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Bacterial Biodiversity in Midguts of *Anopheles* Mosquitoes, Malaria Vectors in Southeast Asia

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Additional information is available at the end of the chapter

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1. Introduction

Factors allowing the development of a pathogen to reach the infecting stage in a mosquito are poorly known. On the 528 species of mosquitoes recorded within the *Anopheles* genus [1], only 70 to 60 are able to transmit parasites responsible for malaria and filariasis [2, 3]. In vector-parasite interactions, the mosquito gut represents the first point of contact between parasites ingested and the vector epithelial surfaces. In the midgut, the parasites will have the opportunity to undergo their life cycle, but of the tens of thousands of *Plasmodium* gametocytes ingested by mosquitoes, less than five oocysts might be produced [4]. The factors responsible for this drastic reduction are still poorly understood. Recent studies showed that one of these factors concerns the primordial role played by the bacteria naturally present in mosquito midgut. Then, there is a growing interest on bacterial biodiversity in *Anopheles* mosquitoes and particularly those based on the identification of bacteria to be used for malaria transmission blocking based on bacterial genetic changes to deliver antiparasite molecules or paratransgenic approach [5-13]. Recent studies reported the presence of symbiotic bacteria, such as *Pantoea agglomerans* or *Asaia* in midgut lumen with anti-*Plasmodium* effector proteins that render host mosquitoes refractory to malaria infection [6, 10, 13]. Engineered *P. agglomerans* strains were able to inhibit *Plasmodium falciparum* development by 98% [13]. Other studies showed that insects with an important microbiota seem more resistant to infections and certain bacteria, such as *Enterobacter* sp. (Esp Z) inhibit partially or totally ookinete, oocyst and sporozoite formation [14-16]. In *Anopheles albimanus*, co-infections with the bacteria, *Serratia marcescens*,

and *Plasmodium vivax* resulted in only 1% of mosquitoes being infected with oocysts, compared with 71% infection for control mosquitoes without bacteria [17]. A recent meta-taxogenomic study provides an in-depth description of the microbial communities in the midgut of *Anopheles gambiae* exposed to *P. falciparum* infection and the links between microbiota and parasitic status by comparing midgut microbiota in *P. falciparum*-positive and *P. falciparum*-negative individuals. Authors found significant correlation between the high enterobacterial content and malaria infection. Despite conflicting results on the role of enterobacteria, it has now clearly been established that bacteria present in *Anopheles* populations have a great influence on parasite transmission [18].

In Thailand and Vietnam, malaria is a public health priority with a strong prevalence of this disease in forested regions, in particular along the international borders with Myanmar and Cambodia respectively. In these malaria endemic areas, another parasitic disease occurs, Bancroftian lymphatic filariasis (BLF) for which only limited data are available [2]. Malaria and BLF are mosquito-borne diseases with *Plasmodium* species, especially *P. falciparum*, *P. vivax*, and rural strains of *Wuchereria bancrofti* sharing the same *Anopheles* vector species. In Southeast Asia, *Anopheles* vectors belong to species complexes with different involvement in the transmission of pathogens [19]. Few sibling species of the Dirus and Minimus Complexes and the Maculatus Group are involved in malaria and BLF, but specific role of each sibling species and factors influencing this role have never been studied due to the lack of reliable methods for species identification, now available [20-22]. As mosquito microbiota is one of the factors influencing pathogen transmission, this chapter is presenting the biodiversity of bacteria in the midgut of field-collected adults of 10 *Anopheles* species, topic less studied compared to the large number of studies presenting bacteria in the defense against parasites in laboratory conditions.

1.1. Midgut microbiome of mosquitoes

Many insects contain large communities of diverse microorganisms that probably exceed the number of cells in the insect itself [23]. More specifically, complex microbiotae have been described in mosquito midgut reporting the presence of numerous Gram-negative rods, including *Serratia marcescens*, *Klebsiella ozaenae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter* spp. [14]. Recently, three metagenomic studies provided a more comprehensive picture of the diversity of midgut microbiota in *Anopheles gambiae*, the main malaria vector in Africa [18, 24, 25]. In wild caught adults of *Anopheles* species, the microbiota showed the common presence of *Pseudomonas* and *Aeromonas* species reported from at least five species among which malaria vectors (Table 1). The following five genera, *Asaia*, *Bacillus*, *Chryseobacterium*, *Klebsiella*, and *Pantoea* have been reported from four field collected *Anopheles* species, while *Serratia* and *Stenotrophomonas* were identified in three species (Table 1). At least three mosquito-specific bacterial species, isolated from the midgut of main malaria vectors of the Gambiae Complex, have been described, such as *Thorsellia anophelis* [26], *Janibacter anophelis* [27] and *Elizabethkingia anophelis* [28]. The first of the three species represents a new genus and species found predominant in the midgut of *Anopheles arabiensis* [29], the same *Anopheles* species in which *J. anophelis* was isolated. The third newly described species is closely related

to *Elizabethkingia meningoseptica* as they share 98.6% similarity, and both species have been found in the midgut of *Anopheles gambiae* [11, 28]. The latter species, *E. meningoseptica*, was also isolated from diseased birds, frogs, turtles, cats, being most likely an agent of zoonotic infections, as well as a human meningitis especially in newborn infants [30]. Bacteria of the genus *Asaia* have also been associated with *Anopheles* species, in particular field-collected *An. gambiae*, *An. funestus*, *An. coustani* and *An. maculipennis* (Table 1), as well as a colony of *An. stephensi* in which *Asaia* bacteria was dominant and stably associated [9]. The presence of *Asaia* species in *Anopheles* could serve as candidate for malaria control based on the production of antiparasite molecules in mosquitoes for use in paratransgenic control of malaria [6, 9, 31]. Other bacterial species have been defined as antimalarial agents, especially those producing prodigiosin, a pigment produced by various bacteria, including *S. marcescens* [14].

The number of bacteria not only varied between individuals but also changed markedly during development, depending on both the stage of development and the blood-feeding status of the mosquitoes [31]. The normal midgut microbiota of *Anopheles* mosquitoes need to be further identified [5] as only few studies have reported the microbiota of wild caught malaria vectors (Table 1) [5-7, 9, 11, 12, 17, 24, 26-29, 31-35]. Further investigations of gut microbiota, especially of wild-caught insect vectors, might contribute to understanding the annual and regional variations recorded for vector transmitted diseases [17] and yield novel vector-control strategies [14].

1.2. Exploring bacterial communities by 16S PCR-TTGE

Bacterial communities are classically assessed through culture-dependent methods based on colony isolation on solid medium, sometimes after enrichment by growth in liquid medium. But, it is now obvious that the microbial diversity is poorly represented by the cultured fraction, and culture have been shown to explore less than 1% of the whole bacterial diversity in environment samples [36]. Thanks to sophisticated biotechnological and computational tools of the metagenomics, molecular ecology offers the potential of determining microbial diversity in an ecosystem by assessing the genetic diversity. The complete metagenomic approach will give the total gene content of a community, thus providing data about biodiversity but also function and interactions [37]. For the purpose of biodiversity studies, metagenomics can focus on one common gene shared by all members of the community. The most commonly used culture-independent method relies on amplification and analysis of the 16S rRNA genes in a microbiota [38].

The 16S rRNA genes are widely used for documentation of the evolutionary history and taxon assignment of individual organisms because they have highly conserved regions for construction of universal primers and highly variable regions for identification of individual species [39]. The notion developed by Woese that rRNA genes could identify living organisms by reconstructing phylogenies resulted in the adoption of 16S rRNA gene in microbiology [39]. Its universality and the huge number of sequences stored in databases have established 16S rRNA gene as the “gold standard” not only in microbial phylogeny, systematics, and identification but also microbial ecology [40].

| Bacteria genera (species) | Anopheles | | | | | | | | Total | |
|---|-----------|------------|----------|----------|--------|----------|----------|--------------|-----------|-------------|
| | albimanus | arabiensis | coustani | darlingi | dureni | funestus | gambiae | maculipennis | stephensi | ele species |
| Achromobacter (A. xylosoxidans) | | | | | | | [31] | | [5, 34] | 2 |
| Acidovorax (A. temperans) | | [12] | | | | | | | | 1 |
| Acinetobacter sp. (A. hemolyticus, A. radioresistens) | | | | | | [24] | | | [34] | 2 |
| Aeromonas sp. (A. hydrophila) | | | [24]* | [35] | | [24] | [12, 24] | | [5] | 5 |
| Anaplasma (A. ovis) | | [12] | | | | | | | | 1 |
| Asaia spp. (A. bogorensis, A. siamensis) | | | [24] | | | [24] | [9, 24] | [9] | | 4 |
| Bacillus spp. (B. cereus, B. coagulans, B. megaterium, B. mucoides, B. silvestris, B. simplex, B. thuringensis) | | [12] | | | | [31] | [31] | | [34] | 4 |
| Bordetella | | | | | | | | | [5] | 1 |
| Brevundimonas (B. diminuta) | | | | | | [31] | | | | 1 |
| Cedecea (C. davisae) | | | | | | [31] | [31] | | | 2 |
| Chryseobacterium (C. indologenes) | | | [24] | | | [24] | [24] | | [34] | 4 |
| Citrobacter (C. freundii) | | | | | | | | | [34] | 1 |
| Enterobacter spp. (E. amnigenus, E. cloacae, E. sakazaki) | [17] | | | | | | | | [34] | 2 |
| Ehrlichia | | [12] | | | | | | | | 1 |
| Erwinia (E. ananas, E. chrysanthenum) | | | | | | [31] | [31] | | | 2 |
| Escherichia (E. coli, E. senegalensis) | | [12] | | | | [31] | | | | 2 |
| Elizabethkingia (E. anophelis, E. meningoseptica) | | | | | | | [11, 28] | | | 1 |
| Flavobacterium (F. resinovorum) | | | | | | [31] | | | | 1 |
| Gluconobacter (G. cerinus) | | | | | | [31] | | | | 1 |
| Janibacter (J. anophelis, J. limosus) | | [12, 27] | | | | | | | | 1 |
| Klebsiella spp. (K. pneumoniae) | | | | | [33] | [31] | [31] | | [32] | 4 |
| Kluyvera (K. cryocrescens) | | | | | | [31] | | | | 1 |
| Leuconostoc (L. citreum) | | | | | | | | | [34] | 1 |
| Leminorella (L. grimontii) | | | | | | | | | [34] | 1 |
| Morganella (M. morganii) | | | | | | | [31] | | | 1 |
| Mycoplasma (M. wenyonii) | | [12] | | | | | | | | 1 |
| Myroides | | | | | | | | | [5] | 1 |
| Nocardia (N. corynebacterioides) | | [12] | | | | | | | | 1 |
| Paenibacillus sp. | | [12] | | | | | | | | 1 |
| Pantoea (P. agglomerans, P. stewartii) | | | | [35] | | [31] | [11, 31] | | [7] | 4 |

| Anopheles | | | | | | | | | | Total Anoph eles species |
|---|-----------|------------|----------|----------|--------|----------|----------|--------------|-----------|-----------------------------------|
| Bacteria genera (species) | albimanus | arabiensis | coustani | darlingi | dureni | funestus | gambiae | maculipennis | stephensi | |
| Pseudomonas spp. (P. aeruginosa, P. mendosina, P. pseudoflava, P. putida, P. stutzeri, P. synxantha, P. testosteroni) | | | | [35] | [33] | [29, 31] | [12, 31] | | [5, 34] | 5 |
| Salmonella spp. (S. choleraesuis, S. enteritidis) | | | | | | [31] | [31] | | | 2 |
| Serratia (S. marcescens, S. nematodiphila, S. odorifera, S. proteamaculans) | [17] | [12] | | | | | | | [32, 34] | 3 |
| Sphingobacterium (S. multivorum) | | | | | | | [11] | | | 1 |
| Spiroplasma sp. | | | | | | [12] | | | | 1 |
| Stenotrophomonas (S. maltophilia) | | [12] | | | | [31] | [11, 12] | | | 3 |
| Thorsellia (T. anophelis) | | [26, 29] | | | | | | | | 1 |
| Vibrio (V. metschnikovii) | | [12] | | | | | | | | 1 |
| Xenorhabdus (X. nematophila) | | | | | | | | | [34] | 1 |
| Zymobacter | | | | | | | [24] | | | 1 |

°, An. gambiae s.l. or s.s.; *, For Osei-Poku et al (2012) [24], genera with low frequency were not considered in this table.

°, *An. gambiae* s.l. or s.s.; *, For Osei-Poku et al (2012) [24], genera with low frequency were not considered in this table.

Table 1. Bacterial genera isolated from the midgut of wild-caught adults of 9 *Anopheles* species linked to the associated reference numbers.

The complete 16S rRNA gene (1500 bp) gives the accurate affiliation to a species in most cases. In metagenomics, the amplified fragments are shorter, ranging from 200 to 400 bp, but contain nine hypervariable regions (V1-V9) [41], which compensate the lack of information due to the small sequence size by a high rate of mutation. In most studies, the V3 region located in the 5' part of the gene is chosen [42]. However, the phylogenetic information is sometimes insufficient to achieve species identification. Depending on the bacterium, sequences provide identification to the genus or family level only. Consequently, the diversity of the community is not described by a list of bacterial species but by a list of operational taxonomic units (OTUs) corresponding to the lower taxonomic level being accurately identified. The 16S rRNA gene, in spite of some recognized pitfalls [43], remains today the most popular marker for studying the specific diversity in a bacterial community. Alternative markers can also be proposed such as *rpoB* [40] but universal *rpoB* PCR primers allowing the exploration of the whole bacterial diversity cannot be designed (Jumas-Bilak E, personal data) and the databases remain poor in *rpo* sequences.

Molecular approaches for assessing biodiversity avoid the bias of cultivability but displayed several pitfalls that should be evaluated and considered for a sound interpretation of the data. Particularly, DNA should be recovered and amplified from all the genotypes in the community, i.e. extraction and PCR should be as universal as possible. Special attention should be given to *Firmicutes* and *Actinobacteria* because they display thick and resistant cell wall. The extraction

efficiency should be tested on a wide panel of bacteria to scan a large range of bacterial types. Extraction is generally improved by the use of large-spectrum lytic enzymes and/or by a mechanical grinding [44, 45]. The PCR itself is another cause of limitations in the molecular approaches. It often praises for its detection sensitivity but this sensitivity can fail when complex samples are analyzed. For example, detection thresholds of 10^3 - 10^4 CFU (colony forming units)/mL are currently described for universal PCR and migration in denaturing gels [44-46]. The detection limit cannot be easily assessed as it depends on both CFU/g count of each OTU and the relative representation of OTUs in the community. Minor populations of less than 1% of total population are generally undetectable for denaturing-gel-based methods used in microbial ecology [45, 47, 48].

In biodiversity studies, the different 16S rRNA genes representative of the community are amplified by PCR and then separated and identified either by cloning and Sanger sequencing or by direct pyro-sequencing [38]. Tools for sequence-specific separation after bulk PCR amplification, such as T-RFLP (Terminal-Restriction Fragment Length Polymorphism) [49], D-HPLC (Denaturing High Performance Liquid Chromatography) [50], CDCE (Constant Denaturing Capillary Electrophoresis) [51], SSCP (Single Stranded Conformation Polymorphism) [52], DGGE (Denaturing Gradient Gel Electrophoresis) [53], TGGE (Temperature Gradient Gel Electrophoresis), [48] and TTGE (Temporal Temperature Gradient Gel Electrophoresis) [47], can also be used. Methods based upon separation in denaturing electrophoresis allow the comparison of microbiotae with low or medium diversity [54]. They easily provide a “fingerprint” of the community diversity and therefore they are suitable for the follow-up of large collection of samples.

PCR-TTGE is a PCR-denaturing gradient gel electrophoresis that allows separation of DNA fragments in a temporal gradient of temperature [47, 55]. PCR amplicons of the same size but with different sequences are separated in the gel. In a denaturing acrylamide gel, DNA denatures in discrete regions called melting domains, each of them displaying a sequence specific melting temperature. When the melting temperature (T_m) of the whole amplicon is reached, the DNA is denatured creating branched molecules. This branching reduces DNA mobility in the gel. Therefore, amplicons of the same size but with different nucleotide compositions can be separated based on differences in the behavior of their melting domains. When DNA is extracted and amplified from a complex community, TTGE leads to the separation of the different amplicons and produces a banding pattern characteristic of the community. Counting bands on the TTGE profile provides a diversity score that roughly corresponds to the number of molecular species in the sample. The banding profile can be further analyzed by measuring distance migration of bands and comparing with patterns from known species. This comparison allows the affiliation of band to some representative species. Affiliation of all bands can be achieved by cutting bands from the gel, extracting DNA from bands and sequencing. A method associating migration distances measurement and sequencing of selected bands has shown its efficiency in describing bacterial communities of low complexity such as the gut microbiota of neonates [45]. Such an approach is simple enough and cost-effective to survey bacterial communities on a wide range of samples [56].

This chapter presents the bacterial biodiversity in the midguts of malaria vectors from Thailand and Vietnam based on the amplification of the V3 region of the 16S rRNA gene, separation of amplicon by TTGE and sequencing. The bacterial biodiversity among specimens and species in relation to the collection site are discussed.

2. Material and methods

2.1. Mosquito collections and species identification

In Thailand, populations of *Anopheles* mosquitoes were collected from three different sites located in malaria endemic area along the Thai-Myanmar border (Figure 1). One study site is in Pu Teuy, a village located in Sai Yok District, Kanchanaburi Province, western Thailand (14° 17'N, 99° 01'E). The rural site is located in mountainous terrain mostly surrounded by forest. The main water body near the collection site is a narrow, slow running stream, bordered with native vegetation [57]. This stream represents the main larval habitat for *An. minimus* s.l. [58]. A total of 1,330 malaria cases were reported in 2011 in the Sai Yok District with a prevalence of 389 cases of *P. falciparum* (44.7%) and 481 cases of *P. vivax* (55.3%) with a mortality rate per 100,000 inhabitants of 0.71 [59]. The second site located in Mae Sod District, Tak Province, is in the northern part of Thailand (16° 67'N, 98° 68'E). This is a forested area associated to agricultural fields and small streams. In 2011, 1,876 malaria cases were reported in this district with 187 cases of *P. falciparum* (28.3%) and 473 cases of *P. vivax* (71.7%). The mortality rate per 100,000 was of 0.56 [59]. The third site in Sop Moei District is the most southern district of Mae Hong Son Province (17° 86'N, 97° 96'E). This mountainous province is located north of Tak Province with a high malaria transmission occurring from June to August, during the rainy season [60]. In 2011, 1,643 malaria cases were found in this district due to *P. falciparum* with 419 cases (45.0%) and *P. vivax* with 511 cases (55.0%) and a mortality rate per 100,000 of 0.41 [59].

The specimens from Vietnam were collected from six sites located in Dak Ngo Commune, Tuy Duc District, Dak Nong Province (11°59'N, 107°42'E - central Highlands) where 848 malaria cases were reported in 2011, of which, 322 cases (54.9%) were caused by *P. falciparum*, 209 cases (35.6%) by *P. vivax* and 56 cases (9.5%) were mixed infections [61]. This province was named in 2004 after integrating parts from northern area of Binh Phuoc Province and southern area of Dak Lak Province. The average temperature in this province is around 24° C with the rainy season ranging from May to October and the dry season from November to April. The climate is favorable for agriculture, especially coffee, pepper and rubber plantations. Crops of coffee, pepper or cashew nuts were normally cultivated around houses. Villages were surrounded by cassava, corn and rice fields and located in the fringe forest. Every year, during harvest period, workers from neighbourhood come to work in the field, which generate high population movements in this area.

Anopheles mosquitoes were morphologically sorted by taxon before using specific AS-PCR assays for species identification within the complex or the group [20-22]. Each individual was split in two pieces, head-thorax for species identification and abdomen for midgut bacteria analysis.



Figure 1. Map of Southeast Asia showing the locations of three provinces in Thailand (blue dots) and the province in Vietnam (red dot) where the mosquito collections were implemented.

2.2. DNA extraction

Mosquitoes stored at -20°C were surface rinsed twice in purified water prepared for injectable solution, and abdomen was thoroughly disrupted using a tissue crusher device in 150 µl of TE buffer. DNA was extracted using the Master Pure Gram Positive DNA purification kit as recommended by the supplier (Epicentre Biotechnologies, Madison, USA).

2.3. PCR

The V2–V3 region of the 16S rRNA gene of bacteria in the samples was amplified using the primers HDA1/HDA2 [45]; HDA1: 5'-ACTC CTA CGG GAG GCA GCA GT-3', HDA2: 5'-GTA TTA CCG CGG CTG CTG GCA-3'. A 40-bp clamp, named GC (5'-CGC CCG GGG CGC GCC

CCG GGC GGG GCG GGG GCA CGG GGG G-3') flanked the 5' extremity of HDA1 [47] in order to form HDA1-GC. PCR was performed using an Eppendorf thermal cycler® (Eppendorf, Le Pecq, France) and 0.5 ml tubes. The reaction mixture (50 µl) contained 2.5 units of Taq DNA Polymerase (FastStart High fidelity PCR system, Roche, Meylan, France), 0.2 mM of each primer and 1 µl of DNA in the appropriate reaction buffer. Amplification was 95°C for 2 min, 35 cycles of 95°C for 1 min, 62°C for 30 s, 72°C for 1 min and 7 min at 72°C for final extension. To avoid contamination, solutions were prepared with sterile DNA-free water and preparation of the mastermix, addition of template DNA and gel electrophoresis of PCR products were carried out in separate rooms. PCR amplification was checked by DNA electrophoresis in 1.5% agarose gels containing ethidium bromide and visualized under ultraviolet light.

2.4. TTGE migration

TTGE was performed using the DCode universal mutation detection system (Bio-Rad Laboratories, Marne-la-Coquette, France) in gels that were 16 cm × 16 cm by 1 mm. The gels (40 ml) were composed of 8% (wt/vol) bisacrylamide (37.5:1), 7 M urea, 40 µl of N,N,N',N'-tetramethylethylenediamine (TEMED), and 40 mg ammonium persulfate (APS). Gels were run with 1X Tris-acetate-EDTA buffer at pH 8.4. The 5 µl of DNA was loaded on gel with 5 µl of in-house dye marker (saccharose 50%, Bromophenol Blue 0.1%) using capillary tips. Denaturing electrophoresis was performed at 46 V with a temperature ramp from 63°C to 70°C during 16 h (0.4°C/h) after a pre-migration of 15 min at 20 V and 63°C. Gels were stained with ethidium bromide solution (5 µg/ml) for 20 minutes, washed with de-ionized water, viewed using a UV transillumination system (Vilbert-Lourmat, France) and photographed.

2.5. TTGE band sequencing and OTU affiliation

TTGE bands were excised and the DNA was eluted with 50 µl of elution buffer (EB) of the Qiaquick PCR purification kit (Qiagen, Courtabeuf, France) overnight at 37°C before PCR amplification with HDA1/HDA2 used without GC clamp. The reaction conditions were identical to those described above. PCR products were sequenced on an ABI 3730xl sequencer (Cogenics, Meylan, France). Each sequencing chromatograph was visually inspected and corrected. The sequences were analyzed by comparison with Genbank (<http://www.ncbi.nlm.nih.gov/>) and RDPII databases (<http://rdp.cme.msu.edu/>) using Basic Local Alignment Search Tool (BLAST) and Seqmatch programs, respectively. The reference sequence with the highest percentage was used for OTU affiliation. A sequence was affiliated to a species-level OTU when the percent of sequence similarity was above 99.0%, as previously proposed [62]. This value is over the recognized cut-off value for the delineation of species [63], but warrants high stringency for species-level OTU affiliation. Below 99.0%, the sequence is affiliated to the genus of the reference sequence with the highest percentage. When several species reference sequences match equally, affiliation was done to the genus level. For example, sequence with 99.5% in similarity to the species *Aeromonas caviae* and *Aeromonas hydrophila* was only assigned to the genus *Aeromonas*. Low cut-off is not defined for the genus delineation since affiliation to a higher taxonomic rank such as family or order will be done considering the taxonomic frame of the clade using Greengenes database [64]. On each TTGE gel, about

50% of the bands were sequenced, the others being affiliated to an OTU by comparison of their migration distance with that of sequenced bands.

2.6. Phylogeny

The sequences for phylogenetic analysis were selected in the GenBank database using BLAST program and taxonomy browser (<http://www.ncbi.nlm.nih.gov>). The sequences were then quality checked using SEQMATCH program in the 16S rDNA-specialized database, RDP-II (<http://rdp.cme.msu.edu>). Sequences were aligned using the ClustalX program [65], and the alignment was manually corrected to exclude gaps and ambiguously aligned regions. Maximum-likelihood (ML) phylogenetic analysis was performed using PhyML v2.4.6 [66], the model being General Time Reversible plus gamma distribution plus invariable site. ML bootstrap support was computed on 100 reiterations using PhyML.

3. Results

3.1. *Anopheles* species

Among the 175 specimens of *Anopheles* collected in Thailand and Vietnam, a total of 10 species were identified including six species per country of which two, *An. maculatus* and *An. dirus*, were common to both countries (Table 2). Eight species out of 10 belong to a group or a complex of which the sibling species were identified using the appropriate PCR assay (see Material and Methods). The Maculatus Group was represented by two species, *An. maculatus* and *An. sawadwongporni*, the latter collected in Thailand only. Within the Dirus Complex, three species were identified, *An. dirus*, *An. baimaii* and *An. scanloni*, the latter two were also collected in Thailand, as well as two species of the Minimus Complex, *An. minimus* and *An. harrisoni*. Three additional species were collected in Vietnam, *An. gigas* belonging to the Gigas Complex, *An. barbumbrosus*, and *An. crawfordi*. Among the 10 collected species, the former seven species of the Maculatus Group, Dirus and Minimus Complexes are defined as important malaria vectors and the latter three species have not been reported as being involved in malaria transmission [19, 67].

3.2. PCR-TTGE profiles and diversity index in midgut bacterial communities of *Anopheles*

The midgut microbiota of 175 specimens of *Anopheles* mosquitoes was investigated by 16S rRNA gene PCR-TTGE anchored in the V3 hypervariable region. A representative gel is given in Figure 2. TTGE profiles were obtained for 144 samples, 31 samples (17.7%) giving no amplification in PCR or a faint PCR signals leading to non-detectable TTGE profiles. Negative results suggested a low bacterial inoculum rather than a total absence of bacteria in the corresponding samples. Most negative samples came from Vietnam mosquitoes (n=26), compared to Thailand (n=5), and seemed to be unrelated to the *Anopheles* species. Finally, V3 16S PCR-TTGE approach led to the description of a microbial community for about 80% of the specimens analyzed and therefore appeared as an efficient tool to investigate midgut bacterial diversity in a large population of mosquitoes.

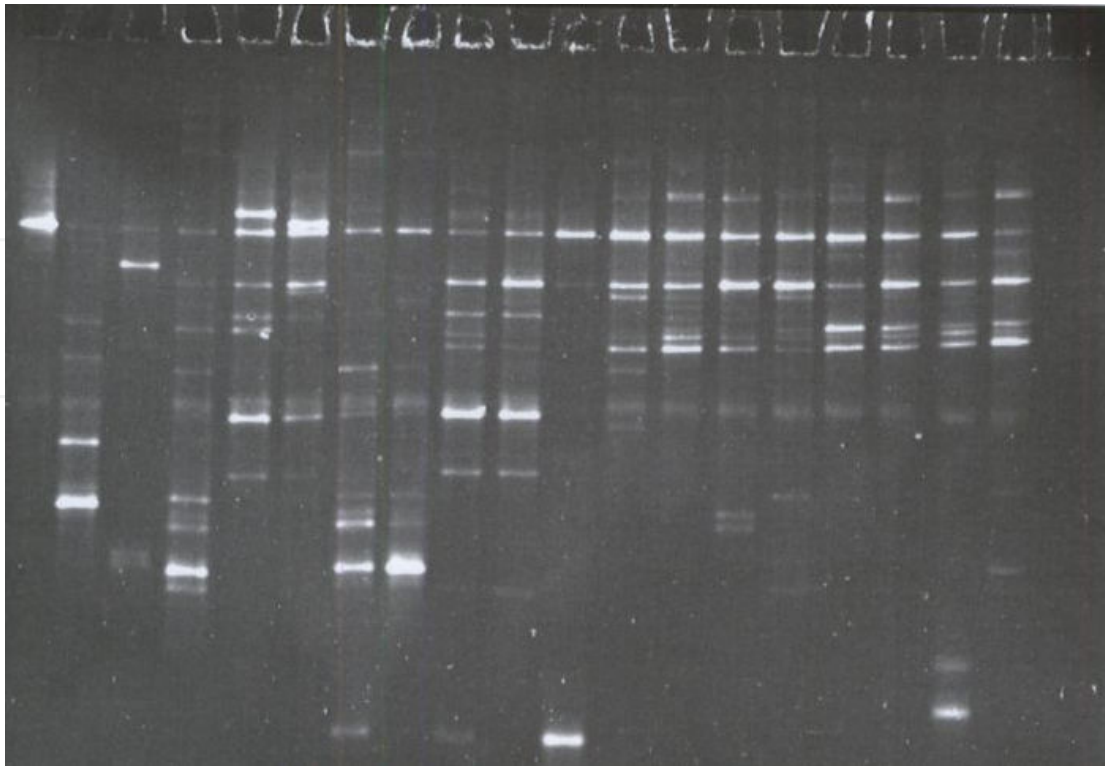


Figure 2. Representative TTGE analysis of V3 16S rRNA gene PCR products amplified from midgut samples of *Anopheles* mosquitoes from Thailand. Each lane corresponded to a specimen microbiota.

A raw diversity index that globally reflects the bacterial diversity in a sample is classically evaluated by counting the bands in TTGE profiles. At a first glance, the number of bands on TTGE profiles (Figure 2) ranged from 1 to 10 suggesting that the bacterial diversity per specimen ranged from 1 to 10 OTUs. However, sequencing showed that bands with different distance of migration could belong to the same OTU. This atypical phenomenon was observed for bacteria displaying sequence heterogeneity among their 16S rRNA gene copies. For instance, members of the genus *Acinetobacter* as well as most members of the genera affiliated to the family *Enterobacteriaceae* displayed a high level of 16S rRNA gene heterogeneity leading to complex banding patterns in V3 16S PCR-TTGE. Considering that *Acinetobacter* and *Enterobacteriaceae* were prevalent in our samples, the raw diversity index drastically overestimated the bacterial diversity. Therefore, a refined diversity index was calculated after affiliation of each band to an OTU by sequencing or by comparative approach (see Material and Methods).

The refined diversity index showed a low bacterial diversity per specimen with an average of 1.5 OTU per specimen. Most positive samples displayed a diversity index of 1 or 2 (Figure 3). Five OTUs is the maximal biodiversity per specimen observed in our population of *Anopheles* mosquitoes. Figure 3 showed that the number of OTUs per specimen differed slightly between populations from different origin, with an average of 1.7 and 1.3 OTU per specimen in Thailand and Vietnam, respectively. Considering mosquito species, the average diversity varied between 0 for *An. sawadwongporni* and 3 for *An. harrisoni* (Table 2).

| | COUNTRY | | VECTORS | | | | | | | | | NON VECTORS | | |
|--------------------|-----------------|-----------------|----------------------|----------------------|--------------------------|---------------------|----------------------|--------------------|--------------------|-----------------|------------------|------------------|------------------------|----------------------|
| | Thailand (n=75) | Vietnam (n=100) | Maculatus | | | Minimus | | Dirus | | | | | | |
| | | | An. maculatus (n=28) | An. maculatus (n=11) | An. sawadwongporni (n=1) | An. minimus (n= 37) | An. harrisoni (n=13) | An. baimaili (n=4) | An. scanloni (n=3) | An. dirus (n=6) | An. dirus (n=23) | An. gigas (n=24) | An. barumbrosus (n=13) | An. crawfordi (n=12) |
| Acinetobacter | | | 14 | | | 10 | 10 | 1 | 1 | | 11 | 18 | 3 | 5 |
| Aeromonas | | | | | | 8 | | | | | | | | |
| Asaia | | | 2 | | | | 4 | | | | | | | |
| Bacillus | | | 1 | | | | | 1 | | | | | | 1 |
| Cellvibrio* | | | | | | | | | | | 1 | | | |
| Chromobacterium* | | | | | | 1 | | | | | | | | |
| Chryseobacterium | | | | | | | 1 | | 1 | | | | 1 | |
| Citrobacter | | | | | | 1 | | | | | | | | |
| Corynebacterium* | | | | | | | | | | | | | | 1 |
| Cronobacter* | | | | | | 5 | | | | | | | | |
| Diaphorobacter* | | | | | | 1 | | | | | | | | |
| Diplorickettsia* | | | | | | | | | | | 1 | 1 | | |
| Elizabethkingia | | | | | | 4 | | | | 1 | | | | |
| Enhydrobacter* | | | | | | | | | | | | | 1 | |
| Enterobacter | | | | 1 | | 11 | | 1 | | 1 | 2 | | | |
| Escherichia | | | | | | 1 | | | | | | | | |
| Gluconacetobacter* | | | | | | | 2 | | | | | | | |
| Kluyvera | | | | | | | 3 | | | | | | | |
| Microbacterium* | | | | | | | | | | | | | 1 | |
| Moraxella* | | | 1 | | | | | | | | 1 | | | 2 |
| Nitrincola* | | | | | | | | | | | | 3 | | |
| Pantoea | | | | | | 1 | | | | | | | | |
| Pseudomonas | | | | 8 | | 1 | 8 | | 1 | 1 | | | | 2 |
| Psychrobacter* | | | | | | | | | | | | 1 | | |
| Raoultella* | | | | | | 2 | 9 | | | | 2 | | | |
| Riemerella* | | | | | | | | | | | | | 1 | |
| Serratia | | | | 8 | | 6 | | | 1 | 2 | | | | 3 |
| Shewanella* | | | | | | | 1 | | | | | | | |
| Sphingomonas* | | | 20 | | | | | | | | 16 | 8 | 1 | 1 |
| Staphylococcus* | | | | | | | | | | | | 1 | 1 | |
| Stenotrophomonas | | | | | | | | | | | 2 | | | |
| Diversity Index | 1.7 | 1.3 | 1.3 | 1.8 | 0 | 1.5 | 3 | 0.6 | 1.3 | 1.2 | 1.7 | 1.4 | 0.6 | 0.3 |

Table 2. Bacterial genera detected in midgut of *Anopheles* species caught in Thailand (blue) and Vietnam (red) with the number of specimens carrying each genus. Diversity index links to *Anopheles* species and origin is given at the bottom of the table. Genera described for the first time in *Anopheles* are marked with asterisk. Vertical lines delineated, from left to right, both countries with their respective number of *Anopheles* specimens, and groupings of *Anopheles* species, such as the Maculatus Group, the Minimus and Dirus Complexes, and the non-vector species including the Gigas Complex and 2 additional species.

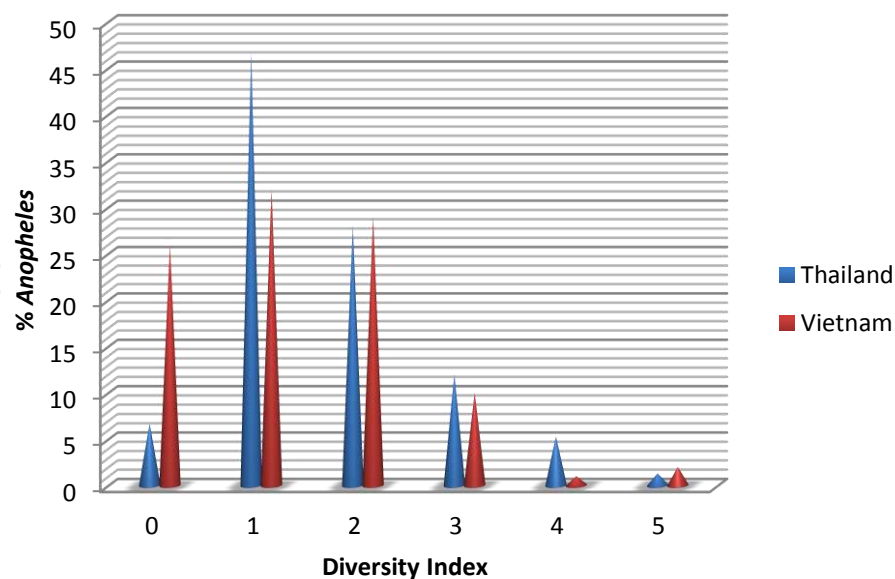


Figure 3. Distribution of the *Anopheles* mosquito populations from Thailand and Vietnam according to their refined diversity index

3.3. Bacterial diversity in the whole population of *Anopheles* mosquitoes

16S rRNA gene PCR-TTGE is focused on hypervariable region V3 produced sequences of about 200 bp, which are generally not informative enough for species affiliation. Consequently, we presented here the bacterial diversity to the genus level. However, probable species affiliation will be proposed for several genera when the phylogenetic signal of the V3 region was significant.

Contrasting with the low diversity per specimen, OTU diversity in the whole population was high with the detection of 31 different bacterial genera (Table 2) distributed in four phyla, *Proteobacteria*, *Bacteroidetes/Chlorobi*, *Firmicutes* and *Actinobacteria*. *Proteobacteria* largely dominated the midgut microbiota of *Anopheles* mosquitoes with 232 OTUs in the population studied. Their diversity encompassed *Alpha*, *Beta*- and *Gamma* superclasses of *Proteobacteria*.

The gamma-proteobacterial genera *Acinetobacter*, *Pseudomonas*, *Enterobacter*, *Serratia* and *Raoultella* were widely detected in our populations. A total of 40% of specimens and 70% of *Anopheles* species were colonized by members of the genus *Acinetobacter*, which therefore could be considered as a 'core genus' of the midgut microbiota of *Anopheles*. The sequences affiliated to the genus *Acinetobacter* were identified to the species level by a phylogenetic approach (Figure 4). The *Anopheles* midgut microbiota included 6 main species, *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, *Acinetobacter johnsonii*, *Acinetobacter soli*, *Acinetobacter guillouiae* and *Acinetobacter junii*, the two latter being more represented. The genus *Acinetobacter* belongs to the order *Pseudomonadales* in gamma-proteobacteria together with *Pseudomonas* (*Pseudomonas fluorescens* and *Pseudomonas alcaligenes*), *Moraxella*, *Enhydrobacter*, *Psychrobacter* and *Cellvibrio*. *Enterobacteriales* was the second main order of gamma-proteobacteria represented in the midgut microbiota of *Anopheles*. In enterobacteria, the species affiliation could not be achieved since genera are very close together in 16S rRNA gene phylogeny, particularly for *Enterobacter* and its rela-

tives *Cronobacter* and *Pantoea*. Members of gamma-proteobacteria of the orders *Legionellales* (*Diplorickettsia*), *Oceanospirillales* (*Nitrincola*), *Alteromonadales* (*Shewanella*), *Xanthomonadales* (*Stenotrophomonas*) and *Aeromonadales* (*Aeromonas*) were also detected showing the very wide diversity of gamma-proteobacteria in the midgut microbiota of *Anopheles*.

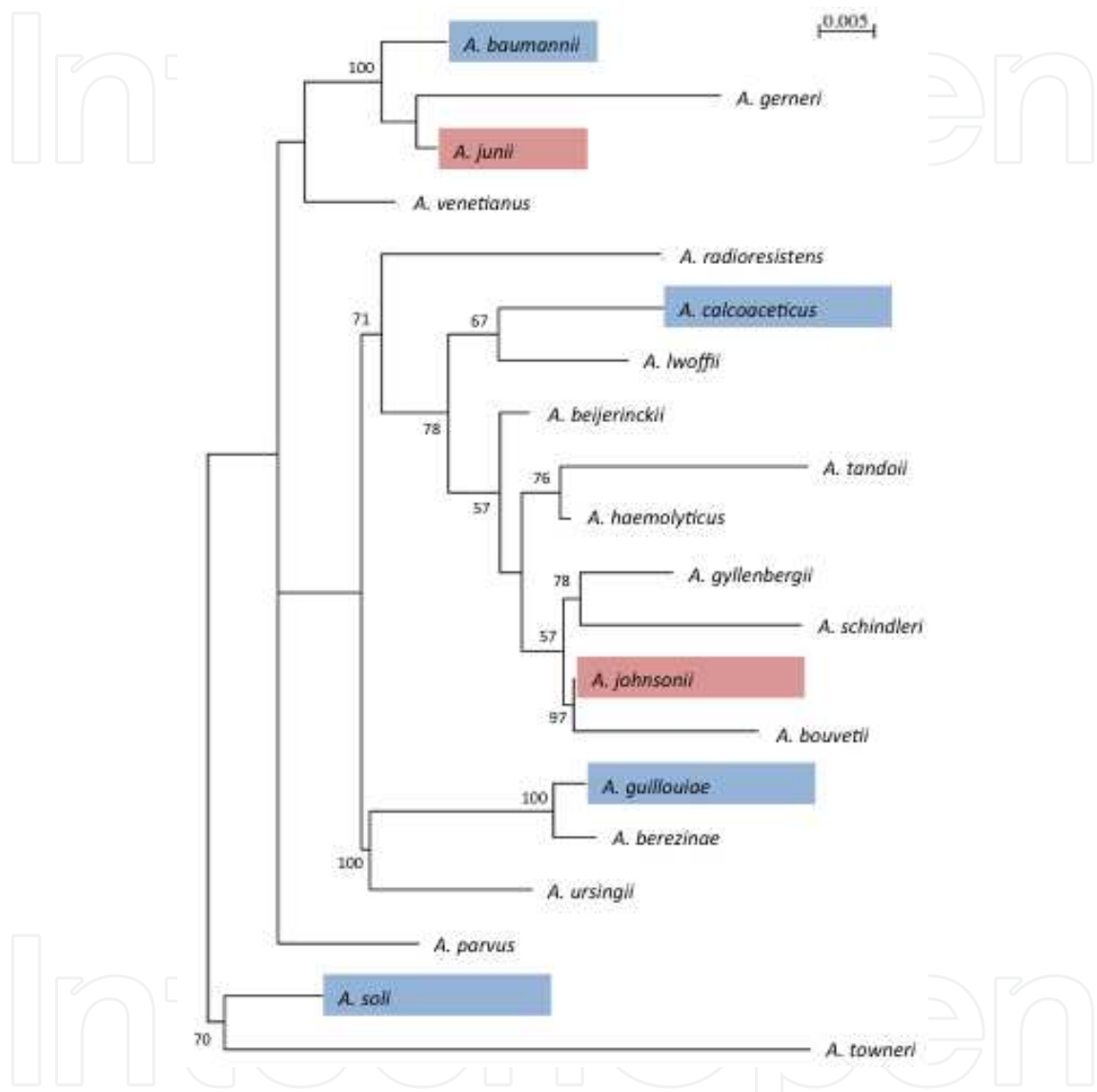


Figure 4. Maximum Likelihood phylogenetic tree of the genus *Acinetobacter*. Lineages of strains detected in the microbiota of *Anopheles* mosquitoes are in color, blue for Thailand, red for Vietnam. Bootstrap percentages (>50 %) after 100 resamplings are shown. Bar: 0.5 % sequence divergence.

The diversity was lower in alpha- and beta-proteobacteria. However, the genus *Sphingomonas* that belonged to *Alphaproteobacteria*, was the second main genus detected in this study (26% of the *Anopheles* species colonized) mostly represented by sequences affiliated or related to the species *Sphingomonas aromaticivorans* and *Sphingomonas glacialis*. Acetic-acid bacteria (*Asaia* and *Gluconacetobacter*) belonged to *Alphaproteobacteria* and were sporadically represented as well as *Chromobacterium* and *Diaphorobacter*, the two members of *Betaproteobacteria*.

Beside *Proteobacteria*, the phyla *Bacteroidetes/Chlorobi*, *Actinobacteria* and *Firmicutes* were represented by only few genera: 15, 9 and 5 respectively. *Chryseobacterium*, *Elizabethkingia* and *Riemerella*, which colonized only 8 mosquitoes, belonged to *Bacteroidetes/Chlorobi* and class *Flavobacteriia*. Sequences affiliated to the genus *Elizabethkingia* could not be related with certainty to *Elizabethkingia anophelis*, because the V3 region did not discriminate between this *Anopheles*-specific species and the human pathogen *Elizabethkingia meningoseptica*. *Bacillus* and *Staphylococcus* (*Firmicutes*), *Corynebacterium* and *Microbacterium* (*Actinobacteria*) were the sole Gram-positive genera found in the population of *Anopheles* mosquitoes. The most related species were *Bacillus cereus*, *Corynebacterium freiburgense* and *Microbacterium trichothecenolyticum*. The *Staphylococcus* sequences found in two mosquitoes were identical to those of a strain isolated in the midgut of the ladybug *Harmonia axyridis* and were linked to the species *Staphylococcus sciuri*.

3.4. Bacterial associations and relationship

Acinetobacter spp. was present in all mosquito specimens except in *An. maculatus* and *An. dirus* from Thailand. Specimens of these two *Anopheles* species were mainly colonized by *Pseudomonas* and *Serratia* (Table 2). When the microbiota of each specimen is considered (data not shown), the pair *Pseudomonas* / *Serratia* never co-habited with *Acinetobacter* in the same midgut. *Pseudomonas* strains associated with *Serratia* were related to the species *P. fluorescens* whereas *P. alcaligenes* was never associated with *Serratia* and inhabited midguts colonized with *Acinetobacter*. These results suggested that the association *P. fluorescens* / *Serratia* might specifically inhibit the colonization of *Anopheles* midgut by *Acinetobacter*.

Negative relationships between *Sphingomonas* and enterobacteria were also suggested in Table 2 for mosquitoes from Vietnam. Considering each specimen, we always observed the absence of enterobacteria when *Sphingomonas* colonized the midgut (data not shown).

3.5. Comparison of bacterial diversity in the midgut of *Anopheles* from Thailand and Vietnam

Table 2 and Figure 5 showed the differential distribution of bacterial genera according to the geographic origin of mosquitoes. Eight genera were shared between specimens from Thailand and Vietnam and corresponded to genera with high prevalence such as *Enterobacter*, *Serratia*, *Pseudomonas* and *Acinetobacter*. In Thailand, each of these four genera colonized more than 10% of specimens, each of the genera *Raoultella*, *Cronobacter*, *Aeromonas*, *Elizabethkingia* and *Asaia* colonized 3 to 10% of the specimens, and 10 other genera colonized 2% or less of the specimens (Figure 5A).

Except for the core genus *Acinetobacter*, main genera found in Thailand were not prevalent in specimens from Vietnam, *Enterobacter*, *Serratia*, *Pseudomonas*, *Raoultella*, and *Asaia* colonizing each 2% or less of the Vietnam specimens (Figure 5B). *Cronobacter*, *Aeromonas* and *Elizabethkingia* were not detected in *Anopheles* mosquitoes from Vietnam. Except for the genus *Acinetobacter* again (40%), the more prevalent genera in specimens from Vietnam appeared origin-specific. Indeed, *Sphingomonas* and *Moraxella* present in *Anopheles* from Vietnam at 36% and 3% respectively, were not detected in mosquitoes from Thailand (Figure 5B). When the species

forming the genus *Acinetobacter* were considered, we observed again an origin-specific distribution with *A. junii* and *A. johnsonii* dominating the microbiota of mosquitoes from Vietnam but absent from the Thailand samples. Gut microbiota of mosquitoes from Thailand displayed a wider *Acinetobacter* diversity with four species represented, *A. baumannii*, *A. calcoaceticus*, *A. soli* and *A. guillouiae* (Figure 4). In the same phylogenetic clade of *Acinetobacter*, bacterial lineages from Thailand mosquitoes differed from bacterial lineages of Vietnam mosquitoes. For instance, the lineages *A. baumannii* and *A. junii* belonged to the same clade in the 16S rRNA gene tree but inside this clade, each lineage was origin-specific (Figure 4).

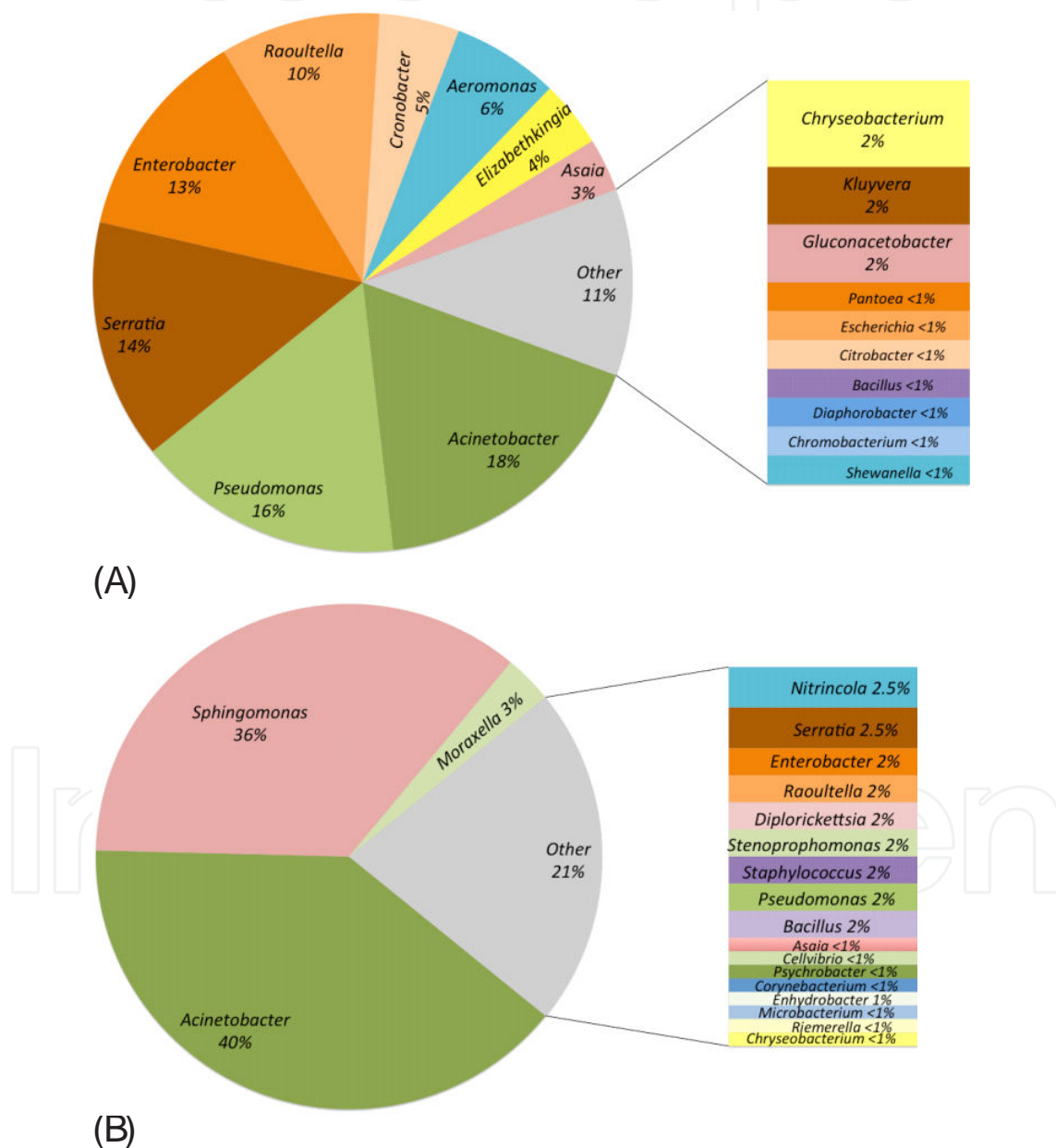
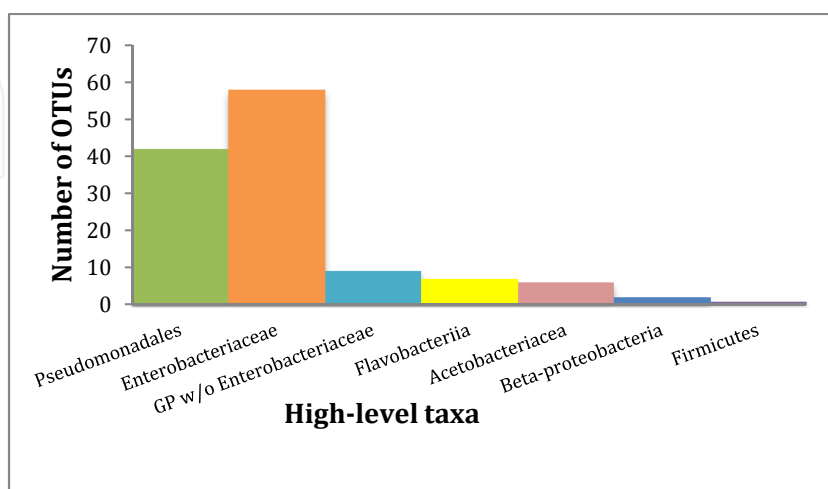
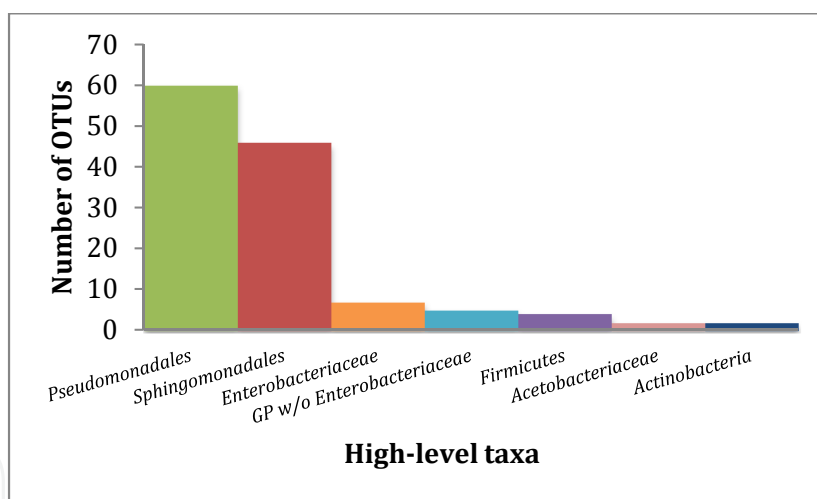


Figure 5. Repartition in genera of OTU assigned bands obtained by PCR-TTGE from 175 specimens of *Anopheles* mosquitoes from Thailand (A) and from Vietnam (B).

Considering bacterial taxa higher than the genus, the microbiotae of *Anopheles* from Thailand and Vietnam were both dominated by *Pseudomonadales* (Figure 6) due to the general high prevalence of *Acinetobacter*. *Enterobacteriaceae* largely dominated the microbiota of *Anopheles* from Thailand but contributed little to bacterial diversity in *Anopheles* from Vietnam.



(A)



(B)

Figure 6. Repartition in significant high-level bacterial taxa of OTU from 175 specimens of *Anopheles* mosquitoes from Thailand (A) and from Vietnam (B). GP for *Gammaproteobacteria*.

This low prevalence of enterobacteria in the midgut of *Anopheles* from Vietnam was particularly noteworthy (Fig. 6B). In opposite, *Sphingomonadales* was the major high-level taxon in Vietnam specimens but absent from Thailand specimens. Therefore, the ratio *Enterobacteriaceae* / *Sphingomonadales* appeared as a signature differentiating Thailand and Vietnam *Anopheles* specimens. Other signatures, which should be confirmed with more specimens, were *Betaproteobacteria* and *Actinobacteria* in specimens from Thailand and Vietnam, respectively.

3.6. Links between microbiota composition and *Anopheles* species or species complexes

Table 2 showed the distribution of bacterial genera according to the species of *Anopheles*. To evaluate the link between bacteria and host species, we first compared the microbiotae of the same mosquito species but from different origins. Specimens of *An. maculatus* gave a good model for this comparison because it was enough represented in both geographic sites (Figure 7). The two groups of microbiotae differed clearly, in particular considering the origin-specific signature, i.e. the ratio *Enterobacteriaceae* / *Sphingomonadales* (Figure 7). Therefore, the case of *An. maculatus* indicated that the microbiota composition was influenced by sampling geographic sites rather than *Anopheles* species. Comparison of the microbiotae between *An. dirus* from Thailand and Vietnam resulted in the same conclusion (Table 2).

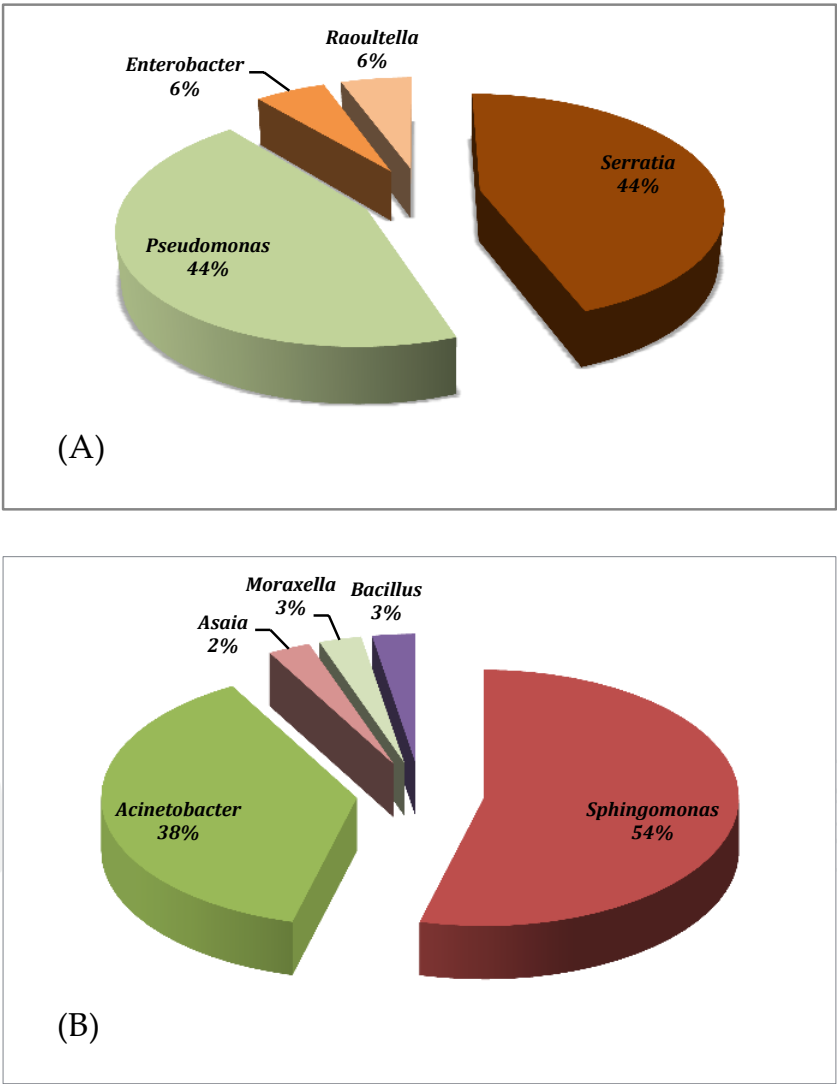


Figure 7. Comparison of the microbiota of *An. maculatus* caught in Thailand (n=11) (A) and in Vietnam (n=28) (B).

Sibling species within a group or a complex have been linked to the microbial content of the midgut. As previously observed for the species *An. maculatus*, the corresponding

complex displayed a non-specific microbiota but its bacterial colonization is influenced by the geographic origin. Similar situation was observed in the Dirus Complex for which the shared bacterial genera were *Acinetobacter* and *Enterobacter*, as well as for *An. gigas* of the Gigas Complex with shared bacteria belonging to the dominating genera *Acinetobacter* and *Enterobacter*.

In the Minimus Complex, *An. minimus* and *An. harrisoni* were colonized by 18 different bacterial genera but only three were shared by both species. Two shared genera corresponded to bacteria widely represented in the whole population, *Acinetobacter* and *Pseudomonas*, while the third one, *Raoultella*, seemed to be more specific, represented in two and nine specimens of *An. minimus* and *An. harrisoni* respectively, showing its higher association to the latter species.

4. Discussion

To our knowledge, this study describes the midgut microbiotae of the largest population of field-collected *Anopheles* species with 10 species (Table 2) when the literature shows 9 analyzed species, *An. gambiae* being the most studied species of all (Table 1). Thereby, 16S rRNA gene PCR-TTGE focused on hypervariable region V3 proves its efficiency to study microbiota of *Anopheles* mosquitoes. This method, that presents a relative low resolution, is efficient to follow bacterial communities with low to moderate diversities. This limit is due to the number of bands that can be separated within the length of the gel. Optimization of TTGE conditions allows separation of bands by a minimum of 0.1 mm over all the gel length. Therefore, TTGE would be difficult to interpret if the diversity exceeds 25 to 30 OTUs [45]. Microbiotae of *Anopheles* displays TTGE profiles that do not exceed 10 bands but the profiles have been interpreted with difficulties due to heterogeneities in rRNA genes for most bacteria in the mosquito midgut ecosystem. At the genomic level, rRNA genes are generally organized in multigene families [68] in which sequences show low variability within species, subspecies or genome [69]. However, intra-genomic heterogeneity in the form of nucleotide differences between 16S rRNA gene copies are described in relation to fine-tuning of the ribosome function to optimize bacterial niche fitness [70]. In PCR-TTGE, heterogeneities lead to multiple bands for a single OTU and then to an overestimation of OTU diversity. This pitfall has been avoided here by band sequencing that led to the definition of a refined diversity index drastically lowered in comparison with the raw diversity index. The level of ribosomal heterogeneity in bacteria genome from midgut of mosquitoes suggested adaptation processes in a rather instable niche.

With the development of high-output sequencing, twenty-one century metagenomics consider fingerprint approaches as obsolete. However, these methods remain of great interest to give a snapshot of microbiota in large populations of hosts. Thereby, we described herein the midgut microbiotae of 175 specimens of 10 *Anopheles* species with a sequencing effort of less than 150 reads compared to 5 millions of reads estimated for the same study by pyrosequencing. A pyrosequencing study of the midgut microbiota of *An. gambiae* (30 laboratory breed and two field-collected mosquitoes) has been recently published [18]. Authors described bacteria

belonging to 26 phyla, among which, five represented more than 99% of the total microbiota: *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, and *Fusobacteria*. Except the latter, four phyla corresponded to those described in this study, suggesting that PCR-TTGE explored the majority of bacterial populations in the microbiota. Among 147 OTUs detected by pyrosequencing, only 28 genera had an abundance of >1% in at least one mosquito midgut [18]. This is in accordance with our results describing 31 bacterial genera in the microbiota of field-collected *Anopheles*. Fourteen of the 31 genera have been previously detected in diverse studies on field-collected *Anopheles* (Table 1). Then, we would like to highlight the fact that 17 bacterial genera were described herein for the first time (Table 2), 6 (32%) and 12 (60%) from the populations of Thailand and Vietnam respectively, suggesting that the bacterial diversity associated to midgut of *Anopheles* remains underestimated. It is noteworthy that twice as many new genera were found in specimens from Vietnam compared to Thailand. Newly described genera were scarcely represented in few specimens except for *Sphingomonas* found in 46 specimens belonging to five species from Vietnam and *Raoultella* found in 13 specimens belonging to 3 species from Thailand and Vietnam. Of note the genus *Sphingomonas* has been detected by pyrosequencing in the midgut of a population of *An. gambiae* maintained in standard insectary conditions [18].

The gut microbiota of mosquitoes presented a large inter-specimen variability but was dominated by few genera, *Acinetobacter*, *Pseudomonas*, *Enterobacter*, *Serratia*, *Raoultella* and *Sphingomonas*. Among them, *Acinetobacter* was considered as a mosquito midgut core genus because it was detected in most specimens. *Acinetobacter*, *Pseudomonas* and *Sphingomonas* also belong to the *An. gambiae* midgut core microbiota defined by Boissière et al. [18]. *Asaia* was found in all samples by pyrosequencing but its relative abundance showed great variations ranging from 0.04 to 98.95% according the *An. gambiae* specimen [18]. We detected *Asaia* in only 6 specimens of *An. maculatus* and *An. harrisoni*. Again, our result compared to pyrosequencing data suggested that PCR-TTGE failed to detect minority and/or low loaded populations. This low resolution is certainly a limit but we also see it as a benefit because the majority taxa detected by TTGE probably corresponds to true colonizers of the midgut and not to transient or contaminant bacteria.

Anopheles-associated bacterial species recently described were not detected or identified with confidence in this study. Members of the genus *Elizabethkingia* detected in *Anopheles* from Thailand could not be identified as *E. anophelis* owing to its relatedness in 16S rRNA gene sequence with *E. meningosepticum*. Of note, *Thorsellia anophelis* has been detected in mosquitoes used in the optimization step of this study but not in mosquitoes included in the study population.

In spite of the large inter-specimen variability, sub-populations from different geographic origins exhibit drastically different midgut microbiotae. High prevalence of *Enterobacteriaceae* and absence of *Sphingomonas* spp. characterize microbiotae of *Anopheles* caught in Thailand whereas *Anopheles* in Vietnam displayed high prevalence of *Sphingomonas* and low rate of enterobacteria. Similar differences in enterobacteria prevalence have been described in *An. gambiae* originating from two sampling sites in Cameroon [18]. Composition of the midgut microbiota seems unrelated to *Anopheles* species, except for *Raoultella* and *An. harrisoni* but their

relationship needs to be confirmed on additional specimens. Some positive and negative associations of bacteria suggested complex interactions in the microbiota. The most striking result was the pair *P. fluorescens* / *Serratia* which never co-habited with *Acinetobacter*. *Pseudomonas fluorescens* is well known as a great anti-microbial and bacteriocin-like substances producer [71] exhibiting negative effect on diverse Gram-negative bacteria and biofilm formation [72]. The bacteriocins are narrow-spectrum toxins that typically kill bacteria related to the producing strain as is the case for *P. fluorescens* and *Acinetobacter*, which both belong to the order *Pseudomonadales*. Moreover, bacteriocins can play an important role in the fitness of a strain by killing or inhibiting bacterial co-inhabitants that compete for the limited resources probably found in the midgut environment [71]. Similar antagonism was observed between *Sphingomonas* and enterobacteria in mosquitoes from Vietnam. *Sphingomonas* is a sparsely known genus but antimicrobial activities against *Candida* have been described recently [73]. Culture of the natural isolates of *P. fluorescens*, *Serratia*, *Acinetobacter* and *Sphingomonas* should confirm these potential antagonisms and give insights about their mechanism.

Antagonism against enterobacteria is of particular interest because it has been suggested that mosquitoes harboring *Enterobacteriaceae* are more likely to be infected by *P. falciparum* [18]. In our collection, *An. minimus* specimen KAN-27 from Pu Teuy, Kanchanaburi was infected by *P. falciparum* and displayed a microbiota containing exclusively enterobacteria that belonged to four genera, *Pantoea*, *Enterobacter*, *Cronobacter* and *Escherichia*. This specimen displayed the highest enterobacterial diversity of the *Anopheles* collection and the core genus *Acinetobacter* was not detected. Identification of the *Enterobacter* species in our samples will be the next step with the search for *Enterobacter* (Esp_Z), which was reported to inhibit *P. falciparum* development in *An. gambiae* [15]. As the microbiota might have an impact on pathogen development in *Anopheles* mosquitoes and disease transmission, more studies need to be done for better understanding the role of some specific bacteria in wild mosquito populations before developing potential method of control.

5. Conclusion

Based on the analysis of the midgut microbiota of 10 field-caught *Anopheles* species from Thailand and Vietnam, we described 17 bacterial genera for the first time in *Anopheles* mosquitoes, suggesting that the bacterial diversity associated to midgut of *Anopheles* remains underestimated. Low bacterial diversity ranging from one to three per specimen was found which contrasted with a high OTU diversity in the whole *Anopheles* population that presented 31 different bacterial genera distributed in four phyla, *Proteobacteria*, *Bacteroidetes/Chlorobi*, *Firmicutes*, and *Acinetobacteria*. More specifically, the association of *Pseudomonas* and *Serratia* never co-habited with *Acinetobacter* in the same mosquito midgut. The same presence/absence was observed between *Sphingomonas* and enterobacteria. Midgut microbiota was drastically different for the *Anopheles* from Thailand compared to those from Vietnam showing the importance of the geographic origin. The ratio *Enterobacteriaceae* / *Sphingomonadales* appeared as a signature differentiating the *Anopheles* specimens from Thailand and Vietnam.

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