

Molecular typing of bacteria of the genus *Asaia* in malaria vector *Anopheles arabiensis* Patton, 1905

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Abstract

The acetic acid bacterium *Asaia* spp. was successfully detected in *Anopheles arabiensis* Patton, 1905, one of the major vector of human malaria in Sub-Saharan Africa. A collection of 45 *Asaia* isolates in cell-free media was established from 20 individuals collected from the field in Burkina Faso. 16S rRNA universal polymerase chain reaction (PCR) and specific qPCR, for the detection of *Asaia* spp. were performed in order to reveal the presence of different bacterial taxa associated with this insect. The isolates were typed by internal transcribed spacer-PCR, BOX-PCR, and randomly amplified polymorphic DNA-PCR, proved the presence of different *Asaia* in *A. arabiensis*.

Introduction

Species of the genus *Anopheles* Meigen, 1818 are the most important vectors of malaria in Sub-Saharan Africa. Within the *Anopheles gambiae* complex, composed of seven morphologically indistinguishable species of mosquitoes, *Anopheles arabiensis* Patton, 1905 and *Anopheles gambiae* Giles, 1902 sensu strictu show the wider distribu-

tion and are the most efficient malaria vectors (Onyabe & Conn, 2001a and 2001b). *A. arabiensis* prefers tropical weather and its typical habitat is the dry Savannah, reproducing in natural and artificial puddles of water, but can fly for more than two kilometers from its place of birth. *A. arabiensis* population density varies depending on climate conditions during the year; the adults show the minimum density between March and June, while after the first rains in July reach the highest levels (*i.e.* August and September) then decrease in October (Robert *et al.*, 1998). The presence of *A. arabiensis* is favored by temperatures of 28-30°C, clean water, high concentrations of HCO₃⁻ and CO₃²⁻, low concentrations of NO₃⁻ and NaCl, absence of predatory invertebrates and fish (as *Gambusia* and *Tilapia*), presence of plants of the genus *Pistia* (water lettuce) and absence of those of the genus *Lemna* (duckweed) (Robert *et al.*, 1998). *A. arabiensis* is the only vector of malaria in Dakar, Senegal, and is predominant in Ethiopia, Sudan and in the Guinean Savannah. *A. gambiae* is vicariant of *A. arabiensis* in the Sub-Saharan region not colonized by the latter. In the last 20 years, *A. arabiensis* has expanded its distribution, spreading even in forests where *A. gambiae* was prevalent, probably because of climate change (Onyabe & Conn, 2001a; Donnelly *et al.*, 2001). Hargreaves and colleagues (2003) demonstrated that *A. arabiensis* is resistant to DDT, one of the major insecticides used in malaria control program. As a result of these data, in order to control the vector, it is important to develop new integrated strategies. Similar to a recently described work (Chouaia *et al.*, 2010), here we present the first description of the symbiont *Asaia* in *A. arabiensis* mosquitoes and its genetic diversity.

Asaia is a Gram-negative acetic acid bacterium associated with different genera of insect (Favia *et al.*, 2007; Crotti *et al.*, 2009; Damiani *et al.*, 2010). It has been found in the gut, salivary glands, and reproductive organs; it is transmitted vertically, horizontally and venereally from males to females. Recently, it has been demonstrated that *Asaia* is not transmitted to humans (Epis *et al.*, 2011) suggesting that *Asaia* could be regarded only as an opportunistic pathogens and a good candidate to potentially vector antiparasitoid factors (Favia *et al.*, 2008; Ricci *et al.*, 2010). Uncovering the level of diversity of the symbiont *Asaia* of *A. arabiensis* would be useful for potential applications by allowing the selection of the dominant genotypes, that in this case represents the most widespread genotype among the insect populations, in order to develop paratransgenic approaches.

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Material and methods

Mosquito strains origin

A. arabiensis was collected from Soumouso and Vallée du Kou (Burkina Faso), immediately stored in enrichment medium, and sent

to the University of Camerino (Italy). Furthermore, 20 females collected from the same localities were reared in Burkina Faso insectary; the laid eggs were sent and maintained in the insectary of the Laboratory of Parasitology at the University of Camerino in order to start the new colony.

Isolation of *Asaia* from *A. arabiensis*

Asaia strains were isolated from adult mosquitoes using the enrichment medium I as described by Lisdiyanti *et al.*, 2001. *A. arabiensis* samples were washed in 1% sodium hypochlorite for 1 min, subsequently washed three times with 0.9% NaCl, and homogenized by grinding in 200 μ L 0.9% NaCl. The homogenate was inoculated into the enrichment medium and allowed to grow at 30°C for 3 days, with shaking. When microbial growth occurred, the microorganisms were streaked on CaCO₃ agar plates (pH 6.8) containing 1.0% (wt/vol) d-glucose, 1.0% (wt/vol) glycerol, 1.0% (vol/vol) ethanol, 1.0% (wt/vol) bactopectone, 0.5% (wt/vol) yeast extract, 0.7% (wt/vol) CaCO₃, and 1.5% (wt/vol) agar as reported in Chouaia *et al.*, 2010. After 3 days growth, the colonies were collected. After the incubation period, 500 μ L of enriched broth containing the *Asaia* have been preserved in a solution of 16% glycerol. The glycerine obtained were placed at -80°C for storage.

DNA extraction and amplification from selected colonies

DNA was extracted from *Asaia* strains and mosquitoes using commercial kit Wizard Genomic DNA Purification (PROMEGA Corp., Fitchburg, WI, USA) and eluted in 100 μ L of elution buffer. The quantitative polymerase chain reaction (qPCR) screening of mosquitoes, for the specific detection of *Asaia* spp., were performed with IQ thermal cycler (Bio-Rad, Hercules, CA, USA) using the previously described primers Asafor (5'-GCGCGTAGGCGGTTTACAC) and Asarev (5'-AGCGTCAGTAATGAGCCAGGTT) (Favia *et al.*, 2007). The 16S rRNA gene was PCR amplified with universal bacterial 16S rRNA gene primers 27F (5'-TCGACATCGTTTACGGCGTG) and 1492R (5'-CTACGGC-TACCTTGTTACGA).

Internal transcribed spacer (ITS)-PCR was performed, with primer ITSF (5'-GCCAAGGCATCCAAC) and ITSr (5'-GTCGTAA CAAGGTAGC-CGTA) (Daffonchio *et al.*, 1998). The BOX-PCR was performed, with BOX-A1 primer (5'-CTACGGCAAGGCGACGCTGAC) according to Urzi *et al.*, 2001; randomly amplified polymorphic DNA (RAPD)-PCRs were performed, using the RAPD OPA4 (5'-AATCGGGCTG) and OPA10 (5'-GTGATCGCAG) primers (Daffonchio *et al.*, 1998).

Sequence analysis

Nucleotide identity searches were performed in the GenBank database using the basic local alignment search tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) program to identify the bacterial strains most closely related to isolates and the best matching DNA sequences of the sequenced amplicons. A phylogenetic tree based on the analysis of the 16S rRNA sequences was inferred using the neighbor-joining method (Saitou *et al.*, 1987) with 1000 replicates as a bootstrap test and visualized using Mega 4 (Tamura *et al.*, 2007), the 16S rRNA sequence of *Acetobacter tropicalis* BA 1.3 was used as an outgroup. The 16S rRNA gene sequences were deposited under accession numbers from HE814602 to HE814621 in the European Molecular Biology Laboratory Nucleotide Sequence Database (<http://www.ebi.ac.uk/>).

Cluster analysis

PCR fingerprintings were obtained by visualization, under UV light, of 1.2% of agarose gel electrophoresis. The profiles were analyzed using the Quantity One® software (version 4.6.5, Bio-Rad). For each strain profile, a matrix, reporting the presence (1) or absence (0) of bands at a specific distance, was generated. The analysis of profiles of

ITS-PCR, BOX-PCR and RAPD-PCRs allowed obtaining a genotypic profile for each strain. To estimate the level of similarity between the strains based on the merged profiles obtained from ITS-PCR, BOX-PCR, and RAPD-PCR, and to create the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree, MVSP software (version 3.13n, Kovach Computing Services, Pentraeth, Isle of Anglesey, UK) was used (Dunn and Everitt, 1982).

Results

Forty-five isolates of *Asaia* spp. were obtained from 20 mosquitoes. The sequences obtained by universal bacterial 16S rRNA PCR confirmed the presence of *Asaia* spp. in *A. arabiensis* mosquitoes. Moreover, specific qPCR carried on *A. arabiensis* showed the presence of this bacterium associated to the studied population; *Asaia* 16S rRNA gene copies was abundant in all DNAs extracted from *A. arabiensis* mosquitoes, with a mean of 7.8×10^5 *Asaia* 16S rRNA gene copies for single individual.

The 45 *Asaia* isolates from the mosquitoes exhibited the same ITS-PCR fingerprinting profiles, presenting an amplification pattern consisting of 3 bands between 500 and 3700 bp (data not shown). The RAPD-PCR using the primer set OPA4 and OPA10 revealed, respectively, four and three genotypes characterized by complex band patterns between 450 and 7300 bp. The analysis obtained from BOX-PCR showed several profiles among the strains examined. BOX-PCR discriminated from 4 to 6 band pattern types, with most of the bands in the range between 350 and 4500 bp.

By using the software Quantity One, for each strain, a band profile was obtained from the analysis of the agarose gels of the amplifications carried out on the genome of *Asaia* from *A. arabiensis*. The merging of the results obtained from all the four PCRs (ITS-PCR, BOX-PCR and RAPD-PCRs) allowed us to distinguish 21 genotype patterns. The tree generated with the UPGMA methods using the Jaccard coefficient allowed clustering the different profiles in a tree (Figure 1). From the analysis of this tree it was possible to identify 9 groups (from I to IX).

Table 1. Different groups and relative profile numbers and *Asaia* strains.

Groups	Profile numbers	<i>Asaia</i> strains
I	1	1
	2	2, 3, 4, 5
	3	12
II	4	7, 8, 9, 11
	5	10
	6	6
III	7	15, 16
	8	14, 17, 18
	9	19, 20, 21, 22
IV	10	13
V	11	36, 37
VI	12	27, 28
	13	29, 30, 31
	14	32, 33, 34
VII	15	35
	16	23, 24, 25
VIII	17	39
	18	40, 41, 43
	19	42
IX	20	45
	21	38, 44

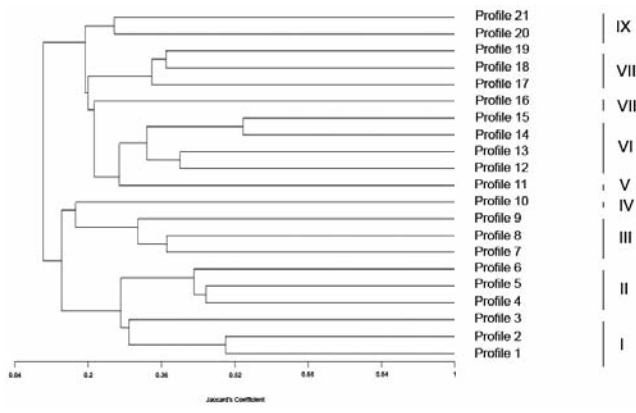


Figure 1. Similarity tree between the profiles of *Asaia* spp. isolated from the *Anopheles arabiensis* mosquitoes. The tree was generated with the Unweighted Pair Group Method with Arithmetic Mean methods using the Jaccard coefficient. We can distinguish 9 groups (from I to IX).

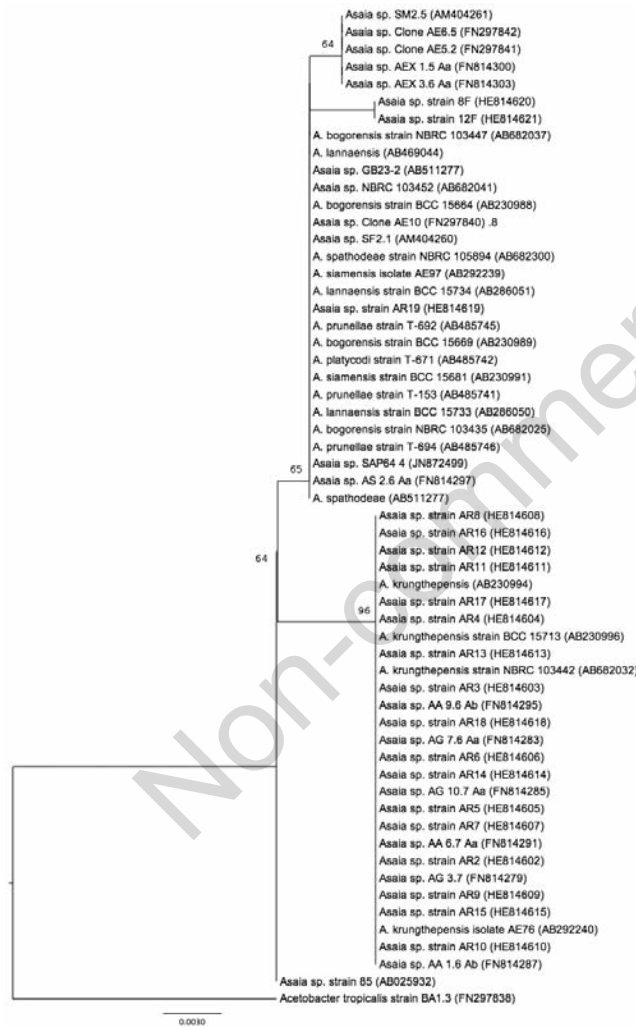


Figure 2. Phylogenetic positions of the strains of *Asaia* isolated from *An arabiensis*, based on 16S rRNA gene sequences (Neighbor joining method; Kimura correction; all positions containing gaps and missing data were eliminated from the data set). Values of bootstrap superior to 60% have been reported in the tree. The scale bar represents sequence divergence. The 16S rRNA sequence of *Acetobacter tropicalis* strain BA 1.3 was used as outgroup.

The analysis of the data allowed also to identify two more abundant strains (groups III and VI) each of them grouping 20% of the total strains (Table 1). Those stains were present in all studied populations of mosquito and not restricted to only one individual. Other strains were also present in more than one mosquito like the groups I, II and VII. Except for the group IV all the other groups were represented by more than one isolate present in more than one mosquito.

The phylogenetic tree based on the partial 16S rRNA sequence of representative strains of *Asaia* isolated from *A. arabiensis* showed that the strains clustered with other strains of *Asaia krungthepensis* (Figure 2). The phylogenetic analysis of the 16S rRNA did not show a high diversity of the strains as compared to the results of the molecular typing based on DNA markers (Figure 1).

Discussion and conclusions

Previous studies have shown that *A. stephensi* (Favia *et al.*, 2007), *Aedes aegypti*, *A. gambiae*, *Ae. albopictus* mosquitoes (Chouaia *et al.*, 2010) and *Scaphoideus titanus* (Crotti *et al.*, 2009) host the bacterium *Asaia*. Here we demonstrate the presence of acetic acid bacteria *Asaia* both in males and in females mosquitoes of *A. arabiensis*. The fact that *Asaia* is also able to be transmitted horizontally and cross colonize different species (Damiani *et al.*, 2008; Crotti *et al.*, 2009) may suggest the capacity of *Asaia* to be environmentally transmitted between *A. arabiensis* and *A. stephensi*.

In order to obtain a genetic profile of each strain of *Asaia*, the DNA was amplified using BOX-PCR, RAPD-PCRs and ITS-PCR. Based on these profiles, a similarity tree was build. This analysis showed that, in *A. arabiensis*, *Asaia* strains clustered in 21 different genotypes that can be grouped into 9 clusters. This study showed that the same *A. arabiensis* mosquito may harbor simultaneously different strains of the symbiont and that each strain of *Asaia* can colonize more than a single specimen of *A. arabiensis*. A similar multiple infection phenomena have been reported in other mosquito species harboring *Asaia* (Chouaia *et al.*, 2010) and insects associated with the symbiont *Wolbachia* (Werren *et al.*, 1995). The strains isolated in this work display a higher diversity (21 genotypes from 45 strains) than those studied in the work of Chouaia *et al.*, 2010 using BOX-PCR, RAPD-PCRs, tRNA-PCR and ITS-PCR (29 genotypes from 284 strains). The detected higher diversity can be explained by the fact that, unlike the mosquito species studied by Chouaia *et al.* (2010), the population of *A. arabiensis* studied in this work is a wild population. The mosquito associated *Asaia* is a secondary symbiont that may be acquired from the environment (Damiani *et al.*, 2008; Chouaia *et al.*, 2012). In spite of the stable lab rearing concentrations, the natural environment present complex interactions both at the biotic and abiotic levels, thus bacterial populations displaying high diversity have higher chance to adapt and survive in new environmental conditions (have higher chance of adaptations and survival in a changing environment). The presence of *Asaia* in *A. Arabiensis*, the most important malaria vector in Sub-Saharan Africa, can lead to the possibility to use this symbiont in a paratransgenesis approach as support for the prevention of malaria transmission.

In devising a paratransgenic approach it is essential to consider the genetic heterogeneity of the bacterium *Asaia* and the lack of a predominant strain in *A. arabiensis*.

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