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Sporobolomyces agrorum sp. nov. and *Sporobolomyces sucorum* sp. nov., two novel basidiomycetous yeast species isolated from grape and apple must in Italy

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Abstract

During a survey of yeast populations associated with grape and apple musts used for wine and cider fermentation, respectively, six pink-coloured ballistoconidia-forming yeasts belonging to the order *Sporidiobolales* (Basidiomycota) were isolated. Phylogenetic analysis inferred using sequences of the internal transcribed spacer (ITS), the D1/D2 domain of the large subunit rRNA gene, the small subunit (SSU) rRNA gene and DNA-directed RNA polymerase II subunit (*RPB2*) indicated that the six isolates were separated in two novel species. One of the new species, *Sporobolomyces agrorum* sp. nov., isolated from grape must, had *Sporobolomyces roseus* and *Sporobolomyces metaroseus* as its closest relatives, but showed four/two and 16 nucleotide substitutions in the D1/D2 and ITS regions, respectively, to these two species. The other novel species, *Sporobolomyces sucorum* sp. nov., was found in apple must and was closely related to *Sporobolomyces pararoseus* and *Sporobolomyces patagonicus*, but showed two/three and five substitutions in those two regions for its closest relatives. We detected additional representatives of this species, most of them isolated from grapes whose sequences were already available on public databases. A sexual stage could not be observed for the novel species.

The genus *Sporobolomyces* has been revised recently and a total of 15 species are currently accepted [1]. The proposed changes were aimed to eliminate polyphyly and acknowledge the principle of 'one fungus=one name' [2–4], thus eliminating the dual nomenclature for asexual and sexual taxa. As a consequence, sexual species like *Sporodiobolus metaroseus* were renamed as *Sporobolomyces* [5]. The genus Sporobolomyces and its sexual counterpart, Sporodiobolus, that also occurs as a supported clade within the Sporodiobolaceae (Sporodiobolaes), contains the type species of *Sporobolomyces*, *Sporobolomyces salmonicolor*, and the type species of *Sporodiobolus*, *Sporodiobolus johnsonii* [1].

In the course of an evaluation of the yeast microbiota of grape and apple musts used to produce wine and artisanal cider in Italy, we detected six isolates that belonged to the genus *Sporobolomyces* based on morphological, phenotypic and phylogenetic analyses, but that could not be classified into any of the existing species. Here we present their formal description as *Sporobolomyces agrorum* sp. nov., found on grape must, and *Sporobolomyces sucorum* sp. nov. found on apple must but with additional representatives from grapes and other sources whose sequences were already available in public databases.

Samples of grape and apple must were collected during wine and cider fermentations carried out in 2011 and 2017, respectively, in Verona (Italy). The samples were directly plated onto Wallerstein laboratory nutrient agar (WL; Oxoid) and yeast extract peptone dextrose agar medium (YPD; 10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} dextrose, 15 g l^{-1} agar). After incubation for 5 days at 25 °C, yeast colonies were isolated and purified through repeated streaking. Isolates were maintained on YPD agar slants and kept at 4 °C. For light microscopy, an Optika B-383 Phi microscope was used (Optika srl). Crosses were made on corn meal agar and potato dextrose agar (Millipore) using all strain pairwise combinations and were incubated at 20 °C for 2 months. The biochemical and physiological characterization of yeast cultures was performed according to Kurtzmann *et al.* [6]. DNA

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Keywords: grape must; apple juice; basidiomycetous yeast; taxonomy; *Sporobolomyces agrorum*; *Sporobolomyces sucorum*.
Abbreviations: D1/D2, D1/D2domain of the largesubunit rRNA gene; ITS, internal transcribed spacer; RPB2, DNA-directed RNA polymerase II subunit; SSU, small subunitrRNA gene; WL, Wallerstein laboratory nutrient agar; YPD, yeast extract peptone dextrose agar medium.
The GenBank/EMBL/DDBJ accession numbers of the ITS, D1/D2 domain, SSU and *RPB2* sequences of *Sporobolomyces agrorum* sp. nov. PYCC 8108^T are MK037438, MK037432, MK037429 and MK040591, and of *Sporobolomyces sucorum* sp. nov. PYCC 8111^T are MK037435, MG478490, MK037426 and MK040588. The MycoBank accession numbers are MB830803 and MB830804, respectively.
Two supplementary figures and one supplementary table are available with the online version of this article.

from selected yeast cultures was obtained using the protocol described by Cocolin *et al.* [7] and was used to amplify the regions/genes used in the phylogenetic analyses [internal transcribed spacer (ITS), D1/D2 domain of the large subunit rRNA gene, small subunit (SSU) rRNA gene and DNA-directed RNA polymerase II subunit (*RPB2*)], using ITS1/ITS4, NL1/NL4, NS1/NS4, LROR/LR7 and RPB2-5F/RPB2-7 cR primers, respectively [1, 8–10]. The amplification products were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) according to the manufacturer's instructions. The sequencing of these products was carried out in both directions using the same primers used in the PCR amplification (Eurofins Genomics). Phylogenetic analyses were made with MEGA7 [11] using the maximum-likelihood algorithm and the Kimura two-parameter evolutionary model, as suggested by the implemented model test.

Preliminary surveys carried out at the Department of Biotechnology of University of Verona, Italy, on grape and apple musts used for wine and cider production yielded 74 yeast isolates that were subsequently identified at species level. Ascomycetous yeasts prevailed over basidiomycetous yeasts in both musts and the most frequent genera were *Hanseniaspora*, *Candida*, *Metschnikowia*, *Zygosaccharomyces* and *Pichia*. Basidiomycetous yeasts were mainly represented by *Rhodotorula* and *Filobasidium*. Among basidiomycetous yeasts, six isolates belonging to *Sporobolomyces* were recovered. Three strains were isolated from grape must (YG1=PYCC 8108^T, YG12=PYCC 8109 and YG13=PYCC 8110) and another three strains from apple must (YR16=PYCC 8111^T, YR18=PYCC 8112 and YR20=PYCC 8113). DNA sequences from these six isolates (Table 1) and relevant reference sequences from species of the genus *Sporobolomyces* retrieved from GenBank (Table S1, available in the online version of this article) were used to establish individual alignments of ITS, D1/D2 domain, SSU and *RPB2* sequences and also concatenated alignments of these regions. The phylogenetic analyses using the D1/D2 domain (Fig. S1) and the D1/D2+ITS (Fig. S2) alignments allowed comparisons with larger numbers of reference sequences than the one presented in Fig. 1. Nevertheless, the combined phylogenetic analysis shown in Fig. 1 benefits from a more robust sequence dataset since it is based on a concatenated alignment (ITS+D1/D2 +SSU+*RPB2*).

For the first novel species, *S. agrorum*, besides the three strains isolated during this study (PYCC 8108, PYCC 8109 and PYCC 8110), we found sequences of two additional representatives of this species deposited in GenBank. One strain, AY-14, was isolated from the leaf of a herbaceous plant in Russia and labelled *Sporobolomyces* cf. *roseus*. The other is CBS 2642, isolated from milk in UK and labelled as *Sporodiobolus metaroseus* (Fig. S1 and Table S1). Based on our phylogenetic analyses (Figs 1 S1 and S2), the closest relatives of *S. agrorum* are *S. metaroseus* and *S. roseus*. The number of nucleotide substitutions from *S. metaroseus* CBS 7683^T is two in the D1/D2 domain region and 16 in the ITS region. The number of nucleotide substitutions from *S. roseus* CBS 486^T (=AS 2.1948^T) is four in the D1/D2 domain region and 16 in the ITS region. The three strains of this novel species studied in detail in this work (PYCC 8108, PYCC 8109 and PYCC 8110) have identical sequences in the four regions that were investigated.

For the second novel species, the D1/D2 domain sequences of strains PYCC 8111, PYCC 8112 and PYCC 8113 were found to be identical to various sequences deposited at GenBank but labelled as *Sporidiobolus pararoseus* (Table S1 and Fig. S1). Therefore, besides the three strains reported here, the novel taxon *S. sucorum* is represented by more than 10 strains, isolated from grapes in France, Germany and Slovenia, grapevines in Austria, flowers in PR China and forest soil in the USA. Based on the concatenated alignment of ITS, D1/D2 domain, SSU and *RPB2* the closest relatives of *S. sucorum* are *S. pararoseus* CBS 491^T and *Sporobolomyces patagonicus* CRUB 1038^T (Fig. 1). The strains of *S. sucorum* (PYCC 8111, PYCC 8112 and PYCC 8113) differ from *S. pararoseus* CBS 491^T by two nucleotide substitutions in the D1/D2 domain region and five substitutions in the ITS region. The number of nucleotide substitutions from *S. patagonicus* CRUB 1038^T are three in the D1/D2 domain region and five the ITS region. The three strains of *S. sucorum* depicted in Fig. 1 have identical D1/D2 domain and SSU sequences but distinct ITS and *RPB2* sequences that differ by two nucleotide substitutions for ITS and between nine and 33 nucleotide substitutions for *RPB2*.

DESCRIPTION OF *SPOROBOLOMYCES AGRORUM* SP. NOV. M. LORENZINI, G. ZAPPAROLI AND J.P. SAMPAIO

Etymology: *Sporobolomyces agrorum* (a.gro′rum. L. gen. pl. n. *agrorum* of fields; relative to the rural environment where the species was isolated such as vineyards/grape and herbaceous plants for forage - of the fields).

MycoBank number: MB830803

After 1 week at 25 °C, cultures are salmon to pink with a smooth and glistening surface, with an entire margin and a mucous texture. Yeast cells after 4 days on YPD agar ovoid to cylindrical, 2.5–5.5×6–13 µm (Fig. 2a-g). Ballistoconidia are infrequently produced on corn meal agar after 4 days at 18 °C, ovoid to reniform, 2.5–4×6–8 µm (Fig. 2e). Crosses of PYCC 8108, PYCC 8109 and PYCC 8110 carried out on corn meal agar and potato dextrose agar and incubated at 20 °C for 8 weeks did not yield mycelium or teliospores. Fermentation of glucose is negative. Carbon compounds assimilated: D-glucose, D-galactose (weak), ethanol and glycerol (weak). No growth on inulin, sucrose, raffinose, melibiose, lactose, trehalose, maltose, melezitose, soluble starch, cellobiose, L-rhamnose, D-xylose, L-arabinose, D-arabinose, D-ribose, methanol, D-mannitol, myo-inositol, DL-lactate and citrate. Nitrogen compounds assimilated: potassium nitrate, ethylamine hydrochloride (weak) and cadaverine dihydrochloride. No growth on L-lysine and sodium nitrite. Growth in the presence of 0.1 % cycloheximide. Growth at 35 °C

Table 1. Strains and sequences of *Sporobolomyces agrorum* sp. nov. and *Sporobolomyces sucorum* sp. nov.

NA, Not available.

Species	Strain	Origin	D1/D2 Accession No.	ITS Accession No.	*RPB2* Accession No.	SSU Accession No.	Reference
Sporobolomyces agrorum	PYCC 8108^T=YG1	Grape must for Amarone wine production, Verona, Italy, 2011	MK037432	MK037438	MK040591	MK037429	This study
Sporobolomyces agrorum	PYCC 8109=YG12	Grape must for Amarone wine production, Verona, Italy, 2011	MK037433	MK037439	MK040592	MK037430	This study
Sporobolomyces agrorum	PYCC 8110=YG13	Grape must for Amarone wine production, Verona, Italy, 2011	MK037434	MK037440	MK040593	MK037431	This study
Sporobolomyces agrorum	CBS 2642	Milk, UK	KY109710	KY105474	NA	NA	[23]
Sporobolomyces agrorum	AY-14	*Taraxum officinale* green leaves, Moscow, Russia	FN357235	NA	NA	NA	[22]
Sporobolomyces sucorum	PYCC 8111^T = YR16	Apple must for artisanal cider production, Verona, Italy, 2017	MG478490	MK037435	MK040588	MK037426	This study
Sporobolomyces sucorum	PYCC 8112=YR18	Apple must for artisanal cider production, Verona, Italy, 2017	MG478489	MK037436	MK040589	MK037427	This study
Sporobolomyces sucorum	PYCC 8113=YR20	Apple must for artisanal cider production, Verona, Italy, 2017	MG478491	MK037437	MK040590	MK037428	This study
Sporobolomyces sucorum	YP-259	Forest soil, North Carolina, USA	KU702525	KU702552	NA	NA	[19]
Sporobolomyces sucorum	CBS 13667	Grapes, Germany	KP346960	KP346988	NA	NA	[20]
Sporobolomyces sucorum	CBS 13692	Grapes, Germany	KP346957	NA	NA	NA	[20]
Sporobolomyces sucorum	CBS 13680	Grapes, Germany	KP346956	KP346984	NA	NA	[20]
Sporobolomyces sucorum	CBS 13677	Grapes, Germany	KP346955	KP346983	NA	NA	20]
Sporobolomyces sucorum	YM25618	Flowers, Yunnan Province, PR China	JQ964221	NA	NA	NA	–
Sporobolomyces sucorum	PuiC5.10	Wine grapes, France	HE802538	NA	NA	NA	–
Sporobolomyces sucorum	PomC5.19	Wine grapes, France	HE802462	NA	NA	NA	–
Sporobolomyces sucorum	PomC5.17	Wine grapes, France	HE802460	NA	NA	NA	–
Sporobolomyces sucorum	PomC5.14	Wine grapes, France	HE802457	NA	NA	NA	–
Sporobolomyces sucorum	PomC5.11	Wine grapes, France	HE802454	NA	NA	NA	–
Sporobolomyces sucorum	PomC5.9	Wine grapes, France	HE802452	NA	NA	NA	–
Sporobolomyces sucorum	I-Y376b	Xylem sap from grapevines, Austria	GU585217	NA	NA	NA	–
Sporobolomyces sucorum	ZIM 631	Grape berries, Slovenia	AM748549	NA	NA	NA	[21]

(normal) and at 37° C (weak). No growth at 42 °C. Growth in the absence of vitamins. Hydrolysis of urea and DBB reaction are positive.

The holotype (PYCC 8108) is maintained in a metabolically inactive state in the Portuguese Yeast Culture Collection, Caparica, Portugal, and the ex-type strain (YG1) was deposited in the same collection and in the collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands (CBS 15629^T). Strains PYCC 8108 (YG1), PYCC 8109 (YG12) and PYCC 8110 (YG13) were isolated by M. Azzolini and G. Zapparoli from grape must employed in Amarone wine production in Verona, Italy, in 2011. Additional strains have been isolated from a plant and milk (Table 1).

DESCRIPTION OF *SPOROBOLOMYCES SUCORUM* SP. NOV. M. LORENZINI, G. ZAPPAROLI AND J.P. SAMPAIO

Ethymology: *Sporobolomyces sucorum* (su.co′rum. L. gen. pl. n. *sucorum*: of juice; apple and grape juice/must is the environment where the species was frequently isolated).

MycoBank number: MB830804

After 1 week at 25 °C, cultures are pale salmon with a smooth and glossy surface with an entire margin and a soft and mucous texture. Yeast cells after 4 days on YPD agar ovoid, 3–6×5–11 µm (Fig. 2h-o). Ballistoconidia are abundantly produced on corn meal agar after 4 days at 18 °C, ovoid to slightly reniform, 2.5–3.5×5–7 µm (Fig. 2l). Crosses of cultures (PYCC 8111, PYCC 8112 and PYCC 8113) carried out on corn meal agar and potato dextrose agar and incubated at 20 °C for 8 weeks did not yield mycelium or teliospores. Fermentation of glucose is negative. Carbon compounds assimilated: D-glucose, D-galactose (weak), cellobiose (weak, variable), ethanol, glycerol (weak, variable) and D-mannitol (weak). No growth on inulin, sucrose, raffinose, melibiose, lactose, trehalose, maltose, melezitose, soluble starch, L-rhamnose, D-xylose, L-arabinose, D-arabinose, D-ribose, methanol, myo-inositol, DL-lactate and citrate. Nitrogen compounds assimilated: potassium nitrate, ethylamine hydrochloride, cadaverine dihydrochloride and L-lysine (variable). Nitrogen compounds not assimilated: sodium nitrite. Growth in the

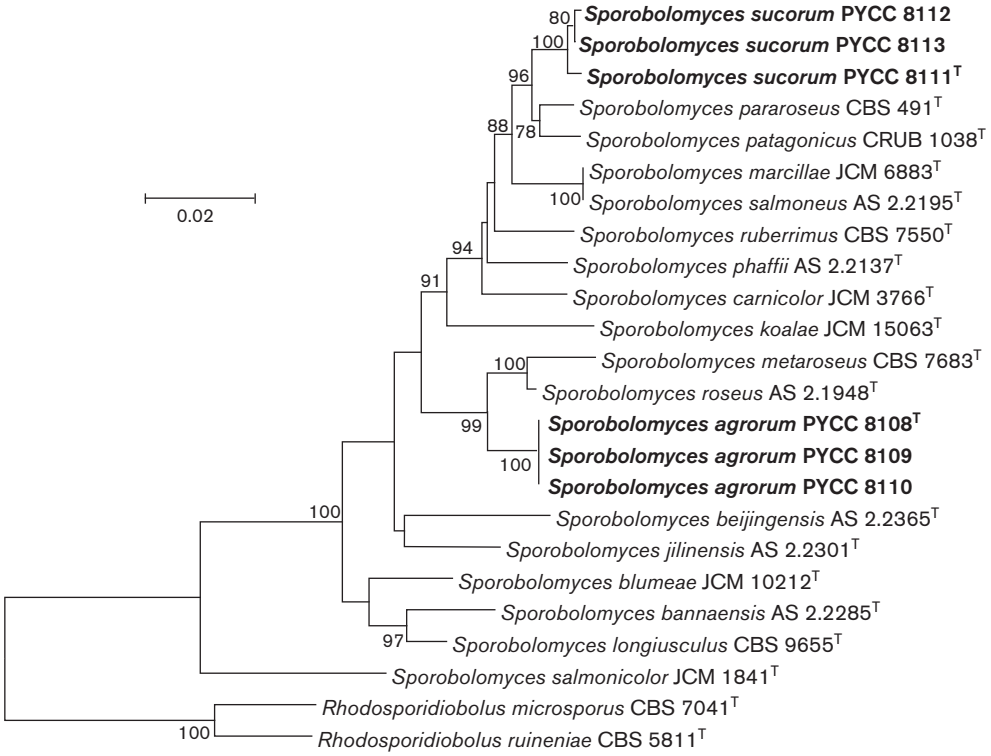

Fig. 1. Phylogenetic placement of *Sporobolomyces agrorum* sp. nov. and *Sporobolomyces sucorum* sp. nov. Maximum-likelihood phylogenetic tree of concatenated D1/D2 domain, ITS, *RPB2* and SSU sequences (3300 bp), computed using the Kimura two-parameter evolution model. Numbers on the branches represent bootstrap percentages (1000 replicates). *Rhodosporidiobolus microsporus* CBS 7041^T and *Rhodosporidiobolus ruineniae* CBS 5811^T were used as outgroups. Bar, number of expected of substitutions per site.

presence of 0.01 % (positive/weak) and 0.1 % cycloheximide (variable). Growth at 35° C (normal) and at 37° C (weak). No growth at 42° C. Growth in the absence of vitamins. Hydrolysis of urea and DBB reaction are positive.

The holotype (PYCC 8111) is maintained in a metabolically inactive state in the Portuguese Yeast Culture Collection, Caparica, Portugal, and the ex-type strain (YR16) was deposited in the same collection and in the collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands (CBS 15628^T). Strains PYCC 8111 (YR16), PYCC 8112 (YR18) and PYCC 8113 (YR20) were isolated by M. Lorenzini and G. Zapparoli from apple must employed in artisanal cider fermentations in Verona, Italy, in 2017. Additional strains of *S. sucorum* have been isolated from grapes and vine plants (Table 1).

Several physiological tests allow the differentiation of the two new species from their closest relatives. Contrary to *S. roseus* and *S. metaroseus, S. agrorum* is unable to assimilate sucrose, raffinose, trehalose, maltose, melezitose, soluble starch, cellobiose, D-arabinose, D-ribose, DL-lactate, citrate and nitrite (Table 2). *Sporobolomyces sucorum* does not utilize sucrose, raffinose, melezitose and soluble starch as carbon sources and grows at 35 °C in contrast to its closest relatives, *S. pararoseus* and *S. patagonicus* (Table 3). *Sporobolomyces* has variable assimilation profiles among species [12], and none displays a limited number of assimilated carbon sources such as those of the two novel species, particularly *S. agrorum*. The capacity to assimilate to glucose, galactose, glycerol and ethanol is consistent with the origin of these strains since apple and grape must contain these compounds, mainly glucose.

In common with other *Sporobolomyces* species, the new taxa described here appear to be ecologically associated with plants and fruits (Table 1). *Sporobolomyces agrorum* contains strains isolated from grape musts and herbaceous plants. Similarly, *S. metaroseus,* and *S. roseus* are known strains isolated from the phylloplane [5]. *Sporobolomyces sucorum* includes several strains isolated from apple musts, grapes and grapevines while strains of the closest related species *S. patagonicus* and *S. pararoseus* were mainly isolated from aquatic environments, soil, leaves and atmosphere [6, 13–15].

In this study we propose the recognition of two novel *Sporobolomyces* species based on molecular and phenotypic analyses of new isolates from apple and grape musts. A detailed multi-gene phylogenetic analysis of new isolates has been performed to obtain a reliable identification at the species level since the mere use of BLASTn for the identification of

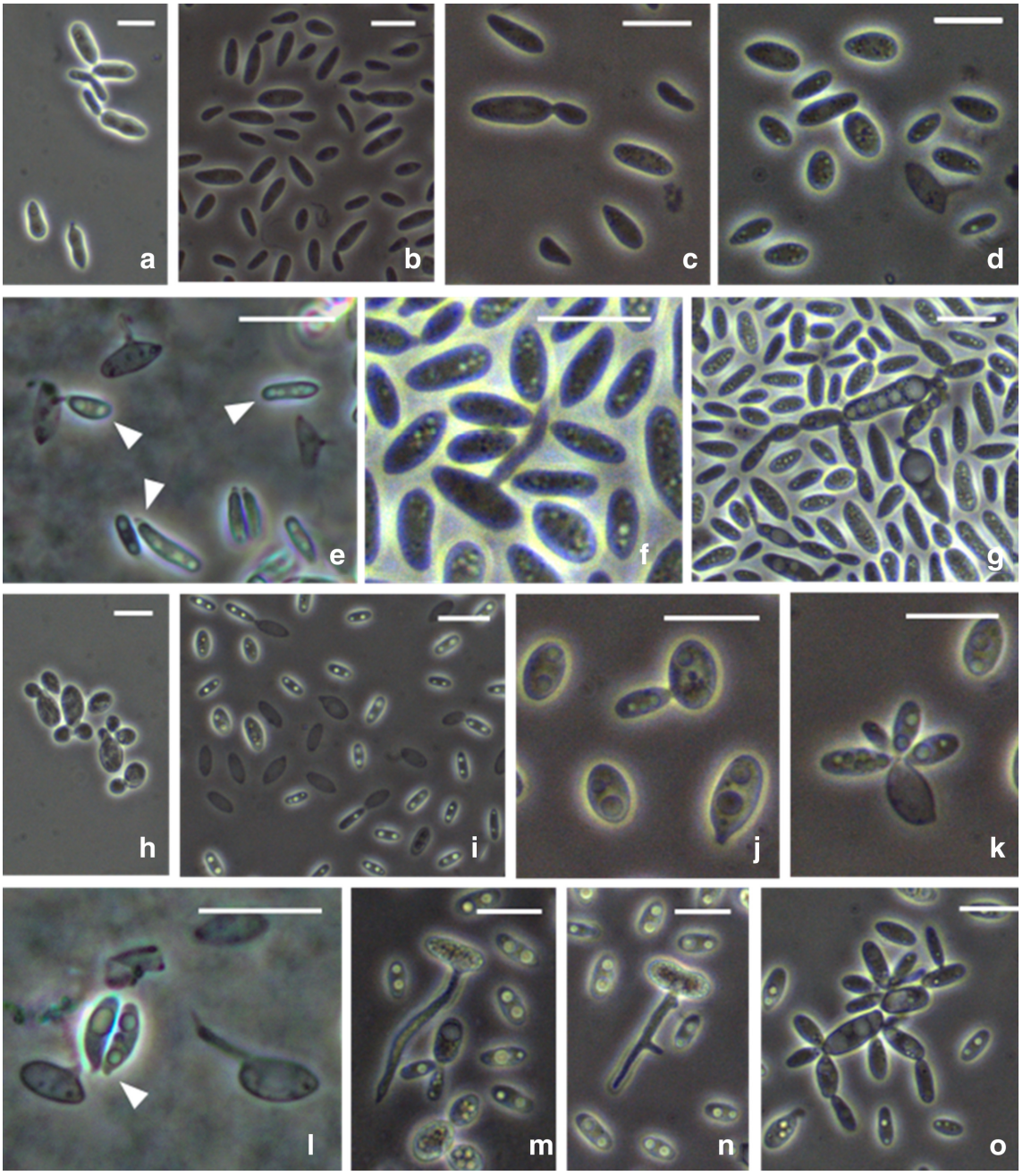

Fig. 2. Micro-morphology of *Sporobolomyces agrorum* sp. nov. (a–g) and *Sporobolomyces sucorum* sp. nov. (h–o) on YPD (a, h) and corn meal agar (b–g, i–o). Budding yeast cells (a-c and h-j), ballistoconidia-forming cells and ballistoconidia (d–f) and (l–n) (ballistoconidia are indicated by arrows), and rudimentary pseudohyphae (g, o). Bars, 10 µm.

Sporobolomyces strains, as reported in some investigations on grape micobiota [16, 17], was not fully appropriate. Phylogenetic trees provided by ITS, D1/D2 and other genes provide focal points for systematic analysis of basidiomycetous yeasts including *Sporobolomyces* [18]. In the present study, this approach allowed the discrimination of two novel species based on the topology of trees reconstructed using four gene sequences of new isolates.

We also included, in our molecular analyses, sequences from strains previously isolated by other researchers that represent additional isolates of the novel species here described. According to the results if phylogenetic and nucleotide substitution analyses, several isolates formerly identified as *S. pararoseus* (i.e. CBS 13667, CBS 13692, CBS 13680, CBS 13677, YM25618, PomC5.19, PomC5.14, PomC5.11, PomC5.9, I-Y376b, ZIM 631), *Sporodiobolus* sp.

Table 2. Salient physiological differences between *Sporobolomyces agrorum* sp. nov. and its closest related species

+, Growth; –, no growth; ND, not determined.

Characteristics*	*Sporobolomyces agrorum* sp. nov.	*Sporobolomyces roseus*	*Sporobolomyces metaroseus*
Sucrose	–	+	+
Raffinose	–	+	+
Trehalose	–	+	+
Maltose	–	+	+
Melezitose	–	+	+
Soluble starch	–	+	+
Cellobiose	–	+	+
D-Arabinose	–	+	+
D-Ribose	–	+	+
DL-Lactate	–	+	+
Citrate	–	+	+
Nitrite	–	+	+
At 35 °C	+	ND	–

*Data for the reference species were taken from Kurtzman *et al.* [6] and Valério *et al.* [5].

(YP-2589), *Sporobolomyces* sp. (PuiC5.10) and *Rhodotorula* sp. (PomC5.17) [19–21] can be considered members of the new species *S. sucorum*. In fact, these isolates display identical or very similar D1/D2 domain and ITS sequences to our three strains from apple must (data not shown). At species level, it is noticeable that the number of nucleotide substitutions between *S. sucorum* and the two closest related species, *S. pararoseus* and *S. patagonicus*, is similar to that observed between these two latter species despite they show more divergent D1/D2 domain (five mismatches) than ITS (zero mismatches) [13]. Similarly, AY-14 [22] and CBS 2642 [23] can be assigned to *S. agrorum* since they show no or few differences between both gene sequences (data not shown). Since most isolates of *S. sucorum* were obtained from grapes, it is conceivable that the grapevine environment may be a preferred habitat of this novel species, although its recovery from forest soil (YP-259), [19] and flowers of an herbaceous plant (YM25618) suggests a wider distribution as a generalist phylloplane yeast. Similarly, the recovery of *S. agrorum* from grape must (this study) and from herbaceous plant leaves (AY-14) indicates that it is likely that this species is a phylloplane yeast as most other *Sporobolomyces* species [24]. Moreover, the isolation of strain CBS 2642 from milk [23] is congruent with the occurrence of these yeasts on leaf surface of herbaceous plants. In fact, the recovery of this strain and other basidiomycetes, including *Sporoidiobolus salmonicolor* and *S. roseus* from dairy products [25], is consistent with yeast contaminations of fresh or ensiled forage to feed animals.

Table 3. Salient physiological differences between *Sporobolomyces sucorum* sp. nov. and its closest related species

+, Growth; –, no growth.

Characteristics*	*Sporobolomyces sucorum* sp. nov.	*Sporobolomyces pararoseus*	*Sporobolomyces patagonicus*
Sucrose	–	+	+
Raffinose	–	+	+
Melezitose	–	+	+
Soluble starch	–	+	+
D-Arabinose	–	+	–
Ethanol	+	+	–
Citrate	–	+	–
Nitrate	+	–	+
At 35 °C	+	–	–

*Data for the reference species were taken from Kurtzman *et al.* [6].

The recovery of *S. agrorum* and *S. sucorum* from grape and apple musts confirms the occurrence of this genus in the carposphere microbiome. *Sporobolomyces* species have already been isolated from apple and grape musts as well as at the beginning of wine fermentation [26–28]. It cannot be excluded that these yeasts can have a role on the production of some metabolites that could affect the aroma of ciders or wines. In fact, *Sporobolomyces* can produce volatile organic compounds including odour-active molecules, such as higher alcohols, acetate esters and thiols [29, 30]30. These yeasts are not involved in the alcoholic fermentation but could be metabolically active at the beginning of this process, and their contribution to the flavour of wine or cider may be not negligible although further investigations on this topic are necessary.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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