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RECEIVED 24 November 2023

ACCEPTED 11 March 2024

PUBLISHED 27 March 2024

CITATION

Gomard Y, Hafsia S, Lebon C, Rabarison P,
Idaroussi A-b, Yssouf A, Boussès P, Mavingui P
and Atyame C (2024) Genetic diversity of
endosymbiotic bacteria *Wolbachia* infecting
two mosquito species of the genus
Eretmapodites occurring in sympatry in the
Comoros archipelago.
Front. Microbiol. 15:1343917.
doi: 10.3389/fmicb.2024.1343917

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Genetic diversity of endosymbiotic bacteria *Wolbachia* infecting two mosquito species of the genus *Eretmapodites* occurring in sympatry in the Comoros archipelago

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Introduction: The influence of *Wolbachia* on mosquito reproduction and vector competence has led to renewed interest in studying the genetic diversity of these bacteria and the phenotypes they induced in mosquito vectors. In this study, we focused on two species of *Eretmapodites*, namely *Eretmapodites quinquevittatus* and *Eretmapodites subsimplicipes*, from three islands in the Comoros archipelago (in the Southwestern Indian Ocean).

Methods: Using the *COI* gene, we examined the mitochondrial genetic diversity of 879 *Eretmapodites* individuals from 54 sites. Additionally, we investigated the presence and genetic diversity of *Wolbachia* using the *wsp* marker and the diversity of five housekeeping genes commonly used for genotyping through Multiple Locus Sequence Typing (MLST).

Results and discussion: Overall, *Er. quinquevittatus* was the most abundant species in the three surveyed islands and both mosquito species occurred in sympatry in most of the investigated sites. We detected a higher mitochondrial genetic diversity in *Er. quinquevittatus* with 35 reported haplotypes ($N = 615$ specimens, $Hd = 0.481$ and $\pi = 0.002$) while 13 haplotypes were found in *Er. subsimplicipes* ($N = 205$ specimens, $Hd = 0.338$ and $\pi = 0.001$), this difference is likely due to the bias in sampling size between the two species. We report for the first time the presence of *Wolbachia* in these two *Eretmapodites* species. The prevalence of *Wolbachia* infection varied significantly between species, with a low prevalence recorded in *Er. quinquevittatus* (0.8%, $N = 5/627$) while infection was close to fixation in *Er. subsimplicipes* (87.7%, $N = 221/252$). Both male and female individuals of the two mosquito species appeared to be infected. The analysis of MLST genes revealed the presence of two *Wolbachia* strains corresponding to two new strain types (STs) within the supergroups A and B, which have been named wEretA and wEretB. These strains were found as mono-infections and are closely related, phylogenetically, to *Wolbachia* strains previously reported in *Drosophila* species.

Finally, we demonstrate that maternal transmission of *Wolbachia* is imperfect in *Er. subsimplicipes*, which could explain the presence of a minority of uninfected individuals in the field.

KEYWORDS

Wolbachia, *Eretmapodites quinquevittatus*, *Eretmapodites subsimplicipes*, mitochondrial genetic diversity, Comoros archipelago

Introduction

Endosymbiotic bacteria are of increasing interest due to their impact on the biology of arthropods. Some of these bacteria are known to be essential for the evolution of their hosts enabling them to adapt to new ecological niches (Douglas, 1998). Other bacteria provide selective advantages depending on the ecological contexts by providing for example protection against predators (Tsuchida et al., 2010) or pathogens (Oliver et al., 2003; Scarborough et al., 2005; Hedges et al., 2008; Teixeira et al., 2008; Jaenike et al., 2010). In addition to these positive effects, endosymbiotic bacteria are also selfish elements that can manipulate the reproduction of their hosts to increase their own fitness (Duron et al., 2008). This is the case for the bacteria *Wolbachia* which are associated with various reproductive manipulation phenotypes in arthropods (Werren et al., 2008).

Wolbachia are maternally inherited alpha-proteobacteria commonly found in arthropods and filarial nematodes (Werren et al., 2008). These bacteria are estimated to be present in up to 66% of insect species (Hilgenboecker et al., 2008; Zug and Hammerstein, 2012; Weinert et al., 2015), thus probably representing the most abundant endosymbiont described to date. *Wolbachia* exhibit high genetic diversity and have been classified into 17 phylogenetic groups or supergroups (A to Q) (Baldo et al., 2006; Paraskevopoulos et al., 2006; Bordenstein et al., 2009; Ros et al., 2009; Glowska et al., 2015). The widespread distribution of *Wolbachia* is primarily attributed to their impact on the reproductive biology of their hosts. In arthropods, *Wolbachia* manipulate host reproduction by biasing the sex ratio toward females (the transmitting sex), or by causing sterility through a phenomenon known as cytoplasmic incompatibility (CI) (Werren et al., 2008). Cytoplasmic incompatibility results from sperm-egg incompatibility occurring when *Wolbachia*-infected males mate with either uninfected females or females infected with an incompatible *Wolbachia* strain, resulting in high embryonic mortality reaching up to 100% in certain mosquito species (Laven, 1951; Werren et al., 2008; Atyame et al., 2014). This phenotype is commonly observed in arthropods (Shropshire et al., 2020; Turelli et al., 2022) including in mosquito vectors (Sicard et al., 2019).

Aside from the manipulation of reproduction, *Wolbachia* can also impact the vector competence of mosquitoes, which refers to their ability to become infected with and transmit a pathogen. *Wolbachia* have shown to provide protection against major mosquito-borne pathogens like Dengue virus (DENV), Chikungunya virus (CHIKV) or *Plasmodium* infections (Moreira et al., 2009; Bian et al., 2010, 2013; Hoffmann et al., 2011; Walker et al., 2011; Aliota et al., 2016; Dutra et al., 2016).

However, *Wolbachia* can also be linked to increased pathogen transmission in some cases (Hughes et al., 2012; Dodson et al., 2014; Zélé et al., 2014). Because of *Wolbachia*'s influence on both mosquito reproduction and vector competence, these bacteria are increasingly seen as promising tools for mosquito and mosquito-borne diseases control (Bourtzis et al., 2014). In recent years, there has a growing number of studies focusing on the genetic diversity of *Wolbachia* and their associated phenotypes in mosquito vectors (Sicard et al., 2019). *Wolbachia* have been well studied in various medically importance mosquito species of such as *Culex pipiens pipiens* and *Culex pipiens quinquefasciatus* (Duron et al., 2005; Atyame et al., 2011, 2014; Dumas et al., 2013), *Aedes albopictus* (Kambhampati et al., 1993; Armbruster et al., 2003; Tortosa et al., 2010; Zouache et al., 2011), as well as more recently in *Anopheles* species (Baldini et al., 2014; Ayala et al., 2019) and *Aedes aegypti* (Coon et al., 2016; Thongsripong et al., 2018). However, there have been limited studies on the presence of *Wolbachia* in mosquitoes of the *Eretmapodites* genus (Tokash-Peters et al., 2022; Osuna et al., 2023), despite their role in the arbovirus transmission (arthropod-borne viruses) (Bamou et al., 2021; Cêtre-Sossah et al., 2023).

Mosquitoes from the *Eretmapodites* genus (Theobald, 1901) (subfamily: Culicinae; tribe: Aedini) are exclusively Afrotropical species occurring in continental Africa (Harbach, 2007), Madagascar (Tantely et al., 2016), and in the islands of the Comoros archipelago (composed of four volcanic islands: Grande Comore, Mohéli, Anjouan and Mayotte) within the Southwestern Indian Ocean (Le Goff et al., 2014; Boussès et al., 2018). A total of 51 *Eretmapodites* species have been described so far (<https://mosquito-taxonomic-inventory>, accessed in November 2023) (Harbach, 2013), most (32 species) from Cameroon (Bamou et al., 2021), while only four species are known in Madagascar (Tantely et al., 2016), and two species are reported in the Comoros archipelago (Le Goff et al., 2014; Boussès et al., 2018). *Eretmapodites* species are mostly found in forested areas but some species are also adapted to rural and peri-urban environments (Le Goff et al., 2014; Boussès et al., 2018; Bamou et al., 2021). Along with their aggressive daytime biting behavior, these mosquitoes are known to bite both animals and humans (Musa et al., 2020); as a result they have the potential to serve as bridge vectors of pathogens between animals and humans. Different arboviruses such as Rift Valley fever virus (RVFV), Semliki Forest virus (SFV), or CHIKV have been detected and/or isolated from *Eretmapodites* mosquitoes [review in Bamou et al. (2021)]. In addition, some studies have described the ability of *Eretmapodites* mosquitoes to transmit arboviruses under laboratory conditions. For example, Bauer (1928) showed that *Eretmapodites chrysogaster* is able to transmit the Yellow Fever virus (YFV). The study of McIntosh

and coworkers (McIntosh et al., 1980) demonstrated the ability of *Eretmapodites quinquevittatus* to transmit the RVFV. Likewise, it has been recently shown that *Eretmapodites subsimplicipes* is a competent vector for the transmission of RVFV (Cêtre-Sossah et al., 2023). Despite the medical interest of *Eretmapodites* species, the biology, ecology and genetics of these mosquitoes remain poorly investigated.

In this study we focused on two *Eretmapodites* species, namely *Er. quinquevittatus* and *Er. subsimplicipes*, from three islands in the Comoros archipelago: Grande Comore, Mohéli and Mayotte. We used molecular identification to determine the abundance of each mosquito species on the surveyed islands. Then, we examined the presence of *Wolbachia* in both *Eretmapodites* species through the presence/absence of the *Wolbachia* surface protein gene *wsp* (Braig et al., 1998). The genetic diversity of the detected *Wolbachia* was further characterized by sequencing the *wsp* gene and the five housekeeping genes developed for the *Wolbachia* typing (MLST) (Baldo et al., 2006). Finally, we examined the vertical transmission of *Wolbachia* in a laboratory colony of *Er. subsimplicipes*. The role of *Wolbachia* in the evolution of *Eretmapodites* species is discussed.

Materials and methods

Mosquito sampling

Adult *Eretmapodites* specimens were collected in 2019 (March to May and November to December) from 54 natural breeding sites on three islands of the Comoros archipelago (in the Southwestern Indian Ocean): Grande Comore (18 sites), Mohéli (eight sites), and Mayotte (28 sites) (Figure 1; Supplementary Table 1). Larvae were also collected in the site Bambo Est on Mayotte in March 2019 and March 2022 to establish a laboratory colony for testing vertical transmission of *Wolbachia* (see below). Adult mosquitoes were captured using portable electric aspirators (BioQuip InsectaVac aspirator, Bioquip, CA). Collected adults were introduced in cages (16 × 16 × 16 cm) and brought to the laboratory where they were sorted by sex and individually stored in 1.5 ml tubes at −80°C (for samples from Mayotte) or in 70% ethanol (for samples from Grande Comore and Mohéli) until morphological identification and molecular analyses.

Mosquito identification

The larvae and adults of *Eretmapodites* from the Bambo Est site (Mayotte) and collected in March 2019 and March 2022 were identified morphologically using taxonomic keys (Edwards, 1941; Hopkins, 1952; Service, 1990). The larvae of *Er. quinquevittatus* have a characteristic thick, heavily chitinized 3-VIII seta and, in adults, the scutum is decorated with five parallel bands of dark scales on a yellow-brown background. The larvae of *Er. subsimplicipes* are easily distinguished from those of *Er. quinquevittatus* by lateral setae of abdominal segments I–VI inserted on a sclerotized conical tubercle and, in adults, a more homogeneous scutum devoid of such dark bands. Molecular identification of species was realized on larvae and all collected adult specimens through PCR amplification and sequencing of

a 658 bp fragment of the mitochondrial cytochrome c oxidase subunit 1 encoding gene (*COI*) (Folmer et al., 1994) (primers listed in Supplementary Table 2). DNA extraction, PCR, and sequencing were performed as described below.

Wolbachia genotyping

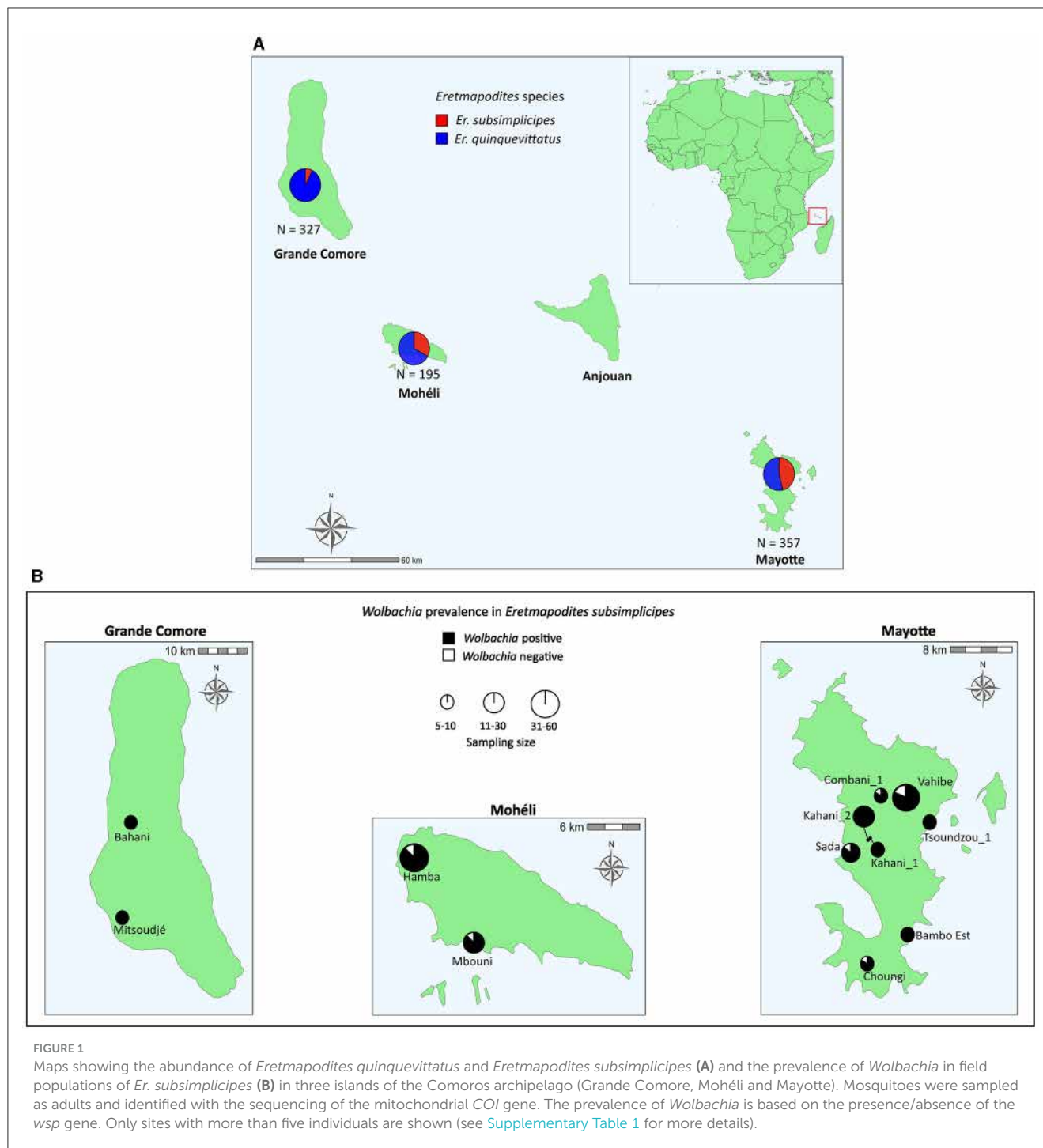
Wolbachia detection was performed in all sampled adults using PCR targeting the surface protein gene *wsp* (Braig et al., 1998) (Supplementary Table 2) which is more variable than the slowly evolving 16S rRNA gene (Zhou et al., 1998). For *wsp*-positive samples, *Wolbachia* were genotyped by sequencing the *wsp* gene and the five MLST loci: *coxA*, *fbpA*, *ftsZ*, *gatB* and *hcpA* (Baldo et al., 2006) (Supplementary Table 2). For all individuals in which *Wolbachia* DNA could not be amplified, the quality of the DNA template was checked by the amplification of the *COI* gene.

PCR amplification and sequencing

DNA was extracted from individual mosquitoes using the QIAcube HT robotic workstation and the associated Cador Pathogen 96 QIAcube HT Kit (Qiagen) following manufacturer's recommendations, eluted with 100 µl AVE buffer (Qiagen) and eventually stored at −20°C until molecular investigations. PCRs were performed with 0.5 ng of genomic DNA in a 25 µl final volume reaction containing 8.5 µl of water, 12.5 µl of GoTaq® G2 HotStart Green Master Mix (Promega), and 1 µl of each primer (10 µM) (Supplementary Table 2). All PCR programs included an initial denaturation step at 95°C for 5 min, followed by 36 cycles (30 cycles for the *COI* gene) at 94°C for 30 s, 52°C–59°C for 60 s and 72°C for 90 s, and a final elongation step at 72°C for 7 min. Amplified DNA fragments were ran on 1.5% agarose gel electrophoresis stained with 1X GelRed™ (Biotium Inc.) and visualized under ultraviolet light. PCR products were Sanger sequenced on both strands (Genoscreen, Lille, France). Only unique generated sequences were submitted to GenBank under the following accession numbers: OR282837–OR282884, OR296528–OR296530, OR296531–OR296533, OR296534–OR296536, OR296537–OR296539, OR296540–OR296543, and OR296544–OR296547 for *COI*, *coxA*, *fbpA*, *ftsZ*, *gatB*, *hcpA*, and *wsp*, respectively.

Sequences

All sequences were visually inspected and manually edited using Geneious Prime v.2022.2.2 (Kearse et al., 2012). For the *COI* gene, comparisons with public sequences were performed using basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST, accessed on 28 July 2023) from GenBank. The mitochondrial haplotype diversity (*Hd*) and nucleotide diversity (π) were calculated in the software DnaSP v6.12.03 (Rozas et al., 2017). For *Wolbachia* genes, the generated



sequences were compared with data available in the *Wolbachia* MLST database (<https://pubmlst.org/organisms/wolbachia-spp>, accessed on 03 August 2023) (Jolley et al., 2018). For each MLST gene, a new allele was considered if there was at least one nucleotide difference with alleles already present in the pubMLST database. Thereafter, the combination of alleles allowed identifying the Sequence Types (STs) among those existing or to propose new STs.

Phylogenetic analysis

Phylogenetic relationships were evaluated for *Eretmapodites COI* and *Wolbachia* genes. Only unique mitochondrial haplotypes and bacterial alleles were included in the analyses. For *Wolbachia*, phylogenetic analyses were conducted for each of the six sequenced genes and on all five MLST concatenated genes (*coxA*, *fbpA*, *ftsZ*, *gatB*, and *hcpA*, in this order). The phylogenies were constructed

with data from Baldo et al. (2006). For each data set, the best-fitting model of sequence evolution was determined using jModelTest v.2.1.4 (Darriba et al., 2012). Then, phylogenetic constructions were performed using MrBayes v.3.2.3 (Ronquist et al., 2012). For each phylogeny, the analysis corresponded to two independent runs of four incrementally heated Metropolis Coupled Markov Chain Monte Carlo (MCMCMC) starting from a random tree. The MCMCMC was run for 10 million generations with trees and associated model parameters sampled every 100 generations. The convergence level was assessed with an average standard deviation of split frequencies inferior to 0.05. The 10% initial trees for each run were discarded as burn-in and the phylogeny along with posterior probabilities were obtained from the remaining trees. The resulting Bayesian phylogeny trees were visualized and annotated with FigTree v.1.4.2 (Rambaut, 2014).

Vertical transmission of *Wolbachia*

Since only the *Er. subsimplicipes* species could be reared from larvae collected in the Bambo Est site (Mayotte) in March 2019, we used it to examine the vertical transmission of *Wolbachia*. Field larvae (F_0 generation) were brought to the laboratory and kept alive in the insectary where they were identified morphologically and maintained under standard rearing conditions (27°C and 80% relative humidity with a 12h:12h photoperiod). Larvae were supplied every 2 days with yeast tablets and adults were fed with 10% sucrose solution. To get eggs and ensure the maintenance of mosquitoes, females were blood-fed using a Hemotek feeding system (Hemotek Limited, GreatHarwood, UK) with defibrinated cow blood. The eggs of the next generation (F_1 generation) were collected and reared to adulthood. The amplification process was performed over four generations (F_4 generation) to increase the number of females for the experiment. Then, the vertical transmission of *Wolbachia* was assessed using females and males from the established laboratory colony. Females of the F_4 generation were allowed to mate in the laboratory with males from the same colony. After mating, the females were blood-fed and individually isolated to lay eggs. Then, the presence of *Wolbachia* was tested for each female by PCR using the *wsp* gene as described above. The offsprings from each *Wolbachia*-infected female were kept alive until adulthood and males and females were screened for the presence of *Wolbachia*.

Results

Eretmapodites quinquevittatus is more abundant than *Eretmapodites subsimplicipes*

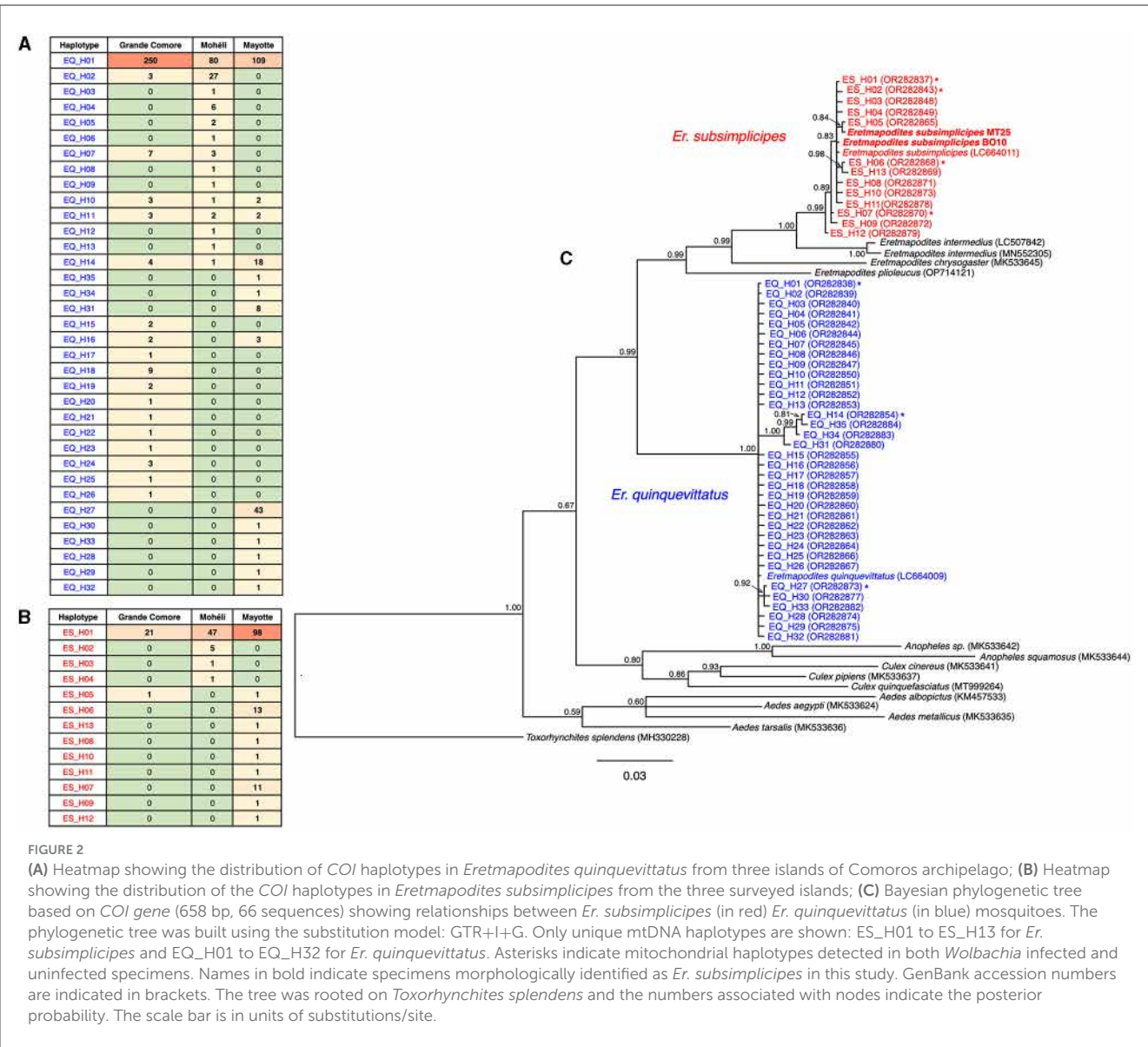
Larvae and adults collected in the site Bambo Est (Mayotte) in March 2019 and March 2022 were morphologically identified as *Er. subsimplicipes*. COI sequencing of these morphologically identified specimens showed a closed match with the published sequence of *Er. subsimplicipes* from Mozambique (GenBank accession number: LC664011, 99.8%–100.0% percentage of identity based on 633 bp), thus confirming the identification of our specimens. We

then sequenced a total of 879 mosquitoes (655 females and 224 males) from Grande Comore ($N = 327$), Mohéli ($N = 195$), and Mayotte ($N = 357$). The comparison of the obtained COI sequences with the GenBank database indicated the presence of two *Eretmapodites* species: *Er. quinquevittatus* (GenBank accession number: LC664009, 98.4%–100.0% percentage of identity based on 629 bp) and *Er. subsimplicipes* (GenBank accession number: LC664011, 99.7%–100.0% percentage of identity based on 633 bp). Among the sequences, 71.3% ($N = 627/879$) and 28.7% ($N = 252/879$) belonged to *Er. quinquevittatus* and *Er. subsimplicipes*, respectively (Supplementary Tables 1, 3). *Eretmapodites quinquevittatus* appeared more common than *Er. subsimplicipes* in all three investigated islands (Figure 1A). Both *Eretmapodites* species were found in sympatry in 31 out of 54 sampled sites (seven sites in Grande Comore, seven sites in Mohéli and 17 sites in Mayotte), while *Er. quinquevittatus* was found alone in 22 sites (11 sites in Grande Comore, one site in Mohéli and ten sites in Mayotte) and *Er. subsimplicipes* alone at one site (Bambo Est) on Mayotte (Supplementary Table 1).

Higher mtDNA polymorphism in *Er. quinquevittatus*

Among the 627 *Er. quinquevittatus* specimens, COI sequences with good qualities (i.e., 658 bp with no ambiguities) were obtained for 615 samples leading to 35 haplotypes (Figure 2A; Supplementary Table 4). Pairwise nucleotide identity between the haplotypes ranged from 98.2% to 99.9%. The overall haplotype diversity (Hd) and nucleotide diversity (π) values were 0.481 and 0.002, respectively. The most frequent haplotype [EQ_H01, found in 71.4% of sequences ($N = 439/615$)] was also the most widespread in all three islands (Figure 2A; Supplementary Table 4). The second most frequent haplotype (EQ_H27, scored in 43 specimens) was geographically restricted to Mayotte (Supplementary Table 4). Of the 35 haplotypes, four haplotypes were shared by all three islands (EQ_H01, EQ_H10, EQ_H11, and EQ_H14), two haplotypes (EQ_H02 and EQ_H07) were shared by Grande Comore and Mohéli, one haplotype (EQ_H16) was common to Grande Comore and Mayotte, and no common haplotype was detected between Mohéli and Mayotte (Supplementary Table 4). On Grande Comore, 18 haplotypes were found whereas the number of haplotypes was similar between Mohéli and Mayotte (14 haplotypes on each island). A total of 11, eight and nine haplotypes were unique on Grande Comore, Mohéli and Mayotte, respectively (Figure 2A; Supplementary Table 4).

COI good quality sequences were obtained for 205 of the 252 *Er. subsimplicipes* samples leading to 13 haplotypes (Figure 2B; Supplementary Table 5). Pairwise nucleotide identity between the haplotypes yielded values ranging from 99.4 to 99.9%. The overall Hd and π values were 0.338 and 0.001, respectively. The haplotype ES_H01 was the most frequently observed in the dataset, with 80.9% of specimens ($N = 166/205$) and the only one common to all three islands (Figure 2B; Supplementary Table 5). The number of haplotypes was higher in Mayotte (ten haplotypes), followed by Mohéli (four haplotypes),



while the lowest diversity was observed in Grande Comore (two haplotypes). Unique haplotypes were only found in Mayotte (eight haplotypes) and Mohéli (three haplotypes) (Figure 2B; Supplementary Table 5).

We assessed phylogenetic relationships between the two *Eretmapodites* species by incorporating the COI haplotypes identified in the present study and those of other mosquito species retrieved from GenBank including sequences of *Eretmapodites* mosquitoes: *Er. quinquevittatus* (GenBank: LC664009), *Er. subsimplicipes* (GenBank: LC664011), *Eretmapodites intermedius* (GenBank: LC507842 and MN552305), *Eretmapodites chrysogaster* (GenBank: MK533645), and *Eretmapodites plioleucus* (GenBank: OP714121). The phylogenetic tree revealed that *Er. quinquevittatus* and *Er. subsimplicipes* formed two well-supported clades (Figure 2C). Although higher haplotype diversity was found in *Er. quinquevittatus*, the genetic cluster formed by *Er. subsimplicipes* appears slightly more diverged than that of *Er. quinquevittatus*.

Lower prevalence of *Wolbachia* in *Er. quinquevittatus* than in *Er. subsimplicipes*

The 879 *Eretmapodites* mosquitoes were screened for *Wolbachia* infection based on the detection of the *wsp* gene by PCR. The overall prevalence of *Wolbachia* was 25.7% ($N = 226/879$), with a significant lower prevalence detected in *Er. quinquevittatus* (0.8%, $N = 5/627$) as compared to *Er. subsimplicipes* (87.7%, $N = 221/252$) (Table 1) (Fisher's exact test, $P < 0.001$). *Wolbachia* infections were detected in both males and females of both mosquito species (Table 1). In *Er. quinquevittatus*, two out of the five *Wolbachia* infected mosquitoes were females and three were males. In contrast, the majority of the *Wolbachia* infected *Er. subsimplicipes* mosquitoes were females, with 201 females and 3 males out of a total of 221 infected mosquitoes. For *Er. quinquevittatus*, the bacterial infection prevalence between sites ranged from 0.0% to 7.7% with the five *Wolbachia*-infected specimens detected from five

TABLE 1 Prevalence of *Wolbachia* in *Eretmapodites quinquevittatus* and *Eretmapodites subsimplicipes* in three islands of the Comoros archipelago, based on presence/absence of the *wsp* gene and according to the sex of mosquitoes.

Island	Sex	N	<i>Eretmapodites quinquevittatus</i>	<i>Eretmapodites subsimplicipes</i>
			Prevalence of <i>Wolbachia</i> in %	Prevalence of <i>Wolbachia</i> in %
Grande Comore	All	327	0.7 (2/303)	97.1 (22/24)
	Females	209	0.5 (1/188)	90.5 (19/21)
	Males	118	0.9 (1/115)	100.0 (3/3)
Mohéli	All	195	0.8 (1/131)	85.9 (55/64)
	Females	126	0.0 (0/62)	85.9 (55/64)
	Males	69	1.4 (1/69)	-
Mayotte	All	357	1.0 (2/193)	87.8 (144/164)
	Females	320	0.6 (1/156)	87.8 (144/164)
	Males	37	2.7 (1/37)	-
Total	All	879	0.8 (5/627)	87.7 (221/252)
	Females	655	0.5 (2/406)	87.6 (218/249)
	Males	224	1.4 (3/221)	100.0 (3/3)

N = total number of mosquitoes examined.

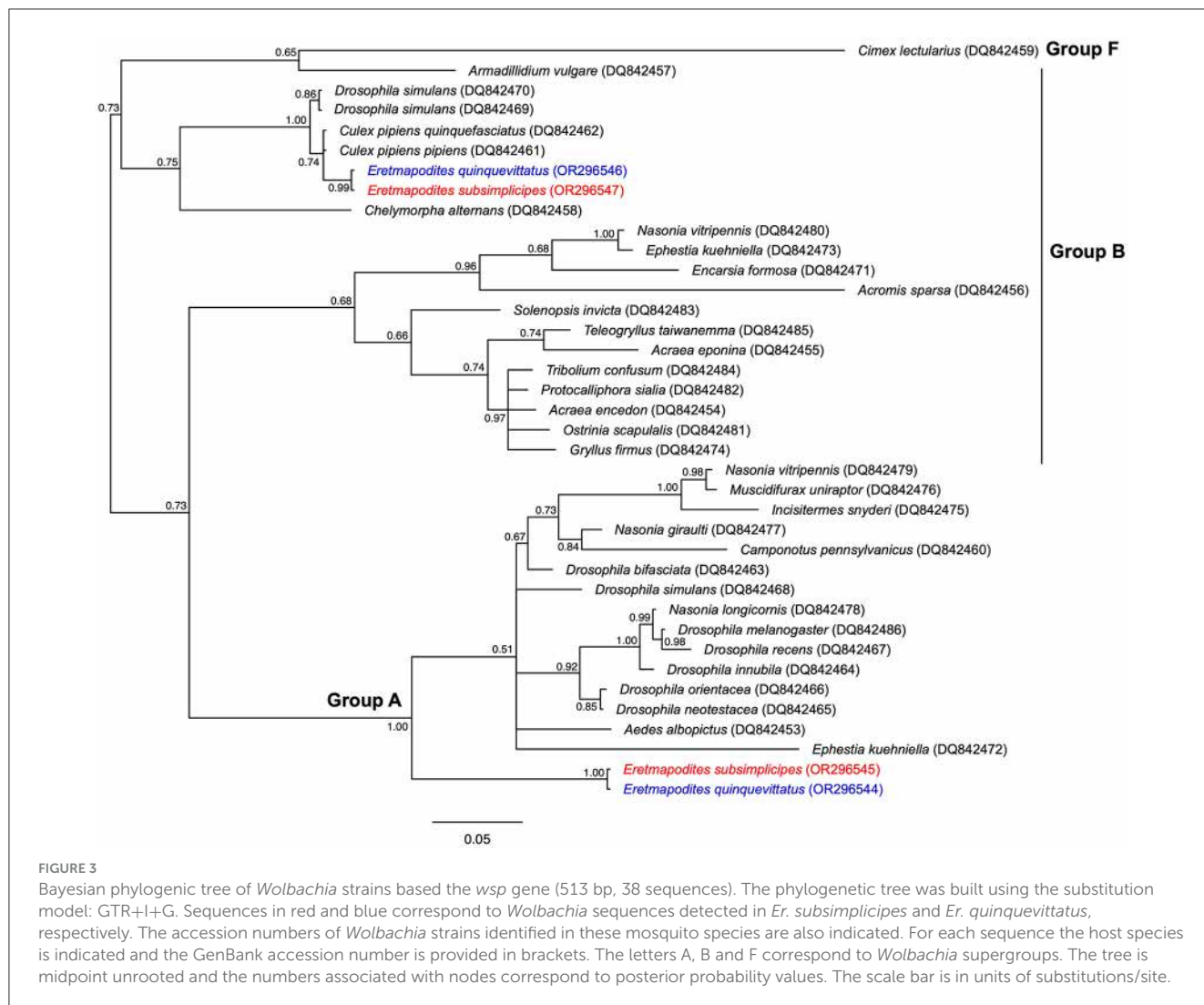
sites: two sites in Grande Comore, one site in Mohéli and two sites in Mayotte (Supplementary Table 1). For *Er. subsimplicipes*, *Wolbachia*-positive specimens were detected in all but one site (the Iconi site on Grande Comore, N = 32 sites with *Er. subsimplicipes* specimens) and infection prevalence ranged from 50.0% to 100.0% including in sites with a large number of samples (Figure 1B; Supplementary Table 1). For both mosquito species, *Wolbachia* infection prevalence did not significantly vary according to the sampled islands (Fisher’s exact tests, all $P > 0.7$) (Supplementary Table 1).

Two *Wolbachia* A and B supergroups occurred in *Er. quinquevittatus* and *Er. subsimplicipes*

The sequencing of the *wsp* gene in *Er. quinquevittatus* and *Er. subsimplicipes* revealed the presence of two *Wolbachia* supergroups A and B in each *Eretmapodites* species (Figure 3). For *Er. quinquevittatus*, three samples out of the five *Wolbachia*-infected were successfully sequenced and one sample belonged to supergroup A while two samples belonged to supergroup B (Figure 3; Supplementary Table 3). Concerning *Er. subsimplicipes*, the sequencing of the *wsp* gene was succesful for 218 out of the 221 samples. Almost all of these samples (N = 217/218) belonged to supergroup A and one sample to supergroup B. When comparing the 218 *wsp* sequences of supergroup A (217 sequences for *Er. subsimplicipes* and one sequence for *Er. quinquevittatus*), no polymorphism was noted, a unique *wsp* allele shared by the two mosquito species was observed (Figure 3; Supplementary Table 3). The analysis of the three *wsp* sequences from supergroup B (two sequences for *Er. quinquevittatus* and one sequence for *Er.*

subsimplicipes) also revealed one *wsp* allele shared by both *Eretmapodites* species.

As the *wsp* gene alone is not relevant for a reliable genotyping of *Wolbachia* strains due to recombination in *Wolbachia* genomes (Jiggins et al., 2001; Bordenstein and Wernegreen, 2004; Baldo et al., 2006; Atyame et al., 2011), we sequenced the five *Wolbachia* MLST genes *coxA*, *fbpA*, *ftsZ*, *gatB* and *hcpA*. The sequences of the five MLST genes were not obtained systematically for each of the 226 *Wolbachia*-infected *Eretmapodites* mosquitoes. Indeed, PCR amplifications have failed for some genes (particularly *fbpA*) in certain samples, possibly due to mutations in the targeted primers sites. Additionally, since we used universal degenerated primers (Baldo et al., 2006), it may have been possible to improve our protocols to increase amplification success for *Eretmapodites*. Ultimately, we obtained 214 sequences for *coxA*, 114 sequences for *fbpA*, 210 sequences for *gatB*, 177 sequences for *hcpA* and 214 sequences for *ftsZ*. We confirmed the presence of *Wolbachia* strains belonging to supergroups A and B with each of the five MLST genes (Supplementary Figures 1–5; Supplementary Table 3). We found two alleles for four of the five genes (*coxA*, *fbpA*, *ftsZ* and *gatB*), one allele belonging to supergroup A and the other one to supergroup B (Supplementary Figures 1–4; Supplementary Table 3). The most polymorphic locus was *hcpA* with three alleles, two alleles for supergroup A and one allele for supergroup B (Supplementary Figure 5; Supplementary Table 3). The two *hcpA* alleles falling in the supergroup A were genetically close, with 99.8% pairwise identity based on 476 bp. Our data do not support co-infection by *Wolbachia* strains from supergroups A and B. None of the five MLST genes could be amplified in the single *Er. quinquevittatus* sample infected with a *Wolbachia* strain from supergroup A. Therefore, using the MLST genes, we detected supergroup A only in *Er. subsimplicipes* and supergroup B in both *Er. subsimplicipes* and *Er. quinquevittatus* (Supplementary Figures 1–5; Supplementary Table 3). As observed



with the *wsp* gene, all MLST alleles were shared by the two mosquito species within supergroup B (Supplementary Figures 1–5).

Comparison of allelic polymorphism with pubMLST database revealed that within the supergroup A, alleles identified in the present study for *coxA*, *fbpA*, *ftsZ*, and *hcpA* are new with the exception of the *gatB* allele matching with allele #49 (Supplementary Table 6). The *coxA* allele showed a close match with allele #173, the *fbpA* allele with allele #60, the *ftsZ* allele with allele #52, and the two *hcpA* alleles were genetically closely related to allele #11 (Supplementary Table 6). The combination of the five alleles resulted in a new *Wolbachia* strain type, which we named “wEretA.” For supergroup B, all observed alleles for the five MLST genes are already present in the pubMLST database. Indeed, *coxA*, *fbpA*, *ftsZ*, *gatB*, and *hcpA* alleles matched with alleles #281, #453, #244, #283, and #309, respectively (Supplementary Table 6). However, no *Wolbachia* strain type was assigned to the combination of these five alleles in the pubMLST database. Hence, we considered this *Wolbachia* strain type as new and named it “wEretB.” The MLST allelic profiles of wEretA and wEretB appeared genetically different from those of a *Wolbachia* strain previously described in the species *Eretmapodites*

chrysogaster from Cameroon for which *coxA* matched with #275, *ftsZ* matched with #106, and *fbpA* matched with #6 (Osuna et al., 2023). Using complete MLST profiles obtained for 84 mosquitoes (83 *Er. subsimplicipes* and one *Er. quinquevittatus*), we performed a phylogenetic analysis based on the 2,079 bp concatenated sequences of the five MLST genes. It appears that *Wolbachia* strains wEretA (infecting *Er. subsimplicipes*) and wEretB (infecting both *Er. subsimplicipes* and *Er. quinquevittatus*) form two robust monophyletic clades within A and B supergroups, respectively (Figure 4). wEretA is genetically closely related to wDori and wDneo infecting *Drosophila orientacea* and *Drosophila neotestacea*, respectively (Figure 4). wEretB is closely related to wMa infecting *Drosophila simulans* (Figure 4). In summary, MLST data revealed that (i) wEretA is restricted to *Er. subsimplicipes* (83 complete MLST allelic profiles) and (ii) wEretB infects both *Er. subsimplicipes* and *Er. quinquevittatus* (1 complete MLST allelic profile each) (Figure 4; Supplementary Table 3). Finally, we examined the evolution of the diversity of *Wolbachia* in their hosts by comparing the concatenated MLST phylogeny and the COI phylogeny from different host species including in *Er. subsimplicipes* and *Er. quinquevittatus*. No congruence

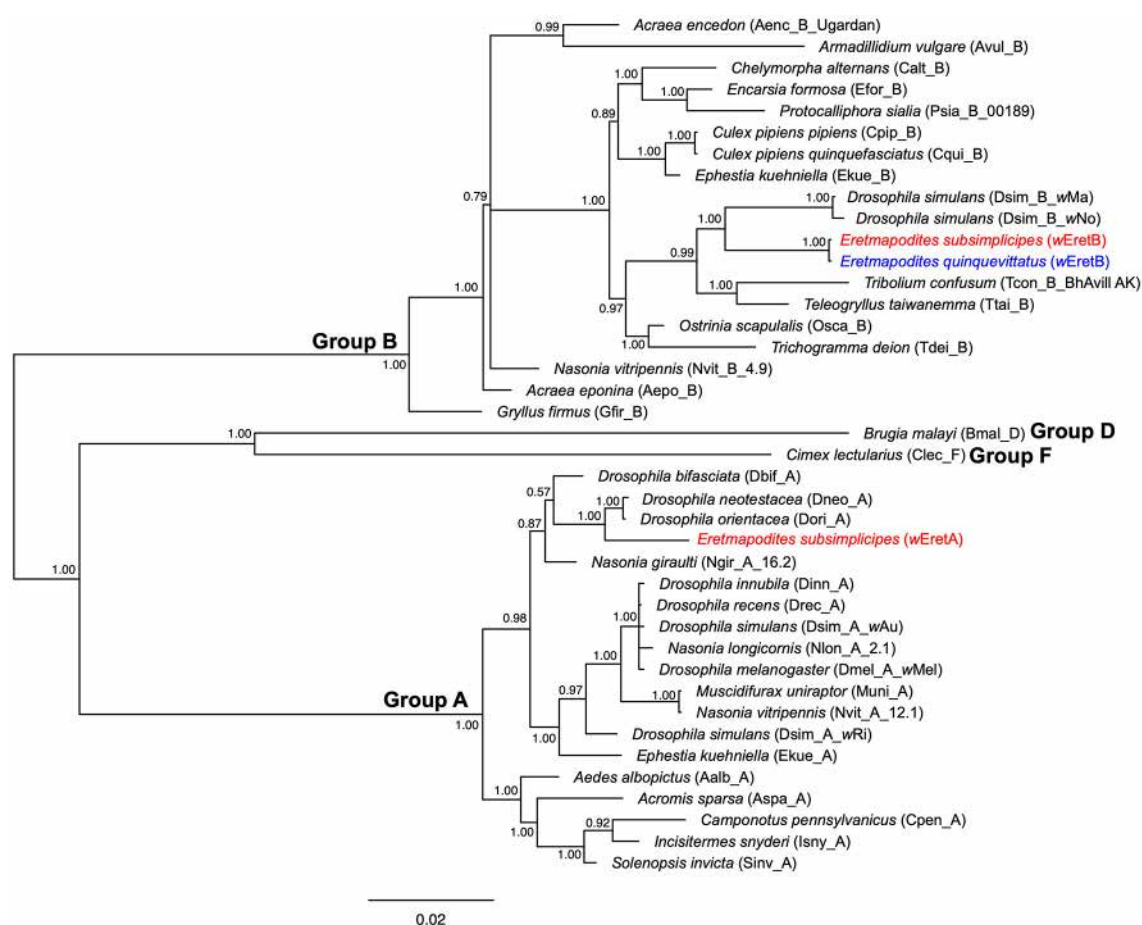


FIGURE 4

Bayesian phylogenetic tree of *Wolbachia* strains obtained from concatenated data set of *MLST* genes (*coxA*, *fbpA*, *ftsZ*, *gatB*, and *hcpA*) (2,079 bp, 40 sequences). The phylogenetic tree was built using the substitution model: GTR+I+G. Sequences in red and blue correspond to *Wolbachia* sequences detected in *Er. subsimplicipes* and *Er. quinquevittatus*, respectively. The names of *Wolbachia* strains identified in these mosquito species (wEretA and wEretB) are indicated in brackets. The letters A, B, D, and F correspond to *Wolbachia* supergroups. The tree is midpoint unrooted and the numbers associated with nodes correspond to posterior probability values. The scale bar is in units of substitutions/site.

between *Wolbachia* and *COI* phylogenies was shown (Figure 5), demonstrating that *Wolbachia* mainly use horizontal transfers to spread in their hosts.

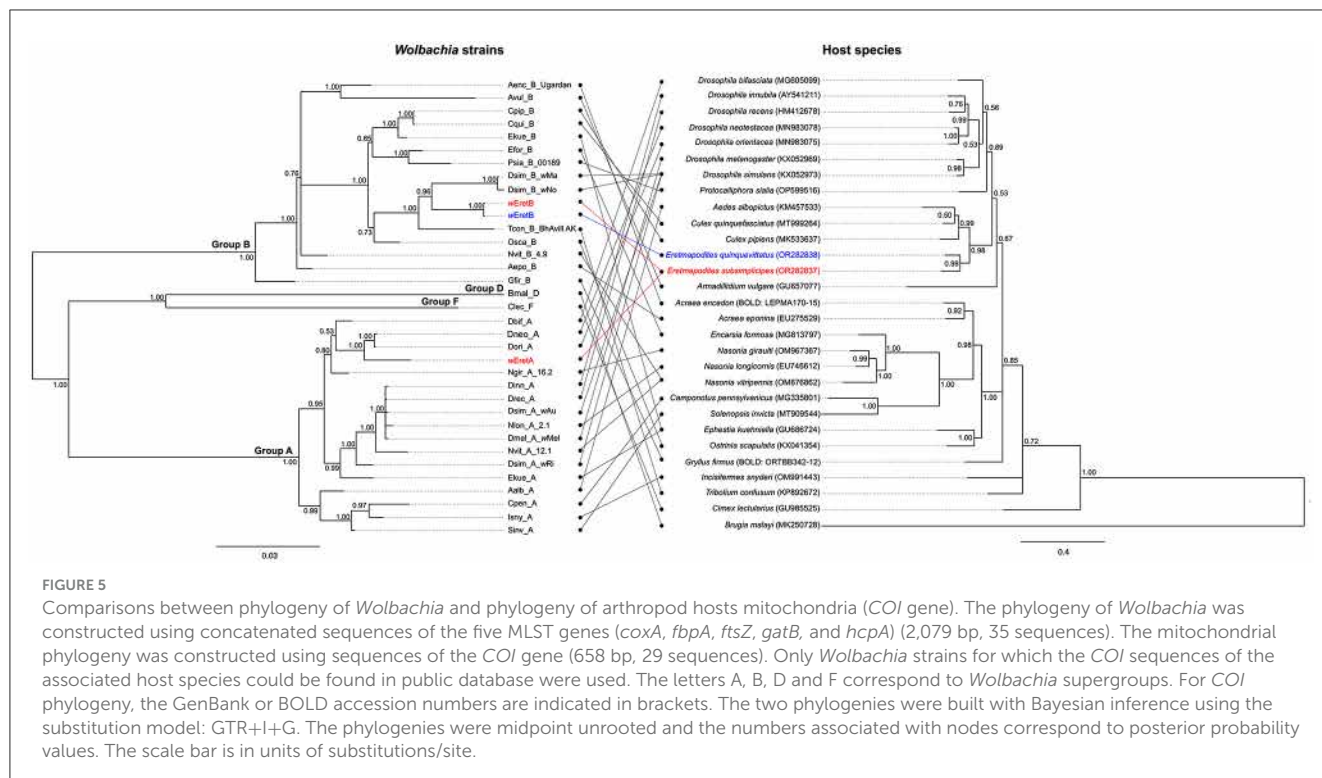
Wolbachia is maternally inherited in *Er. subsimplicipes*

To assess maternal transmission of *Wolbachia* in *Eretmapodites* mosquitoes, we focused on the species *Er. subsimplicipes* as it is the only species for which we currently have a laboratory colony. We examined the progeny of 30 wEretA infected laboratory females (see above) based on the sequencing of the *wsp* gene. In general, the number of eggs per female ranging from 4 to 61 (mean number of 32 eggs per female) (Table 2). The hatching rate of the eggs ranged from 5% to 100%, with a mean rate of 74%. It seems that the number of adults produced by each female is limited, as the mean rates for eggs becoming larvae and larvae reaching the adult stage are only 15% and 26%, respectively (Table 2). A total of 131 offspring (74 males and 57 females) from the 30 investigated females were then

screened for the presence of *Wolbachia*. Sixty per cent ($N = 78/131$) were found infected (Table 2), leading to a maternal transmission of *Wolbachia* ranging from 0% to 100%. Among the 30 females, four females did not transmit *Wolbachia* to their offspring, the transmission of *Wolbachia* was imperfect (between 6% and 88%) for ten females while perfect maternal transmission of *Wolbachia* (100%) was recorded for 16 females (Table 2).

Discussion

Using morphological and molecular methods, we confirmed the presence of two *Eretmapodites* species, *Er. quinquevittatus* and *Er. subsimplicipes*, in three islands of the Comoros archipelago (Grande Comore, Mohéli and Mayotte) (Le Goff et al., 2014; Boussès et al., 2018). The two species occurred in sympatry in the majority of investigated sites but *Er. quinquevittatus* was most commonly found in the three islands. The higher abundance of *Er. quinquevittatus* observed in this study may be due to sampling biases related to the type of samples collected and the method used for collection. In contrast to a previous study



conducted between 2008 and 2012 in Mayotte, which found *Er. subsimplicipes* to be the most frequently encountered mosquito species on the island (Le Goff et al., 2014), our observations were based on adult mosquitoes. The difference between our findings and the previous study could be attributed to the fact that larvae were sampled in the study of Le Goff et al. (2014), whereas we focused on adult collection. It is possible that breeding sites of *Er. quinquevittatus* are less accessible compared to those of *Er. subsimplicipes*, which could result in sampling bias when working with adults that have the ability to fly far away from their breeding sites. However, it is also plausible that the distribution area of *Er. quinquevittatus* in Mayotte has increased over the last past 10 years. Additionally, the sampling method used in our study, which involved portable electric aspirators to collect resting adult mosquitoes in vegetation and flying adults around manipulators, may have better suited the collection of *Er. quinquevittatus* adults compared to *Er. subsimplicipes*. Since the biology and ecology of both species in the field are not well understood, it is possible that this methodological difference influenced our findings. It would be interesting in future investigations to compare the distribution area of both *Eretmapodites* species in Mayotte, but also in the other islands of the Comoros archipelago, using both larval and adult sampling.

The mtDNA polymorphism based on the *COI* gene revealed 13 and 35 haplotypes in *Er. subsimplicipes* and *Er. quinquevittatus*, respectively. In both species, we found unique haplotypes (i.e., encountered in only one island), suggesting different colonization events probably from Madagascar or the east coast of Africa, regions geographically close to the Comoros archipelago and where both *Eretmapodites* species have been also identified

(Harbach, 2007; Tantely et al., 2016). Other mtDNA haplotypes were shared by different islands and could be the result of a single colonization event of *Eretmapodites* mosquitoes (from Madagascar or Africa) either to different islands, or to one island followed by a secondary dispersion event in a stepping stone mode. Such dispersion from a nearby island can be facilitated by frequent trade between the islands of the Comoros archipelago (Roger et al., 2014). For example, it is well known that the spread of the Asian tiger mosquito *Ae. albopictus* worldwide has been facilitated by the international trade of used tires (Reiter and Sprenger, 1987).

The mitochondrial haplotype diversity was higher in *Er. quinquevittatus* (35 haplotypes, $H_d = 0.481$ and $\pi = 0.002$, for $N = 615$ samples) than in *Er. subsimplicipes* (13 haplotypes, $H_d = 0.338$ and $\pi = 0.001$, for $N = 205$ samples). The difference between the two species can be explained by the sampling sizes as we found more *Er. quinquevittatus* specimens in our dataset. Alternatively, a higher mtDNA diversity in *Er. quinquevittatus* could result from a low prevalence of *Wolbachia* infection. Indeed, mitochondria and *Wolbachia* are in linkage disequilibrium, both cytoplasmic elements being linked through maternal cotransmission within egg cytoplasm's (Rasgon et al., 2006; Atyame et al., 2011; Dumas et al., 2013). Therefore, the spread of *Wolbachia* in host populations should result in an indirect selective sweep of the mtDNA leading to a reduction of mitochondrial diversity in *Wolbachia* infected host populations (Rasgon et al., 2006; Atyame et al., 2011; Dumas et al., 2013). In this study, we detected *Wolbachia* for the first time in both *Er. quinquevittatus* and *Er. subsimplicipes*, the lowest *Wolbachia* prevalence occurring in *Er. quinquevittatus* (0.8% vs. 87.7% in *Er. subsimplicipes*).

TABLE 2 Maternal transmission of *Wolbachia* in *Eretmapodites subsimplicipes*.

Female	N eggs	N larvae	% egg hatching	% emergence	% egg reaching to adult	Adult progeny						% maternal transmission
						Number of adults			Wolbachia positive samples			
						Total	♂	♀	Total	♂	♀	
Eret_01	16	16	100%	25%	25%	4	3	1	3	2	1	75%
Eret_02	18	7	39%	43%	17%	3	1	2	2	1	1	67%
Eret_03	21	9	43%	11%	5%	1	1	0	1	1	0	100%
Eret_04	22	20	91%	15%	14%	3	1	2	1	0	1	33%
Eret_05	24	21	88%	5%	4%	1	1	0	0	0	0	0%
Eret_07	51	49	96%	4%	4%	2	0	2	2	0	2	100%
Eret_08	61	60	98%	3%	3%	2	0	2	2	0	2	100%
Eret_09	16	15	94%	7%	6%	1	0	1	1	0	1	100%
Eret_10	4	1	25%	100%	25%	1	1	0	1	1	0	100%
Eret_11	27	6	22%	50%	11%	3	3	0	3	3	0	100%
Eret_12	15	6	40%	17%	7%	1	0	1	1	0	1	100%
Eret_14	39	39	100%	8%	8%	3	0	3	3	0	3	100%
Eret_15	39	30	77%	27%	21%	8	4	4	8	4	4	100%
Eret_16	54	52	96%	8%	7%	4	4	0	4	4	0	100%
Eret_17	38	2	5%	50%	3%	1	0	1	1	0	1	100%
Eret_19	33	18	55%	11%	6%	2	2	0	2	2	0	100%
Eret_20	56	53	95%	2%	2%	1	1	0	1	1	0	100%
Eret_21	39	22	56%	14%	8%	3	3	0	3	3	0	100%
Eret_22	15	15	100%	27%	27%	4	3	1	3	2	1	75%
Eret_23	32	26	81%	65%	53%	17	12	5	1	1	0	6%
Eret_24	55	54	98%	9%	9%	5	2	3	4	1	3	80%
Eret_25	34	33	97%	24%	24%	8	3	5	8	3	5	100%
Eret_26	29	22	76%	36%	28%	8	4	4	7	3	4	88%
Eret_27	10	3	30%	33%	10%	1	0	1	0	0	0	0%
Eret_28	54	46	85%	33%	28%	15	7	8	0	0	0	0%
Eret_29	32	29	91%	21%	19%	6	3	3	0	0	0	0%

(Continued)

TABLE 2 (Continued)

Female	N eggs	N larvae	% egg hatching	% emergence	% egg reaching to adult	Adult progeny						% maternal transmission
						Number of adults			Wolbachia positive samples			
						Total	♂	♀	Total	♂	♀	
Eret_30	43	40	93%	23%	21%	9	4	5	7	2	5	78%
Eret_31	17	10	59%	100%	59%	10	8	2	6	4	2	60%
Eret_32	36	34	94%	6%	6%	2	1	1	1	0	1	50%
Eret_33	34	28	82%	7%	6%	2	2	0	2	2	0	100%
						131	74	57	78	40	38	

The presence of *Wolbachia* in females and their offspring was examined through the presence/absence of the *wsp* gene. For each female, the number of eggs, the number of larvae (first instar larvae), the egg hatching rate (%), the percentage of emerging adults, the percentage of egg reaching to adult and the number of emerging adults (males and females) were provided. Finally, the rate of maternal transmission of *Wolbachia* was determined from adult progeny of each female. N = total number of mosquitoes examined (eggs or larvae).

Wolbachia infection is not fixed in any of the *Eretmapodites* field populations, with *Wolbachia*-infected and uninfected specimens found within the same sampling sites. The presence of *Wolbachia*-infected and uninfected specimens is commonly observed in field populations of other arthropod species and can be associated with low phenotypic manipulation but also to imperfect maternal transmission (Werren et al., 2008). We have monitored maternal transmission of (the most frequent) *wEretA* using a laboratory colony of *Er. subsimplicipes*. It should be noted that it was challenging to rear *Er. subsimplicipes* species under insectary conditions and a reduced number of adult offspring was obtained for each female. Despite this challenge, our results show that maternal transmission of *Wolbachia* is imperfect or non-existent in some females, which could explain why *Wolbachia* infection is not fixed in *Er. subsimplicipes* field populations. COI sequencing data is also consistent with imperfect maternal transmission in *Er. subsimplicipes* since identical mtDNA haplotypes are shared by *Wolbachia*-infected and uninfected mosquitoes (Figure 2C).

The examined phylogenies of *wsp* and each of the five MLST genes showed that both *Er. quinquevittatus* and *Er. subsimplicipes* are infected with two *Wolbachia* supergroups A and B. Within each *Wolbachia* supergroup, the genetic diversity was low, only one allele being detected for almost all loci (except for *hcpA*). The concatenated phylogeny of the five MLST genes also confirmed the presence of two *Wolbachia* supergroups A and B strains (namely *wEretA* and *wEretB*, respectively) in our dataset. In *Er. subsimplicipes*, mosquitoes were infected with either *wEretA* or *wEretB*, although more higher infections by *wEretA* than *wEretB* were observed; while only *wEretB* was observed in *Er. quinquevittatus*. The presence of two divergent *Wolbachia* strains in *Er. subsimplicipes* can be explained by horizontal transfer events from other arthropod species infected with genetically related *Wolbachia* such as *Drosophila spp.* which appeared to be infected with *Wolbachia* strains closely related to the strains *wEretA* and *wEretB* (Figure 4). Interestingly, the strain *wEretB* was shared by *Er. subsimplicipes* and *Er. quinquevittatus*. Several hypotheses can be proposed to explain this pattern. The *wEretB* strain might have been present in the common ancestor of both *Eretmapodites* species, and this *Wolbachia* strain was maintained in both species after their divergence, but the absence of nucleotide diversity between *wEretB* infecting both mosquito species does not support this assumption. For instance, some difference exists between the *Wolbachia* strains Dinn_A and Drec_A (within the supergroup A) infecting the genetically closely related *Drosophila* species *D. innibula* and *D. recens* (Figure 4). Another possibility would be horizontal transfers of *Wolbachia* between both mosquito species or from other host species. The widespread distribution of *Wolbachia* in arthropods is commonly associated with horizontal transfers occurring between closely related or genetically divergent host species (Heath et al., 1999; Ahmed et al., 2016; Tolley et al., 2019). These transfers would take place through mechanisms such as contamination, predation, or parasitism, particularly among species sharing the same ecological niches. Although evidence of horizontal transfers is rare, studies have shown that such transfers can occur in host-parasitoid associations (Huigens et al., 2004; Ahmed et al., 2015). The lack of congruence between *Wolbachia* and hosts phylogenies also support the possibility of horizontal transfers of *Wolbachia* between species (Tolley et al., 2019). In our

study, we compared the phylogenies of concatenated MLST genes and *COI*, and no congruence was found (Figure 5), confirming the potential for horizontal transfers of *Wolbachia* between host species. Furthermore, the success of interspecific transfers of *Wolbachia* via embryonic microinjections (Sasaki and Ishikawa, 2000; McMeniman et al., 2009; Hughes and Rasgon, 2014) also supports the hypothesis of horizontal transfers of *Wolbachia*. Assuming a horizontal transfer of *wEretB* from *Er. subsimplicipes* to *Er. quinquevittatus*, the low prevalence of *Wolbachia* in *Er. quinquevittatus* could be explained either by a recent transfer of *wEretB*, or by differences in phenotypes induced by this *Wolbachia* strain when infecting each mosquito species. This change in phenotype expression of the same *Wolbachia* strain when infecting different host species has been previously described in *Drosophila recens* and *Drosophila subquinaria* (Jaenike, 2007). It would be interesting for future investigations to examine the phenotypes induced by the *Wolbachia* strains *wEretA* and *wEretB* in *Er. subsimplicipes* and *Er. quinquevittatus* to better understand the dynamics of these bacteria in the field. Lastly, a horizontal transfer of *wEretB* might have happened through introgression between both *Eretmapodites* species. Introgressions of *Wolbachia* have been observed in various subspecies of mosquitoes in the *Culex pipiens* complex, such as *Culex pipiens pipiens* and *Culex pipiens quinquefasciatus*, which hybridize in natural environments. This hybridization leads to subspecies sharing the same *Wolbachia* strains, as determined through the *Wolbachia* MLST genotyping method (Atyame et al., 2011; Dumas et al., 2013) (see also Figure 4). This hypothesis could be tested in the future by comparing the polymorphism in nuclear genomes of *Er. subsimplicipes* and *Er. quinquevittatus* mosquitoes.

Conclusion

In the present study, we characterized the mitochondrial genetic diversity of *Er. quinquevittatus* and *Er. subsimplicipes* occurring in sympatry in three islands of the Comoros archipelago. We also characterized the genetic diversity of *Wolbachia* infecting both mosquito species and identified two new *Wolbachia* strains, which have been named *wEretA* and *wEretB*. Experimental rearing of *Er. subsimplicipes* revealed imperfect maternal transmission of *Wolbachia* that might explain the infection patterns found in the field. Future studies will examine the phenotypes induced by these *Wolbachia* in *Er. quinquevittatus* and *Er. subsimplicipes* to better understand their dynamics *in natura*. As *Eretmapodites* mosquitoes are competent vectors for the transmission of arboviruses (Bamou et al., 2021; Cêtre-Sossah et al., 2023), future investigations should also consider the effects of *wEretA* and *wEretB* on vector competence.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/genbank/>, OR282837-OR282884,

OR296528-OR296530, OR296531-OR296533, OR296534-OR296536, OR296537-OR296539, OR296540-OR296543, and OR296544-OR296547.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

YG: Data curation, Formal analysis, Methodology, Writing—original draft, Writing—review & editing. SH: Conceptualization, Methodology, Writing—original draft, Writing—review & editing. CL: Investigation, Methodology, Writing—original draft. PR: Investigation, Resources, Writing—original draft. A-bl: Investigation, Resources, Writing—original draft. AY: Investigation, Resources, Writing—original draft. PB: Formal analysis, Methodology, Writing—original draft. PM: Conceptualization, Funding acquisition, Validation, Writing—review & editing. CA: Conceptualization, Investigation, Methodology, Supervision, Validation, Writing—original draft, Writing—review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the European Regional Development Funds PO INTERREG through the VECTOBIOMES project (number RE0009962) and the multi-years agreement of ARS-Mayotte/2022/n°75. SH was supported by a PhD degree scholarship from the Regional Council of Reunion Island (DIRED/20181182) and the University of Reunion Island.

Acknowledgments

We thank all collaborators from Union of Comoros for their help on the field and in the labs: Mohamed Salim Ben Said Hafi (Regional Director of Health, Anjouan), Djamaldine Mohamed Sambi (Regional Director of Health, Mohéli), Aboubacar Said Anli (Regional Director of Health, Moroni), Daanti Mouhtal Elhad and Idami Ousseni (PNLP, Anjouan), Yousra Ahamada Ali (PNLP, Mohéli), Youssouf Ismainla, and Amblat Ali Ahmed (PNLP, Moroni). We thank all collaborators from ARS Mayotte for their help on the field and in the labs: Ismael Nahouda, Mikidachi Said, Mourssalina Haraouna, Lassadi Hassani Ali, and Madi-Saindou Mouhamadi. We thank Louis Collet (Centre Hospitalier de Mayotte) for his help in the storage and the shipment of samples. We are thankful to Fiona Baudino (UMR PIMIT, Réunion) for her help in the lab. We are grateful to Pablo Tortosa (UMR PIMIT) for his proofreading and relevant remarks on the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1343917/full#supplementary-material>

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