Yeasts vectored by migratory birds collected in the Mediterranean island of Ustica and description of Phaffomyces usticensis f.a. sp. nov., a new species related to the cactus ecoclad

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Abstract

Nine yeast species belonging to genera Candida, Cryptococcus, Phaffomyces, Rhodotorula and Wickerhamomyces, and one species of Aureobasidium genus were isolated from the cloaca of migratory birds. Candida glabrata and C. inconspicua were the species most frequently isolated and Wickerhamomyces sylviae, which has recently been described as a new species isolated from bird cloaca, was again found. The majority of isolates showed the ability to grow up to 40 °C and/or at pH 3.0, two environmental conditions typical of the digestive tract of birds. The phylogenetic analysis of the D1/D2 domain of 26S rRNA gene placed the cultures of Phaffomyces in a new lineage that differed from the closest species, P. opuntiae, by 13 nucleotide substitutions. The new species was able to grow at 40 °C and at pH 2.5, which suggests a possible adaptation to the bird cloaca. Moreover, the ability to grow in the presence of digitonin at pH 3.7 and the assimilation of ethyl acetate indicates a potential cactophilic origin. For the first time, the presence of yeasts belonging to the Phaffomyces clade in Europe and also in non-cactus environments is reported. The new species is formally described as P. usticensis sp. nov. (PYCC 6346T = CBS 12958T).

Introduction

The migration of birds of the order Passeriformes across the Mediterranean Basin has been the subject of several studies regarding the ecology of bird species (Birtas et al., 2013; Maggini et al., 2013), as well as the biodiversity of microorganisms carried by these animals. These and other studies have unveiled birds as potential vectors of yeasts, thus suggesting that animals could contribute to increase the yeast diversity of different ecosystems (Cafarchia et al., 2006a; Francesca et al., 2012). Therefore the findings concerning adaptation and persistence of yeasts in birds and insects, sometimes involving insect endosymbiosis, suggest that animals can play an important role in...
the ecology, distribution and evolution of yeasts (Stevic, 1962; Rosa et al., 2009; Basukriadi et al., 2010; Ricci et al., 2011; Stefanini et al., 2012; Chen et al., 2013; Hui et al., 2013).

Recently we analysed the diversity of yeasts (Francesca et al., 2010, 2012) and filamentous fungi (Alfonzo et al., 2013) isolated from migratory birds in the island of Ustica (Sicily, Italy). Francesca et al. (2012) also showed the persistence of yeasts in migratory birds for a period of 12 h, suggesting that birds can act as long-distance vectors of living yeasts. Such recent findings are promoting interest in the dissemination of microorganisms by animals that could even include transcontinental displacement. Francesca et al. (2013) studied eight strains belonging to a new ascomycetous yeast species, Wickerhamomyces sylviae, found during the 2012 autumnal migration on the island of Ustica. Contrary to its closest relatives, W. sylviae showed a unique phenotypic behaviour, being able to grow at temperatures up to 42 °C. Since birds belonging to the order Passeriformes have body temperatures of 42 ± 1 °C (Gwinner, 1990), the ability of W. sylviae to grow at high temperatures suggests an adaptation to the gastrointestinal tract of birds.

Up to now, most studies on yeasts transported by animals have been focused on Saccharomyces cerevisiae due to its relevance in human activities (Goddard et al., 2010; Stefanini et al., 2012) and it has been shown that social wasps have a preferential role in the dissemination of this yeast (Stefanini et al., 2012). Although important findings have been obtained, it is still unclear how Saccharomyces or other yeasts could be transported between distant places, since social wasps as well as other animal vectors do not travel long distances. Migratory birds, on the contrary, can move between continents and therefore can act as yeast carriers over long distances. In the Mediterranean area, bird migrations involve millions of individuals that, twice a year, in spring and autumn, move between Africa and North Europe. The body fat represents the main energy source during flight and the birds with a value of subcutaneous fat amount (SFA) corresponding to 0 or 1 (SFA) need to stop in resting sites to replenish their fat reserves (Kaiser, 1993). During the flight, and in the places where they stop, birds can ingest yeasts present in their diets such as insects and fruits.

Our working hypothesis is that birds can transport and disseminate yeasts able to withstand the conditions of the animal’s gastrointestinal tract along long distances, during their annual migrations. In the present work we analysed the yeasts transported by birds to Ustica island during the spring migration of 2013. The objectives of our study were: (1) to identify at species level the yeasts isolated from migratory birds; (2) to characterize the yeasts isolated in this study and eight strains of W. sylviae previously isolated from birds; and (3) to describe a novel ascomycetous species of the genus Phaffomyces for which the name Phaffomyces usticensis sp. nov. (type strain PYCC 6346T (CBS 12958T) is proposed.

Materials and methods

Sampling site and analysis of birds

Birds were sampled in April 2013 during the spring migration from sub-Saharan areas to North Europe in Ustica island (38°51’N, 12°58’E, Sicily, Italy), one of the most important stop-over sites in the Mediterranean area (Francesca et al., 2012). Birds were captured and ringed by expert ornithologists, authorized for ringing activity, following the instructions of Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA – Italy) (http://www.isprambiente.gov.it/it/ispra) to avoid injury or stress. All birds were identified at species level as reported by Mullanney et al. (1999) and Svensson (1992), and were classified on the basis of migration strategy (trans-Saharan or partial). The analysis also included the measurement of SFA of the abdominal region (Kaiser, 1993), which was evaluated by visual biometric measurement. Cloaca was plugged with sterile cotton swabs and streaked onto malt extract (ME) agar (Oxoid, Milan, Italy) supplemented with chloramphenicol (0.5 g L⁻¹) and biphenyl (1 g L⁻¹). Petri dishes were incubated at room temperature (25 ± 2 °C) for 48–72 h at the sampling site. Once in the laboratory, all plates without visible growth of yeast colonies were further incubated at 25 ± 2 °C for an additional period of 24–72 h. All samples were inoculated in duplicate.

Isolation and molecular identification of yeasts

After growth, all isolates were picked up from agar plates and purified to homogeneity after several sub-culturing steps onto ME agar. Yeast isolates were identified by molecular methods. DNA was extracted by cell lysis using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. To perform a first discrimination of yeasts, all isolates were analysed by restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The DNA fragments were amplified with the primer pair ITS1/ITS4 according to Esteve-Zarzoso et al. (1999). The generated amplicons were then digested with the endonucleases CfoI, HaeIII and HinfI (MBI Fermentas, St. Leon-Rot, Germany) at 37 °C for 8 h. The ITS ampli-
were analysed on agarose gel using 1.5% and 3% (w/v) agarose in 1× TBE (89 mM Tris-borate, 2 mM EDTA pH 8) buffer, stained with SYBR safe DNA gel stain (Invitrogen, Milan, Italy), visualized by UV transillumination and acquired by Gel Doc 1000 Video Gel Documentation System (BioRad, Richmond, CA). Standard DNA ladders were 1 kb Plus and 50 pb (Invitrogen).

One to three representative isolates of each profile were subjected to an additional enzymatic restriction targeting the 26S rRNA gene. After amplification with the primer pair NL1/LR6, the method of Baleiras-Couto et al. (2005) was conducted, the PCR products were digested with the endonucleases HinfI, MseI and ApaI (MBI Fermentas) and visualized as described above. At least one isolate per group was further processed by sequencing of the D1/D2 region of the 26S rRNA gene (Settanni et al., 2012). The identities of the generated sequences were determined by BLASTN (http://www.ncbi.nlm.nih.gov).

Phenotypic characterization of yeasts

To evaluate the ability to tolerate the conditions of the digestive tract and cloaca of birds, the yeasts isolated in the present study were tested for growth in liquid medium at 37, 40 and 42 °C, in the presence of 50% glucose, 0.1% and 0.01% cycloheximide, 10% NaCl, 16% NaCl, Tween 40, Tween 60, Tween 80, 10% NaCl/5% glucose, on 2-keto-D-glucone, on vitamin free and amino acid free medium, starch formation, gelatin liquefaction and Diazonium Blue B reaction (Kurtzman et al., 2011). All isolates that were phenotypically close to cactophilic yeasts were subjected to specific tests to confirm cactophilic phenotypes such as growth in presence of triterpene glycosides using ME agar supplemented with 8 mg L⁻¹ of digitonin (Sigma) (Stammer et al., 1980), growth on ME agar at pH 3.7 (acidified with HCl) and assimilation of ethyl acetate on yeast nitrogen base (Lachance et al., 1988).

Phylogenetic analyses of new species

The D1/D2 domain of the 26S rRNA gene was used for the phylogenetic analyses. Multi sequence alignments were performed with CLUSTALW (BIOEDIT V7.0.9) (Thompson et al., 1997). All sequences that showed an identity below 99% to the type-strain of the closely related species were further analysed. The phylogenetic trees were obtained using three different methods: (1) Bayesian inference, with MRBAYES 3.1.2 software (Ronquist & Huelsenbeck, 2003), was carried out using 500 000 generations with four independent chains and the generalised time reversible model. Substitution-rate variation among sites was modeled by a discrete approximation of the gamma-distribution with a proportion of invariable sites (I1G). The resultant trees were sampled every 100 generations with trees sampled during the first 50 000 generations discarded as burn-in (the burn-in period was estimated by plotting the likelihood of the sampled trees). Relationships among the remaining trees were summarized using a majority-rule consensus method with clade probabilities determined using MRBAYES 3.1.2. Phylogenetic trees were saved and modified for publication in Adobe PHOTOSHOP CS6 (Adobe Systems Incorporated); (2) maximum-likelihood statistical methods (Saitou & Nei, 1987) with 1000 bootstrap iterations; and (3) neighbor-joining with 1000 bootstrap replications (Felsenstein, 1985) were both carried out using MEGA v5.10 (Tamura et al., 2011). Model parameters were calculated in MODESTEST (Tamura et al., 2011).

Phenotypic characterization of the new species

Colony and cell morphology of the new species were examined after growth on 5% ME media and Glucose Yeast Peptone (GYP) media incubated at 25 °C for 3 and 7 days. Hyphae or pseudohyphae were examined after 7 days of growth on Dalmau plates carried out on bacto yeast morphology agar (Difco Laboratories) (Kurtzman et al., 2011). Formation of ascospores was tested in six different media incubated at 15, 20 and 25 °C (de Garcia et al., 2010): corn meal agar (CMA), potato dextrose agar (PDA), acetate agar (Ac), Gorodkowa agar (Go), Starkey’s modified ethanol medium (St) and glucose ‘soytone’ agar (GSA). The cultures were analysed at 3-day intervals for 2 months. All strains were tested for sporulation independently, in pairs and in mass-mating tests. The glucose fermentation test was carried out in liquid medium (Kurtzman et al., 2011) and assimilation of carbon and nitrogen compounds was tested in microplates (Robert et al., 1997; Robert, 2003; Kurtzman et al., 2011). Additional tests were: growth at 37 °C, in the presence of 50% glucose, 0.1% and 0.01% cycloheximide, 10% NaCl, 16% NaCl, Tween 40, Tween 60, Tween 80, 10% NaCl/5% glucose, on 2-keto-6-glucanate, on vitamin free and amino acid free medium, starch formation, gelatin liquefaction and Diazonium Blue B reaction (Kurtzman et al., 2011). All isolates that were phylogenetically close to cactophilic yeasts were subjected to specific tests to confirm cactophilic phenotypes such as growth in presence of triterpene glycosides using ME agar supplemented with 8 mg L⁻¹ of digitonin (Sigma) (Stammer et al., 1980), growth on ME agar at pH 3.7 (acidified with HCl) and assimilation of ethyl acetate on yeast nitrogen base (Lachance et al., 1988).

Strain typing of the new species

Intraspecific characterization of the isolates belonging to the new species was carried out by two different PCR
Table 1. Birds sampled in Ustica island

<table>
<thead>
<tr>
<th>Bird family</th>
<th>Bird species</th>
<th>Migration type</th>
<th>No. of individuals sampled</th>
<th>No. of individuals carrying yeasts</th>
<th>SFA* of birds carrying yeasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sylviidae</td>
<td>Sylvia borin (garden warbler)</td>
<td>●†</td>
<td>37</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Hippolais icterina (icterine</td>
<td>●</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Acrocephalus schoenobaenus</td>
<td>●</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Phylloscopus sibilatrix</td>
<td>●</td>
<td>19</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Sylvia communis (white-throat)</td>
<td>●</td>
<td>24</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Sylvia cantillans (subalpine</td>
<td>●</td>
<td>9</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Phylloscopus trochilus</td>
<td>●</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Muscipipidae</td>
<td>Ficedula albicollis (collared</td>
<td>●</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ficedula hypoleuca (pied</td>
<td>●</td>
<td>24</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Musciapa striata (spotted</td>
<td>●</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ficedula albicollis (collared</td>
<td>●</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Turdidae</td>
<td>Monticola solitarius (blue</td>
<td>■</td>
<td>15</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Rock-thrush)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saxicola rubetra (whinchat)</td>
<td>●</td>
<td>42</td>
<td>18</td>
<td>7</td>
</tr>
<tr>
<td>Hirundinidae</td>
<td>Delichon urbicum (house</td>
<td>●</td>
<td>7</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>martin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>210</td>
<td>70 (33%)</td>
<td>26</td>
</tr>
</tbody>
</table>

●, Trans-Saharan migratory bird; ■, Partial migratory bird.
*SF A, subcutaneous fat amount.
†Symbols indicate the bird migration strategy.

fingerprinting assays with primers (GTG)₃ (Sampaio et al., 2001) and M13 (Stenlid et al., 1994; Valmorri et al., 2010). All patterns were analysed using the GELCOM- PARE II software version 6.5 (Applied-Maths, Sin Marten Latem, Belgium).

Results

Isolation, molecular characterization and distribution of yeasts

We analysed 210 birds that were identified at species level and classified as reported in Table 1. Birds of the Families Sylviidae and Turdidae showed the highest frequencies of yeast isolation and 33% of the individuals gave positive results. A total of 79 yeasts were isolated from bird cloaca and subjected to molecular characterization. The restriction analysis of ITS1-5.8S-ITS2 and of the 26S rRNA gene separated the isolates into 10 groups (Table 2). Only two groups were successfully identified by comparison of restriction profiles with those reported in the literature (Esteve-Zarzoso et al., 1999; Nisiotou & Nychas, 2007; Tofalo et al., 2009; Francesca et al., 2012; Settanni et al., 2012) and corresponded to Candida glabrata (group III) and C. inconspicua (group IV). The remaining groups could not be identified at species level by RFLP analysis and therefore sequencing of D1/D2 domain of the 26S rRNA gene was required. This procedure allowed the identification of isolates of groups I, II, V, VI, VII, IX and X as Aureobasidium pullulans [to be considered not as yeast but dimorphic member of the Pezizomycotina (euascomycetes)], C. albicans, Candida albida var. kuett- ingii, Cryptococcus magnus, Cryptococcus victoriae, Rhodo- torula mucilaginosa and W. sylviae, respectively. The isolates of group VIII had only 97% sequence identity to P. opuntiae, and were therefore classified as undescribed members of the genus Phaffomyces. The distribution of isolates per each yeast species is reported in Table 2. Can- dida glabrata and C. inconspicua were the most frequently isolated species representing, respectively, 20% and 22% of the total number of isolates, and were followed by R. mucilaginosa (16%) and C. albicans (15%). Table 2 also shows the distribution of the 10 yeast species among birds. Sylvia borin (garden warbler) and Saxicola rubetra (whinchat) showed the highest yeast diversity; the lowest diversity was found for Monticola solitarius (blue rock-thrush) and Delichon urbicum (house martin). Except for M. solitarius, which is a sedentary species at Ustica, all bird species hosting a consistent number of yeasts were ‘trans-Saharan’ migratory birds. Furthermore, 33% of yeasts were isolated from birds showing low (0–1) values of SFA.

The molecular differentiation of yeast strains was carried out on 13 cultures of W. sylviae, five of which were isolated in the present work and the remaining eight collected during an earlier sampling of migratory birds (Fran- cesca et al., 2013). The dendrogram resulting from the analysis both of the RAPD and MSP profiles, suggests that
Table 2. Molecular identification of yeast isolates from birds with RFLP-PCR and distribution of isolates per each bird species

<table>
<thead>
<tr>
<th>R.P.</th>
<th>5.8S-ITS PCR</th>
<th>Size of restriction fragments</th>
<th>26S PCR</th>
<th>Size of restriction fragments</th>
<th>Species (% identity)*</th>
<th>Isolate code</th>
<th>Acc. No.</th>
<th>No. of isolates†</th>
<th>Distribution of isolates per each bird species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CfoI</td>
<td>HaeIII</td>
<td>Hinfl</td>
<td>HinfI</td>
<td>MseI</td>
<td>ApaI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>580</td>
<td>260 + 160 + 80</td>
<td>440 + 150</td>
<td>240 + 180 + 140</td>
<td>1100</td>
<td>470 + 390 + 180 + 55</td>
<td>585 + 210 + 160 + 95 + 60</td>
<td>n.a.</td>
<td>A. pullulans (100)</td>
</tr>
<tr>
<td>II</td>
<td>530</td>
<td>285 + 255</td>
<td>438 + 91</td>
<td>277 + 261</td>
<td>1100</td>
<td>490 + 403 + 186</td>
<td>570 + 405</td>
<td>555 + 410 + 130</td>
<td>B. albicans (99)</td>
</tr>
<tr>
<td>III</td>
<td>880</td>
<td>380 + 160 + 140</td>
<td>650 + 220</td>
<td>350 + 260 + 55</td>
<td>1100</td>
<td>490 + 215 + 195 + 55</td>
<td>675 + 375 + 75</td>
<td>725 + 430</td>
<td>B. albata (99)</td>
</tr>
<tr>
<td>IV</td>
<td>480</td>
<td>105 + 90 + 75 + 56</td>
<td>480</td>
<td>265 + 220</td>
<td>1100</td>
<td>485 + 235 + 180 + 130</td>
<td>n.a.</td>
<td>710 + 385</td>
<td>B. inconspicua (99)</td>
</tr>
<tr>
<td>V</td>
<td>700</td>
<td>325 + 300</td>
<td>510 + 70</td>
<td>280 + 255 + 95</td>
<td>1100</td>
<td>335 + 275 + 215 + 155</td>
<td>400 + 360 + 245 + 65</td>
<td>n.a.</td>
<td>C. albidus var. kuetzingii (99)</td>
</tr>
<tr>
<td>VI</td>
<td>650</td>
<td>350 + 300</td>
<td>520 + 90</td>
<td>280 + 235 + 140</td>
<td>1100</td>
<td>255 + 200 + 175 + 160 + 145 + 75 + 55</td>
<td>400 + 365 + 244</td>
<td>n.a.</td>
<td>C. magnus (99)</td>
</tr>
<tr>
<td>VII</td>
<td>530</td>
<td>290 + 245</td>
<td>360 + 125</td>
<td>269 + 180 + 85</td>
<td>1100</td>
<td>420 + 275 + 210 + 200</td>
<td>405 + 285 + 245</td>
<td>n.a.</td>
<td>C. victoriae (99)</td>
</tr>
<tr>
<td>VIII</td>
<td>480</td>
<td>240 + 220</td>
<td>380 + 115</td>
<td>404</td>
<td>1100</td>
<td>335 + 310 + 210 + 185 + 75</td>
<td>n.a.</td>
<td>n.a.</td>
<td>P. sp. (97)</td>
</tr>
<tr>
<td>IX</td>
<td>640</td>
<td>300 + 225</td>
<td>404 + 217</td>
<td>346 + 215</td>
<td>1100</td>
<td>495 + 410 + 205</td>
<td>355 + 270 + 235 + 140</td>
<td>n.a.</td>
<td>M. mucilaginosa (99)</td>
</tr>
<tr>
<td>X</td>
<td>640</td>
<td>610</td>
<td>560 + 80</td>
<td>330 + 310</td>
<td>1080</td>
<td>500 + 240 + 180 + 160</td>
<td>n.a.</td>
<td>n.a.</td>
<td>W. sylviae (100)</td>
</tr>
</tbody>
</table>

All values for the 5.8S-ITS PCR, 26S PCR and restriction fragments are given in bp.

A, blue rock-thrush; B, garden warbler; C, house martin; D, pied flycatcher; E, subalpine warbler; F, whinchat; G, whitethroat; H, wood warbler; R.P., restriction profile; n.a., not applicable since no restriction fragment was obtained.

*According to a BLASTN search of D1/D2 26S rRNA gene sequences in NCBI database.
†Number of isolates per each yeast species.
‡Percentage based on the total number of isolates.
§The number of isolates is reported between brackets.
the *W. sylviae* cultures represent 13 genetically distinct strains (Fig. 1). The similarity between the *W. sylviae* isolates collected in the two samplings was lower than 30%.

**Phenotypic characterization of yeasts**

All the 87 yeasts studied in the present work were phenotypically characterized by testing their ability to grow at high temperatures, low pH and in the presence of 1% acetic acid (Table 3). Intense growth at 40 and 42 °C was observed for 54% and 24% of the isolates, respectively, and 33% and 25% of the isolates showed intense growth at pH 3.0 at 40 and 42 °C, respectively. At the same temperature conditions, but at pH 2.5, a low percentage of isolates showed intense growth. These percentages increased significantly when the results ‘intense’ and ‘weak’ were considered together. The highest percentage (5%) of growth (albeit weak) on 1% acetic acid agar was observed at 25 °C. No growth was observed for basidiomycetes at any of the other experimental conditions.

**Molecular and phenotypic characterization of *P. usticensis* sp. nov**

Four cultures (967A2 = PYCC 6347, 967A3 = PYCC 6348, 967A4 = PYCC 6349 and 967A5 = PYCC 6346\(^T\)) that were isolated from two individuals of *D. urbicum* (house martin), showed identical D1/D2 sequences. They were assigned to the genus *Phaffomyces* but could not be identified at the species level since they had 97% D1/D2 sequence identity to the closest described species. The results of the phylogenetic analyses performed with neighbor-joining, maximum-likelihood and Bayesian methods are represented in Fig. 2. The analysis showed that the isolates belonged to the *Phaffomyces* clade (Yamada et al., 1997) and that the species that was most closely related was *P. opuntiae*. The D1/D2 sequences of our isolates differed from those of *P. opuntiae* by 13 nucleotide substitutions. As shown in Fig. 2, our isolates were in a separate lineage supported by high bootstrap values, which suggests that these four isolates represent a novel species of the genus *Phaffomyces*. Furthermore, our
isolates differ from their closest relatives by the ability to assimilate D-gluconate, growth on vitamin-free medium, and no assimilation of ethanol. In contrast to the phenotypic behavior reported for their closest species, isolates showed notable growth in liquid medium at 37 and 40 °C, at pH 3.0 at 25 °C, in presence of digitonin, the ability to grow on pH 3.7 agar, and the capability to assimilate ethyl acetate. One isolate (967A2) showed weak growth at pH 2.5 and on 1% acetic acid agar (25 °C), whereas the others were able to grow in these conditions. In addition, phenotypic variability was detected mainly in D-xylose, maltose, α,α-trehalose and xylitol assimilation, but also for grow at 40 °C, at pH 2.5, in the presence of digitonin and on 1% acetic acid medium.

The novel species was also genetically characterized at strain level by RAPD-PCR and MSP-PCR fingerprinting analysis. The dendrograms resulting from these analyses showed that the Phaffomyces isolates were divided into two clusters that had a similarity level of 90% (Fig. 3).

### Description of Phaffomyces usticensis sp. nov

#### Growth on 5% ME agar

After 3 days at 25 °C onto 5% ME agar, colonies are beige, butyrous and without elevation; the margin of colony is entire and pseudohyphae are not formed. Cells are spher-
Cal or ellipsoidal (1.5–4 × 2–5 μm) and occur single or in pairs and multiply by multilateral budding (Fig. 4).

**Dalmau plate culture on morphology agar**

After 15 days at 25 °C on Dalmau plates, no hyphae or pseudophyphae are detected.

**Formation of ascospores**

No asci or signs of conjugation are detected in the sporulation media and conditions tested.

**Fermentation and assimilation tests**

Glucose is not fermented. D-Glucose, DL-lactate, succinate and glycerol assimilations are positive. Assimilation of salicin is positive and occasionally delayed. Citrate and d-gluconate are positive and occasionally weak. D-Mannitol is delayed. D-Glucosamine, D-ribose, L-rhamnose and D-glucuronate are delayed and occasionally negative. D-Xylose, maltose, α, α-trehalose, cellobiose, ribitol, xylitol, L-arabinitol, D-glucitol and L-malic acid are variable. D-Arabinose and inulin are negative and occasionally delayed; sucrose is negative and occasionally weak.

D-Galactose, L-sorbose, L-arabinose, methyl α-d-glucoside, melibiose, lactose, raffinose, melezitose, soluble starch, erythritol, myo-inositol, D-glucono-1,5-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, D-galacturonate, methanol, ethanol, L-tartaric acid, d-tartaric acid, m-tartaric acid, saccharic acid, mucic acid, protocatechuic acid, vanillic acid, ferulic acid, veratric acid, p-hydroxybenzoic acid, m-hydroxybenzoic acid, gallic acid, salicylic acid, gentisic acid, catechol, phenol, hexadecane, N-acetil-D-glucosamide, galactitol and nitrate assimilations are negative.

**Additional physiological tests**

Growth at 37 °C, on vitamin-free medium and amino acid free medium is positive. Growth in the presence of 50% glucose, 0.1% and 0.01% cycloheximide, 10% NaCl, 16% NaCl, Tween 40, Tween 60, Tween 80, 10% NaCl/5% glucose is negative. Starch formation, gelatine liquefaction, Diazonium Blue B reaction and hydrolysis of urea are also negative.

Growth on pH 3.7 agar and assimilation of ethyl acetate is positive; growth in pH 3.0 liquid medium is positive and occasionally delayed; growth at 40 °C and in the presence of digitonin is positive and occasionally negative; growth in liquid medium at pH 2.5 and on 1% acetic acid agar is variable, growth at 42 °C is negative.

**Etymology**

The epithet ‘usticensis’ derives from ‘Ustica’ island (Sicily, Italy), where the strains of *P. usticensis* sp. nov. were collected. The Mycobank deposit is: MB 805761.

**Type strain**

The holotype PYCC 6346T (= CBS 12958T) was isolated from a trans-Saharan migratory bird (*D. urbicum* Linnaeus, 1758 (house martin)) in Ustica island, 38°51’N, 12°58’E, April 2013, Sicily, Italy by C. Sannino.

On the basis of the results reported above and according to the criteria suggested by Kurtzman *et al.* (2011), the name *P. usticensis* (type strain PYCC 6346T = CBS 12958T) is proposed for the new ascymycetous yeast species described in the present study.
Discussion

Although recent studies have shown that birds can play an important role in the dissemination of yeasts and filamentous fungi during their migrations across the Mediterranean (Francesca et al., 2012; Alfonzo et al., 2013), a detailed understanding of the significance and consequences of such dissemination is lacking. In this report we analysed 210 migratory birds captured in Ustica, an island that represents one of the most important stop-over points for birds that migrate from the sub-Saharan Africa to Central–North Europe. Since bird migrations are strongly affected by climatic conditions and by the physiological condition of the animals, the experimental procedure could not be designed to include a given number of individuals of a given bird species because it is not possible to standardize the number of species and/or individuals per species of birds during the sampling. In the present study we observed that 33% of birds surveyed carried yeasts, a value that is comparable to what has been reported in a previous study on migratory birds (Francesca et al., 2012) and higher than the values reported by Cafarchia et al. (2006a).

Among the isolates identified in the present study, only the 9% of yeasts belonged to the Basidiomycetes and, of the three species of Cryptococcus that were found, two of them, C. victoriae and C. albidus var. kuetzingii, had not been isolated from migratory birds before. The Ascomycetes represented 91% of our isolates, which is in accordance with previous results (Cafarchia et al., 2006a; Francesca et al., 2012). The species C. albicans, C. glabrata and C. inconspicua, already isolated from birds (Cafarchia et al., 2006a; Lord et al., 2010; Francesca et al., 2012), together represented more than 56% of the ascomycetous yeasts found. These three Candida species are pathogenic to humans (Papon et al., 2013) and migratory birds may contribute to their dissemination. Hubalek (2004) reported an annotated checklist of potential pathogenic microorganisms carried by migratory birds that included C. albicans and C. tropicalis. According to Hubalek (2004), migratory birds could be involved in the dispersal of potential pathogens as biological and/or mechanical carriers and as transporters of infected ecto-parasites. Another important human pathogen, C. neoformans, was previously found to be carried by non-migratory birds of prey (Cafarchia et al., 2006b), but in our study this yeast was not isolated.

The species W. sylviae has been only found in the cloaca of migratory birds and 13 strains have been isolated from different bird individuals caught in two different years and during two different migratory trajectories, one in October 2012 (bird migration from North Europe to Africa) and the other in April 2013 (bird migration from Africa to North Europe). Therefore we propose that W. sylviae is adapted to the physiological conditions of the bird’s intestinal tract and that migratory birds could represent a long distance vector of this species, in case future studies reveal its presence in the environment, along the migratory routes of its bird hosts.

It is logical to assume that the yeasts detected in the cloaca of birds were first ingested and then survived gastric transit, thus withstanding the high body temperature of 41 ± 2 °C and low pH of the digestive tract and cloaca. We tested the hypothesis that the yeasts isolated from birds are adapted to the gastrointestinal environment by performing specific phenotypic tests. The majority of our isolates, including all the strains belonging to W. sylviae, were able to grow at high temperatures and at low pH, and more than 25% of the isolates were able to grow under the simultaneous effect of these two parameters. Among basidiomycetous yeasts, only C. albidus var. kuetzingii could grow at 37 °C and at low pH, whereas various species of ascomycetous yeasts appear to be well adapted to tolerate the stress conditions that we studied. These results seem to correlate with the higher percentage of isolation of ascomycetes than of basidiomycetes from the cloaca of migratory and non-migratory birds that we and others have observed (Hubalek, 2004; Cafarchia et al., 2006a; Lord et al., 2010; Francesca et al., 2012) and support the claim of an ecological adaptation to the bird’s gastrointestinal niche. Another line of evidence corroborating the hypothesis of bird’s niche adaptation is the finding of yeast species that are specific for this ecosystem. Besides W. sylviae, P. usticensis sp. nov. is the second yeast species whose habitat is the bird’s body.

The isolation of P. usticensis sp. nov. in Ustica is the first finding of a yeast belonging to the Phaffomyces clade in Europe and also represents the first time that Phaffomyces has been isolated from non-cactus environments. So far, all species belonging to this clade have been isolated from cactus hosts and thus they are considered part of the cactus–yeast community (Cardinali et al., 2012). Phaffomyces opuntiae, the closest relative to P. usticensis sp. nov., has been isolated only in Australia, where cacti are one of the largest family of plants. Furthermore, C. coquimbonensis (Cardinali et al., 2012), the second closest relative of P. usticensis sp. nov., has only been collected in native (Chile) and non-native (Australia) cactus habitats.

Phylogenetic studies can provide important insights not only the ecological history but also the origin of populations associated to specific hosts, such as cactus–yeast community (Starmer et al., 2001, 2003; Anderson et al., 2004). Most yeasts isolated from cactus tissue, namely from decaying stems of cactus, do not overlap with other yeast hosts confined to the same area (Starmer et al.,
2003). Furthermore, as noted by Starmer et al. (2003) the origin of cactus-yeasts is clearly polyphyletic and the different species of cactophilic yeasts seem to have adapted independently to cactus habitats according to the ‘independent origin’ model suggested to describe this yeast community. In particular, the members of the Phaffomyces clade do not overlap any yeast populations originated from native cactus habitats and this clade is probably characterized by one of the most disjunctive distribution of all cactophilic yeast populations (Cardinali et al., 2012). In this sense, birds could represent additional factors promoting the dispersal of yeasts belonging to the cactophilic community. The remarkable ability of the new species to grow at low pH, in the presence of digitonin and to assimilate ethyl acetate suggest a cactophilic adaptation sensu Starmer et al. (1980) and Lachance et al. (1988). On the other hand, since P. usticensis sp. nov. is a cactophilic yeast found in birds for the first time, the possibility that P. usticensis sp. nov. is a bird-associated yeast accidentally found in cacti must be considered. In this sense, it might be interesting to test other species and/or strains belonging to Phaffomyces clade for their ability to tolerate the specific conditions of bird body. Further studies should clarify the relationship between the bird’s niche and cactophilic adaptations aiming at detecting their present coexistence or determining whether one is ancestral to the other.

The presence of P. usticensis sp. nov. in bird cloaca could be attributed to the food ingested. Fruits, seeds and insects are commonly ingested by birds during their migration (Snow & Perrins, 1998), thus they could represent the primary source of colonization of bird’s digestive tract by yeasts. Presently, the cactus-specific drosophilids are viewed as the most important vector to transport yeasts from one cactus-habitat to another (Fellows & Heed, 1972; Starmer et al., 1988). Adults of Drosophila were found feeding on cactus-stems but also on decaying cactus fruits and the cactus-yeast community would appear to establish a mutualistic relationship with this vectors (Starmer et al., 2003). Since the birds analysed in the present work began their migration from sub-Saharan desert areas where cactus are present (Stocker, 1976), the finding of cactophilic yeasts in bird cloaca could be due to the feeding habits of birds.

Body fat is the first energy source dissipated by birds during migration and when SFA reaches a value of 0 or 1, a stop is necessary (Goymann et al., 2010). The low values of SFA (between 0 and 1) detected in birds positive for P. usticensis sp. nov. supports the hypothesis that migratory birds carried these yeasts from cactus plants in sub-Saharan areas far from our sampling site in Ustica island. Although in our previous study we showed the persistence of a wine yeast strain in a bird’s digestive tract for around 12 h after ingestion (Francesca et al., 2013), up to now no additional results on the persistence of strains directly isolated from birds have been obtained. Another argument supporting the long distance transport of yeasts is that birds were analysed soon after landing, which excludes the colonization of bird cloaca with Phaffomyces acquired with food sources ingested in Ustica.

Asci or signs of conjugation were not detected in P. usticensis sp. nov. We follow recent proposals to reject the dual nomenclature that assigns different names for the sexual and asexual form of fungal species (Hibbett & Taylor, 2013). Article 4.1 of the Melbourne Code (McNeill et al., 2011) as well as data reported by Badotti et al. (2012) and Lachance & Kurtzman (2013) support the ending of this dual nomenclature and a more sequence-based taxonomy. On the basis of these considerations, the designation ‘forma asexualis’ (‘f.a.’) has been associated to the name of the new species.

In conclusion, the present work provides additional insights into yeast diversity associated with migratory birds and preliminary results on the potential adaptation of yeasts found in bird cloae. For the first time, cactophilic yeasts belonging to Phaffomyces have been isolated in Europe and from birds and, on the basis of a phylogenetic and phenotypic analysis, the novel yeast species P. usticensis is proposed. Further analyses of birds sampled in different years and sites should allow a deeper knowledge of the diversity of yeasts carried by migratory birds and specific experiments on persistence of yeasts into bird cloaca could provide additional information on yeast adaptation to the conditions of the intestinal tract of birds.

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**References**


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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Phenotypic characterization of the 10 yeast species isolated from birds (results shown in percentage of the total number of isolates as follows: positive/weak/negative).