.l. is a malaria vector in coastal areas (Fig. 9) that extend from northeastern India eastwards to southern Vietnam (south of the 11th parallel) and southwards to the Andaman and Nicobar Islands (India), Malaysia (peninsular and Borneo), and Indonesia (Java, Sumatra, Sulawesi) (Dusfour et al., 2004a). Adult females are mainly anthropophilic and endophilic, and exhibit both endophagy and exophagy. This taxon is responsible for regular malaria outbreaks in certain areas where it occurs in great numbers (Oo et al., 2004). The availability of a reliable PCR identification method will allow future investigators to determine more precisely the behavior (and the distribution) of each species of the complex.

Due to its ecological and behavioral plasticity, *An. sundaicus* s.l. has adapted to a range of coastal and inland environmental situations. It is regarded mainly as a brackish water taxon, but larvae tolerate a wide range of salinity from freshwater to sea water (Nguyen Tang Am et al., 1993). The immature stages require sunlit habitats with stagnant fresh or brackish water, floating algae, and non-invasive vegetation. Filamentous floating algae and aquatic plants appear to be crucial for the development of the larvae. Aquatic flora provides food (micro-algae and bacteria) and protection against predators. Particularly favorable habitats are coastal shrimp/fish ponds or irrigated inland sea-water canals, but immature stages also inhabit ponds, swamps, mangrove, and rock pools (Chang et al., 2001; Dusfour et al., 2004a; Harinasuta et al., 1974; Ikemoto et al., 1986; Kalra, 1978; Nguyen Tang Am et al., 1993). The affinity of *An. epiroticus* with aquaculture, particularly shrimp and fish ponds in southern Vietnam (Nguyen Tang Am et al., 1993; Trung et al., 2004), needs to be monitored on a larger scale as this economic activity is growing throughout Southeast Asia with an increasing risk of malaria epidemics.

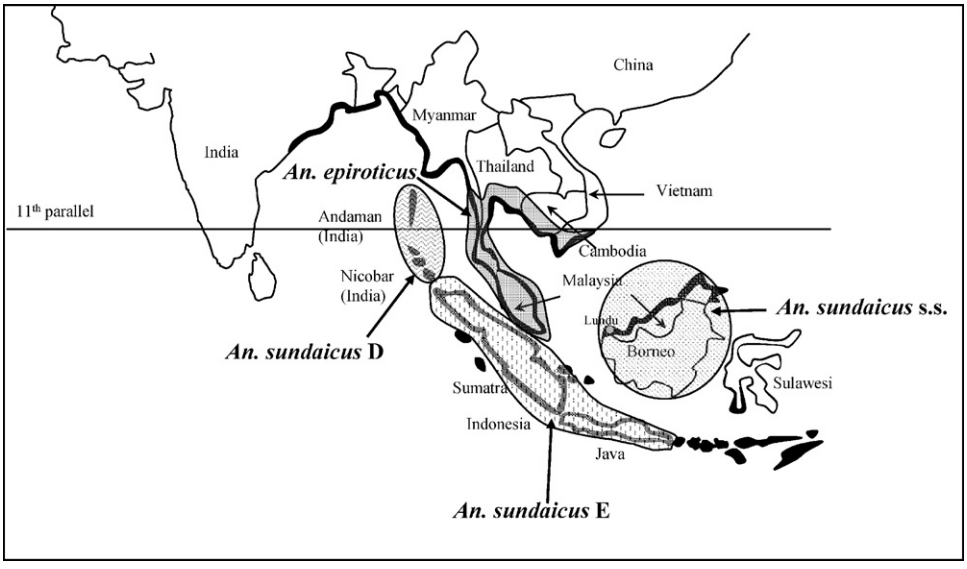

Fig. 9. Distribution of the four members of the Sundaicus Complex.

9. Implications for vector control

Malaria vector control programs in Southeast Asia are largely based on the use of insecticides for indoor residual spraying (IRS) and, to a lesser extent, insecticide treated nets (ITNs). IRS programs were first organized in the 1950s and implemented for decades in the Southeast Asian countries. Later, ITNs were required to supplement the IRS campaigns. Nowadays, IRS is mainly used to control focal epidemics (WHO, 2007c). Until recently, coverage of ITN and IRS only reached 10–20% of the populations at risk, but with the recent support of the Global Fund to the malaria endemic countries, the use of ITNs has increased significantly. However, more training and resources are required. The current trend in Southeast Asian is to rely on the increasing use of ITN based on several actions: (i) expansion to a maximum coverage of ITN, including re-treatments; (ii) promotion of long-lasting ITNs to avoid re-treatments; (iii) lowering the cost of bed nets to a minimum through tax exemption; and (iv) increased access to nets through the commercial sector and targeted subsidies for the poorest people (WHO, 2007d).

These actions will most likely improve the malaria situation in Southeast Asia but they should be closely associated with a better knowledge of the targeted malaria vectors. The success of vector control programs is highly dependant on a thorough knowledge of behavior and bionomics of the vector, which must be precisely identified to ensure the application of appropriate control measures (Trung et al., 2005). Precise information about ecological changes, the behavioral plasticity of most *Anopheles* species as mentioned above, and the accurate identification of the vector species is required for the development of effective vector control strategies that are based principally on IRS and ITN. For instance, IRS is more efficient where the vector is endophilic and bites late at night, such as *An. minimus* and *An. epiroticus*, whereas ITNs seem more effective than other techniques for the control of exophagic and exophilic mosquitoes such as *An. dirus* s.l. (Trung et al., 2005). For the protection of people at occupational risk, such as forest workers, the use of treated hammock nets needs to be implemented, although their efficacy against this vector is sometimes questionable (Trung et al., 2004). Rapid ecological changes, such as those occurring in Southeast Asia, especially deforestation, are modifying the cohort of malaria vector species, as noted for the Minimus and Dirus Complexes, and surveillance needs to be done on a regular basis because vector control programs are highly dependant on the vector species and its behavior. This is necessary for appropriately targeting the species involved in malaria transmission. Vector control should also be adapted to seasonal variations. In hilly forested areas of Southeast Asia, it is common knowledge that malaria transmission is perennial due to the presence of species of the Dirus and Minimus Complexes, the first being present mainly during the rainy season and the latter during the drier periods of the year (Harbach et al., 1987; Ismail et al., 1978; Phan, 1998; Rattanarithikul et al., 1996a).

Beside the use of chemical insecticides through IRS and ITN, efforts to minimize this dependency have been undertaken while searching and developing eco-friendly alternative methods for the control of vector mosquitoes. Instead of controlling adult mosquitoes, these alternative methods target immature stages, particularly larvae. Application of environmental management and biological control need to be utilized wherever it is cost effective and feasible (WHO, 2007d). Nowadays, biological control methods are once again receiving much research focus for malaria vector control. Larvivorous fish have been used for over 100 years for controlling mosquito densities and malaria incidence in many countries, including India, Malaysia, Papua New Guinea, and Thailand (Rozendaal, 1997). *Gambusia affinis* and *Poecilia reticulata* are the most successful and effective larvivorous fish for vector control (Ghosh and Dash, 2007). In India, remarkable results have been achieved for the control of malaria vectors like *An. culicifacies* species A that breed predominantly in ponds and wells (Ghosh et al., 2005). Larvivorous fish have also been used to control *An. sundaicus* s.l. in Indonesia (Ikemoto et al., 1986) and *An. dirus* s.l. in gem pits in Thailand (Kitthawee et al., 1993). In certain areas where *An. dirus* s.l. utilizes small habitats, for example wells in Myanmar, this biological control method should be applied and may give good results (Oo et al., 2004). However, the effectiveness of this strategy is questionable (Meek, 1995), particularly in large wetlands where its efficacy has not been demonstrated. Its implementation requires some baseline knowledge of vector biology, and should be included as part of an integrated malaria control program (Ghosh and Dash, 2007; Meek, 1995). Another potential biological control agent, tested in Japan, involves the use of copepod species as predators in rice fields during the summer (Dieng et al., 2003). Results showed that copepods are efficient biological control agents against mosquito larvae. However, the reduction of larval densities is temporary if not properly managed; hence, the method only has a limited effect on malaria transmission (Subbarao and Sharma, 1997).

Various biolarvicides have also been thoroughly investigated, especially strains of the bacteria *Bacillus sphaericus* and *B. thuringiensis* var. *israelensis* H-14 (*Bti*), which are highly effective against mosquito larvae at very low doses and safe to other non-target organisms. Formulations of *B. sphaericus* have been used against *An. stephensi*, *An. subpictus*, and *An. culicifacies* s.l., but repeated applications in the same habitat resulted in the development of resistance in the larvae of the targeted species (Mittal, 2003). Therefore, *B. sphaericus* has limited prospects for the control of malaria vectors. *Bti* formulations have a broader spectrum of activity against *Aedes*, *Culex*, and *Anopheles* species, but it was found less effective against *Anopheles* due to many limitations (exposure to sunlight reduces efficacy, weekly application required in most habitats, etc.) (Mittal, 2003).

Biological control may have an impact on malaria vectors in certain specific situations but in most cases it has proven to be too tedious for general use because the types of larval habitats of the main malaria vectors are not conducive of this kind of strategy (Meek, 1995). However, biological control can still be considered within an integrated vector management strategy based on selective application of various control measures determined by the eco-epidemiological situation of malaria. For instance, in certain rural communities, biological control

may be a helpful supplement to IRS or ITN, particularly during the dry season when larvae of vector species are concentrated in relatively few habitats (Walker and Lynch, 2007).

Environmental management for larval control is another option, especially against *An. sundaicus* s.l. Some success in controlling this taxon was achieved in Malaysia by building bunds and digging drains for excluding brackish water (Moorhouse and Wharton, 1965). More recently, larvicide by clearance of algae has been used successfully in Indonesia against this malaria vector species (Kirnowardoyo, 1988; Soekirno et al., 1983). In Malaysia, larvae of *An. maculatus* s.l. have been controlled by periodic flushing of streams using small dams fitted with siphons (Williamson and Scharff, 1936), or by drainage (Moorhouse and Wharton, 1965). These larvicidal methods are opportunities to complement adulticiding along with other components of integrated vector management, and have a direct bearing on concerns about insecticide resistance, environmental impact, rising costs of IRS, and logistical constraints (Walker and Lynch, 2007).

The lesson learned over the years is that malaria control is too complex to be addressed by a single approach (Shiff, 2002). It is important to tailor the strategy to the prevailing malaria vector species, as well as ecological and epidemiological conditions (Mouchet and Carnevale, 1998). We now understand the ecological conditions that affect and regulate the distribution and abundance of mosquito populations (Gillies, 2001), and reliable and easy molecular methods have been developed to supplement morphological identification of closely related and isomorphic species (Table 1). Therefore, combined sustainable and appropriate vector control measures in relation to the targeted vector species and prophylaxis must be implemented to achieve the goal of the revised strategy of the Southeast Asian Regional Committee, which aims to reduce the level of malaria morbidity and mortality recorded in 2000 by 50% before 2010 (WHO, 2007a).

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