RESEARCH ARTICLE

Prevalence, genomic and metabolic profiles of *Acinetobacter* and *Asaia* associated with field-caught *Aedes albopictus* from Madagascar

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Abstract

The presence of cultivable bacteria *Acinetobacter* and *Asaia* was recently demonstrated in the mosquito vector *Aedes albopictus*. However, it is not known how prevalent these bacteria are in field populations. Here, the presence of these bacteria in *Ae. albopictus* populations from Madagascar was diagnosed by amplification of 16S rRNA gene fragments. Both genera were detected at relatively high frequencies, 46% for *Asaia* and 74% for *Acinetobacter*. The prevalence of *Acinetobacter* correlated significantly with mosquito gender, and the prevalence of *Asaia* with the interaction between mosquito gender and the sampling site. For each bacterial genus, more male than female mosquitoes were infected. Using pulse field gel electrophoresis, no significant difference in genome size was found between *Acinetobacter* isolates from mosquitoes compared with free-living *Acinetobacter*. However, a great diversity was observed in plasmid numbers (from 1 to 12) and sizes (from < 8 to 690 kb). Mosquito isolates utilized fewer substrates than free-living isolates, but some substrates known as blood or plant components were specifically utilized by mosquito isolates. Therefore it is likely that a specific subpopulation of *Acinetobacter* is selected by *Ae. albopictus*. Overall, this study emphasizes the need to gain a global view on the bacterial partners in mosquito vectors.

Introduction

Mosquitoes are hematophagous insects that transmit many pathogens, such as those causing malaria, filariasis and arbovirosis, leading to millions of deaths every year (Christodoulou, 2011). Madagascar and its neighbouring islands have experienced severe epidemics of arboviruses, notably of chikungunya and dengue, and the mosquito species *Aedes albopictus* was identified as the primary vector responsible for these outbreaks (Ratsitorahina *et al.*, 2008). *Aedes albopictus* is able to transmit no fewer than 22 different viruses, so there have been many efforts to limit its expansion (Reiter, 1998) mainly by using chemical insecticides. However, negative consequences like the emergence of insecticide-resistant mosquitoes, environmental contamination and the extermination of non-target organisms have called chemical-based methods of control into question (David *et al.*, 2010). Alternative strategies of controlling insect-borne diseases have been proposed based on the use of symbiotic bacteria (Christodoulou, 2011; Hoffmann *et al.*, 2011). For a symbiont to be amenable as a biological control agent in this context, it needs to exhibit a certain level of prevalence, dominance and stability in the insect body (Riehle & Jacobs-Lorena, 2005).

Wolbachia is the most studied bacterial symbiont in *Ae. albopictus*, but recently two cultivable *Acinetobacter* and *Asaia* bacteria were found in this mosquito species (Crotti *et al.*, 2009; Zouache *et al.*, 2011), thus enlarging

the scope for research into possible biological control strategies. Members of the genus *Asaia* are *Alphaproteobacteria* able to produce acetic acid (Crotti *et al.*, 2010). The occurrence of *Asaia* was already reported in different insect species including the mosquitoes *Ae. albopictus* and *Aedes aegypti* (Crotti *et al.*, 2009; Chouaia *et al.*, 2010; Damiani *et al.*, 2010). In *Anopheles stephensi*, the Asian malaria mosquito vector, these bacteria are able rapidly to cross tissue barriers and disperse throughout the whole body of insect host (Favia *et al.,* 2007). Members of *Acinetobacter* are hosted by a number of insects (Dillon & Dillon, 2004), but their occurrence in hematophagous groups is poorly documented. Recently, different *Acinetobacter* species were found in natural populations of tsetse fly *Glossina* spp. (Geiger *et al.*, 2009) and *Ae. albopictus* (Zouache *et al.*, 2011). Both *Asaia* and *Acinetobacter* are found in the mosquito organs where viruses replicate (Crotti *et al.*, 2009; Zouache *et al.*, 2009) and they can be cultured and genetically transformed, so it may be possible to exploit these properties to study hematophagous insect-microorganism interactions in more depth.

The objective of this study was to survey the prevalence of *Asaia* and *Acinetobacter* in natural populations of *Ae. albopictus* from Madagascar with respect to insect gender and ecological behaviour. To discover possible bacterial signatures of genomic adaptation, *Acinetobacter* isolates obtained in the survey were compared by plasmid and metabolic profiling.

Materials and methods

Sampling areas and mosquito collection

The sampling areas and capture procedure were approved by Madagascar National Parks. Mosquito samples were collected in December 2010 at four sites in three regions of Madagascar: Analamanga, Antsinanana and Vatovavy-Fitovivany. Samples that had been collected from another site in the Diana region in December 2009 were also included in the survey. The main characteristics of the sampling sites are given in Table 1. Butterfly netting was used to collect both female and male mosquitoes flying just above grass or the ground. Some laboratory-reared insects that had been collected as pupae 1 year before at Tsimbazaza Park were also studied. *Aedes albopictus* males and females were identified using morphological characteristics keys (Ravaonjanahary, 1978). Only non-blooded mosquitoes were used for the analysis. Most mosquitoes were stored in tubes containing 70% ethanol while the remainder were kept alive and transported to the microbiology laboratory for bacterial isolation.

Genomic DNA extraction and diagnostic PCR of the *rrs* gene

DNA was extracted from 30 male and 30 female mosquitoes selected from the samples collected at each site as previously described (Zouache *et al.*, 2011). PCR amplification of bacterial 16S rRNA-encoding *rrs* genes was done using 30 ng of DNA template in 25 μL of reaction mixture containing 1× buffer (Roche, Switzerland), 40 μM of each deoxynucleoside triphosphate, 0.2 μM each of primers pA and pH (primer details in Supporting Information, Table S1), 0.06 mg mL^{-1} T4 gene protein 32 (Roche) and 0.028 U of Expand DNA polymerase (Roche, France). Nested PCR was then used to screen for the presence of *Asaia* and *Acinetobacter* DNA in positive *rrs* PCR products (1 μL). Reactions (25 μL) containing 1× polymerase reaction buffer (Invitrogen, France), 1.5 mM $MgCl_2$, 40 μM of each deoxynucleoside triphosphate,

Table 1. Ecoclimatic characteristics of mosquito *Aedes albopictus* sampling sites

Region	Site (GPS coordinates)	Zone	Climate	Vegetation	Potentially bitten hosts
Antsinanana	Toamasina (18°8′59.64″S, 49°24′8.312″E)	City on coast	Hot, humid	Bushes, fruit trees	Humans, chickens, ducks
Vatovavy-Fitovivany	Mananjary (21°13′52″S, 48°20′30.999″E)	City on coast	Hot, humid	Bushes, fruit trees	Humans, chickens, ducks
Analamanga	Tsimbazaza Park (18°55′40.395″S, 47°31′38.5″E)	City	Highland	Bushes, fruit trees	Humans, lemurs, reptiles, birds
	Ankazobe (18°18′58.1″S, 47°7′6.351″E)	Village outskirts	Wet, cool	Bamboo forest (with tyres on ground)	Humans, chickens
Diana	Diego-Suarez (12°16′51.24″S, 49°17′27.092″E)	City on coast	Hot, humid	Bushes, fruit trees	Humans, chickens, ducks

0.2 μM of each primer pair (Table S1) and 0.5 U of Taq DNA polymerase (Invitrogen) were carried out in a T-Gradient Thermocycler (Biometra, France). *Acinetobacter* and *Asaia* DNA was amplified as previously described (Crotti *et al.*, 2009; Zouache *et al.*, 2009). For each set of PCR reactions, bacterial DNA extracts from reference strains *Acinetobacter* ATCC 23055 and *Asaia* ATCC BAA-21 were used as positive controls. Amplified DNA fragments were separated by electrophoresis through 1% or 1.5% agarose gels stained with ethidium bromide and visualized under ultraviolet illumination.

Sequencing bacterial *rrs* and mitochondrial genes and phylogenetic analyses

From the diagnostic PCR described above, five positive PCR products were randomly selected for both male and female mosquitoes from each bacterial genus and for each sampling site. Mitochondrial genes encoding cytochrome oxidase I (COI) and NADH dehydrogenase subunit 5 (ND5) were also sequenced from three males and three females per site, as previously described (Raharimalala *et al.*, 2012). PCR products were sequenced at Biofidal-DTAMB (FR Bio-Environment and Health, Lyon, France) and the sequences were analysed with the BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *Acinetobacter* sequences were used to construct phylogenetic trees, but *Asaia* sequences were too short to be analysed at the species level (Table S1). Sequences were aligned using CLUSTALW and then adjusted manually using BIOEDIT software (Tom Hall). The resulting alignment was used to construct a maximum-likelihood tree using SEAVIEW v.4.2.12. (http://pbil.univ-lyon1.fr/software/seaview.html). The tree topology was tested by bootstrap analysis with 1000 resamplings. The sequence of *Moraxella lacunata* (AF005160) was used as an outgroup.

Enrichment, isolation and identification of *Acinetobacter*

Field-caught live mosquito specimens were anaesthetized with ether. Six adult mosquitoes per site (three males and three females) were surface-disinfected and crushed individually in 150 μL sterile 0.8% NaCl. Homogenates (10 μL) were streaked directly on plates containing a modified rich solid Luria–Bertani medium containing NaCl at 5 mg mL^{-1} (LBm) then incubated at 26 °C. Homogenates (20 μL) were also added to 1 mL of enrichment medium (0.2% KNO_3, 0.02% $MgSO_4{\cdot}7H_2O$, 0.2% sodium acetate, 0.04 M KH_2PO_4, pH 6.0; Bouvet & Joly-Guillou, 2000) and incubated at 30 °C for 24–48 h with shaking. When microbial growth occurred, an 10-μL aliquot of the culture was streaked onto Herellea agar plates (Biolife, Italy; Mandel *et al.*, 1964) and incubated at 37 °C for 24–48 h. Single distinct colonies of different colours or shapes were selected and re-inoculated onto fresh agar plates of the corresponding medium. Resultant colonies were streaked out again to check for purity and samples were stored in 25% glycerol at −80 °C. All isolates were screened by colony PCR amplification of the *rrs* gene using specific primers for *Acinetobacter*. For this, a single colony was suspended in 25 μL sterile water and boiled for 2 min, then placed on ice for 2 min. The boiling and cooling steps were repeated followed by centrifugation at 16 100 ***g*** for 5 min. Each 50-μL reaction contained 2 μL supernatant, 1× PCR buffer, 2 mM $MgCl_2$, 0.5 mM dNTP, 0.5 μM of each primer Acin1 and Ac (Table S1) and 1 U Taq Polymerase (Invitrogen). PCR conditions, sequencing of positive PCR products and analysis were as described above. Complete *rrs* genes of *Acinetobacter* isolates were amplified by colony PCR using the universal bacterial primers pA and pH (Table S1).

Assays for carbon and nitrogen source utilization

The Biolog (Hayward, CA) Phenotype MicroArrays (PM) system and the Omnilog reader (Hayward, CA) were used to study the metabolic profiles of the different *Acinetobacter* isolates and reference strains (Table S2). Standard commercial microplates (PM1–3) were used to simultaneously test for the ability of the microorganism to oxidize a panel of 190 different carbon sources (PM1 and PM2) and 95 different nitrogen sources (PM3), following the manufacturer's standard operating conditions. The PM plates were placed in an aerobic OmniLog incubator at 28 °C to collect signal data every 15 min for 48 h. The signal data (conversion of pixel density to a signal value reflecting cell growth) were analysed using BIOLOG MICRO-LOG3 4.20 software (Biolog, Inc.) and the average signal value for each well was exported to MICROSOFT OFFICE EXCEL (Microsoft) to be compiled. Substrate utilization was scored as positive when its growth value was at least 20% of the growth value recorded on D-alanine, a substrate used by all the strains (i.e. growth value of 100%).

Plasmid content

Two different methods were used to search for the presence of plasmids in *Acinetobacter*. To identify high molecular-weight plasmids, the procedure of Eckhardt (1978) was used with the modifications previously described (Mavingui *et al.*, 2002). For this, *Acinetobacter* strains were grown in LBm until the optical density of the culture at 600 nm reached 0.5, then 300 μL of culture

was analysed. Electrophoresis was carried out at 10 V for 20 min and 85 V for 210 min. To identify lower molecular-weight plasmids, a second method described by Seifert *et al.* (1994) was used. The size of a plasmid was estimated by comparing its mobility in agarose gels to the mobility of plasmids from sequenced *Azospirillum* genomes (Caballero-Mellado *et al.*, 1999; Wisniewski-Dyé *et al.*, 2011) and standard supercoiled plasmids (Life Technologies, Inc.).

Pulse field gel electrophoresis

The undigested genomes of *Acinetobacter* isolates were analysed by pulse field gel electrophoresis (PFGE) according to published protocols with some modifications (Schwartz & Cantor, 1984). Briefly, isolates were grown in LBm liquid medium for 18 h at 30 °C. Bacteria were subcultured to ensure they were pure. Cell cultures were centrifuged at 5000 ***g*** for 20 min at 4 °C. The pellet was washed once and resuspended in 1× Tris-EDTA buffer. The optical density of the suspension at 600 nm was adjusted to 1.8 and 2.0. An equal volume of a 1.6% low melting-point agarose (Bio-Rad) was mixed with cell suspensions and 150 μL of the mixture was placed in a plug mould (Bio-Rad). Cells were lysed using a lysis solution (2× Tris NaCl EDTA, 10% sodium lauroyl sarcosinate, 1.4 mg mL^{-1} lysozyme) at 37 °C for 24 h, then proteins digested with 0.5 mg mL^{-1} proteinase K (Euromedex) in 0.5 M EDTA pH 8 and 1% *N*-lauryl-sarcosine at 37 °C for 48 h. Electrophoresis was carried out in 0.8% chromosomal-grade agarose in 1× TAE buffer using a CHEF Mapper XA (Biorad, France) at 14 °C, a constant pulse of 500 ms and a field angle of 106° for 48 h at 3 V cm^{-1}.

Statistical analysis

To investigate the effect of sampling site and mosquito gender on the prevalence of each bacterial genus in the field populations, logistic regression models were built considering all possible combinations of additive effects and interactions. The most appropriate model was selected for each type of bacterium using the Akaike information criterion (AICc) adjusted for small sample size (Anderson *et al.*, 1994). The models were ranked according to the smallest AICc differences (denoted ΔAICc) between the focal model and the lowest AICc model. When ΔAICc was larger than 2, the model with the smallest AICc was selected. Parameter estimates were obtained by the method of maximum likelihood. Possible interactions between both bacteria were sought using the corrected chi-squared method (Hellard *et al.*, 2012). To take account of factors that could potentially create statistical associations (i.e. false interactions), both identified risk factors for the two bacteria (i.e. the mosquito sex and the sampling site) were included in the logistic regression model to estimate the expected frequencies. All analyses were performed in R software (R Development Core Team, 2009).

Nucleotide sequence accession numbers

The CO1 and ND5 gene sequences were submitted to GenBank under accession numbers JN406664–JN406666, JN406668–JN406670, JN406694, JN406697, JN406700–JN406705, JN406709–JN406711, JN406714, JN406715, JN406727–JN406732, JN406738–JN406740, JN406742–JN406744, JN406763–JN406767, JN406771–JN406776, JN406779–JN406783, JN406789–JN406794, JN406804–JN406809, and JN406840–JN406844. The rRNA gene sequences obtained from direct PCR amplification (JN379293–JN379351) and from *Acinetobacter* isolates (JQ408696–JQ408701 and JQ627634) are also available in GenBank.

Results

Prevalence of *Acinetobacter* and *Asaia* in *Ae. albopictus*

Alignment of all COI and ND5 gene sequences of *Ae. albopictus* showed polymorphism of only 0.01% and 0.004%, respectively (not shown), revealing a strong phylogenetic relationship between mosquito populations from the five sampling sites. Diagnostic PCR amplification of the 16S rRNA-encoding *rrs* gene was used to assess the prevalence of *Acinetobacter* and *Asaia* in field and laboratory-reared populations of *Ae. albopictus*. The overall infection frequencies were quite similar between field and laboratory populations (Table 2) with a higher infection frequency

Table 2. Prevalence of *Acinetobacter* and *Asaia* in *Aedes albopictus*

	Total		Male		Female	
	Acinetobacter	*Asaia*	*Acinetobacter*	*Asaia*	*Acinetobacter*	*Asaia*
Laboratory populations	70% (35/50)	46% (23/50)	59% (19/32)	28% (9/32)	89% (16/18)	78% (14/18)
Field populations	74% (184/249)	46% (117/252)	79% (96/121)	43% (53/122)	69% (88/128)	49% (64/130)

Number of infected mosquito individuals vs. number of mosquito individuals analysed is indicated in parentheses.

for *Acinetobacter* (around 70%) than for *Asaia* (46%). Infection frequencies were compared between field populations sampled in the same year, i.e. data from specimens from the Diego site in the Diana region were excluded from the statistical analysis. According to the AICc, the best logistic regression model to explain *Acinetobacter* prevalence included the gender effect (Table S3), which is in keeping with the statistical analysis showing a significant difference in infection frequencies according to the sex of the mosquito. Males are two times more infected than females ($P = 0.04$, Table S3), contrary to what was observed in laboratory populations. For *Asaia*, the selected model included the interaction between the sex and the sampling site ($P = 3.84 \times 10^{-11}$; Table S3). Males tended to be more infected than females except in Mananjary, where the opposite was true (Table S4). When the identified risk factors (i.e. mosquito sex and sampling site) were taken into account, a probable interaction between the two bacteria was also revealed $\chi^2_{corr} = 6.45124$, $P = 0.001$). Double-infected mosquitoes account for 50% (119/237) of the total caught, with proportionally more double-infection of females (54%) than of males (47%). This interaction seems to be synergistic because more double-infected mosquitoes were observed than would be expected if the bacteria acted independently.

The partial sequences of *rrs* genes amplified from the *Acinetobacter*-positive mosquito samples allowed them to be presumptively assigned to seven species with percentages of identity ranging from 96% to 100%: *Acinetobacter baumanii*, *Acinetobacter junii*, *Acinetobacter johnsonii*, *Acinetobacter schindlerii*, *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. A phylogenetic tree clusters the sequences of the different species of *Acinetobacter* with those obtained after colony isolation and those from corresponding reference strains (Fig. 1).

Molecular characterization of *Acinetobacter* isolates from *Ae. albopictus*

Attempts were made to culture bacteria from *Ae. albopictus* mosquito samples. Diagnostic PCR allowed to identify four isolates potentially affiliated with *Acinetobacter*. On the basis of the nearly complete *rrs* gene sequences obtained (1409–1467 bp long), two isolates cultured on Herellea agar plates were judged to be affiliated to *A. baumanii* and two isolates cultured on LBm to *A. lwoffii*. To further characterize these isolates from mosquitoes, they were compared to five additional strains: one from *A. calcoaceticus*, two from *A. johnsonii* that had been isolated from *Ae. albopictus* in our previous study (Zouache *et al.*, 2011), and two other *Acinetobacter* sp. isolates recovered from water in which *Ae. albopictus* larvae were breeding in the laboratory (Table S2).

PFGE was used to analyse the genetic material of isolates. All isolates contained a high molecular-weight replicon (*c.* 3 Mb) which considered alongside sequenced reference genomes probably corresponds to the bacterial chromosome (Fig. 2). A second replicon (*c.* 1 Mb) is also seen in the PFGE pattern from mosquito isolates 1b, 3a and 8 and reference strains Ref-1, Ref-3 and Ref-4. Intra-specific comparisons showed that mosquito isolates of *A. lwoffii* (2a and 2b) and *A. calcoaceticus* (3a) had a similar pattern to one of their corresponding reference strains. For *Acinetobacter baumannii*, only one of the two mosquito isolates (1b) had a similar PFGE pattern to that of the reference strain. By contrast, the PFGE patterns produced from mosquito isolates of *A. johnsonii* (4a and 4b) were similar to each other yet distinct from their reference strain. Isolates 8 and 9 from medium in which laboratory mosquitoes had been bred had PFGE profiles closely related to that of the *A. johnsonii* reference strain. A great diversity in both the number and size of plasmids was observed both between isolates and between isolates and their respective reference strains (Fig. 3). With the Eckhardt procedure for lysis and plasmid extraction it was possible to detect low molecular-weight (up to 75 kb) and high molecular-weight (up to 690 kb) plasmids (Fig. 3). The mosquito isolates of *A. lwoffii* contained the most plasmids (> 12 replicons).

Metabolic characteristics of *Acinetobacter* isolates

The metabolic capabilities of *Acinetobacter* isolates were explored using BIOLOG assays. Two independent experiments were done giving similar results. The bacterial strains were found to have different capacities for substrate utilization with both broad and narrow spectra suggesting variability in endogenous enzymatic activities (Table S5). The reference strains tend to utilize a broader spectrum of substrates than isolates from mosquitoes: 36.3% vs. 28.5% for *A. calcoaceticus*, 20.7% vs. 18.8% for *A. johnsonii*, 22.2% vs. 15.1% for *A. lwoffii* and 28.5% vs. 29.1% for *A. baumannii*, respectively. At the intra-species level, some substrates were specifically used by reference strains whereas others were catabolized only by mosquito isolates (Table 3).

Discussion

This study provides an estimate of the prevalence of *Acinetobacter* and *Asaia* in natural populations of the mosquito *Ae. albopictus* in Madagascar. The overall infection frequency of *Acinetobacter* (*c.* 70%) was higher than that of *Asaia* (*c.* 46%). High prevalence is one of the criteria that may indicate there is an intimate symbiotic

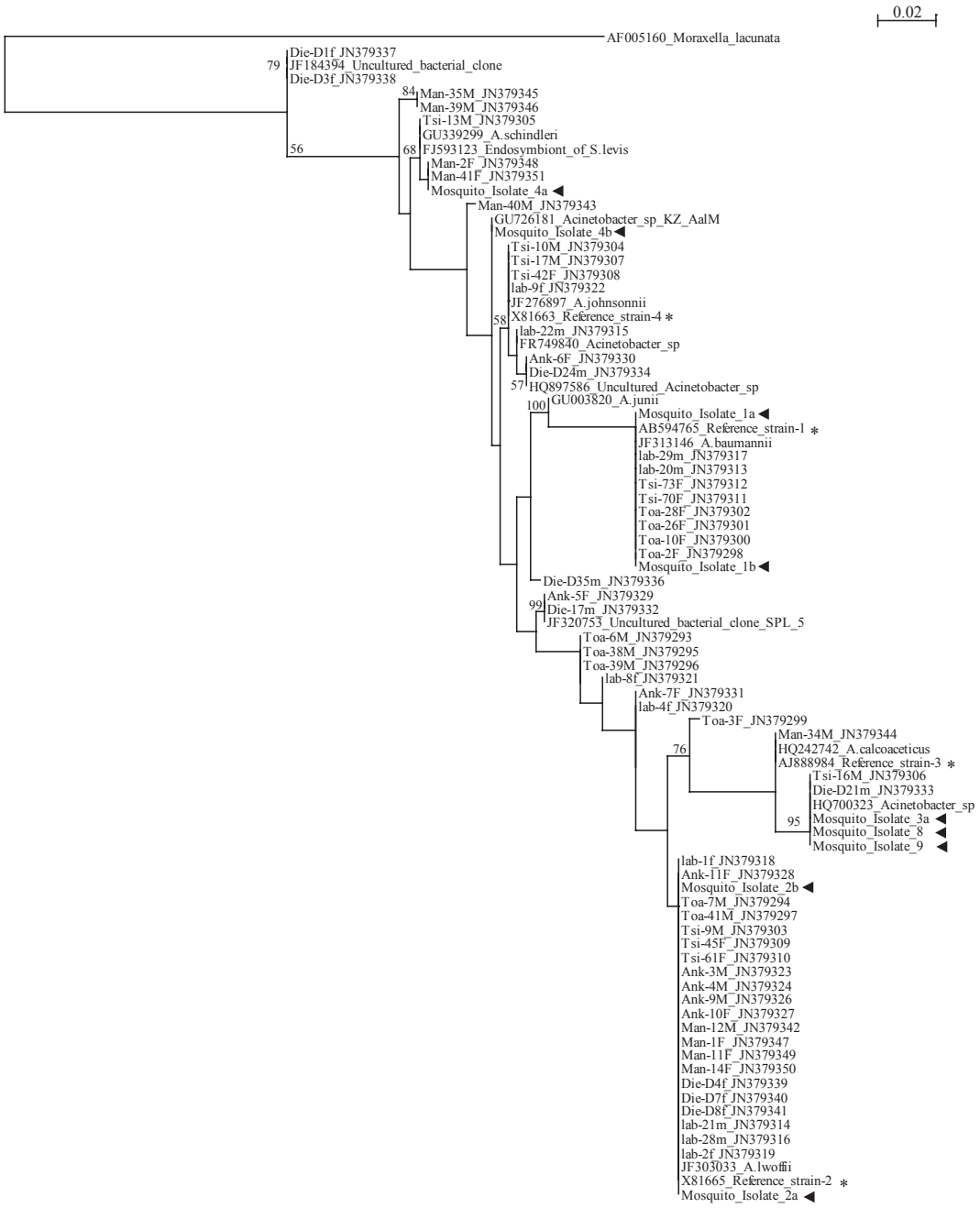

Fig. 1. Maximum-likelihood phylogenetic tree comparing a region of bacterial 16S rRNA gene sequences. Samples are named according to the sampling site (Man, Mananjary; Ank, Ankazobe; Tsi, Tsimbazaza; Toa, Toamasina; Die, Diego; lab, laboratory-reared populations) followed by the number of the sample and the sex of the mosquito (M, male; F, female). Sequence accession numbers are indicated next to the sequence name. The sequence of *Moraxella lacunata* (AF005160) was used as an outgroup and bootstrap values (1000 tree replications) higher than 50% are indicated at the nodes of the tree. Mosquito isolates 1a, 1b, 2a, 2b, 3a, 4a, 4c are indicated by arrows and reference strains are indicated by asterisks.

relationship between a given bacterium and its host. Previous studies demonstrated that the *Asaia* genus was 100% prevalent in laboratory populations of *Ae. albopictus* (Chouaia *et al.*, 2010). Here we report the much lower prevalence of *Asaia* in natural populations of *Ae. albopictus* from Madagascar. This difference suggests that environmental factors strongly influence prevalence. Members of the genus *Asaia* are widely distributed in nectar-bearing flowers (Yamada & Yukphan, 2008), whereas *Acinetobacter* are mostly free-living saprophytes

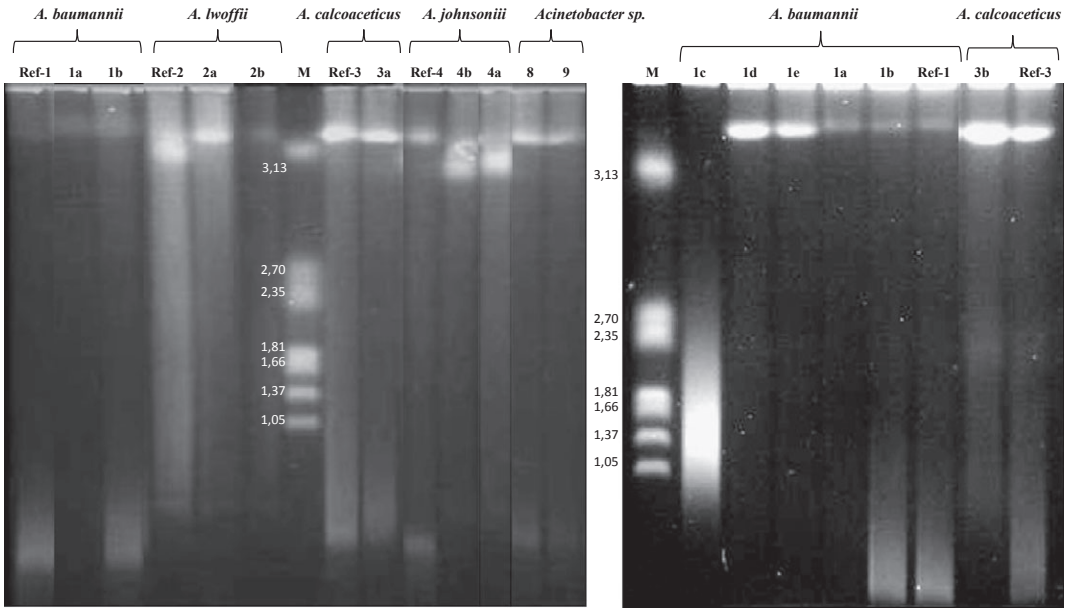

Fig. 2. PFGE of undigested genomic DNA of *Acinetobacter* mosquito isolates and their reference strains. Chromosomal DNA from *Hansenula wingei* (M) was used as a reference (BioRad). Characteristics of the samples are given in Table S2.

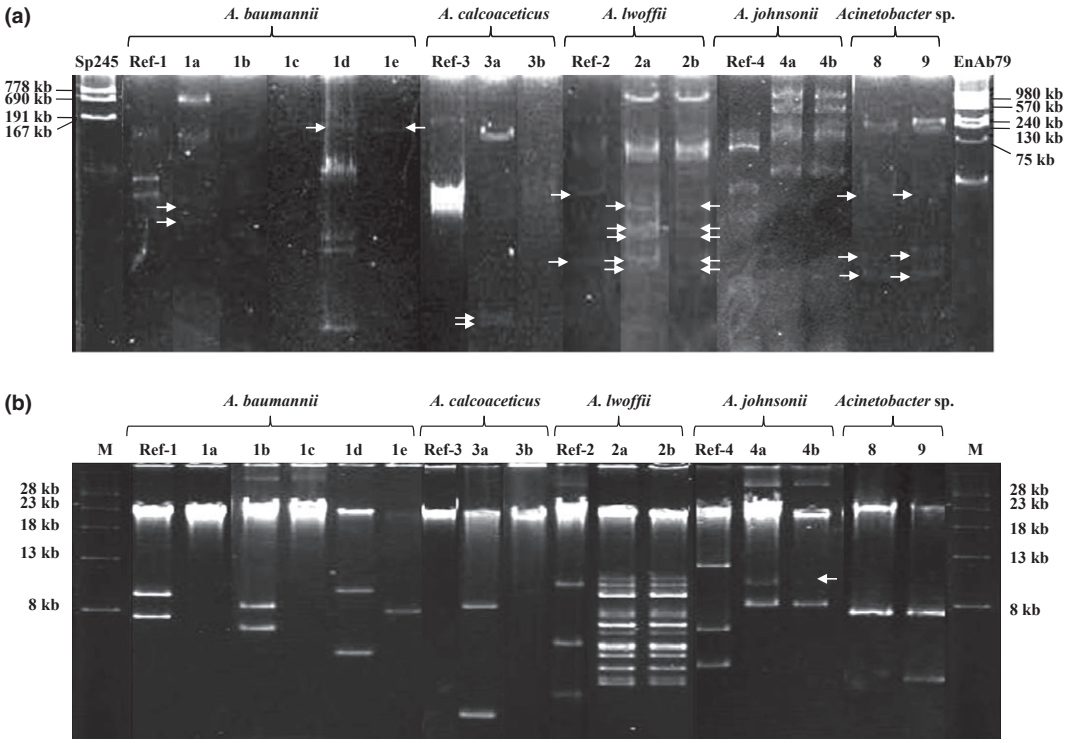

Fig. 3. Electrophoretic profiles of plasmids from *Acinetobacter*. Characteristics of the samples are given in Table S2. (a) Electrophoretic profiles of high molecular-weight plasmids obtained by a modified Eckhardt procedure. Plasmids from *Azospirillum brazilense* strains En-Ab79 and Sp245 were used as references (Caballero-Mellado *et al.*, 1999; Wisniewski-Dyé *et al.*, 2011). (b) Electrophoretic profiles of low molecular-weight plasmids obtained by the procedure of Seifert *et al.* (1994). Standard supercoiled plasmids (Life Technologies, Inc.) were used as references (M). Photograph quality was improved using ADOBE PHOTOSHOP ELEMENTS 6.0 (Adobe).

Table 3. Differential substrate utilization between *Acinetobacter* mosquito isolates and their reference strains

	Acinetobacter calcoaceticus	*Acinetobacter baumannii*	*Acinetobacter johnsonii*	*Acinetobacter* sp.
Carbohydrates	D-galactose, D-mannose, D-melibiose, L-lyxose, glucuronamide, β-D-allose, D-fucose, gentiobiose, D-glucosamine, D-hydroxyacétone, D-saccharic acid, mucic acid dextrin, D-ribono-1,4-lactone	α-D-glucose	L-arabinose, D,L-lactamide	L-arabinose, D-xylose
Carboxylates	Formic acid, oxalomalic acid D-L carnitine	D-malic acid, α-keto valeric acid, D- L carnitine	γ-amino butyric acid capric acid, β-hydoxy butyric acid 4-hydroxy-benzoic acid sebacic acid	Mono-methyl-succinate, β-hydroxy-butyric acid acetoacetic acid
Amino acids	D-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-threonine, L-alanyl-glycyl, glycyl-L-proline, L-isoleucine, L-leucine, L-valine, alanine-asparagine, alanine-glutamine, alanine-glycine, glycine-asparagine, glycine-glutamine, glycine-methionine alanine-threonine	L-isoleucine, L-ornithine, glycine, D-asparagine	L-aspartic acid, L-glutamic acid, L-asparagine, L-glutamine, L-pyroglutamic acid, L-citrulline, D,L-α-amino-N-butyric acid, γ-amino-N-butyric acid, δ-amino-N-valeric acid tyramine	δ-amino-N-valeric acid, alanine-glutamine, methionine-alanine D,L-α-amino-caprylic acid
Alcohol	2,3-butanediol			
Amide			Formamide	
Amine	Ethalonamine		Methylamine	
Nitrogen				Nitrate
Nucleic acids	Adenine, guanine, xanthine, inosine		Biuret guanosine, xanthine	Biuret, guanine, guanosine, uracil

Not underlined, substrates only used by the reference strain.
Underlined, substrates only used by *Acinetobacter* mosquito isolates.

often present in the rhizosphere, in water, or on the skin of vertebrates (Doughari *et al.*, 2011). Interestingly, these ecological niches can also all be used by mosquitoes as food sources or for laying eggs, hatching larvae, or resting. Sharing such environments may thus favour encounters between mosquitoes and these bacteria. Sex effects on the composition and diversity of mosquito-associated bacteria have already been reported in natural populations of *Ae. albopictus* (Zouache *et al.*, 2011). Both intrinsic genetic characteristics and ecological behaviour may account for such differences. In particular, only females are hematophagous. Previous studies reported that members of *Asaia* and *Acinetobacter* may cause opportunistic infections in humans (Alauzet *et al.*, 2010; Doughari *et al.*, 2011), raising the possibility that cross-infection could occur when mosquitoes bite hosts. In addition, females scatter over a wider area than males when looking for a breeding site, increasing the chance of encountering these bacteria. Our findings revealed a possible synergy between the two types of bacteria, although we cannot exclude the possibility that they share an unknown risk factor that could create a statistical association between the bacteria or that some mosquito individuals are particularly sensitive to multiple infections.

The high prevalence of *Acinetobacter* found in our study raises questions as to how the bacterium is acquired and transmitted. Using fluorescent *in situ* hybridization, *Acinetobacter* has been localized in the midgut but not in the ovaries of *Ae. albopictus* (Zouache *et al.*, 2009), so the bacteria are likely to be acquired from the environment. However, this does not exclude the possibility of vertical transmission by other mechanisms such as coprophagy, paternal transmission or egg-smearing (Kikuchi, 2009). Experimental infection showed that *Asaia* could be transmitted both vertically and horizontally from parents to offspring (Crotti *et al.*, 2009). Surprisingly, the *Asaia* infection prevalence reported here does not strongly support the assumption of a high vertical transmission rate. On the contrary, the high prevalence of *Acinetobacter* suggests efficient transmission and maintenance within host populations, indicating a possible role in the biology of the mosquito in nature. As yet, the role of *Acinetobacter* in insect biology has only been demonstrated in *Stomoxys calcitrans*, which need these bacteria to ensure complete

development of the fly larvae (Lysyk *et al.*, 1999). It has already been shown that some *Acinetobacter* species are efficient pollutant degraders (Lal & Khanna, 1996). As *Ae. albopictus* is able to invade suburban areas and survive in polluted zones (Mousson *et al.*, 2005), it is possible that *Acinetobacter*-associated mosquitoes have an improved capacity to colonize and adapt to anthropized environments. A potential role for *Asaia* in the biosynthesis of vitamins in the mosquito species *Anopheles stephensi* was suggested by Crotti *et al.* (2009).

To gain more insight into the mosquito–bacteria interactions, the genetic contents and metabolic capabilities of *Acinetobacter* mosquito isolates and their free-living relatives were compared. Despite considerable effort, only a few *Acinetobacter* were isolated from field-caught *Ae. albopictus*, contradicting somewhat the high prevalence of *Acinetobacter* detected by molecular methods. The seven *Acinetobacter* isolated from *Ae. albopictus* were phylogenetically closely related to the species *A. calcoaceticus*, *A. baumanii*, *A. lwoffii* and *A. johnsonii*. The estimated genome sizes of *Acinetobacter* isolates (*c.* 3 Mb) were similar to those of *Acinetobacter* strains reported in published databases ranging from 3.59 to 4.05 Mb (Fondi *et al.*, 2010). It is well known that plasmids are among the most important elements in the evolution of prokaryotes and their adaptation to fluctuating environmental conditions (Eberhard, 1990). The presence and wide diversity of plasmids in *Acinetobacter* isolates may suggest these genetic elements have a biological role. The functions of many plasmids of *Acinetobacter* isolates have yet to be identified but some have been associated with metabolic activities or metal resistance (Winstanley *et al.*, 1987; Schembri *et al.*, 1994; Mengoni *et al.*, 2007). Information on phenotypic traits is a useful complement to genomic data (Bochner *et al.*, 2010), so substrate utilization assays were performed to investigate whether the variation observed at the DNA level also reflected variation in the metabolic potential of *Acinetobacter* isolates. Fairly large metabolic differences were found between the *Acinetobacter* isolates, and this may related to their phylogenetic relationships given that the nine isolates belonged to distinct genotypes (Geiger *et al.*, 2011). Some *Acinetobacter* isolates from mosquitoes were unable to utilize certain substrates metabolized by the reference strains, suggesting that substantial changes in gene content or regulation or both had occurred. Conversely, some substrates were only used by mosquito-associated strains. For example, amino acids α-keto valeric acid and glycine, which are blood components, were only metabolized by the isolates of *A. baumannii* from mosquitoes, whereas 4-hydroxybenzoic acid and xylose, which are common constituents of plants, were only metabolized by *A. johnsonii* isolates from mosquitoes. Possibly these bacterial isolates improve blood digestion and nectar assimilation respectively by the insect host. Using this approach, a phenotypic or genotypic subpopulation of *Acinetobacter* from mosquitoes appears to be selected from the surrounding environment.

In conclusion, this study demonstrated the relatively high occurrence of *Acinetobacter* and *Asaia* within the mosquito *Ae. albopictus*. This bacterial prevalence significantly correlated with the sex of the mosquito and environmental factors, notably the climate and ecology. The variability observed emphasizes the need to gain a global view of the diversity and genetic potential of bacterial partners in mosquito vectors.

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References

Alauzet C, Teyssier C, Jumas-Bilak E, Gouby A, Chiron R, Rabaud C, Counil F, Lozniewski A & Marchandin H (2010) *Gluconobacter* as well as *Asaia* species, newly emerging opportunistic human pathogens among acetic acid bacteria. *J Clin Microbiol* **48**: 3935–3942.

Anderson DR, Burnham KP & White GC (1994) AIC model selection in overdispersed capture-recapture data. *Ecology* **75**: 1780–1793.

Bochner B, Gomez V, Ziman M, Yang S & Brown SD (2010) Phenotype microarray profiling of *Zymomonas mobilis* ZM4. *Appl Biochem Biotechnol* **161**: 116–123.

Bouvet PJM & Joly-Guillou ML (2000) *Acinetobacter* (Freney J, Renaud F, Hansen W & Bollet C, eds), pp. 1239–1258. Précis de bactériologie Clinique, Editions ESKA, Paris.

Caballero-Mellado J, López-Reyes L & Bustillos R (1999) Presence of 16S rRNA genes in multiple replicons in *Azospirillum brasilense*. *FEMS Microbiol Lett* **178**: 283–288.

Chouaia B, Rossi P, Montagna M *et al.* (2010) Molecular evidence for multiple infections as revealed by typing of *Asaia* bacterial symbionts of four mosquito species. *Appl Environ Microbiol* **76**: 7444–7450.

Christodoulou M (2011) Biological vector control of mosquito-borne diseases. *Lancet Infect Dis* **11**: 84–85.

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.

Crotti E, Rizzi A, Chouaia B *et al.* (2010) Acetic acid bacteria, newly emerging symbionts of insects. *Appl Environ Microbiol* **76**: 6963–6970.

Damiani C, Ricci I, Crotti E *et al.* (2010) Mosquito-bacteria symbiosis: the case of *Anopheles gambiae* and *Asaia*. *Microb Ecol* **60**: 644–654.

David J-P, Coissac E, Melodelima C, Poupardin R, Riaz MA, Chandor-Proust A & Reynaud S (2010) Transcriptome response to pollutants and insecticides in the dengue vector *Aedes aegypti* using next-generation sequencing technology. *BMC Genomics* **11**: 216.

Dillon RJ & Dillon VM (2004) The gut bacteria of insects: nonpathogenic interactions. *Annu Rev Entomol* **49**: 71–92.

Doughari HJ, Ndakidemi PA, Human IS & Benade S (2011) The ecology, biology and pathogenesis of *Acinetobacter* spp.: an overview. *Microbes Environ* **26**: 101–112.

Eberhard WG (1990) Evolution in bacterial plasmids and levels of selection. *Q Rev Biol* **65**: 3–22.

Eckhardt T (1978) A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. *Plasmid* **1**: 584–588.

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

Fondi M, Bacci G, Brilli M, Papaleo MC, Mengoni A, Vaneechoutte M, Dijkshoorn L & Fani R (2010) Exploring the evolutionary dynamics of plasmids: the *Acinetobacter* pan-plasmidome. *BMC Evol Biol* **10**: 59.

Geiger A, Fardeau M-L, Grebaut P, Vatunga G, Josénando T, Herder S, Cuny G, Truc P & Ollivier B (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.

Geiger A, Fardeau M-L, Njiokou F, Joseph M, Asonganyi T, Ollivier B & Cuny G (2011) Bacterial diversity associated with populations of *Glossina* spp. from Cameroon and distribution within the Campo sleeping sickness focus. *Microb Ecol* **62**: 632–643.

Hellard E, Pontier D, Sauvage F, Poulet H & Fouchet D (2012) True versus false parasite interactions: a robust method to take risk factors into account and its application to feline viruses. *PLoS ONE* **7**: e29618.

Hoffmann AA, Montgomery BL, Popovici J *et al.* (2011) Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* **476**: 454–457.

Kikuchi Y (2009) Endosymbiotic bacteria in insects: their diversity and culturability. *Microbes Environ* **24**: 195–204.

Lal B & Khanna S (1996) Degradation of crude oil by *Acinetobacter calcoaceticus* and *Alcaligenes odorans*. *J Appl Bacteriol* **81**: 355–362.

Lysyk TJ, Kalischuk-Tymensen L, Selinger LB, Lancaster RC, Wever L & Cheng KJ (1999) Rearing stable fly larvae (Diptera: Muscidae) on an egg yolk medium. *J Med Entomol* **36**: 382–388.

Mandel AD, Wright K & McKinnon JM (1964) Selective medium for isolation of mima and herellea organisms. *J Bacteriol* **88**: 1524–1525.

Mavingui P, Flores M, Guo X, Dávila G, Perret X, Broughton WJ & Palacios R (2002) Dynamics of genome architecture in *Rhizobium* sp. strain NGR234. *J Bacteriol* **184**: 171–176.

Mengoni A, Ricci S, Brilli M, Baldi F & Fani R (2007) Sequencing and analysis of plasmids pAV1 and pAV2 of *Acinetobacter venetianus* VE-C3 involved in diesel fuel degradation. *Ann Microbiol* **57**: 521–526.

Mousson L, Dauga C, Garrigues T, Schaffner F, Vazeille M & Failloux A-B (2005) Phylogeography of *Aedes* (Stegomyia) *aegypti* (L.) and *Aedes* (Stegomyia) *albopictus* (Skuse) (Diptera: Culicidae) based on mitochondrial DNA variations. *Genet Res* **86**: 1–11.

R Development Core Team (2009) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. Available at: http://www.R-project.org.

Raharimalala FN, Ravaomanarivo LH, Ravelonandro P *et al.* (2012) Biogeography of the two major arbovirus mosquito vectors, *Aedes aegypti* and *Aedes albopictus* (Diptera, Culicidae), in Madagascar. *Parasit Vectors* **20**: 56.

Ratsitorahina M, Harisoa J, Ratovonjato J, Biacabe S, Reynes JM, Zeller H, Raoelina Y, Talarmin A, Richard V & Soares JL (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.

Reiter P (1998) *Aedes albopictus* and the world trade in used tires, 1988–1995: the shape of things to come? *J Am Mosq Control Assoc* **14**: 83–94.

Riehle MA & Jacobs-Lorena M (2005) Using bacteria to express and display anti-parasite molecules in mosquitoes: current and future strategies. *Insect Biochem Mol Biol* **35**: 699–707.

Schembri MA, Bayly RC & Davies JK (1994) Cloning and analysis of the polyhydroxyalkanoic acid synthase gene from an *Acinetobacter* sp.: evidence that the gene is both plasmid and chromosomally located. *FEMS Microbiol Lett* **118**: 145–152.

Schwartz DC & Cantor CR (1984) Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* **37**: 67–75.

Seifert H, Boullion B, Schulze A & Pulverer G (1994) Plasmid DNA profiles of *Acinetobacter baumannii*: clinical application in a complex endemic setting. *Infect Control Hosp Epidemiol* **15**: 520–528.

Winstanley C, Taylor SC & Williams PA (1987) pWW174: a large plasmid from *Acinetobacter calcoaceticus* encoding

benzene catabolism by the beta-ketoadipate pathway. *Mol Microbiol* **1**: 219–227.

Wisniewski-Dyé F, Borziak K, Khalsa-Moyers G *et al.* (2011) *Azospirillum* genomes reveal transition of bacteria from aquatic to terrestrial environments. *PLoS Genet* **7**: e1002430.

Yamada Y & Yukphan P (2008) Genera and species in acetic acid bacteria. *Int J Food Microbiol* **125**: 15–24.

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

Zouache K, Raharimalala FN, Raquin V, Tran-Van V, Raveloson LHR, Ravelonandro P & Mavingui P (2011) Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. *FEMS Microbiol Ecol* **75**: 377–389.

Supporting Information

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

Table S1. Primers used in this study.

Table S2. Characteristics of *Acinetobacter* isolates and reference strains used in this study.

Table S3. Best logistic regression models for *Acinetobacter* and *Asaia* prevalence data according to Akaike information criterion (AICc).

Table S4. Final logistic-regression models of mosquito infection risk factors for the two bacteria: variables and variable categories.

Table S5. Substrate utilization profiles of the *Acinetobacter* mosquito isolates and their reference strains.

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