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 L1–L4 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.

**Introduction**

Studies on the microbiome associated with haematophagous 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.
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. 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).

**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.

<table>
<thead>
<tr>
<th>Fungal identification (% identity)</th>
<th>N. of clones/total clones</th>
<th>N. of clones/total clones</th>
<th>N. of clones/total clones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female individuals</td>
<td>Male individuals</td>
<td>Pools of female guts</td>
</tr>
<tr>
<td>Candida sp. AY520257 (94–98%)</td>
<td>3/9</td>
<td>1/6</td>
<td>2/4</td>
</tr>
<tr>
<td>Pichia sp. AB022439 (95–98%)</td>
<td>2/9</td>
<td>1/6</td>
<td>1/4</td>
</tr>
<tr>
<td>Wickerhamomyces anomalus EF427893 (96–100%)</td>
<td>1/9</td>
<td>1/6</td>
<td>0/4</td>
</tr>
<tr>
<td>Wickerhamomyces anomalus EF427893 (96–98%)</td>
<td>1/9</td>
<td>1/6</td>
<td>0/4</td>
</tr>
<tr>
<td>Wallemia sebi AF548107 (99–100%)</td>
<td>1/9</td>
<td>1/6</td>
<td>0/4</td>
</tr>
<tr>
<td>Uncultured fungus AM114819 (98–99%)</td>
<td>1/9</td>
<td>1/6</td>
<td>1/4</td>
</tr>
<tr>
<td>Uncultured fungus AB248107 (96–98%)</td>
<td>1/9</td>
<td>1/6</td>
<td>0/4</td>
</tr>
</tbody>
</table>

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 analyzed 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.

<table>
<thead>
<tr>
<th>N. isolates</th>
<th>Closest relative</th>
<th>ITS</th>
<th>D1/D2</th>
</tr>
</thead>
<tbody>
<tr>
<td>77</td>
<td>Candida intermedia</td>
<td>FJ515166, (98–99%)</td>
<td>FJ455102, (99–100%)</td>
</tr>
<tr>
<td>15</td>
<td>Hanseniaspora uvarum</td>
<td>FJ515178, (99%)</td>
<td>GU585885, (99–100%)</td>
</tr>
<tr>
<td>8</td>
<td>Wickerhamomyces anomalus</td>
<td>EU330185, (99–100%)</td>
<td>FM178296, (98–100%)</td>
</tr>
</tbody>
</table>

The percentages of identity of 18S–26S rRNA ITS and D1/D2 domain of the 26S rRNA gene are presented.
generations were tested (Table 3). We examined larval stages L₁–L₃ and pupae as pools, and L₄ 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 L₄ larvae out of 45, 29 out of 45 pupae, 107 out of 150 adults (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

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

<table>
<thead>
<tr>
<th>DNA source</th>
<th>Developmental stages (days post emergence)</th>
<th>N. samples analysed per mosquito generation</th>
<th>N. of generations examined</th>
<th>Positives/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole mosquitoes</td>
<td>Larvae-L₁</td>
<td>1 pool (15–20 individuals)</td>
<td>3</td>
<td>3/3 pools</td>
</tr>
<tr>
<td></td>
<td>Larvae-L₂</td>
<td>1 pool (10 individuals)</td>
<td>3</td>
<td>3/3 pools</td>
</tr>
<tr>
<td></td>
<td>Larvae-L₃</td>
<td>1 pool (5 individuals)</td>
<td>3</td>
<td>3/3 pools</td>
</tr>
<tr>
<td></td>
<td>Larvae-L₄</td>
<td>15 individuals</td>
<td>3</td>
<td>30/45 individuals</td>
</tr>
<tr>
<td></td>
<td>Pupae</td>
<td>15 individuals</td>
<td>3</td>
<td>29/45 individuals</td>
</tr>
<tr>
<td></td>
<td>Adults (0 day)</td>
<td>50 individuals (25♀, 25♂)</td>
<td>3</td>
<td>107/150 individuals</td>
</tr>
<tr>
<td>♀ Guts</td>
<td>Adults (2 days)</td>
<td>1 pool (5 guts)</td>
<td>3</td>
<td>3/3 pools</td>
</tr>
<tr>
<td>♀ Guts</td>
<td>Adults (10 days)</td>
<td>1 pool (5 guts)</td>
<td>3</td>
<td>3/3 pools</td>
</tr>
<tr>
<td>♂ Guts</td>
<td>Adults (2 days)</td>
<td>1 pool (5 guts)</td>
<td>3</td>
<td>3/3 pools</td>
</tr>
<tr>
<td>♂ Guts</td>
<td>Adults (10 days)</td>
<td>1 pool (5 guts)</td>
<td>3</td>
<td>3/3 pools</td>
</tr>
<tr>
<td>♂ Gonads</td>
<td>Adults (2 days)</td>
<td>1 pool (7 gonads)</td>
<td>3</td>
<td>3/3 pools</td>
</tr>
<tr>
<td>♂ Gonads</td>
<td>Adults (10 days)</td>
<td>1 pool (7 gonads)</td>
<td>3</td>
<td>3/3 pools</td>
</tr>
<tr>
<td>♂ Gonads</td>
<td>Adults (2 days)</td>
<td>1 pool (7 gonads)</td>
<td>3</td>
<td>3/3 pools</td>
</tr>
<tr>
<td>♂ Gonads</td>
<td>Adults (10 days)</td>
<td>1 pool (7 gonads)</td>
<td>3</td>
<td>3/3 pools</td>
</tr>
</tbody>
</table>

Whole adult mosquitoes, larvae-L₄ and pupae have been tested individually, the earlier larval stages (L₁, L₂, L₃) 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.
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 (Kagiyma 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...
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.,

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**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.
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. anomalous* 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 mosquitoes (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% OsO4 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.
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 lyticas 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 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 rDNA gene and ITS2; together with the D1/D2 region of 26S rDNA 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′-CTTGTGTTAGAGGAAGTAA-3′) and ITS4 (5′-TCTTGGCTTATGATATGC-3′) under the following amplification conditions in a final volume of 50 μl: 1× Taq polymerase buffer, 1.8 mM MgCl₂, 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 rDNA gene was carried out using primers NL1 (5′-CATATCATAAGCGGAGTAAAAG-3′) and NL4 (5′-GGTCGTTTAAGACGG-3′). PCR was performed using 1× Taq polymerase buffer, 1.8 mM MgCl₂, 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 rDNA 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′-GTCAAATCGGAATGGCTCAT

Wickerhamomyces anomalus in Anopheles stephensi 919

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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