RESEARCH ARTICLE

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 ecoclade

Nicola Francesca[1,2], Cláudia Carvalho[2], Ciro Sannino[1], Marco A. Guerreiro[2], Pedro M. Almeida[2], Luca Settanni[1], Bruno Massa[1], José P. Sampaio[2] & Giancarlo Moschetti[1]

[1]Department of Agricultural and Forest Science, University of Palermo, Palermo, Italy; and [2]Centro de Recursos Microbiológicos, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal

Correspondence: Giancarlo Moschetti, Department of Agricultural and Forestry Science, University of Palermo, Viale delle Scienze 4, 90128 Palermo, Italy.
Tel.: + 39 091 23896050;
fax: + 39 091 6515531;
e-mail: giancarlo.moschetti@unipa.it

The GenBank/EMBL/DDBJ accession numbers for the sequences of D1/D2 domain of 26S gene of strains PYCC 6346^T (= CBS 12958^T), 967A2, 967A3 and 967A4 are KF719195, KF719192, KF719193 and KF719194, respectively. The Mycobank accession name number is MB 805761. f.a., for *forma asexualis* asexual form: this term has been added to the name of species in the title and abstract of this publication.

Received 13 February 2014; revised 8 June 2014; accepted 26 June 2014. Final version published online 28 July 2014.

DOI: 10.1111/1567-1364.12179

Editor: Cletus Kurtzman

Keywords
cactus-yeast; *Delichon urbicum*; novel species; *Phaffomyces usticensis* sp. nov.; phenotypic characterization; phylogenetic analysis.

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 6346^T = CBS 12958^T).

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 (Birtsas *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 across 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 6346^T (CBS 12958^T) 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 Mullarney *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 amplicons, as well as the respectively restriction fragments,

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, growth at pH 2.5 and pH 3.0 (medium was acidified with 1 N hydrochloric acid), and growth on 1% acetic acid agar (Yarrow, 1998). The eight strains belonging to *W. sylviae*, previously isolated by Francesca *et al.* (2013), were also characterized. The tests were carried out individually and by combining the temperatures of 25, 37, 40 and 42 °C with low pH and presence of acetic acid. The results of the tests were evaluated as 'positive' in presence of an intense growth with strong cell deposit; 'weak' when a growth with limited cell deposit was registered; and 'negative' when no growth occurred.

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 (Ronquisit & 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 MODELTEST (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-D-gluconate, 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) (Starmer *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

Bird family	Bird species	Migration type	No. of individuals sampled	No. of individuals carrying yeasts	SFA* of birds carrying yeasts	
					0–1	2–7
Sylviidae	*Sylvia borin* (garden warbler)	●†	37	20	8	12
	Hippolais icterina (icterine warbler)	●	8	0	0	0
	Acrocephalus schoenobaenus (sedge warbler)	●	6	0	0	0
	Phylloscopus sibilatrix (wood warbler)	●	19	6	3	3
	Sylvia communis (whitethroat)	●	24	11	2	9
	Sylvia cantillans (subalpine warbler)	●	9	2	0	2
	Phylloscopus trochilus (willow warbler)	●	4	0	0	0
Muscicapidae	*Ficedula albicollis* (collared flycatcher)	●	7	0	0	0
	Ficedula hypoleuca (pied flycatcher)	●	24	5	2	3
	Muscicapa striata (spotted flycatcher)	●	5	0	0	0
	Ficedula albicollis (collared flycatcher)	●	3	0	0	0
Turdidae	*Monticola solitarius* (blue rock-thrush)	■	15	4	2	2
	Saxicola rubetra (whinchat)	●	42	18	7	11
Hirundinidae	*Delichon urbicum* (house martin)	●	7	4	2	2
Total			210	70 (33%)	26	44

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

fingerprinting assays with primers $(GTG)_5$ (Sampaio *et al.*, 2001) and M13 (Stenlid *et al.*, 1994; Valmorri *et al.*, 2010). All patterns were analysed using the GELCOMPARE 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 albidus* var. *kuetzingii*, *Cryptococcus magnus*, *Cryptococcus victoriae*, *Rhodotorula 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. *Candida 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 (Francesca *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

		Size of restriction fragments				Size of restriction fragments							
R.P.	5.8S-ITS PCR	CfoI	HaeIII	HinfI	26S PCR	HinfI	MseI	ApaI	Species (% identity)*	Isolate code	Acc. No.	No. of isolates† (%)‡	Distribution of isolates per each bird species
I	580	260 + 160 + 80	440 + 150	240 + 180 + 140	1100	470 + 390 + 180 + 55	585 + 210 + 160 + 95 + 60	n. a.	*Aureobasidium pullulans* (100)	852A	KF880791	5 (6)	B (2)§; H (3)
II	530	285 + 255	438 + 91	277 + 261	1100	490 + 403 + 186	570 + 405	555 + 410 + 130	*Candida albicans* (99)	801B	KF880792	12 (15)	A (6); E (2); F (4)
III	880	380 + 160 + 140	650 + 220	350 + 260 + 55	1100	490 + 215 + 195 + 55	675 + 375 + 75	725 + 430	*Candida glabrata* (99)	766A	KF880793	16 (20)	B (3); F (2); D (6); G (5)
IV	480	105 + 90 + 75 + 56	480	265 + 220	1100	485 + 235 + 180 + 130	n.a.	710 + 385	*Candida inconspicua* (99)	791A	KF880794	17 (22)	B (7); E (1); F (6); G (3)
V	700	325 + 300	510 + 70	280 + 255 + 95	1100	335 + 275 + 215 + 155	400 + 360 + 245 + 65	n. a.	*C. albidus var. kuetzingii* (99)	820GYP	KF880795	2 (3)	F (2)
VI	650	350 + 300	520 + 90	280 + 235 + 140	1100	255 + 200 + 175 + 160 + 145 + 75 + 55	400 + 365 + 244	n. a.	*Cryptococcus magnus* (99)	660A	KF880796	2 (3)	B (2)
VII	530	290 + 245	360 + 125	269 + 180 + 85	1100	420 + 275 + 210 + 200	405 + 285 + 245	n. a.	*Cryptococcus victoriae* (99)	551B2	KF880797	3 (4)	H (3)
VIII	480	240 + 220	380 + 115	404	1100	335 + 310 + 210 + 185 + 75	n.a.	n. a.	*Phaffomyces* sp. (97)	967A5	KF719195	4 (5)	C (4)
IX	640	300 + 225	404 + 217	346 + 215	1100	495 + 410 + 205	355 + 270 + 235 + 140	n. a.	*Rhodotorula mucilaginosa* (99)	L42	KF880798	13 (16)	B (9); F (4)
X	640	610	560 + 80	330 + 310	1080	500 + 240 + 180 + 160	n. a.	n. a.	*Wickerhamomyces sylviae* (100)	692A	KF880799	5 (6)	G (5)

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 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.

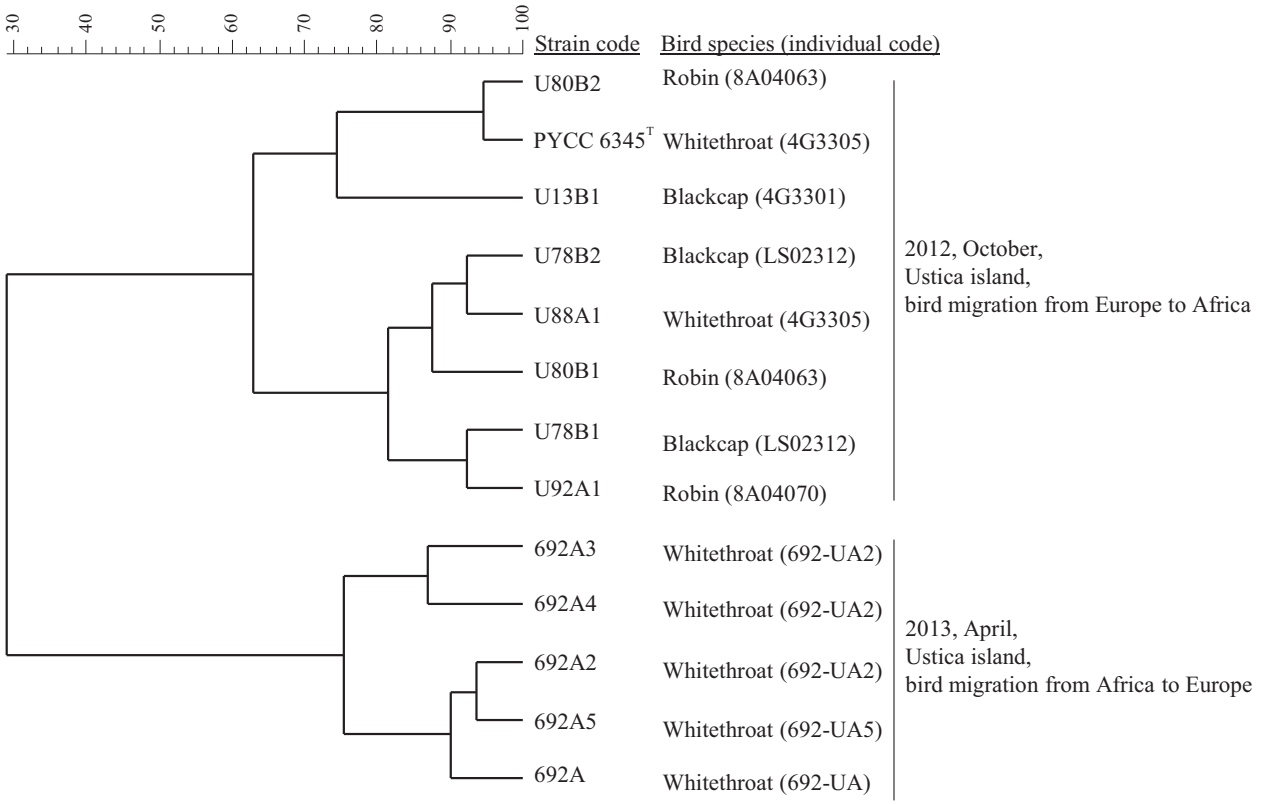

Fig. 1. Dendrograms obtained from combined RAPD-PCR and MSP-PCR profiles of the *Wickerhamomyces sylviae* strains. Upper line indicates the percentage of similarity.

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. The isolates belonging to *C. albicans*, *C. glabrata*, *A. pullulans* and *W. sylviae* showed intense or weak growth for the majority of the tests (Supporting Information, Table S1). On the other hand, among the basidiomycetous species, only *C. albidus* var. *kuetzingii* showed positive growth at 37 °C, and at low pH (3.0 and 2.5) 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

Table 3. Phenotypic characterization of the 87 yeast isolates collected from birds

Characteristics	Positive	Weak	Negative
Growth at:			
37 °C	79	13	8
40 °C	54	2	44
42 °C	24	7	69
pH 3.0 and 25 °C	84	10	6
pH 3.0 and 37 °C	51	17	32
pH 3.0 and 40 °C	33	16	51
pH 3.0 and 42 °C	25	6	69
pH 2.5 and 25 °C	57	18	25
pH 2.5 and 37 °C	32	14	54
pH 2.5 and 40 °C	30	6	64
pH 2.5 and 42 °C	22	5	74
On 1% acetic acid agar at 25 °C	–	5	95
On 1% acetic acid agar at 37 °C	–	2	98
On 1% acetic acid agar at 40 °C	–	–	100
On 1% acetic acid agar at 42 °C	–	–	100

The results are shown in percentage and highlighted in black boxes when the majority (> 50%) of isolates showed intense growth (positive) and in gray boxes when a minority (< 50%) of isolates showed intense or weak results.

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 *P. usticensis* Francesca, Carvalho, Settanni, Sampaio & Moschetti 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 spheri-

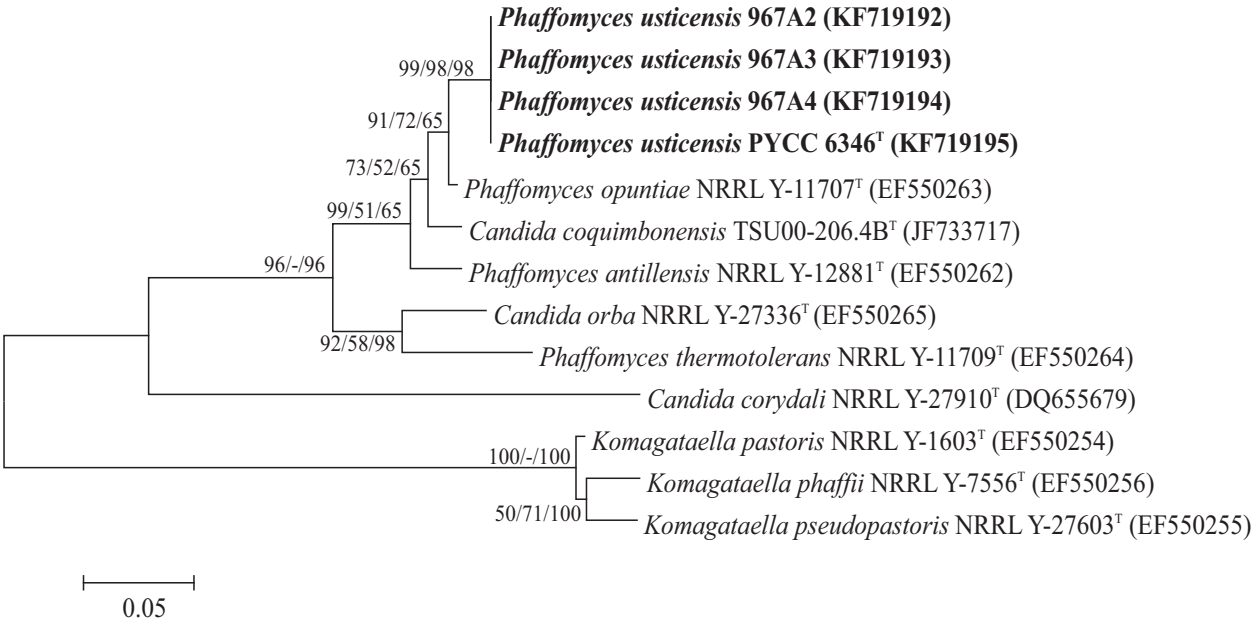

Fig. 2. Neighbour-joining phylogenetic tree based on the the D1/D2 domains of 26S rRNA gene sequences of the four strains of *Phaffomyces usticensis* sp. nov. and its closest relatives. Support values of nodes are reported as follows: neighbor-joining bootstrap values/maximum-likelihood bootstrap values/MRBAYES posterior probabilities. Values below 50% are shown as a minus sign on the branches. The GenBank accession numbers are reported between brackets after strain codes. *Komagataella pastoris*, *K. phaffii* and *K. pseudopastoris* are used as the outgroup species. Scale bar: 5% sequence difference.

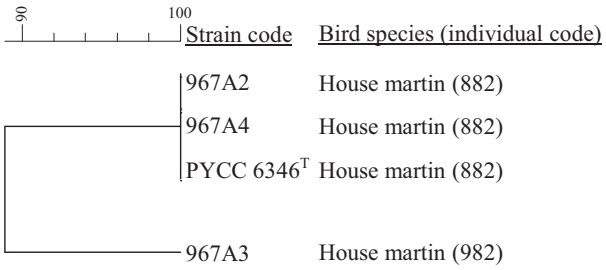

Fig. 3. Dendrograms obtained from combined RAPD-PCR and MSP-PCR profiles of the *Phaffomyces usticensis* strains. Upper line indicates the percentage of similarity.

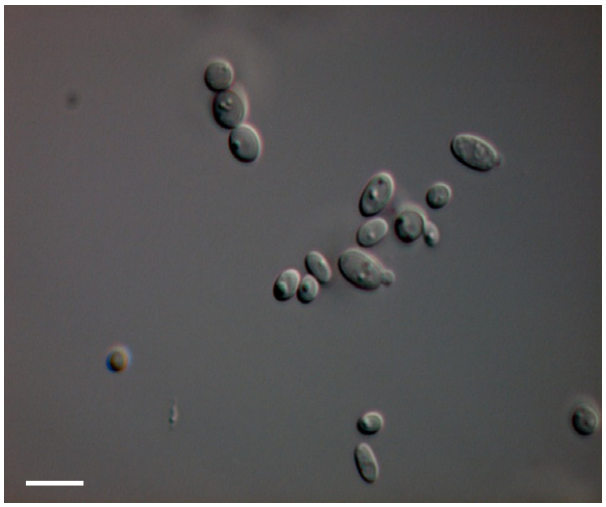

Fig. 4. Vegetative cells of *Phaffomyces usticensis* sp. nov. PYCC 6346^T (= CBS 12958^T) after 3 days at 25 °C on 5% ME agar. Scale bars: 10 μm.

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, mucid 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 6346^T (= CBS 12958^T) 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 6346^T = CBS 12958^T) is proposed for the new ascomycetous 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 stopover 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 ectoparasites. 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 $\pm$ 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 the 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 cloacae 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 cloacae. 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 cloacae could provide additional information on yeast adaptation to the conditions of the intestinal tract of birds.

Acknowledgements

This work was partially financially supported by FCT (Portugal) through project Pest-OE/BIA/UI0457/2011 and by Provincia Regionale of Palermo (Italy) for ringing of birds in Ustica. Ringing activity was authorized by ISPRA and Assessorato alle Risorse Agricole della Regione Siciliana.

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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).