Medical and Veterinary Entomology

Medical and Veterinary Entomology (2016) **30**, 101–106 doi: 10.1111/mve.12149

SHORT COMMUNICATION

Isolation of a *Wickerhamomyces anomalus* yeast strain from the sandfly *Phlebotomus perniciosus*, displaying the killer phenotype

E. MARTIN[1], G. BONGIORNO[2], L. GIOVATI[3], M. MONTAGNA[4], E. CROTTI[5], C. DAMIANI[6], L. GRADONI[2], L. POLONELLI[3], I. RICCI[6], G. FAVIA[6] and S. EPIS[1]

[1]Department of Veterinary Science and Public Health, University of Milan, Milan, Italy, [2]Unit of Vector-Borne Diseases and International Health, MIPI Department, Istituto Superiore di Sanità, Rome, Italy, [3]Microbiology and Virology Unit, Department of Biomedical, Biotechnological and Translational Sciences, University of Parma, Parma, Italy, [4]Department of Agricultural and Environmental Sciences, University of Milan, Milan, Italy, [5]Department of Food, Environmental and Evolutionary Sciences, University of Milan, Milan, Italy and [6]School of Biosciences and Veterinary Medicine, University of Camerino, Camerino, Italy

Abstract. The yeast *Wickerhamomyces anomalus* has been studied for its wide biotechnological potential, mainly for applications in the food industry. Different strains of *W. anomalus* have been isolated from diverse habitats and recently from insects, including mosquitoes of medical importance. This paper reports the isolation and phylogenetic characterization of *W. anomalus* from laboratory-reared adults and larvae of *Phlebotomus perniciosus* (Diptera: Psychodidae), a main phlebotomine vector of human and canine leishmaniasis. Of 65 yeast strains isolated from *P. perniciosus*, 15 strains were identified as *W. anomalus*; one of these was tested for the killer phenotype and demonstrated inhibitory activity against four yeast sensitive strains, as reported for mosquito-isolated strains. The association between *P. perniciosus* and *W. anomalus* deserves further investigation in order to explore the possibility that this yeast may exert inhibitory/killing activity against *Leishmania* spp.

Key words. *Leishmania*, biocontrol, phlebotomine sandflies, yeast community.

Wickerhamomyces anomalus (also known as *Pichia anomala* or *Hansenula anomala*) is a Saccharomycetes yeast of great interest for its biotechnological applications. Like other yeast species, strains of *W. anomalus* are known to produce killer toxins (*Wa*KT) with antimicrobial activity against fungi, including other yeasts, and bacteria (Passoth *et al.*, 2011). Given these characteristics, strains of *W. anomalus* have been exploited in food production and preservation, environmental bioremediation, and the production of therapeutic molecules and biofuel (Walker, 2011). Although some cases of human infection caused by *W. anomalus* have been reported (Kalkanci *et al.*, 2010; Oliveira *et al.*, 2014), this yeast can only be considered as an opportunistic pathogen (Epis *et al.*, 2015) and is classed at biosafety level 1 by the European Food Safety Authority (De Hoog, 1996).

Wickerhamomyces anomalus has been isolated from different substrates and habitats, including fruits, plants, dairy and baked food products, cereal silos, oil-contaminated soil and marine environments (Walker, 2011). Recently, *W. anomalus* was also isolated from arthropods, in particular from *Drosophila* sp. (Diptera: Drosophilidae) (Zacchi & Vaughan-Martini, 2002), *Doubledaya bucculenta* beetles (Coleoptera: Erotylidae) (Toki *et al.*, 2012) and from different mosquito species (Ricci *et al.*, 2011a). Interestingly, one of the strains isolated from the malaria mosquito vector *Anopheles stephensi* (Diptera: Culicidae) has been shown to produce a killer toxin active against other yeast species (Cappelli *et al.*, 2014). This result may suggest a potential involvement of *W. anomalus* in protection against pathogens in mosquitoes, as previously reported for the crab *Portunus trituberculatus* (Decapoda: Portunidae) (Wang *et al.*, 2008)

Correspondence: Sara Epis, Department of Veterinary Science and Public Health, University of Milan, Via Celoria 10, 20133 Milan, Italy. Tel.: + 39 02 5031 8101; Fax: + 39 02 5031 8095; E-mail: sara.epis@unimi.it

The present paper reports the isolation and characterization of strains of *W. anomalus* from laboratory-reared *Phlebotomus perniciosus*, the main phlebotomine vector in the western Mediterranean area of the protozoan parasite *Leishmania infantum* (Trypanosomatida: Trypanosomatidae), the causative agent of canine and human visceral leishmaniasis (Killick-Kendrick, 1990).

A laboratory colony of *P. perniciosus*, originating from samples collected in the Madrid area (Spain) and established at the Institute of Health Carlos III of Madrid in 1983, was employed. The colony has been maintained since June 2012 at the Unit of Vector-Borne Diseases of Istituto Superiore di Sanità, Rome, and at the time of this study was in its 27th generation. Adults were routinely maintained in thin mesh cages in thermostat-controlled cabinets at standard conditions of temperature (28 ± 1 °C) and relative humidity (95–100%), under a photoperiod of LD 7 : 17 h and were provided with 30% sugar solution daily (Maroli *et al.*, 1987). Larvae were grown in plaster jars under the same standard conditions and were supplemented with larval food according to the mass-rearing technique of Modi & Tesh (1983), with strict regulation of food quantity and moisture.

A total of seven females, seven males and seven fourth-stage larvae were preserved alive until microbiological analysis. In order to isolate yeasts associated with *P. perniciosus*, two different media, universally used for the isolation and cultivation of yeasts, were employed: (a) yeast malt (YM) agar medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L dextrose, 20 g/L agar, pH 6.2), and (b) potato dextrose agar (PDA) medium (Sigma-Aldrich Corp., St Louis, MO, U.S.A.), both supplemented with chloramphenicol 100 mg/L to avoid bacterial growth. Adult sandflies were surface-sterilized in 100% ethanol and washed twice in 1× phosphate-buffered saline (PBS) supplemented with detergent. Whole bodies were individually homogenized with a sterile pestle by grinding each body in 200 μL of sterile 0.9% NaCl water solution. A sample of 100 μL of each homogenate was spread directly on to a YM agar plate supplemented with chloramphenicol and the remaining 100 μL of homogenate were spread on a PDA plate supplemented with the same antibiotic. The plates were incubated for 48–72 h at 30 °C. The L4 larvae were dissected in a drop of sterile 1× PBS using sterile needles under a stereomicroscope (Leica M50; Leica Microsystems GmbH, Wetzlar, Germany). The midguts of larvae were individually homogenized with a sterile micropestle by grinding them in 100 μL of sterile 0.9% NaCl solution, spread directly on PDA (50 μL) and YM (50 μL) plates supplemented with chloramphenicol and incubated for 48–72 h at 30 °C. Growing yeast colonies were then selected and collected based on colony morphology (Kurtzman & Fell, 2000), re-plated twice in order to purify them, and pure cultures of yeast strains [grown in liquid potato dextrose broth (PDB) medium; Sigma-Aldrich Corp.] were stored in 20% glycerol (Sigma-Aldrich Corp.) at − 80 °C.

DNA from each yeast colony was extracted using the enzyme lyticase (Sigma-Aldrich Corp.) and the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) following the supplementary protocol for purification of total DNA from yeasts. *Wickerhamomyces anomalus*-specific polymerase chain reaction (PCR), targeted on the *β*-tubulin gene, was performed on all DNAs from selected yeast colonies using the specific primers SpWanom-170F (5′-TTATCCATCCACCAATTG-3′) and SpWanom-374R (5′-GGAACTAAGTTCACAGCTA-3′) (Huang *et al.*, 2012) in a total volume of 25 μL containing 1× Green GoTaq Reaction Buffer, 0.2 mM dNTPs, 0.5 μM of each primer, 1.25 U GoTaq G2 (Promega Corp., Madison, WI, U.S.A.) and 7–10 ng of template DNA, following the thermal protocol reported in Huang *et al.* (2012), except that the annealing temperature was decreased to 52 °C. Subsequently, in order to characterize the isolated strains, PCRs for the amplification of the 18S–26S rRNA internal transcribed spacer (ITS) and the 26S rRNA gene were carried out, as suggested in Schoch *et al.* (2012), for samples that tested positive in the previous *β*-tubulin-targeted PCR. This PCR amplification of the ITS fragment was carried out using primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Manter & Vivanco, 2007), whereas PCR amplification of the D1/D2 domain of the 26S rRNA gene was conducted using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Ferreira *et al.*, 2010). All amplicons obtained from the ITS and 26S rRNA gene were sequenced.

Before DNA extraction, 17 adults and 10 L4 larvae of *P. perniciosus*, previously stored in 100% ethanol, were carefully washed in sterile 1× PBS. DNA extraction was performed in an extractor hood using a commercial kit, following the protocol previously described for yeasts. DNAs were eluted in 25 μL of AE buffer. A *W. anomalus*-specific PCR was carried out on DNA from whole insects using the previously described *β*-tubulin-targeted PCR (Huang *et al.*, 2012).

In order to perform molecular identification, the sequences obtained from 18S–26S rRNA ITS and 26S rRNA gene were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/blast) and compared with sequences available in GenBank (nucleotide collection nr/nt; http://www.ncbi.nlm.nih.gov/genbank/). In addition, the clustering pattern among the sequences amplified in the present study and previous data was determined through phylogenetic analyses. Homologous sequences of both markers were retrieved from GenBank. Sequences from previous studies were included (Toki *et al.*, 2012, 2013) and two sets of data were produced, referring to, respectively, 18S–26S rRNA ITS and 26S rRNA gene sequences.

The two datasets of sequences were aligned using MAFFT Version 6.0 (http://mafft.cbrc.jp/alignment/server/) (Katoh *et al.*, 2005) implementing a G-INS-i search strategy (Katoh & Toh, 2008). The appropriate model of nucleotide substitution was estimated with jModelTest 2 (Darriba *et al.*, 2012) using Akaike's information criterion (AIC) to select the best-fit model. The best model of nucleotide evolution comprised a general time reversible (GTR) model (Lanave *et al.*, 1984) with gamma distribution for both sets of data. Maximum likelihood phylogenetic inference and the hierarchical cluster analysis adopting the unweighted pair group method with arithmetic mean (UPGMA) were performed on the aligned datasets using, respectively, PhyML Version 3.0 (Guindon *et al.*, 2010) and MEGA 6 (Tamura *et al.*, 2013). Branch support was estimated using the approximate likelihood ratio test (aLRT) approach (Anisimova

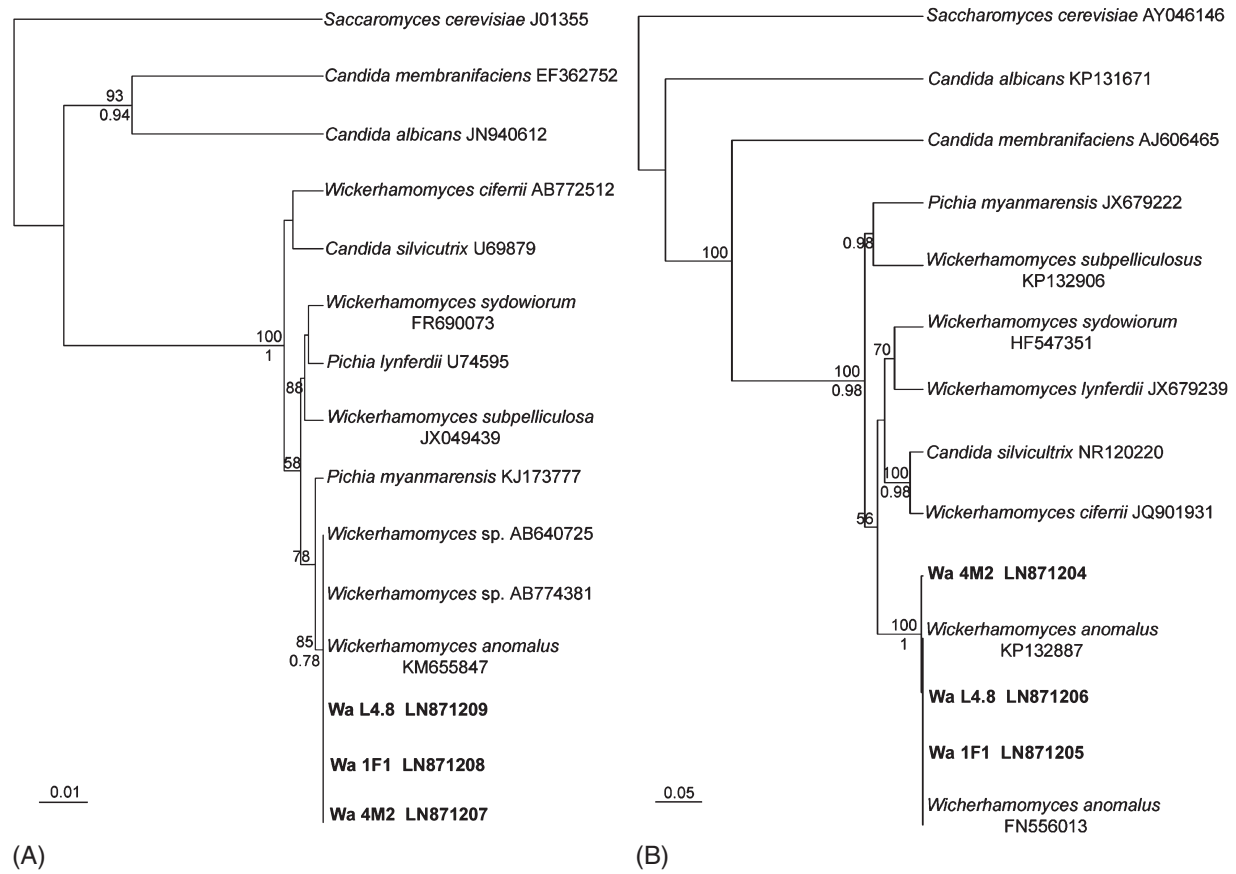

Fig. 1. UPGMA dendrograms inferred from (A) 26S rRNA and (B) 18S–26S rRNA ITS gene sequence datasets. Support values, respectively, the approximate likelihood ratio values for the maximum likelihood tree (A) and UPGMA bootstrap percentages (B) are reported on the branches. Support values below 0.5 and 50% are not reported. The dendrograms obtained are rooted on the outgroup, represented by *Saccaromyces cerevisiae*. Isolates obtained in this study are reported in bold. The scale bar indicates the distance in substitutions per site.

& Gascuel, 2006) in maximum likelihood analyses and by bootstrap analysis (1000 replicates) in UPGMA.

An assay to test for the killer phenotype was performed on one of the 15 isolates of *W. anomalus* recovered from *P. perniciosus* (isolate *Wa*_1F1) using the killer-sensitive reference strains NEQAS 8706 (*Candida glabrata*), NEQAS 6208 (*Candida lusitaniae*), NCYC 1006 (*Saccharomyces cerevisiae*) and *Wa*UM3 [*W. anomalus* (a strain not producing toxins)] (Polonelli *et al.*, 1997). The killer strain *Wa*ATCC 96603 was used as a *Wa*KT-producing positive control (Guyard *et al.*, 2001). The killing assays were performed following a previously described method with minor modifications (Polonelli *et al.*, 1983). Briefly, each reference strain was resuspended in water to a final concentration of 0.5 McFarland and plated (100 μL) on methylene blue agar (MBA: 2% peptone, 2% glucose, 1% yeast extract, 2% agar, 0.003% methylene blue, buffered at pH 4.5 with 0.1 M citric acid and 0.2 M Na_2HPO_4, and autoclaved at 121 °C for 15 min). Strains *Wa*_1F1 and *Wa*ATCC 96603 reactivated in Sabouraud dextrose agar were spotted (5 mm diameter) on to the surface of previously prepared MBA, and the plate was incubated at 25 °C for 48 h. The killer-positive strain was characterized by the presence of a clear surrounding zone.

To date, most scientific research on the microbial community that colonizes haematophagous insects has been performed in mosquitoes (Dillon & Dillon, 2004). Very little is known about sandfly fungi microflora and their possible impact on the biology and reproduction of sandflies and sandfly–pathogen interaction (Zacchi & Vaughan-Martini, 2002; Sant'Anna *et al.*, 2014).

In particular, among insect vectors, the yeast *W. anomalus* has been described only in mosquitoes of public health importance; in these arthropod species, the yeast was observed in the midgut as well as in the gonads (Ricci *et al.*, 2011b). The present paper reports the first isolation o*f W. anomalus* from the sandfl*y P. perniciosus* and its antimicrobial activity against susceptible yeast strains.

Culture-based screening for yeasts was effected using 21 *P. perniciosus* sandfly individuals (14 whole adults and seven midguts of larvae) and led to the recovery of 65 yeast isolates from 12 flies (four females, two males, six larvae). Two media were used in order to increase the likelihood of obtaining yeast colonies; more yeast colonies were obtained from the YM agar medium than from the PDA medium, from which moulds were also retrieved. Specific PCR, for the amplification of the β-tubulin gene, identified 15 of the 65 isolates as

W. anomalus. These *W. anomalus* isolates derived from both whole adults (four isolates from two males and four isolates from three females) and from the midguts of the L4 larvae (seven isolates from three larvae). In summary, *W. anomalus* isolates were obtained from a total of eight *P. perniciosus* individuals. The 15 isolates identified as *W. anomalus* were further characterized by amplification and sequencing of the 18S–26S rRNA ITS and the D1/D2 domain of the 26S rRNA gene in order to confirm identification and to perform a phylogenetic analysis. Concerning the 26S rRNA gene, all sequences were identical at nucleotide level and showed 100% identity (E-value: 0) with *W. anomalus*, as revealed by the BLAST search. With respect to the ITS marker, sequences of the 15 isolates from larvae, females and most males were identical (represented by *Wa*_L4.8 and *Wa*_1F1) (Fig. 1); one of the sequences from male isolates (*Wa*_4M2) showed one nucleotide deletion and one nucleotide substitution in comparison with the above sequences. BLAST analysis performed on ITS sequences confirmed the previous results, showing 100% and 99% identity, respectively, (E-value: 0) with *W. anomalus*. The 26S rRNA and ITS sequences of *W. anomalus*, obtained from isolates from one male (*Wa*_4M2), one female (*Wa*_1F1) and one larva (*Wa*_L4.8), were deposited in the database [European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database, accession nos. LN871204–LN871209] and used for phylogenetic analysis. Phylogenetic analyses, performed on the two markers in order to elucidate the relationships of the new fungal isolates (*Wa*_4M2, *Wa*_1F1, *Wa*_L4.8) from the sandfly *P. perniciosus*, confirmed their clustering with *Wickerhamomyces* spp. and the identification as *W. anomalus* (Fig. 1). Based on the analyses performed on the 26S rRNA gene sequence dataset, sandfly isolates cluster with *W. anomalus* and with a *Wickerhamomyces* sp. isolated from a fungus-growing beetle [accession nos. AB640725 and AB774381 (Toki *et al.*, 2012, 2013)]. On the basis of the ITS marker, the new isolated strains cluster in a well-supported clade (node support of 0.99 aLRT and 100% of bootstrap) with two specimens of *W. anomalus*, one of which was isolated from the mosquito *An. stephensi* (Fig. 1) [accession no. FN556013 (Ricci *et al.*, 2011a)].

In addition, to further verify the presence of *W. anomalus* in the analysed population of *P. perniciosus*, a *W. anomalus*-specific PCR assay was carried out on DNA from whole insects; one of nine females, two of eight males and three of 10 L4 larvae tested positive for the presence of *W. anomalus*. As in other cases of arthropod–symbiont associations, this suggests that yeasts associated with sandflies provide the host with some benefit. Recently, Sant'Anna *et al.* (2014) reported that pre-feeding yeast or bacteria to the sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae) (bacteria *Asaia* sp. and *Ochrobactrum intermedium*, and yeast *Pseudozyma* sp.) can prevent the establishment of *Leishmania mexicana* infection within the insect vector. As demonstrated in plant systems, the yeast *Pseudozyma* can secrete extracellular metabolites that inhibit the pathogen, but may also prime the plant immune system to induce a local and systemic immune response towards the pathogen. Insect associations with yeasts may also benefit insects by promoting the development of other mutually beneficial organisms in the community while decreasing the presence of insect pathogens. Davis *et al.* (2011) reported that volatiles produced by the yeast *Ogatea*

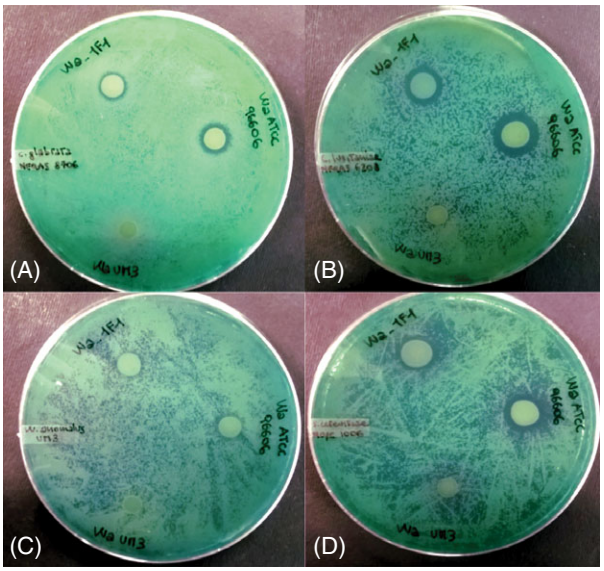

Fig. 2. Evidence of growth inhibition of the environmental strain *Wickeramomyces anomalus Wa*ATCC 96603 (right of the plates) and the strain *W. anomalus* Wa_1F1 isolated from *Plebotomus perniciosus* (left of the plates) determined by a transparent halo, in the plates with the sensitive strains: (A) *Candida glabrata* NEQAS 8706; (B) *Candida lusitaniae* NEQAS 6208; (C) *Wickeramomyces anomalus Wa*UM3, and (D) *Saccharomyces cerevisiae* NCYC 1006. No growth inhibition was determined by the *Wa*UM3 (*W. anomalus*, a strain not producing toxins) in the plates with the sensitive strains (bottom left of the plates).

pini found in the western pine beetle inhibited the development of the entomopathogenic fungi and increased the development of mutualist fungi, demonstrating that yeasts can selectively shape the microbial community.

As has been reported for mosquitoes, *W. anomalus* is not present in 100% of *P. perniciosus* individuals. This supports the speculation that as the yeast is not fixed in the population, it is not required for host survival. Nevertheless, non-obligate symbionts can exert beneficial roles and can be acquired by ingestion or through interaction with co-specifics and the environment (Engel & Moran, 2013).

Some *W. anomalus* strains have demonstrated antifungal activity and, more generally, have been shown to inhibit harmful microorganisms in a great variety of habitats (Polonelli, 2000; Cappelli *et al.*, 2014). To establish whether the strain *Wa*_1F1 isolated from *P. perniciosus* possesses antifungal activity, susceptible yeast strains were submitted to specific killer phenotype assays. Results are shown in Fig. 2 and present evidence of the growth inhibition of tested microorganisms, determined by a transparent halo, in plates with susceptible strains (*C. glabrata* NEQAS 8706, *C. lusitaniae* NEQAS 6208, *W. anomalus Wa*UM3 and *S. cerevisiae* NCYC 1006). The transparent halo was similar to that generated by *Wa*ATCC 96606, the positive strain, known to produce the killer toxin (Guyard *et al.*, 2001). As previously reported for the mosquito *An. stephensi* (Cappelli *et al.*, 2014), the present results show that *W. anomalus* from *P. perniciosus* inhibits the growth of selected fungi, and can thus be regarded as a killer yeast that is probably able to produce a killer toxin. Further studies should investigate whether

killer toxin-producing strains of *W. anomalus* are commonly found in wild populations of *P. perniciosus* and if they possess anti-*Leishmania* activity.

It is important to note that Adler & Theodor (1929) have proposed that females of *Phlebotomus papatasi* infected by fungi were significantly more resistant to *Leishmania major* infection than uninfected females. Using the same study model, Schlein *et al.* (1985) reported a high prevalence of microbial infection in the digestive tract of laboratory-reared *P. papatasi* and hypothesized that these might have a negative effect on *Leishmania* transmission in endemic areas. In conclusion, the present discovery of *W. anomalus* yeasts displaying the killer phenotype in the sandfly *P. perniciosus*, the main Mediterranean vector of *L. infantum*, opens new avenues for research aimed at investigating the potential of this yeast as an agent for the biocontrol of leishmaniasis.

Acknowledgements

The authors are grateful to Prof. Stefania Conti, Department of Biomedical, Biotechnological and Translational Sciences, University of Parma, Parma, Italy, Claudia Santinoli, Department of Biomedical, Biotechnological and Translational Sciences, University of Parma, Parma, Italy and Prof. Claudio Bandi, Department of Veterinary Science and Public Health, University of Milan, Milan, Italy, for their suggestions. This research was supported by the Italian Ministry of Education, University and Research FIRB 2013 [RBFR136GFF (to SE)] and by the European Union Seventh Framework Programme ERC 2011 [FP7/2007-2013_FP7/2007-2011 under grant agreement no. 281222 (to IR)].

References

Adler, S. & Theodor, O. (1929) Attempts to transmit *Leishmania tropica*: the transmission of *L. tropica* by *Phlebotomus sergenti*. *Annals of Tropical Medicine and Parasitology*, **23**, 1–18.

Anisimova, M. & Gascuel, O. (2006) Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. *Systematic Biology*, **55**, 539–552.

Cappelli, A., Ulissi, U., Valzano, M. *et al.* (2014) A *Wickerhamomyces anomalus* killer strain in the malaria vector *Anopheles stephensi*. *PLoS ONE*, **9**, e95988.

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, **9**, 772.

Davis, T.S., Hofstetter, R.W., Foster, J.T., Foote, N.E. & Keim, P. (2011) Interactions between the yeast *Ogataea pini* and filamentous fungi associated with the western pine beetle. *Microbial Ecology*, **61**, 626–634.

De Hoog, G.S. (1996) Risk assessment of fungi reported from humans and animals. *Mycoses*, **39**, 407–417.

Dillon, R.J. & Dillon, V.M. (2004) The gut bacteria of insects: non-pathogenic interactions. *Annual Review of Entomology*, **49**, 71–92.

Engel, P. & Moran, N.A. (2013) The gut microbiota of insects – diversity in structure and function. *FEMS Microbiology Reviews*, **37**, 699–735.

Epis, S., Capone, A., Martin, E. *et al.* (2015) A rapid qPCR method to investigate the circulation of the yeast *Wickerhamomyces anomalus* in humans. *New Microbiologica*, **38**, 577–581.

Ferreira, N., Belloch, C., Querol, A., Manzanares, P., Vallez, S. & Santos, A. (2010) Yeast microflora isolated from Brazilian cassava roots: taxonomical classification based on molecular identification. *Current Microbiology*, **60**, 287–293.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, **59**, 307–321.

Guyard, C., Evrard, P., Corbisier-Colson, A.M. *et al.* (2001) Immuno-crossreactivity of an anti-*Pichia anomala* killer toxin monoclonal antibody with a *Williopsis saturnus* var. *mrakii* killer toxin. *Medical Mycology*, **39**, 395–400.

Huang, C.H., Chang, M.T. & Huang, L. (2012) Species identification of *Wickerhamomyces anomalus* and related taxa using β-tubulin (β-tub) DNA barcode marker. *Yeast*, **29**, 531–535.

Kalkanci, A., Dizbay, M., Turan, O. *et al.* (2010) Nosocomial transmission of *Candida pelliculosa* fungemia in a pediatric intensive care unit and review of the literature. *Turkish Journal of Pediatrics*, **52**, 42–49.

Katoh, K. & Toh, H. (2008) Improved accuracy of multiple ncRNA alignment by incorporating structural information into a MAFFT-based framework. *BMC Bioinformatics*, **9**, 212.

Katoh, K., Kuma, K., Toh, H. & Miyata, T. (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research*, **33**, 511–518.

Killick-Kendrick, R. (1990) Phlebotomine vectors of the leishmaniases: a review. *Medical and Veterinary Entomology*, **4**, 1–24.

Kurtzman, C.P. & Fell, J.W. (2000) *The Yeasts, A Taxonomic Study*, 4th edn. Elsevier Science, Amsterdam.

Lanave, C., Preparata, G., Saccone, C. & Serio, G. (1984) A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution*, **20**, 86–93.

Manter, D.K. & Vivanco, J.M. (2007) Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by qPCR and length heterogeneity analysis. *Journal of Microbiological Methods*, **71**, 7–14.

Maroli, M., Fiorentino, S. & Guandalini, E. (1987) Biology of a laboratory colony of *Phlebotomus perniciosus* (Diptera: Psychodidae). *Journal of Medical Entomology*, **24**, 547–551.

Modi, G.B. & Tesh, R.B. (1983) A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. *Journal of Medical Entomology*, **20**, 568–569.

Oliveira, V.K., Ruiz Lda, S., Oliveira, N.A. *et al.* (2014) Fungemia caused by *Candida* species in a children's public hospital in the city of São Paulo, Brazil: study in the period 2007–2010. *Revista do Instituto de Medicina Tropical de São Paulo*, **56**, 301–305.

Passoth, V., Olstorpe, M. & Schnürer, J. (2011) Past, present and future research directions with *Pichia anomala*. *Antonie Van Leeuwenhoek*, **99**, 121–125.

Polonelli, L. (2000) Human, monoclonal and recombinant candidacidal, pneumocysticidal and mycobactericidal antibodies. *Indian Journal of Chest Diseases and Allied Sciences*, **42**, 259–263.

Polonelli, L., Archibusacci, C., Sestito, M. & Morace, G. (1983) Killer system: a simple method for differentiating *Candida albicans* strains. *Journal of Clinical Microbiology*, **17**, 774–780.

Polonelli, L., Séguy, N., Conti, S. *et al.* (1997) Monoclonal yeast killer toxin-like candidacidal anti-idiotypic antibodies. *Clinical and Diagnostic Laboratory Immunology*, **4**, 142–146.

Ricci, I., Mosca, M., Valzano, M. *et al.* (2011a) Different mosquito species host *Wickerhamomyces anomalus* (*Pichia anomala*): perspectives on vector-borne diseases symbiotic control. *Antonie Van Leeuwenhoek*, **99**, 43–50.

Ricci, I., Damiani, C., Scuppa, P. *et al.* (2011b) The yeast *Wickerhamomyces anomalus* (*Pichia anomala*) inhabits the midgut and reproductive system of the Asian malaria vector *Anopheles stephensi*. *Environmental Microbiology*, **13**, 911–921.

Sant'Anna, M.R., Diaz-Albiter, H., Aguiar-Martins, K. *et al.* (2014) Colonization resistance in the sand fly gut: *Leishmania* protects *Lutzomyia longipalpis* from bacterial infection. *Parasites and Vectors*, **7**, 329.

Schlein, Y., Polacheck, I. & Yuva, B. (1985) Mycoses, bacterial infections and antibacterial activity in sand flies (Psychodidae) and their possible role in the transmission of leishmaniasis. *Parasitology*, **90**, 57–66.

Schoch, C.L., Seifert, K.A., Huhndorf, S. *et al.* (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 6241–6246.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, **30**, 2725–2729.

Toki, W., Tanahashi, M., Togashi, K. & Fukatsu, T. (2012) Fungal farming in a non-social beetle. *PLoS ONE*, **7**, e41893.

Toki, W., Takahashi, Y. & Togashi, K. (2013) Fungal garden making inside bamboos by a non-social fungus-growing beetle. *PLoS ONE*, **8**, e79515.

Walker, G.M. (2011) *Pichia anomala*: cell physiology and biotechnology relative to other yeasts. *Antonie Van Leeuwenhoek*, **99**, 25–34.

Wang, L., Yue, L., Chi, Z. & Wang, X. (2008) Marine killer yeasts active against a yeast strain pathogenic to crab *Portunus trituberculatus*. *Diseases of Aquatic Organisms*, **80**, 211–218.

Zacchi, L. & Vaughan-Martini, A. (2002) Yeasts associated with insects in agricultural areas of Perugia, Italy. *Annals of Microbiology*, **52**, 237–244.

Accepted 6 August 2015
First published online 6 November 2015