Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar

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**Introduction**

All arthropod pests and vectors harbor a number of commensal and mutualistic microorganisms that have an impact on the ecology and behavior of their hosts (Buchner, 1965; Moran *et al*., 2008; Moya *et al*., 2008). Indeed, it is well-known that microbial communities associated with insects can contribute to host reproduction and survival, community interactions, protection against natural enemies and vectorial competence (Buchner, 1965; Moran *et al*., 2008; Moya *et al*., 2008; Gottlieb *et al*., 2010; Oliver *et al*., 2010). However, such extended phenotypes were mostly shown in phytophagous arthropods, whereas research on hematophagous insects has been limited. Historically, this unawareness was partly due to the lack of data on the composition of native bacterial communities associated with the later group of insects. A few studies have, however, reported a number of bacterial species in some medically important hematophagous insects. A relevant example is the tsetse fly *Glossina*, which harbors the secondary symbiont *Sodalis glossinidius*, suspected to enhance vectorial competence (Cheng & Aksoy, 1999; Aksoy & Rio, 2005; Farikou *et al*., 2010). More recently, bacteria belonging to genera *Enterobacter*, *Entero- coccius* and *Acinetobacter* were isolated in *Glossina palpalis palpalis*, but their role in the tsetse fly biology remains to be determined (Geiger *et al*., 2009).

Mosquitoes are vectors of a large number of animal and human pathogens, including parasites and viruses. During the last few years, Madagascar and other neighboring islands have experienced severe epidemics of arboviruses, notably chikungunya and dengue. The species *Aedes albopictus* and *Aedes aegypti* have expanded over the Indian Ocean Islands (Fontenille & Rodhain, 1989; Salvan & Mouchet, 1994; Delatte *et al*., 2008; Sang *et al*., 2008; Bagney *et al*., 2009a, b)

**Abstract**

Symbiotic bacteria are known to play important roles in the biology of insects, but the current knowledge of bacterial communities associated with mosquitoes is very limited and consequently their contribution to host behaviors is mostly unknown. In this study, we explored the composition and diversity of mosquito-associated bacteria in relation with mosquitoes’ habitats. Wild *Aedes albopictus* and *Aedes aegypti* were collected in three different geographic regions of Madagascar. Culturing methods and denaturing gradient gel electrophoresis (DGGE) and sequencing of the rrs amplicons revealed that *Proteobacteria* and *Firmicutes* were the major phyla. Isolated bacterial genera were dominated by *Bacillus*, followed by *Enterobacter*, *Agrobacterium* and *Acinetobacter*. Common DGGE bands belonged to *Aci netobacter*, *Asaia*, *Delftia*, *Pseudomonas*, *Enterobacteriaceae* and an uncultured *Gammaproteobacterium*. Double infection by maternally inherited *Wolbachia* *pipiensis* prevailed in 98% of males (*n* = 272) and 99% of females (*n* = 413); few individuals were found to be monoinfected with *Wolbachia* wAlbB strain. Bacterial diversity (Shannon–Weaver and Simpson indices) differed significantly per habitat whereas evenness (Pielou index) was similar. Overall, the bacterial composition and diversity were influenced both by the sex of individuals and by the environment inhabited by the mosquitoes; the latter might be related to both the vegetation and the animal host populations that *Aedes* used as food sources.
and have been identified as the primary vectors responsible for these outbreaks (Schuffenecker et al., 2006; Vazeille et al., 2007; Delatte et al., 2008; Ratsitorahina et al., 2008; Sang et al., 2008). As for all insects, the successful spreading of mosquitoes worldwide might be partly linked to their symbiosis with microorganisms, notably with bacteria. However, little is known about the current composition of mosquito-associated microbial communities, and consequently, their potential contribution to the host behaviors is mostly ignored. Investigations have been performed to screen bacterial communities in mosquitoes reared under laboratory conditions or collected in the fields, using culture and nonculture methods. These studies have focused mainly on the gut microbial communities of two mosquitoes, *Anopheles* and *Culex*, and these revealed the presence of diverse bacterial groups including known genera such as *Acinetobacter*, *Aeromonas*, *Asaia*, *Bacillus*, *Enterobacter*, *Flavobacterium*, *Lactococcus*, *Pantoea*, *Pseudomonas*, *Microbacterium*, *Staphylococcus* and *Stenotrophomas* (Pumpuni et al., 1996; Strait et al., 1998; Pidiyar et al., 2004; Favia et al., 2007; Terenius et al., 2008; Rani et al., 2009). These surveys highlighted that the relative abundance and the composition of mosquito-associated bacteria varied depending on the developmental stages and laboratory-reared or wild targeted populations. For *Aedes* mosquitoes, Demaio et al. (1996) reported the occurrence of cultivable bacteria belonging to *Enterobacter*, *Klebsiella*, *Pseudomonas* and *Serratia* in the midgut of wild *Aedes triseriatus*. Most recently, this inventory was extended to *Acinetobacter*, *Asaia*, *Bacillus*, *Comamonas*, *Delftia*, *Pantoea* and *Wollbachia* detected in *A. aegypti* or *A. albopictus*, reared in insectaries (Gusmao et al., 2007, 2010; Crotti et al., 2009; Zouache et al., 2009b).

The aim of this study was to survey the composition of bacterial communities associated with wild *Aedes* mosquitoes and to explore whether the bacterial diversity is related to host ecology. To that end, we used culture and nonculture methods to describe the bacterial composition and diversity of *A. albopictus* and *A. aegypti*, males and females, caught from ecologically contrasted regions of Madagascar.

### Materials and methods

#### Location and characteristics of survey areas

The sampling regions were selected for their different ecoclimatic characteristics (Table 1) and because they were sites of chikungunya or/and dengue epidemics (Ratsitorahina et al., 2008; Randrianasolo et al., 2010), although no such virus infection was detected in the sampled population (data not shown). Vegetation and animals of the sampling sites are reported in Table 1.

The Analamanga region (Tsimbazaza Park, Ambohidratrimo and Ankazobe) is located in the centre of Madagascar at an altitude of 1200–1500 m. This region has a highland climate with two seasons: a hot and rainy period from October to March (21 °C average and about 200 mm of precipitation per month), followed by a cold and dry period (with temperatures down to 10 °C and rainfall not exceeding 20 mm month⁻¹). The mean relative humidity in this region is high (77.5% in 2008). The Zoological and Botanic Park of Tsimbazaza is located in the centre of Antananarivo town at 1250 m altitude. Ambohidratrimo Hill is located 25 km to the northwest of Antananarivo with an altitude of 1300 m. Ankazobe is 80 km from the northern limits of Antananarivo at 1500 m altitude. This site is transitional, connecting the central and the western regions. It is surrounded by the nature reserve of Ambohitantely. The climate is wetter and colder than the other towns in the centre.

The Atsinanana region (Toamasina) is on the east coast of Madagascar at sea level. The climate is particularly hot and humid: the mean annual rainfall is about 3200 mm with rain all year, the mean annual temperature is 25 °C with a minimum of 18 °C from June to August, and relative humidity is around 87% all year.

The Boeny region (Mahajanga, Andranofasika and Ankarafantsika natural reserve) has an arid tropical climate characterized by a warm summer (mean temperature of 27 °C) with moderate rainfall (mean precipitation is about 400 mm year⁻¹) and high relative humidity (81%) from November to March. Mahajanga is in the northwest of Madagascar.

<table>
<thead>
<tr>
<th>Region</th>
<th>Site</th>
<th>Zone</th>
<th>Potentially bitten hosts</th>
<th>Vegetation</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analamanga</td>
<td>Ambohidratrimo</td>
<td>Village outskirts</td>
<td>Humans, birds, reptiles</td>
<td>Bamboo hedge</td>
<td>137</td>
<td>35</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Tsimbazaza Park</td>
<td>City</td>
<td>Humans, lernus, reptiles, birds</td>
<td>Bamboo, bushes</td>
<td>823</td>
<td>62</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ankazobe</td>
<td>Village outskirts</td>
<td>Humans, chickens</td>
<td>Bamboo forest</td>
<td>93</td>
<td>95</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Boeny</td>
<td>Mahajanga Town</td>
<td>City</td>
<td>Humans, ovine (sheep), bovine (zebu)</td>
<td>Fruit trees, bushes</td>
<td>290</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Andranofasika</td>
<td>Village</td>
<td>Humans, birds</td>
<td>Mango trees</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Ankarafantsika</td>
<td>Natural reserve</td>
<td>Humans, lernus, birds, reptiles</td>
<td>Forest</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Atsinanana</td>
<td>Toamasina Town</td>
<td>City</td>
<td>Humans, chickens, ducks</td>
<td>Fruit trees, bamboo hedge, bushes</td>
<td>320</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Numbers of individuals collected at each site between February and May 2008.*
Madagascar, 600 km from Antananarivo in Edge Sea at a 22 m altitude. There are mango trees, bushes and flowers near dwellings in the town. The Andranofasika village is about 110 km from Mahajanga town and 5 km from the National Park of Ankarafantsika.

**Mosquito collection**
Mosquitoes were collected between February and May 2008. Two methods were used to capture adult mosquitoes: during peaks of biting activity, a tube was used to capture insects landing on the human body or nets were used to capture insects near the grass. Aedes specimens, males and females, were identified using morphological characteristic keys (Ravaonjanahary, 1978). Captured adults were separated according to species and sex and stored in tubes containing silica gel. For each tube, the species, date, location, geographical position, and type of site was recorded. Only non-blooded mosquitoes were used for the analysis.

**Bacterial isolation**
Only live mosquito specimens from the field were used. Individuals were anaesthetized at 4 °C, rinsed three times in sterile water, surface disinfected in 70% ethanol for 10 min and rinsed five times in sterile water and once in sterile 0.8% NaCl. Two adult mosquitoes per sample were crushed in 150 μL sterile 0.8% NaCl. Homogenates (10 μL) were streaked on plates of modified Luria–Bertani and PYC agar media (Zouache et al., 2009b). After incubation at 26 °C, single distinct colonies were reincubated onto fresh agar plates of the corresponding medium. Colonies were streaked to check for purity and stored in 25% glycerol at −80 °C until use.

**Genomic and plasmid DNA extractions**
Mosquitoes were surface disinfected as described above, and then individually crushed in 200 μL of extraction buffer (2% hexadecyltrimethyl ammonium bromide, 1.4 M NaCl, 0.02 M EDTA, 0.1 M Tris pH 8, 0.2% 2-β-mercaptoethanol) heated to 60 °C. Homogenates were incubated for 15 min at 60 °C and proteins were extracted with chloroform:isoamyl alcohol (24:1, v/v). DNA was precipitated with isopropyl alcohol, pelleted by centrifugation for 15 min at 12,000 g, washed with 75% ethanol, dried and then dissolved in 30 μL of sterile water.

For bacterial isolates, genomic and plasmid DNA were extracted using the DNeasy Tissue Kit and QIAprep Spin Miniprep Kit, respectively (Qiagen, France). Reactions (25 or 50 μL volumes) contained genomic DNA template (1 μL), 200 μM of each dNTP, 500 nM of each primer, 0.025 mg mL⁻¹ of T4 gene 32 protein (Roche, France) and 0.5 U of Expand polymerase in 1 × reaction buffer (Roche). PCR products were purified using QIAquick PCR Purification Kit (Qiagen). ARDRA was performed to screen the rrs genes of bacterial isolates in 20 μL reactions containing 200 ng of DNA, 1 × Buffer Tango™ and 10 U of each endonuclease RsaI and HhaI (Fermentas, France). DNA fragments were separated on 1% or 2% agarose gels stained with ethidium bromide.

Real-time quantitative PCR was performed using the LightCycler apparatus (Roche). The 20-μL reaction mixture contained 1 × LightCycler DNA Master SYBR Green I (Roche), primers at 300 nM (for wsp) or 200 nM (for actin) (see Table 2) and 10 ng of template DNA. The amplification program was 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C and 30 s at 72 °C. Standard curves were constructed using a dilution series (10⁵–10⁶ molecules) of the pQuantAlb plasmid (Tortosa et al., 2008) containing wsp and actin fragments.

**Denaturing gradient gel electrophoresis (DGGE)**
Ingény PhorU (Apollo Instruments, Compiègne, France) systems were used for DGGE analysis of the V3 PCR products as published (Zouache et al., 2009a). The 6% acrylamide gels contained a linear chemical gradient of urea and formamide from 35% to 65% urea and 40% deionized formamide (v/v). PCR products (2 μg) were run in 1 × TAE at 60 °C for 17 h at 100 V, and then gels were immersed in SYBR Green for 30 min, rinsed in distilled water and photographed under UV. Bands were excised, washed three times with sterilized water and then 30 μL of water was added to the tubes, which were heated to 60 °C for 30 min and kept overnight at 4 °C. The eluate (2 μL) was used for PCR amplification, and then amplicons were cloned and sequenced as described below.

**Cloning and sequencing**
PCR products were purified using the MinElute PCR Purification Kit (Qiagen), and cloned in the PCR® 2.1-TOPO® vector according to the TOPO TA 2.1 Kit (Invitrogen, France). Clones containing DNA inserts were sequenced at Genoscreen (Lille, France). Sequences were analyzed with the BLASTN program at NCBI (http://www.ncbi.nlm.nih.gov/).

**DGGE fingerprints and statistical analyses**
Each band was considered as an operational taxonomic unit (OTU). Images acquired with Fisher Bioblock Scientific System (Fisher, Ilkirch, France) were analyzed using GELCOMPAR II version 5.1 packages (Applied Maths, Kortrijk, Belgium). The software carries out a density profile analysis for
each lane and calculates the relative contribution of each band to the total band intensity in the lane, with a reference pattern included in all gels. Relative intensity in the profile of each band or OTU (\( Ni/f \)) was calculated (PRIMER v6 software) by the relative contribution of each band or OTU (\( ni/g \)). Diversity indices:

\[
S = \frac{N}{N_i} (1 - \frac{1}{\log N_i})
\]

\[
\log S = H'_{\text{max}}
\]

Statistical analyses were performed using SPSS software and/or R packages.

### Results

#### Collection of mosquitoes and bioecology

To collect *Aedes* adult mosquitoes, larval development sites were used as indicators (Table 1). Larvae refuges of *A. aegypti* consisted of natural sites (holes in trees or rocks, wet leaves of bamboo or palm trees and coconuts) outside cities and villages, whereas larvae refuges of *A. albopictus* were natural and artificial sites (containers or flowerpots) near habitations. Adults were collected around these larvae breeding sites.

Both species were found to be exophilic (which do not enter inside habitations). Except for natural reserves of the Ankafantsika and the Andranofasika villages, *A. albopictus* was predominant in all sites sampled (Table 1). Indeed, a total of 137 females and 35 males were caught in the neighborhood of the tourist attraction site of Ambobihitrino, named ‘Le Palais des Rois’. In Tsimbazaza Park, the presence of bamboo, bushes and many animals creates a favorable environment for *A. albopictus* development; a total of 823 females and 62 males were captured in this site. In contrast, only 93 females and 95 males were trapped in Ankazobe that has a colder climate (Table 1). The rainy and hot climate throughout the year in Toamasina allows uninterrupted development of *A. albopictus*, but it was difficult to capture adults during the active rainy season: 320 females and 30 males were trapped in the town itself. The two *Aedes* species were found in the Boeny region, but in different sites (Table 1). *Aedes albopictus* was predominant in urban areas, with 290 females and 20 males. *Aedes aegypti* was the major species found in village (Andranofasika) and forest zones (Ankafantsika), although few individuals were caught: 13 females and 12 males.

#### Cultivable bacteria

To search for cultivable bacteria in mosquitoes, insects originating from the Boeny region were chosen as the two species under study were both present in the area: *A. albopictus* at the Mahajanga site and *A. aegypti* at the Ankafantsika site. For the two media used, 22 colony types were obtained from males and 10 from females of...
Table 3. Phylogenetic affiliation of isolates and sequences obtained from Aedes sp.

<table>
<thead>
<tr>
<th>Species (Sex)</th>
<th>Origin</th>
<th>Name of isolates</th>
<th>Accession number</th>
<th>Phylogenetic affiliation</th>
<th>Most closely related organism</th>
<th>Accession number</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aedes albopictus</td>
<td>Mahajanga</td>
<td>KZ_Aal_F_Mm1</td>
<td>1477</td>
<td>GU726172</td>
<td>Alphaproteobacteria</td>
<td>Gu726172</td>
<td>1476/1477 (99)</td>
</tr>
<tr>
<td></td>
<td>(Female) (town)</td>
<td>KZ_Aal_F_Mm2</td>
<td>1541</td>
<td>GU726171</td>
<td>Firmicutes Bacillus sp. 41KB</td>
<td>FJ615523.1</td>
<td>1538/1541 (99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KZ_Aal_F_Mm3</td>
<td>1544</td>
<td>GU726173</td>
<td>Firmicutes Bacillus cereus strain : Pa-1</td>
<td>AB247137.1</td>
<td>1541/1544 (99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KZ_Aal_F_Mm4</td>
<td>1540</td>
<td>GU726174</td>
<td>Firmicutes Bacillus sp. NN106 1</td>
<td>AY973278.1</td>
<td>1531/1540 (99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KZ_Aal_M_Mm1</td>
<td>1543</td>
<td>GU726176</td>
<td>Firmicutes Bacillus sp. No.49</td>
<td>AB066347.1</td>
<td>1543/1543 (100)</td>
</tr>
<tr>
<td>Aedes albopictus</td>
<td>Mahajanga</td>
<td>KZ_Aal_M_Mm2</td>
<td>1540</td>
<td>GU726177</td>
<td>Firmicutes Bacillus sp. NN106 1</td>
<td>AY973278.1</td>
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<td>(Male) (town)</td>
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<td>1541</td>
<td>GU726185</td>
<td>Firmicutes Bacillus sp. 41KB</td>
<td>FJ615523.1</td>
<td>1540/1541 (99)</td>
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<td>GU726178</td>
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<td>EU703817.1</td>
<td>1477/1483 (98)</td>
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<td>GU726180</td>
<td>Gammaproteobacteria Acinetobacter sp. SH-94B</td>
<td>FN377701.1</td>
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<td></td>
<td></td>
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<td>1530</td>
<td>GU726181</td>
<td>Gammaproteobacteria Acinetobacter johnsonii strain S35</td>
<td>AB099655.1</td>
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<td></td>
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<td>KZ_Aal_M_Mm8</td>
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<td>EF175731.1</td>
<td>1499/1503 (99)</td>
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<td>Gammaproteobacteria Enterobacter cloacae isolate 766</td>
<td>AM778415.1</td>
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<tr>
<td>Aedes aegypti</td>
<td>Ankarafantsika</td>
<td>KZ_Aae_F_Ma1</td>
<td>1544</td>
<td>GU726175</td>
<td>Firmicutes Bacillus sp. G2DM-51</td>
<td>DQ416786.1</td>
<td>1539/1544 (99)</td>
</tr>
<tr>
<td>(Female)</td>
<td>Natural Reserve</td>
<td>KZ_Aae_F_Ma2</td>
<td>1544</td>
<td>GU726179</td>
<td>Firmicutes Bacillus megaterium strain MPF-906</td>
<td>DQ660362.1</td>
<td>1540/1544 (99)</td>
</tr>
</tbody>
</table>

A. albopictus. Only four colony types were recovered from A. aegypti females. Two to four representatives of each colony type were used for genomic DNA extraction and PCR amplification of the rrs gene. ARDRA of entire rrs gene amplicons revealed a total of 13 distinct patterns (not shown). Sequencing of the rrs gene of each isolate and BLASTN analysis allowed identifying two phyla: Proteobacteria and Firmicutes (Table 3). Bacteria belonging to the genus \textit{Bacillus} were present in all the specimens of both sexes and species. In addition, one isolate from an \textit{A. albopictus} female was an \textit{Agrobacterium} sp. whereas isolates of the genera \textit{Acinetobacter} and \textit{Enterobacter} were found in \textit{A. albopictus} males. For all isolates, the sequence similarities were between 98% and 100% with respect to the \textit{rrs} sequences of type strains reported in databases.

**DGGE fingerprints and phylogenetic affiliation of bacterial sequences**

To investigate the whole bacterial community of the two \textit{Aedes} species, PCR-DGGE fingerprints of hypervariable V3 regions were produced. For each sampling site, females and five males (four males for \textit{A. aegypti}) were analyzed individually. DGGE profiles varied between individuals of the same sex whether from the same site or not (Fig. 1). Banding patterns also differed between females and males of both \textit{A. albopictus} and \textit{A. aegypti}. To compare the DGGE profiles better, we analyzed them with \textsc{gelcompar} software and then by principal component analysis (PCA) using \textsc{r} software. In terms of the bacterial communities they host, females and males of \textit{A. albopictus} from all collection sites are distinct, the first two axes explaining > 43.8% of the total variability in PCA (Fig. 2).

To explore whether the mosquitoes’ environment influences the bacteria they host, PCA was performed on the DGGE band profiles from males and females separately. For males (Fig. 3a and c), the type of vegetation (Table 1) may explain the differences because (1) individuals from urban areas (Mahajanga, Antananarivo and Toamasina) characterized by bushes and fruit trees are different from those from suburban areas (Ambohitrarotina and Ankazobe) surrounded by bamboo (PCA1, 17% of total variability); and (2) individuals from Ankazobe that is mainly a natural habitat are distinct from those from the touristic site of Ambohitrarotina (PCA3, 9.9% of variability). Although weaker (PCA3, 9.8% of total variability) for females, in addition to vegetation, differences between sites (Fig. 3b and c) can be linked to the hosts available to bite (Table 1). For instance, poultry were currently found in Toamasina and Ankazobe whereas Mahajanga is the only site where there is extensive ovine and bovine rearing. In contrast, Tsimbazaza Park is well-frequented by tourists and hosts a diverse range of vertebrates. In addition to humans, Ambohitrarotina may host natural fauna.

To identify the bacterial community in these mosquito samples, representative DGGE bands were excised from the gel, cloned and sequenced as numbered in Fig. 1. The V3 fragment size obtained varies from 165 to 196 bp, giving only an indication of bacterial phylogenetic affiliation. BLAST analyses indicated that sequences belonged to \textit{Bacteroidetes} (2.6% of the sequenced bands), \textit{Firmicutes} (10.5%) and \textit{Proteobacteria} (86.9%). At the genus level, sequences were affiliated mostly with \textit{Acinetobacter}, \textit{Asaia}, \textit{Pseudomonas} and an uncultured \textit{Gammaproteobacterium} (Table 4). Some other bacteria detected included the genera \textit{Bradyrhizobium} sp., \textit{Delftia} sp., \textit{Herbaspirillum} sp., \textit{Rhizobium} sp. and...
Stenotrophomonas sp. as well as members of the Enterobacteriaceae (uncultured Citrobacter sp., Enterobacter sp., Pantoea sp., Shigella sp. and Yokenella sp.). An uncultured Streptococcaceae bacterium and members of the genus Staphylococcus were also identified (Table 4). As expected, sequences of the control bands corresponding to Wolbachia V3 amplicons were seen exclusively in *A. albopictus* (Fig. 1a–f).

**Bacterial diversity analysis**

We evaluated the bacterial diversity and evenness in *A. Albopictus* from the different sampling sites. Considering all the sampling sites, the Shannon–Weaver \( (H') \) index varied from 1.16 to 2.45 and the Simpson diversity \( (1 - \lambda') \) index varied from 0.63 to 0.89. The Pielou's index \( (J') \) was between 0.80 and 0.86 (Table 5). Statistical analyses for all indices showed that there was a significant difference \( (P < 0.01, \text{Tukey}) \) linked to the sex for individuals from Tsimbazaza Park only. In addition, Shannon–Weaver and Simpson diversity indices varied between sampling sites. In particular, significant differences \( (P < 0.01, \text{Tukey}) \) were found between samples from Ankazobe, Mahajanga and Tsimbazaza Park. The regions Ambohidratrimo and Toamasina had intermediary values (Table 5). No differences in evenness between sampling sites were observed with Pielou's index.

**Wolbachia prevalence and density in *A. albopictus***

Usually, *A. albopictus* harbors two Wolbachia strains named wAlbA and wAlbB (Sinkins *et al*., 1995). Diagnostic PCR using wsp primers against the subset (685 of a total of 1905) of wild *A. albopictus* revealed double infection in 99% females \( (n = 413) \) and 98% males \( (n = 272) \); four females and six males found were singly infected with wAlbB strain (not shown).

Wolbachia's density was estimated by quantitative PCR targeting the wsp gene with primers designed to be strain specific toward wAlbA and wAlbB strains and the host gene encoding the cytoskeleton protein actin (Table 2). The relative numbers of bacterial genes per host gene are given as the copy number ratio of Wolbachia wsp to host actin. Overall, the relative numbers of the wAlbA strain varied from 0 to 5.19 per female (Fig. 4) and from 0 to \( 1.67 \times 10^{-2} \) per male (Supporting Information, Fig. S1). The wAlbB
density was also extremely variable, between $4.56 \times 10^{-4}$ and 5.16 per female (Fig. 4) and from $9.42 \times 10^{-3}$ to 1.16 per male (Fig. S2). In general, Wolbachia strains \( \text{wAlbA} \) and \( \text{wAlbB} \) were significantly \((P < 0.05, \text{Tukey})\) more abundant in females than in males. Interestingly, Wolbachia’s density in females varied depending on either the bacterial strains present or the mosquitoes’ geographical origin (Fig. 4). The relative density of strain \( \text{wAlbA} \) was significantly higher \((P < 0.05, \text{Tukey})\) than that of \( \text{wAlbB} \) in females from Tsimbazaza Park only. The densities of each Wolbachia strain in females were compared between sampling sites. Results indicated that \( \text{wAlbA} \) strain was more abundant \((P < 0.05, \text{Tukey})\) in Tsimbazaza Park than in Mahajanga, whereas \( \text{wAlbB} \) strain predominated \((P < 0.05)\) in Ambohidratrimo compared with Mahajanga and Tsimbazaza Park. Differences in Wolbachia densities in males were not statistically significant between sites, probably due to a high interindividual variability.

**Discussion**

Our data illustrate the current distribution and preferential habitats of \( \text{A. albopictus} \) and \( \text{A. aegypti} \), two major mosquito vectors of arbovirus, in seven localities of Madagascar (Table 1 and Fig. 3c). \( \text{Aedes albopictus} \) was found to be predominant in urban and suburban areas, whereas \( \text{A. aegypti} \) specimens were exclusively recovered in sylvan habitats showing weakly anthropophilic behavior (Table 1). In contrast to previous reports showing a high prevalence of \( \text{A. aegypti} \) in Mahajanga (Ravaonjanahary, 1978; Fontenille & Rodhain, 1989), we noted the current dominance of \( \text{A. albopictus} \) in this region. These data are in line with what is known on the undercurrent expansion of \( \text{A. albopictus} \) in Indian Ocean Islands and worldwide, affecting the density of sister taxon \( \text{A. aegypti} \) concomitantly (Salvan & Mouchet, 1994; O’Meara et al., 1995; Delatte et al., 2008; Bagny et al., 2009a, b, c; Paupy et al., 2010).

To examine whether the environment inhabited by the mosquitoes influenced the diversity of bacterial communities associated with wild mosquitoes, DGGE analysis was performed. Profiles varied between individuals and capture sites. This variation could be linked to environmental features, suggesting that some bacterial species that colonize mosquitoes may originate from the environment. Thus, vegetation used as food sources or resting and potential hosts for biting appear to be factors influencing the bacterial community associated with \( \text{A. albopictus} \) and \( \text{A. aegypti} \). Bacterial communities associated with mosquitoes were mainly studied from laboratory-reared populations, which may not reflect those of wild populations. Indeed, it was shown that field-caught Anopheles mosquitoes harbor a greater bacterial diversity than laboratory populations (Rani...
et al., 2009). Studies on other insects such as the ground beetle *Poecilus chalcites* have also shown a higher bacterial diversity in wild populations in comparison with those from laboratories (Lehman et al., 2009). In addition, it was demonstrated that either nutrition regime or breeding technique could affect the composition of insects’ commensal microbial community (Rani et al., 2009; Zouache et al., 2009a). Conversely, the bacterial populations can influence the behavior and the biology of insect hosts as well (Tsuchida et al., 2004; Moran & Degnan, 2006). Generally, such extended phenotypes issuing from these reciprocal interactions are evidenced in symbioses between insects and their vertically transmitted endosymbiotic bacteria (Buchner, 1965; Moran et al., 2008). Actually, only a few bacterial symbionts horizontally acquired from the environment have been shown to significantly impact the insects’ fitness. This is the case of the heteropteran stinkbug *Riptortus clavatus* which acquires the beneficial gut bacterial symbiont...
### Table 4. Phylogenetic affiliation of sequences obtained from Aedes sp. in DGGE analysis

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Bands</th>
<th>Size (bp)</th>
<th>Accession number</th>
<th>Phenylogenetic affiliation</th>
<th>Most closely related organism</th>
<th>Accession number</th>
<th>Similarity (%)</th>
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<tr>
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importance of taking into account environmental factors clearly established. Altogether, these studies highlighted the albeit the environmental origin of these microbiota was not the protection against pathogens in *Lepidoptera* or desert locust (Dillon & Charnley, 2002; Raymond et al., 2008, 2009), albeit the environmental origin of these microbiota was not clearly established. Altogether, these studies highlighted the importance of taking into account environmental factors such as ecological niches when analyzing symbiotic microbiota associated with wild animal populations. Whether the bacterial communities found here may contribute to adaptive behavior and successful invasion of *A. albopictus* is under investigation.

At the genus level, several bacteria detected in this study are commonly described in soil and some have been found in hematophagous species of *Culicidae*, including *A. triseriatus* (Demaio et al., 1996), *Culex quinquefasciatus* (Pidiyar et al., 2004), *Culex quinquefasciatus* (Favia et al., 2008), *Anopheles darlingi* (Terenius et al., 2008), *Anopheles gambiae* (Dong et al., 2009), *A. albopictus* (Zouache et al., 2009b) and *A. aegypti* (Gusmao et al., 2007, 2010; Crotti et al., 2009). Intriguingly, three genera, *Acinetobacter, Asaia* and *Pseudomonas*, that are known to contain cultivable species were constantly found in the two species studied here. This suggests either a continuous acquisition through the environment or a vertical inheritance through generations. Interestingly, the genus *Asaia* was previously found in laboratory-reared *Anopheles stephensi* and *A. aegypti*, as well as wild *A. gambiae* where it was demonstrated to be transmitted vertically (Favia et al., 2007; Crotti et al., 2009; Damiani et al., 2010). Our results are the first description of *Asaia* sp. in natural populations of both *A. albopictus* and *A. aegypti*. The ability of *Asaia* to be inherited both paternally and maternally is attracting attention as a potential candidate for blocking transmission of mosquito-borne pathogens through paratransgenesis (Favia et al., 2008). Functions have been suggested for some of the other bacterial genera isolated here. The genus *Bacillus* may probably be involved in cellulose and hemicellulose degradation in termites (reviewed in Konig, 2006). Members of the *Enterobacteriaceae*

![Fig. 4. Relative density of Wolbachia in Aedes albopictus females from different sites in Madagascar. The relative numbers of Wolbachia are given as the copy number ratio of wsp to host actin. wAlbA (black) and wAlbB (grey) strains were measured in five female individuals per sampling site. Bars indicate SEs.](image)
family are thought to provide an additional nitrogen source to the fruit fly Ceratitis capitata (Behar et al., 2005). A recent study has shown that an Acinetobacter sp. strain is able to inhibit a tobacco mosaic virus by producing an antiviral compound (Lee et al., 2009). Many other groups of bacteria detected for the first time in mosquitoes perform unknown functions. A better knowledge of the mosquito-associated bacteria will allow investigating their role in the host biology.

Usually, natural populations of A. albopictus have been found singly or doubly infected with Wolbachia (Kittayapong et al., 2000, 2002; Tortosa et al., 2010). When associated with A. albopictus, Wolbachia manipulates the reproduction of its host, inducing a density-dependent cytoplasmic incompatibility phenomenon, which increases the proportion of infected individuals in the population (Sinkins et al., 1995; Dobson et al., 2001). Interestingly, Wolbachia was recently demonstrated to inhibit mosquito-borne pathogens in some circumstances (Moreira et al., 2009; Bian et al., 2010; Glaser & Meola, 2010). Here, the survey of Wolbachia in A. albopictus wild populations revealed a high rate of double infection by Wolbachia wAlbA and wAlbB strains in both sexes. The densities of the two Wolbachia strains varied depending on the sex and the sampling region. These results are in accordance with previous data on high variability in Wolbachia densities in field populations (Ahantarig et al., 2008; Unkless et al., 2009). A few cases of single infection by wAlbB were also detected both in males and in females (Fig. 4). Loss of wAlbA strain in A. albopictus males’ aging in the laboratory was recently reported in previously doubly infected populations from the Reunion island (Tortosa et al., 2010). Surprisingly, a different pattern was found in field populations of A. albopictus from Thailand, where single infection consists of either Wolbachia wAlbA or wAlbB strains (Kittayapong et al., 2000; Ahantarig et al., 2008), suggesting that different factors may account for the prevalence of Wolbachia in this mosquito species, which in turn could potentially interfere with the extended population phenotype.

In conclusion, the results presented here highlight the link between the habitats and the bacterial diversity of wild mosquitoes. As pathogens transmitted by mosquitoes coexist with associated bacteria that can affect insect population dynamics and vectorial competence, characterizing the bacterial composition and diversity of A. albopictus and A. aegypti in their environment is a step forward in understanding the ecology and the multipartite interactions occurring in these two major vectors of arbovirus.

Acknowledgements

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References


Bacterial communities of wild Aedes mosquito vectors


Raymond B, Johnston PR, Wright DJ, Ellis RJ, Crickmore N & Bonsall MB (2009) A mid-gut microbiota is not required for the pathogenicity of Bacillus thuringiensis to diamondback moth larvae. Environ Microbiol 11: 2556–2563.


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Relative density of Wolbachia wAlbA in Aedes albopictus males from different collection sites in Madagascar.

Fig. S2. Relative density of Wolbachia wAlbB in Aedes albopictus males from different sites in Madagascar.

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