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# Fast method for identifying inter- and intra-species *Saccharomyces* hybrids in extensive genetic improvement programs based on yeast breeding

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**Running title**: **Fast identification of yeast hybrids**

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**Abstract**

**Aims**: The present work proposes a two-step molecular strategy to select inter- and intra-species *Saccharomyces* hybrids obtained by spore-to-spore mating, one of the most used methods for generating improved hybrids from homothallic wine yeasts.

**Methods and Results**: Since low spore viability and haplo-selfing are the main causes of failed mating, at first we used colony screening PCR (csPCR) of discriminative gene markers to select hybrids directly on dissection plate and discard homozygous diploid colonies arisen from one auto-diploidized progenitor. Then, pre-selected candidates were submitted to

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recursive streaking and conventional PCR in order to discriminate between the hybrids with stable genomic background and the false-positive admixtures of progenitor cells both undergone haplo-selfing. csPCRs of internal transcribed spacer (ITS) 1 or 2, and the subsequent digestion with diagnostic endonucleases *Hae*III and *Rsa*I, respectively, were efficient to select 6 new *Saccharomyces cerevisiae* x *Saccharomyces uvarum* hybrids from 64 crosses. Intragenic minisatellite regions in *PIR3*, *HSP150*, and *DAN4* genes showed high inter-strain size variation detectable by cost-effective agarose gel electrophoresis and were successful to validate 6 new intra-species *S. cerevisiae* hybrids from 34 crosses.

**Conclusions**: Both protocols reduce significantly the number of massive DNA extractions, prevent misinterpretations caused by one or both progenitors undergone haplo-selfing, and can be easily implemented in yeast labs without any specific instrumentation.

**Significance and Impact of the study**: The study provides a method for the marker-assisted selection of several inter- and intra-species yeast hybrids in a cost effective, rapid and reproducible manner.

**Keywords**: hybrid, *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, spore, mating, colony PCR, rDNA Internal Transcribed Spacer, minisatellite regions

## Introduction

Genetic modifications of industrially relevant microorganisms have gained beneficial effects from combinatorial approaches. These approaches can alter the entire cellular milieu in a global fashion and rely on strain randomization and subsequent selection/screening for the improved phenotype (Santos and Stephanopoulos 2008). Among methodologies for strain randomization (reviewed by Giudici *et al.* 2005; Steensels *et al.* 2014a), meiosis and mating between members of genetically distinct populations (globally referred to as hybridization) represent a genetic engineering (GE)-free method that produces non genetically modified organisms (GMO) with phenotypic novelty, heterosis and changes in gene expression (Chen 2013). Hybridization has been hypothesized to provide evolutionary benefits due to the

purging of deleterious mutations via meiosis (Mortimer *et al.* 1994) and the combination of beneficial alleles into one genome via mating (Fisher 1930).

From ancient time, *Saccharomyces cerevisiae* and its relatives belonging to the *Saccharomyces sensu stricto* group (*Saccharomyces bayanus* var. *uvarum*, *Saccharomyces cariocanus*, *Saccharomyces kudriavzevii*, *Saccharomyces mikatae*, *Saccharomyces paradoxus*, and *Saccharomyces pastorianus*) served as the most important yeast starters in food and beverage fermentation. In winemaking, artificial and natural hybrids bring sensorial complexity to wine and are more resistant to environmental fluctuations, compared to their parental strains (Sipiczki 2008 and references therein). Hybrids between non-cryotolerant *S. cerevisiae* and cryotolerant *S. uvarum* strains showed growth at an increased range of temperature and to synthesize by-products at midway concentrations compared to their progenitors (Zambonelli *et al.* 1993; Kishimoto 1994; Zambonelli *et al*. 1997; Rainieri *et al*. 1998a; Solieri *et al.* 2008). Similarly, *S. cerevisiae* x *S. uvarum* hybrids with high flocculence phenotype positively impacted the sparkling wine production (Coloretti *et al.* 2006). Other works demonstrated that natural hybrids between *S. cerevisiae* and S*. kudriazevii* (González *et al.* 2006; Gangl et al. 2009), as well as artificial hybrids between *S. cerevisiae* and *S. paradoxus* (Bellon *et al.* 2011; Steensels *et al.* 2014b) and between *S. cerevisiae* and *S. mikatae* (Bellon *et al*. 2013) are promising wine strarter cultures. Targeted intra-species hybridizations were successful to create *S. cerevisiae* hybrids with thermo-tolerance (Rainieri *et al*. 1998b), killer phenotype (Hammond and Eckersley 1984), and the enhanced ability to flocculate (Javadekar *et al*. 1995), to reduce $H_2S$ production (Romano *et al.* 1985), and to improve wine quality (Marullo *et al.* 2006; Steensels *et al.* 2014b).

Spore-to-spore mating is one of the most used methods to perform sexual hybridization of homothallic wild-type strains and to obtain improved wine yeast hybrids (as reviewed by Steensels *et al.* 2014a). Haploid genomes are directly forced to engage in mating with haploid genomes from other tetrads due to physical attachments between gametes through micromanipulation. Spores remain dormant until they detect new nutrients in the

environment, when they germinate into metabolically active haploid cells that can then attempt to mate. Two mating types, *MAT*α and *MAT*a, are monomorphic and produce attractive sexual pheromones called α-factor and a-factor, respectively. Spore attachment should eventually stimulate zygote formation, if gametes with opposite mating types are randomly sorted. Main drawbacks in spore-to-spore mating are: 1) it is only applicable to sporulation-efficient strains; 2) some relevant oenological traits can be lost after meiosis (Gimeno-Alcañiz and Matallana 2001; Marullo *et al.* 2006); 3) the recovery of hybrids is low due to the opportunity for haploid homothallic segregants to mate with their own mitotic progeny after mating-type switching (a process globally referred to as haplo-selfing) (as reviewed by Knop 2006; Greig and Leu 2009).

In breeding, rigorous validation of hybrids relies on pairs of either molecular or phenotypic markers, which have to be mutually exclusive in parental strains and both present in hybrid progeny. Different methodologies have been described to discriminate between parents and their hybrids, such as karyotyping (Giudici *et al.* 1998; Le Jeune *et al.* 2007), microsatellite analysis (Erny *et al*. 2012), PCR-RFLP or sequencing of divergent rRNA gene regions or housekeeping markers (González *et al*. 2006; Solieri *et al.* 2008; Bellon *et al.* 2013). All these techniques rely on extensive DNA manipulation procedures, which make hybrid confirmation a time-consuming and expensive step. Alternatively, complementation of selectable markers (either auxotrophic or antibiotic resistance) can be employed. Homothallic parental strains can be converted to heterothallism by deletion of *HO* gene (Bakalinsky and Snow 1990; Tamai *et al.* 2001; Walker *et al.* 2003). The resulting deletion mutants are mating-competent and give rise to hybrids selected by complementation of antibiotic resistance genes. Although this method is effective in addressing genetic issues (Albertin *et al.* 2013), it is inapplicable to GE-free programs for improving wine yeasts. Alternatively, spontaneous *ura3*$^{-}$ and *lys2*$^{-}$ auxotrophic mutants were generated from prototrophic industrial strains through 5-FOA and α-AA mutagenesis (Akada 2002; Nakazawa and Iwano 2004; Pérez-Través *et al.* 2012). Parental wine strains were also

screened at large-scale for identifying auxotrophic markers, often without success because they are generally prototrophic and diploid/aneuploid (Bizaj *et al.* 2012; Fernández-González *et al.* 2014). None of these methods can be successfully applied in rapid, large-scale breeding programs.

As a solution, in this paper we propose a fast two-step procedure for the marker-assisted selection of inter- and intra-species hybrid colonies constructed by spore-to-spore method. The strategy provides the following improvements: 1) minimal use of target gene specific primers, 2) optimized protocol for direct colony screening PCR (csPCR) that eliminates the need to extract genomic DNA. The effectiveness of this strategy was proven by constructing 6 new inter-species *S. cerevisiae* x *S. uvarum* hybrids and 6 new intra-species *S. cerevisiae* hybrids using spore-to-spore mating.

## Materials and Methods

### *Strains and growth conditions*

*Saccharomyces cerevisiae*, *S. uvarum*, and the hybrid strains used in this work are listed in **Table 1**. All yeasts were grown on YPDA (10 g $l^{-1}$ yeast extract, 10 g $l^{-1}$ peptone, 20 g $l^{-1}$ dextrose, 15 $l^{-1}$ agar) medium for 48 h at 28°C, and then stored at 4°C until use. For long term storage, strains were stored at -80°C in YPD medium supplemented with 25% (v/v) glycerol as cryo-preservative. All the strains are deposited in the Unimore Microbial Culture Collection (www.unimore.umcc.it). Sporulation was induced by sub-culturing exponentially grown cells from YPDA to ACK (15 g $l^{-1}$ potassium acetate, 20 g $l^{-1}$ agar) medium. The plates were incubated at 28°C for up to 2 weeks and asci formation was microscopically checked after 3, 7, and 14 days, respectively. Melibiose fermentation was tested in Durham tubes filled with YPM (YPD containing 20 g $l^{-1}$ melibiose instead of glucose) at 25 °C and yeast growth was checked after 3 and 7 days. Temperature sensitivity was tested on YPDA plates incubated at 37 °C for 3 days. *S. cerevisiae* and *S. uvarum* parental strains were used as reference strains.

***Hybrid constitution***

Tetrad dissection, intra- and inter-species hybrid constitution were performed as previously described (Solieri *et al*. 2008). Briefly, after parental strains underwent meiosis, asci were partially digested by Zymoliase T100 (Shigaku, Japan) at the final concentration of 2 mg $ml^{-1}$ and haploid gametes were directly mated by using micromanipulator (Singer Instruments, United Kingdom) in order to constitute diploid hybrids. Dissection plates were incubated at 28°C for 24 h to allow the growth of putative hybrid colonies.

***In silico analysis and restriction enzyme selection***

The full length rDNA region spanning ITS1, ITS2 and the 5.8S rRNA gene (so called 5.8S-ITS region) of *S. cerevisiae* and *S. uvarum* were retrieved from NCBI (http://www.ncbi.nlm.nih.gov). The sequences were subjected to *in silico* PCR amplification using primers ITS1/ITS2 and ITS3/ITS4 (**Table S1**) to trim off the untargeted regions on both 5′ and 3′ ends of the sequences using the online Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/pcr_products). Multiple sequence alignments of ITS1 and ITS2 sequences were performed using Clustal X, version 2.0 (http://www.clustal.org/clustal2). Using NEBcutter, version 2.0 (http://tools.neb.com/NEBcutter), ITS amplicon sequences were subjected to *in silico* restriction digestion with the commercially available type-II restriction endonucleases listed in the REBASE database (http://rebase.neb.com) (Roberts *et al*. 2010) to select the enzymes which cut *S. cerevisiae* and *S. uvarum* sequences differently at not more than 5 cleavage sites.

***DNA manipulations***

Genomic DNA was extracted through phenol-based method from exponentially grown cells after mechanical lysis (Hoffman and Winston 1987). Both conventional PCR and csPCR reactions were carried out on a BioRad thermalcycler (BioRad, Hercules, California, USA) using rTaq DNA polymerase (Takara, Japan), in 25 µl final volume according to

manufacturer's conditions. All gene markers used in this work and the corresponding primer pairs are listed in **Table S1**, whereas PCR conditions and final concentrations of used primers are reported in supplementary **Table S2**. PCR fragment sizes were analyzed by electrophoresis on 2.0% (w/v) agarose gel in presence of ethidium bromide, using GeneRuler 100 bp or 100 bp Plus DNA ladder (Fermentas, Thermo Fisher Inc., MA, USA) as molecular size markers. After the run at 90 V for 2 h, the gels were examined under UV light and the images were digitally captured using the BioDocAnalyze gel imaging and analysis system (Biometra, Göttingen, Germany). For conventional PCR gDNA was used as template at the final concentration of 8 ng $\mu l^{-1}$. The csPCR was performed as follows. Twenty-four hour old hybrid colonies, directly grown on dissection plates, were picked with a sterile loop and resuspended into 10 µl aliquots of milliQ $H_2O$ containing forward and reverse primers at the concentrations reported in **Table S2**. After thermal lysis at 98°C for 15 min, 15 µl aliquots of the PCR reaction mix were added to each diluted colony and underwent thermal PCR conditions (**Table S2**). PCR efficiency was calculated as follows: efficiency= (number of amplifications obtained/number of colonies tested) *100.

ITS1 and ITS2 markers were digested using the endonucleases *Hae*III and *Rsa*I (Fermentas, Thermo Fisher Inc., MA, USA), respectively, according to the manufacturer's instructions. Restriction fragments were separated on 2.0% (w/v) agarose gel as reported above. Restriction analysis of 5.8S-ITS regions was performed according to Esteve-Zarzoso *et al*. (1999).

***PFGE-karyotyping***

Karyotype analysis of hybrids and their corresponding progenitors was performed using pulse-field gel electrophoresis (PFGE). Briefly, chromosomal DNA was prepared from overnight cultures in agarose plugs as described by Sheehan and Weiss (1990). Chromosomes were separated with a CHEF DRII apparatus (Bio-Rad, Richmond, CA, USA) on a 1% agarose gel (Qbiogene, Carlsbad, CA, USA), using 0.5X TBE as running buffer.

Electrophoresis was carried out according to a two-block program, *i.e.* block 1 (switch time, 60 sec; run time, 14 h; voltage gradient, 6 V $cm^{-1}$; included angle, 120') and the block 2 program (switch time, 90 sec; run time, 10 h; voltage gradient, 6 V $cm^{-1}$; included angle, 120'). Agarose gel was stained with ethidium bromide (0.5 µg $ml^{-1}$; 30 min), washed in 0.5X TBE buffer for 5 min, and visualized as reported above.

**Results**

Figure 1 depicts the two-step strategy designed to validate both inter- and intra-species hybrids. Spore-to-spore mating provides three possible outputs: i) true hybrid colonies resulted from mating between haploids cells with opposite mating types; ii) failed mating due to the death of one haploid cell, while the remaining one undergoes haplo-selfing; iii) mixed progenitor cells both undergone haplo-selfing (**Fig. 1** panel A). Therefore, in the first step the colony screening PCR (csPCR) was set up on inter- and intra-species discriminatory gene markers to identify putative hybrid colonies directly on the dissection plate used for spore-to-spore mating. The second step consists of sub-culturing the hybrid candidates positively pre-selected in the former step in order to eliminate unmated colonies represented by mixtures of progenitors both undergone haplo-selfing, and to confirm true hybrids through conventional PCR (**Fig. 1** panel B).

***ITS1- and ITS2-csPCR for inter-species hybrid validation***

We performed 4 crossing schemes of *S. cerevisiae* strains to *S. uvarum* strains (IperR.4B x CRY13.2A, 4003.7C x CRY13.2A, 4003.7C x UMCC 2575.2A, and 2001.6B x UMCC 2575.2A), resulting in 75 putative hybrid colonies (64 viable) on the dissection plates. rDNA 5.8S-ITS region is one of the most used phylogenetic marker for discriminating *S. cerevisiae* and *S. uvarum*. Thus, our first attempt was to test 27 randomly selected hybrid colonies by using csPCR amplification of 880 bp long 5.8S-ITS region with the primer set ITS1/ITS4. The PCR efficiency was 63%, with 37% of the csPCR reactions failing in giving visible 5.8S-ITS PCR amplicons (**Table 2**). The application of different PCR conditions (extension time,

denaturation temperature, $MgCl_2$ concentration) did not increase amplification efficiency beyond 63% (unpublished observations).

Reduction in the length of target gene sequence positively affects the robustness and reproducibility of csPCR (Sambrook *et al*. 1989). In order to improve the amplification efficiency of csPCR assay, we shortened the size of target DNA by csPCR amplifying only either the variable ITS1 region by using universal primers ITS1 and ITS2, or the variable ITS2 region by using universal primers ITS3 and ITS4, respectively. *S. cerevisiae* and *S. uvarum* ITS1 and ITS2 sequences were retrieved from full-length 5.8S-ITS sequences deposited in NCBI and subjected to multiple sequence alignments. *S. cerevisiae* and *S. uvarum* ITS1 regions were 361 and 360 bp long, respectively, and displayed 95.79% identity, whereas the *S. cerevisiae* and *S. uvarum* ITS2 regions were 232 and 231 bp long, with a 98.70% identity. csPCRs with primer pairs ITS1/ITS2 and ITS3/ITS4 resulted in PCR fragments of about 450 and 430 bp for both *S. cerevisiae* and *S. uvarum* species, respectively, which were submitted to *in silico* restriction analysis. Considering the length and the number of polymorphic fragments with sizes greater than 100 bp (for easy analysis in normal agarose gel), among restriction enzymes (which cut the variable regions differently), we chose *Hae*III and *Rsa*I as diagnostic endonucleases for ITS1 and ITS2 csPCR-RFLP assays, respectively.

The ITS1 and ITS2 csPCR-RFLP protocols were validated on 26 and 38 putative hybrid colonies, respectively. In both cases we obtained 100% of success in csPCR (**Table 2**). Detection experiments were repeated three times with the same results, confirming reproducibility. ITS1 and ITS2 polymorphisms between *S. cerevisiae* and *S. uvarum* were successfully assessed by RFLP with the diagnostic restriction enzymes *Hae*III and *Rsa*I, respectively. Three colonies arisen from the spore mating between *S. cerevisiae* IperR.4B and *S. uvarum* CRY13.2A were screened through ITS2-csPCR followed by *Rsa*I digestion. One IperR.4Bx CRY13.2A colony showed the diagnostic hybridization pattern made of complementary *S. cerevisiae* and *S. uvarum*-specific profiles (260, 130, 90 bp) (**Table 2**).

Putative hybrid colonies obtained from crossing *S. cerevisiae* 4003.7C to either *S. uvarum* CRY13.2A or *S. uvarum* UMCC 2575.2A were confirmed by ITS1 and ITS2-csPCR followed by RFLP with the diagnostic endonucleases *Hae*III and *Rsa*I, respectively (**Fig. 2**). Out of 54 screened colonies, 4 showed complementary patterns expected for hybridization. We performed 8 crosses between *S. cerevisiae* 2001.6B and *S. uvarum* UMCC 2575.2A, but only 7 colonies were viable and one showed the complementary pattern expected for hybridization based on *Rsa*I restriction of ITS2 csPCR products (**Table 2**).

The complementary hybridization patterns obtained in step 1 could be false positive results arisen from admixture of *S. cerevisiae* and *S. uvarum* cells both undergone haplo-selfing. According to the strategy reported in **Fig. 1**, the subset of 6 positive inter-species hybrids was submitted to recursive steps of sub-culturing on YPDA plate. This step assures that stable cell populations grown from single hybrid cells were submitted to classical gDNA extraction for the final hybrid confirmation by full-length 5.8S-ITS region PCR-RFLP, which was proved to be a reliable rDNA segment for the examination of *S. cerevisiae* x *S. uvarum* hybrids (Antunovics *et al*. 2005). As results, out of 6 pre-selected hybrid colonies, all were definitely confirmed as new *S. cerevisiae* x *S. uvarum* hybrids, namely IperR.4B x CRY13.2A, 4003.7C x CRY13.2A I, 4003.7C x CRY13.2A II, 4003.7C x UMCC 2575.2A I, 4003.7C x UMCC 2575.2A II, and 2001.6B x UMCC 2575.2A (**Table 2**).

Finally, the effectiveness of the method was proven by confirming the selected hybrid strains through two alternative phenotypic and molecular tests frequently used for *S. cerevisiae* x *S. uvarum* hybrid validation. According to Pfliegler *et al.* (2012), the hybrids were tested for the ability to grow at 37°C and to ferment melibiose (*S. cerevisiae* is described as melibiose-negative fermenter, whereas *S. uvarum* should be sensitive to 37 °C). All hybrids showed complementation of both parental phenotypes, i.e. they fermented melibiose and grew at 37°C (data not shown). The hybrids were also confirmed by karyotyping. As expected, CHEF analysis showed that hybrids possess genetic background comprised of the

chromosome bands from both parental strains (**Fig. S1).** These results supported the inter-species hybridization events originating the strains IperR.4B x CRY13.2A, 4003.7C x CRY13.2A I, 4003.7C x CRY13.2A II, 4003.7C x UMCC 2575.2A I, 4003.7C x UMCC 2575.2A II, and 2001.6B x UMCC 2575.2A, and confirmed the suitability of the method for validating *S. cerevisiae* x *S. uvarum* hybrids.

***Validation of intra-species variable markers***

For intra-species outcrossing, the molecular characterization of parental strains must be preliminary established to differentiate each parental strain involved in hybridization events and the resulting true F1 diploid hybrids. For this purpose, we chose 5 nuclear genes containing tandem repeats within their coding regions, namely *PIR3*, *AGA1*, *SED1*, *DAN4*, and *HSP150*. These genes were previously showed to be highly variable within a pool of wild type strains, owing to expansion or contraction of these repeats (Mannazzu *et al*. 2002; Marinangeli *et al*. 2004; Verstrepen *et al*. 2005). Variation in number of tandem repeats was so high to be easily checked in low-cost agarose gel electrophoresis (Verstrepen *et al.* 2005). We amplified each of the 5 repeat regions by conventional PCR and compared their sizes in 10 *S. cerevisiae* strains, which are either commercial wine yeasts or promising parental candidates for hybrid constitution on the basis of our previous works (De Vero *et al.* 2011; Mezzetti *et al.* 2014). A complete overview of PCR amplicon size variation was reported in supplementary **Table S3**. Intragenic repetitive regions of *AGA1* and *SED1* marker genes did not show any inter-strain variation in length, resulting in approximately 1200-bp long PCR amplicons in both cases. In contrast, the marker genes *DAN4*, *PIR3* and *HSP150* displayed higher degree of size variation. In particular, *DAN4* and *HSP150* markers varied significantly from strain to strain and gave different PCR bands with size ranging from 1100 to 1600 and from 1600 to 1900 bp, respectively. Marker gene *PIR3* displayed significant size reduction in strain PB2033 compared to the other strains, and, thus, it could be effective in detecting hybridization events involving strain PB2033 as progenitor.

Furthermore, strains PB2033, 16003, PB2010, and PB2590 yielded two different PCR amplicons for one or more markers, suggesting that their genome contains two size variants for each marker gene. Since spore-to-spore conjugation relies on mating between haploid meiotic segregants, we analyzed how these size variants segregate in monosporic clones derived from these strains. Segregation pattern of *HSP150* marker showed that the monosporic segregants of strains 16003 and PB2033 harbor only one of the two size variants (**Fig. 3**). Similar results were observed for genes *PIR3* and *DAN4* in monosporic segregants of strain PB2010 (**Fig. 3**) and for the gene *DAN4* in monosporic segregants of strain PB2590 (data not shown). These results indicate that size variants are on a single locus which segregates according to Mendelian pattern during meiosis. As a consequence, true diploid F1 hybrids should inherit only one single size variant from each progenitor, leading to two-band profile. Based on these results, the most discriminative marker was suited for each possible combination of progenitors to be exploited in intra-species outcrossing (**Table 3**).

***Implementation of intra-species variable markers in csPCR***

The *PIR3*, *DAN4* and *HSP150* markers were implemented into a two-step csPCR assay, according to **Fig. 1**. As previously reported for inter-species hybrids, the goal was to obtain a robust and fast method for the direct amplification of both parental size variants of these markers in putative hybrid colonies grown on YPDA after spore-to-spore mating (step 1). Hybrid candidates selected in step 1 were then submitted to streaking, gDNA extraction and conventional PCR targeting the same DNA molecular markers (step 2). As a proof of concept the method was tested on 36 (34 viable) putative hybrid colonies generated by mating PB2590 and Mo21T2-10 (20 crosses); IperR and PB2033 (9 crosses); IperR and V1F1-B (7 crosses). *DAN4*-csPCR assay was used to validate the outcrossing between PB2590 and Mo21T2-10. Out of 18 viable colonies, all gave visible PCR bands (100% PCR efficiency), but only one showed the two-band hybridization patterns corresponding to both the parental *DAN4* size variants, whereas the remaining ones showed only single parental

size variant. *HSP150*-csPCR assay was tested on hybrid colonies between strains IperR and V1F1-B (**Table 2**). The first attempt was to amplify *HSP150* gene with primer set HSP150F1/HSP150F1 from the 7 putative hybrid colonies, it failed to give PCR bands with expected length of 1800 and 1900 bp in all the reactions. When the amplicon size was shortened by using the primer set HSP150_shortF1/ HSP150_shortF1, all the 7 PCR reactions yielded positive amplification bands (PCR efficiency of 100%). Two colonies were identified as hybrids on the basis of two-band hybridization pattern (800 and 700 bp) (**Fig. 4**). *PIR3*-csPCR was effective to amplify all the putative hybrids colonies obtained by mating strains IperR and PB2033 (**Fig. 4**). Three hybrid candidates were identified, which showed hybridization patterns consisting of 550 and 700 bp long PCR bands. All the 6 hybrid candidates selected in step 1 (PB2590 x Mo21T2-10_1, IperR x PB2033_1, IperR x PB2033_2, IperR x PB2033_8, IperR x V1F1B_6 and IperR x V1F1B_7) were successfully confirmed in step 2 based on conventional PCR against the same gene markers.

Finally, we confirmed the hybrid background of the selected strains by PFGE-karyotyping. For parental pairs PB2590/Mo21T2-10 and IperR/V1F1-B, no significant differences in chromosomal band patterns were detected, resulting in F1 hybrids with karyotypes almost identical to those of the corresponding progenitors (Supplementary **Fig. S2**). For progenitors IperR and PB2033, slight inter-strain variations in chromosomal bands were distinguished and, as expected, the corresponding F1 hybrids IperR x PB2033_1, IperR x PB2033_2, and IperR x PB2033_8 showed complementary chromosomal bands, confirming that their genomes consist of both the chromosomal haplotypes of the progenitors (Supplementary **Fig. S2**).

**Discussion**

The main cost factor in spore mating is the low yield in hybrids and the high number of manually created crosses needed to obtain them. As a result, numerous putative hybrid colonies require to be screened in order to identify selectively cultures harboring both

parental chromosomal complements, and discard the others originated from failed mating. Here, we demonstrated that the marker-assisted selection of hybrids via csPCR is a time- and resource-saving solution to this bottleneck. Since its introduction in the early 90's (Sathe *et al.* 1991), csPCR became a cornerstone, both in the screening of yeast cell recombinants (Amberg *et al.* 2006) and in fast diagnostics of pathogenic yeast and other fungi (Lau *et al.* 2008). Our strategy for detecting inter- and intra-species hybrids is based on csPCR of colonies directly on the dissection plate. Differently from rare/mass mating (Spencer and Spencer 1996), in spore-to-spore mating tetrads were isolated and hybrids were constituted in a manner to allow the spore relationships of all the meiotic products to be recovered. Single colonies derived from hybridization are picked from the micromanipulation agar plate, lysed by thermal treatment and used as a template for gene marker csPCR. The resulting PCR products are digested with diagnostic endonucleases (inter-species hybrids) or directly visualized by gel electrophoresis (intra-species hybrids) to discriminate hybrids containing both parental markers from colonies of one progenitor undergone haplo-selfing (**Fig. 1**). In the second step false-positive colonies represented by mixtures of progenitors both undergone haplo-selfing, are discarded thanks to repetitive streaking of previously selected cultures (**Fig. 1**).

We demonstrated the reliability of this strategy in screening 64 inter- and 34 intra-species colonies, with a hybridization yield of 9.4% and 17.6 %, respectively. Several factors account for this low yield. Tetrads contain 2 spores of each mating type (*MAT*a and *MAT*α), which cannot be discriminated until the spores germinate and express the pheromone specific to each type. Combinations do not allow any mating when all spores are of the same mating type (either all *MAT*a or all *MAT*α). Furthermore, highly heterozygous industrial strains show low spore viability, which correlates negatively with the hybridization yield (Mortimer *et al.* 1994; Johnston *et al.* 2000). Differences in mating and germination timing (Murphy *et al.* 2006; Maclean and Greig 2008; Murphy and Zeyl 2012), as well as in spore size (Smith *et al.* 2014), can redirect diploidization from mating to haplo-selfing, leading to assortative mating

in intra-species outcrossing or to prezygotic isolation in inter-species outcrossing. These factors contribute to reduce the overall yield in artificial hybrid generation and make spore mating poorly effective in breeding plan for improving wine yeasts.

Our method relies on the genetic marker-assisted selection. An important step in non-GE hybrid constitution is the choice of gene marker or genotyping technique for discriminating between parental strains and the resulting hybrid progeny, which bears intermixed parental chromosomal sets. For inter-species outcrossing, the parents *S. cerevisiae* and *S. uvarum* are the most phylogenetically distant species within the *Saccharomyces sensu stricto* clade. Their genomes are collinear and display an estimated genome divergence of about 8-20% (Scannell *et al*. 2011). We focused on rRNA region encompassing ITS1, ITS2 and 5.8S rRNA gene that is recognized as hallmark barcode in hemiascomycetes taxonomy (Kurtzman and Robnett 1998), and we implemented this region in direct csPCR assay. However, csPCR of 5.8S-ITS regions showed poor amplification efficiency, resulting in low number of positive PCR amplifications per assay. The amplification failure depended neither on various parameters concerning PCR nor on cellular lysis protocols. We found that reduction in size of PCR template increases the PCR efficiency to 100%. Based on this observation csPCR was targeted towards either ITS1 or ITS2 region, followed by *Hae*III and *Rsa*I-based restriction analysis, respectively. This improved protocol was successful in selecting 6 newly constituted *S. cerevisiae* x *S. uvarum* hybrids.

Gene markers for extensive screening of intra-species *S. cerevisiae* hybrids entail the following requirements: 1) markers should be highly variable within the pool of progenitor strains; 2) the detection of marker variation should be rapid and cost-effective. Several hypervariable microsatellite loci have been used for strain typing in *S. cerevisiae* (Legras *et al.* 2005, 2007; Bradbury *et al*. 2006; Ezov *et al*. 2006; Goddard *et al*. 2010; Schuller *et al*. 2012). These methods rely on capillary electrophoresis or polyacrylamide gel electrophoresis as detection techniques and were discarded for our purpose. Similarly, multi-

gene sequencing has been proven to effectively describe *S. cerevisiae* populations (Fay and Benavides 2005; Aa *et al.* 2006; Ramazzotti *et al.* 2012; Stefanini *et al*. 2012; Wang *et al*. 2012), but it is impracticable for rapid and cost-effective screening of hybrids candidates. In contrasts, inter δ-element polymorphism analysis (Legras and Karst 2003) and RAPD (Ramírez-Castrillón *et al*. 2014) are fast and cost-effective techniques, but they are not suitable for the direct discrimination of hybrid colonies. Furthermore, meiotic recombination events may change inter-δ/RAPD patterns between progenitors and their corresponding haploid segregants, resulting in misleading hybrid identification when crossing is performed by direct mating of parental spore (no monosporic segregant; Solieri, unpublished results). Minisatellites are less studied than microsatellites and consist of tandem repeats composed of bigger repeat units generally localized on conserved coding regions (Richard and Dujon 2006). Using gene-specific primer pairs flanking the intragenic repetitive domains, strain-specific differences in the repeats determine size variation in PCR amplicons easily detectable by a simple agarose gel electrophoresis (Mannazzu *et al.* 2002; Marinangeli *et al*. 2004; Boveri *et al.* 2012). Among 44 minisatellite-containing genes identified in *S. cerevisiae* genome (Verstrepen *et al.* 2005), five gene candidates (*AGA1*, *SED1*, *HSP150*, *DAN4*, and *PIR3*) showed both small intragenic repetitive domains and high inter-strain degree of repeat length variation. Relatively small intragenic repetitive domains increase the csPCR efficiency, enabling the direct amplificationof hybrid colonies without DNA extraction. Differently from that previously found (Mannazzu *et al.* 2002; Boveri *et al*. 2012), we detected low size variation in intragenic repeats of *AGA1* and *SED1* genes within the pool of wine strains considered in this work. In contrast *HSP150*, *DAN4*, and *PIR3* genes displayed sufficient size differences across the parental set to be applied in the hybridization screening procedure (**Table 3**). Although the repeats were described as mitotic recombination hotspot (Verstrepen *et al*. 2005), we demonstrated that *HSP150*, *DAN4* and *PIR3* genes are stably inherited in the meiotic segregants of heterozygous strains harboring two size variants.

In conclusion, the two-step strategy developed in this work is a reliable and fast method for validating *S. cerevisiae* x *S. uvarum* inter-species and *S. cerevisiae* intra-species hybrids obtained by spore-to-spore mating. This approach will be easily adapted to any other *Saccharomyces* hybrid, if either appropriate diagnostic endonucleases (inter-species hybrids) or intragenic tandem repeats (intra-species hybrids) are chosen. Currently, we are successfully implementing this method into our routine laboratory tests in order to assist extensive inter- and intra-species breeding programs for tailoring new wine yeasts.

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**Conflict of Interest**

None declared

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## Table captions

**Table 1.** *Saccharomyces* strains used in this study

**Table 2**. Summary of validation results obtained for intra- and inter-species crosses by two-step csPCR method. Positive csPCR indicates presence of visible PCR amplicons compared with the total number of csPCR reactions. Non-hybrid colonies indicate failed hybridization crosses with colonies showing only one parental haplotype. Sc, *S. cerevisiae*; Su, *S. uvarum*.

**Table 3.** Selection of the most suitable DNA molecular markers to validate intra-species hybrids for all the possible combination of progenitors. *HSP150* designates both the long (HSP150F1/HSP150R1) and short versions (HSP150F_shortF1/HSP150_shortR1) of the marker.

## Figure captions

**Fig. 1.** Summary of two-step strategy designated for validating inter- and intra-species *Saccharomyces* hybrids obtained by spore-to-spore mating. Panel A represents possible outputs: I, true hybrids originating from mating between parental haploids cells with opposite mating type; II, failed mating events where one progenitor underwent haplo-selfing and the other died; III, cell mixture of progenitors both undergone haplo-selfing. In panel B, P1 and P2 indicate parental strains, while H represents hybrid progenies. H colonies grown on micromanipulation YPDA plate are screened by direct colony screening PCR (csPCR). Amplification using primer pairs targeted either ITS1 or ITS2 followed by digestion with diagnostic endonucleases enables the distinction between H candidates and parental cells undergone haplo-selfing. Similarly, size variation of in gene markers *DAN4*, *PIR3*, and

*HSP150* in P1 and P2 allows identifying *S. cerevisiae* x *S. cerevisiae* H candidates. Selected H candidates are submitted to repetitive rounds of streaking onto YPDA and further screened by conventional PCR. False positive H colonies (admixtures of unmated P1 and P2 cultures) are discarded.

**Fig. 2.** csPCR amplification of putative inter-species hybrid colonies picked directly from dissection plates after spore-to-spore mating between *S. cerevisiae* 4003.7C and *S. uvarum* CRY13.2A (A) and between *S. cerevisiae* 4003.7C and *S. uvarum* UMCC 2575.2A (B). Panel A represents ITS1-csPCR products (left) and their *Hae*III-digestion (right), both visualized on a 2% agarose gel stained with ethidium bromide. Putative hybrid colonies are in lane 1 to 14, whereas *S. uvarum* CRY13.2A and *S. cerevisiae* 4003.7C are used as control in lanes 15 and 16, respectively. Panel B represents ITS2-csPCR products (left) and their *Rsa*I-digestion (right), both visualized on a 2% agarose gel stained with ethidium bromide. Putative hybrid colonies are in lane 1 to 17, whereas *S. cerevisiae* 4003.7C and *S. uvarum* UMCC 2575.2A are used as control in lanes 18 and 19, respectively. Positive colonies with hybridization restriction patterns are indicated with asterisk. M, GeneRuler 100 bp DNA Ladder (Fermentas, Thermo Fisher Inc., MA, USA).

**Fig. 3.** Segregation of markers containing intragenic tandem repeats in monosporic meiotic segregants of *S. cerevisiae* heterozygous progenitors. H1 to H4 indicate *HSP150* PCR amplicons of meiotic segregants arisen form a single tetrad dissection of strain 16003 (P1H); P1 and P2 indicate *PIR3* PCR amplicons of two meiotic segregants arisen form a single tetrad dissection of strain PB2010 (P2P) (only two viable meiotic segregants out of four dissected spores); D1 and D2 indicate *DAN4* PCR amplicons of two meiotic segregants arisen form a single tetrad dissection of strain PB2010 (P2D) (only two viable meiotic segregants out of four dissected spores). M, GeneRuler 100 bp Plus DNA Ladder (Fermentas, Thermo Fisher Inc., MA, USA).

**Fig. 4.** csPCR amplification of putative intra-species hybrid colonies picked directly from dissection plates after spore-to-spore mating between *S. cerevisiae* IperR and V1F1 (A) and between *S. cerevisiae* IperR and PB2033 (B). Panels A and B represent *HSP150*_short- and *PIR3*-csPCR products visualized on a 2% agarose gel stained with ethidium bromide, respectively. Putative hybrid colonies are labeled as numbers, while parental strains as P1 and P2. Positive colonies with hybridization patterns are indicated with asterisks. M, GeneRuler 100 bp Plus DNA Ladder (Fermentas, Thermo Fisher Inc., MA, USA). Remaining screened colonies are omitted from the picture.

**Supporting Information**

**Table S1.** Molecular markers and oligonucleotide primers used in the study.

**Table S2**. PCR conditions used for amplification of gene markers.

**Table S3.** Size variation in PCR amplicons for gene markers containing intragenic repeats.

**Figure S1.** PFGE karyotyping of *S. cerevisiae* x *S. uvarum* hybrids and their corresponding progenitors. Lanes are as follows: 1, *S. cerevisiae* 2001.6B; 2, 2001.6B x UMCC 2575.2A; 3, *S. uvarum* UMCC 2575.2A; 4, *S. cerevisiae* IperR.4B; 5, IperR.4B x CRY13.2A; 6, *S. uvarum* CRY13.2A; 7. 4003.7C x CRY13.2A I; 8, 4003.7C x CRY13.2A II; 9, *S. cerevisiae* 4003.7C; 10, 4003.7C x UMCC 2575.2A I; 11, *S. uvarum* UMCC 2575.2A. Lane M: *S. cerevisiae* PFGE marker (Bio-Rad). Asterisks and circles in progenitors and hybrids haplotypes indicate *S. cerevisiae* and *S. uvarum*-specific chromosomal bands, respectively.

**Figure S2**. PFGE electrophoretic patterns of parental strains PB2033 (1) and IperR (5), and their hybrids IperR X PB2033_1 (2), IperR X PB2033_2 (3) and IperR X PB2033_8 (4). Lane M: *S. cerevisiae* PFGE marker (Bio-Rad). Asterisks and circles indicate the strain-specific chromosomal bands from progenitors PB2033 and IperR, respectively.

**Table 1**.

| Species | Strains | Description/source | Reference |
|---|---|---|---|
| *S. cerevisiae* | Mo21-3 | evolved strain from21T2 | De Vero *et al.* 2011 |
| | IperR | commercial name Elegance | AEB Group |
| | V1F1-B | commercial name Fermol Grand Rouge | AEB Group |
| | PB2590 | commercial name Fermol Mediteranée | AEB Group |
| | PB2033 | commercial name Fermol | AEB Group |
| | 16003 | wild strain isolated from Sicilian grape must | Giudici *et al.* 1997 |
| | 20001 | wild strain isolated from Sicilian grape must | Giudici *et al.* 1997 |
| | 21T2 | evolved strain from 20001 | De Vero *et al.* 2011; Mezzetti *et al.* 2014 |
| | Mo21T2-10 | evolved strain from21T2 | Mezzetti *et al.* 2014 |
| | PB2010 | commercial name Fermol Arome Plus | AEB Group |
| | 4003 | wild strain isolated from Sicilian grape must | Giudici *et al.* 1997 |
| | 4003.7C | monosporic segregant of 4003 | this study |
| | 2001 | wild strain isolated from Sicilian grape must | Giudici *et al.* 1997 |
| | 2001.6B | monosporic segregant of 2001 | this study |
| *S. uvarum* | CRY13 | wild strain isolated from white grape must | this study |
| | CRY13.2A | monosporic segregant of CRY13 | this study |
| | UMCC 2575 | wild strain isolated from 'Lambrusco' sparkiling wine | this study |
| | UMCC 2575.2A | monosporic segregant of UMCC 2575 | this study |
| Inter- species hybrids | 4003 x CRY13 I | *S. cerevisiae* x *S. uvarum* | this study |
| | 4003.7C x CRY13.2A I | *S. cerevisiae* x *S. uvarum* | this study |
| | 4003.7C x CRY13.2A II | *S. cerevisiae x S. uvarum* | this study |
| | 4003.7C x UMCC 2575.2A I | *S. cerevisiae x S. uvarum* | this study |
| | 4003.7C x UMCC 2575.2A II | *S. cerevisiae x S. uvarum* | this study |
| | 2001.6B x UMCC 2575.2A | *S. cerevisiae* x *S. uvarum* | this study |
| | IperR.4B x CRY13.2A | *S. cerevisiae* x *S. uvarum* | this study |
| Intra-species hybrids | IperR x PB2033_1 | *S. cerevisiae* x *S. cerevisiae* | this study |
| | IperR x PB2033_2 | *S. cerevisiae* x *S. cerevisiae* | this study |
| | IperR x PB2033_8 | *S. cerevisiae* x *S. cerevisiae* | this study |
| | IperR x V1F1-B_6 | *S. cerevisiae* x *S. cerevisiae* | this study |
| | IperR x V1F1-B_7 | *S. cerevisiae* x *S. cerevisiae* | this study |
| | PB2590 x Mo21T2 10_1 | *S. cerevisiae* x *S. cerevisiae* | this study |

**Table 2.**

| Type | Progenitor combinations | N of crosses | N° viable colonies | Marker | csPCR (step 1) | | | N of validated hybrids (step 2) |
|---|---|---|---|---|---|---|---|---|
| | | | | | csPCR efficiency (%) | N of non-hybrid colonies | N of hybrid colonies | |
| Sc x Sc | PB2590 x Mo21T2-10 | 20 | 18 | *DAN4* | 100 | 17 | 1 | 1 |
| | IperR x PB2033 | 9 | 9 | *PIR3* | 100 | 6 | 3 | 3 |
| | IperR x V1F1-B | 7 | 7 | *HSP150*_long* | 0 | na | na | na |
| | | | | *HSP150*_short† | 100 | 5 | 2 | 2 |
| Sc x Su | 4003.7C x UMCC 2575.2A | 32 | 28 | 5.8S-ITS PCR-RFLP§ | 63 | na | na | na |
| | | | | *Rsa*I-digested ITS2 | 100 | 26 | 2 | 2 |
| | 4003.7C x CRY13.2A | 32 | 26 | *Hae*III-digested ITS1 | 100 | 24 | 2 | 2 |
| | IperR.4B x CRY13.2A | 3 | 3 | *Rsa*I-digested ITS2 | 100 | 3 | 3 | 1 |
| | 2001.6B x UMCC 2575.2A | 8 | 7 | *Rsa*I-digested ITS2 | 100 | 6 | 1 | 1 |

*referred to as PCR amplicons obtained with primer pair HSP150F/HSP150R; † referred to as PCR amplicons obtained with primer pair HSP150shortF/HSP150shortR; § According to Esteve-Zarzoso *et al.* (1999).

**Table 3.**

| Strains | PB2033 | IperR | 16003 | 21T2 | PB2010 | Mo 21-3 | 20001 | PB2590 | Mo21T2-10 | V1F1-B |
|---|---|---|---|---|---|---|---|---|---|---|
| **PB2033** | - | *HSP150, DAN4 PIR3* | *PIR3* | *DAN4 PIR3* | *HSP150, PIR3* | *DAN4 PIR3* | *HSP150, DAN4, PIR3* | *HSP150, PIR3* | *DAN4, PIR3* | *PIR3* |
| **IperR** | - | - | *HSP150, DAN4* | *HSP150, DAN4* | *DAN4* | *HSP150, DAN4* | *HSP150, DAN4* | *DAN4* | *HSP150 DAN4* | *HSP150, DAN4* |
| **16003** | - | - | - | *NA* | *HSP150* | *DAN4* | *NA* | *HSP150* | *NA* | *HSP150* |
| **21T2** | - | - | - | - | *HSP150, DAN4* | *DAN4* | *NA* | *HSP150, DAN4* | *NA* | *HSP150, DAN4* |
| **PB2010** | - | - | - | - | - | *HSP150, DAN4* | *HSP150, DAN4* | *NA* | *HSP150, DAN4* | *HSP150* |
| **Mo 21-3** | - | - | - | - | - | - | *HSP150, DAN4* | *HSP150, DAN4* | *DAN4* | *DAN4* |
| **20001** | - | - | - | - | - | - | - | *HSP150 DAN4* | *NA* | *HSP150, DAN4* |
| **PB2590** | - | - | - | - | - | - | - | - | *HSP150, DAN4* | *HSP150* |
| **Mo21T2-10** | - | - | - | - | - | - | - | - | - | *HSP150, DAN4* |
| **V1F1-B** | - | - | - | - | - | - | - | - | - | - |