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Short title: Mycobiota across populations of the Asian tiger mosquito

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Abstract

The Asian tiger mosquito *Aedes albopictus,* native to Southeast Asia, has invaded a wide range of tropical and temperate areas worldwide. Recent studies pointed out that invasive populations from Europe harbored reduced bacterial microbiota compared to the native populations. Beside bacteria, mosquitoes also contain fungal communities that have so far been largely ignored. To investigate whether the mosquito invasion process displays a similar impact on fungal diversity, we compared the mycobiota structure of three autochthonous mosquito populations in Vietnam and six populations recently introduced in France and Madagascar. All mosquito populations host a locally structured fungal community and carry a "core mycobiota" dominated by yeasts. However, invasive populations from France and Madagascar harbor a lower fungal diversity compared to Vietnamese populations. These results suggest that similar factors shape the overall composition of the mosquito-associated microbiota during the invasion process as bacterial and fungal communities demonstrate a loss of diversity.

Keywords: Asian tiger mosquito; fungal ecology; mycobiota; metataxogenomic; biogeography

Introduction

The Asian tiger mosquito *Aedes albopictus* is an important disease vector, which transmits pathogens of medical importance such as dengue and chikungunya viruses (Leta et al., 2018). This species is also considered to be one of the most invasive species worldwide (Bonizzoni et al., 2013). Originated from Southeast Asia, the mosquito rapidly spread throughout a wide range of eco-climatic regions worldwide (Kraemer et al. 2015). The mosquito invasive success has been facilitated by the additive impact of globalization, climate change (Semenza and Suk, 2018) and mosquito intrinsic capacities, such as ecological and physiological plasticity (Paupy et al., 2009; Medlock et al., 2012). This translates into its ability to colonize both natural and man-made breeding sites in both sylvatic and anthropic habitats (Hawley, 1988; Juliano and Loubinos, 2005, Medlock et al., 2015). With the lack of vaccines against most of *Ae. albopictus*-vector borne diseases, a particular attention is given to the global spread of this mosquito in order to prevent the emergence or re-emergence of arboviral outbreaks (Lambrechts et al., 2010).

Recent findings highlighted new hypotheses on mosquito holobiont functioning, with mosquito-microbiota interactions being key features of the vector pathosystem (Guégan et al., 2018). Some studies have demonstrated the importance of the mosquito microbiota for disease transmission (Dennison et al., 2014) and pointed out a role of some bacteria in extended phenotypes of mosquitoes (i.e. hosts' phenotypes that are impacted by the symbiotic interaction) such as nutrition, reproduction and development (Ricci et al., 2012; Minard et al., 2013; Mitraka et al., 2013; Coon et al., 2014). Given the close relationships between mosquito and its microbiota, a growing number of studies have considered the use of symbionts as a promising vector control approach to contain mosquito populations (Ricci et al., 2012). Two main approaches were developed for the symbiotic control of mosquitoes. The first one also

called paratransgenesis is based on the manipulation of symbionts in order to express molecules targeting pathogens in the insect vector (Wilke and Marrelli, 2015). The second one relies on microorganisms that reduce mosquito's life span and vector competence (Calvitti et al., 2012; Hedge et al., 2015). Others studies also suggested a potential role of the microbiota in ecological success, adaptive processes and global spread of mosquitoes (Minard et al., 2015; Guégan et al., 2018), as already demonstrated in other insect species (Dunbar et al., 2012; Kikuchi et al., 2012; Romano, 2017).

To date, studies on the mosquito-associated microbiota have mainly focused on bacterial communities and factors susceptible to modulate their composition and diversity (Minard et al., 2013; Minard et al., 2015; Minard et al., 2018; Muturi et al., 2018). Beside bacteria, mosquitoes also carry fungal communities (Thongsripong et al., 2017). However, the contribution of these microbiota members to host phenotypes and how mycobiota diversity is shaped, either by environmental factors or host dynamics, remains poorly studied (Guégan et al., 2018). In a previous study, we showed that the average bacterial diversity associated with invasive populations of the Asian tiger mosquito that recently colonized France was reduced compared to populations collected in Vietnam, the native area of *Ae. albopictus* (Minard et al., 2015). Using the same experimental design, a recent study confirmed this tendency and showed that *Ae. albopictus* collected in Italy (another recently invaded country) harbored a reduced diversity of bacterial microbiota compared to individuals collected in Vietnam (Rosso et al., 2018).

Thus, we hypothesized that such microbiota diversity reduction shown for bacteria in invasive populations of the Asian tiger mosquito could also be observed for the mycobiota. To test this hypothesis, we used previously collected *Ae. albopictus* from Vietnam, Madagascar and Metropolitan France; three different countries with distinct histories of colonization and contrasting eco-climatic features. Indeed, the populations from Vietnam are

part of the native area of the Asian tiger mosquito. Those from Madagascar and France were invasive and respectively collected more than 30 yr and less than 10 yr after the first introduction of the mosquito (Raharimalala et al., 2012; Minard et al., 2015). Moreover, to our knowledge, the present study is the first to explore the fungal diversity in distinct *Ae. albopictus* populations. Therefore, this work might also bring important information concerning the composition and structure of fungal communities associated with mosquitoes.

Materials and Methods

Sampling areas and mosquito collection

As mentioned above, mosquitoes were sampled in Vietnam, Madagascar and France. These three countries exhibited different climatic conditions ranging from tropical or sub-tropical for Vietnam and Madagascar to temperate for France (Table 1). Vietnam is located in the South East Asia and refers to an ancient colonization. Whereas *Ae. albopictus* was introduced in Madagascar in the 1980s (Raharimalala et al., 2012), France is a recent invaded zone with Nice as the first colonized site in 2004 (Medlock et al., 2012). For each country, three sampling sites, separated by at least 40 km, were selected to ensure that mosquitoes collected in two different sites did not belong to the same population. Distances between sites within each country are indicated in Fig. S1. Sampling in Madagascar was performed in December 2010 at Mananjary (MA), Toamasina (TO) and Tsimbazaza Park (TS) whereas sampling in Metropolitan France was performed between August and September 2012 at Nice (NC), Portes-lès-Valence (PL) and Saint-Priest (SP). Mosquito sampling in Vietnam was performed during October 2012 at Bình Dương (BD), Hồ Chí Minh City (HC) and Vũng Tàu City (VT). Adult females were caught alive with nets or BG-Sentinel traps (Roiz et al., 2015) and then identified using morphological characteristics (Rueda, 2004). Only females that did

not contain any blood were retained for analysis. Mosquitoes were stored in 100% ethanol at -80°C until DNA extraction.

Genomic DNA extraction, library preparation and sequencing

A total of 95 female individuals were analyzed (Table 1): 33 from France (i.e. n=11 for NC, n=11 for PL, n=11 for SP), 30 from Madagascar (i.e. n=10 for MA, n=10 for TO, n=10 for TS) and 32 from Vietnam (i.e. n=10 for BD, n=11 for HC, n=11 for VT). Prior to DNA extraction, female specimens were surface-disinfected with 70% ethanol and rinsed with sterile water. Genomic DNA was extracted from whole body mosquitoes of at least 10 females per sampling site using the procedure previously described (Zouache et al., 2011). Briefly, individual mosquitoes were crushed in 200 µL of extraction buffer (2% hexadecyltrimethyl ammonium bromide, 1.4 M NaCl, 0.02 M EDTA, 0.1 M Tris pH 8, 0.2% 2-β-mercaptoethanol). Homogenates were incubated for 15 min at 60 °C and proteins were extracted with chloroform: isoamyl alcohol (24: 1, v/v). DNA was precipitated with isopropyl alcohol, washed with 75% ethanol and then dissolved in 30 µL of sterile water. Additionally, a DNA extraction was carried out without any biological matrix and considered as a negative control to evaluate ambient contaminations.

To provide a complete picture of the mosquito mycobiota, we used the nuclear ribosomal internal transcribed spacer (ITS) region, which has the highest probability of successful identification for the broadest range of fungi (Schoch et al., 2012). Indeed, Thongsripong et al. (2017) recently showed that the 18S marker was not suitable for in-depth description of the fungal community in mosquitoes as it is largely dominated by *Ascogregarina;* a dominant eukaryote within *Aedes* spp. The fungal-specific PCR primers target the ITS-2 region and do not hybridize to either the host DNA nor the DNA of the obligatory eukaryotic symbiont *Ascogregarina taiwanensis* (Dos Passos and Tadei, 2008;

Thongsripong et al., 2017). Modified gITS7 (5'- α GTG AAT CAT CGA RTC TTT G -3') (Ihrmark et al., 2012) and ITS4 primers (5'- β TCC TCC GCT TAT TGA TAT GC-3') (White et al., 1990) were used to construct amplicon libraries by a two-step PCR. In these primers, α and β represent the two Illumina overhanging adapter sequences (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG and GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G, for α and β, respectively). PCR amplifications were carried out in a 25 µl reaction mix containing 30 ng of DNA template, 1X Encyclo buffer with $MgCl_2$ (Encyclo PCR Kit, Evrogen, Moscow, Russia), 0.2 mM of each dNTP (Encyclo PCR Kit, Evrogen), 1 µM of each primer (Life Technologies, Saint-Aubin, France), 0.2 mg mL^{-1} of bovine serum albumin (New England Biolabs, Evry, France), 0.06 mg mL^{-1} of T4 gene 32 protein (New England Biolabs) and 1X of Encyclo polymerase mix (Encyclo PCR Kit, Evrogen). All amplifications were carried out in triplicates on a Biorad C1000 thermal cycler (Biorad, CA, USA) with one cycle of 3 min at 94°C, followed by 40 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 30 s, and a final extension step of 5 min at 72°C. The three PCR replicates from each sample were pooled, purified with Agencourt AMPure XP PCR Purification kit (Beckman Coulter, Villepinte, France), and quantified using the Quant-iT Picogreen dsDNA Assay Kit (Life Technologies, NY, USA). Construction of amplicon libraries (second-step PCR) and sequencing on the Illumina MiSeq platform (2 x 250-bp paired-end reads) were performed by Biofidal (Vaulx-en-Velin, France).

Sequence analysis and taxonomy assignment

The raw dataset consisted of 8,441,658 reads that were demultiplexed by Biofidal. Paired-end reads were then merged using FLASH (Magoč and Salzberg, 2011). Denoising procedures consisted of discarding sequences outside of the expected length (i.e. expected size between 200 and 500 bp) and those containing ambiguous bases (N). Sequences were

clustered into Operational Taxonomic Units (OTUs) using SWARM (Mahé et al., 2014). SWARM is a *de novo* clustering based on an unsupervised single-linkage-clustering method that reduces the impact of clustering parameters on the resulting OTUs by avoiding arbitrary global clustering thresholds that presumes that all species evolve at the same rate and input sequence ordering dependences. SWARM builds OTUs in two steps: (i) an initial set of OTUs is constructed by iteratively agglomerating similar amplicons, and (ii) amplicon abundance values are used to reveal OTUs internal structures and to break them into sub-OTUs if necessary. Compared to user-defined threshold methods, *de novo* clustering algorithms like SWARM were demonstrated to perform well on environmental samples and mock communities where the ground truth was well established (Kopylova et al., 2016). In the present work, the SWARM aggregation distance equaled 3. Chimeras were removed using VSEARCH (Rognes et al., 2016) and, as recommended by Bokulich et al. (2013), low abundance sequences accounting for less than 0.005% of the dataset were filtered out. The taxonomic assignation of OTUs was performed using the RDP Classifier (Wang et al., 2007) against the curate fungal ITS UNITE database (Version 7.2 released on the 2017-12-01; Kõljalg et al., 2013). The advantage of using this Bayesian Classifier over BLAST is not only that it is more accurate in finding the most similar sequences, it also provides a bootstrap confidence score for each of the levels of taxonomic assignment for the best matching taxa (Gweon et al., 2015). Except for the taxonomic assignation of OTUs, which was performed on Mothur v.1.36.1. (Schloss et al., 2009), all other processes were automated within the FROGS pipeline (Escudié et al., 2018). Furthermore, OTUs were removed from further analyses if they were detected in the negative control sample and their relative abundance was not at least 3 times greater than the one observed in the negative control in more than 5% of the samples. A total of 15 OTUs were then discarded from further analyses. To compare samples, a normalization procedure was applied to randomly resample down to 25,357

sequences per individual, discarding 11 samples from the dataset. Fastq files were deposited at the EMBL-ENA public database (http://www.ebi.ac.uk/ena) under the accession number **ERP106695/PRJEB24837**.

Statistical analysis

All statistical analyses were computed with the software R Version 3.1.2. (R Development Core Team, 2009). Alpha-diversity indices aim to describe general properties of communities (typically species diversity) present in environmental samples that allow us to compare them (Morris et al., 2014). The Shannon index is the most commonly used alpha-diversity metric for ecological applications. It quantifies species diversity for each collected sample, combines measures of richness and evenness (Shannon, 1948). To evaluate the difference in α-diversity (Shannon index) between countries, a linear mixed model was applied which included the index as a response variable, the country as a fixed effect and the sampling site as a random variable. The models were performed with the package lme4 and their likelihoods were evaluated with the Akaike Information Criterion (Bates et al., 2015). Multiple comparisons analyses were performed between the different countries with a Tukey's all-pair comparisons test implemented in the package *multcomp* (Bretz et al., 2010). Fungal community compositions between mosquito populations (β-diversity) were primarily analyzed by non-metric multidimensional scaling (NMDS). A stress value was calculated to measure the difference between the ranks on the ordination configuration and the ranks in the original dissimilarity matrix for each repetition.

An Analysis of Molecular Variance (AMOVA) was conducted with the package *pegas* to test the differences in overall fungal community composition among the three countries and among the three sites within each country (Excoffier et al., 1992; Paradis et al., 2010). The AMOVA estimates the level of differentiation (variance) of the mycobiota associated with the

Ae. albopictus among different countries, populations and individuals. All these analyses were based on dissimilarity matrices calculated with the Bray-Curtis dissimilarity index and were performed with *vegan* (Oksanen et al., 2018).

Results

Fungal species diversity in *Aedes albopictus* mosquitoes

The dataset consisted of 2,129,988 sequences representing 695 OTUs. Depending on the sampling site, the number of fungal OTUs detected per individual mosquito varied from 56 ± 25 (Nice) to 93 ± 15 (Saint-Priest) concerning the specimens sampled in France, from 61 ± 9 (Tsimbazaza Park) to 73 ± 16 (Toamasina) for the ones collected in Madagascar and from 55 ± 13 (Hồ Chí Minh City) to 72 ± 14 (Bình Dương) for the specimens sampled in Vietnam (Table S1). A total of 291 OTUs (i.e. 41.9% of the total OTUs) was shared by mosquito populations at the country level (Fig. 1). This means that these OTUs were found at least in one individual mosquito of each of the three countries. Less than 25% of the fungal OTUs were shared by at least one individual from each sampling site (i.e. 120, 113 and 102 OTUs for France, Madagascar and Vietnam, respectively). Multiple comparison analysis performed with the Shannon index (Table S1) indicated that populations from Vietnam harbored a more diverse mycobiota than populations from France ($P = 0.032$) and Madagascar ($P = 0.015$) with a mean difference of 0.79 ± 0.31 and 0.86 ± 0.31, respectively (Fig. 2; Table S2). The populations sampled in France and Madagascar did not show any significant differences of their fungal diversity (Table 2).

At the country level, a total of 515, 470 and 535 fungal OTUs were detected among the three sampling sites localized in France, Madagascar and Vietnam, respectively (Fig. S2). Less that 25% of the fungal OTUs were shared by at least one individual from each sampling

site (i.e. 120, 113 and 102 OTUs for France, Madagascar and Vietnam, respectively). Site-specific OTUs represented from 14 to 19% of the total number of fungal OTUs detected within the French mosquito populations, from 13 to 18% and 13 to 25% for the populations of Madagascar and Vietnam, respectively (Fig. S2). Fungal diversity, estimated through the Shannon index was significantly different between sites within Madagascar and Vietnam. Indeed, for the Malagasy populations a higher diversity was observed in Mananjary compared to Toamasina (Mananjary vs Toamasina; U = 78, Z = 1.01, P = 0.006) or Tsimbazaza Park (Mananjary vs Tsimbazaza; U = 85.5, Z = 0.85, P = 0.008). Similarly, for the Vietnamese populations a higher diversity was observed in Bình Dương compared to Hồ Chí Minh City (Bình Dương vs Hồ Chí Minh City; U = 85, Z = 0.68, P = 0.009).

Taxonomic composition of fungal communities in *Aedes albopictus* mosquitoes

NMDS ordination of the Bray-Curtis distances showed that fungal communities were more similar among individuals at the site level than among countries (Fig. 3). This observation was supported by an AMOVA analysis that depicts more structure among sites within countries ($\sigma^2 = 0.13$; df. = 6,75; $P < 10^{-4}$) than among countries ($\sigma^2 = 0.01$; df. = 2,75; P = 0.04; Table 3). However, there was also a high variability of fungal communities among mosquito individuals ($\sigma^2 = 0.24$; Table 2), which was more important for the Vietnamese populations than Malagasy ones (Fig. 3).

The percentage of unclassified fungal sequences represented less than 1.5% irrespective of the sampling site considered (Fig. 4). Fungal communities detected in *Ae. albopictus* are largely dominated by Ascomycota as they represented $92.4 \pm 4.8\%$, $91.2 \pm 6.6\%$ and $73.3 \pm 11\%$ of the total number of sequences detected in mosquito populations originating from French, Malagasy and Vietnamese sampling sites, respectively (Fig. 4). In comparison, Basidiomycota represented only $7.6 \pm 4.8\%$, $8.8 \pm 6.6\%$ and

24.6 ± 13.8% of the sequences detected in French, Malagasy and Vietnamese sampling sites, respectively. Most of the Ascomycota sequences (91 to 100%) were assigned to the *Pezizomycotina* and *Saccharomycotina* sub-phyla. *Saccharomycotina,* exclusively composed of yeast species, were more abundant in mosquito specimens collected among the Malagasy sampling sites Mananjary and Toamasina (43.4 and 83.9% of the total number of sequences, respectively) than in mosquito populations from Vietnam and France. Basidiomycota sequences were mainly affiliated to the *Agaricomycotina* sub-phylum (Fig. 4) and corresponded either to yeasts or filamentous fungi. Yeasts represented 32.1 ± 33.8%, 24.8 ± 19.1%, and 20.6 ± 20.6% of the total number of *Agaricomycotina* sequences detected in Vietnamese, French and Malagasy sampling sites, respectively. Furthermore, yeasts or yeast-like fungi belonging to the sub-phyla *Ustilaginomycotina*, *Pucciniomycotina*, *Agaricomycotina, Pezizomycotina* and *Saccharomycotina* are an important component of the mosquito mycobiota as they represented 18.9 ± 17.6%, 19.1 ± 13.1% and 46.8 ± 42.5% of the total number of sequences detected in French, Vietnamese and Malagasy populations.

The taxonomic affiliation of fungal sequences varied from the species to the phylum level (Table S3). A total of 196 and 142 different species of Ascomycota and Basidiomycota, distributed among 131 and 101 genera, were detected within these *Ae. albopictus* populations, respectively. Despite the variability in fungal composition among mosquito individuals, irrespective of their country of origin, the analyzed mosquito populations shared 109 Ascomycota and 72 Basidiomycota species (of which 16% and 40% of yeast species, respectively) (Table S3). Only 27 Ascomycota and 24 Basidiomycota species were specifically associated with mosquitoes from a single country and were mostly associated with Vietnamese populations (i.e. 19 and 9 species, respectively).

Among the fungal OTUs, five were widespread in almost all individuals (> 90%) regardless the population origin (Fig. 5). These five OTUs corresponded to the filamentous

fungi *Cladosporium* sp. and *Aspergillus puulaauensi* (present in 100 and 98% of the mosquito individuals, respectively) and to the yeast species *Candida* sp., *Aureobasidium pullulans*, and *Hyphopichia burtonii* (detected in 90.5 and 96% of the mosquito specimens, respectively). It was not possible to obtain a deeper taxonomic affiliation of the OTU sequence related to *Cladosporium* sp. as the amplified ITS region showed 100% of identity with different species (*C. cladosporioides*, *C. asperulatum* and *C. perangustum*). The OTU sequence assigned as *Candida* sp. was closely related to the species *C. oleophila* and most likely belonged to the *Kurtzmaniella* clade. Beside this prevalence, these five OTUs corresponded to the most abundant ones in term of sequences as they represented from 3.4 to 27.2% of the total number of sequences detected in mosquito populations originating from France, Madagascar and Vietnam (Fig. 5).

The other most abundant OTUs (those that represent > 10% of the total sequences in one sample) varied depending on sampling sites and mosquito individuals (Fig. 6). Among them, 20.7% (12 of 58) were assigned to yeast species and belonged to either Ascomycota (e.g. *Candida*, *Lachancea*, *Torulaspora*) or Basidiomycota (e.g. *Hannaella*, *Papiliotrema*, *Kwoniella*) genera. The remaining 79.3% of these most abundant OTUs corresponded to filamentous fungal species, which many are considered as plant pathogens (e.g. *Stemphylium vesicarium, Mycosphaerella etlingerae, Phaeophleospora hymenocallidicola*) or saprotrophs (e.g. *Schizophyllum commune, Phlebia radiata, Aspergillus gracilis*).

Discussion

Our results suggest that the mosquito invasion process has a similar impact on fungi as on bacteria (Minard et al., 2015): we found a reduction of fungal diversity in recently established *Ae. albopictus* populations compared to the autochthonous ones. These results suggest that common factors may shape different communities in the mosquito microbiota. To

date, the studies dealing with the origin of microbes colonizing mosquitoes were mainly focused on the bacterial component. It was shown that mosquitoes acquire a part of their bacteria from their aquatic habitat during larvae feeding (Coon et al., 2016). The authors also showed that bacteria covering the egg surface could also be acquired by the larvae and preserved until they hatch into adults. This suggests a potential vertical transmission of the microbiota through egg smearing. Likewise, the flower nectar would be also a source of bacterial acquisition for adult mosquitoes in nature (Kenney et al., 2017). In contrast to what is know for bacteria, few studies have described the whole fungal communities associated with mosquitoes in the field and even less their mode of acquisition. A study recently suggested that the diversity and composition of mosquito mycobiota would be determined at least partially by the diversity and composition of microbes in their habitats (Thongsripong et al., 2017). Our results also suggest that environmental conditions could be important factors that shape the fungal communities in mosquitoes. Indeed, the fungal community composition, which remained variable among mosquito individuals, was more similar among individuals at the site level than among countries. The fact that many yeast genera identified in the nectar of different flowering plants (Pozo et al., 2011; Canto et al., 2017) belong to abundant fungal OTUs detected in our *Ae. albopictus* populations (e.g. *Aureobasidium, Candida, Papilotrema*, *Vishniacozyma*, *Kwoniella, Hannaella*) suggests that nectar feeding could also contribute to the acquisition of environmental yeast by mosquitoes.

Following these observations, the reduction of diversity in the bacterial and fungal microbiota could be explained by the loss of diversity in environmentally associated microbial communities. Indeed, it has already been shown that anthropogenic environmental changes could induce a loss of diversity in the bacterial microbiota of some primate species (Barelli et al., 2015). On the other hand, immediately after an invasion, invasive populations often endure a reduction in their density (bottleneck) associated with a modification of their genetic

structure (genetic drift). Such phenomena, also referred to the founder effect, might drastically impact the diversity and community structure of vertically and horizontally acquired microorganisms in invasive mosquito populations (as previously suggested for mosquito-associated bacterial microbiota by Minard et al., 2015). The impact of an experimentally induced genetic bottleneck on *Aedes albopictus* bacterial microbiota was previously explored (Minard et al., 2018). However, no differences were observed in the alpha-diversity of the microbiota but the composition of the bacterial microbiota of females differed after the bottleneck. Such controlled experiments could be conducted in the future to explore the potential impact of mosquito population dynamics on their associated mycobiota.

Rich, diverse and variable fungal communities were identified. The presence of five abundant fungal OTUs in almost all individuals (> 90%), regardless of their site and country of origin, could reflect a continuous acquisition from the environment and/or close interactions with mosquitoes. Given that the core microbiota may be defined as the microbial species most prevalent in the hosts (≥ 90%) (Segata et al., 2016), these five fungal OTUs could be considered as members of the 'core mycobiota'. They were affiliated to the filamentous fungi *Cladosporium* sp. and *Aspergillus puulaauensi* and to the yeast species *Candida* sp., *Aureobasidium pullulans*, and *Hyphopichia burtonii*. The most prevalent and abundant species *Cladosporium* sp. (i.e. which was present in 100% of the mosquito individuals and represented 27.2% of the whole sequence dataset) was also one of the most common fungi identified in *Culex pipiens* populations from California (Chandler et al., 2015) and *Cx. quinquefasciatus* populations from Thailand (Thongsripong et al., 2017). In this latter study, the authors also analyzed *Ae. aegypti* and *Ae. albopictus* populations but they probably failed to detect this filamentous fungi as the taxonomic marker they used (18S rRNA) also amplifies the dominant *Aedes*-specific gregarine symbionts *Ascogregarina* (Thongsripong et al., 2017). All of these studies demonstrated that *Cladosporium* sp. occurred frequently in

mosquitoes. However, as this fungal genus is one of most widespread genera worldwide (Bensch et al., 2012), it seems much more likely that it is present in mosquitoes because it is commonly found in mosquito environments.

Yeast growth, which can be permanent or temporary in those fungi that can switch between yeast and filamentous growth, is a trait of many fungi that are closely related to or obligatory-associated with arthropods (Blackwell, 2017). However, not all yeasts are documented to be arthropod-associated and some yeasts such as *Scheffersomyces stipitis* (*Pichia stipitis*) isolated from the gut of the passalid beetle *Odontotaenius disjunctus* grows in hyphal form attached by a specialized cell to the hindgut wall (Suh et al., 2004). Therefore, it is not surprising that 60% of the 'core mycobiota' corresponded to yeast species (*Candida* sp., *Aureobasidium pullulans* and *Hyphopichia burtonii*) and that yeasts or yeast-like fungi belonging to the sub-phyla *Ustilaginomycotina*, *Pucciniomycotina*, *Agaricomycotina, Pezizomycotina* and *Saccharomycotina* are an important component of the mosquito mycobiota (20.7% of the most abundant OTUs and from 18.9 to 46.8% in mean of the total number of sequences detected in French, Vietnamese and Malagasy populations). In contrast to other *Pichia* species, to our knowledge, *Hyphopichia burtonii* (*Pichia burtonii*) has never been demonstrated to be associated with mosquitoes. However, this yeast species has been shown to be associated with the gut of certain insects such as the coffee berry borer *Hypothenemus hampei* (Vega et al., 2003). Frants et al. (1986) showed that *Pichia* was the most prevalent genus isolated from *Aedes* mosquitoes sampled in Russia. The species *Pichia caribbica* was isolated from the gut diverticulum of the closely related mosquito species *Ae. aegypti* (Gusmão et al., 2007). Concerning *Candida* species and *Aureobasidium pullulans*, they have previously been described in adult *Aedes* mosquitoes using either culture-dependent or culture-independent methods (Frants et al., 1986; Gusmão et al*.,* 2010; Bozic et al., 2017). *Candida* species were identified in the midgut and ovary of *Ae. aegypti* (Gusmão et al*.,* 2010)

but also in larvae of *Aedes*, *Anopheles* and *Culex* mosquitoes (Ignatova et al., 1996). However, Steyn et al. (2016) showed a lower abundance of yeast isolates in adults compared to larvae, and suggested that a microbial reduction would have occurred during adult emergence.

All of these observations raise questions about the ecology of mosquito-fungi relationships and its relevance for both partners. Based on what is known for other insects, the challenge for mosquitoes that use plant nectar for their nutrition is to find such a sugar source, which tends to occur in small and ephemeral patches. Similarly, key challenges for dispersal-limited yeasts (i.e. absence of sexual reproduction or limited to the production of few spores with no sporocarp formation) include getting to these patches as well as leaving them for other new patches (Madden et al., 2018). By generating volatile chemicals as by-products of the sugar fermentation, yeasts present in nectar give an indication of the presence of the sugar resource for many insects. In turn, the attracted insects transport yeasts to another patch of sugar (Becher et al., 2018). It has been also shown that yeasts and other fungi are important for insect development as they provide sterols, vitamins and enzymes to either detoxify toxic plant components or to recycle the nitrogen wastes of insects (Blackwell, 2017). In the case of mosquitoes, Díaz-Nieto et al. (2016) and Valzania et al. (2018) showed that *Saccharomyces cerevisiae* promotes the development of *Cx. pipiens* and *Ae. aegypti*, respectively. Another study, which used some yeast species as sole food source for *Cx. pipiens* larvae, demonstrated that yeast strains differently impacted larval growth, survival and pupation (Steyn et al., 2016). Despite these examples, there is no report of mutualistic interactions between fungi and mosquitoes. It was recently demonstrated for another blood-feeding *Diptera* species, the sand fly *Phlebotomus perniciosus*, that its yeast symbiont *Wickerhamomyces anomalus* (*Pichia anomalus*) might contribute to the removal of nitrogenous wastes after the blood meal

(Martin et al., 2018). Interestingly, this yeast species was also detected in the *Ae. albopictus* populations of the present study.

Certain filamentous fungi detected during the present study, particularly in the *Agaricomycotina* (e.g. *Corticium* sp., *Laccaria* sp., *Schizophyllum commune*) are probably passively transported on the surface of the mosquito body. However, some other detected filamentous fungi (not known as entomopathogenic species) were demonstrated to have a negative impact on mosquito fitness and could be promising candidates for the biological control of mosquitoes. For example, the saprotrophic Ascomycota *Penicillium citrinum* causes mortality of the mosquito *Culex quinquefasciatus* (Maketon et al., 2014). Further experimental studies are needed to disentangle potential interactions between these filamentous fungi and their mosquito host since some species, such as *Talaromyces* sp. and *Penicillium chrysogenum,* are able to interfere with vector competence (Angleró-Rodrígues et al., 2016; Angleró-Rodrígues et al., 2017).

In summary, the present study provides some of the first insights into the geographical patterns of fungal communities associated with the Asian tiger mosquito populations in the field. Our results show that invasive populations from France and Madagascar harbor a lower fungal diversity compared to Vietnamese populations. This observation suggests that similar factors shape the overall composition of the mosquito-associated microbiota during the invasion process as the same loss of diversity was observed for bacteria. Moreover, a rich and diverse fungal community has been identified in adult mosquitoes opening the way to further experimental studies to disentangle potential interactions between those communities and their mosquito host. Such understanding of fungal ecology in a vector and invasive species could bear long-term consequences in the development of effective vector control strategies. To develop and extend such bio-control approaches, a prior knowledge of (i) the fungal communities harbored by field mosquito populations, (ii) the factors likely to influence their

composition and (iii) their potential functions *in insecta* is required. Our study has begun to fill these knowledge gaps by providing a picture of the composition and diversity of fungal communities associated with the tiger mosquito in the field, with the identification of many yeasts species. Interestingly, because they can be genetically engineered, yeasts are considered as promising candidates for paratransgenesis. Additionally, further studies should be undertaken to analyze the mycobiota associated with males as genetic control methods of *Aedes* mosquitoes, such as the sterile insect technique or the incompatible insect technique, are based on population suppression strategies through the release of male mosquitoes (Lees et al., 2015; Zhang et al., 2016).

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Figure legends

Figure 1. Venn-diagram depicting the fungal ITS-2 Operational Taxonomic Units (OTUs) overlap between *Aedes albopictus* specimens sampled in Metropolitan France, Madagascar and Vietnam. A total of 695 fungal OTUs (based on the sequencing of the ITS-2 regions) were detected among the three countries.

Figure 2. Alpha diversity of the fungal communities within field collected *Aedes albopictus*. The alpha-diversity was estimated with the Shannon index for different populations collected in France (NC, Nice; PL, Porte-lès-Valence; SP, Saint-Priest), in Madagascar (MA, Mananjary; TO, Toamasina; TS, Tsimbazaza) and in Vietnam (BD, Bình Dương; HC, Hồ Chí Minh City; VT, Vũng Tàu City).

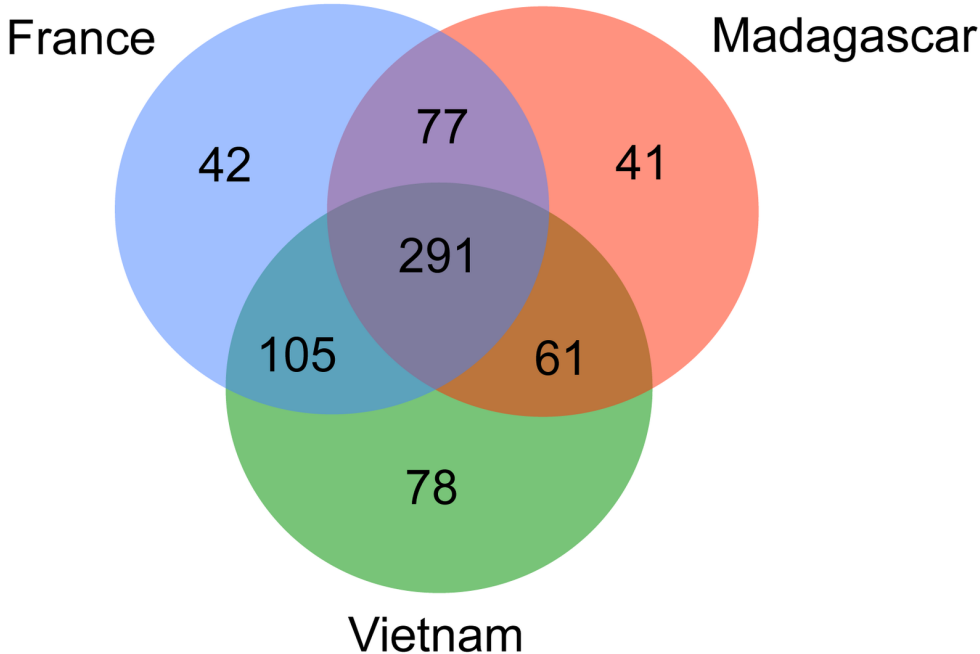
Figure 3. Non-metric multidimensional scaling (NMDS) ordination displaying fungal ITS-2 Operational Taxonomic Units (OTUs) composition across the mosquito specimens collected within three sampling sites in Metropolitan France, Madagascar and Vietnam. NMDS ordination of mosquito samples was based on a Bray-Curtis dissimilarity matrix of the square root-transformed abundance data obtained from sequence counts. Sampling sites in France are Nice (NC), Portes-lès-Valence (PL) and Saint Priest (SP) for which each individual mosquito is symbolized by a green dot. Mananjary (MA), Toamasina (TO) and Tsimbazaza (TS) correspond to the sampling sites in Madagascar for which each mosquito specimen is represented by a red dot. Finally, sampling sites in Vietnam are Bình Dương (BD), Hồ Chí Minh (HC) and Vũng Tàu (VT) for which each individual mosquito is symbolized by a blue dot. For a given country (France, Madagascar or Vietnam), to better distinguish the mosquito samples issued from the three collected sites, the shade of the corresponding colour (green,

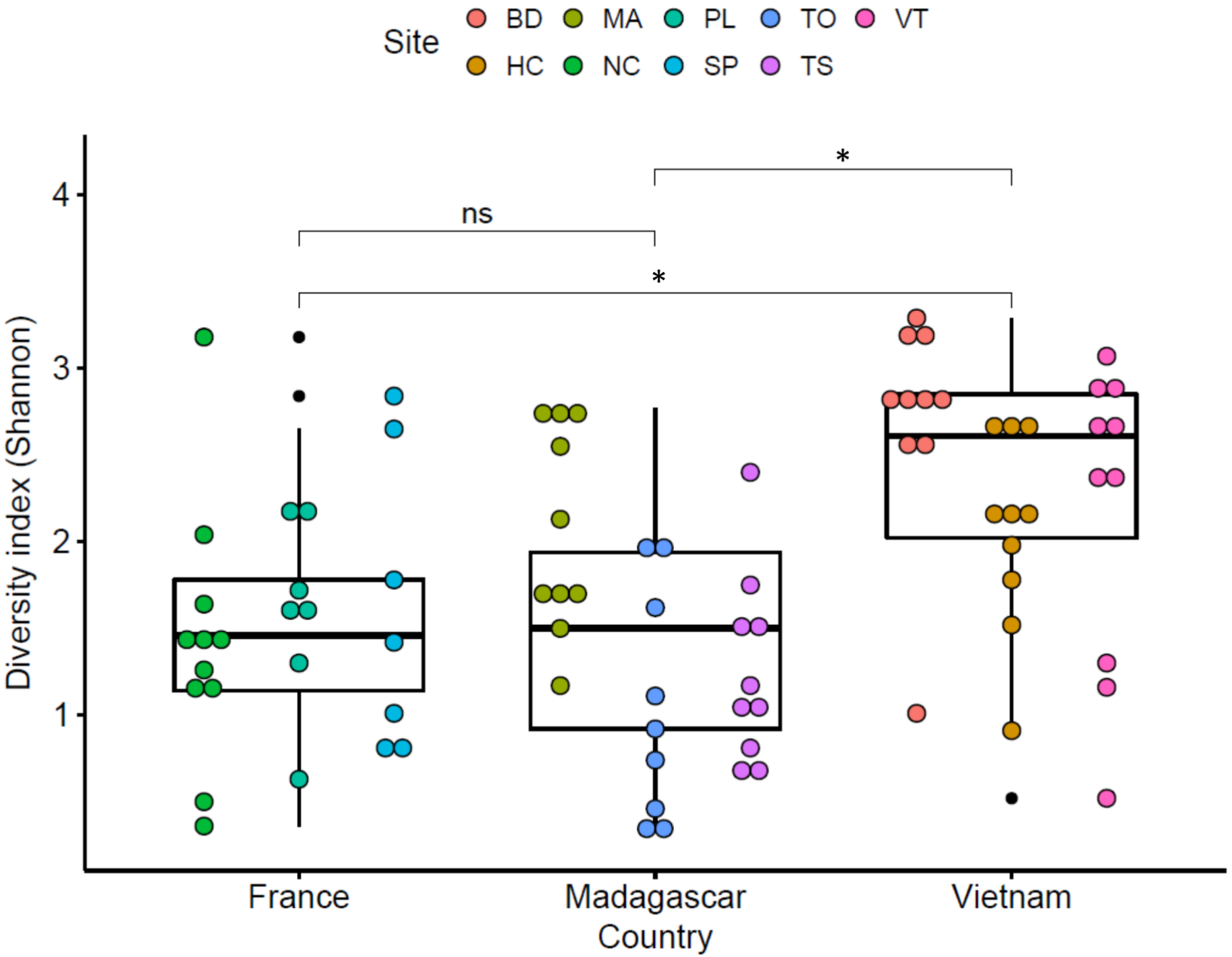
red or blue) is slightly different from a site to another. *stress* = 0.3; $\rho = 0.91$.

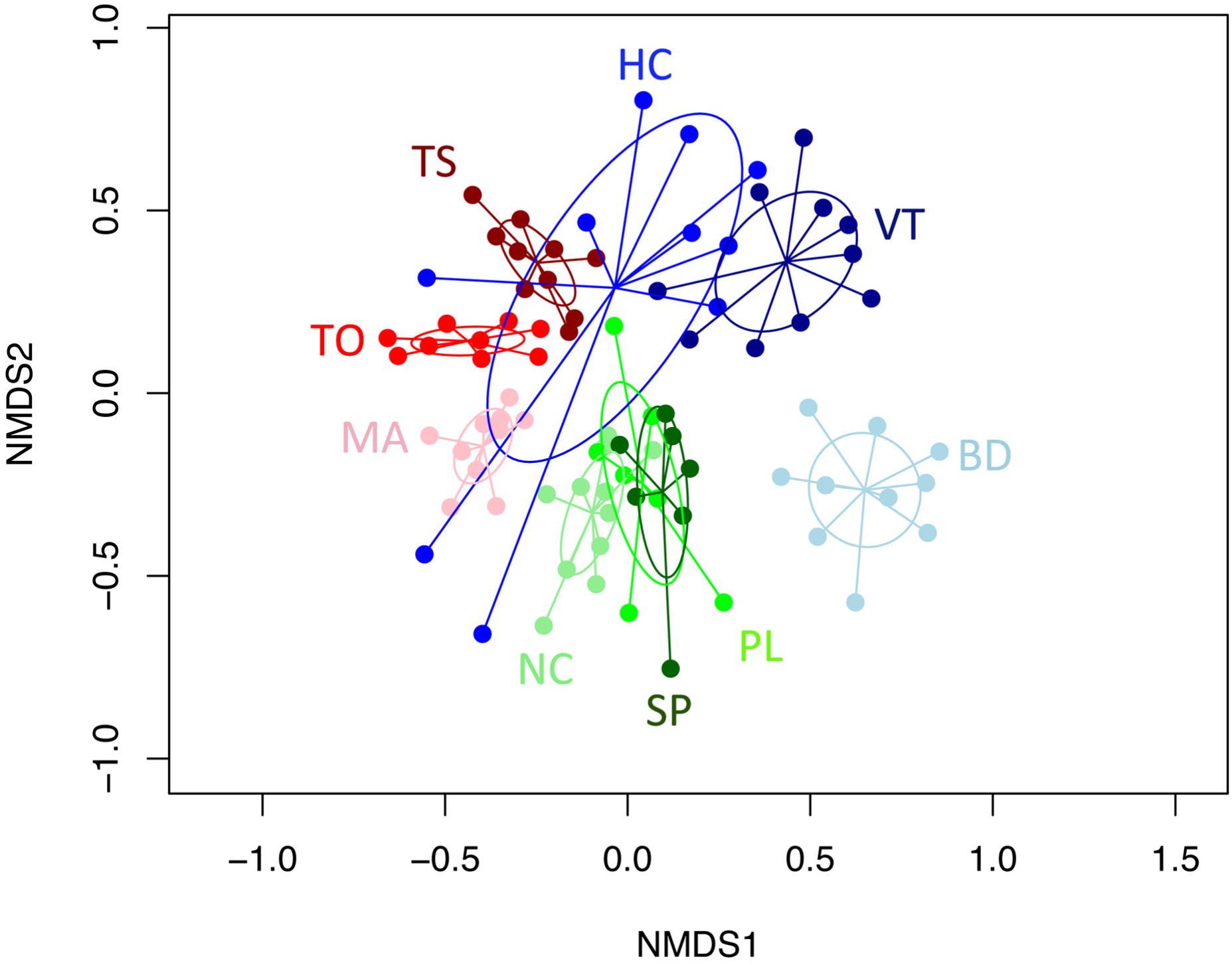
Figure 4. Relative abundance of the fungal taxa at the phylum and subphylum levels. *Aedes albopictus* specimens were collected within the three sampling sites in Metropolitan France, Madagascar and Vietnam. Sampling sites in France are Nice (NC), Portes-lès-Valence (PL) and Saint Priest (SP). Mananjary (MA), Toamasina (TO) and Tsimbazaza (TS) correspond to the sampling sites in Madagascar and the ones in Vietnam are Bình Dương (BD), Hồ Chí Minh (HC) and Vũng Tàu (VT).

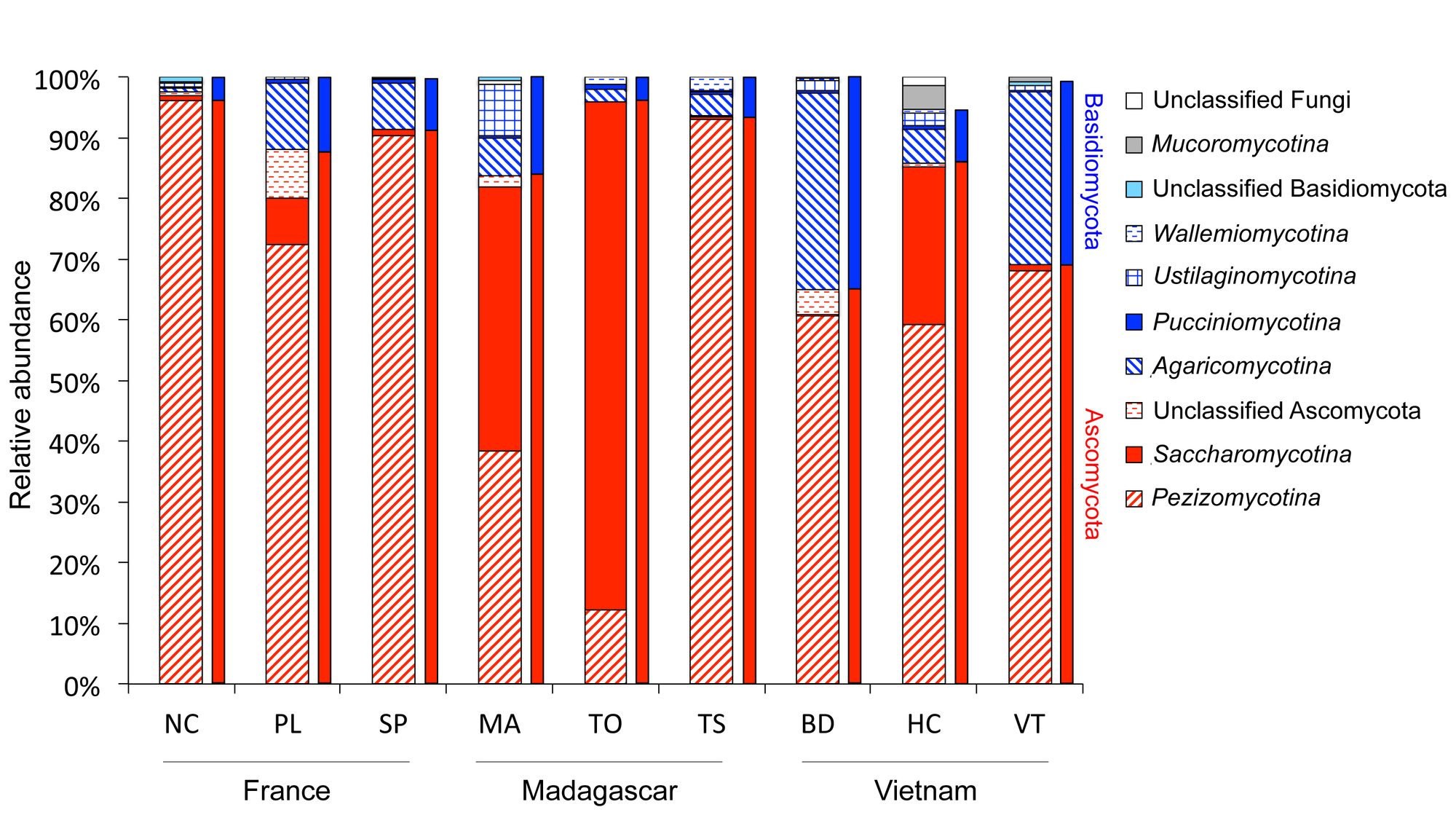
Figure 5. Prevalence and average relative abundance of the fungal OTUs detected in *Aedes albopictus* populations sampled in Metropolitan France, Madagascar and Vietnam. OTUs with a prevalence > 90% across the 84 mosquito samples are highlighted in red and labelled with their OTU ID and their closest matching fungal affiliation.

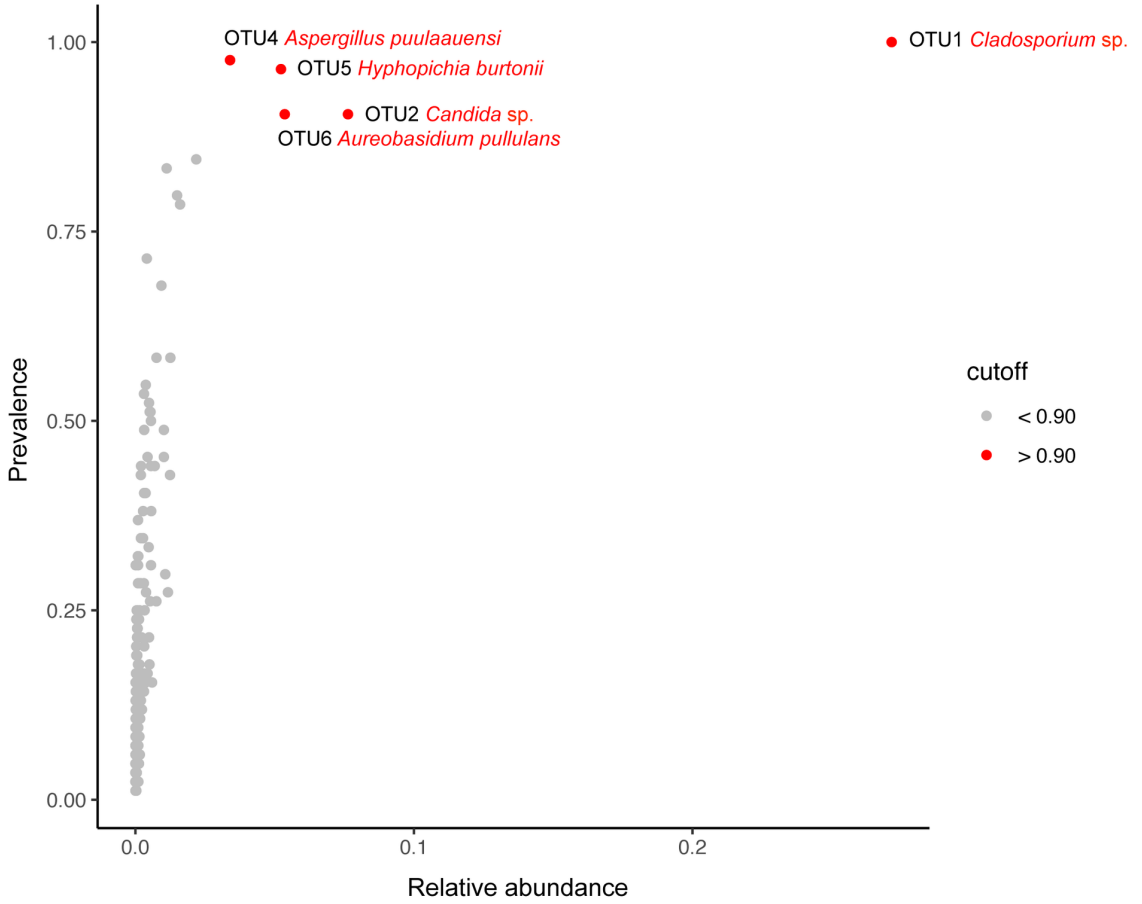
Figure 6. Relative abundance of the most abundant ITS-2 Operational Taxonomic Units (OTUs) detected in *Aedes albopictus* populations sampled in Metropolitan France (F), Madagascar (M) and Vietnam (V). The most abundant OTUs are those that represent > 10% of the total sequences in one sample. Names of sampling sites were abbreviated as follow: Nice (NC), Portes-lès-Valence (PL), Saint-Priest (SP), Mananjary (MA), Toamasina (TO), Tsimbazaza Park (TS), Bình Dương (BD), Hồ Chí Minh City (HC), Vũng Tàu City (VT). All OTUs affiliated to Ascomycota, Basidiomycota and Mucoromycota phylum are presented in red, blue and black respectively. Asterisks indicate yeast species. The dendrogram, obtained by hierarchical clustering, represents the correlations of OTUs abundances among samples.


France
Madagascar
Vietnam
42
77
41
291
105
61
78


Site
BD
MA
PL
TO
VT
HC
NC
SP
TS
ns
*
*
Diversity index (Shannon)
4
3
2
1
France
Madagascar
Vietnam
Country


HC
TS
VT
TO
MA
BD
NC
PL
SP
NMDS1
NMDS2
1.0
0.5
0.0
−0.5
−1.0
−1.0
−0.5
0.0
0.5
1.0
1.5


Relative abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
NC
PL
SP
MA
TO
TS
BD
HC
VT
France
Madagascar
Vietnam
Basidiomycota
Ascomycota
Unclassified Fungi
Mucoromycotina
Unclassified Basidiomycota
Wallemiomycotina
Ustilaginomycotina
Pucciniomycotina
Agaricomycotina
Unclassified Ascomycota
Saccharomycotina
Pezizomycotina


OTU4 Aspergillus puulaauensi
OTU5 Hyphopichia burtonii
OTU2 Candida sp.
OTU6 Aureobasidium pullulans
OTU1 Cladosporium sp.
cutoff
< 0.90
> 0.90
Prevalence
1.00
0.75
0.50
0.25
0.00
0.0
0.1
0.2
Relative abundance

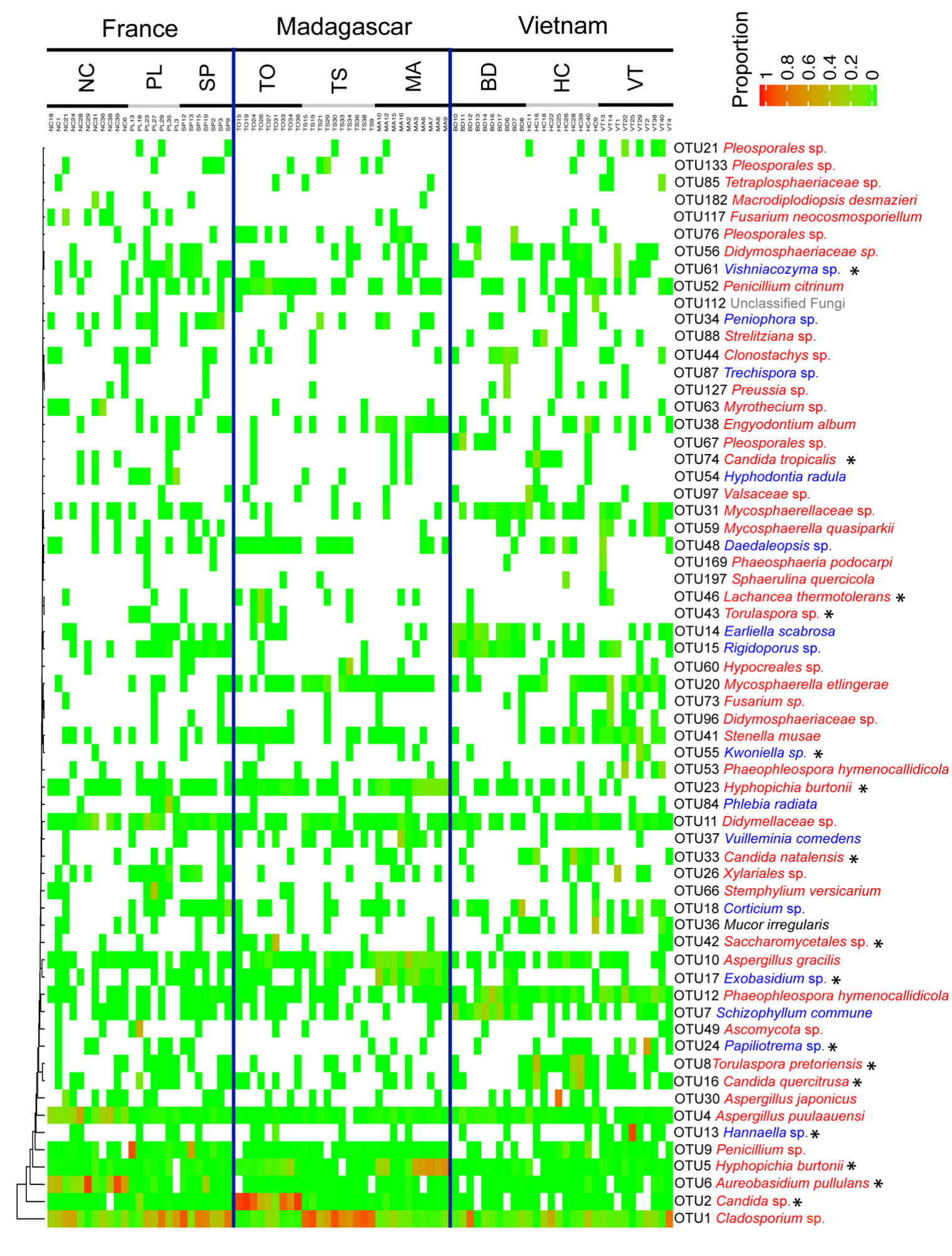

France
Madagascar
Vietnam
NC
PL
SP
TO
TS
MA
BD
HC
VT
Proportion
1
0.8
0.6
0.4
0.2
0
OTU21 Pleosporales sp.
OTU133 Pleosporales sp.
OTU85 Tetraplosphaeriaceae sp.
OTU182 Macrodiplodiopsis desmazieri
OTU117 Fusarium neocosmosporiellum
OTU76 Pleosporales sp.
OTU56 Didymosphaeriaceae sp.
OTU61 Vishniacozyma sp. *
OTU52 Penicillium citrinum
OTU112 Unclassified Fungi
OTU34 Peniophora sp.
OTU88 Strelitziana sp.
OTU44 Clonostachys sp.
OTU87 Trechispora sp.
OTU127 Preussia sp.
OTU63 Myrothecium sp.
OTU38 Engyodontium album
OTU67 Pleosporales sp.
OTU74 Candida tropicalis *
OTU54 Hyphodontia radula
OTU97 Valsaceae sp.
OTU31 Mycosphaerellaceae sp.
OTU59 Mycosphaerella quasiparkii
OTU48 Daedaleopsis sp.
OTU169 Phaeosphaeria podocarpi
OTU197 Sphaerulina quercicola
OTU46 Lachancea thermotolerans *
OTU43 Torulaspora sp. *
OTU14 Earliella scabrosa
OTU15 Rigidoporus sp.
OTU60 Hypocreales sp.
OTU20 Mycosphaerella etlingerae
OTU73 Fusarium sp.
OTU96 Didymosphaeriaceae sp.
OTU41 Stenella musae
OTU55 Kwoniella sp. *
OTU53 Phaeophleospora hymenocallidicola
OTU23 Hyphopichia burtonii *
OTU84 Phlebia radiata
OTU11 Didymellaceae sp.
OTU37 Vuilleminia comedens
OTU33 Candida natalensis *
OTU26 Xylariales sp.
OTU66 Stemphylium versicarium
OTU18 Corticium sp.
OTU36 Mucor irregularis
OTU42 Saccharomycetales sp. *
OTU10 Aspergillus gracilis
OTU17 Exobasidium sp. *
OTU12 Phaeophleospora hymenocallidicola
OTU7 Schizophyllum commune
OTU49 Ascomycota sp.
OTU24 Papiliotrema sp. *
OTU8 Torulaspora pretoriensis *
OTU16 Candida quercitrusa *
OTU30 Aspergillus japonicus
OTU4 Aspergillus puulaauensi
OTU13 Hannaella sp. *
OTU9 Penicillium sp.
OTU5 Hyphopichia burtonii *
OTU6 Aureobasidium pullulans *
OTU2 Candida sp. *
OTU1 Cladosporium sp.

Table 1. Characteristics of sampling sites and number of mosquito *Aedes albopictus* analyzed in the present study.

Country	Region	Site (GPS coordinates)	Climate	Habitat	Number of analyzed females
Metropolitan France	Provence Alpes Côtes d'Azur	Nice (43°41'60''N, 7°17'33''E)	Temperate	Suburban	11
	Rhône-Alpes	Portes-lès-Valence (44°52'8''N, 4°52'9''E)	Temperate	Suburban	11
	Rhône-Alpes	Saint Priest (45°41'49''N, 4°58'50''E)	Temperate	Suburban	11
Madagascar	Vatovavy-Fitovivany	Mananjary (21°13′52″S, 48°20′30.999″E)	Tropical	Suburban	10
	Antsinanana	Toamasina (18°8′59.64″S, 49°24′8.312″E)	Tropical	Suburban	10
	Analamanga	Tsimbazaza Park (18°55′40.395″S, 47°31′38.5″E)	Tropical	Suburban	10
Vietnam	South-East	Bình Dương (10°57'13''N, 106°41'50''E)	Tropical	Suburban	10
	South-East	Hồ Chí Minh City (10°47'19''N, 106°42'19''E)	Tropical	Suburban	11
	South	Vũng Tàu City (10°22'26''N, 107°4'13''E)	Tropical	Suburban	11

Table 2. Multiple comparison of the richness and the α-diversity among the countries.

Response variable	Fixed effect	Multiple comparisons	Estimate	s.e. [(1)]	z-value	P-value [(2)]
Schao index	Country	Madagascar vs. France	0.0026	0.0061	0.443	0.898
		Vietnam vs. France	0.0031	0.0060	0.512	0.866
		Vietnam vs. Madagascar	0.0004	0.0060	0.069	0.997
Shannon index	Country	Madagascar vs. France	-0.0730	0.3137	-0.233	0.971
		Vietnam vs. France	0.7863	0.3128	2.514	**0.032**
		Vietnam vs. Madagascar	0.8593	0.3079	2.791	**0.015**

[(1)]Standard error

[(2)] Adjusted for multiple comparisons (single step method); the sampling site was used as a random effect

Table 3. Analysis of Molecular Variance highlighting the structure of mycobiota dissimilarities.

Levels	**SSD**	**MSD**	**df.**[(1)]	**σ^2**	**P-value**[(2)]
Among countries	3.8040	1.9020	2	0.0146	**0.0374**
Among sites within countries	8.8069	1.4678	6	0.1326	**$<10^{-4}$**
Error	18.0231	0.2403	75	0.2403	

(1) Degrees of freedom

(2) Obtained with 9,999 permutations of the Bray-Curtis distance matrix