RESEARCH ARTICLE

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

Karima Zouache[1,2], Fara Nantenaina Raharimalala[1,2,3], Vincent Raquin[1,2], Van Tran-Van[1,2], Lala Harivelo Ravaomanarivo Raveloson[3], Pierre Ravelonandro[4] & Patrick Mavingui[1,2]

[1]Université de Lyon, Lyon, France; [2]UMR CNRS 5557 Ecologie Microbienne, Université Lyon 1, Villeurbanne, France; [3]Département d'Entomologie de la Faculté des Sciences d'Antananarivo, Madagascar; and [4]Centre National de Recherche sur l'Environnement, Madagascar

Correspondence: Patrick Mavingui, UMR CNRS 5557 Ecologie Microbienne, Université Lyon 1, 43 boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex, France. Tel.: +33 4 72 43 11 43; fax: +33 4 72 43 12 23; e-mail: patrick.mavingui@univ-lyon1.fr

Received 30 August 2010; revised 11 November 2010; accepted 11 November 2010.
Final version published online 22 December 2010.

DOI:10.1111/j.1574-6941.2010.01012.x

Editor: Christoph Tebbe

Keywords
bacterial community; DGGE; quantitative PCR; *Wolbachia*.

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 *Acinetobacter*, *Agrobacterium* and *Enterobacter*. Common DGGE bands belonged to *Acinetobacter, Asaia*, *Delftia, Pseudomonas*, *Enterobacteriaceae* and an uncultured *Gammaproteobacterium*. Double infection by maternally inherited *Wolbachia pipientis* prevailed in 98% of males ($n = 272$) and 99% of females ($n = 413$); few individuals were found to be monoinfected with *Wolbachia w*AlbB 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.

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 glossidinius*, suspected to enhance vectorial competence (Cheng & Aksoy, 1999; Aksoy & Rio, 2005; Farikou *et al.*, 2010). More recently, bacteria belonging to genera *Enterobacter*, *Enterococcus* 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; Bagny *et al.*, 2009a, b)

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*, *Lactoccocus*, *Pantoea*, *Pseudomonas*, *Microbacterium*, *Staphylococcus* and *Stenotrophomas* (Pumpuni *et al.*, 1996; Straif *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 *Wolbachia* 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 cost 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

Table 1. Ecological characteristics of mosquito *Aedes* sp. capture sites

					*A. albopictus**		*A. aegypti**	
Region	Site	Zone	Potentially bitten hosts	Vegetation	Female	Male	Female	Male
Analamanga	Ambohidratrimo	Village outskirts	Humans, birds, reptiles	Bamboo hedge	137	35	0	0
	Tsimbazaza Park	City	Humans, lemurs, reptiles, birds	Bamboo, bushes	823	62	0	0
	Ankazobe	Village outskirts	Humans, chickens	Bamboo forest	93	95	0	0
Boeny	Mahajanga Town	City	Humans, ovine (sheep), bovine (zebu)	Fruit trees, bushes	290	20	0	0
	Andranofasika	Village	Humans, birds	Mango trees	0	0	10	7
	Ankarafantsika	Natural reserve	Humans, lemurs, birds, reptiles	Forest	0	0	3	5
Atsinanana	Toamasina Town	City	Humans, chickens, ducks	Fruit trees, bamboo hedge, bushes	320	30	0	0

*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 reinoculated 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).

Diagnostic PCR, amplified ribosomal DNA restriction analysis (ARDRA) and quantitative PCR amplification

Diagnostic PCR amplification was performed with primers listed in Table 2 using a T Gradient Thermocycler (Biometra, 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 (10^1–10^8 molecules) of the pQuantAlb plasmid (Tortosa *et al.*, 2008) containing *wsp* and *actin* fragments.

Denaturing gradient gel electrophoresis (DGGE)

Ingeny 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

Table 2. Primers used in this study

Samples	Gene	Primer name	Primer sequence (5′–3′)	Amplicon size (bp)/T_m (°C)	References
Organism					
Bacteria	*rrs*	pA	5′AGAGTTTGATCCTGGCTCAG3′	About 1500/55	Bruce *et al*. (1992)
		pH	5′AAGGAGGTGATCCAGCCGCA3′		
	V3 region *rrs*	16S (V3) 338F	5′GCCGCCCGCCGCGCGCGGC GGGCGGGGCGGGGGCACGGGG GGACTCCTACGGGAGGCAGCAG3′	About 200/55	Muyzer *et al*. (1993)
		16S (V3) 520R	5′ATTACCGCGGCTGCTGG3′		
Wolbachia	*rrs*	99F	5′TTGTAGCCTGCTATGGTATAACT3′	864/52	O'Neill *et al*. (1992)
		994R	5′GAATAGGTATGATTTTCATGT3′		
	wsp	81F	5′TGGTCCAATAAGTGATGAAGAAAC3′	600/55	Zhou *et al*. (1998)
		328F	5′CCAGCAGATACTATTGCG3′	379/52	Zhou *et al*. (1998)
		183F	5′AAGGAACCGAAGTTCATG3′	501/52	Zhou *et al*. (1998)
		691R	5′AAAAATTAAACGCTACTCCA3′		
Plasmid					
TOPO 2.1		M13F	5′GTAAAACGACGGCCAG3′	Variable/Variable	
		M13R	5′CAGGAAACAGCTATGAC3′		
pQuantAlb	*wsp* A group	QAdir1	5′GGGTTGATGTTGAAGGAG3′	264/60	Tortosa *et al*. (2008)
		QArev2	5′CACCAGCTTTTACTTGACC3′		
	wsp B group	183F	5′AAGGAACCGAAGTTCATG3′	112/60	Tortosa *et al*. (2008)
		QBrev2	5′AGTTGTGAGTAAAGTCCC3′		
	actin	ActAlb-dir	5′GCAAACGTGGTATCCTGAC3′	139/60	Tortosa *et al*. (2008)
		ActAlb-rev	5′GTCAGGAGAACTGGGTGCT3′		

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 (*Pi*) was calculated by the relative area under the peak in the profile ($Pi = n_i/N$, where n_i is the area under the peak i, and N is the sum of the areas for all peaks within the profile). The relative intensity of each band was used to calculate (PRIMER v6 software) Shannon–Weaver ($H' = -\Sigma P_i \log P_i$ where $P_i = n_i/N$) and Simpson ($1 - \lambda' = 1 - \{\Sigma_i N_i(N_i - 1)\}/\{N(N-1)\}$) diversity indices. We estimated the evenness of the numbers of bacterial species in each sample using Pielou's index ($J' = H'/\log S$, where $\log S = H'_{\max}$). Statistical analyses were performed using SPLUS 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 Ankarafantsika 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 Ambohidratrimo, 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 (Ankarafantsika), 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 Ankarafantsika 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.

Species (Sex)	Origin	Name of isolates	Size (bp)	Accession number	Phylogenetic affiliation	Most closely related organism	Accession number	Similarity (%)
Aedes albopictus	Mahajanga	KZ_Aal_F_Mm1	1477	GU726172	*Alphaproteobacteria*	*Agrobacterium* sp. JS71	AY174112.1	1476/1477 (99)
(Female)	(town)	KZ_Aal_F_Mm2	1541	GU726171	*Firmicutes*	*Bacillus* sp. 41KB	FJ615523.1	1538/1541 (99)
		KZ_Aal_F_Mm3	1544	GU726173	*Firmicutes*	*Bacillus cereus* strain : PDa-1	AB247137.1	1541/1544 (99)
		KZ_Aal_F_Mm4	1540	GU726174	*Firmicutes*	*Bacillus* sp. NN106 1	AJ973278.1	1531/1540 (99)
Aedes albopictus	Mahajanga	KZ_Aal_M_Mm1	1543	GU726176	*Firmicutes*	*Bacillus* sp. No.49	AB066347.1	1543/1543 (100)
(Male)	(town)	KZ_Aal_M_Mm2	1540	GU726177	*Firmicutes*	*Bacillus* sp. NN106 1	AJ973278.1	1533/1540 (99)
		KZ_Aal_M_Mm3	1541	GU726185	*Firmicutes*	*Bacillus* sp. 41KB	FJ615523.1	1540/1541 (99)
		KZ_Aal_M_Mm4	1532	GU726178	*Gammaproteobacteria*	*Acinetobacter* sp. EH 28	EU703817.1	1477/1483 (98)
		KZ_Aal_M_Mm5	1530	GU726180	*Gammaproteobacteria*	*Acinetobacter* sp. SH-94B	FN377701.1	1505/1512(99)
		KZ_Aal_M_Mm6	1530	GU726181	*Gammaproteobacteria*	*Acinetobacter johnsonii* strain S35	AB099655.1	1505/1532 (98)
		KZ_Aal_M_Mm7	1534	GU726182	*Gammaproteobacteria*	*Enterobacter* sp. NJ-1	AM396909.1	1491/1505 (99)
		KZ_Aal_M_Mm8	1534	GU726183	*Gammaproteobacteria*	*Enterobacter* sp. Px6-4	EF175731.1	1499/1503 (99)
		KZ_Aal_M_Mm9	1537	GU726184	*Gammaproteobacteria*	*Enterobacter cloacae* isolate 766	AM778415.1	1515/1534 (98)
Aedes aegypti	Ankarafantsika	KZ_Aae_F_Ma1	1544	GU726175	*Firmicutes*	*Bacillus* sp. G2DM-51	DQ416786.1	1539/1544 (99)
(Female)	Natural Reserve	KZ_Aae_F_Ma2	1544	GU726179	*Firmicutes*	*Bacillus megaterium* strain MPF-906	DQ660362.1	1540/1544 (99)

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 *Bacillus* were present in all the specimens of both sexes and species. In addition, one isolate from an *A. albopictus* female was an *Agrobacterium* sp. whereas isolates of the genera *Acinetobacter* and *Enterobacter* were found in *A. albopictus* males. For all isolates, the sequence similarities were between 98% and 100% with respect to the *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 *Aedes* species, PCR-DGGE fingerprints of hypervariable V3 regions were produced. For each sampling site, females and five males (four males for *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 *A. albopictus* and *A. aegypti*. To compare the DGGE profiles better, we analyzed them with GELCOMPAR software and then by principal component analysis (PCA) using R software. In terms of the bacterial communities they host, females and males of *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 (Ambohidratrimo 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 Ambohidratrimo (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, Tzimbazaza Park is well-frequented by tourists and hosts a diverse range of vertebrates. In addition to humans, Ambohidratrimo 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 *Bacteroidetes* (2.6% of the sequenced bands), *Firmicutes* (10.5%) and *Proteobacteria* (86.9%). At the genus level, sequences were affiliated mostly with *Acinetobacter, Asaia*, *Pseudomonas* and an uncultured *Gammaproteobacterium* (Table 4). Some other bacteria detected included the genera *Bradyrhizobium* sp., *Delftia* sp., *Herbaspirillum* sp., *Rhizhobium* sp. and

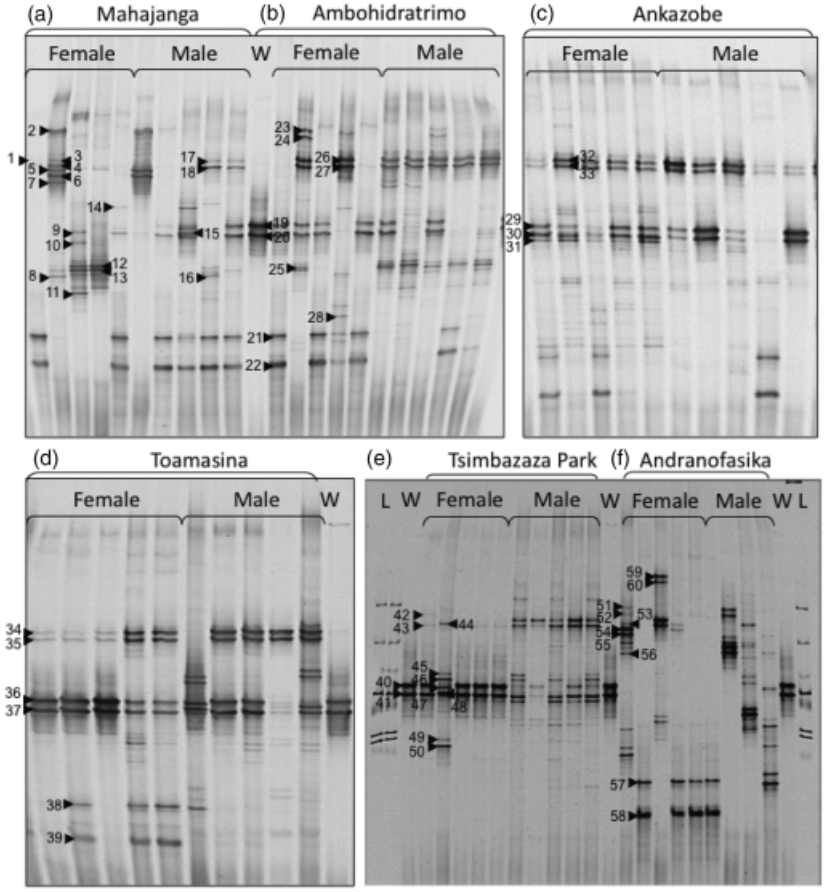

Fig. 1. DGGE profiles of bacterial communities of *Aedes albopictus* (a–e) and *Aedes aegypti* (f) from different regions of Madagascar. W, *Wolbachia* strain *w*AlbB from the Aa23 cell line used as an internal gel migration control. L, ladder used as an external gel migration control. Numbers correspond to cloned and sequenced bands (Table 4).

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 ($P < 0.01$, 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 ($P < 0.01$, 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 *w*AlbA and *w*AlbB (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 *w*AlbB strain (not shown).

Wolbachia's density was estimated by quantitative PCR targeting the *wsp* gene with primers designed to be strain specific toward *w*AlbA and *w*AlbB 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 *w*AlbA strain varied from 0 to 5.19 per female (Fig. 4) and from 0 to 1.67×10^{-2} per male (Supporting Information, Fig. S1). The *w*AlbB

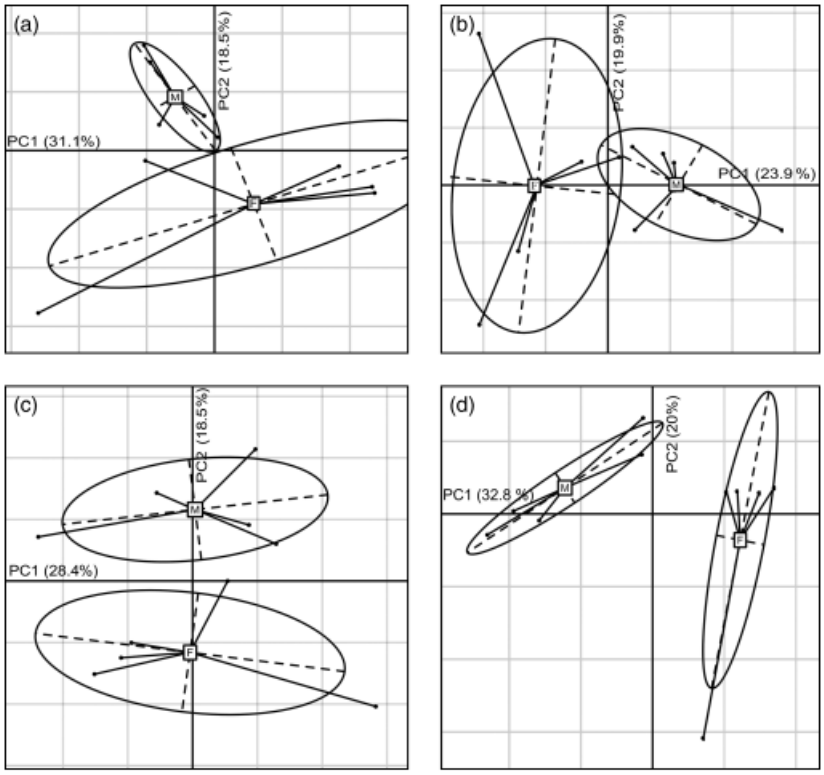

Fig. 2. Principal component analysis (PCA) of male and female *Aedes albopictus* from the same collection site (a–d). F, females; M, males. (a) PCA of individuals from Ambohidratrimo. (b) PCA of individuals from Ankazobe. (c) PCA of individuals from Toamasina. (d) PCA of individuals from Tsimbazaza Park. Individuals are represented by dots. Individuals of the same sex are encircled. The percentage indicated within parentheses corresponds to the variance explained by each principal component.

density was also extremely variable, between 4.56×10^{-4} and 5.16 per female (Fig. 4) and from 9.42×10^{-3} to 1.16 per male (Fig. S2). In general, *Wolbachia* strains *w*AlbA and *w*AlbB were significantly ($P < 0.05$, 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 *w*AlbA was significantly higher ($P < 0.05$, Tukey) than that of *w*AlbB in females from Tsimbazaza Park only. The densities of each *Wolbachia* strain in females were compared between sampling sites. Results indicated that *w*AlbA strain was more abundant ($P < 0.05$, Tukey) in Tsimbazaza Park than in Mahajanga, whereas *w*AlbB 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 *A. albopictus* and *A. aegypti*, two major mosquito vectors of arbovirus, in seven localities of Madagascar (Table 1 and Fig. 3c). *Aedes albopictus* was found to be predominant in urban and suburban areas, whereas *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 *A. aegypti* in Mahajanga (Ravaonjanahary, 1978; Fontenille & Rodhain, 1989), we noted the current dominance of *A. albopictus* in this region. These data are in line with what is known on the undercurrent expansion of *A. albopictus* in Indian Ocean Islands and worldwide, affecting the density of sister taxon *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 *A. albopictus* and *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

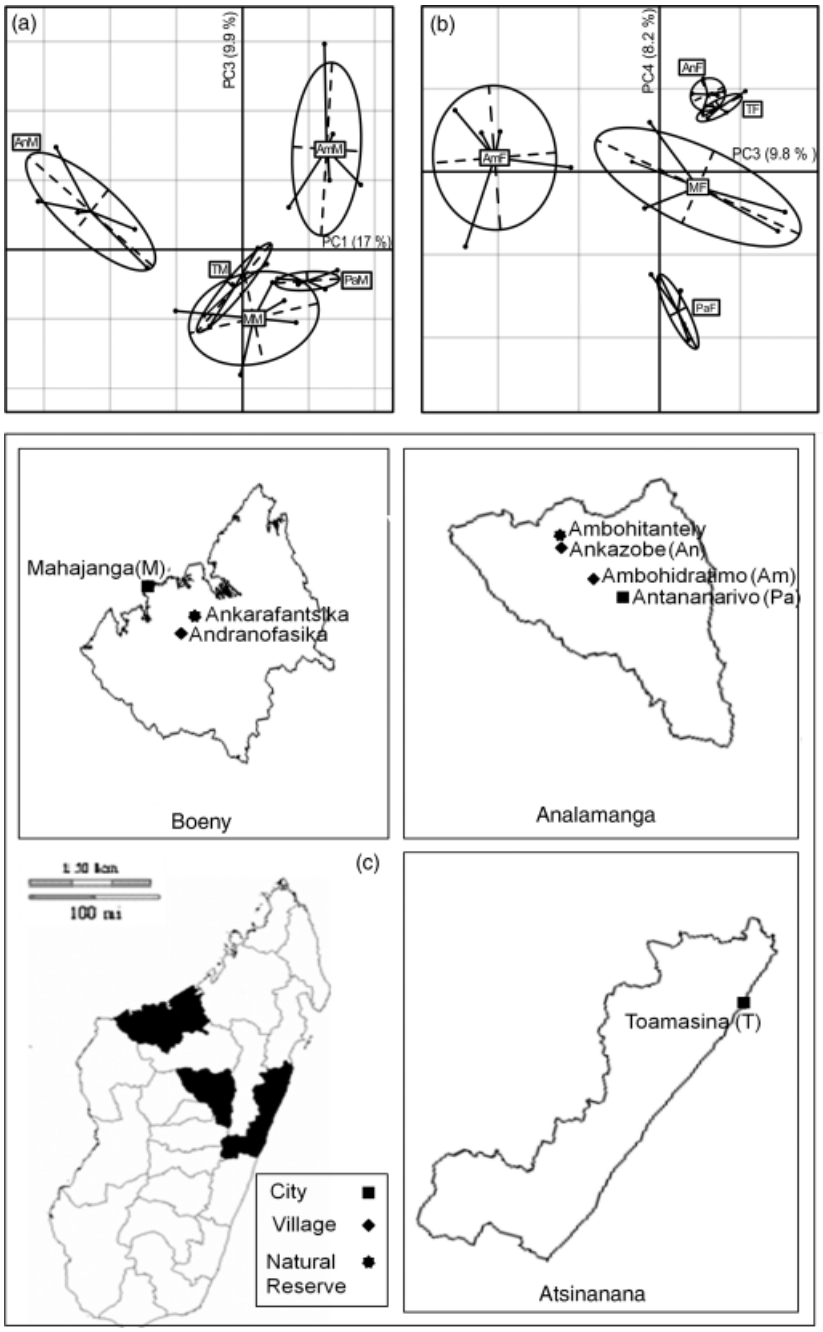

Fig. 3. Principal component analysis (PCA) of *Aedes albopictus* collected from different sites in Madagascar. Individuals are represented by dots. Individuals from the same collection site are encircled. Percentages correspond to the variance explained by each principal component (PC). (a) PCA of *A. albopictus* females. AmF, females from Ambohidratrimo (birds, reptiles); AnF, females from Ankazobe (poultry); MF, females from Mahajanga (ovine and bovine); PaF, females from Tsimbazaza Park (lemurs, birds and reptiles); TF, females from Toamasina (poultry). The two axes explain 17% (PC1) and 9.9% (PC3) of the total variability. (b) PCA of *A. albopictus* males. AmM, males from Ambohidratrimo (bamboo hedge); AnM, males from Ankazobe (bamboo forest); MM, males from Mahajanga; PaM, males from Tsimbazaza Park; TM, males from Toamasina (vegetation of the three cities corresponds to bushes and fruit trees). The two axes explained 9.8% (PC3) and 8.2% (PC4) of the total variability. (c) Map of Madagascar showing sites of *Aedes* collection. The abbreviations used in the map, after names of collection sites, correspond to those used in PCA panels.

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

Mosquito species	Bands	Size (bp)	Accession number	Phylogenetic affiliation	Most closely related organism	Accession number	Similarity (%)
Aedes albopictus	1; 3; 4; 8a; 42a; 43a	195	GU985109	*Gammaproteobacteria*	*Acinetobacter genomosp.* 3 strain bpoe135	FN563421.1	195/195 (100)
	2; 7a	194	GU985110	*Gammaproteobacteria*	*Pseudomonas putida* strain S5	AB512773.1	194/194 (100)
	5; 6; 7b	195	GU985111	*Gammaproteobacteria*	*Pseudomonas* sp. XL-NA	GU290043.1	195/195 (100)
	8b	194	GU985112	*Gammaproteobacteria*	*Pantoea agglomerans* strain 14	GQ494018.1	191/194 (98)
	8c	194	GU985113	*Gammaproteobacteria*	*Stenotrophomonas maltophilia* strain Y1	GQ268318.1	194/194 (100)
	9a; 44a	194	GU985114	*Gammaproteobacteria*	Uncultured *Citrobacter* sp. clone GASP-WA1W3_F04	AB485746.1	193/194 (99)
	9b; 11a; 12; 13	194	GU985115	*Gammaproteobacteria*	*Yokenella regensburgei*	AB519797.1	194/194 (100)
	10a	194	GU985116	*Gammaproteobacteria*	Uncultured *Citrobacter* sp. clone GASP-WA1W3_F04	AB485746.1	194/194 (100)
	10b; 22; 25a; 38; 39	169	GU985117	*Alphaproteobacteria*	*Asaia* sp. T-694	AB485746.1	169/169 (100)
	11b	165	GU985119	*Alphaproteobacteria*	*Asaia* sp. T-694	AB485746.1	165/165 (100)
	11c	194	GU985120	*Gammaproteobacteria*	*Pseudomonas* sp. XL-NA	GU290043.1	191/194 (98)
	14a; 15a; 45c; 47	194	GU985121	*Betaproteobacteria*	*Delftia* sp. LP2MM	GU272362.1	194/194 (100)
	14b	169	GU985122	*Alphaproteobacteria*	Uncultured *Bradyrhizobium* sp. clone PSB011.C21 G01	GU300452.1	169/169 (100)
	14c	195	GU985123	*Firmicutes*	Uncultured *Streptococcaceae* bacterium clone Cat008D_G01A	EU5725351.1	195/195 (100)
	15b	194	GU985124	*Betaproteobacteria*	*Delftia* sp. LP2MM	GU272362.1	193/194 (99)
	16a; 21	168	GU985125	*Alphaproteobacteria*	*Asaia* sp. T-694	AB485746.1	168/168 (100)
	16b	194	GU985126	*Gammaproteobacteria*	*Shigella flexneri* 2002017	CP001383.1	194/194 (100)
	17; 18; 24; 26; 27; 32-35	194	GU985127	*Gammaproteobacteria*	Uncultured bacterium clone 100p7	FJ934840.1	194/194 (100)
	19; 20; 29; 30; 36; 37; 40; 41	169	GU985150	*Alphaproteobacteria*	*Wolbachia pipientis* (host *Aedes albopictus*)	X61767	169/169 (100)
	23	194	GU985129	*Firmicutes*	Uncultured bacterium clone FFCH17137	EU134660.1	182/196 (92)
	25b	171	GU985131	*Alphaproteobacteria*	*Rickettsia* endosymbiont of *Hemiclepsis marginata*	FJ562342.1	171/171 (100)
	28a	169	GU985132	*Alphaproteobacteria*	Uncultured *Rhizobium* sp. clone PSB011.C21	GU300273.1	169/169 (100)
	28b	189	GU985133	*Bacteroidetes*	Uncultured bacterium clone AFYEL_aaj68h06	EU465158.1	182/190 (95)
	31	194	GU985134	*Betaproteobacteria*	*Herbaspirillum* sp. AU13533	EU549851.1	186/194 (95)
	42c; 43c	194	GU985135	*Firmicutes*	*Staphylococcus* sp. NT15I3.2B	GQ365194.1	194/194 (100)
	44b	194	GU985136	*Gammaproteobacteria*	*Enterobacter* sp. GA47-2	GQ114854.1	193/194 (99)
	45a; 46; 49b	194	GU985137	*Gammaproteobacteria*	*Pseudomonas* sp. XL-NA	GU290043.1	193/194 (99)
	45b	194	GU985138	*Gammaproteobacteria*	Uncultured *Citrobacter* sp. clone GASP-WA1W3_F04	AB485746.1	191/194 (98)
	48a	194	GU985139	*Gammaproteobacteria*	Uncultured *Citrobacter* sp. clone GASP-WA1W3_F04	AB485746.1	192/194 (98)
	48b	194	GU985140	*Betaproteobacteria*	*Delftia* sp. LP2MM	GU272362.1	192/194 (98)
	49a	194	GU985141	*Gammaproteobacteria*	*Enterobacter* sp. GA47-2	GQ114854.1	194/194 (100)
		189	GU985142	*Gammaproteobacteria*	*Enterobacter* sp. GA47-2	GQ114854.1	189/189 (100)
Aedes aegypti	50	195	GU985143	*Gammaproteobacteria*	*Acinetobacter genomosp.* 13TU strain BL Ac12	FJ860871.1	194/195 (99)
	51	194	GU985144	*Firmicutes*	*Staphylococcus saprophyticus* strain YSY1-8	GU197539.1	194/195 (99)
	52; 54	194	GU985145	*Gammaproteobacteria*	*Pseudomonas* sp. ND6	AY589689.1	189/194 (97)
	53	194	GU985146	*Gammaproteobacteria*	*Acinetobacter* sp. CCGE2017	EU867306.1	193/196 (98)
	55a	194	GU985147	*Gammaproteobacteria*	*Enterobacter* sp. DgG7	FJ599777.1	194/194 (100)
	55b	194	GU985148	*Gammaproteobacteria*	*Pseudomonas* sp. PRB5	GU223119.1	194/194 (100)
	56a	194	GU985149	*Gammaproteobacteria*	*Pseudomonas* sp. YM-M-129	GU220065.1	194/194 (100)
	57; 58	169	GU985118	*Alphaproteobacteria*	*Asaia* sp. T-694	AB485746.1	169/169 (100)
	59	194	GU985130	*Firmicutes*	Uncultured bacterium clone FFCH17137	EU134660.1	182/196 (92)
	60	194	GU985128	*Gammaproteobacteria*	Uncultured bacterium clone 100p7	FJ934840.1	194/194 (100)

Burkolderia from the environment in each generation (Kikuchi *et al.*, 2007). Other examples consist of gut microbiota that may contribute to nutrition and detoxification of some insects such as termites and the beetle *Tenebrio molitor* (Genta *et al.*, 2006; Warnecke *et al.*, 2007), or provide 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.

Table 5. Diversity indices and evenness values of *Aedes albopictus*

Location	Sex	H'*	$1-\lambda'$†	J'‡
Mahajanga	F	1.82 ± 0.53	0.78 ± 0.13	0.81 ± 0.01
	M	1.54 ± 0.30	0.73 ± 0.10	0.81 ± 0.07
Ambohidratrimo	F	1.97 ± 0.24	0.83 ± 0.04	0.84 ± 0.02
	M	2.04 ± 0.08	0.85 ± 0.01	0.86 ± 0.04
Ankazobe	F	2.07 ± 0.12	0.84 ± 0.03	0.83 ± 0.04
	M	2.45 ± 0.24	0.89 ± 0.03	0.86 ± 0.02
Toamasina	F	1.89 ± 0.34	0.80 ± 0.07	0.80 ± 0.04
	M	1.97 ± 0.14	0.83 ± 0.03	0.86 ± 0.07
Tsimbazaza Park	F	1.16 ± 0.47	0.63 ± 0.01	0.76 ± 0.07
	M	1.95 ± 0.33	0.82 ± 0.07	0.83 ± 0.04

Values are mean values ± SEs.

*Shannon–Weaver diversity index ($H' = -\Sigma P_i \log P_i N$).

†Simpson diversity index ($1-\lambda' = 1 - \{\Sigma_i N_i (N_i - 1)\} \{N (N-1)\}$).

‡Estimation of the evenness of the number of bacterial species in each sample.

Pielou's index ($J' = H'/\log$ where $\log S = H'_{max}$).

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), *Culicoides sonorensis* (Campbell *et al.*, 2004), *Culex quinquefasciatus* (Pidiyar *et al.*, 2004), *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 in 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*

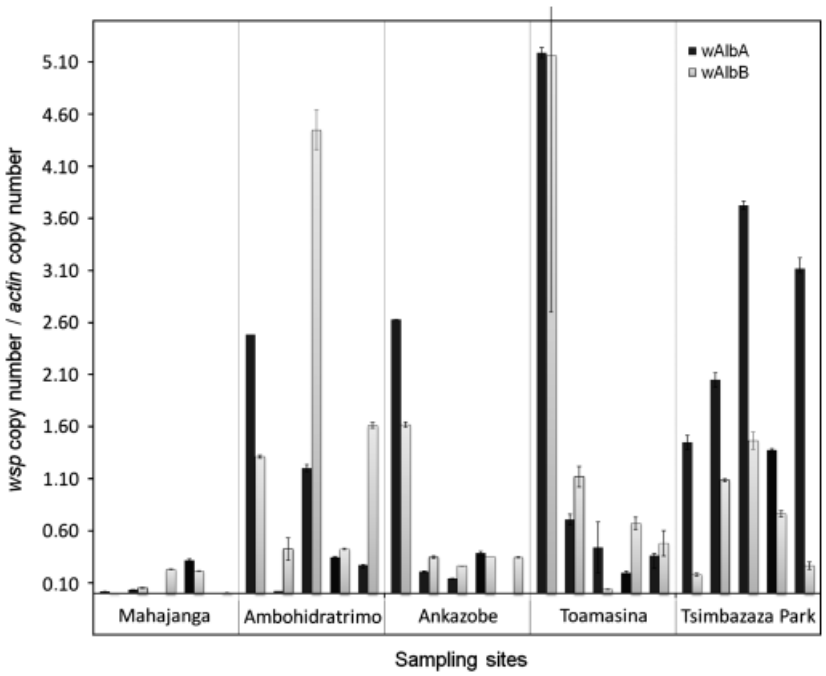

Fig. 4. Relative density of *Wolbachia* in *Aedes albopictus* females from different sites in Madagascar. The relative numbers of *Wolbachia* are given as the copy number ratio of *wsp* to host *actin*. *w*AlbA (black) and *w*AlbB (grey) strains were measured in five female individuals per sampling site. Bars indicate SEs.

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 w*AlbA and *w*AlbB 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; Unckless *et al.*, 2009). A few cases of single infection by *w*AlbB were also detected both in males and in females (Fig. 4). Loss of *w*AlbA 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 w*AlbA or *w*AlbB 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

This paper is dedicated to the memory of Dr Jesus Caballero-Mellado (Centro de Ciencia Genómica, Cuernavaca, Morelos, Mexico) who left us in October 2010. We are grateful to Madagascar National Parks (formerly ANGAP) for authorizing collection of wild mosquitoes and to Biofidal-DTAMB Laboratory of IFR41 in University Lyon 1 for technical assistance. K.Z. was supported by PhD fellowships from the French Ministère de l'Education Nationale, de la Recherche et des Nouvelles Technologies. F.N.R. was supported by the Fondation pour la Recherche sur la Biodiversité (FRB, formerly IFB). This work was funded by grants ANR-06-SEST07 and FRB-CD-AOOI-07-012, and was carried out within the frameworks of GDRI 'Biodiversité et Développement Durable à Madagascar' and COST action F0701 'Arthropod Symbioses: from fundamental to pest disease management'.

References

Ahantarig A, Trinachartvanit W & Kittayapong P (2008) Relative *Wolbachia* density of field-collected *Aedes albopictus* mosquitoes in Thailand. *J Vector Ecol* **33**: 173–177.

Aksoy S & Rio RV (2005) Interactions among multiple genomes: tsetse, its symbionts and trypanosomes. *Insect Biochem Molec* **35**: 691–698.

Bagny L, Delatte H, Elissa N, Quilici S & Fontenille D (2009a) *Aedes* (Diptera: Culicidae) vectors of arboviruses in Mayotte (Indian Ocean): distribution area and larval habitats. *J Med Entomol* **46**: 198–207.

Bagny L, Delatte H, Quilici S & Fontenille D (2009b) Progressive decrease in *Aedes aegypti* distribution in Reunion Island since the 1900s. *J Med Entomol* **46**: 1541–1545.

Bagny L, Freulon M & Delatte H (2009c) First record of *Aedes albopictus*, vector of arboviruses in the Eparse Islands of the Mozambique Channel and updating of the inventory of *Culicidae*. *B Soc Pathol Exot* **102**: 193–198.

Behar A, Yuval B & Jurkevitch E (2005) *Enterobacteria*-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata*. *Mol Ecol* **14**: 2637–2643.

Bian G, Xu Y, Lu P, Xie Y & Xi Z (2010) The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PLoS Pathog* **6**: e1000833.

Bruce KD, Hiorns WD, Hobman JL, Osborn AM, Strike P & Ritchie DA (1992) Amplification of DNA from native populations of soil bacteria by using the polymerase chain reaction. *Appl Environ Microb* **58**: 3413–3416.

Buchner P (1965) *Endosymbiosis of Animals with Plant Microorganisms*. Interscience, New York.

Campbell CL, Mummey DL, Schmidtmann ET & Wilson WC (2004) Culture-independent analysis of midgut microbiota in the arbovirus vector *Culicoides sonorensis* (Diptera: Ceratopogonidae). *J Med Entomol* **41**: 340–348.

Cheng Q & Aksoy S (1999) Tissue tropism, transmission and expression of foreign genes *in vivo* in midgut symbionts of tsetse flies. *Insect Mol Biol* **8**: 125–132.

Crotti E, Damiani C, Pajoro M *et al.* (2009) *Asaia*, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders. *Environ Microbiol* **11**: 3252–3264.

Damiani C, Ricci I, Crotti E *et al.* (2010) Mosquito–bacteria symbiosis: the case of *Anopheles gambiae* and *Asaia*. *Microb Ecol*.

Delatte H, Dehecq JS, Thiria J, Domerg C, Paupy C & Fontenille D (2008) Geographic distribution and developmental sites of *Aedes albopictus* (Diptera: Culicidae) during a Chikungunya epidemic event. *Vector Borne Zoonot* **8**: 25–34.

Demaio J, Pumpuni CB, Kent M & Beier JC (1996) The midgut bacterial flora of wild *Aedes triseriatus*, *Culex pipiens*, and *Psorophora columbiae* mosquitoes. *Am J Trop Med Hyg* **54**: 219–223.

Dillon R & Charnley K (2002) Mutualism between the desert locust *Schistocerca gregaria* and its gut microbiota. *Res Microbiol* **153**: 503–509.

Dobson SL, Marsland EJ & Rattanadechakul W (2001) *Wolbachia*-induced cytoplasmic incompatibility in single- and superinfected *Aedes albopictus* (Diptera: Culicidae). *J Med Entomol* **38**: 382–387.

Dong Y, Manfredini F & Dimopoulos G (2009) Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathog* **5**: e1000423.

Farikou O, Njiokou F, Mbida Mbida JA *et al.* (2010) Tripartite interactions between tsetse flies, *Sodalis glossinidius* and trypanosomes – an epidemiological approach in two historical human African trypanosomiasis foci in Cameroon. *Infect Genet Evol* **10**: 115–121.

Favia G, Ricci I, Damiani C *et al.* (2007) Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. *P Natl Acad Sci USA* **104**: 9047–9051.

Favia G, Ricci I, Marzorati M, Negri I, Alma A, Sacchi L, Bandi C & Daffonchio D (2008) Bacteria of the genus *Asaia*: a potential paratransgenic weapon against malaria. *Adv Exp Med Biol* **627**: 49–59.

Fontenille D & Rodhain F (1989) Biology and distribution of *Aedes albopictus* and *Aedes aegypti* in Madagascar. *J Am Mosqito Contr* **5**: 219–225.

Geiger A, Fardeau ML, Grebaut P *et al.* (2009) First isolation of *Enterobacter*, *Enterococcus*, and *Acinetobacter* spp. as inhabitants of the tsetse fly (*Glossina palpalis palpalis*) midgut. *Infect Genet Evol* **9**: 1364–1370.

Genta FA, Dillon RJ, Terra WR & Ferreira C (2006) Potential role for gut microbiota in cell wall digestion and glucoside detoxification in *Tenebrio molitor* larvae. *J Insect Physiol* **52**: 593–601.

Glaser RL & Meola MA (2010) The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. *PLoS One* **5**: e11977.

Gottlieb Y, Zchori-Fein E, Mozes-Daube N *et al.* (2010) The transmission efficiency of Tomato yellow leaf curl virus by the whitefly *Bemisia tabaci* is correlated with the presence of a specific symbiotic bacterium species. *J Virol* **84**: 9310–9317.

Gusmao DS, Santos AV, Marini DC, Russo Ede S, Peixoto AM, Bacci Junior M, Berbert-Molina MA & Lemos FJ (2007) First isolation of microorganisms from the gut diverticulum of *Aedes aegypti* (Diptera: Culicidae): new perspectives for an insect–bacteria association. *Mem I Oswaldo Cruz* **102**: 919–924.

Gusmao DS, Santos AV, Marini DC, Bacci M Jr, Berbert-Molina MA & Lemos FJ (2010) Culture-dependent and culture-independent characterization of microorganisms associated with *Aedes aegypti* (Diptera: Culicidae) (L.) and dynamics of bacterial colonization in the midgut. *Acta Trop* **115**: 275–281.

Kikuchi Y, Hosokawa T & Fukatsu T (2007) Insect–microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. *Appl Environ Microb* **73**: 4308–4316.

Kittayapong P, Baisley KJ, Baimai V & O'Neill SL (2000) Distribution and diversity of *Wolbachia* infections in Southeast Asian mosquitoes (Diptera: Culicidae). *J Med Entomol* **37**: 340–345.

Kittayapong P, Baimai V & O'Neill SL (2002) Field prevalence of *Wolbachia* in the mosquito vector *Aedes albopictus*. *Am J Trop Med Hyg* **66**: 108–111.

Konig H (2006) *Bacillus* species in the intestine of termites and other soil invertebrates. *J Appl Microbiol* **101**: 620–627.

Lee JS, Lee KC, Kim KK, Hwang IC, Jang C, Kim NG, Yeo WH, Kim BS, Yu YM & Ahn JS (2009) *Acinetobacter antiviralis* sp. nov., from Tobacco plant roots. *J Microbiol Biotechn* **19**: 250–256.

Lehman RM, Lundgren JG & Petzke LM (2009) Bacterial communities associated with the digestive tract of the predatory ground beetle, *Poecilus chalcites*, and their modification by laboratory rearing and antibiotic treatment. *Microb Ecol* **57**: 349–358.

Moran NA & Degnan PH (2006) Functional genomics of *Buchnera* and the ecology of aphid hosts. *Mol Ecol* **15**: 1251–1261.

Moran NA, McCutcheon JP & Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts. *Annu Rev Genet* **42**: 165–190.

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA *et al.* (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium. *Cell* **139**: 1268–1278.

Moya A, Pereto J, Gil R & Latorre A (2008) Learning how to live together: genomic insights into prokaryote–animal symbioses. *Nat Rev Genet* **9**: 218–229.

Muyzer G, de Waal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microb* **59**: 695–700.

Oliver KM, Degnan PH, Burke GR & Moran NA (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu Rev Entomol* **55**: 247–266.

O'Meara GF, Evans LF Jr, Gettman AD & Cuda JP (1995) Spread of *Aedes albopictus* and decline of *Ae. aegypti* (Diptera: Culicidae) in Florida. *J Med Entomol* **32**: 554–562.

O'Neill SL, Giordano R, Colbert AM, Karr TL & Robertson HM (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *P Natl Acad Sci USA* **89**: 2699–2702.

Paupy C, Ollomo B, Kamgang B *et al.* (2010) Comparative role of *Aedes albopictus* and *Aedes aegypti* in the emergence of Dengue and Chikungunya in central Africa. *Vector Borne Zoonot* **10**: 259–266.

Pidiyar VJ, Jangid K, Patole MS & Shouche YS (2004) Studies on cultured and uncultured microbiota of wild *Culex quinquefasciatus* mosquito midgut based on 16s ribosomal RNA gene analysis. *Am J Trop Med Hyg* **70**: 597–603.

Pumpuni CB, Demaio J, Kent M, Davis JR & Beier JC (1996) Bacterial population dynamics in three anopheline species: the impact on Plasmodium sporogonic development. *Am J Trop Med Hyg* **54**: 214–218.

Randrianasolo L, Raoelina Y, Ratsitorahina M, Ravolomanana L, Andriamandimby S, Heraud JM, Rakotomanana F, Ramanjato R, Randrianarivo-Solofoniaina AE & Richard V (2010) Sentinel surveillance system for early outbreak detection in Madagascar. *BMC Public Health* **10**: 31.

Rani A, Sharma A, Rajagopal R, Adak T & Bhatnagar RK (2009) Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi* – an Asian malarial vector. *BMC Microbiol* **9**: 96.

Ratsitorahina M, Harisoa J, Ratovonjato J *et al.* (2008) Outbreak of dengue and Chikungunya fevers, Toamasina, Madagascar, 2006. *Emerg Infect Dis* **14**: 1135–1137.

Ravaonjanahary C (1978) *Les Aedes de Madagascar*. Travaux et documents de 1'ORSTOM, France.

Raymond B, Lijek RS, Griffiths RI & Bonsall MB (2008) Ecological consequences of ingestion of *Bacillus cereus* on *Bacillus thuringiensis* infections and on the gut flora of a lepidopteran host. *J Invertebr Pathol* **99**: 103–111.

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.

Salvan M & Mouchet J (1994) *Aedes albopictus* and *Aedes aegypti* at Ile de la Reunion. *Ann Soc Belg Med Tr* **74**: 323–326.

Sang RC, Ahmed O, Faye O *et al.* (2008) Entomologic investigations of a chikungunya virus epidemic in the Union of the Comoros, 2005. *Am J Trop Med Hyg* **78**: 77–82.

Schuffenecker I, Iteman I, Michault A *et al.* (2006) Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med* **3**: e263.

Sinkins SP, Braig HR & O'Neill SL (1995) *Wolbachia* superinfections and the expression of cytoplasmic incompatibility. *Proc Biol Sci* **261**: 325–330.

Straif SC, Mbogo CN, Toure AM, Walker ED, Kaufman M, Toure YT & Beier JC (1998) Midgut bacteria in *Anopheles gambiae* and *An. funestus* (Diptera: Culicidae) from Kenya and Mali. *J Med Entomol* **35**: 222–226.

Terenius O, de Oliveira CD, Pinheiro WD, Tadei WP, James AA & Marinotti O (2008) 16S rRNA gene sequences from bacteria associated with adult *Anopheles darlingi* (Diptera: Culicidae) mosquitoes. *J Med Entomol* **45**: 172–175.

Tortosa P, Courtiol A, Moutailler S, Failloux AB & Weill M (2008) Chikungunya–*Wolbachia* interplay in *Aedes albopictus*. *Insect Mol Biol* **17**: 677–684.

Tortosa P, Charlat S, Labbe P, Dehecq JS, Barre H & Weill M (2010) *Wolbachia* age-sex-specific density in *Aedes albopictus*: a host evolutionary response to cytoplasmic incompatibility? *PLoS One* **5**: e9700.

Tsuchida T, Koga R & Fukatsu T (2004) Host plant specialization governed by facultative symbiont. *Science* **303**: 1989.

Unckless RL, Boelio LM, Herren JK & Jaenike J (2009) *Wolbachia* as populations within individual insects: causes and consequences of density variation in natural populations. *Proc Biol Sci* **276**: 2805–2811.

Vazeille M, Moutailler S, Coudrier D *et al.* (2007) Two Chikungunya isolates from the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, *Aedes albopictus*. *PLoS One* **2**: e1168.

Warnecke F, Luginbuhl P, Ivanova N *et al.* (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* **450**: 560–565.

Zhou W, Rousset F & O'Neil S (1998) Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. *Proc Biol Sci* **265**: 509–515.

Zouache K, Voronin D, Tran-Van V & Mavingui P (2009a) Composition of bacterial communities associated with natural and laboratory populations of *Asobara tabida* infected with *Wolbachia*. *Appl Environ Microb* **75**: 3755–3764.

Zouache K, Voronin D, Tran-Van V, Mousson L, Failloux AB & Mavingui P (2009b) Persistent *Wolbachia* and cultivable bacteria infection in the reproductive and somatic tissues of the mosquito vector *Aedes albopictus*. *PLoS One* **4**: e6388.

Supporting Information

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

Fig. S1. Relative density of *Wolbachia w*AlbA in *Aedes albopictus* males from different collection sites in Madagascar.
Fig. S2. Relative density of *Wolbachia w*AlbB in *Aedes albopictus* males from different sites in Madagascar.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.