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ORIGINAL ARTICLE

Isolation, identification and oenological characterization of non-*Saccharomyces* yeasts in a Mediterranean island

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Significance and Impact of the Study: In recent years, interest is growing for wine production by non-*Saccharomyces* yeasts, both in research and in the industry. This study describes the yeast population of the grapes in a small-secluded island in the Mediterranean Sea, useful site for the search of new strains. Evaluation of fundamental oenological characters identifies potential best yeasts to assay in experimental vinifications. We also describe, for the first time, 14 new colony morphologies on WL Nutrient Agar, culture medium used to monitor the yeast population dynamics.

Keywords
diversity, identification, non-*Saccharomyces*, wine, yeasts.

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Abstract

We isolated, identified and characterized yeast strains from grapes, and their fermented musts, sampled in the small island of Linosa, where there are no wineries and therefore the possibility of territory contamination by industrial strains is minimal. By traditional culture-dependent methods, we isolated 3805 colonies, distinguished by molecular methods in 17 different species. Five hundred and forty-four isolates were analysed for the main oenological characteristics such as fermentative vigour with and without sulphites, sugar consumption and production of alcohol, volatile acidity, hydrogen sulphide, glycerol and β-glucosidase. This analysis identified *Kluyveromyces marxianus* (seldomly used in winemaking) as the most interesting candidate yeast for the production of innovative wines.

Introduction

Non-*Saccharomyces* yeasts include several species responsible for the early stages of spontaneous alcoholic fermentation (Jolly *et al.* 2014). Grapes are the main source of non-*Saccharomyces* yeasts, with same species typically found worldwide (Jolly *et al.* 2006; Barata *et al.* 2012). In recent years, interest in these yeasts has been growing because they can improve wine quality: (i) lowering the alcohol content; (ii) increasing the final concentrations of glycerol; (iii) increasing the range of flavour compounds, through the production of esters, higher alcohols and β-glucosidase; (iv) producing proteolytic and pectinolytic activities; (v) affecting the concentration of polysaccharides (Jolly *et al.* 2014). For these reasons, researchers all over the world agree on the need to increase our knowledge of non-*Saccharomyces* yeasts and of their oenological characteristics (Ciani *et al.* 2010; Jolly *et al.* 2014; Steensels and Verstrepen 2014).

In the last years, yeast industry has started to produce selected non-*Saccharomyces* strains (Lu *et al.* 2016). However, the use of commercial yeasts can cause their dissemination, although limited, in the areas surrounding the wineries (Valero *et al.* 2005). In order to find new non-*Saccharomyces* yeast strains for wine production, we studied yeast population found on grapes in a small Mediterranean island, Linosa, where there are no wineries and where the possibility of yeast strains exchanges from other territories is limited.

Results and discussion

Yeast population

We report the yeast population found on grapes in two new vineyards (A and B) and in some old vines on the island of Linosa. This is a small, secluded island in the Mediterranean Sea (Fig. 1), where there are no cellars and

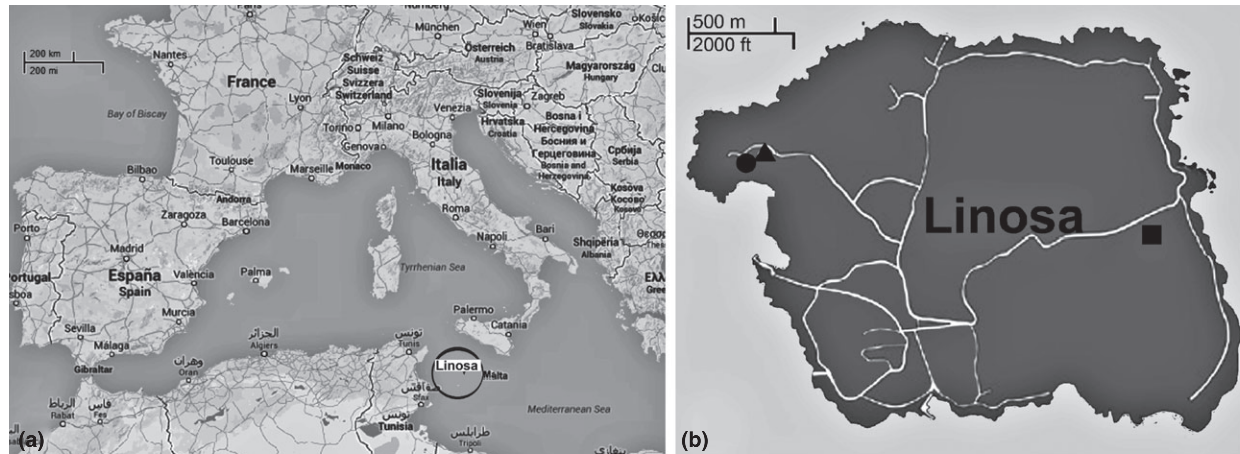

Figure 1 Geographic localization of the Linosa Island (a) and location of the grape sampling sites (b): (●) new vineyard A, (■) new vineyard B and (▲) old vines.

no import of commercial yeasts, except for one *Saccharomyces cerevisiae* strain used in the only one bakery of the island. Although the introduction of yeast strains from outside remains possible, for example, via insects (Stefanini *et al.* 2012) or migratory birds (Francesca *et al.* 2012), we expected that the contamination by commercial yeasts would be lower in comparison to areas where wineries are present.

For an initial evaluation, a total of 3805 yeast colonies (from grapes and, to increase the ability to isolate any alcohol tolerant yeast, from their musts fermented in the laboratory) were analysed and divided into 16 different groups (Table 1, Fig. 2) on the basis of their morphotype on WL Nutrient agar, which allows a preliminary discrimination between yeast species by colony morphology and colour (Pallmann *et al.* 2001; Romancino *et al.* 2008; Li *et al.* 2011). To the best of our knowledge, except for the genus *Hanseniaspora* and the species *Issatchenkia orientalis*, the remaining colony morphologies of Table 1 have not been previously described.

The ITS rDNA of representative 553 isolates were analysed by PCR-RFLP (Table 1). We found distinctive restriction patterns for each of the 16 groups, except for morphotypes I and II, which showed the same patterns; moreover, morphotype IX was resolved into three different restriction patterns, for a total of 17 different patterns. The sequencing of D1/D2 region of the 26S rDNA allowed the unequivocal identification of 15 species, while further tests were required in two cases of uncertainty. Morphotypes I and II were identified as two different varieties of yeast-like fungus *Aureobasidium pullulans*. PCR-RFLP patterns were consistent with at least one of the following authors: Esteve-Zarzoso *et al.* (1999), Granchi *et al.* (1999), Villa-Carvajal *et al.* (2006), Nisiotou and Nychas (2007), Nisiotou *et al.* (2007), Barata *et al.* (2008), Jatmiko *et al.* (2012), Merín *et al.* (2013). Since in the region D1/D2 there are no differences between *Kluyveromyces lactis* var. *lactis* and *Kluyveromyces marxianus*, for morphology XIII further sequencing of the ITS region of rDNA was carried out. Results revealed only 92% identity with *K. lactis* var. *lactis* (type strain CBS683, accession number AJ229069) and 100% with *K. marxianus* (type strain CBS712, accession number EF568057), leading to the unequivocal assignment of this morphology to the latter species. Finally, since in the region D1/D2 there are no differences between *Candida albicans* and *Candida africana*, the phenotypic identification of morphology III has led to its unequivocal assignment to the species *C. albicans* which can occasionally be found on the grapes (Jolly *et al.* 2006).

We found most of the species only in samples from old vines; *A. pullulans* var. *pullulans* and *Hanseniaspora opuntiae* also in samples from vineyards A and B; *Pichia norvegensis* even in samples of the vineyard A; *A. pullulans* var. *melanigenum*, *Cryptococcus albidus*, *Hanseniaspora uvarum* and *Kodamaea ohmeri* only in samples from the vineyard B, located on the opposite side of the island than the other two sampling sites. The greatest biodiversity found on old vines is probably due to lack of phytosanitation and/or to the increased accessibility of the grapes to the animals of the island, due to the lack of fences.

Table 1 also shows that we found most of the yeasts only or mainly on grapes, with *Hanseniaspora guilliermondii*, *H. opuntiae*, *H. uvarum* and *I. orientalis* the most abundant species. We recovered *A. pullulans* var. *pullulans*, *H. guilliermondii* and *H. opuntiae* from samples at all stages of ripeness; the occurrence of all the other species was restricted to specific times during grape maturation.

Table 1 Colony morphologies of isolated yeast, results of molecular identification and percentage distribution

WL morphotype (number of detected colonies)	Colony colour	Colony topography	ITS1-ITS4 Amplicon size (bp)	ITS1-ITS4 Restriction fragments (bp)	Sequencing of D1/D2 region of 26S rDNA (type strain-identity %- acces sion number)	Species	% on grapes	% in fermented musts
I (6)	Light green/dark at the center over time	Convex; surface:rough/glossy	590	HhaI:190-180-100	CBS123·37-99%-FJ150917	*Aureobasidium pullulans* var. *melanigenum*	0·27	0·00
II (41)	Light green	Convex; surface:rough/glossy	590	HhaI:190-180-100	CBS584·75-100%-FJ150942	*Aureobasidium pullulans* var. *pullulans*	1·86	0·00
III (9)	White/yellowish medium	Flat; surface:smooth/glossy	550	HaeIII:460-90	CBS562-99%-U45776	*Candida albicans**	0·41	0·00
IV (6)	Cream/yellowish medium	Convex; surface:smooth/glossy	490	HaeIII:400-60	CBS2868-100%-U45703	*Candida apicola*	0·27	0·00
V (169)	Light green in the center/yellowish medium	Convex; surface:smooth/glossy	880	HaeIII:670-220	CBS138-99%-U44808	*Candida glabrata*	0·05	10·13
VI (85)	Bright green/yellowish medium	Convex; surface:smooth/glossy	580	HinfI:300-230	CBS7853-99%-EF550286	*Candida stellimalicola*	3·86	0·00
VII (1)	Light green/yellowish medium	Convex; surface:smooth/glossy	380	HinfI:190-190	CBS6936-99%-U44817	*Clavispora lusitaniae*	0·05	0·00
VIII (18)	Light green in the center/cream at the periphery	Convex; surface:smooth/glossy	630	HinfI:350-160-120	CBS142-100%-AF075474	*Cryptococcus albidus*	0·82	0·00
IX (1749)	Intense green/yellowish medium	Flat; surface:smooth/opaque	750	DdeI:360-180-85-70-50	CBS465-99%-AF399797	*Hanseniaspora guilliermondii*	10·65	1·27
				DraI:420-300-30	CBS8733-99%-AJ512453	*Hanseniaspora opuntiae*	50·22	3·25
				DdeI:290-180-90-85-75-50	CBS314-99%-AF399798	*Hanseniaspora uvarum*	15·22	0·00
X (216)	Grey-green	Convex; surface:opaque/wrinkled	450	HinfI:250-200	CBS2352-100%-U45712	*Hyphopichia (Pichia) burtonii*	0·00	13·02
XI (673)	Cream to light green/yellowish medium	Convex; surface:opaque/fuzzy, with volcanic center	510	HinfI:220-150-140	CBS5147-100%-EF550222	*Issatchenkia orientalis*	15·50	20·01
XII (312)	Cream to light green/yellowish medium	Flat; surface:smooth/opaque	410	HaeIII:290-120	CBS2617-100%-EF550233	*Issatchenkia terricola*	0·00	18·81
XIII (5)	Light green, green at the periphery/yellowish medium	Knoblike; surface:smooth/glossy	750	HinfI:250-185-120-75-65-50	CBS712-100%-AY497692	*Kluyveromyces marxianus**	0·23	0·00
XIV (5)	Green, darker in the center/yellowish medium	Elevated and convex; surface: opaque/wrinkled	420	HinfI:210-180	CBS5367-100%-U45702	*Kodamaea (ex Pichia) ohmeri*	0·23	0·00
XV (530)	Very light green/yellowish medium	Convex; surface:smooth/glossy	620	HaeIII:400-120-80	NRRL Y-27274-100%-EU348786	*Meyerozyma (ex Pichia) caribbica*	0·00	31·95
XVI (34)	White to cream/yellowish medium	Flat; surface:opaque/wrinkled	500	HinfI:275-230	CBS6564-99%-EF550239	*Pichia norvegensis*	0·36	1·57

*For the identification of this species further assays were performed as described in the text.

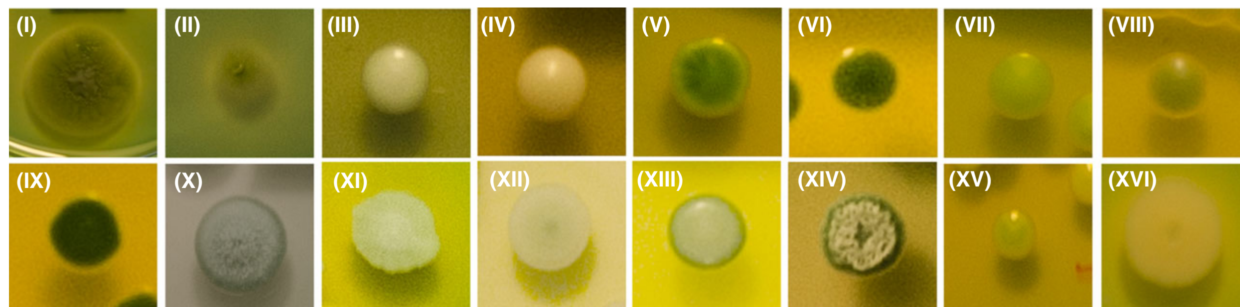

Figure 2 The 16 colony morphotypes on WL Nutrient Agar. I: *Aureobasidium pullulans* var. *melanigenum*; II: *Aureobasidium pullulans* var. *pullulans*; III: *Candida albicans*; IV: *Candida apicola*; V: *Candida glabrata*; VI: *Candida stellimalicola*; VII: *Clavispora lusitaniae*; VIII: *Cryptococcus albidus*; IX: *Hanseniaspora* spp.; X: *Hyphopichia (Pichia) burtonii*; XI: *Issatchenkia orientalis*; XII: *Issatchenkia terricola*; XIII: *Kluyveromyces marxianus*; XIV: *Kodamaea (ex Pichia) ohmeri*; XV: *Meyerozyma* (ex *Pichia*) *caribbica*; XVI: *Pichia norvegensis.*

Since the opportunistic pathogenicity of *C. albicans* is well known (Papon *et al.* 2013), all the nine isolates of this species were eliminated and we did not carry out further investigations on them.

Oenological characterization of the yeast species

Overall, 10 important oenological characteristics were assayed on 544 isolates belonging to the remaining 16 species: results of the analysis for eight of these parameters are shown in Table 2; measured values for a strain of *S. cerevisiae* and for unfermented must are also shown respectively as positive and negative control. Fermentative vigour without sulphites after two and 7 days, residual reducing sugar and ethanol produced after 15 days are all indicators of the fermentative ability of the different yeasts (Caridi *et al.* 2002; Luna-Solano *et al.* 2003). As expected, all the non-*Saccharomyces* yeasts isolated in Linosa show fermentative ability lower than *S. cerevisiae*. In addition, if we take into account the amounts of fermented sugar, they are higher producers of volatile acidity compared to *S. cerevisiae*, with the largest amount ever produced by the genus *Hanseniaspora*. Furthermore, as demonstrated by the differences in the fermentative vigour without and with SO_2, except *Candida glabrata*, they are all sensitive to sulphites, a preservative widely used in winemaking. Four species, *Candida apicola*, *C. glabrata*, *I. orientalis* and *K. marxianus*, ferment sugar quantities between 65 and 91 g l^{-1} and produce alcohol between 3·76% and 5·39% (v/v). Three of them (*C. apicola*, *C. glabrata* and *I. orientalis*) produce significant amounts of glycerol: this compound, above certain thresholds, can positively contribute to the taste, smoothness, consistency and overall body of wine (Scanes *et al.* 1998). In particular, *C. apicola* yeasts isolated in Linosa produce two and a half times more glycerol per gram of fermented sugar compared to the *S. cerevisiae* control strain.

Table 3 shows the production of H_2S and of β-glucosidase. Six species (including *C. apicola* and *I. orientalis*) produce high amounts of hydrogen sulphide, incompatible with their oenological use because above 50–80 $\mu g\ l^{-1}$, H_2S produces an off-flavour of rotten eggs (Rauhut 1993). However, several isolates of *C. glabrata* and *K. marxianus* produce quantities of sulphide compatible with winemaking. Table 3 shows also that isolates of *K. marxianus* found in Linosa are among the higher producers of β-glucosidase, an enzyme able to release monoterpenols, important in wine flavour and aroma, from their flavourless, nonvolatile glycosidic complexes (Jolly *et al.* 2014).

In conclusion, several isolates of the species *C. glabrata* and *K. marxianus* could be useful in the production of experimental wines richer, respectively, in glycerol and aromatic compounds. The presence of *C. glabrata* on grapes and musts has been widely reported (see Jolly *et al.* 2006 for review), but its possible pathogenicity and resistance to azoles and polyenes (Papon *et al.* 2013) make its use impossible in winemaking (EFSA 2013). *Kluyveromyces marxianus* has instead a much lower pathogenicity, is sensitive to antimycotics (Papon *et al.* 2013) and has a qualified presumption of safety (EFSA, European Food Safety Authority 2013). This microorganism has been isolated from a large variety of habitats, mostly from foods and beverages, especially dairy products, as well as decaying plant tissue and associated insects (Lachance 2011). It is widely studied for its ability to produce enzymes of industrial interest as inulinase, β-galactosidase, β-glucosidase, endopolygalacturonases, protein phosphatases, carboxypeptidases and aminopeptidases (Fonseca *et al.* 2008), but its direct use in winemaking has been very limited so far (Kourkoutas *et al.* 2004). Thanks to their fermentative ability and β-glucosidase activity, *K. marxianus* yeasts isolated in Linosa may therefore represent an important starting point for the production of new and more aromatic wines.

Table 2 Oenological parameters measured in grape must fermented by the isolated yeasts

Species (number of analysed yeast isolates)	Fermentative vigour after 2 days without SO_2 (gCO_2 per 100 ml)	Fermentative vigour after 2 days with SO_2 (gCO_2 per 100 ml)	Fermentative vigour after 7 days without SO_2 (gCO_2 per 100 ml)	Fermentative vigour after 7 days with SO_2 (gCO_2 per 100 ml)	Ethanol% after 15 days (v/v)	Reducing Sugar after 15 days (g l^{-1})	Volatile Acidity after 15 days (g l^{-1})	Glycerol after 15 days (g l^{-1})
Aureobasidium pullulans var. *melanigenum* (6)	0·01 ± 0·01a	0·01 ± 0·02a	0·03 ± 0·02a	0·02 ± 0·02a	0·12 ± 0·01ab	201·96 ± 0·36hi	0·10 ± 0·01a	0·00 ± 0·00a
Aureobasidium pullulans var. *pullulans* (21)	0·01 ± 0·02a	0·02 ± 0·04a	0·05 ± 0·03a	0·04 ± 0·05a	0·24 ± 0·02ab	198·77 ± 0·82hi	0·15 ± 0·02ab	0·00 ± 0·00a
Candida apicola (6)	0·22 ± 0·07c	0·04 ± 0·06a	1·51 ± 0·14fg	0·04 ± 0·05a	4·04 ± 0·13f	141·32 ± 2·73d	0·49 ± 0·08de	5·98 ± 0·34g
Candida glabrata (42)	0·69 ± 0·17e	0·31 ± 0·08b	2·34 ± 0·68h	2·05 ± 0·51b	5·39 ± 1·08g	115·26 ± 17·45b	0·45 ± 0·04cd	4·37 ± 0·82f
Candida stellimalicola (43)	0·05 ± 0·03ab	0·05 ± 0·02a	0·16 ± 0·04ab	0·07 ± 0·04a	0·69 ± 0·08bc	193·18 ± 1·29gh	0·41 ± 0·03cd	0·87 ± 0·17bc
Clavispora lusitaniae (1)	0·09 ± 0·01abc	0·02 ± 0·01a	0·95 ± 0·04de	0·02 ± 0·01a	1·64 ± 0·02de	176·69 ± 0·71ef	0·22 ± 0·00b	0·69 ± 0·03b
Cryptococcus albidus (18)	0·03 ± 0·02ab	0·02 ± 0·01a	0·02 ± 0·01a	0·01 ± 0·01a	0·08 ± 0·06ab	207·36 ± 2·40i	0·25 ± 0·03b	0·09 ± 0·06a
Hanseniaspora guilliermondii (24)	0·04 ± 0·04ab	0·01 ± 0·01a	0·65 ± 0·10cd	0·03 ± 0·02a	1·38 ± 0·18d	187·46 ± 3·35fg	0·73 ± 0·06g	1·57 ± 0·35d
Hanseniaspora opuntiae (116)	0·04 ± 0·04ab	0·01 ± 0·02a	0·52 ± 0·16bc	0·02 ± 0·02a	1·03 ± 0·18cd	194·22 ± 3·02gh	0·58 ± 0·11ef	0·73 ± 0·32b
Hanseniaspora uvarum (35)	0·08 ± 0·03ab	0·03 ± 0·03a	0·47 ± 0·14bc	0·03 ± 0·06a	2·10 ± 0·97e	174·74 ± 18·08e	0·73 ± 0·13g	1·38 ± 0·82cd
Hyphopichia (Pichia) burtonii (54)	0·12 ± 0·04abc	0·02 ± 0·02a	1·20 ± 0·14ef	0·03 ± 0·01a	1·63 ± 0·18de	174·18 ± 6·63e	0·25 ± 0·09b	1·37 ± 0·37cd
Issatchenkia orientalis (67)	0·37 ± 0·07d	0·03 ± 0·04a	2·37 ± 0·29h	0·13 ± 0·15a	4·32 ± 0·31f	129·65 ± 5·66c	0·50 ± 0·04def	3·73 ± 0·44e
Issatchenkia terricola (31)	0·02 ± 0·01ab	0·01 ± 0·01a	0·18 ± 0·08ab	0·03 ± 0·02a	0·41 ± 0·04ab	196·85 ± 0·40ghi	0·20 ± 0·02ab	0·01 ± 0·02a
Kluyveromyces marxianus (5)	0·15 ± 0·02bc	0·02 ± 0·01a	1·78 ± 0·07g	0·02 ± 0·01a	3·76 ± 0·04f	136·01 ± 0·72cd	0·38 ± 0·07c	1·84 ± 0·25d
Kodamaea (ex Pichia) ohmeri (5)	0·04 ± 0·01ab	0·02 ± 0·01a	0·79 ± 0·10cd	0·04 ± 0·02a	1·33 ± 0·20d	180·62 ± 4·85ef	0·25 ± 0·04b	0·05 ± 0·05a
Meyerozyma (ex *Pichia*) *caribbica* (53)	0·11 ± 0·07abc	0·03 ± 0·04a	1·23 ± 0·49ef	0·04 ± 0·02a	1·44 ± 0·20d	181·47 ± 3·11ef	0·25 ± 0·05b	0·46 ± 0·30ab
Pichia norvegensis (17)	0·04 ± 0·02ab	0·04 ± 0·02a	0·06 ± 0·03a	0·05 ± 0·02a	0·06 ± 0·01a	207·18 ± 0·16i	0·24 ± 0·01b	0·00 ± 0·00a
*Saccharomyces cerevisiae*L404	3·22 ± 0·99f	2·95 ± 0·93c	9·97 ± 0·74i	9·35 ± 0·76c	11·42 ± 0·20h	9·99 ± 1·75a	0·60 ± 0·05f	7·15 ± 0·41h
Not-inoculated must	0·01 ± 0·01a	0·01 ± 0·01a	0·03 ± 0·02a	0·03 ± 0·03a	0·09 ± 0·05ab	206·66 ± 2·04i	0·20 ± 0·04ab	0·01 ± 0·02a

In each column, different letters indicate significant differences between different species. Tukey's HSD test, $P < 0.01$.

Table 3 Distribution of yeast isolates in scales from zero to three and from white to black, respectively, for β-glucosidase and sulphide increasing production

	β-glucosidase				H_2S production				
Species	0	1	2	3	w	ph	h	dh	b
Aureobasidium pullulans var. *melanigenum*			6			6			
Aureobasidium pullulans var. *pullulans*			14	7	21				
Candida apicola			6					6	
Candida glabrata	42					38		4	
Candida stellimalicola		36		7	32	7		4	
Clavispora lusitaniae		1						1	
Cryptococcus albidus			18		18				
Hanseniaspora guilliermondii			24			24			
Hanseniaspora opuntiae			116		2	114			
Hanseniaspora uvarum			34	1	31	4			
Hyphopichia (Pichia) burtonii				54				54	
Issatchenkia orientalis	67							67	
Issatchenkia terricola		31							31
Kluyveromyces marxianus				5			5		
Kodamaea (ex Pichia) ohmeri			5					5	
Meyerozyma (ex *Pichia*) *caribbica*		51	2			28	24	1	
Pichia norvegensis		17				3		14	

W, white; ph, pale hazel; h, hazel; dh, dark hazel; b, black.

Materials and methods

Sampling sites

In 2007, we established two experimental vineyards (A and B) of the Muscat of Alexandria cultivar on Linosa island (433 inhabitants; 5·43 km^2; 35°52′00″North; 12°52′00″East; Fig. 1a), about 160 km away from Tunisia and Sicily (Italy). We used Bordeaux mixture for phytosanitation and protected the vineyards with fences. Some unprotected scattered plants of Ansonica cultivar were also present (Fig. 1b); we did not applied any phytosanitation treatment to these 'old vines'.

Grape sampling

During 2009 summer, we carried out aseptically five samplings in duplicate, once every 2 weeks, from each vineyard and from the old vines. Samples were kept cold and transferred to the laboratory within 12 h.

Microbiological analyses of 'grapes' and 'fermented musts'

One hundred millilitres of grape juices was poured into sterile bottles and left to ferment at 28°C ('fermented musts') until no further effervescence could be seen and no further reduction in glucose concentration was measured using a Keto Diabur Test (Roche, Mannheim, Germany). We plated aliquots of grape juices and fermented musts, eventually serially diluted in duplicate with sterile peptone 1 g l^{-1}, on WL Nutrient Agar (Oxoid, Basingstoke, England) (Pallmann *et al.* 2001) and on Lysine Agar (Oxoid) (Fowell 1965), with Diphenyl 0·1 g l^{-1} to slow-down moulds (Kurtzman *et al.* 2011). After 5 days at 28°C, we analysed plates with 20–200 colonies. When the number of colonies from undiluted samples was low, we analysed a high number of plates. We streaked yeasts in single colonies on WL Nutrient Agar to better recognize their morphotypes. Based on colony morphology on WL Nutrient Agar and microscopic analyses (data not shown), morphotypes of 3805 yeast colonies were identified. A number of representative colonies were isolated and purified: 553 yeast isolates were stored at 4°C on Malt Agar (30 g l^{-1} Malt extract, 15 g l^{-1} Agar; Oxoid) and used for further investigations.

Molecular analyses

Five hundred and fifty-three yeast isolates were analysed by PCR-RFLP (Esteve-Zarzoso *et al.* 1999) using the primers ITS1 and ITS4 (White *et al.* 1990). We digested amplicons with HinfI, HaeIII, HhaI and, in case, DdeI and RsaI (New England BioLabs, Ipswich, MA, USA). The PCR products and their restriction fragments were separated on 1·5–3% agarose gels with 1xTBE buffer; Table 1 shows only one polymorphism per species. One–four isolates per each group were analysed by amplification and

sequencing of the D1/D2 region of the 26S rDNA (Kurtzman and Robnett 1997) using the primers NL-1 and NL-4 (O'Donnell 1993). Amplification and sequencing of the region ITS1-5·8S-ITS2 were performed using the primers ITS1 and ITS4 (White *et al.* 1990). Except for morphology IX, the D1/D2 sequence analysis along with the sequence analysis of the *K. marxianus* ITS region were performed at the Industrial Yeasts Collection (www.dbvpg.unipg.it) of the University of Perugia (Italy).

Phenotypic identification

For the unequivocal discrimination between *C. africana* and *C. albicans*, we assayed physiological (growth at 45°C) and biochemical (assimilation of *N*-acetylglucosamine, threalose and DL-lactate) abilities in triplicate (Romeo and Criseo 2011). These assays were performed at the Industrial Yeasts Collection of the University of Perugia (Italy).

Phenotypic characterization

Fermentative vigour without and with sulphites were measured according to Caridi *et al.* (2002): flasks containing 100 ml of sterile white must (20°Brix, pH 3.20, filtered by Stericup vacuum filtration system, Millipore, Billerica, MA, USA), with and without SO_2 (100 mg l^{-1}) and covered with 10 ml of sterile liquid paraffin to prevent evaporation, were inoculated in triplicate with 5 ml of 48 h precultures of each isolated yeast and incubated at 25°C. Fermentative vigour was measured as weight loss caused by CO_2 production (g CO_2 per 100 ml) after 2 and 7 days; *S. cerevisiae* L404 (DIPROVAL collection of the University of Bologna, Italy) was used as a positive control, not-inoculated must as negative control. Reducing sugar, ethanol, glycerol and volatile acidity were measured in must prepared as described above (without SO_2) using a WineScan™ apparatus (FOSS, Hilleroed, Denmark) after 15 days of fermentation. We estimated H_2S production on BiGGY agar (Oxoid) recording the biomass colour after 48 h at 25°C (Nickerson 1953). β-glucosidase production was assayed as in Strauss *et al.* (2001) onto selective medium containing 6·7 g l^{-1} Yeast Nitrogen Base (Difco, Detroit, MI, USA), 5 g l^{-1} arbutin (Sigma-Aldrich, Saint Louis, MO, USA), 0·2 g l^{-1} ammonium ferric citrate and 20 g l^{-1} agar (pH 5·0).

Statistical analysis

We used Analysis of Variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test to calculate significant differences between oenological parameters of different yeast species reported in Table 2. All tests were performed at a significances level of $P < 0.01$ using the statistical program SPSS (ver. 13, IBM, Armonk, NY, USA).

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Conflict of Interest

Under Italian law, the industrial property of the yeast strains described in this paper belong to D. Oliva and to the RIWO.

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