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Molecular Evidence for Multiple Infections as Revealed by Typing of *Asaia* Bacterial Symbionts of Four Mosquito Species[▽]

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The recent increased detection of acetic acid bacteria (AAB) of the genus *Asaia* as symbionts of mosquitoes, such as *Anopheles* spp. and *Aedes* spp., prompted us to investigate the diversity of these symbionts and their relationships in different mosquito species and populations. Following cultivation-dependent and -independent techniques, we investigated the microbiota associated with four mosquito species, *Anopheles stephensi*, *Anopheles gambiae*, *Aedes aegypti*, and *Aedes albopictus*, which are important vectors of human and/or animal pathogens. Denaturing gradient gel electrophoresis (DGGE) analysis based on the 16S rRNA gene revealed the presence of several bacterial taxa, among which *Asaia* sequences were among the dominant in most of the samples. A collection of 281 *Asaia* isolates in cell-free media was established from individuals belonging to the four species. The isolates were typed by internal transcribed spacer (ITS)-PCR, tRNA-PCR, BOX-PCR, and randomly amplified polymorphic DNA (RAPD)-PCR, revealing that different *Asaia* strains are present in different mosquito populations, and even in single individuals.

As defined by Anton de Bary (8), symbiosis is the living together of organisms belonging to different species. In the medical entomology area, investigations of the interactions between arthropods and microorganisms focused mainly on the negative interactions, i.e., on pathogenic microorganisms transmitted to vertebrate hosts or on microorganisms pathogenic to the arthropod itself. In recent decades, more interest has been directed toward the study of other forms of arthropod symbiosis, including mutualistic interactions and reproductive parasitism (21). Recently, from the study of mutualistic primary symbionts (e.g., *Wigglesworthia* in *Glossina* spp.) and manipulators of reproduction (e.g., *Wolbachia*), there has been a switch of research toward secondary mutualistic symbionts. Several studies have shown that these secondary symbionts play important roles, even if not vital, in the physiology of their insect hosts (23, 24).

An issue which is still almost unexplored regards the diversity of symbionts belonging to a given taxon within individual hosts. For example, by analyzing the symbionts of different insect hosts, *Wolbachia* has been shown to encompass a wide molecular diversity (27, 31), but limited information is available for the symbiont diversity in single individual hosts.

Acetic acid bacteria (AAB) are environmental bacteria that can be found in different niches, like the flower of the orchid tree *Bauhinia purpurea* (34) or are involved in processes like wine fermentation or spoilage (16). In the last decade, several AAB were discovered in association with arthropods, as was *Gluconobacter morbifer* associated with *Drosophila melanogaster* (23) or *Acetobacter tropicalis* associated with the olive fruit fly *Bactrocera oleae* (17). AAB seem to play a role in the stimulation of the immune system and the protection of the host against pathogens (24). AAB of the genus *Asaia* (34) have recently been found associated with different insect species (4), like the malaria mosquito vectors *Anopheles stephensi* (*An. stephensi*) (11) and *Anopheles gambiae* (*An. gambiae*) (7) or the virus vector *Aedes aegypti* (*Ae. aegypti*) (4). *Asaia* has been found as an extracellular bacterium in the gut, the salivary glands, and reproductive organs (11), from which it is transmitted vertically to the progeny (11) by egg smearing (4) and venereally from males to females, and then to the offspring (6). *Asaia* is also transmitted horizontally, through the cofeeding of mosquitoes on the same food source (11).

The diverse mode of transmission of *Asaia* spp. among mosquitoes indicates that these insects are potentially exposed to diverse strains, suggesting that differently from symbionts transmitted only (or predominantly) vertically, several strains could coexist in different populations and even within single insect individuals. This aspect of insect symbiosis has rarely been investigated and would be of interest for a better under-

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TABLE 1. Screening for *Asaia* in mosquitoes: summary of the results from culture-dependent and -independent investigations

Mosquito strain/species	*Asaia* detected by DGGE	*Asaia* detected by specific PCR	No. of *Asaia* isolates
Anopheles gambiae strains			
Burkina	Yes	Yes	73
G3	Yes	Yes	No isolates
Anopheles stephensi	Yes	Yes	72
Aedes aegypti strains			
Milan	Yes	Yes	No isolates
Camerino	Yes	Yes	71
Aedes albopictus	Yes	Yes	65
Total			281

standing of several biological facets of the symbiosis, like the adaptation of host populations to their specific environment or the efficiency of cross-breeding between populations. We should also consider that *Asaia* has been proposed as a potential vector of antiplasmodial factors, through an approach known as paratransgenesis (12). Uncovering the level of diversity of *Asaia* in mosquitoes would be useful also for this potential application, to select the dominant genotype(s) for developing paratransgenetic approaches (2).

Here we present a picture of the diversity of *Asaia* in mosquitoes, obtained after an investigation on the distribution, isolation, and molecular typing of these symbionts in different populations of four mosquitoes species (*An. stephensi*, *An. gambiae*, *Ae. aegypti*, and *Aedes albopictus* [*Ae. albopictus*]), which are implicated in the transmission of infectious diseases to humans and animals.

MATERIALS AND METHODS

Mosquito strains. For the denaturing gradient gel electrophoresis (DGGE) analysis (see below), the study was carried out with 6 mosquito strains representing 4 species (Table 1). Two strains of *An. gambiae*, including the G3 strain maintained at the Department of Genetics, University of Stockholm, and a strain maintained at the Centre National de Recherche et de Formation sur le Paludisme, Burkina Faso, were used. The strain Liston of *An. stephensi* was obtained from a colony reared in the insectary of the Laboratory of Parasitology (University of Camerino, Italy) since 1988. One strain of *Ae. aegypti* was maintained at the Suisse Tropical Institute (Basel, Switzerland) and reared in the laboratory at the University of Camerino (Italy) since 2006. The other strain of *Ae. aegypti* came from the rearing facility at the Sezione di Parassitologia, DIPAV University of Milan, Italy. The strain of *Ae. albopictus* was a wild strain sampled in San Benedetto del Tronto (Italy) and reared in the insectary of the Laboratory of Parasitology (University of Camerino, Italy) since 2008. After DGGE, the following part of the study was carried out with strains listed above, except the *An. gambiae* G3 strain and the Milan *Ae. aegypti* strain.

***Asaia* isolation from mosquitoes and DNA extraction.** *Asaia* strains were isolated from adult mosquitoes using an enrichment medium, named enrichment medium I, as indicated by Lisdiyanti et al. (19). Adults were sterilized in 1% sodium hypochlorite for 2 min, washed three times with 0.9% NaCl, and homogenized by grinding in 200 μl 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 $CaCO_3$ 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) Bacto peptone, 0.5% (wt/vol) yeast extract, 0.7% (wt/vol) $CaCO_3$, and 1.5% (wt/vol) agar. After 3 days growth, circular nonpigmented colonies capable of causing clearing of the $CaCO_3$ were selected and streaked for further studies.

Chromosomal DNA was extracted from both *Asaia* strains and the mosquito microbiome using the cetyltrimethylammonium bromide (CTAB) method with a prior cell lysis by enzymatic methods and followed by an isopropanol precipitation of the DNA as described by Jara et al. (15).

PCR-DGGE and band sequence analysis. 16S rRNA gene amplification by PCR for DGGE analysis was carried out by using primers 357f (5′-CCTACGG GAGGCAGCAG) and 907r (5′-CCGTCAATTCCTTTRAGTTT) that target a portion of the 16S rRNA gene, including the hypervariable V3 regions, which have been shown to be useful for analyzing bacterial communities (3, 35). Primer 357f incorporated a GC clamp for the first amplification.

DGGE analysis was carried out on each PCR amplicon using a DCode universal mutation detection system (Bio-Rad, Milan, Italy), according to the procedure described previously (9). Electrophoresis was performed in a 0.5-mm polyacrylamide gel (7% [wt/vol] acrylamide-bisacrylamide, 37.5:1) containing a 35 to 60% urea-formamide denaturing gradient (100% corresponds to 7 M urea and 40% [vol/vol] formamide) according to the method of Muyzer et al. (22), increasing in the electrophoretic run direction. The gel was subjected to a constant voltage of 90 V for 15 h at 60°C in 1× Tris-acetate-EDTA (TAE) buffer (50× TAE stock solution consisting of 2 M Tris base, 1 M glacial acetic acid, 50 mM EDTA). After electrophoresis, the DGGE gels were stained in 1× TAE solution containing SYBR green (Molecular Probes, Leiden, Netherlands) for 45 min and photographed under a UV illumination using a GelDoc 2000 apparatus (Bio-Rad). For the sequencing of DGGE bands, bands of interest were excised from the gels with a sterile blade, mixed with 50 μl of sterile water, and incubated overnight at 4°C to allow the DNA of the bands to diffuse out of the polyacrylamide gel blocks. Two microliters of this aqueous solution was used to reamplify the PCR products with the same primers, excluding the GC clamp, according to Sanchez et al. (26).

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 (25) with 2,000 replicates as a bootstrap test. The phylogenetic analyses were conducted by using Mega 4 (28).

PCR typing of *Asaia* isolates. The 16S rRNA gene was amplified with universal bacterial 16S rRNA gene primers 27F (5′-TCGACATCGTTTACGGCGTG) and 1492R (5′-CTACGGCTACCTTGTTACGA). Internal transcribed spacer (ITS)-PCR was performed, with primer ITSF (5′-GCCAAGGCATCCAAC) and ITSR (5′-GTCGTAACAAGGTAGCCGTA), according to Daffonchio et al. (5). tRNA-PCR was performed, with tRNA primer T3A (5′-GGGGGTTCGAATT CCCGCCGGCCCCA) according to Borin et al. (1). BOX-PCR was performed, with BOX-A1 primer (5′-CTACGGCAAGGCGACGCTGAC), according to Urzì et al. (29). Randomly amplified polymorphic DNA (RAPD)-PCRs were performed, using RAPD OPA4 (5′-AATCGGGCTG), OPA7 (5′-GAAACG GGTC), and OPA10 (5′-GTGATCGCAG) primers according to Daffonchio et al. (5).

PCR fingerprintings were resolved by agarose gel electrophoresis under UV light. Band profiles were analyzed using the Quantity One software (version 4.6.5). MVSP software (version 3.13n) was used to estimate levels of similarity between strains based on the merged profiles obtained from ITS-PCR, tRNA-PCR, BOX-PCR, and RAPD-PCR and to generate unweighted pair group method with arithmetic average (UPGMA) trees.

Nucleotide accession number. The 16S rRNA gene sequences were deposited under accession numbers FN814275 to FN814281, FN814283 to FN814297, FN814299 to FN814301, and FN814303.

RESULTS

PCR-DGGE from the whole insect body. Bacterial PCR-DGGE fingerprints were obtained from the whole body of *Anopheles* and *Aedes* mosquitoes by the use of insects reared in laboratories but having different origins (Table 1). For each population, six individuals were tested independently. Whole-insect-body samples from the same origin had similar DGGE profiles (Fig. 1). All of the profiles from all of the individuals showed some common bands and some bands specific to certain populations. Other bands were found only in individuals having the same origin. Some other profiles were specific to a genus and absent from the other. Sequences of selected bands representative of most of the different migration classes in the
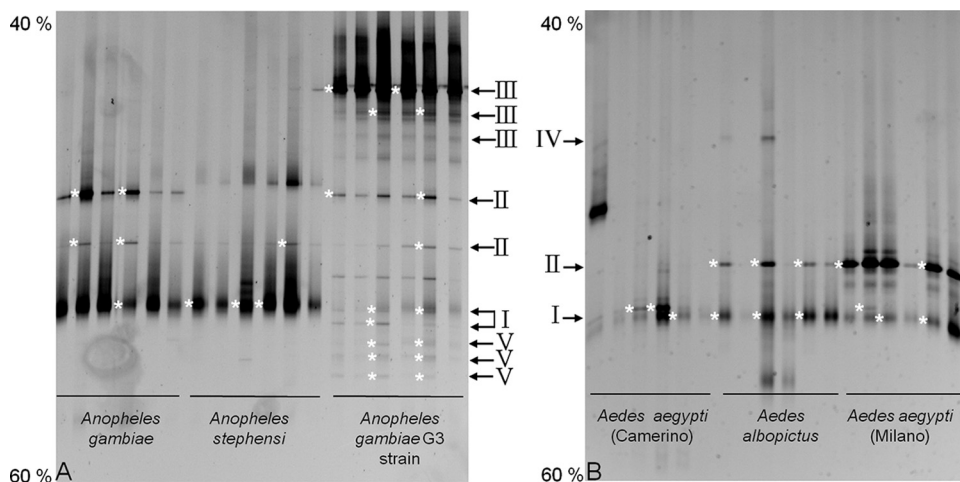
FIG. 1. DGGE profiles of 16S rRNA gene fragments amplified by PCR using general primers (357f and 907r) from whole-mosquito DNA templates. (A) DGGE profiles from mosquitoes of the genus *Anopheles*. Bands showing sequence identity with *Asaia*, *Burkholderia*, *Elizabethkingia*, and *Bacillus* spp. are indicated with I, II, III, and V, respectively. (B) DGGE profiles from mosquitoes of the genus *Aedes*. Bands with sequence identity with *Asaia* and *Burkholderia* spp. are indicated with I and II, respectively. IV indicates a band that was not identified, due to unreadable sequences. The numbers (40% and 60%) indicate the percentage of denaturing condition at the respective gel positions. *Anopheles gambiae* strain G3 indicates a laboratory strain reared in Stockholm, Sweden. *Aedes aegypti* (Camerino) and *Aedes aegypti* (Milano) indicate individuals reared in the laboratories in Camerino and Milan. Asterisks on the left of the bands indicate the bands that were cut and sequenced.

gels showed that a major band in the middle of the gel and common to all of the profiles matched with *Asaia* spp. We emphasize that banding patterns were well conserved within mosquito species, even between individuals of different sex, with the exception of a few minor bands (Fig. 1). Considering only the *Anopheles* genus (Fig. 1A), in addition to *Asaia* spp., another band matching *Burkholderia* sp. was found in all the samples. A second band identified as *Asaia* sp. was present in *An. gambiae*. The individuals from the G3 strain of *An. gambiae* also presented bands that were identified after sequencing as *Elizabethkingia* sp. In addition, further bands were identified as deriving from *Burkholderia* sp. It is worth noticing that sequences identified as belonging to the genus *Bacillus* were also found in the G3 strain (lowest faint bands). For the profiles of individuals of the *Aedes* genus (Fig. 1B), the already mentioned band common to all of the specimens was identified as deriving from *Asaia*. In these mosquitoes, however, this band was intense for some individuals but faint for some others. The bands in the middle of the gel were identified as belonging to *Burkholderia* sp. This bacterium was mostly associated with the strain of *Ae. aegypti* from Milan and with *Ae. albopictus*. Another band was observed in the upper part of the gel, but it produced unreadable sequences, possibly due to the presence of comigrating amplicons.

***Asaia* isolation and typing.** Two hundred eighty-one isolates of *Asaia* spp. were obtained from individuals belonging to all four mosquito species. Even if *Asaia* was detected in all of the strains by DGGE, it was not possible to get it in culture from the *Ae. aegypti* strain reared in Milan (Table 1). Along with *Asaia*, seven isolates identified as *Gluconacetobacter liquefaciens* were also obtained.

The diversity of the *Asaia* symbionts was assayed with different fingerprinting approaches: ITS-PCR, tRNA-PCR, BOX-PCR, and RAPD-PCR (5). The 281 *Asaia* isolates from the mosquitoes exhibited the same ITS-PCR and tRNA-PCR

fingerprinting profiles, presenting single bands of about 850 and 1,000 bp, respectively (data not shown). BOX-PCR and RAPD-PCR were able to distinguish several profiles among the strains examined. BOX-PCR discriminated four band pattern types, with more than 10 bands in the range between 200 and 3,000 bp. The RAPD-PCR using the primers OPA4, OPA7, and OPA10 unraveled, respectively, four, three, and five genotypes which were characterized by complex band patterns between 400 and 4,000 bp. The merging of the results obtained from all the six PCRs allowed us to distinguish 29 genotype patterns. The dominant profile (profile 1.1.1.1), represented by 65 strains (Table 2), was present in all of the mosquito species except *Ae. aegypti*. Some rarer profiles (profiles 1.2.2.4, 2.2.1.1, and 2.1.1.3) were present in only one species, while other patterns (profiles 1.1.2.2, 2.1.1.1, and 4.1.1.3) were present in more than one species. The analysis of these profiles showed that they were very similar, sharing more than 50% similarity (Fig. 2). When a threshold of 75% similarity is adopted, only eight groups can be distinguished, the first group (group I) is composed of eight profiles and includes 156 strains representing 55.51% of the isolates. Different *Asaia* types were found to be simultaneously associated with single mosquito individuals, indicating that multiple infections can occur during the mosquito life span. A similar situation has been recorded for *Wolbachia*, for which individual insect specimens have been found to harbor more than one strain of this bacterium (27, 30).

Phylogenetic analysis. Almost the entire 16S rRNA gene was sequenced for 26 *Asaia* isolates. The sequences obtained were compared to the databases using BLAST (Table 3) and used for phylogenetic analysis. Phylogenetic analysis included AAB sequences retrieved from the databases, which in most cases derive from strains not associated with insects (hereafter indicated as environmental strains). The phylogenetic tree that was generated groups AAB strains of environmental origin

TABLE 2. Number of strains of *Asaia* for each profile pattern and their distribution among the mosquito populations

Profile	No. of strains	Mosquito species harboring the *Asaia* profile[a]
1.1.1.1	65	*Ae. albopictus* (34), *An. gambiae* (22), *An. stephensi* (9)
1.1.1.3	29	*An. stephensi* (26), *Ae. aegypti* (3)
1.1.2.1	10	*Ae. albopictus* (10)
1.1.2.2	51	*Ae. aegypti* (48), *An. gambiae* (3)
1.2.1.1	9	*An. gambiae* (7), *Ae. albopictus* (2)
1.2.2.1	8	*An. gambiae* (6), *Ae. albopictus* (2)
1.2.2.2	5	*Ae. albopictus* (3), *An. gambiae* (1), *Ae. aegypti* (1)
1.2.2.3	4	*An. stephensi* (4)
1.2.2.4	11	*Ae. aegypti* (11)
1.3.1.1	2	*Ae. albopictus* (2)
1.3.1.2	1	*Ae. albopictus* (1)
1.3.1.5	1	*Ae. aegypti* (1)
1.4.1.1	2	*Ae. albopictus* (2)
2.1.1.1	9	*An. gambiae* (6), *Ae. albopictus* (3)
2.1.1.2	4	*An. gambiae* (3), *Ae. albopictus* (1)
2.1.1.3	8	*An. stephensi* (8)
2.1.2.1	6	*An. gambiae* (6)
2.1.2.2	4	*An. gambiae* (3), *Ae. aegypti* (1)
2.2.1.1	13	*An. gambiae* (13)
2.2.2.1	3	*An. stephensi* (3)
2.2.2.2	1	*An. gambiae* (1)
2.2.2.3	1	*Ae. aegypti* (1)
3.1.1.1	3	*An. stephensi* (3)
3.1.2.2	1	*Ae. aegypti* (1)
3.2.1.2	1	*Ae. albopictus* (1)
3.3.2.2	3	*An. gambiae* (3)
4.1.1.3	21	*An. stephensi* (19), *Ae. albopictus* (2)
4.2.1.2	2	*An. gambiae* (2)
4.3.3.2	3	*Ae. albopictus* (3)

[a] The numbers in parentheses indicate the number of strains obtained for each profile from the various mosquito species.

with the strains isolated from mosquitoes. Indeed, mosquito strains clustered into two groups within the *Asaia* genus, one clustering with *Asaia krungthepensis* and one clustering with *Asaia bogorensis*, together with isolates from environmental niches (Fig. 3), and this was not dependent on the origin of the strain. It is interesting to note that the clustering of *Asaia* strains from mosquitoes in the 16S rRNA-based phylogenetic tree is not congruent with the clustering in the UPGMA tree based on the PCR banding profiles (Fig. 2).

DISCUSSION

The analysis of the 16S rRNA gene sequences of the DGGE bands indicates that the microbial community associated with mosquitoes is characterized by a limited bacterial diversity. This observation is comparable to similar observations made for *Drosophila* by Ryu et al. (24), in which seven bacterial species were found to be associated with a laboratory strain. This is in agreement with experimental evidence showing that the transfer of wild insects to laboratory insectaries leads to a reduction of the diversity of the associated microflora, likely due to an adaptation to the rearing conditions (18).

Culture methods allowed us to identify another species from the AAB associated with mosquitoes (*G. liquefaciens*), which was not detected by DGGE. This could be due to the detection limits of the PCR used to amplify the total DNA extracted from the insect and to the enrichment steps that may have led to the selection of some species at the expense of others. The fact that *Asaia* was detected with both culture-independent and -dependent techniques in all of the mosquito species examined suggests that this bacterium is a truly constant component of

FIG. 2. Similarity tree between the various profile patterns of *Asaia* spp. isolated from the mosquitoes. The tree, generated with the UPGMA methods using the Jaccard coefficient, allowed us to cluster the different profiles in a tree, presenting a similarity higher than 50% among all of the patterns. We can distinguish 8 groups (from I to VIII) that share a similarity higher than 75%.

TABLE 3. Identification of strains based on the 16S rRNA gene sequences

Isolate	Closest relative[a]	% Similarity	Accession no. of the closest relative	Profile
AG 1.5 α	Asaia krungthepensis	100	AB292240	1.1.1.1
AG 2.6 β	Asaia sp. SF2.1*	100	AM404260	3.3.2.2
AG 2.7 β	Asaia sp. SF2.1*	100	AM404260	3.3.2.2
AG 3.5 α	Asaia krungthepensis	99	AB292240	2.2.1.1
AG 3.7 α	Asaia krungthepensis	100	AB292240	2.2.1.1
AG 4.5 α	Asaia krungthepensis	99	AB292240	1.2.1.1
AG 6.6 α	Asaia krungthepensis	100	AB292240	1.1.1.1
AG 7.6 α	Asaia krungthepensis	99	AB292240	1.2.1.1
AG 9.5 α	Asaia krungthepensis	99	AB292240	2.1.2.1
AG 10.7 α	Asaia krungthepensis	100	AB292240	4.2.1.1
AA 1.5 β	Asaia krungthepensis	100	AB292240	1.1.1.1
AA 1.6 β	Asaia krungthepensis	100	AB292240	1.1.1.1
AA 3.7 α	Asaia krungthepensis	100	AB292240	1.1.1.1
AA 4.6 β	Asaia krungthepensis	99	AB292240	1.4.1.1
AA 5.6 α	Asaia krungthepensis	100	AB292240	1.1.2.1
AA 6.7 α	Asaia krungthepensis	99	AB292240	1.1.2.1
AA 7.5 α	Asaia sp. SF2.1*	100	AM404260	4.4.3.2
AA 8.6 β	Asaia krungthepensis	100	AB292240	1.1.1.1
AA 8.7 β	Asaia krungthepensis	100	AB292240	1.1.1.1
AA 9.6 β	Asaia krungthepensis	100	AB292240	2.1.1.1
AS 1.7 α	Asaia sp. SF2.1*	100	AM404260	1.1.1.3
AS 2.6 α	Asaia sp. SF2.1*	100	AM404260	4.1.1.3
AS 3.6 α	Asaia sp. SF2.1*	100	AM404260	1.1.1.3
AE α 1.5 α	Asaia sp. SM2.5*	100	AM404261	1.1.2.2
AE α 1.7 α	Asaia sp. SM2.5*	99	AM404261	1.1.2.4
AE α 3.6 α	Asaia sp. SM2.5*	99	AM404261	1.1.2.2

[a] *, strains clustering along with Asaia bogorensis.

the microbial community associated with these insects. This also agrees with the experimental evidence that *Asaia* sp. is able to cross-colonize different species of mosquitoes (4).

Asaia was detected in the Milan *Ae. aegypti* population by culture-independent tools, but all attempts at isolation failed. This situation is similar to the case described by Crotti et al. (4) in which they were able to detect *Asaia* in the leafhopper *Scaphoideus titanus* but not to isolate it in culture. This is possibly due to the fact that some AAB can be found in a viable but not culturable state, as was described by Millet and Lonvaud-Funel (20). This state is characterized by the ability of the cells to perform most metabolic functions but not to grow.

The present study and other studies (4) demonstrate that *Asaia* is capable of colonizing rather diverse insect species. Here, different strains of *Asaia*, possibly representing different species, were shown to be able to colonize the same insect species. Such an occurrence is similar to what was observed with *D. melanogaster*, which can be colonized by two species of *Gluconacetobacter*, *Gluconacetobacter intermedius* and *Gluconacetobacter rhaeticus* (24). These diverse species could be selected by the capability to colonize slightly different microniches in the host gut.

Despite *Asaia* typing experiments indicating a rather high heterogeneity associated with mosquitoes, the 16S rRNA gene-based phylogenetic analysis indicated that all of the strains fall inside two of the validly described species, *A. bogorensis* and *A. krungthepensis*. Such an apparent incoherence can be explained by considering that the phylogenetic analysis of *Asaia* was based on a 16S rRNA gene which is a highly conserved gene (33), while the typing is based mostly on randomly distributed regions (14, 32) that may be subject to a higher rate of substi-

tution. However, typing results showed a rather high similarity of the profiles (over 50%).

It is interesting to note that a single mosquito individual can harbor up to five strains of *Asaia*. For example, we recorded individuals harboring strains with profiles 1.1.1.1, 1.2.1.1, 1.3.1.2, 2.1.1.1, and 4.4.3.2. It is generally thought that symbionts evolve toward reduced virulence and even to mutualistic interaction with the hosts where the symbiont transmission is mainly vertical and the symbiotic population is made up of closely related individuals, a condition that might promote virulence reduction through kin selection (10, 13). In the case of mosquitoes, *Asaia* does not apparently exert any pathogenic effect on the host, but rather, considering its widespread presence in mosquitoes, it could be beneficial indeed. Considering that *Asaia* can follow different ways for horizontal transmission (4, 6, 11), as indirectly evidenced by the results reported here showing the clustering of symbiotic and environmental isolates, vertical transmission does not appear to be the main mechanism for the maintenance of this bacterium in the insect hosts. Indeed, horizontal transmission tends to lead single hosts to be infected by multiple strains, a case reported here for several mosquito individuals. Despite the above evidence for frequent horizontal transmission and multiple infections of single hosts, *Asaia* is likely useful to the mosquito, otherwise it would be difficult to explain its 100% prevalence and its preservation in laboratory conditions. Current models to explain the evolution of virulence in host-symbiont interactions are thus probably not adequate to interpret the *Asaia*-mosquito symbiotic system. While primary symbionts in insects are generally vertically transmitted to the progeny, and likely form clones, it is perhaps more common for secondary symbionts to experience horizon-
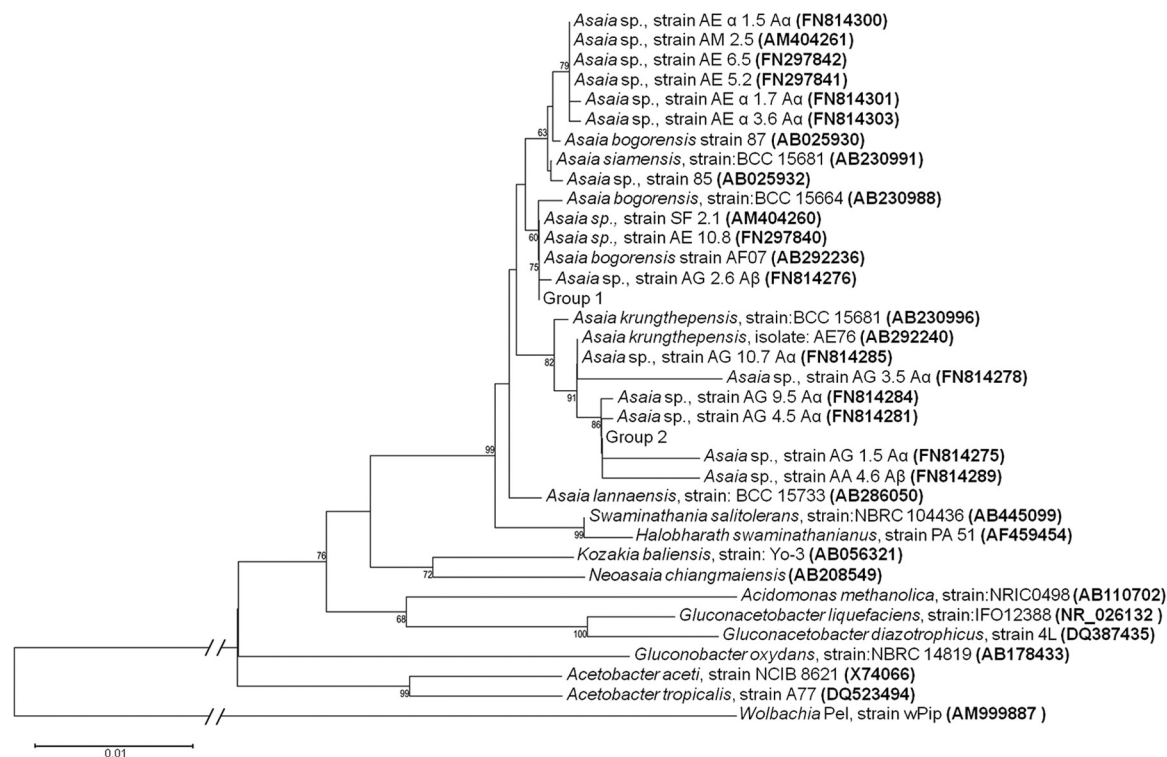
FIG. 3. Phylogenetic positions of the strains of *Asaia* indicated in Table 3, based on 16S rRNA gene sequences (neighbor-joining method; Kimura correction; all positions containing gaps and missing data were eliminated from the data set). Numbers at each node represent the bootstrap percentages of replications calculated from 2,000 replicated trees. The scale bar represents sequence divergence. Group 1 includes in the same cluster *Asaia* strains AG2.7 Aβ (FN814277), AA7.5 Aα (FN814292), AS1.7 Aα (FN814296), AS2.6 Aα (FN814297), and AS3.6Aα (FN814299). Group 2 includes in the same cluster *Asaia* strains: AG3.7 Aα (FN814279), AG6.6 Aα (FN814281), AG7.6 Aα (FN814283), AA1.5 Aβ (FN814286), AA1.6 Aβ (FN814287), AA3.7 Aα (FN814288), AA5.6 Aα (FN814290), AA 6.7 Aα (FN814291), AA 8.6Aβ (FN814293), AA8.7 Aβ (FN814294), and AA9.6 Aβ (FN814295).

tal transmission. We would thus suggest that investigations of the diversity of symbionts are extended to those secondary symbionts that are subjected to horizontal transmission.

REFERENCES

1. **Borin, S., D. Daffonchio, and C. Sorlini.** 1997. Single strand conformation polymorphism analysis of PCR-tDNA fingerprinting to address the identification of *Bacillus* species. FEMS Microbiol. Lett. **157:**87–93.
2. **Bourtzis, K.** 2008. *Wolbachia*-based technologies for insect pest population control. Adv. Exp. Med. Biol. **627:**104–113.
3. **Chakravorty, S., D. Helb, M. Burday, N. Connell, and D. Alland.** 2007. A detailed analysis of 16S ribosomal gene segments for the diagnosis of pathogenic bacteria. J. Microbiol. Methods **69:**330–339.
4. **Crotti, E., C. Damiani, M. Pajoro, E. Gonella, A. Rizzi, I. Ricci, I. Negri, P. Scuppa, P. Rossi, P. Ballarini, N. Raddadi, M. Marzorati, L. Sacchi, E. Clementi, M. Genchi, M. Mandrioli, C. Bandi, G. Favia, A. Alma, and D. Daffonchio.** 2009. *Asaia*, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders. Environ. Microbiol. **11:**3252–3264.
5. **Daffonchio, D., S. Borin, G. Frova, P. L. Manachini, and C. Sorlini.** 1998. PCR fingerprinting of the whole genome, the spacers between the 16S and 23S rRNA genes and of the intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. Int. J. Syst. Bacteriol. **48:**107–116.
6. **Damiani, C., I. Ricci, E. Crotti, P. Rossi, A. Rizzi, P. Scuppa, F. Esposito, C.** Bandi, D. Daffonchio, and G. Favia. 2008. Paternal transmission of symbiotic bacteria in malaria vectors. Curr. Biol. **18:**1087–1088.
7. **Damiani, C., I. Ricci, E. Crotti, P. Rossi, A. Rizzi, P. Scuppa, A. Capone, U. Ulissi, S. Epis, M. Genchi, N. Sagnon, I. Faye, A. Kang, B. Chouaia, C. Whitehorn, G. W. Moussa, M. Mandrioli, F. Esposito, L. Sacchi, C. Bandi, D. Daffonchio, and G. Favia.** 23 June 2010. Mosquito-bacteria symbiosis: the case of *Anopheles gambiae* and *Asaia*. Microb. Ecol. doi:10.1007/s00248-010-9704-8.
8. **de Bary, A.** 1879. The phenomenon of symbiosis. Karl J. Trubner, Strasbourg, Germany.
9. **De Vero, L., E. Gala, M. Gullo, L. Solieri, S. Landi, and P. Giudici.** 2006. Application of denaturing gradient gel electrophoresis (DGGE) analysis to evaluate acetic acid bacteria in traditional balsamic vinegar. Food Microbiol. **23:**809–813.
10. **Ebert, D., and E. A. Herre.** 1996. The evolution of parasitic diseases. Parasitol. Today **12:**96–101.
11. **Favia, G., I. Ricci, C. Damiani, N. Raddadi, E. Crotti, M. Marzorati, A. Rizzi, R. Urso, L. Brusetti, S. Borin, D. Mora, P. Scuppa, L. Pasqualini, E. Clementi, M. Genchi, S. Corona, I. Negri, G. Grandi, A. Alma, L. Kramer, F. Esposito, C. Bandi, L. Sacchi, and D. Daffonchio.** 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.
12. **Favia, G., I. Ricci, M. Marzorati, I. Negri, A. Alma, L. Sacchi, C. Bandi, and D. Daffonchio.** 2008. Bacteria of the genus *Asaia*: a potential paratransgenic weapon against malaria. Adv. Exp. Med. Biol. **627:**49–59.
13. **Griffin, A. S., S. A. West, and A. Buckling.** 2004. Cooperation and competition in pathogenic bacteria. Nature **430:**1024–1027.
14. **Hadrys, H., M. Balick, and B. Schierwater.** 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol. **1:**55–63.
15. **Jara, C., E. Mateo, J. M. Guillamón, M. J. Torija, and A. Mas.** 2008. Analysis of several methods for the extraction of high quality DNA from acetic acid bacteria in wine and vinegar for characterization by PCR-based methods. Int. J. Food Microbiol. **128:**336–341.
16. **Kersters, K., P. Lisdiyanti, K. Komagata, and J. Swings.** 2006. The family

Acetobacteraceae: the genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconaceto-bacter*, *Gluconobacter*, and *Kozakia*, p. 163–200. *In* M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (ed.), The prokaryotes, 3rd ed., vol. 5. Springer, New York, NY.

17. **Kounatidis, I., E. Crotti, P. Sapountzis, L. Sacchi, A. Rizzi, B. Chouaia, C. Bandi, A. Alma, D. Daffonchio, P. Mavragani-Tsipidou, and K. Bourtzis.** 2009. *Acetobacter tropicalis* is a major symbiont of the olive fruit fly (*Bactrocera oleae*). Appl. Environ. Microbiol. **75:**3281–3288.

18. **Lehman, R. M., J. G. Lundgren, and L. M. Petzke.** 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.

19. **Lisdiyanti, P., H. Kawasaki, T. Seki, Y. Yamada, T. Uchimura, and K. Komagata.** 2001. Identification of *Acetobacter* strains isolated from Indonesian sources, and proposals of *Acetobacter syzygii* sp. nov., *Acetobacter cibinongensis* sp. nov., and *Acetobacter orientalis* sp. nov. J. Gen. Appl. Microbiol. **47:**119–131.

20. **Millet, V., and A. Lonvaud-Funel.** 2000. The viable but non-culturable state of wine micro-organisms during storage. Lett. Appl. Microbiol. **30:**136–141.

21. **Moran, N. A.** 2006. Symbiosis. Curr. Biol. **24:**R866–R871.

22. **Muyzer, G., T. Brinkhoff, U. Nubel, C. Santegoeds, H. Schafer, and C. Wawer.** 1998. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, p. 3.4.4/1–3.4.4/27. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. Bruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, Netherlands.

23. **Roh, S. W., Y. D. Nam, H. W. Chang, K. H. Kim, M. S. Kim, J. H. Ryu, S. H. Kim, W. J. Lee, and J. W. Bae.** 2008. Phylogenetic characterization of two novel commensal bacteria involved with innate immune homeostasis in *Drosophila melanogaster*. Appl. Environ. Microbiol. **74:**6171–6177.

24. **Ryu, J. H., S. H. Kim, H. Y. Lee, J. Y. Bai, Y. D. Nam, J. W. Bae, D. G. Lee, S. C. Shin, E. M. Ha, and W. J. Lee.** 2008. Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophila*. Science **319:**777–782.

25. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.

26. **Sánchez, O., J. M. Gasol, R. Massana, J. Mas, and C. Pedros-Alio.** 2007. Comparison of different denaturing gradient gel electrophoresis primer sets for the study of marine bacterioplankton communities. Appl. Environ. Microbiol. **73:**5962–5967.

27. **Sinkins, S. P., T. Walker, A. R. Lynd, A. R. Steven, B. L. Makepeace, H. C. Godfray, and J. Parkhill.** 2005. *Wolbachia* variability and host effects on crossing type in *Culex* mosquitoes. Nature **436:**257–260.

28. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.

29. **Urzì, C., L. Brusetti, P. Salamone, C. Sorlini, E. Stackebrandt, and D. Daffonchio.** 2001. Biodiversity of Geodermatophilaceae isolated from altered stones and monuments in the Mediterranean basin. Environ. Microbiol. **3:**471–479.

30. **Werren, J. H., D. Windsor, and L. R. Guo.** 1995. Distribution of *Wolbachia* among neotropical arthropods. Proc. R. Soc. Lond. B **262:**197–204.

31. **Werren, J. H., L. Baldo, and M. E. Clark.** 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Microbiol. **6:**741–751.

32. **Williams, J. G., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18:**6531–6535.

33. **Woese, C. R., G. E. Fox, L. Zablen, T. Uchida, L. Bonen, K. Pechman, B. J. Lewis, and D. Stahl.** 1975. Conservation of primary structure in 16S ribosomal RNA. Nature **254:**83–86.

34. **Yamada, Y., K. Katsura, H. Kawasaki, Y. Widyastuti, S. Saono, T. Seki, T. Uchimura, and K. Komagata.** 2000. *Asaia bogorensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the alpha-Proteobacteria. Int. J. Syst. Evol. Microbiol. **50:**823–829.

35. **Yu, Z., and M. Morrison.** 2004. Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **70:**4800–4806.