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ORIGINAL PAPER

Molecular detection of six (endo-) symbiotic bacteria in Belgian mosquitoes: first step towards the selection of appropriate paratransgenesis candidates

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Abstract Actually, the use of symbiotic bacteria is one of alternative solution to avoid vector resistance to pesticides. In Belgium, among 31 identified mosquito species, 10 were considered as potential vectors. Given to introduction risks of arbovirosis, the purpose of this study was to investigate the presence of symbiosis bacteria in potential mosquito vectors. Eleven species caught from 12 sites in Belgium were used: *Culex pipiens* s.l., *Culex torrentium*, *Culex hortensis*, *Anopheles claviger*, *Anopheles maculipennis* s.l., *Anopheles plumbeus*, *Culiseta annulata*, *Ochlerotatus geniculatus*, *Ochlerotatus dorsalis*, *Aedes albopictus*, and *Coquillettidia richiardii*. Six genera of symbiotic bacteria were screened: *Wolbachia* sp., *Comamonas* sp, *Delftia* sp., *Pseudomonas* sp., *Acinetobacter* sp., and *Asaia* sp. A total of 173 mosquito individuals (144 larvae and 29 adults) were used for the polymerase chain reaction screening. *Wolbachia* was not found in any *Anopheles* species nor *Cx. torrentium*. A total absence of *Comamonas* and *Delftia* was observed in all species. *Acinetobacter*, *Pseudomonas*, and *Asaia* were found in most of species with a high prevalence for *Pseudomonas*. These results were discussed to develop potential strategy and exploit the variable occurrence of symbiotic bacteria to focus on them to propose biological ways of mosquito control.

Keywords Mosquitoes · Symbiotic bacteria · Vector-borne diseases · Belgium

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Introduction

The living world emerges from the interaction of different organisms that maintains between them complex and diverse interactions (Chow et al. 2010). Among them, symbiotic relationship was defined as the sustainable coexistence of two or more organisms (Dheilly 2014) that concerns a part or the whole life cycle of such organisms, regardless of the nature of trade arising between them (Brossard et al. 1991). Microorganisms have colonized and predominated throughout the world and demonstrated an extraordinary variety. In terms of adaptation, few microorganisms could even survive in extreme conditions, and settle and grow in hostile environments (Minard et al. 2013a). Then, this ability to live everywhere allowed them to establish special relationships with living hosts. Especially, the symbiotic relationships that different types of microorganisms have established with mosquitoes have probably played a key role in the evolutionary success of these insects. Symbiotic microorganisms influenced various biological functions and integrated those under the control of the host genome, providing to mosquitoes an improved adaptability to their environment (Ricci et al. 2012a). Currently, more than 3,500 species of Culicidae were described in the World, classified in two subfamilies and 112 genera. The subfamily Anophelinae has three genera while the Culicinae has 109 genera segregated into 11 tribes (Harbach 2013). Studying mosquito biology, ecology, and control is important because females of many species are

potential vectors of parasites leading to infectious diseases such as malaria, dengue fever, Chikungunya disease, West Nile fever, and dirofilariosis affecting animals including humans (Bawin et al. 2015; Ricci et al. 2012b). Mosquitoes are the indirect cause of more morbidity and mortality among humans than any other group of organisms. The extension of human activity, trade, and tourism contributed to the emergence of new strains of diseases in areas previously free of these diseases (Medlock et al. 2015). Indeed, the development of transport networks, the rapidity of travel, and the facilitation of human movement increased the risk of vector invasion (Manguin and Boëte 2011). The emergence or re-emergence of a pathogen could have a significant impact on human and animal health in naive environments and countries. Moreover, the role of climate change had a significant impact in the spread of invasive mosquito species (Slenning 2010). The loss of the biodiversity also increases the risk of human exposure to vector-borne diseases (Ostfeld 2009). The absence of a vaccine against most of arbovirosis implies efficient vector control strategies, which are up to date mainly based on the use of chemical insecticides. However, most vectors develop resistance against currently used products (Corbel and N'Guessan 2013) whereas new chemical compounds was limited firstly by financial costs in research and development, and secondly by the toxicity of derivatives to human population, biodiversity, and environment. The exploitation of new methods, such as the use of microbial communities as a bio-pesticide for vector control, will be therefore a promoted strategy at the global level (Bawin et al. 2015).

In Belgium, the introduction and spread of mosquito-borne viruses could be prevented yet. Although there was no case of autochthonous transmission of arbovirosis by mosquitoes in the last decades, the risk for the introduction of new pathogens is increasing. Belgium has multiple relationships with neighboring countries which have competent vectors and where autochthonous cases of arbovirosis were recently recorded (e.g., Chikungunya and dengue in France; Vega-Rua et al. 2013). Unfortunately, the existence of competent vectors in Belgium increases the risk of contingency. In parallel, the country is not protected with the increase of vector resistance due to the widespread use of insecticides. The aims of this study were (1) to check the presence of the six symbiotic bacteria in field mosquito species in Belgium and (2) to suggest new candidates for future paratransgenesis strategies.

Material and method

Sampling areas and mosquito collection

The mosquito individuals used for this study was originated from recent inventories in Belgium of (Boukraa et al. 2013), 2015b (submitted), Raharimalala et al. (2015; submitted), and also some specimens from the present study. In total, we examined mosquitoes from 13 sites in Belgium (Fig. 1) belonging to various environments including equestrian farm, cattle farm, company of import/recycling used tires, and quarry of recycling of materials from construction. The collection of mosquitoes was performed in 2011–2013 where both larval sampling (dipping) and adult trapping (CO2 baited trap Mosquito Magnet Liberty Plus) were used. To avoid breeding site influence on the screening, larvae were collected in various environments differing by their type (artificial/natural), kinds (buckets/water troughs/puddle/pond/used tires), state water (permanent/temporary and standing/running), and vegetation situation (presence/absence of floating and/or emergent vegetation). All specimens were stored either in 70–95 % ethanol (larval stages) or in a freezer at −20 °C (adult stages) until being used for polymerase chain reaction (PCR) analyses. Mosquito species identification was based on morphotaxonomy (larvae, adults, and genitalia analyses), by using identification keys (Becker et al. 2010; Schaffner 2001).

Nucleic acid extraction

DNeasy Blood & Tissue Kits (Qiagen) were used to extract genomic DNA following the instructions of the manufacturer. Briefly, all 173 samples were washed two times in sterile distilled water and one time in 70 % alcohol to avoid contamination. The samples were then individually crushed with plastic pestles in 1.5 ml tubes with 180 μl of buffer ATL, and 20 μl of proteinase K were added. Mixtures were vortexed thoroughly and incubated 3 h in a thermomixer (56 °C, 450 rpm). After processing and washing with adequate product in the kit, total DNA was eluted with 70 μl of buffer AE. The purity and quantity of DNA contained in each sample were assessed using a spectrophotometer (Nanodrop ®), and DNA concentration was adjusted to 30 ng μl^{-1} before being stored at −20 °C until used.

PCR amplification and sequencing data

A conventional PCR was used to screen the presence of six genera of (endo-) symbiotic bacteria (*Wolbachia* sp., *Comamonas* sp., *Delftia* sp., *Pseudomonas* sp., *Acinetobacter* sp., and *Asaia* sp.) among the 173 specimens belonging to 11 mosquito species from 13 sites in Belgium (Table 1). In a first time, universal eubacteria primers pA: 5′-AGAGTTTGATCCTGGCTCAG-3′ and pH: 5′-AAGGAGGTGATCCAGCCGCA-3′ that target for 16S rRNA gene sequences were used (Bruce et al. 1992) to check the presence of bacterial DNA in the samples. PCR conditions were set as an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s, extension at 72 °C for 1 min 15 s, and a final extension at 72 °C for 10 min. Then, each positive sample was checked with six specific primers that target (endo-) symbiotic bacteria (Table 2). For all process, PCR was performed using

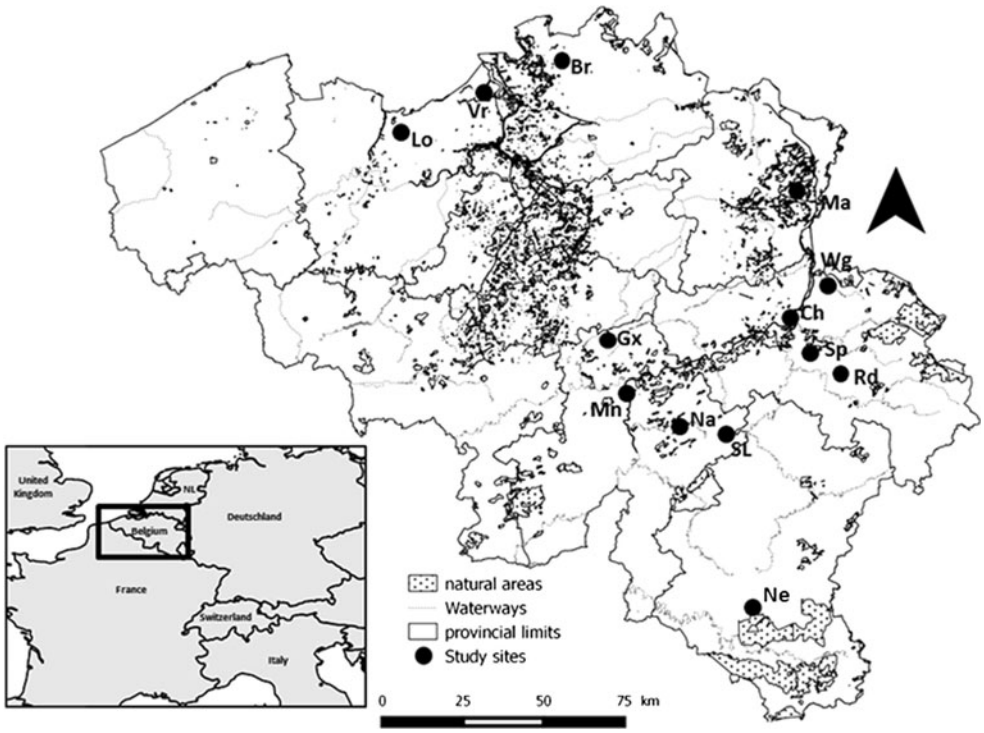

Fig. 1 Localization map of study sites used for mosquito collections: *Br* Brecht, *Ch* Chénée, *Gx* Gembloux, *Ma* Maasmechelen, *Mn* Malonne, *Na* Nattoye, *Ne* Neufchateau, *Lo* Lochristi, *Rd* La Reide, *SL* Somme-Louze, *Sp* Sprimont, *Vr* Vrasene, *Wg* Warsage

60 ng of genomic DNA per microliter in a final volume mix of 50 μl, with 5 μl buffer ×1, 1 μl dNTP 2 mM, 0.7 μl of each primer (forward/reverse) to 10 μM of final concentration, and 0.5 μl of 0.4 U Taq DNA polymerase (Eurogentec). Results were checked by electrophoresis on 2 % agarose gels (70 V, 45 min), stained with SYBR® Safe DNA gel stain

Table 1 Mosquito species screened and sampling sites

Species	Type of stations	Study sites	Breeding sites	Nb. Indiv. larvae	adults	References
An. claviger	EF	Gx	Water trough, backwater, and pond	6	0	[A]
An. maculipennis s. l.	EF	Gx, Ch, and Wg	Water trough, pond, and puddle	18	0	[A]
An . plumbeus	CIRUT	Vr	/	0	8	Boukraa et al. (2013)
Ae. albopictus	CIRUT	Vr	/	0	1	Boukraa et al. (2013)
Oc. dorsalis	CIRUT	Lo	/	0	1	Boukraa et al. (2013)
Oc. geniculatus	QRMC	Ma	/	0	1	[B]
	CIRUT	Vr	/	0	8	Boukraa et al. (2013)
Cq. richiardii	CIRUT	Vr	/	0	1	Boukraa et al. (2013)
Cs. annulata	EF	Sp, Ch, and Mn	Backwater, water trough, rut, and ditch	18	0	[A]
	CF	Br	Ditch	6	0	This study
Cx. pipiens s.l.	EF	Gx, Rd, Sp, Ch, Mn, and Wg	Tin, water trough, backwater, rut, and pond	36	0	[A]
	CF	Br, SL, and Ne	Ditch and used tire	12	0	This study
	CIRUT	Lo and Na	/	0	9	Boukraa et al. (2013)
Cx. torrentium	EF	Gx, Rd, Sp, Ch, Mn, and Wg	Water trough, rut, ditch, tin, used tire, and pond	36	0	[A]
	CF	SL	Pond	6	0	This study
Cx. hortensis	EF	Ch	Water trough and backwater	6	0	[A]

Br brecht, *Ch* Chénée, *Gx* Gembloux, *Ma* Maasmechelen, *Mn* Malonne, *Na* Nattoye, *Ne* Neufchateau, *Lo* Lochristi, *Rd* La Reide, *SL* Somme-Louze, *Sp* Sprimont, *Vr* Vrasene, *Wg* Warsage, *EF* equestrian farm, *CF* cattle farm, *CIRUT* company of import/recycling used tires, *QRMC* quarry of recycling of materials from construction, *[A]* Boukraa et al. 2015b (submitted), *[B]* Raharimalala et al. 2015 (submitted)

Table 2 Primers used in this study to target potential symbiotic bacteria in sampled field mosquitoes

Genera	Genes	Primers	Sequences (5′-3′)	Base number	Bp amplification size	Tm (°C)	References
Wolbachia	*srrs*	16S (V3) 99 F 994R	5′-TTGTAGCCTGCTATGGTATAACT-3′ 5′-GAATAGGTATGATTTTCATGT-3 ′	23 21	895	52	O'Neill et al. (1992)
Comamonas	rss	Com199F Com614R	5′-CCTTGTGCTACTAGAGC-3′ 5′-GCAGTCACAATGGCAGTT-3′	17 18	433	53	Zouache et al. (2009)
Delftia	rss	Delf63F Delf440R	5′-TAACAGGTCTTCGGACGC-3′ 5′-CCCCTGTATTAGAAGAAGCT-3′	18 20	397	56	Zouache et al. (2009)
Pseudomonas	rss	Ps For Ps Rev	5′-GGTCTGAGAGGATGATCAGT-3′ 5′-TTAGCTCCACCTCGCGGC-3′	20 18	990	52	Widmer et al. (1998)
Acinetobacter	rss	Acin1	5′-ACTTTAAGCGAGGAGGAGGCT-3′	21	426	58	Sanguin et al. (2006)
		Ac	5′-GCGCCACTAAAGCCTCAAAGGCC-3′	23			Kenzaka et al. (1998)
Asaia	rss	Asarev Asafor	5′-AGCGTCAGTAATGAGCCAGGTT-3′ 5′-GCGCGTAGGCGGTTTACAC-3′	22 19	180	60	Crotti et al. (2009)

Bp base pair, *Tm* melting time

(Invitrogen) and photographed with Gel Doc 2000 system (BioRad). A part of positive PCR products to the specific primers were sequenced (GATC Biotech AG, Germany), and sequences data were edited and aligned with BioEdit and Multialn softwares. All sequences were blasted in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Results were compared with data available in GenBank to confirm the identity of bacteria and deposited under accession numbers [GenBank: KJ512993–KJ512997].

Statistical analysis

Chi-square tests of independence were applied to compare the presence of symbiotic bacteria between mosquito species in larvae and adults. All tests were performed using Minitab® v.16 software (http://www.minitab.com/fr-FR/default.aspx).

Results

Eleven mosquito species (144 larvae and 29 adults) belonging to five genera were used for the screening (Table 1): *Culex pipiens* s.l. L, *Culex torrentium* (Martini, 1925), *Culex hortensis* (Ficalbi, 1889), *Anopheles claviger* (Meigen 1804), *Anopheles maculipennis* s.l. (Meigen, 1818), *Anopheles plumbeus* (Stephens 1828), *Culiseta annulata* (Schrank, 1776), *Ochlerotatus geniculatus* (Olivier, 1791), *Ochlerotatus dorsalis* (Meigen, 1830), *Aedes albopictus* (Skuse, 1894), and *Coquillettidia richiardii* (Dyar, 1905). Mosquito species were selected for the study because of their susceptibility as vectors.

The presence of symbiotic bacteria statistically differed between mosquito species in both larvae (Table 3) and adults (Table 4). Considering larvae, *Wolbachia* sp. was only found in *Cx. pipiens* s.l. (54.2 %) and *Cx. hortensis* (16.7 %; $\chi^2_{(5)}=60.3$; $P<0.001$; Table 3). *Pseudomonas* sp. was found in all mosquito species (Fig. 2), and dominated in *Cx. pipiens* s.l. (93.8 %) and *Cx. torrentium* (83.3 %), followed by *Cs. annulata* (75.0 %), *An. maculipennis* s.l. (72.2 %), *An. claviger* (50.0 %), and *Cx. hortensis* (33.3 %; $\chi^2_{(5)}=19.9$; $P=0.001$; Table 3). *Acinetobacter* sp. was also found in all tested species and dominated in *Cx. hortensis* (100.0 %), followed by *Cs. annulata* (54.2 %), *Cx. pipiens* s.l. (52.1 %), *An. maculipennis* s.l. (50.0 %), *Cx. torrentium*

Table 3 PCR results for the screening of the six symbiotic bacteria among six mosquito species (larvae) from Belgium

Genera	*Cx. pipiens* s.l. ($n=48$)	*Cx. torrentium* ($n=42$)	*Cx. hortensis* ($n=6$)	*An. claviger* ($n=6$)	*An. maculipennis s.l.* ($n=18$)	*Cs. annulata* ($n=24$)	Statistic ($\chi^2_{(5)}$)	*P* value
Wolbachia	26 (54.2 %) a	0 (0.0 %) b	1 (16.7 %) ac	0 (0.0 %) bc	0 (0.0 %) bc	0 (0.0 %) b	60.3	<0.001
Comamonas	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	–	–
Delftia	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	–	–
Pseudomonas	45 (93.8 %) a	36 (83.3 %) ab	2 (33.3 %) c	3 (50.0 %) c	13 (72.2 %) bc	18 (75.0 %) bc	19.9	0.001
Acinetobacter	25 (52.1 %) b	13 (36.1 %) c	6 (100.0 %) a	2 (33.3 %) c	9 (50.0 %) bc	13 (54.2 %) b	12.6	0.027
Asaia	0 (0.0 %) b	7 (19.4 %) a	0 (0.0 %) b	1 (16.7 %) a	6 (33.3 %) a	7 (29.2 %) a	18.6	0.002

Values that do not share a common letter are significantly different

Table 4 PCR results for the screening of the six symbiotic bacteria among six mosquito species (adults) from Belgium

Genera	*Cx. pipiens* s.l. ($n=9$)	*An. plumbeus* ($n=8$)	*Oc. geniculatus* ($n=9$)	*Oc. dorsalis* ($n=1$)	*Ae. albopictus* ($n=1$)	*Cq. richiardii* ($n=1$)	Statistic ($\chi^2_{(5)}$)	*P* value
Wolbachia	9 (100.0 %) a	0 (0.0 %) b	0 (0.0 %) b	1 (100.0 %) a	1 (100.0 %) a	1 (100.0 %) a	29.0	<0.001
Comamonas	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	–	–
Delftia	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	–	–
Pseudomonas	6 (66.7 %) a	6 (75.0 %) a	8 (88.9 %) a	1 (100.0 %) a	1 (100.0 %) a	1 (100.0 %) a	2.3	0.814
Acinetobacter	1 (11.1 %) b	0 (0.0 %) b	0 (0.0 %) b	0 (0.0 %) b	1 (100.0 %) a	0 (0.0 %) b	15.2	0.010
Asaia	1 (11.1 %) b	0 (0.0 %) b	0 (0.0 %) b	0 (0.0 %) b	1 (100.0 %) a	0 (0.0 %) b	15.2	0.010

Values that do not share a common letter are significantly different

(36.1 %), and *An. claviger* (33.3 %; $\chi^2_{(5)}=12.6$; $P=0.027$; Table 3). *Asaia* sp. was found in *An. maculipennis* s.l. (33.3 %), *Cs. annulata* (29.2 %), *Cx. torrentium* (19.4 %), and *An. claviger* (16.7 %) but not in *Cx. pipiens* s.l. (0.0 %) and *Cx. hortensis* (0.0 %; $\chi^2_{(5)}=18.6$; $P=0.002$; Table 3). No *Comamonas* sp. and *Delftia* sp. were found in larvae.

Considering adults, *Wolbachia* sp. was found in all *Cx. pipiens*, *Oc. dorsalis*, *Ae. albopictus*, and *Cq. richiardii* individuals but not in *An. plumbeus* and *Oc. geniculatus* ($\chi^2_{(5)}=29.0$; $P<0.001$; Table 4). *Pseudomonas* sp. was found in *Cq. richiardii* (100.0 %), *Ae. albopictus* (100.0 %), *Oc. dorsalis* (100.0 %), *Oc. geniculatus* (88.9 %), *An. plumbeus* (75.0 %), and *Cx. pipiens* s.l. (66.7 %) but no statistical differences between species was found ($\chi^2_{(5)}=2.3$; $P=0.814$; Table 4). *Acinetobacter* sp. was only found in *Ae. albopictus* (100.0 %) and *Cx. pipiens* (11.1 %; $\chi^2_{(5)}=15.2$; $P=0.010$; Table 4). *Asaia* sp. was only found in *Ae. albopictus* (100.0 %) and *Cx. pipiens* (11.1 %; $\chi^2_{(5)}=15.2$; $P=0.010$; Table 4). No *Comamonas* sp. and *Delftia* sp. were found in adults.

Concerning the distribution of bacteria in mosquito species, *Pseudomonas* was largely predominated in each species, in larvae and adults, and in different biotopes (Fig. 3a–d).

Discussion

In this study, we tested six symbiotic bacteria genera in eleven potential vector mosquito species in Belgium. The objective of this study was to find potential candidates for a symbiotic bacteria paratransgenic control. The study choice of these six symbiotic bacteria genera was due to the known of their ability to manipulate part or entire biology of their host. Our results have shown that four out of the six bacteria studied (*Wolbachia*, *Pseudomonas*, *Acinetobacter*, and *Asaia*) were found while *Comamonas* and *Delftia* were not.

Decision to study *Comamonas* genera was due to its possible involvement in decreasing pathogen transmission, mainly in *Anopheles* species (Minard et al. 2013a) while *Delftia* genera could principally act in delaying larval development,

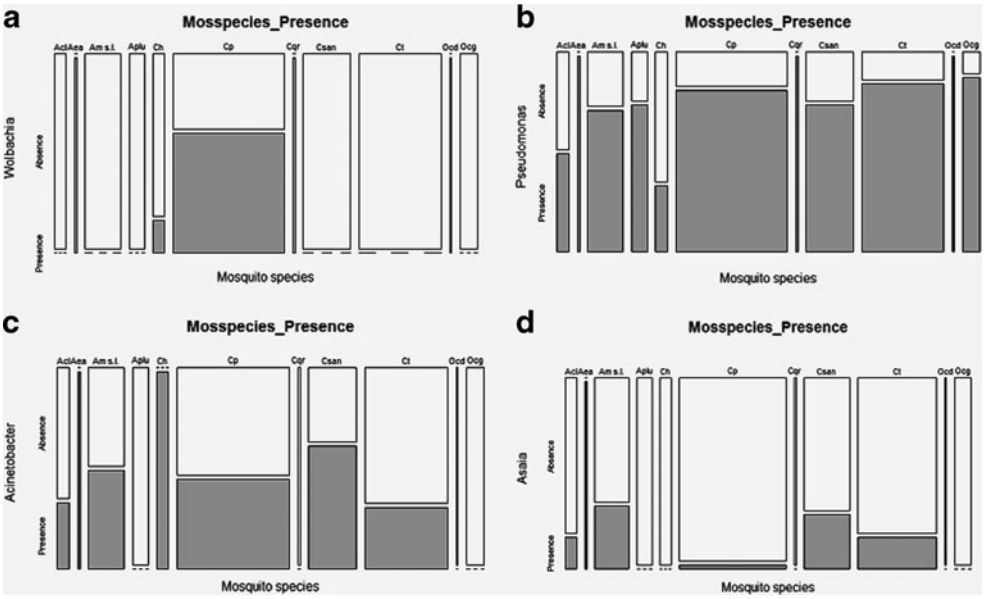

Fig. 2 Distribution of symbiotic bacteria in mosquito species: **a–d** *Wolbachia*, *Pseudomonas*, *Acinetobacter*, and *Asaia* distribution, respectively. *Acl Anophles claviger*, *Aea Aedes albopictus*, *Am* s.l. *Anopheles maculipennis* s.l., *Aplu Anopheles plumbeus*, *Ch Culex hortensis*, *Cp Culex pipiens* s.l., *Cqr Coquillettidia richiardii*, *Csan Culiseta annulata*, *Ct Culex torrentium*, *Ocd Ochlerotatus dorsalis*, *Ocg Ochlerotatus geniculatus*, *Mossp* mosquito species

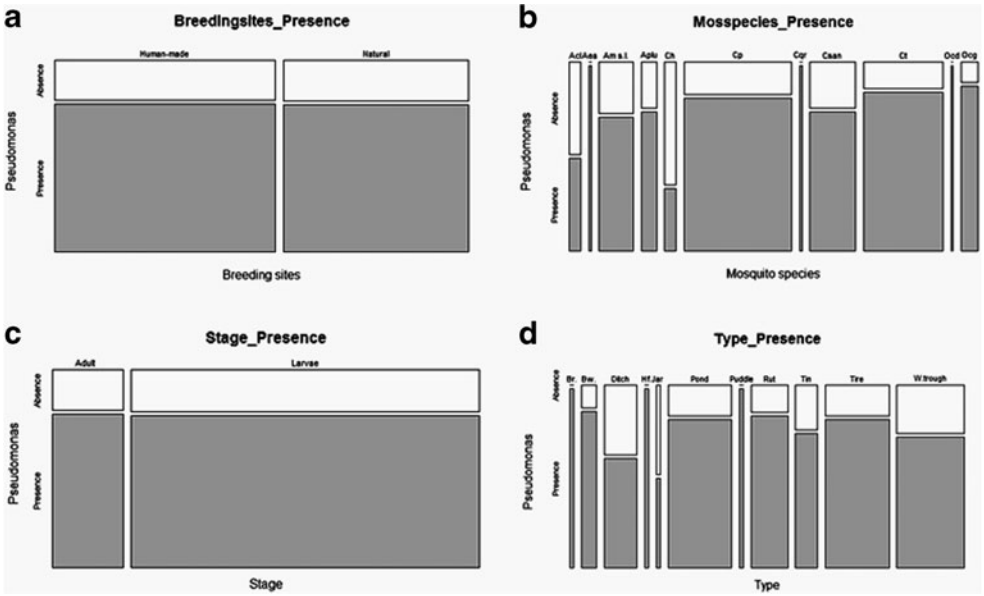

Fig. 3 Pseudomonas repartition in different biotopes: **a–d** Breeding sites, mosquito species, stade of mosquito, and type of sites, respectively. *Acl Anophles claviger*, *Aea Aedes albopictus*, *Am* s.l. *Anopheles maculipennis* s.l., *Aplu Anopheles plumbeus*, *Ch Culex hortensis*, *Cp Culex pipiens* s.l., *Cqr Coquillettidia richiardii*, *Csan Culiseta annulata*, *Ct Culex torrentium*, *Ocd Ochlerotatus dorsalis*, *Ocg Ochlerotatus geniculatus*, *Mossp* mosquito species, *Br* bovine farm, *Bw* backwater, *Hf* horse feetprint, *W.trough* water trough

also mainly in *Anopheles* species (Chavshin et al. 2014). These two genera were often detected in some *Anopheles* species, *Cx. pipiens*, *Aedes* (Lindh 2007), and recently, Zouache et al. detected them in *Aedes albopictus* of Madagascar (Zouache et al. 2011). But surprisingly, we did not find them including the three *Anopheles* species and in *Cx. pipiens* s.l. studied here. Comparing into the previous study, these bacteria were found in every tropical countries (Chavshin et al. 2014; Lindh 2007; Minard et al. 2013a) but never detected in temperate country. We supposed that the main cause could be that the environment is too hostile for these bacteria and limited their growth in Belgium. For that, we could not suggest their uses as paratrangenesis tool in Belgium.

Wolbachia genera constituted one of the symbiotic bacteria that we have used in this study. These endosymbiotic bacteria are well studied and reduce the life span of the mosquito species (Almeida et al. 2011; Atyame et al. 2014) or interfere with pathogen replication and dissemination in mosquito species (Bian et al. 2013; Caragata et al. 2014). In this research, *Wolbachia* is in low proportion in the studied mosquitoes. We supposed that this lowest proportion has the same reason of the case of *Delftia* and *Comamonas*, as the environment is not favorable for them. Despite that these bacteria were already used elsewhere in the fight against *Culex* and *Aedes* (van den Hurk et al. 2012; Zélé et al. 2014), we do not advocate its use in Belgium.

Pseudomonas was largely predominated in all stages and mosquito species studied. This high prevalence could suggest that *Pseudomonas* would be a best candidate to paratransgenesis because his grow in cell-free and ordinary culture media is facilitate and suitability for genetic transformation (Chavshin et al. 2014). In the literature, three key components were required for used bacteria as paratrangenesis: an existing of effector molecule that could achieves the desired effect, a mechanism to display or excrete the effector molecule on the surface of the bacteria, and bacteria that can survive in the mosquito long enough to produce the expected amount of effector molecules and thereby achieve the desired effect in the mosquito (Wilke and Marrelli 2015). These three parameters seem to be complete in *Pseudomonas*. Anterior study attested the use of chitinolytic activity of extra cellular mosquitocidal proteins of *P. fluorescens* (Brammacharry and Paily 2012). Moreover, the high prevalence of *Pseudomonas* may indicate existence of intimate symbiotic relationship between a given bacterium and its host (Minard et al. 2013b). This relationship would be exploited to use these bacteria as symbiotic control by exploiting its influence in mosquito fitness with introduction of a modified gene in the bacteria. Secondly, the fact that *Pseudomonas* could be found in both larvae and adults may suggest a transstadiale transmission. Or, another way to perform paratrangenesis approach was to target bacteria that are well established in mosquitoes and transmitted to the next generation (Wilke and Marrelli 2015). The studies conducted by Chavshin et al. (2012, 2014, 2015) confirmed this transstadial passage based of phenotypic marker as used for *Asaia* bacterium, such as green fluorescent protein (GFP; Favia et al. 2007). Normally, most bacteria are lost during the metamorphosis from larval to adult stage and only a few bacteria are transmitted from the larva to the adult (Moll et al. 2001). Indeed, during emergence and transformation of adult mosquitoes, a sterilization mechanism is set up to sequester bacteria in the larval gut. Just a few bacteria could cross the barrier of the meconial peritrophic membranes and be transmitted to adult stages (Chavshin et al. 2012). Most of transstadial bacteria (as the case of *Pseudomonas*) are Gram negative. These bacteria are generally transformable for useful in paratransgenic method (Wirth

et al. 1989) and could affect the parasite load in the infected mosquitoes. They could dramatically reduce either *Plasmodium falciparum* Welch, 1897 or *Plasmodium vivax* Grassi and Feletti, 1890, parasite prevalence and density within vectors (Chavshin et al. 2012).

In the other part of our results, *Acinetobacter* sp. represented a non-negligible value in all larval species studied, likewise the case of *Asaia* sp. with the lowest prevalence. The same results were observed by Minard et al. (2013b) and Zouache et al. (2011) during a prevalence study of symbiotic bacterial in *Ae. albopictus* and *Ae. aegypti* L. of Madagascar. Considering their high prevalence, candidature of these two symbiotic bacteria to paratransgenetic bacteria could also pay attention. Firstly, high prevalence of *Acinetobacter* could indicate and suggest that these bacteria could play a role in the biology of the mosquito in the field (Minard et al. 2013b); even this role in insect biology has been only confirmed in *Stomoxys calcitrans* L. where it ensured complete development of the fly larvae (Lysyk et al. 1999). Anterior study conducted by Mweresa et al. (2015) demonstrated that *Acinetobacter baumannii* played a role in mosquito attractiveness during host biting. Its presence is also detected to have increased considerably after feeding on blood meals than feeding on sugar of the adult female mosquitoes (Zayed and Bream 2004). But most of the species of *Acinetobacter* are associated with bacteraemia, pulmonary infections, meningitis, diarrhea, and notorious nosocomial infections and constituted a veritable danger for human and are responsible of rapid development of resistance to a wide range of antimicrobials (Doughari et al. 2011). Secondly, candidature of *Asaia* for use in mosquito paratransgenesis may pay attention also. These bacteria have a peculiar relationship with mosquitoes because of its localization in the gut, in the salivary glands, and in the reproductive organs of both sexes (Favia et al. 2007). Anterior study (Ricci et al. 2012b) found *Asaia* in all the developmental stages of all tested malaria vectors and in *Aedes aegypti* with high prevalence. It was demonstrated that like *Pseudomonas*, this bacteria is easily cultivable outside in cell-free media that facilitates his transformation.

Moreover, this bacteria could use multiple ways of transmission within and between population hosts like vertically transmission (by progeny by maternal or paternal), horizontal routes by mating and co-feeding (Ricci et al. 2012b). However, even the three symbiotic bacteria were promising as a good candidate; some characteristics such as transstadial transmission, non-pathogenicity, immunological and physiological adaptation to mosquito midgut conditions, colonization in the mosquito midgut including effective competition with resident bacteria, and persistency in the gut for a reasonable time (Chavshin et al. 2014) should be well examined. In fact, anterior studies reported that members of *Acinetobacter* and *Asaia* may cause opportunistic infections in humans and their presence could provoke cross-infection when mosquitoes bite hosts (Alauzet et al. 2010; Doughari et al. 2011; Minard et al. 2013b). These reason motivated us to suggest *Pseudomonas* as the best candidate latest.

Conclusion

This study permitted to demonstrate the prevalence of four symbiotic bacteria, among 6, in 11 mosquito species in Belgium. It also allowed the highlighting of the possible role of three bacteria genera for paratransgenesis in Belgium. Due to its omnipresence, *Pseudomonas* seemed to be the most interesting candidate for paratransgenic bacteria in order to reduce and/or inhibit the transmission of pathogens.

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Authors' contributions FNR conceived the study. FNR, SBoukraa, TB contributed to material collection, data analysis, interpretation and manuscript writing. FNR and SBoukraa conducted PCR studies and PCR analysis. SBoyer and FF contributed to data interpretation and manuscript writing. All authors read and approved the final version of the manuscript.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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