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The yeast *Wickerhamomyces anomalus* (*Pichia anomala*) inhabits the midgut and reproductive system of the Asian malaria vector *Anopheles stephensi*

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Summary

While symbiosis between bacteria and insects has been thoroughly investigated in the last two decades, investments on the study of yeasts associated with insects have been limited. Insect-associated yeasts are placed on different branches of the phylogenetic tree of fungi, indicating that these associations evolved independently on several occasions. Isolation of yeasts is frequently reported from insect habitats, and in some cases yeasts have been detected in the insect gut and in other organs/tissues. Here we show that the yeast *Wickerhamomyces anomalus*, previously known as *Pichia anomala*, is stably associated with the mosquito *Anopheles stephensi*, a main vector of malaria in Asia. *Wickerhamomyces anomalus* colonized pre-adult stages (larvae L_1–L_4 and pupae) and adults of different sex and age and could be isolated in pure culture. By a combination of transmission electron microscopy and fluorescent *in situ* hybridization techniques, *W. anomalus* was shown to localize in the midgut and in both the male and female reproductive systems, suggesting multiple transmission patterns.

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Introduction

Studies on the microbiome associated with haematophagus insects have mainly focused on the bacterial component rather than the eukaryotic one. In particular, only a few reports have been published on the yeast microbiota associated with mosquitoes. Ignatova and colleagues (1996) documented the isolation of *Candida* sp. from mosquitoes and more recently *Pichia caribbica* was isolated from the gut diverticulum of the dengue and yellow fever mosquito vector *Aedes aegypti* (Gusmão *et al.*, 2007).

In recent years, the relationship between symbionts and mosquitoes has attracted a great deal of attention, for the perspective of exploiting the symbionts for blocking the transmission of parasites, for example, through the production of antagonistic factors (Favia *et al.*, 2008; Moreira *et al.*, 2009). 'Paratransgenesis' is defined as the genetic manipulation of midgut symbionts to express antiparasite effector molecules that could exert a direct action on the parasite (Riehle and Jacobs-Lorena, 2005). Yeast symbionts would offer an attractive alternative to bacteria for their potential to express antiparasite factors with folding properties suited to eukaryotic targets like the plasmodia.

We recently carried out research aimed at characterizing the microbiota in different mosquitoes involved in malaria transmission and identified bacteria of the genus *Asaia* as symbionts inhabiting the mosquito's midgut and gonads (Favia *et al.*, 2007; Damiani *et al.*, 2008; Crotti *et al.*, 2009). At the microscope level, we observed that, beyond *Asaia* sp., yeast-like organisms were also present in the mosquito midgut and in reproductive systems.

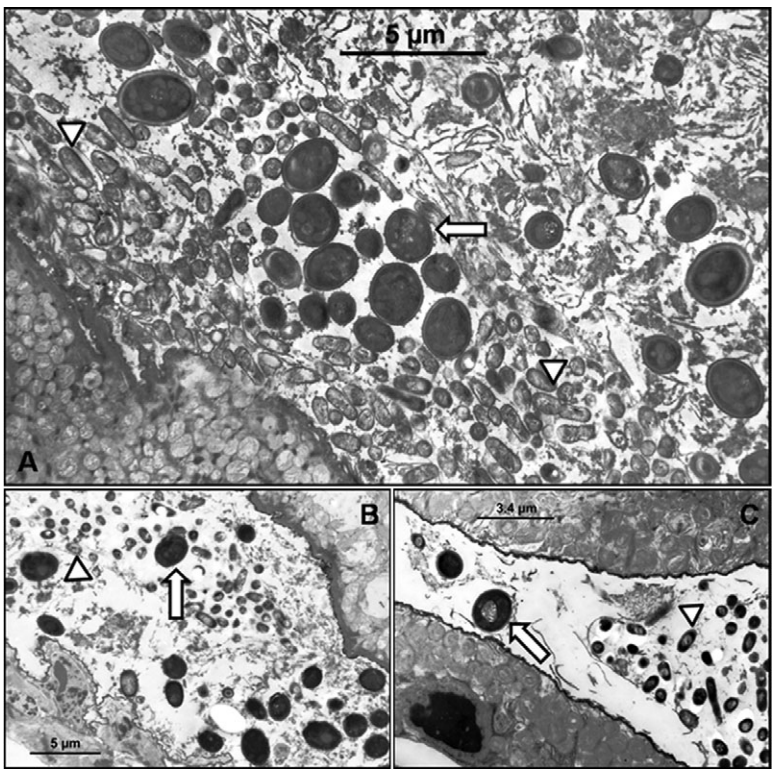

Fig. 1. Transmission electron microscopy micrographs of yeasts in *Anopheles stephensi*. Yeasts (arrows) and bacteria (triangles) are visible in the female mosquito midgut lumen (A) and in the male gonoduct (B, C).

We report here the characterization of the yeasts associated with the malaria vector *Anopheles stephensi* (Diptera: Culicidae), identifying *Wickerhamomyces anomalus* (Saccharomycetales) as an important component of the yeast microbiome in this mosquito species.

Results

Analysis of yeasts in A. stephensi

Transmission electron microscopy analysis performed on mosquito specimens belonging to a laboratory-reared colony of *A. stephensi*, detected yeasts in the female midgut lumen and male gonoduct (Fig. 1). We frequently noticed yeasts in active division, especially at the level of the male reproductive system (Fig. 2), revealing that mosquito's tissues represent a favourable environment for these organisms. In order to identify these yeasts, we carried out an analysis of the mosquito-associated yeast community on both male and female individuals, using molecular and culture-dependent methods.

We identified six and three fungal clades/species, respectively, by culture-independent (Table 1) and plating analysis (Table 2). Twenty-two out of 34 sequences obtained by polymerase chain reaction (PCR)-based analysis from whole insects were phylogenetically related to *Candida* sp., *Pichia* sp. or *W. anomalus* according to 18S rRNA gene sequence analysis (Table 1). Twelve additional sequences matched with uncultured fungi from environmental samples and the fungal species *Wallemia sebi*, rarely associated with arthropods and never with insects. *Wickerhamomyces anomalus*-related sequences appeared to dominate in the gut followed by *Pichia* sp.

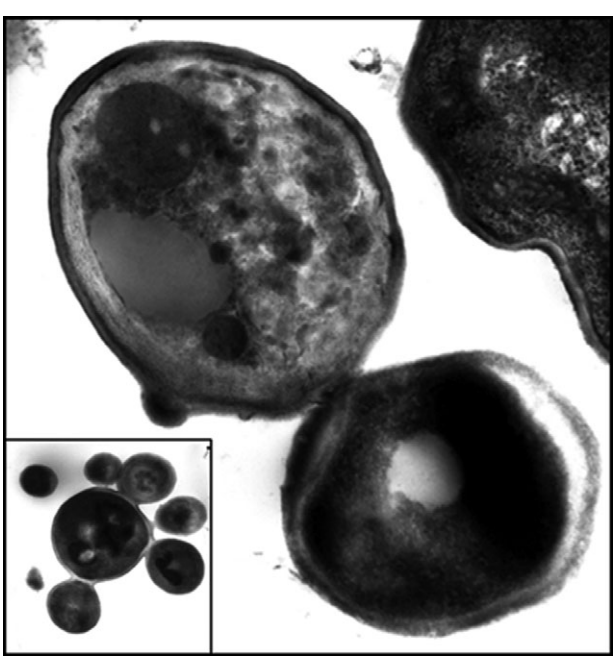

Fig. 2. Transmission electron microscopy micrographs revealing budding yeasts in active division in *Anopheles stephensi* male gonoducts.

Table 1. Fungal sequences obtained from *Anopheles stephensi* by PCR-based screening with oligonucleotides designed on conserved sequences of 18S rRNA.

	N. of clones/total clones for each DNA preparation									
	Female individuals			Male individuals			Pools of female guts[b]			
Fungal identification (% identity[a])	1	2	3	1	2	3	1	2	3	N. of clones/ total clones
Candida sp. AY520257 (94–98%)	3/9	1/6	2/4	1/7	1/6	2/2	1/6	1/4	0/2	12/46
Pichia sp. AB022439 (95–98%)	2/9	1/6	1/4	1/7	3/6	0/2	2/6	1/4	0/2	11/46
Wickerhamomyces anomalus EF427893 (96–100%)	1/9	1/6	0/4	1/7	1/6	0/2	3/6	2/4	2/2	11/46
Wallemia sebi AF548107 (99–100%)	1/9	1/6	0/4	1/7	0/6	0/2	0/6	0/4	0/2	3/46
Uncultured fungus AM114819 (98–99%)	1/9	1/6	1/4	2/7	1/6	0/2	0/6	0/4	0/2	6/46
Uncultured fungus AB248107 (96–98%)	1/9	1/6	0/4	1/7	0/6	0/2	0/6	0/4	0/2	3/46

a. % identity range values of clones are reported per each fungal identification.
b. Each preparation consists of a pool of five guts.
Forty-six clones have been listed into six groups: *Candida* sp., *Pichia* sp., uncultured fungus (AM114819), uncultured fungus (AB248107), *Wallemia sebi* and *Wickerhamomyces anomalus* (total sequences and numbers of identified clones are reported per each group and sample).

and *Candida* sp. (Table 1). Analysis of *Candida* sp. and *Pichia* sp. sequences did not allow the identification at the species level.

Ten newly emerged lab-reared individuals (five males and five females) were examined for the diversity of the associated yeasts by cultivation. A total of 100 isolates have been collected and clustered according to the colony morphology and identified by sequencing the 18S–26S rRNA internal transcribed spacer (ITS) and the D1/D2 domain of the 26S rRNA gene. Three yeast species have been identified: 77 isolates as *Candida intermedia*, 15 as *Hanseniaspora uvarum* and 8 as *W. anomalus* (Table 2).

The relatively small number of yeasts identified by both cultivation-dependent and independent approaches could reflect a limited diversity in the studied lab-reared colony (Rani *et al*., 2009) or could be due to the technical limitations of the approaches used, such as the choice of the cultivation media or the PCR primers (Zacchi and Vaughan-Martini, 2002; Hong *et al*., 2009).

The isolation of some *Candida* sp. from different mosquito species has been already documented (Ignatova *et al*., 1996). *Candida intermedia*, has been previously isolated from the gut of other insects (Nguyen *et al*., 2007). Yeast clades belonging to *Pichia* sp. have been previously associated to the intestinal tract of various insects (Nakase *et al*., 2007; Rao *et al*., 2007) including mosquitoes (Gusmão *et al*., 2007; 2010). However, *Candida* and *Pichia* appear to be ubiquitous in insects and were not associated with a specific host, which may suggest a non-exclusive relationship with mosquitoes. None of the sequences obtained by PCR-based analysis matched to the genus *Hanseniaspora*, even though we identified several isolates on plate as *H. uvarum*. *Hanseniaspora* strains have been isolated from insects belonging to different species (Pérez *et al*., 2003; Nguyen *et al*., 2007) supporting a wide distribution of these yeasts too.

Wickerhamomyces anomalus was identified both by molecular ecology analyses and by isolation. It was detected in both entire male and female mosquitoes, and in the female gut, where it has been revealed in all the samples. The observation that *W. anomalus* is not widely distributed among insects or insect habitats (Zacchi and Vaughan-Martini, 2002), suggested us that it may represent a symbiont specifically associated with *A. stephensi* and not just a transient commensal.

Prevalence of W. anomalus *in* A. stephensi *and localization in mosquito organs*

Insects reared in near-axenic conditions have been analysed by both specific PCR tests and fluorescence *in situ* hybridization (FISH) for further investigating the presence of *W. anomalus*. A survey using a semi-nested PCR assay specific for *W. anomalus* was performed on *A. stephensi* individuals from the laboratory colony. Pre-adult stages and adults of different sex and age from three mosquito

Table 2. Yeast species associated to *Anopheles stephensi* identified following isolation in pure culture.

		GenBank Accession N. (% identity)	
N. isolates	Closest relative	ITS	D1/D2
77	*Candida intermedia*	FJ515166, (98–99%)	FJ455102, (99–100%)
15	*Hanseniaspora uvarum*	FJ515178, (99%)	GU585885, (99–100%)
8	*Wickerhamomyces anomalus*	EU330185, (99–100%)	FM178296, (98–100%)

The percentages of identity of 18S–26S rRNA ITS and D1/D2 domain of the 26S rRNA gene are presented.

Table 3. Prevalence of *Wickerhamomyces anomalus* in different developmental stages and organs of *Anopheles stephensi* revealed by specific semi-nested PCR.

DNA source	Developmental stages (days post emergence)	N. samples analysed per mosquito generation	N. of generations examined	Positives/total
Whole mosquitoes	Larvae-L_1	1 pool (15–20 individuals)	3	3/3 pools
	Larvae-L_2	1 pool (10 individuals)	3	3/3 pools
	Larvae-L_3	1 pool (5 individuals)	3	3/3 pools
	Larvae-L_4	15 individuals	3	30/45 individuals
	Pupae	15 individuals	3	29/45 individuals
	Adults (0 day)	50 individuals (25♀, 25♂)	3	107/150 individuals
♀ Guts	Adults (2 days)	1 pool (5 guts)	3	3/3 pools
	Adults (10 days)	1 pool (5 guts)	3	3/3 pools
♂ Guts	Adults (2 days)	1 pool (5 guts)	3	3/3 pools
	Adults (10 days)	1 pool (5 guts)	3	3/3 pools
♀ Gonads	Adults (2 days)	1 pool (7 gonads)	3	3/3 pools
	Adults (10 days)	1 pool (7 gonads)	3	3/3 pools
♂ Gonads	Adults (2 days)	1 pool (7 gonads)	3	3/3 pools
	Adults (10 days)	1 pool (7 gonads)	3	3/3 pools

Whole adult mosquitoes, larvae-L_4 and pupae have been tested individually, the earlier larval stages (L_1, L_2, L_3) and dissected organs have been analysed as pools. Larval breeding water and adult sucrose solution have been used as negative controls for each PCR run. Number of dissected organs corresponded to 48 per generation and total of 144 mosquitoes.

generations were tested (Table 3). We examined larval stages L_1–L_3 and pupae as pools, and L_4 larvae and male and female adults as single individuals. Adults of different ages (0, 2 and 10 days post emergence) were examined. The presence of *W. anomalus* was confirmed throughout the mosquito development and in the adult insect up to at least 10 days post emergence. To establish the prevalence of *W. anomalus* in adults, both female and male mosquitoes were sampled almost immediately after emergence and before sucrose solution was added to the cage. PCR experiments tested positive on all larvae and pupae pools (Table 3). The presence of *W. anomalus* was confirmed by PCR in 69% of the specimens tested individually, i.e. 30 positive L_4 larvae out of 45, 29 out of 45 pupae, 107 out of 150 adults (Table 3).

Finding *W. anomalus* in newly emerged non-fed adults excluded the possibility that the yeast symbiont was acquired through food consumption or following a protracted permanence in the insectary. To clarify if the yeast can enter the environment and be ingested by the larvae, the breeding water was screened by the *W. anomalus*-specific PCR and tested negative.

Polymerase chain reaction analysis to identify yeast localization in specific tissues was performed on dissected organs from young (2 days post emergence) and old (10 days post emergence) adult mosquitoes in order to check for the stability of the association (Table 3). Newly emerged mosquitoes were allowed to feed on sterile sucrose solution for 2 days to eliminate meconium, thus preventing tissue contamination from possible leaking of the intestinal content during the organ dissection. A total of 24 organ preparations (eight per generation) were analysed consisting in guts and gonads from both males and females. *Wickerhamomyces anomalus* was detected in all the organ preparations analysed (Table 3). Particularly, the presence of the yeast in the gonads reinforced other evidences, i.e. detection in pre-adults and in newly emerged mosquitoes, that *W. anomalus* is not an occasional environmental contaminant and suggest that it could possibly undergo a vertical transmission route.

Wickerhamomyces anomalus localization in tissues found by semi-nested PCR analysis was confirmed by FISH experiments. FISH analysis indicated the localization of the yeast in the midgut of both female and male mosquitoes (Fig. 3) and in their gonads (Figs 4 and 5). *Wickerhamomyces anomalus* was particularly abundant in both the ovaries (Fig. 4) and the male reproductive system, displaying a wide distribution throughout the male apparatus, including testes, gonoducts and accessory glands (Fig. 5). Analysis of non-fed newly emerged individuals showed a weak fluorescence signal in comparison with older individuals (10 days post emergence), supporting the hypothesis that the yeast benefits of nutrients from the mosquito diet.

Discussion

The present study was conceived after transmission electron microscopy observation of yeasts in the gut and the reproductive system of *A. stephensi*. It was indeed their presence in the mosquito reproductive apparatus that suggested a specific association with the insect host, rather than an occasional presence, related for example to acquisition with food, or to other forms of acquisition from the environment. The subsequent molecular, microbiological and microscopy work that we performed on a laboratory-reared colony of *A. stephensi* confirmed that at

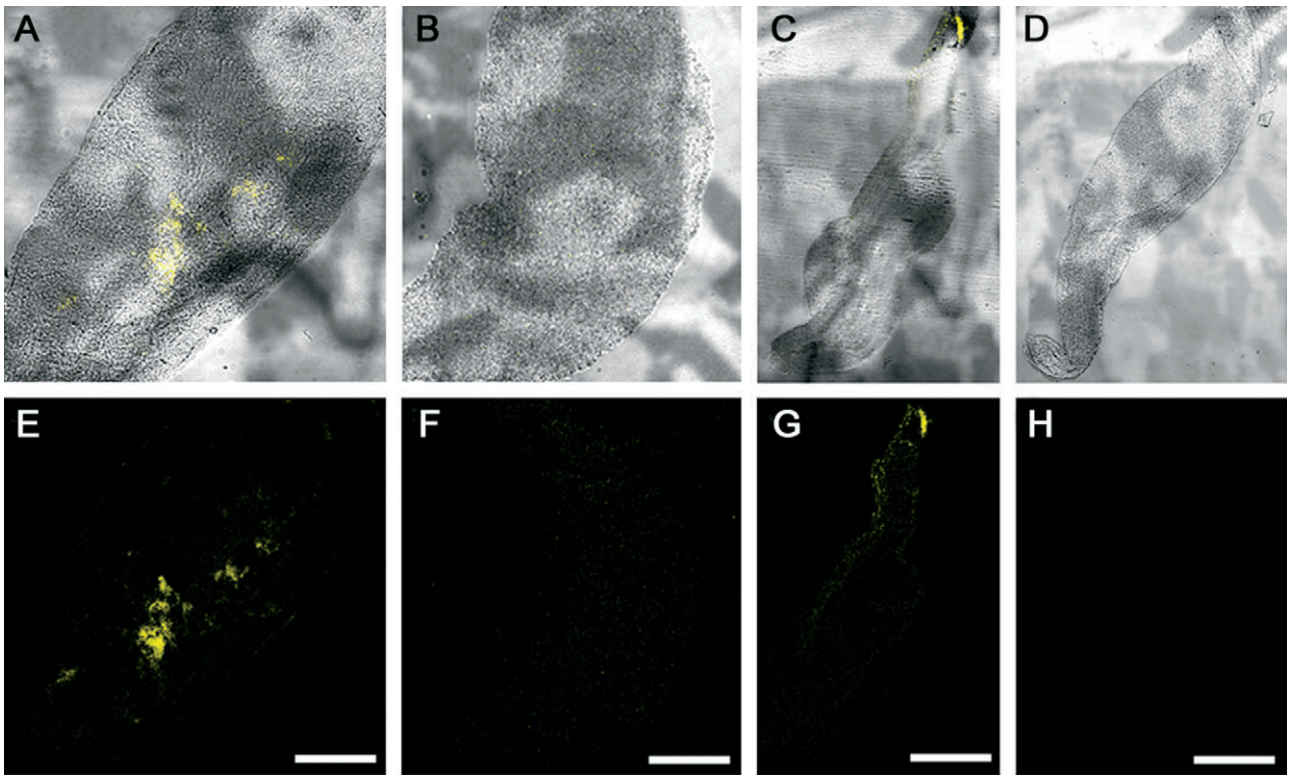

Fig. 3. FISH localization of *Wickerhamomyces anomalus* in *Anopheles stephensi* female (A, B) and male (C, D) midguts 10 days post emergence, observed by confocal laser scanning microscope analysis revealing bright signals (E, G). No fluorescence has been observed in the controls in the absence of the probes (F, H). Bars correspond to 210 μm in A, B, E, F and to 140 μm in C, D, G, H.

least one yeast species, *W. anomalus*, is specifically associated with these mosquitoes. The evidence for the association between *W. anomalus* and *A. stephensi* derives from the following results: (i) *W. anomalus* was identified by molecular and cultivation-based methods, (ii) *W. anomalus* was PCR-detected in mosquitoes from all development stages, with an average prevalence of about 70% in individuals tested at different ages and from different generations and (iii) *W. anomalus* was detected by using specific FISH probes in both male and female guts and reproductive systems.

The ascomycete *W. anomalus*, previously known as *Pichia anomala* (Kurtzman *et al*., 2008), is an heterothallic yeast, forming one to four shaped ascospores (Hansen, 1904), belonging to the so-called non-*Saccharomyces* wine yeasts (Rojas *et al*., 2003). Fredlund and colleagues (2002) reported that *W. anomalus* is physiologically versatile being capable of growing under a quite large pH range, under high osmotic pressure and anaerobic conditions, while it shows low tolerance to ethanol and acetate (Kalathenos *et al*., 1995).

The different portions of the gastrointestinal tract of adult mosquitoes have different pH according to the specific compartments. For example, pH is acidic in the lumen of the ventral diverticulum, named crop, that enlarges into the abdomen and which stores the sugar meal before slowly moving it to the midgut for the digestion step (Thompson, 1905). Moreover, *Anopheles* species are known to host in the gut acetic acid bacteria like *Asaia* (Favia *et al*., 2007; 2008; Crotti *et al*., 2009; Damiani *et al*., 2010) whose metabolism converts sugar to acids and that are capable to decrease the pH of the medium down to 3. Considering the quite wide variations of the environmental conditions in the gastrointestinal tract like pH, redox potential, ionic strength and osmolarity, microbial symbionts living there should be capable to tolerate such environmental gradients. Yeasts like *W. anomalus*, that are capable of resisting different pH, salinity and redox conditions, have high potential to be selected for living in the mosquito gut.

Wickerhamomyces anomalus has been isolated from a wide variety of habitats (Kurtzman and Dien 1998) and is considered a common spoilage organism in several products such as beer, silage, baking and dairy products (Kagiyama *et al*., 1988; Kitamoto *et al*., 1999; Dufour *et al*., 2003; Passoth *et al*., 2006). Although substantial work on the physiology and genetics of this yeast has been carried out (Passoth *et al*., 2006), there is a lack of information about its relationship with invertebrate organisms. In 2001, Kurtzman isolated this yeast from insect

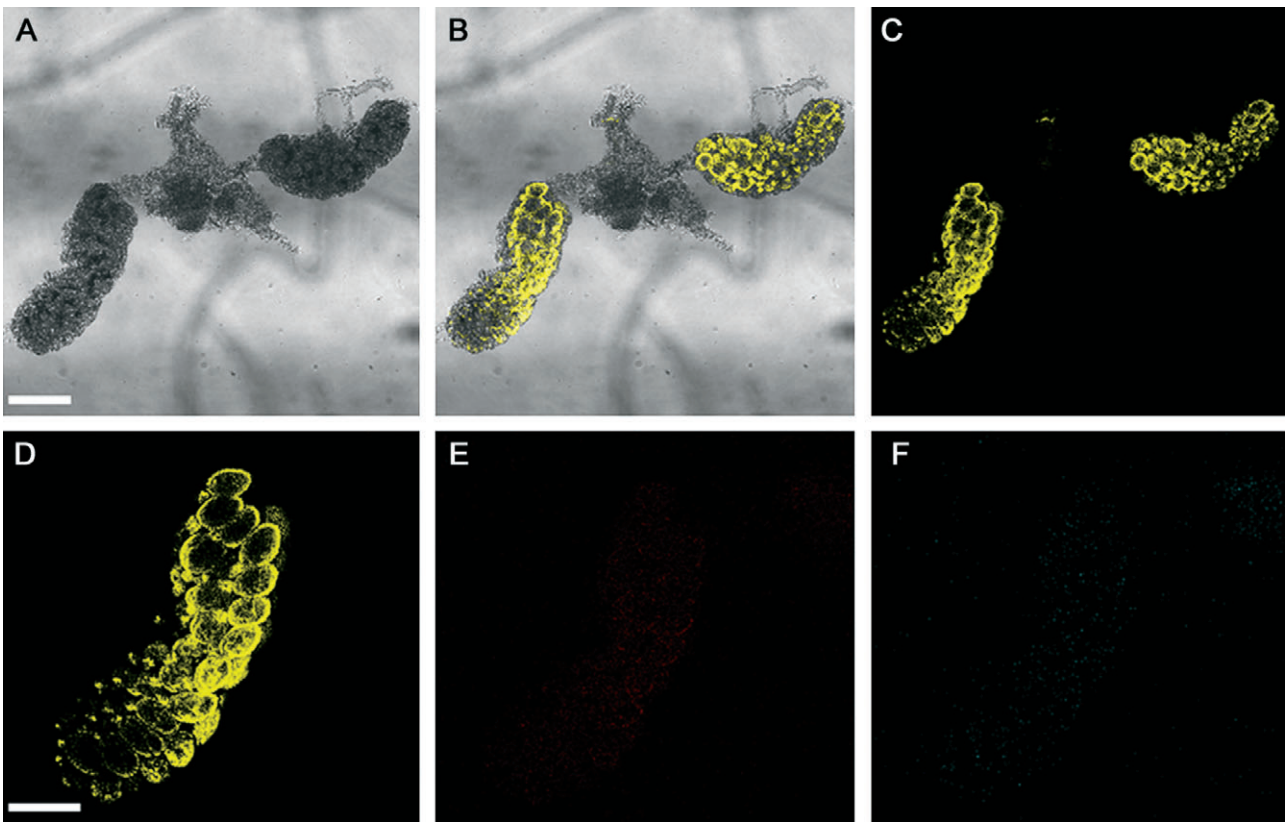

Fig. 4. FISH localization of *Wickerhamomyces anomalus* in *Anopheles stephensi* female gonad (A), observed by confocal laser scanning microscope analysis, revealed bright signals (B, C). Magnification of an ovary portion (D–F) showed the presence of a strong fluorescence due to the *W. anomalus*-specific probes (D) and the absence of autofluorescence in the observed organ (E, F). Bars correspond to 280 μm in A–C and to 140 μm in D–F.

frass from a Ponderosa pine (Kurtzman, 2001), and, more recently, a marine strain of *W. anomalus* has been identified as a killer yeast, acting against pathogenic fungi in the crab *Portunus trituberculatus* (Wang *et al*., 2007). Interestingly, a previous large screening for yeasts associated with different insects led to the detection of *W. anomalus* only in the inner body of *Drosophila* sp. (Zacchi and Vaughan-Martini, 2002). These authors suggested that while other Saccharomycetales (such as *Pichia guilliermondii*, *P. membranifaciens*, etc.) have an ubiquitous distribution among different insect species, not being associated with specific hosts, *W. anomalus*, having a more restricted distribution, could play a more specific biological role within the insect. Indeed, according to the wide environmental distribution of *W. anomalus*, that include plant material and natural sugar solutions, it might share environmental niches with larvae and adult mosquitoes, increasing the chance to be exposed to the insect body surface or to be ingested with the sugar-based meals.

The combination of the niche preference by *W. anomalus*, together with its capacity to retain growth capability under the variable environmental conditions encountered in the mosquito gastrointestinal tract, makes this yeast pre-adapted to colonize the mosquito host body. The finding of *W. anomalus* not only in the gastrointestinal tract, but intimately associated to the mosquito reproductive systems, suggests that this yeast is not only ecologically pre-adapted, but can use some of its biological properties for moving among the different body compartments of the host, and has the capacity to escape the host immune barriers.

The presence of this yeast in mosquito and its location in specific organs raise questions on its functional role in the insect. Gut and gonads are characterized by the presence of nutrients available for yeasts and they represent excellent niches for nutritional mutualism, with supplementation of substances missing in carbohydrate diet of adult mosquitoes, as already described for other insects (Gibson and Hunter, 2009). Among the possible roles played by a symbiont like *W. anomalus* that should be explored in the future are the provision of nutrients to the host and its protection against pathogens (Dillon and Dillon, 2004; Moran *et al*., 2008). Considering that marine strains of *W. anomalus* have already been proposed to protect the host crab against pathogens (Wang *et al*.,

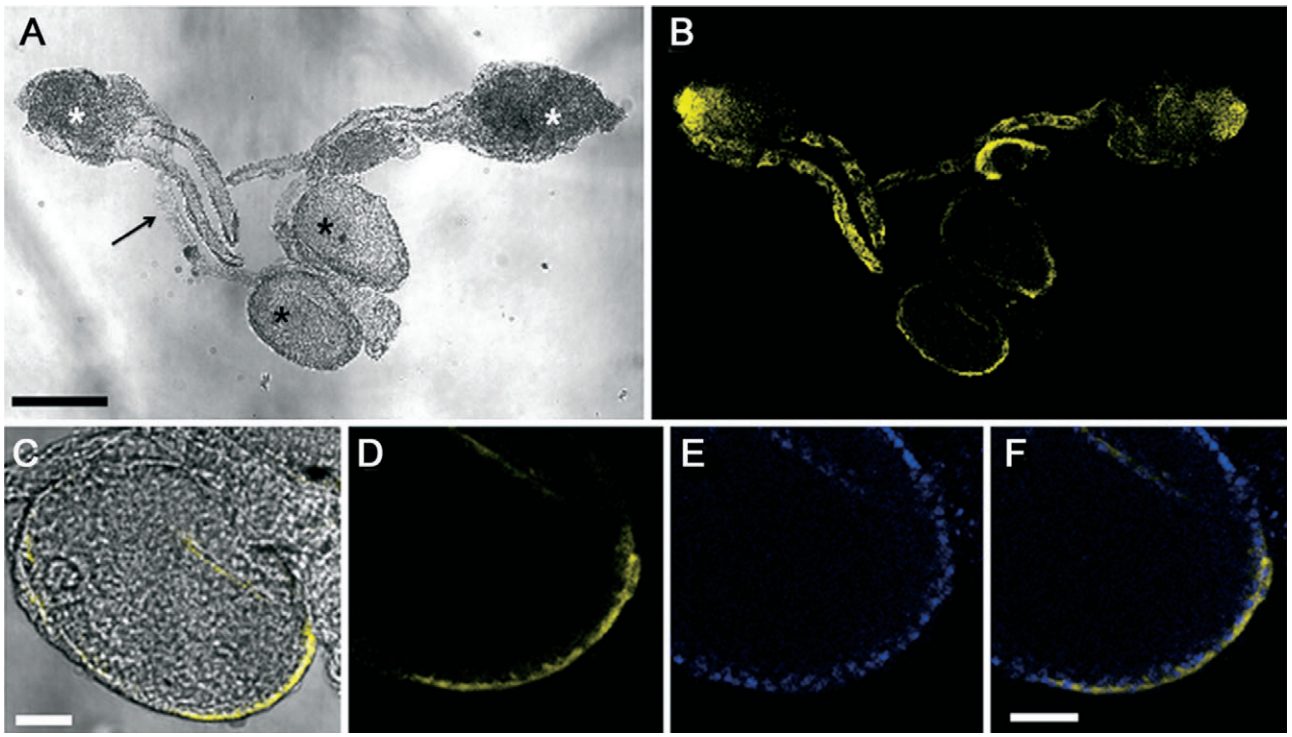

Fig. 5. FISH localization of *Wickerhamomyces anomalus* in *Anopheles stephensi* male reproductive system (A), observed by confocal laser scanning microscope analysis, reveals bright signals (B). White and black asterisks indicate testes and accessory glands respectively; arrow indicates gonoducts. Magnification of an accessory gland (C, D, F) showed a precise localization of the yeast (in yellow) at the periphery of the organ, as deduced also by DAPI staining (blue) of the nuclei (E, F). Bars correspond to 230 μm in A, B and to 30 μm in C–F.

2007; 2008), such a possibility should be also considered for *A. stephensi*.

Several reports indicate that bacteria and yeasts can localize both in the gut and in the reproductive apparatus of insect hosts (Noel and Atibalentja, 2006; Favia *et al*., 2007; Bressan *et al*., 2009). The localization of *W. anomalus* in the reproductive organs suggests a vertical transmission route through generations. Such a possibility is supported by the occurrence of *W. anomalus* in specimens from all life stages. Other transmission routes among adults could obviously be envisioned, including venereal transmission, as already reported for bacterial symbionts in *A. stephensi* (Favia *et al*., 2007; Damiani *et al*., 2008). Until a few decades ago, horizontal transmission was regarded as the most common route of transmission for yeasts associated with insects (Hagen *et al*., 1970). Several cases of yeast–insect associations have now been described, and maternal transmission of these symbionts is now frequently reported (Gibson and Hunter, 2005; 2009; Sacchi *et al*., 2008).

Experimental procedures

Laboratory-reared mosquitoes

Analysed mosquitoes were obtained from a colony of *A. stephensi* (Liston) reared for tens of years in the insectary of the Laboratory of Parasitology of the University of Camerino (Italy). They were maintained at standard setting of 30°C and 80 ± 5% humidity, in near-axenic conditions during both the developmental and adult stages. After eggs eclosion, larvae were grown in clean tanks filled with deionized water containing sterile minced commercial mouse food. After rinsing, the pupae were transferred to clean dishes filled with sterile water and maintained in separate cages until adult emergence. Newly emerged mosquitoes not immediately analysed fed on sterilized 5% sucrose solution supplied on a hygienic cotton pad (renewed every 8 h) for 2 days, thus allowing complete meconium evacuation before tissue dissection. Tissues from older mosquito (10 days post emergence) were analysed as well. Dissected organs were utilized for both DNA extraction and FISH analysis.

Transmission electron microscopy

Adults of *A. stephensi* were dissected in saline. Organs were harvested and prefixed in Karnovsky in cacodylate buffer (pH 7.2) (dos Santos and Gregório, 2002). After post-fixation in 2% OsO_4 for 1.5 h, samples were washed in cacodylate buffer, dehydrated through an ethanol series, transferred in propylene oxide and embedded in Epon 812. Semi-thin sections were stained with 1% borate methylene blue and examined by light microscope. Thin sections were stained with saturated uranyl acetate, followed by Reynolds lead citrate and examined with a Zeiss EM 900 transmission electron microscope at 80 kV.

DNA extraction

Before DNA extraction, adults mosquitoes were carefully washed as follows in order to sterilize the insect surface: samples were rinsed sequentially in sodium hypochlorite (1%), chloridric acid (0.5%), washed in 1× PBS between treatments and finally rinsed with 70% ethanol. Alcohol evaporation was allowed before further processing of the samples. In case of pre-adult stages, the specimens have been washed several times using 1× PBS and finally distilled water. DNA extraction was performed both from whole insects (all developmental stages) and adult specific organs. Dissections were carried out using sterile needles under a stereomicroscope, on a slide rinsed with a drop of sterile 1× PBS. To avoid cross-contamination among tissues, one anatomic portion from a single mosquito (gut or gonads) was recovered. DNA extraction was conducted under sterile conditions in a sterile cabinet (Bioair, Euroclone, Italy). Samples were homogenized in 50 μl of digestion solution (50 mM EDTA and 1.25 μl of lyticase 0.5 mg ml^{-1}) using sterile polypropylene pestles and incubated over night at 37°C. After incubation, 100 μl of extraction buffer (1% SDS, 50 mM Tris-HCl pH 8, 25 mM NaCl, 25 mM EDTA pH 8) was added to the samples and placed 30 min at 65°C. Then 14 μl of 8 M potassium acetate pH 7.2 was added; the homogenates were placed on ice for 30 min and then centrifuged for 10 min at 13 000 r.p.m. DNA was precipitated in 100% ethanol. DNA pellets were dried and then rehydrated in 50 μl of ultra-pure water for PCR applications and the nucleic acid concentration has been estimated by spectrophotometer NanoDrop 1000.

Yeast identification by PCR-based analysis

Polymerase chain reaction screening for mosquito-associated yeasts was performed using the primers yeast-F1 (5′-TAATTCTAGAGCTAATACATG-3′) and yeast-R1 (5′-GGAAACGTCCTTGGCAAA-3′) designed on conserved sequences of the locus 18S rRNA resulting from the alignment of yeasts from different orders. The genomic DNA from six whole mosquitoes (three males and three females) and three female gut preparations (five guts each) was used as template for the PCR amplifications; to avoid meconium leak, guts were dissected 2 days post emergence, whereas whole mosquitoes were analysed at the emergence and before the meal. Each PCR reaction was carried out, under sterile conditions, in a total volume of 25 μl containing: 1× PCR Gold Buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 μM of each primer, 1 U Ampli Taq Gold (Applied Biosystems, USA) and 10 ng template DNA. PCR was carried on a Veriti Thermal Cycler (Applied Biosystems, USA) under these conditions: 94°C/10 min; 30× (94°C/30 s, 50°C/45 s $\Delta t + 0.2$°C, 72°C/45 s); 72°C/10 min. After amplification, 5 μl of PCR products were visualized by electrophoresis on ethidium bromide agarose gel (1.5%) under UV light. Remaining volumes were purified by centrifugation in 1 V of isopropanol, rehydrated in 10 μl of water and cloned using the pGem-T Easy Vector System kit (Promega, USA) in *Escherichia coli* JM109 competent cells. Purified recombinant plasmids were sequenced at the MWG-Genomic Company (Ebersberg, Germany). Sequences were compared with the database at the National Centre for Biotechnology Information by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Yeast isolation and characterization

To isolate yeasts associated with *A. stephensi*, two different media, universally used for the isolation and cultivation of yeasts, were used: YM agar medium (Difco, Milano, Italy) and modified PDB liquid medium (glucose 20 g l^{-1}, yeast extract 10 g l^{-1}, peptone 10 g l^{-1}, potato extract 8 g l^{-1}, pH 5.7). Ten newly emerged non-fed mosquitoes (five females and five males) were surface sterilized in 100% ethanol and washed twice in 0.9% NaCl. The insects were individually homogenized, with a sterile pestle, by grinding in 200 μl of 0.9% NaCl. Approximately 100 μl of each homogenate was spread directly on YM agar plates supplemented with rifampicin (40 μg ml^{-1}) to avoid bacterial growth. The plates were incubated overnight at 30°C. The remaining half homogenate was inoculated in modified PDB medium and grown overnight at 30°C. After growth, the microorganisms were spread on YM agar and incubated overnight at 30°C. Yeast colonies were categorized and selected based on colony morphology and 100 pure cultures of yeast strains were stored in 15% glycerol at −80°C.

18S–26S rRNA ITS and 26S rRNA gene analyses were carried out for all the isolated yeasts. ITS fragments covering ITS1, 5.8S rRNA gene and ITS2; together with the D1/D2 region of 26S rRNA gene are usually used for yeast identification (Manter and Vivanco, 2007; Ferreira *et al*., 2010). For the amplification of ITS fragment, the primers used were ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) under the following amplification conditions in a final volume of 50 μl: 1× *Taq* polymerase buffer, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer and 2 U of *Taq* DNA polymerase (Invitrogen, Milan, Italy). Thermal conditions of PCR reactions were as follows: a denaturation step of 7 min, followed by 35 cycles of 45 s at 94°C, 45 s at 55°C and 1 min at 72°C, with a final extension step of 10 min at 72°C (Sun *et al*., 2009). PCR amplification of the D1/D2 domain of 26S rRNA gene was carried out using primers NL1 (5′-GCATATCAATAAGCG GAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGAC GG-3′). PCR was performed using 1× *Taq* polymerase buffer, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer and 2 U of *Taq* DNA polymerase (Invitrogen) in a final volume of 50 μl. The reaction conditions were: 7 min at 94°C, 35 cycles of 45 s at 94°C, 1 min at 59°C, 1 min at 72°C and a final extension step of 10 min at 72°C (Sun *et al*., 2009). Amplicons obtained from ITS and 26S rRNA gene PCRs were clustered according to the fingerprinting profile. Representative amplicons from each group were sequenced, analysed and aligned using the BLAST program in the National Centre for Biotechnology Information database.

W. anomalus*-specific semi-nested PCR assay*

Semi-nested PCR amplifications, specific for *W. anomalus,* were carried out on three generations (every second generation) of our laboratory-reared colony of *A. stephensi*, using three oligonucleotides designed on the yeast sequence isolated from mosquitoes, which shared 100% homology with the locus 18S rRNA of *W. anomalus* present in the database with Accession Number EF427893. The oligonucleotides employed were *Wa*-for (5′-GTGAAACTGCGAATGGCTCAT

TAAATC-3′), *Wa*-rev (5′-GCCTTCCTTGGATGTGGTAGC-3′) and *Wa*-in (5′-TTTTCGAATCGCATGACTTCGTGTC-3′). The outer oligos (*Wa*-for and *Wa*-rev) were designed on conserved sequences of the gene 18S rRNA, whereas the inner oligo (*Wa*-in) is *W. anomalus*-specific; primers have been chosen after the sequences alignment analysis of different Saccharomycetales by the ClustalW program (http://www.ebi.ac.uk/Tools). Amplification pattern included two PCR products, of, respectively, 356 bp and 167 bp, obtained from both isolate pure cultures (used as positive controls) and mosquito metagenomic DNA; randomly chosen 167 bp products have been sequenced and confirmed to be *W. anomalus*-specific. PCR reactions were carried out as previously described (see PCR-based method), except for the oligos concentration that was 0.2 μM *Wa*-for, 0.5 μM *Wa*-in and 0.6 μM *Wa*-rev. PCR amplification was performed in a Veriti Thermal Cycler (Applied Biosystems, USA) under these conditions: 94°C/10 min; 35× (94°C/30 s; 60°C/30 s; 72°C/30 s); 72°C/7 min. In each amplification larval breeding water and sucrose solution were used as negative controls. Approximately 150 ml of larval breeding water at L_4 stage (around 1 week after the eggs transfer into the tank) was filtered by a Whatman filter paper. Filters were then washed twice in sterile 1× PBS and the recovered PBS was centrifuged at maximum speed for 10 min. DNA from the pellets was extracted using the protocol previously described.

W. anomalus *localization in* A. stephensi *by FISH*

For whole mount FISH, analysis on pure yeast cultures was performed to test the specificity level of the designed probes; *W. anomalus* and *C. intermedia* strains (the last being a negative control) isolated from *A. stephensi* were employed. Once the probes were confirmed to be target-specific and the experimental conditions were established, we carried out experiments on dissected organs from both male and female mosquitoes (newly emerged and 10 days post emergence). Hybridization was carried out on guts and gonads using two *W. anomalus*-specific fluorescent probes targeting the 18S rRNA gene: *Wa*1 (5′-TATGAATCATCAAAGAGCTCAGAAGA-3′) and *Wa*2 (5′-GACACGAAGTCATGCGATTCGAAAA-3′). Probes were labelled at the 5′ end with fluorochrome Cy5 (indodicarbocyanine, absorption/emission at 650/670 nm). A FISH protocol was adapted from Xufre and colleagues (2006), with 2 h hybridization at 40°C. Before hybridization, dissected tissues were fixed overnight at 4°C in ethanol/1× PBS (1:1), then 5 min at 4°C in 4% paraformaldehyde and washed in 1× PBS. Dissected organs were then incubated for 10 min at a temperature of 37°C with a 10 μg ml^{-1} pepsin solution and washed again in 1× PBS. Hybridization was conducted in dark conditions at 40°C for 2 h, with 100 μl of hybridization buffer (2× SSC, 50% formamide, 1% dextran sulfate, 0.1 μg ml^{-1} probes). After hybridization, dissections were washed in 100 μl of washing buffer (2× SSC, 50% formamide, 1% dextran sulfate) at 40°C for 15 min. Subsequently two washes in 0.1× SSC (at room temperature for 10 min) and then two washes in 1× SSC (at room temperature for 10 min) were performed, followed by two washes in 1× PBS at 37°C. Then incubation with 50 ng of DAPI (4′, 6′-diamidino-2-phenylindole) for 5 min at room temperature was performed, followed by two washes in 1× PBS for 5 min at room temperature. Finally, samples were mounted in anti-fading medium then observed using laser scanning confocal microscope SP2-AOBS (Leica). Control experiments involved treatment of slides in absence of probes.

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