Eco-Ethological Heterogeneity of the Members of the *Anopheles minimus* Complex (Diptera: Culicidae) in Southeast Asia and Its Consequences for Vector Control

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J. Med. Entomol. 41(3): 366–374 (2004)

ABSTRACT The presence of cryptic species within *Anopheles minimus* s.l. Theobald, one of the most widespread malaria vectors in Southeast Asia, was suggested on the basis of behavioral heterogeneities observed within this taxon. Subsequently, two species, A and C, were recognized. However, the existence of these cryptic species did not explain all observed behavioral heterogeneities within this complex. Besides, data on the behavior of vectors are essential to understand the dynamics of disease transmission and thus evaluate the appropriateness of vector control measures. Different collection methods were used to collect *Anopheles* species from several localities in Southeast Asia to assess the inter- and intraspecific behavioral divergences of *An. minimus* A and C. Collection results were subjected to a correspondence analysis. The members of the *An. minimus* complex were identified by use of the *octanol dehydrogenase* allozyme profiles or the polymerase chain reaction-restriction fragment length polymorphism assay. Large intraspecific behavioral differences were observed among populations of *An. minimus* A. These populations belong to the same species on the basis of the applied genetic markers. In northern Vietnam, species A tended to be more zoophilic, whereas in the study sites of south central Vietnam, Cambodia, and Laos it showed marked antropophilic behavior when cattle were scarce. In the most northern study site, *An. minimus* A showed noteworthy endophilic behavior. *An. minimus* C was primarily zoophilic and based on this behavior, its role in malaria transmission is questionable. However, it was only found in one locality, so that intraspecific behavioral variation could not be assessed. *An. minimus* A is able to change its host preference in function of local situations in host availability. Hence, its role in malaria transmission can differ from region to region. Similarly, the impact of vector control on this species may differ between localities. In conclusion, intraspecific behavioral differences in *Anopheles* species can occur and these behavioral heterogeneities, albeit important for disease transmission and control, are not a priori indicative for the presence of cryptic species.

KEY WORDS *Anopheles minimus*, behavior, species recognition, Southeast Asia, malaria control

THE BEHAVIOR OF *Anopheles* species largely determines their status as malaria vectors (Klowden 1996, Costantini et al. 1998). Consequently, insights into vector behavior have direct practical implications for malaria control by giving essential information on disease dynamics and on the appropriateness of existing control measures. Moreover, behavioral heterogeneities relevant for disease transmission are often used as a first indication for the existence of cryptic *Anopheles* species (Coluzzi et al. 1979, Foley et al. 1993).

The adult behavior of the Southeast Asian malaria vector *Anopheles minimus* s.l. Theobald is highly diverse. In India, Nepal, and Bangladesh, the species is an anthropophilic and endophilic vector that responds well to vector control measures (Covell 1944, Khan and Talibi 1972, Parajuli et al. 1981, Jana-Kara et al. 1995, Dev 1996). In southern China, however, it is predominantly exophilic (Covell 1944), whereas the species was eliminated from the plain regions of Thailand, where it was primarily known as an endophilic and endophagic vector (Ismail et al. 1974, Suthas et al. 1986). Yet, the use of DDT in forested hilly regions in Thailand provoked changes from endophagy to exophagy and from endophily to exophily (Ismail et al. 1974). Ismail et al. (1974) suggested that two cryptic species were involved: the endophilic and endophagic became scarce after DDT pressure, whereas the ex-

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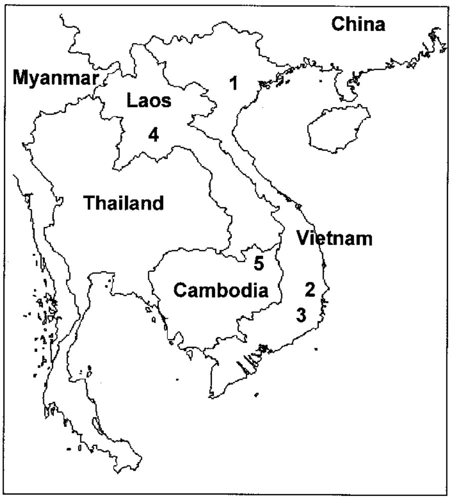

Fig. 1. Map of Southeast Asia, indicating the five study sites. 1) Khoi, Hoa Binh Province. 2) Lang Nhot, Khanh Hoa Province. 3) Village 3, Binh Thuan Province. 4) Na Ang, Vientiane Province. 5) Char Ong Chan, Rattanakiry Province.

ophagic and exophilic species remained abundant in forest fringes and foothills. Likewise, in Vietnam and Myanmar, exophilic *An. minimus* s.l. populations occurred after DDT pressure (Myo Paing et al. 1988, Vu Thi Phan 1998). Subsequently, on the basis of a population genetic study, two species, A and C, were indeed recognized in Thailand (Green et al. 1990). Yet, the recognition of these two species did not explain all behavioral heterogeneities within *An. minimus* s.l. (Green et al. 1990).

An. minimus A and C have also been recognized in Vietnam, where both species are attracted to humans and cattle (Van Bortel et al. 1999, 2000). In northern Vietnam, however, species A is more anthropophilic and endophilic than species C, indicating that *An. minimus* A may play a more important role in malaria transmission than *An. minimus* C (Van Bortel et al. 1999). However, little work has been done to assess behavioral variation within and between *An. minimus* A and C elsewhere in Southeast Asia. Therefore, in the current study, different collection methods were used to collect *Anopheles* species from several localities in Southeast Asia to survey intraspecific behavioral variation in the *An. minimus* complex in relation to other *Anopheles* species.

Materials and Methods

Study Villages. *Anopheles* populations were sampled from three localities in Vietnam, one in Laos, and one in Cambodia (Fig. 1).

Khoi village (20° 38′ N, 105° 18′ E), Hoa Binh Province, northern Vietnam, is located at 80 km southwest of Hanoi. It is situated in a large U-shaped valley at an altitude of 330 m, where rice is cultivated. The village is surrounded by forests. Houses are generally large, well closed, and built on stilts at ≈1.0–1.5 m above the ground. Cattle are kept under the houses at night. Small rivers, the typical breeding places for *An. minimus*, are omnipresent in this area. The annual rainfall, mainly from May to October, is between 1,500 and 2,500 mm. Average monthly temperatures range from 15°C to ≈30°C. The coldest months are December until March–April, and the hottest are June until September. Actually, there is no malaria transmission in Hoa Binh Province, but outbreaks are still possible (Verlé et al. 1998).

Lang Nhot (12° 14′ N, 108° 56′ E), Khanh Hoa Province in south central Vietnam, is a small village situated at 60-m altitude and is surrounded by forested hills. Most of the houses are built on the ground and are largely open, with thatch roofs and incomplete walls of split bamboo. Cattle are scarce in this village, but as in Khoi, the typical larval habitats of *An. minimus* s.l., are ubiquitous. The average monthly temperatures range from 23°C (December and January) to 27°C (April to August). The yearly precipitation varies between 1,400 and 2,800 mm, with most of the rainfall between September and December. The dry season lasts from January until April. Malaria transmission is high in these villages, with *Anopheles dirus* Peyton & Harrison and *Anopheles minimus* Theobald as vectors.

Village 3 (10° 50′ N, 107° 40′ E) is in Binh Thuan Province, south central Vietnam. The village is situated in a hilly, forested area 50 km from the provincial capital. Most houses are built on the ground and are mainly made from wood. Cattle are scarce in this village. Malaria transmission is high and the main vector is *An. dirus* (Van Bortel et al. 2001). The annual average temperature at the district town is 27°C. The coldest months are December through February; the hottest are from April until August. The yearly average precipitation is 1,650 mm. The dry season lasts from December through April, with almost no rain during the first 3 mo of the season. During the rainy season from May through November, monthly average precipitation is 250 mm.

Na Ang (18° 33′ N, 101° 59′ E) is in Province Vientiane, Laos. This village is situated 80 km northwest from the capital Vientiane. Rice fields separate the village from the surrounding forest. Houses are generally large, built on stilts at ≈2 m above the ground. Most houses are made from wood. Cattle are absent most of the time in the village. The wet season prevails from May to October with average rainfall of 1,780 mm. The dry season lasts from November until February. In Vientiane, temperature in April, the warmest month averages 29°C. Malaria transmission in this village is generally low.

Char Ong Chan (13° 43′ N, 106° 59′ E, Bong Long nearest town) is in Rattanakiry Province, northeast Cambodia. This village is located on the top of a hill surrounded by streams, forests and rubber plantations. Malaria transmission in this village is high. The houses are built on stilts with thatch roofs and walls of split bamboo or leaves. Animals such as dogs, pigs and chickens are present in the village. Cattle do not stay permanently in the village. From November until

March, they stay in the village at night. In April and May, cattle are only sporadically present at night, whereas most cattle stay permanently in the field from June until October. Temperature in this region ranges from 21 to 36°C. The rainy season lasts from April until October. The average annual rainfall is 3,300 mm.

Mosquito Collections and Identification. In Vietnam, adult mosquitoes were captured every 4 mo from May 1998 to April 1999 during 10 nights and mornings each time. Human landing collections were made by one collector inside and two collectors outside each of two houses. Houses were the same for the entire study. One person collected resting mosquitoes during the night both inside and outside of a third house. The night collections were made between 1800 and 0600 hours. Two collectors sampled on cattle between 2100 and 2400 hours. Morning collections of indoor resting mosquitoes took place in 10 different houses between 0600 and 0830 hours. In Laos and Cambodia, the same standard protocol was applied, though only from March 1999 until November 1999.

Adult mosquitoes were identified morphologically in the field by use of a standardized key for the medically important anophelines of Southeast Asia (modified from IMPE 1987). After identification, they were put individually in BEEM capsules (Beem, Bronx, NY) and stored in liquid nitrogen (Vietnam) or in a plastic bag containing silica gel (Laos and Cambodia). The identifications of the specimens belonging to the *An. minimus* and *An. dirus* complexes were further verified applying genetic markers. *An. minimus* A and C were identified using the *octanol dehydrogenase* (*Odh*) allozyme profiles (Van Bortel et al. 1999) or the polymerase chain reaction (PCR)-restriction fragment length polymorphism assay (Van Bortel et al. 2000). The species verification of *An. dirus* s.l. was done by the allele specific PCR of Walton et al. (1999) and the multiplex PCR of Manguin et al. (2002). Both methods showed difficulties in separating *An. dirus* A from *An. dirus* C. Hence, results of both assays were combined to distinguish both species.

Data Analysis. The association between the *Anopheles* species with the collection methods was evaluated by applying a correspondence analysis (CA) on the contingency table of the collection data. CA is a multivariate technique most similar to factor analysis but applicable on a two-way contingency table of observed frequencies. Chi-square distances provide a standardized measure of association between the rows and columns of the contingency table. CA transforms these association measures into metric distances and creates orthogonal dimensions upon which the categories can be projected to best account for the strength of association represented by the chi-square distances (Hair et al. 1998). The purpose of CA is to produce a simultaneous ordination of "objects" (here "species") and "variables" (a set of descriptive characteristics or attributes; here, the behavioral characteristics implied by the collection methods) such that variable positions best reflect object positions and vice versa, i.e., to reveal their mutual correspondence or degree of association (Podani 1994, Hair et al. 1998). This degree of association or correspondence is reflected by the metric distances in the ordination plot, so that the smaller the distances between points, the "stronger" their association (Hair et al. 1998). If the chi-square test does not reject the independence model, it would not make sense to apply CA because there would be no significant association to describe (Weller and Romney 1990). CA provides a multivariate representation of interdependence for nonmetric data that cannot be obtained with other methods. In this way, CA allows for the visual exploration of complex data sets. CA was performed using NTSYS, version 1.80 (Rohlf 1993). Each row represented an *Anopheles* species collected at a certain study site during a certain survey. The columns represented the collection methods. The resting night collections were excluded because they yielded too low numbers of mosquitoes. Moreover, surveys 1 and 3 from Laos were excluded from the analysis because no cattle collections were done (no cattle was present at that time). The associations between *Anopheles* species (row categories) and collection methods (column categories) were visualized in three-dimensional ordinations. *Anopheles s*pecies that were associated with the human landing collections (indoor or outdoor) were considered as anthropophilic, those associated with cattle collection were considered as zoophilic species and the species associated with the indoor resting morning collection were considered as endophilic. To evaluate "village effects" upon the collections, the overall ordination was decomposed in five separate ordinations, each displaying the *Anopheles* species of a particular site. Overall intraspecific variation was also evaluated at species level for *An. minimus* A and C, and two closely related species, namely, *aconitus* Dönitz (Myzomyia Series, Minimus group) and *An. jeyporiensis* James (Myzomyia Series). Finally, two further species were evaluated, i.e., *An. dirus* A Peyton & Harrison, a very efficient malaria vector in Southeast Asia, and *Anopheles vagus* Dönitz, a species found in all collection sites.

Results

The collection yields in the five study sites differed largely, both in the numbers of specimens and in numbers of *Anopheles* species (Table 1). A significant association between *Anopheles* species and collection method was observed (chi-square test, $P < 0.0001$). In all but one village, Na Ang, the cattle collections yielded most mosquitoes. Few different *Anopheles* species were found in the indoor resting morning collections. Khoi village differed from all other villages because of the association of mosquitoes with the indoor resting morning collection method and the lack of such an association with the indoor human landing collection (Fig. 2B). The association between mosquitoes and the indoor resting morning collections was mainly caused by *An. minimus* A and *An. vagus* (Fig. 3A and F). Also *An. minimus* C and *An. jeyporiensis* showed association with this collection type

Table 1. Number of *Anopheles* mosquitoes collected by different collection methods

Collection method		Vietnam			Laos	Cambodia
		Khoi	Lang Nhot	Village 3	Na Ang[a]	Char Ong Chan
Indoor human landing	Person-nights	80	83	77	20	60
	No./person-night					
	Anopheles	2.56	4.63	1.54	78.10	2.48
	An. minimus A	1.55	4.31	0.00	69.05	0.05
	An. minimus C	0.41	0.00	0.00	0.00	0.00
	No. of different *Anopheles* species collected	12	6	13	13	12
Outdoor human landing	Person-nights	160	160	155	40	120
	No./person-night					
	Anopheles	6.76	1.33	3.49	66.70	7.41
	An. minimus A	1.09	0.55	0.01	36.23	0.24
	An. minimus C	0.86	0.00	0.00	0.00	0.00
	No. of different *Anopheles* species collected	12	9	16	17	15
Collected on cattle	Nights[b]	40	40	40	10	30
	No./night by one collector					
	Anopheles	46.55	29.85	15.72	22.15	20.08
	An. minimus A	10.39	0.08	0.02	3.55	0.48
	An. minimus C	9.00	0.00	0.00	0.00	0.00
	No. of different *Anopheles* species collected	12	11	15	16	16
Indoor resting morning	House-mornings	400	404	380	100	245
	No./house-morning					
	Anopheles	5.52	0.09	1.30	1.38	0.67
	An. minimus A	1.64	0.08	0.00	1.36	0.04
	An. minimus C	0.33	0.00	0.00	0.00	0.00
	No. of different *Anopheles* species collected	6	3	1	2	4
Total number of different *Anopheles* species collected during entire study		12	13	18	17	20

[a] Based on collections from second survey only.
[b] Collections made during 3 h of the night (2100–2400 hours).

in Khoi, but only in survey 4 (Fig. 3B and D, survey number not indicated).

An. minimus A was associated with all collection methods. The association, however, differed by study site and by survey (Fig. 3A). In Khoi, its variation was explained by the indoor resting morning collections and by the cattle collections, with almost no variation between the four surveys. In Lang Nhot and Na Ang, *An. minimus* A was associated with the indoor and outdoor human landing collection method, whereas the cattle collections yielded hardly any *An. minimus* A mosquito (Table 1). In Char Ong Chan, the anthropophilic behavior of species A changed with season and was related to the presence of cattle. It was associated with cattle collections in the first survey, during which cattle stayed permanently inside the village overnight. In the other surveys cattle did not stay permanently in the village and *An. minimus* A was more associated with human collections (Fig. 3A, survey number not shown). In Binh Thuan, only three *An. minimus* A specimens were collected over the entire study period.

An. minimus C was only found in the study site Khoi, northern Vietnam, where it was mostly associated with the cattle collections (Fig. 3B). However, in survey 4 (March–April 1999, i.e., the end of the cool period), it was also associated with the indoor resting morning collections. Likewise, *An. jeyporiensis* was only associated with indoor resting morning collections in survey 4, whereas it was associated with cattle in the other surveys (Fig. 3D). *An. aconitus* was mainly caught by the cattle collections. It showed, however, close association with human landing collections in Na Ang and in one survey of Char Ong Chan, corresponding with the period that bovines stayed outside the village (Fig. 3C). The widespread species *An. vagus* showed a remarkable diversity in its associations with the collection methods (Fig. 3F). In the northern site, the species was associated with indoor resting morning collections, whereas in village 3 and Char Ong Chan this was only the case in certain surveys. In other villages and surveys, it showed clear association with the cattle collections and even with the human landing collections outdoors. The efficient vector *An. dirus* A was clearly associated with human landing collections in the four villages where it was found (Fig. 3E).

Discussion

Comparable collection data were obtained by applying a standard protocol in the five study sites. This is crucial because the observed behavioral traits are the outcome of a complex interaction of internal and external stimuli (Costantini et al. 1999, Takken and Knols 1999, Dekker et al. 2001). Hence, inferences of behavioral traits through collection data do not indicate the innate preferences of the studied species, but they do reveal the different behavioral responses of a species in the selected environments. CA allowed us to represent the degree of association between the collection data, the species, and the study sites in single ordinations. This made it possible to evaluate inter- and intraspecific behavioral differences in the study sites by looking for associations between *Anopheles* species and collection methods.

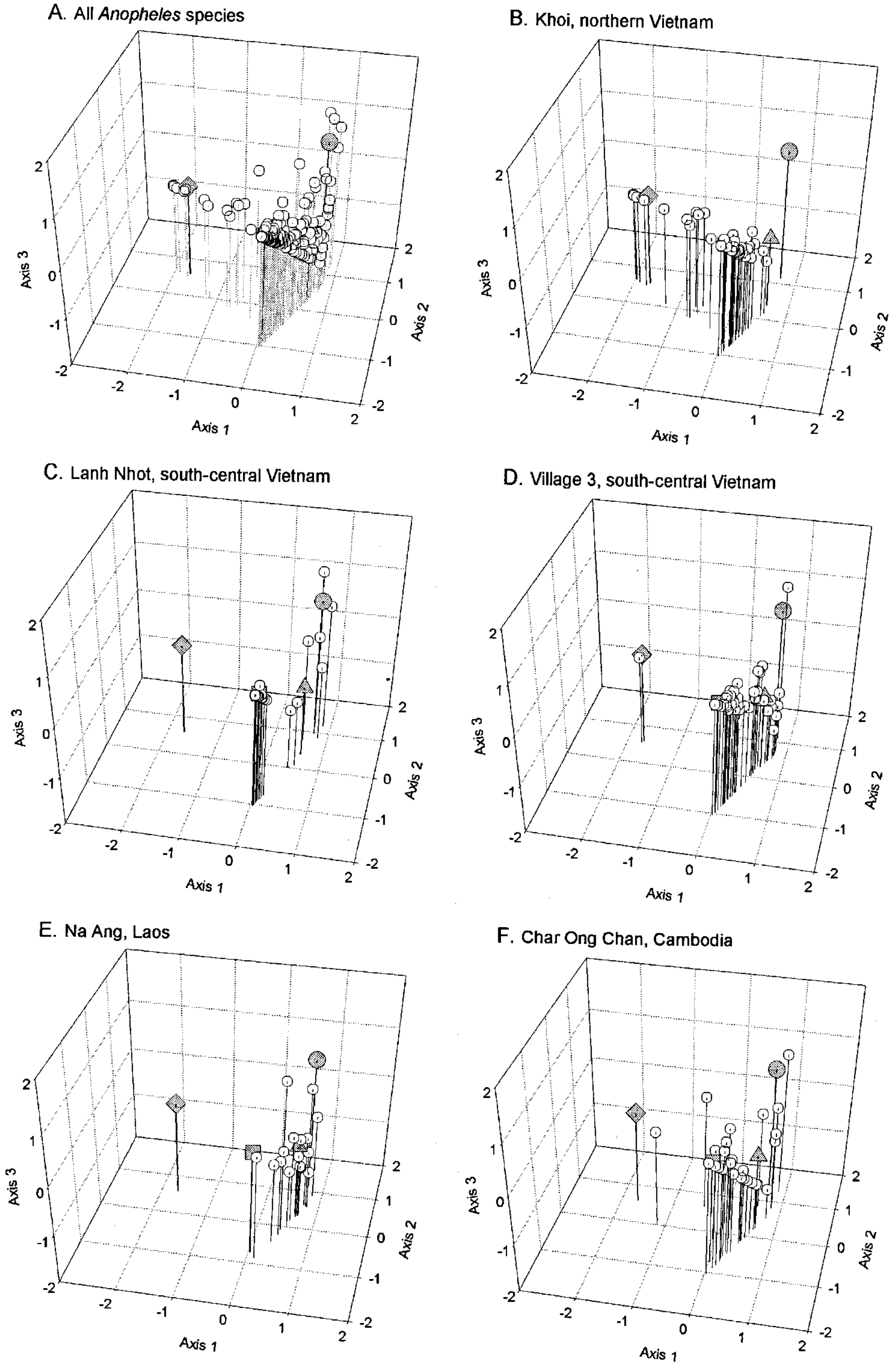

Fig. 2. (Map A) Three-dimensional ordinations of the correspondence analysis showing all *Anopheles* species. (Maps B–F) Decomposed ordination showing only *Anopheles* species (row categories) of a particular study site to evaluate the village effect. (B) Khoi (northern Vietnam). (C) Lang Nhot (south central Vietnam). (D) Village three (south central Vietnam). (E) Na Ang (Laos). (F) Char Ong Chan (Cambodia). The proximity between the species and the collection methods on the graph reveals their mutual degree of association. Collection methods (column categories) in gray: ●, indoor human landing collection; ▲, outdoor human landing collection; ■, cattle collection; ♦, indoor resting morning collection. *Anopheles* species (row categories) in white, ○.

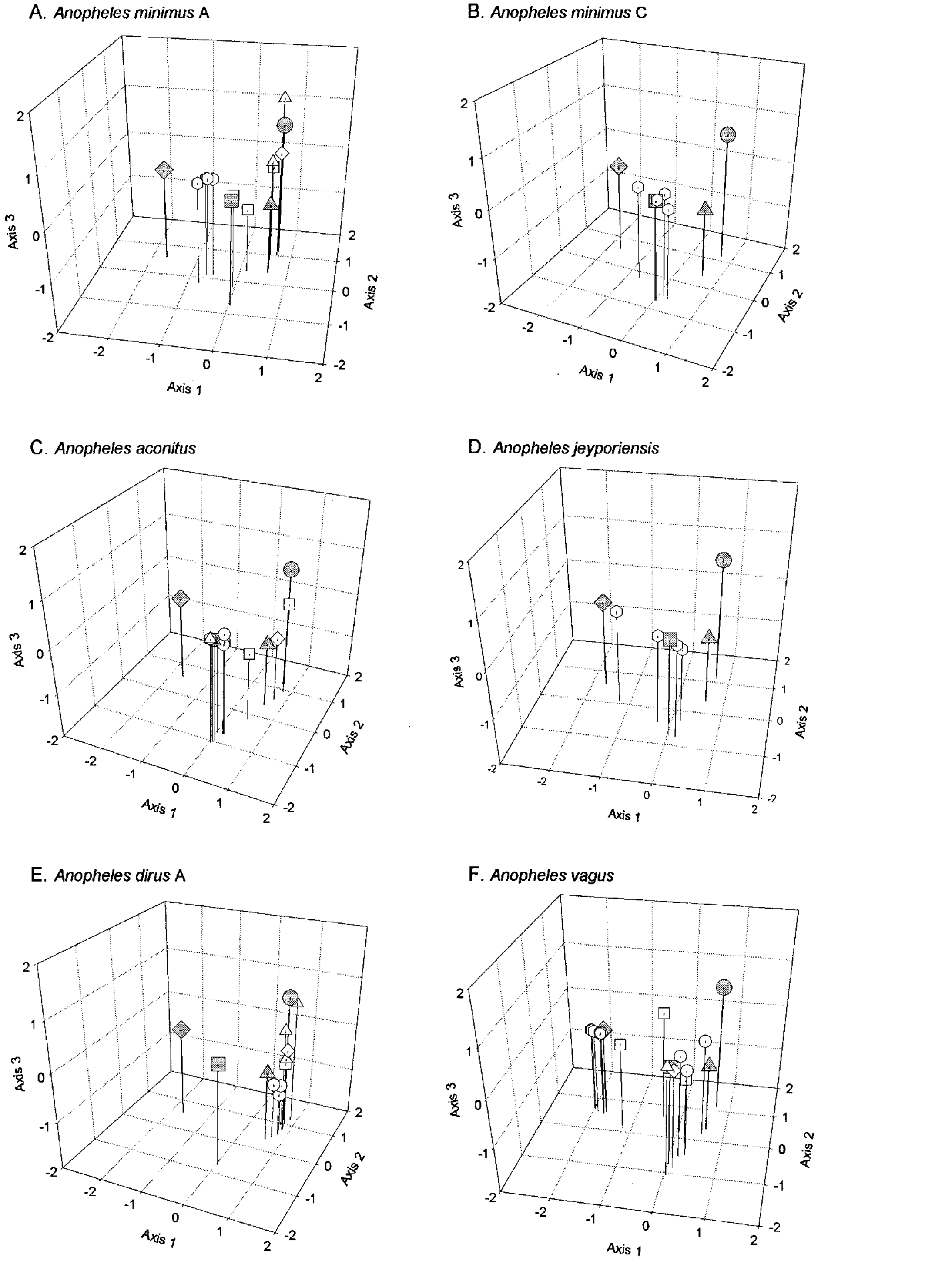

Fig. 3. Decomposed ordination of the correspondence analysis showing particular *Anopheles* species to evaluate intraspecific variation. Map A, *An. minimus* A; B, *An. Minimus*; C, C. *An. aconitus*; D, *An. jeyporiensis*; E, *An. dirus* A; and F, *An. vagus*. Multiple data points from the same village and the same species represent different surveys. The proximity between the species and the collection methods on the graph reveals their mutual degree of association. Collection methods (column categories) in gray: ●, indoor human landing collection; ▲, outdoor human landing collection; ■, cattle collection; ♦, indoor resting morning collection. *Anopheles* species (row categories) in white collected in ⬡, Khoi (northern Vietnam); △, Lang Nhot (south central Vietnam); ○, village 3 (south central Vietnam); ◇, Na Ang (Laos); □, Char Ong Chan (Cambodia).

Large intraspecific behavioral differences were observed among populations of *An. minimus* A, ranging from very anthropophilic and endophagic to exophilic, and even zoophilic. They correspond to the heterogeneities observed previously in *An. minimus* s.l. (Harrison 1980). Behavioral traits often exhibit geographic variation, and the variation is often indicative of underlying genetic differentiation (Foster 1999, Schaffner et al. 2000) or even of cryptic species (Ismail et al. 1974, Coluzzi et al. 1979, Suthas et al. 1986, Foley et al. 1993). However, all concerned populations were identified as *An. minimus* A by the Odh diagnostic enzyme locus (Van Bortel et al. 1999) or by the PCR-restriction fragment-length polymorphism identification method (Van Bortel et al. 2000). Hence, these behavioral divergences were not indicative of the presence of cryptic species. Moreover, on the basis of allozyme electrophoresis, only a limited macrogeographical variation was observed among Vietnamese *An. minimus* A populations (Van Bortel et al. 2003) and no genetic differentiation was observed between different "behavioral types" (i.e., Mosquito Collections) (Van Bortel et al. 1999, Van Bortel et al. 2003).

In the northernmost site, Khoi, there was a strong association between *An. minimus* A and the indoor resting morning collection. Previously, Van Bortel et al. (1999) found that *An. minimus* A was much more abundant in indoor resting morning collections than species C. However, also *An. minimus* C and *An. jeyporiensis* were associated with indoor resting morning collections, but only in the cool period of March–April (survey 4). Moreover, in the northernmost part of its distribution, India, Nepal, and Bangladesh, *An. minimus* s.l. was only recorded as endophilic (Covell 1944, Khan and Talibi 1972, Parajuli et al. 1981, Jana-Kara et al. 1995, Dev 1996). In Vietnam, temperature variation between winter and summer in the northern site is much more important than in the southern sites, whereas little differences in relative humidity exists between the study sites. This could indicate that ambient temperature influences the endophilic behavior and that human dwellings act as a protective microenvironment. The endophilic behavior of *An. minimus* s.l., however, was not restricted to the northern part of its distribution. In the plain regions of Thailand, a less typical environment for *An. minimus s.l.*, it was known as a very endophilic vector (Suthas et al. 1986). Likewise, the association of *An. vagus* with the indoor resting morning collections was not restricted to the northern site, but it is was most pronounced in that area. Other factors as well seemed to influence the *Anopheles* behavior. *An. minimus* A was mainly collected on man in two villages where bovines were scarce. Likewise, species A was associated with man in Char Ong Chan (Cambodia), when cattle stayed outside the village. This is consistent with the observation of Ismail et al. (1978), that *An. minimus* s.l. deviated more to human in the absence or scarcity of cattle. Bovines are abundant in Khoi, northern Vietnam, where they are mainly used to cultivate the fields. Our results suggest that the risk exists that *An. minimus* mosquitoes may also here deviate to humans if bovines would be replaced by mechanical traction. Recently, Rowland et al. (2001) suggested on the basis of results obtained to control transmission by *Anopheles culicifacies* James that cattle sponging with insecticides would be an alternative control method for *An. minimus.* Our results indicate that such treatment may be effective, even though it can be expected that on the long-term *An. minimus* A might deviate from treated cattle to humans.

The efficient vector *An. dirus* A showed a remarkable constant anthropophilic behavior in all villages where it was collected. Likewise, the reputed African malaria vector, *Anopheles gambiae* Giles, is highly anthropophilic throughout its distribution (Costantini et al. 1999). Much more variation was found in *An. aconitus* and *An. vagus,* two species of which the vector status differs from region to region (Covell 1944, Reisen et al. 1971, Vu Thi Phan 1998). In south central Vietnam and Cambodia, where *An. minimus* A exhibited a more anthropophilic behavior, it was found infected by *Plasmodium falciparum* and *Plasmodium vivax* (Trung et al. 2004), which clearly indicates its role as malaria vector in these localities. Yet, in Khoi, where this species showed a more zoophilic behavior, malaria could effectively be controlled and transmission is nowadays absent (Verlé et al. 1998). On the basis of behavior, the vector status of *An. minimus* C in Vietnam is questionable. However, the currently available information is too limited to be conclusive. In Guizhou and Guangxi provinces, southern China, for example, *An. minimus* C is suspected to be responsible for malaria transmission (Chen et al. 2002).

This study clearly shows that the behavior of *Anopheles* species can only be described by evaluating the behavioral variation at various geographical scales. Hence, efforts are still needed to unravel the behavior of *An. minimus* C. *An. minimus* A clearly shows intraspecific behavioral variation. This species is able to change its host preference in response to the local availability of hosts. Hence, its role in malaria transmission can differ from region to region. Moreover, the impact of control on *An. minimus* A may differ between localities. The observed intraspecific behavioral differences in *An. minimus* A are consistent with the earlier conclusion of Green et al. (1990) that the existence of the cryptic species A and C did not resolve all observed behavioral heterogeneities within *An. minimus* s.l. Consequently, it is important to recognize that intraspecific behavioral differences in *Anopheles* species can occur and that these behavioral heterogeneities, albeit important for disease transmission and control, are not a priori indicative of the presence of cryptic species.

Acknowledgments

We acknowledge the excellent technical support of the entomology teams of the National Institute of Malariology, Parasitology and Entomology (NIMPE, Hanoi, Vietnam); the National Center of Malariology, Parasitology, and Entomology (NCMP, Vientiane, Laos); and the National Center for Malaria Control, Parasitology, and Entomology (NCMC, Phnom Phen, Cambodia). We are grateful to the provincial antimalaria teams of Hoa Binh, Lang Nhot, Binh Thuan, Vientiane, and Rattanakiry. We acknowledge the Ministries of Public Health of Vietnam, Laos and Cambodia, for facilitating this research. We thank D. Schrijvers and T. Hoefyzers for excellent technical assistance. This work was carried out within the framework of the INCO-DC research project ERBIC18CT970211 and the Institutional Collaboration between NIMPE and the Institute of Tropical Medicine, Belgium, supported by the Belgian Co-operation (Directorate-General for Development Co-operation, DGDC).

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Received for publication 7 May 2003; accepted 3 November 2003.