OBTENCIÓN, ESTABILIZACIÓN Y SELECCIÓN DE LEVADURAS HÍBRIDAS DE *Saccharomyces* DE INTERÉS ENOLÓGICO

Tesis doctoral presentada por:

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para optar al grado de doctor por la Universidad de Valencia dentro del programa de doctorado de Biología Molecular, Celular y Genética.

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Valencia, 15 de Julio 2015

La Dra. Amparo Querol Simón, Profesora de investigación del Consejo Superior de Investigaciones Científicas (CSIC) en el Instituto de Agroquímica y Tecnología de los Alimentos (IATA) y el Dr. Eladio Barrio Esparducer, Profesor Titular de Departamento de Genética de la Universitat de València

INFORMAN

Que la presente memoria "Obtención, estabilización y selección de levaduras híbridas de *Saccharomyces* de interés enológico" constituye la tesis doctoral de Doña Laura Pérez Través para optar al grado de doctor en Biología Molecular, Celular y Genética por la Universitat de València. Asimismo, certifican haber dirigido y supervisado tanto los distintos aspectos del trabajo como su redacción.

Y para que conste a los efectos oportunos, firmamos el presente informe en Valencia a 12 de Junio de 2015

Fdo. Amparo Querol Simón

Fdo. Eladio Barrio Esparducer

Agradecimientos

Cuando uno comienza a escribir la tesis le parece que se enfrenta a un trabajo que nunca terminará, donde no se ve el final del túnel, y deja los agradecimientos para el final, pensando que será la parte más fácil. Pero con el tiempo, horas de dedicación y paciencia, un día se llega al final de ese túnel y al momento en el que escribir estas líneas, y es ahí cuando nos damos cuenta de que no es una tarea fácil!!!! Durante la tesis son muchas las personas que se cruzan en tu camino y que comparten trabajo, almuerzos, conversaciones y tiempo de relax contigo, y no te quieres olvidar de ninguna de ellas. Por eso, mis primeras líneas van dedicadas a todas esas personas a las que pueda olvidarme de nombrar.

Quisiera agradecer a mi director de tesis, el Dr. Eladio Barrio, la oportunidad que me dio al acogerme en su laboratorio, allá por la prehistoria (porque todavía no había terminado la carrera), como alumna colaboradora, sabiendo que el tiempo de inversión en un alumno de este tipo es, a menudo, tiempo perdido, pero dije que continuaría y así lo he hecho!! Y a mi directora, la Dra. Amparo Querol, por darme la oportunidad de continuar en investigación, trabajando en su laboratorio con una beca bicicleta, antes de embarcarme en este proyecto de tesis doctoral. Creo que al final hemos sabido entendernos y hemos hecho un buen trabajo juntas.

También quisiera agradecer a Ramón González, Pilar Morales y Manolo Quirós por acogerme en su laboratorio durante mi estancia en Logroño, casi sin haber llegado a instalarse ellos allí, y hacerme sentir como en casa. Siempre guardaré un gran recuerdo de esos meses en Logroño.

Ya dentro del laboratorio, tanto en el Cavanilles como en el 307, los compañeros han sido muchos y muy variados (siempre ha parecido la ONU); de cada uno de ellos aprendes algo y sin su aportación (personal o científica) esta tesis no sería lo mismo. Aprendí la forma de trabajar en un laboratorio con Sara, aunque las clases solo me dejaran acudir a deshoras. Con Armando compartí toda la tesis, comencé enseñándole a funcionar en el laboratorio y acabé aprendiendo de él. Silvia siempre ha sido mi "compañera", comenzamos con un año de diferencia y aunque pasáramos la mayor parte del tiempo a un piso de distancia (porque en el 307 siempre faltaba sitio y en el Cavanilles teníamos mucho más) eso no hizo que fuéramos menos compañeras, tampoco ahora que estamos en edificios diferentes y nos vemos poco. Y después llegó Peris…y el laboratorio dejó de ser un lugar tranquilo…

Agradecimientos

Todavía me acuerdo de cuando llegaste, te enseñé a leer geles de RFLPs y te dormías… También pasaron por el Cavanilles Noé, Sandy, Laurette, Fernanda, Chuy… Y Adri, que llegó cuando había terminado mi parte experimental e invadió mi bancada, y con la que sigo compartiendo aventuras y desventuras, ahora en el IATA, en la etapa final de su tesis. Mientras, en el 307 estaban Jordi y Amparito, compañeros de cenas y fiestas. Con Clara (uno de los tres miembros del corralito) y Eli (del grupo de los transformers) también compartí muy buenos momentos, ánimo a las dos, seguro que acabáis enseguida y muy pronto estáis leyendo vosotras! Y sé que os irá muy bien!! Entonces llegó Bruno al 307 y se acabó la calma y el orden. Menos mal que Roberto, su 'jefe' (que paciencia tienes…!!! y ya que estamos, gracias por ayudarme siempre que te lo he pedido) y Jiri, su otra mitad de la pareja 'Zipi y Zape' intentaban mantenerlo a raya. Jiri, compañero al GC, ánimo a ti también en esta recta final, cuando acabes y vuelvas a casa te vamos a echar de menos. Y Lupita, que cogió el relevo de los híbridos y consiguió que se cruzaran, también mucho ánimo en esta fase final. No me quiero olvidar de Marta, Miguel, Liz, Rebeca o la 'loca' de Euge, que aunque han estado durante periodos cortos en el lab, también han formado parte de esta tesis. Para terminar están Javi, quien me está tomando relevo con las fermentaciones y el HPLC, y la recién aterrizada María, aprovechad al máximo este tiempo, y sobre todo disfrutad!!! María, estoy segura de que con Javi tendrás a un buen maestro!!

Este apartado no estaría completo sin nombrar a David y a Christian. Ambos han sido muy importantes en el transcurso de estos años. En primer lugar Christian, sin el que esta tesis no habría llegado a buen puerto, solo se necesitó la combinación de 'paciencia Argentina-Española' para que todo comenzara a salir rodado!!! Formamos un buen tándem!!! Te echamos de menos desde este lado del charco!! Y como no, David, mi apoyo en el trabajo en el día a día, alguien de quien aprender (y mucho) y dispuesto a aprender lo que haga falta (siempre que no le cambies el voltaje de los geles, el tipo de marcador y alguna que otra cosilla más...). Espero seguir trabajando contigo durante mucho tiempo!!!

Y no me quiero olvidar de 'los estudiantes' con los que he compartido-tutelado trabajo de master o prácticas, especialmente a Cristina, Vicente e Ismael, ha sido una gran experiencia trabajar con vosotros (Ah!!! aunque Fani lo diga, ella saca mucho más el látigo!!!).

Agradecimientos

Ni de los laboratorios vecinos!!! No quería dejar de nombrar tanto a los viejos como a los nuevos compañeros: Rosana, María, Ali, José Manuel, Paloma, Carmela, Fani, Sara (mi nueva compañera de experimentos a la que llevo a la carrera estas últimas semanas….), Lucía, Paula, Alba, Anto……

Y para terminar, me gustaría agradecer a toda mi familia: mis padres, mi hermano, mis tíos, mis primos, mi abuela, mis suegros… (ya que, aunque caigan rayos de punta, ninguno se pierde nunca nada…), que siempre me ha apoyado y me ha animado a hacer lo que me gustaba, y a los que espero haber respondido de la misma manera. Por fin he acabado de escribirla!!! Veis como me quedaba poco!!!?? Papá, aunque ya nos hayamos comido el jamón que tenías guardado, seguro que podemos sacar otro, no crees?; Mamá, ya está todo listo!! Tienes preparado el vestido verde??? y Fran, aguanta que sólo te queda una presentación más por escuchar, aunque de esta no te puedes escaquear!!!

Y a Miguel, que siempre ha estado ahí, cuando veía el final muy lejos, cuando me agobiaba por pasar el día entero en el ordenador y ahora cuando por fin estoy terminado, dándome la tranquilidad que necesito en cada momento. Gracias por tu apoyo y paciencia, y por hacer que pudiera desconectar cuando me subía por las paredes!!!

Agradecimientos

A mis abuelos.

A mi tía Paqui.

A mi hijo Mario.

Agradecimientos

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1. Introducción.

Actualmente, el género *Saccharomyces* se compone de 7 especies: *S. arboricolus, S. cerevisiae, S. eubayanus, S. kudriavzevii, S. mikatae, S. paradoxus* y *S. uvarum.* Además, en el género *Saccharomyces* podemos encontrar dos grandes grupos de híbridos *S. pastorianus* (*S. cerevisiae x S. uvarum x S. eubayanus*) y *S. bayanus* (*S. cerevisiae x S. uvarum x S. eubayanus*), así como un número menor de híbridos que poseen porciones de *S. kudriavzevii.* Entre estas, se encuentran las especies de levaduras más importantes implicadas en procesos fermentativos.

En el caso del género *Saccharomyces*, uno de los mecanismos más interesantes observados en la adaptación de las levaduras a procesos industriales es la formación de híbridos entre especies de este grupo. En los últimos años se han descrito híbridos en el género, entre *S. cerevisiae*, *S. uvarum*, *S. eubayanus* y *S. kudriavzevii*. Estos híbridos están presentes en diferentes procesos fermentativos, como la producción de cerveza, vino y sidra. Se han encontrado en Italia, la Bretaña francesa, Suiza, Austria, España, Bélgica, Inglaterra, Alemania y Nueva Zelanda entre otros lugares. El ejemplo mejor descrito de una levadura híbrida es la levadura productora de cerveza lager *S. pastorianus* (sin. *S. carlsbergensis*). La cepa tipo de *S. bayanus* ha sido descrita como poseedora de genoma nuclear tanto de *S. cerevisiae* como de *S. uvarum* y *S. eubayanus*, aunque no está claro que se trate de un triple híbrido, ya que las secuencias de *S. cerevisiae* encontradas son subteloméricas y podrían estar allí debido a una introgresión.

Los híbridos están menos adaptados que sus parentales a condiciones ambientales específicas, pero pueden adaptarse mejor a condiciones fluctuantes intermedias, lo que les proporciona una ventaja selectiva. Por otro lado, los híbridos adquieren propiedades fisiológicas de ambos parentales, por ejemplo, la tolerancia al alcohol y a la glucosa de *S. cerevisiae*, la tolerancia a bajas temperaturas de *S. kudriavzevii*, la mayor producción de compuestos aromáticos de *S. bayanus* o la mayor producción de glicerol de estos últimos.

Resumen Amplio

La gran diversidad de híbridos dentro del género *Saccharomyces* encontrados en diversos orígenes y hábitats, indica que es más frecuente de lo esperado. Los mecanismos propuestos para explicar la generación de híbridos interespecíficos entre las especies de levaduras del género *Saccharomyces* son, bien la conjugación de esporas de distintas especies en el tracto digestivo de invertebrados o bien un suceso de "rare mating" entre cepas diploides de distintas especies del género *Saccharomyces*.

En la producción de vino, La utilización de levaduras seleccionadas produce fermentaciones controladas y, como consecuencia de esta práctica, el vino mantiene sus características sensoriales año tras año. Toda levadura comercial debe poseer una buena capacidad fermentativa y producir vinos con cierta calidad organoléptica. En la industria, cada vez más, se buscan aquellas levaduras que posean, además de estas, otras características fisiológicas que las hagan interesantes desde el punto de visa enológico, más aún si permiten resolver alguna de las exigencias actuales de las bodegas.

Una de las principales demandas del sector vitivinícola está asociada a resolver los problemas planteados con el cambio climático. El disponer de levaduras con un menor rendimiento en etanol, o que incrementen el contenido en glicerol en los vinos pueden ser buenas alternativas para resolver este tipo de problemas. Además de las características fisiológicas mencionadas, las levaduras también deben adaptarse a las actuales prácticas enológicas. Entre las prácticas más comunes encontramos las fermentaciones a bajas temperaturas, cuya finalidad es producir vinos altamente aromáticos, o la adición de manoproteínas al vino, debido a las numerosas propiedades beneficiosas que estas aportan. Las características enológicas más apreciadas, como el vigor fermentativo, el perfil de temperaturas de crecimiento y el rendimiento y tolerancia al etanol, son fenotipos cuantitativos determinados por la contribución acumulativa de múltiples loci polimórficos (QTLs).

Dado el carácter multigénico de las características a mejorar en las cepas vínicas, se tomó la hibridación como un buen mecanismo para abordar esta mejora.

2. Objetivos y Metodología

Para obtener una levadura mejorada, aplicable a un proceso industrial tan complejo como es la vinificación se llevaron a cabo varios análisis. El objetivo de cada uno y la metodología empleada para ellos se resumen a continuación.

2.1. Estudio de la complejidad del antiguo taxón *S. bayanus*.

De los híbridos naturales que conocemos, el parental que más controversia suscita es el antiguo *S. bayanus*, que actualmente se ha separado en *S. uvarum* y *S. bayanus*; además *S. bayanus* (el antiguo *S. bayanus var bayanus*) se ha definido finalmente como un taxón híbrido entre *S. uvarum* y *S. eubayanus*. Consideramos que descifrar la complejidad de este grupo era un buen punto de partida para nuestro trabajo.

Con la finalidad de reconstruir un hipotético genoma *S. bayanus var. bayanus* se secuenciaron 34 regiones génicas de la cepa NBRC1948 (la cual era considerada como una cepa pura antes de definir este taxón como híbrido) y se compararon con las mismas regiones de las cepas CBS7001 (*S. uvarum*) y la parte no-cerevisiae del genoma de la cepa *S. pastorianus* Weihenstephan 30/70 (parte del genoma correspondiente a *S. eubayanus*). Varias de estas secuencias (EPL1, GSY1, JIP5, KIN82, MRC1, PEX2, MAG2, NPR2 y ORC1) se obtuvieron también para otras cepas (CECT 11186 y CBS 424), consiguiendo así un juego completo de alelos de *S. eubayanus*.

Para descifrar la complejidad de esta especie se analizaron 46 cepas clasificadas como *S. bayanus* o *S. pastorianus*. El análisis se llevó a cabo mediante PCR-RFLP para 33 de los 34 genes analizados (ya que se encontraron enzimas de restricción que diferenciaran los alelos '*uvarum*' y '*eubayanus*'). El gen nuclear *MNL1* se analizó mediante secuenciación, al igual que se hizo con el gen mitocondrial *COX2*.

Posteriormente se secuenciaron los genes *CAT8*, *CYC3*, *CYR1*, *GAL4*, *MET6*, *BRE5* y *EGT2*. En los análisis de secuenciación se incluyeron cepas pertenecientes a *S. eubayanus* como cepas control. Con este análisis, y con los datos de procedencia de las cepas, se crearon grupos de alelos (*uvarum* y *eubayanus*) y se estimó la divergencia nucleotídica entre ellos. Esta divergencia nucleotídica se comparó con la existente entre *S. cerevisiae* y *S. paradoxus* y entre las distintas poblaciones de *S. paradoxus*.

A las cepas control de *S. eubayanus*, a varias cepas *S. uvarum* (*S. bayanus var uvarum*) y las cepas pertenecientes a *S. bayanus* (*S. bayanus var bayanus*) se les analizó su capacidad de esporulación y viabilidad mediante crecimiento en un medio con acetato y posterior disección de las ascas utilizando un micromanipulador. También se analizó su ploidía, tiñendo el DNA con PI (yoduro de propidio) y se observó su cariotipo corriendo los cromosomas intactos en un gel de agarosa durante 27h. En ambos análisis la cepa de referencia fue CBS 7001. Combinando el análisis de cariotipos con el análisis de RFLPs se intentó descifrar la composición cromosómica de las cepas pertenecientes a *S. bayanus* (cepas híbridas entre *S. uvarum* y *S. eubayanus*), ya que entre ambos parentales hay dos translocaciones recíprocas.

2.2. Evaluación de diferentes procedimientos para generar híbridos de interés en enología.

A la hora de obtener híbridos en el laboratorio se seleccionaron tres cepas parentales, dos pertenecientes a *S. cerevisiae* (Sc1 y Sc2) y una perteneciente a *S. kudriavzevii* (Sk). Se seleccionaron individuos auxótrofos (*ura3*$^{-}$ y *lys2*$^{-}$) de estas cepas utilizando para ello los medios 5-FOA y αAA y se analizaron marcadores moleculares para poder diferenciar a los parentales de los híbridos obtenidos. Se plantearon dos tipos de cruces, un cruce intraespecífico (Sc1 x Sc2) y otro interespecífico (Sc1 x Sk), y los marcadores moleculares que nos permitieron detectar los híbridos fueron el análisis microsatélites (microsatélites O y P), en el

cruce intraespecífico, y la PCR-RFLP de genes nucleares (*PPR*1 y *BRE*5) en el cruce interespecífico.

Para obtener híbridos se utilizaron tres metodologías: la fusión de protoplastos (P), la técnica de rare mating (RM) y el cruce de esporas. La técnica P se utilizó a modo comparativo, ya que su uso genera organismos modificados genéticamente (GMOs). A los híbridos obtenidos se les analizaron una serie de marcadores moleculares que nos permitieron ver la diversidad de híbridos obtenidos. Estos marcadores, que se seleccionaron por su capacidad de dar una visión amplia del genoma y por presentar distintos perfiles entre las cepas parentales, fueron la PCR de elementos delta y del RAPD R3 para el genoma nuclear y el análisis de mtDNA-RFLP con el enzima de restricción *Hin*F I para el DNA mitocondrial. También se evaluó el contenido en DNA de las cepas parentales y de los híbridos marcando las cepas con PI. Los resultados obtenidos para cada una de las técnicas se compararon entre sí, al igual que entre los distintos cruces.

También se evaluó la necesidad de un proceso de estabilización de estos híbridos recién obtenidos antes de poder ser utilizados para vinificación.

2.3. Estudio del proceso de estabilización de híbridos intraespecíficos e interespecíficos en condiciones de fermentación.

Se propuso un protocolo de estabilización basado en 5 rondas de fermentación. Las condiciones se fijaron en 10mL de mosto sintético incubado a 20ºC y sin agitación. Tras finalizar cada fermentación se utilizaría, como inóculo, una alícuota de la fermentación anterior. Al terminar cada una de las rondas de fermentación se seleccionaron 10 colonias al azar, de cada cepa, y se analizaron los marcadores moleculares (el perfil de elementos delta, el RAPD R3, el perfil de mtDNA-RFLP) y el contenido en DNA.

Para este proceso se utilizaron varios híbridos obtenidos en el trabajo anterior. Del cruce intraespecífico se seleccionaron dos híbridos obtenidos por rare

mating (R2 y R8) y dos obtenidos mediante cruce de esporas (S2 y S7). Del cruce interespecífico se seleccionaron otros cuatro híbridos, R1 y R3 (RM) y S5 y S8 (S).

Para cada uno de los híbridos sometidos a este proceso de estabilización se seleccionaron todos los perfiles diferentes que fueron apareciendo durante las 5 rondas de fermentación y se sometieron a una nueva fermentación (cada fermentación se inoculó con una única colonia) con el fin de evaluar si cada uno de los perfiles era estable. Esta evaluación se realizó con los mismos marcadores que se habían utilizado (elementos delta, RAPD R3, mtDNA-RFLP y análisis del contenido en DNA). Se compararon entre si la estabilización de híbridos de un mismo cruce obtenidos por distinta metodología como la estabilización de híbridos intra e interespecíficos, remarcando las diferencias encontradas.

Finalmente se sometió a dos aislados intraespecíficos (R2 IVo y R8 IIa) al proceso de producción de LSA, ya que es un punto estresante al que se ven sometidas las levaduras vínicas. Estos aislados se seleccionaron porque los híbridos intraespecíficos obtenidos por RM demostraron ser los más variables. Se analizaron los marcadores moleculares antes y después de someter a los aislados a este proceso y se determinó su estabilidad.

El híbrido estable R2 IVo se analizó por Hibridación Genómica Comparada (aCGH). En este análisis se enfrentó a este aislado antes y después de someterse al proceso de LSA para ver si se había producido algún cambio a nivel genómico durante el proceso. Dado que los chips utilizados están basados en el genoma de *S. cerevisiae*, la información obtenida por la técnica de aCGH está limitada al estudio del genoma parental de *S. cerevisiae,* por esta razón se eligió al híbrido intraespecífico (Sc1 x Sc2) R2 IVo.

Tras este análisis se evaluó la capacidad fermentativa de esta cepa, en un mosto natural, antes y después de la producción de LSA. La cinética de fermentación se siguió mediante la pérdida de peso, los datos se convirtieron a % de azúcar consumido y se ajustaron a la ecuación de Gompertz reparametrizada. Se

evaluó la velocidad máxima de fermentación y le periodo de latencia de las cepas. La concentración de metabolitos al final de la fermentación (glucosa, fructosa, glicerol y etanol) se midió con un HPLC equipado con un detector de índice de refracción.

2.4. Caracterización fisiológica y genómica de híbridos artificiales de *S. cerevisiae* con mejoras en su capacidad fermentativa y en la capacidad de liberar manoproteínas.

Diecinueve perfiles estables del cruce intraespecífico (15 derivados de los híbridos R2 y R8 obtenidos por RM y 3 derivados de los híbridos S2 y S7 obtenidos por S), seleccionados en el apartado anterior, se sometieron a una caracterización fisiológica con el fin de seleccionar aquel aislado que mejorara las características de sus cepas parentales: Sc1 – seleccionada por su capacidad de liberación de manoproteínas – y Sc2 – seleccionada por su excelente capacidad fermentativa. En todos los estudios se utilizaron las cepas parentales como cepas control.

El primer estudio fisiológico se llevó a cabo en mosto sintético, la fermentación se siguió mediante el análisis de azúcares presentes en el mosto cada dos días. Los datos se ajustaron a una ecuación exponencial de descenso y se calcularon la tasa de fermentación máxima (K) y el tiempo que tardaron en consumir el 50% y el 98% de los azúcares presentes en el mosto. Se evaluó también la concentración de metabolitos (glucosa, fructosa, glicerol y etanol) en el punto final de la fermentación mediante un análisis de HPLC. A las cepas que consiguieron terminar la fermentación se les midió su producción de polisacáridos mediante la técnica del fenol sulfúrico y se evaluó su perfil de manoproteínas tras correr los sobrenadantes en un gel de acrilamida, transferirlo a una membrana e hibridar esta con concanvalina A marcada con peroxidasa.

Las tres mejores cepas (en cuanto a una elevada producción de polisacáridos y manoproteínas y una buena capacidad fermentativa) se pusieron a fermentar un mosto Sauvignon Blanc. En este caso, una vez terminada la fermentación, se realizó

un estudio de quiebra protéica. Para ello a alícuotas del vino fermentado se le añadieron concentraciones crecientes de bentonita (entre 0 y 60 g/hL), se incubaron durante media hora y, tras eliminar la bentonita, y se hicieron quebrar proteicamente sometiéndolas a calor (80ºC) y posteriormente enfriándolas bruscamente.

La muestra que mejor respondió al tratamiento con bentonita (la cepa que necesitó menor cantidad de bentonita para estabilizar el vino) se evaluó fisiológicamente en una fermentación en mosto natural Verdejo. La fermentación se siguió por pérdida de peso y los datos, una vez transformados a % de azúcar consumido, se ajustaron a la ecuación de Gompertz reparametrizada. Se evaluó la velocidad máxima de fermentación, el periodo de latencia de las cepas y el tiempo que tardan en terminar la fermentación. La concentración de metabolitos al final de la fermentación (glucosa, fructosa, glicerol y etanol) se midió con un HPLC. Además, se analizó la concentración de manoproteínas. Para ello se sometió la fracción macromolecular del sobrenadante a una hidrólisis ácida. Tras neutralizar las muestras, las manoproteínas se cuantificaron en el HPLC.

Para finalizar se decidió realizar un análisis de aCGH sobre la cepa que mejoró las características de ambos parentales. El DNA de esta cepa se hibridó de forma competitiva junto al DNA de cada uno de sus parentales, en un chip de *S. cerevisiae*. Con este análisis se pretendía observar si se podía asociar el aumento en el número de copias de algún gen, en el híbrido mejorado, con las mejoras observadas. Los genes que se consideraron como posibles causantes de esta mejora se analizaron por qRT-PCR para determinar exactamente el número de copias con respecto a sus parentales. También se evaluó la expresión de estos genes durante la fermentación de mosto sintético por qRT-PCR.

3. Conclusiones

3.1. Estudio de la complejidad del antiguo taxón *S. bayanus*.

Todos los aislados clasificados como *S. bayanus* (antiguo *S. bayanus var bayanus*) resultaron ser híbridos entre *S. uvarum* y *S. eubayanus*, no se encontró ninguna cepa pura *S. eubayanus*, y se demostró la presencia de alelos '*uvarum*' en la cepa NBRC 1948. Los híbridos *S. bayanus* pueden dividirse en dos grupos, híbridos tipo I (homocigtos para todos los genes) e híbridos tipo II (heterocigotos para alguno de los genes analizados).

El origen de las cepas pertenecientes a *S. bayanus* se encuentra en múltiples eventos de hibridación entre cepas de *S. uvarum* y *S. eubayanus*, aunque estas últimas estarían relacionadas con las cepas patagónicas pero no serían iguales, de modo que debería existir una población *S. eubayanus* europea que todavía no se ha localizado. Al menos, se necesitaron dos cepas *S. uvarum* implicadas en este proceso para dar lugar a todas las cepas *S. bayanus* analizadas. Tras la hibridación inicial de las dos cepas, un suceso de esporulación y "haplo-selfing" daría lugar a los híbridos tipo I, mientras que un suceso de esporulación y anfimixis daría lugar a los híbridos tipo II.

Entre *S. uvarum* y *S. eubayanus*, la heterocigosidad es responsable de parte del aislamiento reproductivo observado. La antirrecombinación parece ser una de las principales causas de aislamiento reproductivo en especies colineales del género *Saccharomyces*, aunque no debe ser la responsable del proceso de especiación entre *S. uvarum* y *S. eubayanus*, ya que los resultados obtenidos en esta tesis nos muestran que la recombinación en el híbrido ancestral *S. uvarum*/*S. eubayanus* estaba permitida. *S. uvarum* y *S. eubayanus* son las especies hermanas más cercanas del género *Saccharomyces*, se encuentran en la base del árbol filogenético y son las especies que más tiempo han tenido para diferenciarse entre ellas, pero los datos de divergencia nucleotídica indican que no lo han hecho. Ambos datos indican que hay otros factores implicados en la especiación entre *S. uvarum* and *S. eubayanus.*

Otro factor que actúa en la especiación son las translocaciones. Entre *S. uvarum* y *S. eubayanus* hay tres translocaciones, lo que disminuiría la viabilidad de sus esporas en un 87.5%, datos previos de aislamiento entre *S. uvarum* y *S. eubayanus* muestran valores de viabilidad mayores a los esperados (llegando al 19% en algunos casos), por lo que la composición cromosómica de *S. eubayanus* debería ser estudiada en profundidad.

Las barreras ecológicas tampoco parecen ser muy importantes en el proceso de especiación de estas dos especies, ya que se ha observado mayor aislamiento reproductivo entre poblaciones simpátricas que entre poblaciones alopátricas.

El género *Saccharomyces*, debido a sus características (grandes poblaciones, capacidad de cambiar de tipo sexual…) es susceptible de sufrir especiación híbrida homoploide, y eso parece que ha sucedido con los híbridos tipo I pertenecientes a *S. bayanus*. Analizando estos híbridos podemos observar que cumplen los tres criterios asociados a este tipo de especiación: i) están aislados de sus parentales, este aislamiento es parcial pero se ve reforzado por barreras ecológicas (ocupan distinto nicho ecológico). ii) S. bayanus se originó por un suceso de hibridación, como se puede observar mediante los datos de PCR-RFLP, secuenciación, cariotipado… iii) El aislamiento reproductivo es consecuencia del suceso de hibridación, por ejemplo, la combinación de translocaciones en el híbrido da lugar a un aislamiento reproductivo parcial entre estos y los parentales.

La hibridación entre *S. uvarum* y *S. eubayanus* debió darse en ambientes naturales. Estos híbridos colonizaron ambientes fermentativos y proliferaron en ellos. Durante este proceso, se dieron cambios en el genoma de estos híbridos ancestrales y aparecieron los híbridos tipo II, llegando a alcanzar grandes tamaños poblacionales. Los híbridos tipo II esporularon y alguna de las pocas esporas viables autodiploidizaron, dando lugar a la aparición de los híbridos tipo I. Estos híbridos homocigotos recuperaron la fertilidad y se convirtieron en una potencial especie híbrida homoploide. Los híbridos tipo II son el reservorio de los híbridos tipo I.

3.2. Evaluación de diferentes procedimientos para generar híbridos de interés en enología.

Se obtuvieron un elevado número de híbridos artificiales, inter e interespecíficos, mediante las tres metodologías. Aun así la técnica con la que se obtuvo una mayor frecuencia de híbridos fue el cruce de esporas. Al trabajar con parentales auxótrofos, la frecuencia de recuperación de híbridos se mejoró cuando los cultivos se mantuvieron unas horas en medio de ayuno antes de inocular en el medio de selección.

Entre los híbridos recién obtenidos, independientemente del tipo de cruce, se encontró una gran variabilidad, observada en el estudio de los marcadores moleculares (delta, R3 y mtDNA-RFLP). Varios de los híbridos presentaron patrones recombinantes de mtDNA. El hecho de que un mismo patrón recombinante se detectara en diferentes híbridos (obtenidos por el mismo o por distinto protocolo de hibridación) parece indicar la presencia de puntos calientes de recombinación en el mtDNA. La mayor frecuencia de moléculas de mtDNA recombinantes encontradas en el cruce intraespecífico se puede explicar por la mayor similaridad genética entre las moléculas de mtDNA de las cepas parentales.

El análisis de microsatélites (en los híbridos intraespecíficos) y de contenido en DNA (en ambos híbridos), junto con la variabilidad genética encontrada en los marcadores nucleares, sugieren que, durante momentos tempranos de la generación de híbridos se producen sucesos de recombinación y de pérdida de material genético. Este proceso fue especialmente evidente en híbridos generados por P y RM, lo que indica una mayor plasticidad de estos híbridos. Esta mayor plasticidad en híbridos generados por P y RM permitiría una mayor adaptación de los híbridos al ambiente.

La pérdida de material genético hace necesario llevar a cabo un proceso de estabilización sobre los híbridos recién obtenidos para asegurar su invariabilidad cuando se utilicen en procesos industriales. Estudios realizados tras varios ciclos de

crecimiento indican que entre los híbridos recién obtenidos se pueden dar perdidas de material genético y reorganizaciones de su genoma.

3.3. Estudio del proceso de estabilización de híbridos intraespecíficos e interespecíficos en condiciones de fermentación.

La necesidad de estabilización de los híbridos recién formados es un hecho, y la plasticidad de estos genomas (principalmente de aquellos poliploides, obtenidos por RM) realza la importancia del medio y de las condiciones de estabilización, ya que durante este periodo los híbridos tendrían la capacidad de adaptarse. Estudiar como ocurre este proceso puede ayudar a desarrollar protocolos de estabilización para híbridos implicados en otros procesos.

En híbridos poliploides se observa una reducción del contenido en DNA a lo largo de todo el proceso, ocurriendo antes y de forma más rápida en los híbridos interespecíficos. Los valores de ploidía a los que se estabilizan los híbridos son valores similares a los que presentaban sus cepas parentales.

Durante el proceso de estabilización también se observaron reorganizaciones en los marcadores moleculares. Estas reorganizaciones fueron más frecuentes en los híbridos intraespecíficos, y fueron los únicos que mostraron reorganizaciones en el genoma nuclear. En híbridos interespecíficos solo se observaron reorganizaciones en su genoma mitocondrial. Durante la estabilización de los híbridos intraespecíficos, las reorganizaciones del genoma dieron lugar a una elevada diversidad de perfiles derivados de un único perfil inicial.

Dado que la producción de levadura seca activa (LSA) es un proceso estresante, se estudió si durante este proceso los híbridos derivados también pudieran sufrir reorganizaciones. De los dos híbridos analizados uno resultó no ser completamente estable, pero el otro sí que lo fue. Esto resalta la necesidad de

evaluar la estabilidad de los híbridos tras someterlos a cada uno de los puntos estresantes del proceso.

Para resumir, la estabilización de híbridos artificiales se puede dar de varias formas: i) estabilización mediante la pérdida gradual de material genético, sin cambios a nivel nuclear o mitocondrial; ii) estabilización tras la reordenación del genoma nuclear y la pérdida de material genético, con o sin cambios a nivel mitocondrial; iii) estabilización tras una pérdida rápida de material genético, con reordenaciones a nivel mitocondrial.

Los marcadores empleados permiten evaluar el proceso de estabilización y confirmar la estabilidad de los aislados. Según nuestros resultados, 30-50 generaciones (3-5 rondas de fermentación) son suficientes para estabilizar híbridos inter e intraespecíficos respectivamente.

3.4. Caracterización fisiológica y genómica de híbridos artificiales de *S. cerevisiae* con mejoras en su capacidad fermentativa y en la capacidad de liberar manoproteínas.

Varios de los híbridos analizados igualaron o superaron los parámetros cinéticos del parental Sc2 en una fermentación en mosto sintético. Todos ellos produjeron mayor cantidad de polisacáridos que el parental Sc1 y varios presentaron bandas de manoproteínas más intensas que este. En cuanto a la quiebra proteica, uno de los híbridos analizados requirió menores concentraciones de bentonita que el parental Sc1 para estabilizar el vino. Y en fermentación en mosto Verdejo, presentó características cinéticas similares al parental Sc2 y una mayor producción de manoproteínas que el parental Sc1.

Esto demostró que la hibridación es un buen método de mejora de cepas.

El análisis genómico de esta cepa frente a sus parentales, indicó una serie de duplicaciones génicas que podrían estar implicadas en la mejora. El análisis de qRT-PCR para analizar el número de copias de DNA de los genes *MNN*10, *YPS*7 y *HXK*1 no

fue concluyente, por lo que se procedió a estudiar la expresión de estos genes a lo largo de una fermentación en mosto sintético. El análisis de expresión mostró que las mejoras observadas en producción de manoproteínas y capacidad fermentativa podrían estar asociadas a estos genes, confirmando los resultados obtenidos en el análisis genómico.

Introducción

1.1. Las levaduras.

Las levaduras son los organismos eucariotas más simples que existen. Son hongos unicelulares con forma redonda, ovoide o cilíndrica, y, bajo determinadas condiciones, algunas especies pueden formar filamentos. Se reproducen asexualmente por gemación o bipartición y, si existe reproducción sexual, las esporas, denominadas ascosporas o basidiosporas, no se forman dentro de un cuerpo fructífero. Son organismos saprofitos que crecen sobre una gran variedad de sustratos de origen vegetal o animal, o bien son parásitos de plantas y animales. Son habitantes normales de nichos donde hay azúcares (flores, frutos y corteza de árboles). Son responsables de muchas actividades beneficiosas para el hombre, por lo que se les ha utilizado durante milenios para producir alimentos fermentados y bebidas alcohólicas, e incluso antibióticos, vitaminas y enzimas. Las levaduras también son responsables de actividades perjudiciales que incluyen el deterioro de alimentos y productos manufacturados, y la producción de enfermedades infecciosas en el hombre y los animales.

Las levaduras pertenecen al Reino Fungi y dentro de él a la división Eumicota que agrupa a los hongos verdaderos. Las levaduras se incluyen en las subdivisiones Ascomycotina, representada por las levaduras capaces de producir ascosporas, (o levaduras esporógenas), y la Deuteromycotina, representadas por las levaduras incapaces de formar esporas (no esporógenas).

2. El género *Saccharomyces*.

2.1. Clasificación y taxonomía.

Las levaduras del género *Saccharomyces* pertenecen a la subdivisión *Ascomycotina*, ya que su modo de reproducción sexual se basa en la formación de ascosporas. Dentro de esta subdivisión pertenecen a la clase de los H*emiascomycetes,* al orden de los *Saccharomycetales* y a la familia *Saccharomycetaceae,* compuesta por 12 clados (Kurtzman and Robnett 1991; Kurtzman and Robnett 1998; Kurtzman and Robnett 2003; Kurtzman 2003; Kurtzman and Piškur 2005; Wu et al. 2008).

2.2. Características generales.

Las levaduras del género *Saccharomyces* se aíslan tanto de ambientes naturales como de procesos industriales. Son organismos anaerobios facultativos, capaces de desarrollar un metabolismo oxidativo, en presencia de oxigeno, y fermentativo en su ausencia. Presentan el fenómeno de represión catabólica mediante el cual, en presencia de glucosa o de algún otro azúcar fermentable como fructosa, se encuentran reprimidos, entre otros, los genes necesarios para la respiración. En presencia de oxígeno su metabolismo pasa por las siguientes fases: durante la fase exponencial del crecimiento, obtiene la energía procedente de la fermentación; cuando los azúcares se agotan, tiene lugar la desrepresión de los genes implicados en la respiración y la célula sufre una adaptación hacia un metabolismo respiratorio (denominada pausa diáuxica); durante la fase postdiáuxica, las células obtienen energía de la respiración reproduciéndose a una velocidad menor que durante la fase exponencial; una vez agotados los nutrientes, la célula deja de dividirse y entra en fase estacionaria (Werner-Washburne et al. 1993).

2.3. Ciclo de vida.

Las levaduras del género *Saccharomyces* se encuentran en la naturaleza como organismos unicelulares, generalmente diploides (a/α), que se dividen asexualmente por gemación (crecimiento vegetativo). La célula hija inicia su crecimiento formando una yema en la célula madre, posteriormente ocurre la división nuclear, la síntesis de la pared y finalmente la separación de las dos células. La división es asimétrica siendo la célula madre de mayor tamaño que la hija (Gerton et al. 2000). Este ciclo asexual se puede dar tanto en cultivos de células diploides como haploides. En condiciones de ayuno, la célula diploide, puede esporular y originar, mediante meiosis, cuatro ascosporas haploides. Las esporas pueden presentar dos tipos sexuales a ó α. Las esporas germinan y crecen vegetativamente hasta que encuentran una célula de tipo sexual opuesto. (Figura I1).

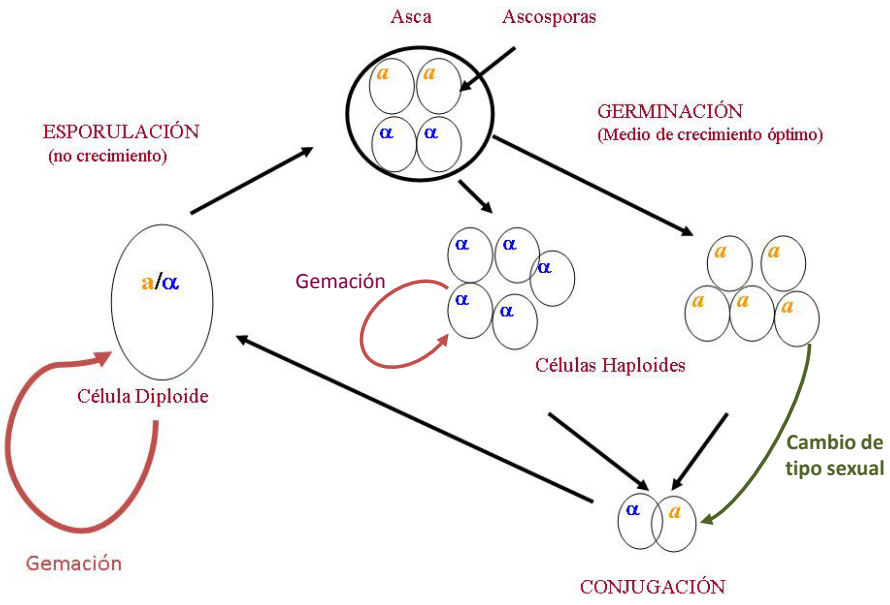

Figura I1. Ciclo de vida de *Saccharomyces.*

El tipo sexual de una célula haploide está determinado por su genotipo en el locus *MAT* ("mating–type") situado en el cromosoma III. Las dos formas del locus *MAT*, *MATa* y *MATα*, difieren tanto en la secuencia nucleotídica y el tamaño, como

en los genes que incluyen (Metzenberg and Glass 1990). Además del locus MAT activo, las células poseen copias del locus *MAT* que están silenciadas y que no interfieren en la determinación sexual; estas copias silenciosas son *HMR* (copia silenciosa del alelo *MATa)* y *HML* (copia silenciosa del alelo *MATα*), y se encuentran en el cromosoma III, a derecha e izquierda del locus activo. Existen levaduras capaces de cambiar de tipo sexual, estas se denominan homotálicas; las levaduras que no son capaces de sufrir este cambio se denominan heterotálicas. En el género Saccharomyces encontramos tanto cepas homotálicas como heterotálicas.

El proceso de cambio en el tipo sexual (figura I2) es el resultado de una translocación de la información no expresada del locus *HMLα* o *HMRa* al locus *MAT*. Se inicia cuando la endonucleasa, producto del locus *HO,* realiza un corte en un sitio específico del locus *MAT*, que posteriormente es degradado por exonucleasas. Tras la degradación, se activan los sistemas de reparación del DNA y reemplazan el locus *MAT* por una de las copias adicionales *HMRa* o *HMLα.* La reparación del locus *MAT* permite el cambio sexual ya que este se reemplaza por el alelo contrario al que estaba en un principio (Herskowitz 1988).

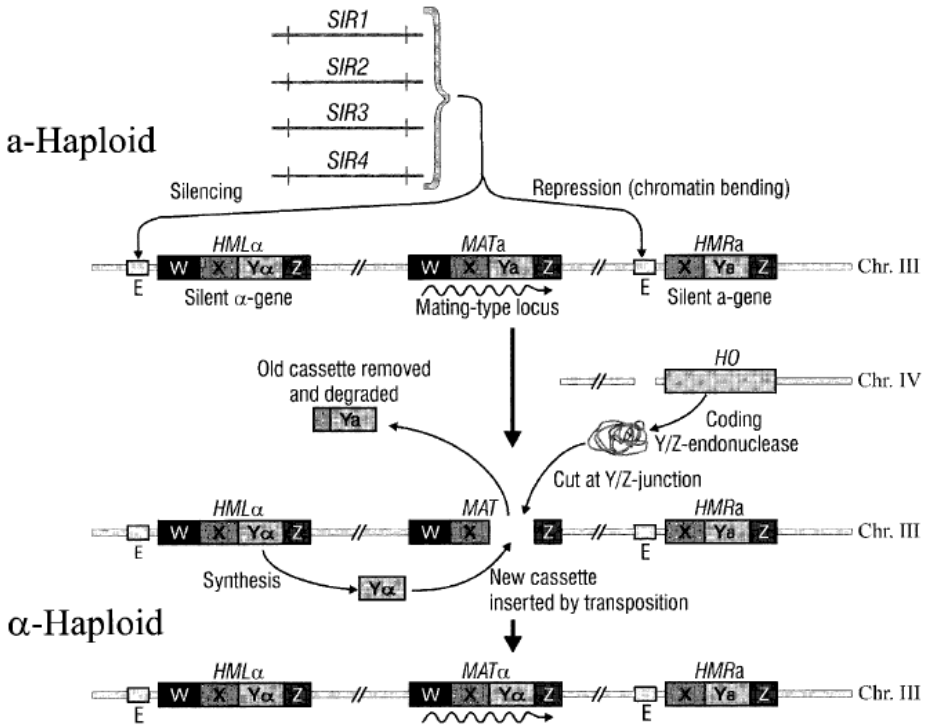

Figura I2. Cambio de tipo sexual (Pretorius 2000). Este proceso se da en cepas homotálicas.

La reproducción sexual en *Saccharomyces* es versátil, incluyendo tres tipos diferentes de apareamiento: la anfimixis, la automixis y el "haplo-selfing" (Figura I3) (Knop 2006). El suceso clásico de apareamiento entre dos células haploides, que provienen de diferentes células diploides no relacionadas, con diferente tipo sexual, se conoce como anfimixis o cruzamiento inter-tétrada. La automixis o cruzamiento intra-tétrada, es el suceso en el cual dos esporas de tipo opuesto, producto de una misma meiosis, se aparean para la formación del individuo diploide; el apareamiento puede ocurrir en el asca antes de que se rompa su pared, o cuando las esporas son liberadas. Por último, se puede dar "haplo-selfing", o autodiploidización, que ocurre sólo en cepas homotálicas, es el apareamiento entre una célula y una de sus células hijas después del cambio del tipo sexual de una de ellas.

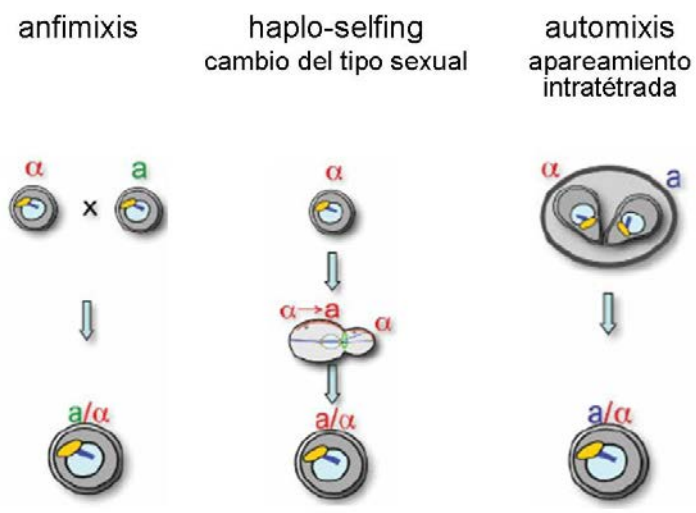

Figura I3. Diferentes tipos de apareamiento de *Saccharomyces cerevisiae*: anfimixis, haplo-selfing y automixis (Knop 2006).

2.4. Estructura celular.

Las células de levadura están formadas por las envueltas celulares, un citoplasma con diversos orgánulos y un núcleo (figura I4).

El núcleo está rodeado por una membrana; allí se encuentran los cromosomas.

El citoplasma es el espacio que se encuentra entre la membrana nuclear y la membrana plasmática. Contiene el citosol, que es una solución acuosa, de pH entre 5 y 6, en la que se encuentran enzimas solubles, carbohidratos de reserva, ribosomas y orgánulos membranosos (retículo endoplásmico, aparato de Golgi, vacuolas y mitocondrias).

Al igual que las plantas, las levaduras poseen dos envueltas celulares, la membrana plasmática y la pared celular. El espacio entre estas dos membranas es el periplasma.

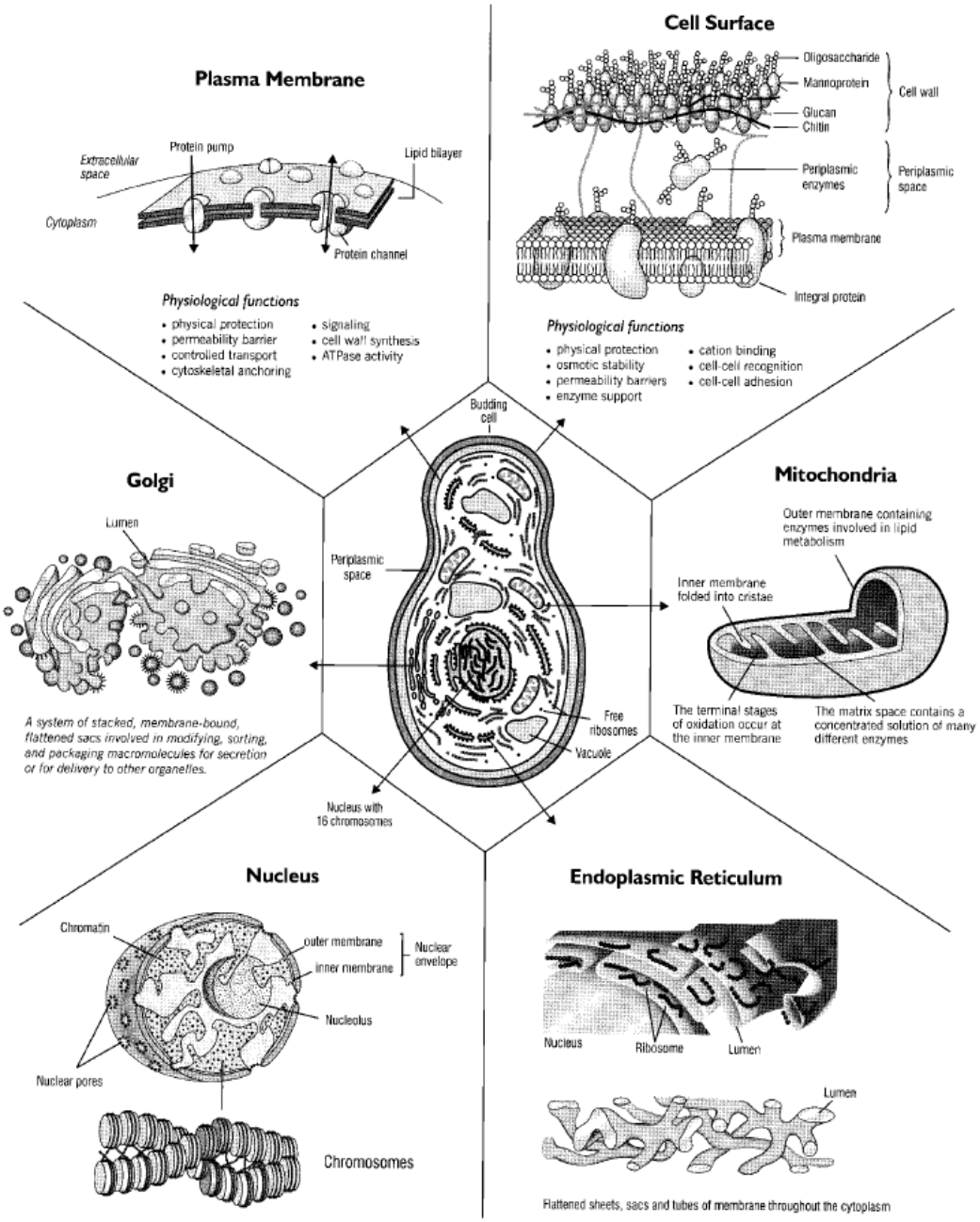

Figura I4. Estructura celular en *Saccharomyces* (Pretorius 2000).

La membrana plasmática constituye una barrera hidrofóbica estable entre el citoplasma y el medio externo. Está construida, principalmente, por lípidos

(fosfolípidos y esteroles) y proteínas (integrales y periféricas). La composición de la membrana en ácidos grasos y su proporción en esteroles, controlan su fluidez.

La pared celular es esencial para la supervivencia de estas células (Cabib *et al.* 2001). Es una estructura de naturaleza dinámica, su composición y arquitectura pueden variar ampliamente en respuesta a cambios del medio de cultivo y también a lo largo de la fase de crecimiento. Dependiendo de las condiciones de crecimiento y de cultivo, puede llegar a constituir hasta el 30% de su peso seco (Klis 1994; Klis et al. 2006; Lesage and Bussey 2006).

En células vegetativas, la pared está compuesta típicamente por b-1,3-glucano (50-55% del peso seco de la pared), quitina (1-2%), b-1,6-glucano (3-14%) y manoproteínas (35-40%). Estudios de microscopía electrónica han revelado una estructura con dos capas bien diferenciadas. La capa interna es la responsable de la resistencia mecánica de la pared y está compuesta por b-1,3-glucano y quitina. La capa externa es responsable de la permeabilidad celular y está compuesta por b-1,6-glucano y manoproteínas(Klis et al. 2002).

3. Especies que forman el género *Saccharomyces.*

3.1. El concepto de especie.

El concepto de especie es fundamental para las ciencias biológicas (Mallet 1995; Coyne and Orr 1998), pero su definición siempre ha sido muy complicada.

El concepto biológico de especie (CBE) es el más aceptado desde que Ernest Mayr lo propuso en 1942, y se aplica en organismos con reproducción sexual. Así, se considera que una especie se compone de organismos que son capaces de producir una progenie híbrida viable y fértil, y que muestran aislamiento reproductivo con

individuos de otras especies, a través de diversos mecanismos, tanto prezigóticos como postzigóticos. Por tanto, una especie es una población de organismos aislada reproductivamente de otra.

Los problemas al aplicar el concepto biológico de especie ocurren en organismos con reproducción asexual, como algunos hongos (Taylor et al. 2000) y plantas (Rieseberg 1997); así como en bacterias, que reciben genes por transferencia horizontal (Gogarten and Townsend 2005). Debido a los problemas en aplicar el concepto biológico de especie, se han propuesto varios conceptos de especie distintos (Hey 2001).

Algunas de las alternativas al concepto de especie biológica comprenden conceptos de especies basados en una cohesión genética o fenotípica. El concepto de especie como agrupamientos genotípicos (CEAG) (Mallet 1995) considera una especie como un grupo de individuos morfológica o genéticamente diferentes de otros grupos, y cuando están en contacto, hay pocos o ningún grupo intermedio entre ellos. El concepto de especie por reconocimiento (CER) (Paterson 1985) comprende una población incluyente de individuos biparentales que comparten un sistema común de fertilización. El concepto de especie por cohesión (CEC) (Templeton 1989) considera a la población más incluyente de individuos con un potencial de cohesión fenotípica, la cual se mantiene mediante diversos mecanismos intrínsecos de cohesión. Estos mecanismos están clasificados en reproductivos, que limitan el intercambio genético, y en demográficos que definen los límites de la dispersión de nuevas variantes a través de la deriva genética y/o la selección natural.

Otras alternativas al concepto de especie biológica son los conceptos de especie basados en una cohesión evolutiva. Entre ellos, está el concepto ecológico de especie (CEE) (Van Valen 1976), el cual define una especie como un linaje o conjunto de linajes afines que evolucionan separadamente de otros, y que ocupan una zona adaptativa o nicho ecológico. El concepto evolutivo de especie (CEvE)

(Wiley 1978), define una especie como un linaje de organismos o poblaciones descendientes de un ancestro común que mantienen su identidad en relación a otros linajes, que evoluciona independientemente y que tiene su propio destino evolutivo.

Por otro lado, están los conceptos de especie basados en la historia evolutiva de los individuos que la componen. El concepto filogenético de especie (CFE) (Cracraft 1989) define a la especie como un grupo monofilético diferenciable al considerar su historia evolutiva y su distribución geográfica. El problema de éste concepto de especie es el conocimiento incompleto de la historia evolutiva puede llevar a una delimitación arbitraria de las especies (Hudson 2002).

3.2. Identificación y clasificación de especies en el género *Saccharomyces*.

La identificación y clasificación de especies dentro del género *Saccharomyces* (Kurtzman 2003; Wu et al. 2008) ha sido controvertida a lo largo del tiempo y todavía no ha sido completamente resuelta, ya que no se ve una clara separación entre estas especies.

Las primeras clasificaciones se basaban en características morfológicas y fisiológicas, como la capacidad para fermentar y asimilar distintas fuentes de carbono y nitrógeno (Guillermond 1912), pero en muchos casos los resultados eran variables lo que llevaba a clasificaciones que diferían de un autor a otro. Además, a ello hay que añadir que la fuente de aislamiento, la aplicación tecnológica de la cepa o el origen geográfico de los aislados podía llegar a ser utilizado como criterio en la definición de especies (Barnett 1992).

Las especies del género *Saccharomyces* presentan reproducción sexual, con un aislamiento reproductivo postzigótico (Naumov 1996), por lo que se aplica, en su identificación, el CBE. Sin embargo, y sobre todo en aislados procedentes de

procesos fermentativos, se han encontrado cepas que no producen esporas, por defectos en la meiosis, lo que ocasiona errores en la aplicación del CBE.

Con el progreso de la biología molecular, se han desarrollado nuevas técnicas para la identificación y caracterización de levaduras (James et al. 1997; Esteve-Zarzoso et al. 1999; Kurtzman and Robnett 2003). La taxonomía molecular, que es el estudio de la clasificación de especies mediante técnicas de biología molecular, se desarrolló como consecuencia de las limitaciones de la taxonomía convencional y a los problemas derivados al aplicar el CBE (Rainieri et al. 2003).

Las primeras técnicas moleculares utilizadas para la identificación de especies de levaduras fueron la determinación de la similitud de genomas basada en su composición nucleotídica (% G+C) o en la reasociación del DNA, en donde una similitud mayor del 80% indica conespecificidad (Price 1978; Vaughan-Martini and Kurtzman 1985).

Otros marcadores moleculares, muy populares a la hora de diferenciar especies y cepas de levaduras, pero no aceptados a la hora de definir especies son: cariotipado electroforético (Johnston 1986); la secuenciación o restricción del DNA mitocondrial (Guillamón et al. 1994; Querol et al. 1994; Belloch et al. 1997; Guillamón et al. 1997); los polimorfismos de longitud de fragmentos de restricción o RFLP de regiones ribosomales (Belloch et al. 1998; Guillamón et al. 1998); los métodos de genotipado basados en la reacción en cadena de la polimerasa o PCR (Barros Lopes et al. 1998; Ryu et al. 1998), el DNA polimórfico amplificado al azar o RAPDs (Fernández-Espinar et al. 2003), los polimorfismos de longitud de fragmentos amplificados o AFLP (de Barros Lopes et al. 1999; Azumi and Goto-Yamamoto 2001); los microsatélites (Bradbury et al. 2006) o la secuenciación de genes nucleares (Valente et al. 1999). Actualmente se han producido grandes avances en el mundo de la biología molecular gracias al desarrollo de las plataformas de secuenciación de alto rendimiento, o técnicas de secuenciación masiva (Next-generation sequencing; NGS). A diferencia de los sistemas de secuenciación tradicionales, estas plataformas

son capaces de generar paralelamente, y de forma masiva, millones de fragmentos de ADN en un único proceso de secuenciación. La aplicación de estas técnicas nos permite la caracterización de la variabilidad genética entre diferentes cepas de levaduras (Zhang *et al.* 2011; Borneman *et al.* 2011).

La utilización del CBE (Naumov 1996) y los análisis de reasociación de DNA (Vaughan-Martini and Kurtzman 1985) llevaron a la simplificación del número de especies del género *Saccharomyces*. Se pasó de 21 especies, en 1970, a 4, en 1998: *S. cerevisiae, S. bayanus, S. paradoxus* y *S. pastorianus*. A ellas se añadieron 3 nuevas especies (Naumov et al. 2000a) *S. cariocanus, S. kudriavzevii* y *S. mikatae*. Posteriormente se describió *S. arboricolus* (o *S. arboricola*) (Wang and Bai 2008). Recientemente se ha descrito una nueva especie, *S. eubayanus* (Libkind et al. 2011); tras su descripción se reconsideró el estatus de la especie *S. bayanus* y se subdividió en dos: *S. bayanus* y *S. uvarum*.

Actualmente, el género *Saccharomyces* se compone de 7 especies: *S. arboricolus, S. cerevisiae, S. eubayanus, S. kudriavzevii, S. mikatae, S. paradoxus* y *S. uvarum* (Almeida et al. 2014; Boynton and Greig 2014). Además, en el género *Saccharomyces* podemos encontrar dos grandes grupos de híbridos *S. pastorianus* (*S. cerevisiae x S. uvarum x S. eubayanus*) y *S. bayanus (S. cerevisiae x S. uvarum x S. eubayanus*), así como un número menor de híbridos que poseen porciones de *S. kudriavzevii* (Lopes et al. 2010; Peris et al. 2012).

S. cerevisiae es la especie predominante en diversos procesos de fermentación industriales como son la producción de pan, elaboración de cerveza, obtención de bebidas destiladas, elaboración de vinos, producción de sidra, sake, así como de bebidas fermentadas tradicionales de diversas regiones del mundo (pulque, masato, chicha, cerveza de sorgo, vino de palma, etc.). Las actividades metabólicas de *S. cerevisiae* han sido explotadas por el hombre desde el desarrollo de la agricultura, por lo que se puede considerar el microorganismo más importante desde el punto de vista económico. (van der Aa Kuhle et al. 2001; Guerra et al. 2001;

Naumova et al. 2002; Jespersen 2003; Cavalieri et al. 2003; Clemente-Jimenez et al. 2004; Hammes et al. 2005; Glover et al. 2005; Legras et al. 2007; Jeyaram et al. 2008; Lodolo et al. 2008).

S. bayanus era considerado como un complejo de cepas agrupadas en 2 variedades (Naumov 2000), *S. bayanus* var. *bayanus* y *S. bayanus* var. *uvarum*, aunque algunos autores ya las elevaban al rango de especie (Pulvirenti et al. 2000; Nguyen and Gaillardin 2005). Todas ellas comparten dos características fisiológicas únicas dentro del género *Saccharomyces* como son un sistema activo de transporte de fructosa y la 'criofilia', es decir, su capacidad para crecer a bajas temperaturas, ambas características de gran interés para el sector enológico (Serra et al. 2005). Estas cepas están implicadas en la fermentación de cerveza de tipo 'lager', de vino y de sidra. En vinos y sidra sólo se aísla la variedad *uvarum*, mientras que en cerveza se encuentra la variedad *bayanus*. Actualmente, tras la descripción de la especie *S. eubayanus* (Libkind *et al.* 2011), estos dos grupos se han dividido definitivamente, de modo que la variedad *uvarum* ha subido al rango de especie (*S. uvarum*) y la variedad *bayanus* (*S. bayanus*) se considera un conjunto de cepas híbridas entre *S. uvarum* y *S. eubayanus,* con mayor o menor proporción de estos genomas.

S. pastorianus es el principal microorganismo productor de cerveza 'lager', sin embargo, se trata de cepas híbridas que son alotetraploides parciales entre *S. cerevisiae* y *S. bayanus* (Hansen and Kielland-Brandt 1994; Nguyen et al. 2000; Casaregola et al. 2001).

S. paradoxus es considerada una especie natural de amplia distribución, formada por distintas variedades geográficas (Naumov et al. 1997; Kuehne et al. 2007); se ha encontrado en procesos de fermentación, llegando a ser la levadura predominante en viñedos croatas (Redžepovic et al. 2002). También se ha descrito como la levadura responsable de la fermentación de pulque y de aguamiel (Sanchez-Marroquin and Hope 1953).

Por último, *S. arboricolus, S. cariocanus, S. eubayanus, S. kudriavzevii* y *S. mikatae* se describieron a partir de cepas aisladas de ambientes naturales. *S. arboricolus* se aisló de árboles de China (Wang and Bai 2008). *S. cariocanus* se aisló de *Drosophila* en Brasil (Naumov et al. 2000a). *S. eubayanus* se aisló de corteza de árboles en Argentina (Libkind et al. 2011). Y *S. kudriavzevii* y *S. mikatae* se aislaron de hojas en descomposición y suelo de Japón (Naumov et al. 2000a).

4. Papel de las especies del género *Saccharomyces* implicadas en procesos fermentativos.

Desde el inicio de la civilización humana, existe una relación estrecha entre el hombre y la actividad fermentativa de los microorganismos. La fermentación es uno de los métodos más antiguos y económicos para la producción y conservación de alimentos, ya que no es necesario el conocimiento del papel de los microorganismos involucrados (Blandino et al. 2003).

Las bebidas y los alimentos fermentados, se definen como productos que han estado bajo el efecto de microorganismos o enzimas, para la obtención de un cambio bioquímico deseable en el sustrato. Los microorganismos responsables de la fermentación son la microbiota indígena que está presente en el sustrato, o los cultivos puros si se añaden como iniciadores. Aunque las bacterias y los hongos filamentosos participan en la producción de alimentos y bebidas fermentadas, en una fermentación alcohólica las levaduras son los microorganismos predominantes.

Las levaduras contribuyen positivamente en el sabor de los productos fermentados (Henschke 1997). Para ello: (i) utilizan los constituyentes del zumo a fermentar, (ii) producen etanol, (iii) producen enzimas que transforman compuestos neutrales del jugo en compuestos activos en el sabor, (iv) producen metabolitos secundarios (por ejemplo ácidos, alcoholes, ésteres, aldehídos, etc.), y,

tras su muerte, (v) las células se degradan autolíticamente liberando productos de su medio interno (Lambrechts and Pretorius 2000; Fleet 2003).

Hay aproximadamente 700 especies descritas de levaduras (Kurtzman and Fell 2000), y solo algunas de ellas son utilizadas en procesos industriales para la obtención de metabolitos y productos fermentados. Uno de los géneros más utilizados es *Saccharomyces*, y en especial *S. cerevisiae*.

La fermentación vínica es un proceso complejo en el que está involucrada una sucesión de diferentes especies de levaduras. Las especies de levaduras predominantes en las uvas son *Hanseniaspora uvarum* y su forma anamórfica *Kloeckera apiculata*, así como levaduras oxidativas de los géneros *Candida, Pichia, Rhodotorula, Torulospora, Kluyveromyces* y en ocasiones *Hansenula*. Estas levaduras, comúnmente denominadas "no-*Saccharomyces*" crecen al inicio de la fermentación pero, cuando se incrementa la concentración de etanol, son reemplazadas por levaduras *Saccharomyces,* ya que éstas son más tolerantes (Fleet and Heard 1993). Aun cuando existe una gran diversidad de géneros y especies de levaduras en el mosto, *S. cerevisiae* es la especie responsable de la fermentación alcohólica (Pretorius 2000). *S. bayanus var. uvarum* también puede llevar a cabo este proceso.

El proceso de fermentación natural de la sidra también involucra una sucesión de levaduras muy semejante a la vínica y se puede dividir en tres fases secuenciales según las especies de levaduras encontradas. Al inicio del proceso predominan las levaduras *Kloeckera*/*Hanseniaspora uvarum*, pero también se encontró *S. cerevisae*. Cuando se lleva a cabo la fermentación alcohólica *S. cerevisiae* y *S. bayanus* son las especies dominantes, y se impone una u otra dependiendo de la estación en la que se realice la fermentación, debido a la tempertura. En la última fase de maduración sólo se encuentran *Dekkera* y *Brettanomyces* (Morrissey et al. 2004; Coton et al. 2006).

Introducción

La cerveza es una de las bebidas alcohólicas con mayor tasa de producción y consumo en el mundo. Desde su desarrollo, la producción de cerveza ha sido un proceso tradicional basado en la fermentación espontánea o en la inoculación con levaduras procedentes de una fermentación anterior. Aunque existe una gran variedad de cervezas, estas se pueden dividir en dos grandes grupos, de acuerdo con las levaduras implicadas y con las condiciones de fermentación. La cerveza tipo "ale" es producida por levaduras que fermentan en la parte superior de los tanques (levaduras "top-fermenting" o "ale"); la fermentación se lleva a cabo a 20-25ºC y en ocasiones está seguida de un periodo de envejecimiento. La cerveza tipo "lager" es producida por levaduras que fermentan en el fondo de los tanques (levaduras "bottom-fermenting" o "lager"), a temperaturas entre 4 y 15ºC y seguido de un largo periodo de maduración a bajas temperaturas ("lagering"). Las levaduras tipo "ale" pertenecen a la especie *Saccharomyces cerevisiae,* mientras que las levaduras tipo "lager" pertenecen bien a la especie *S. bayanus* o bien a la especie *S. pastorianus* (ambas taxones híbridos entre *Saccharomyces uvarum* y *S. eubayanus* y *S. cerevisiae* y *S. eubayanus* respectivamente) (Kielland-Brandt 1981; Libkind et al. 2011).

Las levaduras también están involucradas, desde hace más de 9000 años (McGovern et al. 2009) en la producción de diversos tipos de bebidas fermentadas tradicionales de África (Ezeronye and Legras 2009), Asia y Latinoamérica (Haard et al. 1999; Jespersen 2003; Aidoo et al. 2006; Lappe-Olivera et al. 2008). Estos productos fermentados juegan un papel muy importante en la dieta de países en desarrollo, ya que proporcionan los nutrientes necesarios para una alimentación adecuada. Sin embargo, el papel que juegan las levaduras en la elaboración de estos productos está poco estudiado. La chicha, el pozol, el mudai, el masato, el tequila, el mezcal y la cachaça son algunos ejemplos de bebidas fermentadas tradicionales que se consumen en Latinoamérica. En Asia encontramos el saké, el tapai, el jnard y el kombuche entre otros; algunas bebidas tradicionales de África son el pito, el dolo, el burukutu, el sekete, el amasi, el nono y el rob. *S. cerevisiae*, y en ocasiones *S.*

paradoxus son las levaduras responsables de la fermentación alcohólica de estos productos, aunque pueden estar presentes otras bacterias, levaduras y hongos (Naumova et al. 2003; Jespersen 2003; Nout 2003; Jespersen et al. 2005; Nielsen et al. 2005; Aidoo et al. 2006; Romano et al. 2006).

5. Características genéticas de las levaduras industriales.

El genoma de *S. cerevisiae* es relativamente pequeño, con un gran número de cromosomas, poco DNA repetitivo y genes mayoritariamente sin intrones. Una cepa haploide contiene aproximadamente 12–13 megabases de DNA nuclear, distribuidos en 16 cromosomas, y estos varían en tamaños desde 250 a 2000 kb (Barre et al. 1992; Pretorius 2000). Sin embargo, el número de bandas observadas en los cariotipos electroforéticos, varía de 14 para cepas de laboratorio a más de 20 en las cepas industriales (Codón et al. 1998), debido tanto a diferencias de tamaño entre cromosomas homólogos (polimorfismo de longitud) como a la presencia de reordenaciones y aneuploidias.

Aunque la mayoría de las cepas de laboratorio, de *S. cerevisiae,* son haploides o diploides; las cepas industriales pueden ser diploides, aneuploides o poliploides (Codón and Benítez 1995). La presencia de aneuploidias o poliploidias puede otorgar ciertas ventajas adaptativas en ambientes muy variables, o puede ser una forma de incrementar la dosis génica de algunos genes importantes para la fermentación (Bakalinsky and Snow 1990).

El elevado grado de polimorfismo que presentan las levaduras vínicas, en la longitud de sus cromosomas, es generado por recombinación heteróloga, mediada por los transposones Ty o por las secuencias repetidas subteloméricas (Bidenne et al. 1992; Rachidi et al. 1999). Esto ocasiona, además, una gran variabilidad en la capacidad de esporulación (entre 0 y 75%) y en la viabilidad de las esporas (de 0 hasta 98%) (Mortimer et al. 1994; Codón and Benítez 1995).Este elevado grado de

polimorfismo también ha permitido una diferenciación de las cepas mediante cariotipos (Guillamón et al. 1996).

La mayoría de las cepas industriales son homotálicas, de manera que tras la esporulación se produce una división mitótica en la que la célula hija cambia de sexo y conjuga con la célula madre, de tipo sexual contrario, por lo que el zigoto diploide formado será homocigoto para todos los loci (autodiploidización). Sin embargo se ha observado un cierto/elevado nivel de heterocigosidad tanto en cepas heterotálicas como homotálicas (Barre et al. 1992; Codón and Benítez 1995).

Además del DNA cromosómico, existen varios elementos genéticos extracromosómicos en el núcleo, el citoplasma y la mitocondria de *S. cerevisiae*.

El plásmido nuclear que se encuentra en *S. cerevisiae* se mantiene estable, presenta una longitud de 2 µm y es circular. Este elemento extracromosómico de 6.3 kb está presente entre 50-100 copias por célula; sin embargo, su función biológica se desconoce.

Saccharomyces contiene el DNA mitocondrial más grande entre las levaduras siendo una molécula circular de 75 kb, rica en A+T, que presenta grandes regiones intergénicas e intrones autocatalíticos.

En general las cepas de *Saccharomyces,* que se utilizan industrialmente, son organismos muy especializados, que se han adaptado a diferentes ambientes o nichos ecológicos proporcionados por las diferentes actividades humanas. Este proceso, descrito como "domesticación", es responsable de las características genéticas particulares de las levaduras industriales.

6. Mecanismos de adaptación a condiciones industriales.

El hombre ha seleccionado inconscientemente cepas de *Saccharomyces* para la elaboración de diferentes productos tales como el pan, el vino, la cerveza, entre otros, mediante el proceso de fermentación. Las diversas cepas de levaduras presentes en cada tipo de fermentación se han ido seleccionado de acuerdo con las diferentes características requeridas para una fermentación eficiente. Por ello, la presión selectiva ha favorecido que las cepas de *Saccharomyces* mantengan un elevado metabolismo fermentativo (Pretorius 2000).

En un ambiente fermentativo como el de la producción de vino y otras bebidas alcohólicas, se dan una serie de factores que producen estrés para las células de levaduras (Attfield 1997; Bauer and Pretorius 2000; Querol et al. 2003b) y que afectan significativamente a la capacidad de supervivencia de estas (Fleet and Heard 1993). Entre ellos se encuentra el estrés osmótico, que es ocasionado por una alta concentración de azúcares al inicio de la fermentación alcohólica. En esta etapa de la fermentación, también podemos hablar de otra condición de crecimiento no óptima como es el bajo pH del medio. Al final de la fermentación, la producción de etanol y el agotamiento de algunos nutrientes, como la fuente de nitrógeno, de carbono y las vitaminas, son los principales factores que ocasionan estrés a las células. La temperatura también puede ser considerada como otro factor de estrés (Cardona et al. 2007) ya que, en procesos donde no se dé un control de la temperatura, esta puede elevarse hasta valores superiores a 30ºC.

S. cerevisiae es la principal especie encontrada entre las levaduras fermentativas. Sin embargo, el hecho de que otras especies del género *Saccharomyces,* como *S. bayanus, S. uvarum* o *S. paradoxus* también hayan sido aisladas de fermentaciones hace pensar que el proceso de "domesticación", o adaptación a las condiciones encontradas en los procesos fermentativos, consecuencia de la actividad humana, ocurrió varias veces (Fay and Benavides 2005; Legras et al. 2007).

Algunos trabajos de investigación se han dirigido a estudiar cuales pudieron ser los mecanismos moleculares involucrados en la adaptación de las levaduras a procesos industriales, y el cambio de las características genómicas de las levaduras que han sido seleccionadas a través de billones de generaciones (Querol *et al.* 2003a; Querol *et al.* 2003b).

Dentro de los posibles mecanismos de adaptación descritos para levaduras, cabe destacar los siguientes:

6.1. Renovación genómica.

Mortimer y colaboradores (1994) analizaron poblaciones naturales de *S. cerevisiae* de fermentaciones espontáneas de vinos y encontraron una diversidad genética muy alta. Sin embargo, todas las cepas eran homocigotas para los genes analizados y presentaban una alta fertilidad, ello les llevó a proponer un proceso de evolución para las levaduras vínicas denominado "genome renewal" o renovación genómica.

Para que este fenómeno ocurra, las cepas deben ser homotálicas, es decir, deben ser capaces de cambiar de sexo y conjugar con una célula proveniente de la misma espora. De este modo se produce un diploide, homocigoto para todos los genes, a excepción del locus *MAT*. Con una propagación asexual, continua, las cepas acumulan mutaciones recesivas en heterocigosis, pero pueden cambiar a diploides completamente homocigotos mediante la capacidad de esporulación y el cambio de sexo de las esporas haploides. Esto hace que la selección pueda actuar sobre la fase diploide eliminando genes deletéreos recesivos y/o fijando los alelos beneficiosos o ventajosos; así se promueve la adaptación de las levaduras a las condiciones ambientales.

Sin embargo, se ha demostrado que la homocigosis también es posible mediante recombinación mitótica o conversión génica durante el crecimiento vegetativo (Puig *et al.* 2000b).

6.2. Duplicaciones.

La duplicación es la fuente más importante para la generación de nuevos genes. Esta se puede producir en un solo gen o grupos de genes adyacentes (Puig *et al.* 2000a), en cromosomas, originando aneuploidias (Hughes *et al.* 2000), o en todo el genoma, generando poliploidía (Wolfe and Shields 1997).

La redundancia de genes se mantiene si proporciona una ventaja evolutiva, tal como un incremento en la dosis génica, o si uno de los genes duplicados mantiene su función y el otro adquiere mutaciones que generan una función ventajosa (neo-funcionalización) (Force et al. 1999; Lynch and Force 2000). Sin embargo, esto es muy poco probable (Wagner 1998), pues al acumular mutaciones lo más probable es que se de una pérdida de función convirtiéndose en un pseudogen (pseudogenización o no-funcionalización) (Wagner 2000). Para que los dos miembros de un par complementario de genes se mantengan, se propuso un proceso alternativo, la subfuncionalización (Force *et al.* 1999). Este proceso implica que los dos miembros de cada uno de los duplicados adquieran mutaciones degenerativas, de pérdida de función, que sean complementarias. Así, los dos duplicados se deben mantener para realizar la función que presentaba un solo gen ancestral.

6.2.1. Duplicaciones de un gen o de segmentos génicos.

La duplicación génica se puede dar en un gen, o en un grupo de genes adyacentes, lo que se llama una duplicación segmental. En el genoma de *S. cerevisiae* se han identificado 265 familias multigénicas o grupos de genes idénticos o semejantes. Incluso se ha detectado una familia con 108 miembros (Llorente *et al.* 2000), lo que indica que se han producido una serie de duplicaciones génicas sucesivas.

Existen varios mecanismos propuestos para la generación de este tipo de duplicaciones en tándem o segmentales. El punto más crítico es la generación de la

primera duplicación, ya que es necesario la presencia de secuencias nucleotídicas iguales que flanqueen la región duplicada. La recombinación ectópica entre cromosomas homólogos o el intercambio desigual de cromátidas hermanas en unas secuencias flanqueantes dará lugar a la primera duplicación. Las siguientes duplicaciones pueden ocurrir por una recombinación entre las regiones duplicadas.

Muchos de los genes duplicados mantienen casi idéntica o idéntica la secuencia entre ellos, lo que indica una forma de conservar la función génica, y con ello, un incremento en la dosis génica. La evolución concertada en los miembros de una familia génica se mantiene por una recombinación ectópica y conversión génica (Li 1997).

La mayoría de las duplicaciones de un gen y las familias multigénicas corresponden a duplicaciones de repeticiones directas en tándem; en general, las familias multigénicas están localizadas en regiones subteloméricas, pero hay algunas dispersas en el genoma. Como ejemplo de este tipo de familias multigénicas son los genes involucrados en la asimilación y utilización de azúcares: *MEL, SUC, MGL* y *MAL*. La mayoría de las familias multigénicas están involucradas en el metabolismo secundario, por lo que los genes no son esenciales pero tienen un papel importante en la adaptación a nuevos ambientes (Harrison and Gerstein 2002).

6.2.2. Duplicación cromosómica: aneuploidías.

Otro mecanismo que puede generar nuevos genes es la duplicación cromosómica, que da lugar a aneuploidías. Este mecanismo proporciona una fuente potencial de nuevos genes, pero su consecuencia más importante e inmediata es el aumento de la dosis génica.

Las aneuploidías se producen por una segregación cromosómica errónea durante la meiosis o la mitosis y generan un incremento en el número de genes. Se ha postulado que las aneuploidías podrían conferir ventajas en la adaptación a ambientes externos variables, debido a un aumento de copias de genes

beneficiosos o a la acción protectora de ese "exceso" de copias frente a mutaciones deletéreas (Bakalinsky and Snow 1990; Guijo et al. 1997; Salmon 1997). En general las cepas vínicas de *S. cerevisiae* son aneuploides, con dos, tres y en menor frecuencia cuatro copias de cromosomas (disomías, trisomías y tetrasomías) (Bakalinsky and Snow 1990).

6.2.3. Duplicación genómica: Poliploidía.

El suceso más importante en la evolución del linaje *Saccharomyces* fue la duplicación genómica que aconteció en uno de sus ancestros (Wolfe and Shields 1997). La duplicación genómica generó un incremento del flujo glicolítico, causante del efecto Crabtree, debido en general a la duplicación de los genes de las enzimas de la glicólisis, y particularmente los de los transportadores de hexosas cuyo efecto es el más importante (Conant and Wolfe 2007). La mayor capacidad de consumo de hexosas y producción en condiciones aeróbicas de etanol, tóxico para muchos microorganismos, debió suponer una gran ventaja competitiva para el ancestro de *Saccharomyces*, como lo sigue siendo en la actualidad.

Esta hipótesis permite explicar la ventaja inicial que pudo suponer la repentina duplicación del genoma en el ancestro de *Saccharomyces* que posibilitó un subsiguiente reajuste de los niveles de expresión y la consecuente aparición de sistemas más eficientes de respuesta y regulación del metabolismo respiro-fermentativo (Wolfe 2004).

En el genoma de *S. cerevisiae* se ha observado la presencia de 376 pares de genes distribuidos en 55 regiones duplicadas, por lo que se ha sugerido que un suceso de duplicación genómica o poliploidización tuvo lugar en un ancestro de *S. cerevisiae* (Wolfe and Shields 1997). Esta duplicación del genoma ocasionó la adquisición rápida de una copia extra de cada gen, pero el 88% de los genes adquiridos se perdieron por el proceso de pseudogenización. Cerca de 460 pares de genes se mantienen en el genoma de *S. cerevisiae*, pero, aproximadamente 60 pares

de genes presentan signos de conversión génica, poco más de 100 pares de genes muestran neo-funcionalización, y el resto, probablemente, se mantuvieron por subfuncionalización (Kellis et al. 2004b).

Los mecanismos que pueden generar la poliploidización en levaduras son: a) un error durante la meiosis, que genere esporas diploides, y la conjugación entre estas células, b) un error durante la mitosis en organismos unicelulares, c) apareamiento raro ("rare-mating") entre dos células diploides de la misma especie, que se han convertido en competentes para cruzarse (Kellis et al. 2004a). En estos casos se generan individuos autopoliploides.

Puede darse la generación de levaduras alopoliploides, en donde los núcleos contienen pares de cromosomas homeólogos, es decir, que provienen de especies diferentes por varias formas: a) por una hibridación interespecifica mediante la conjugación de esporas de diferentes especies y una duplicación genómica por errores de la mitosis o meiosis, y b) por un "rare-mating" entre dos cepas diploides de diferentes especies pero que son competentes para cruzarse (de Barros Lopes *et al.* 2002).

La poliploidía, y particularmente la alopoliploidía, pueden ser mecanismos importantes para aumentar la flexibilidad genética y conferir capacidad de adaptación a diversas condiciones ambientales. Además, pueden ser una forma de incrementar la dosis de algunos genes importantes para la fermentación (Conant and Wolfe 2007).

La poliploidización proporcionó las bases evolutivas para que las levaduras obtuvieran genes con nuevas funciones al competir por colonizar los substratos ricos en azúcares proporcionados por las plantas que producen frutos. Un metabolismo fermentativo incluso en presencia de oxígeno (efecto "crabtree"), con un crecimiento rápido y la producción de un compuesto tóxico, el etanol, dio ventajas competitivas al ancestro de *S. cerevisiae (Conant and Wolfe 2007)*.

6.3. Reordenaciones cromosómicas

Se producen debido a la recombinación entre pequeñas secuencias (regiones de microhomología) que se encuentran presentes en genes no homólogos. Este proceso puede generar nuevos genes quiméricos con una función diferente o puede cambiar el patrón de expresión de estos genes. Las reordenaciones cromosómicas pueden estar implicadas en los mecanismos de adaptación de las levaduras a condiciones industriales, sobre todo, en el caso de que genes importantes para el proceso industrial que la levadura lleve a cabo se "sitúen", tras la reordenación cromosómica, bajo el efecto de promotores más fuertes que el que tenían.

Por ejemplo, en la cepa vínica T73, existe un aumento de la expresión del gen *SSU1*, transportador del anhídrido sulfuroso (antiséptico ampliamente utilizado en enología desde tiempo inmemorial) del interior celular al exterior. Este fenómeno se debe a una translocación entre los cromosomas VIII y XVI que ha situado la región codificante de *SSU1* bajo el control del promotor fuerte del gen *ECM34*, lo que lleva a un incremento de su expresión y, por tanto, confiere una mayor resistencia al sulfuroso a las levaduras portadoras de esa translocación (Pérez-Ortín *et al.* 2002). Esta translocación es muy frecuente, tanto en homocigosis como en heterocigosis entre las levaduras vínicas, pero está ausente en otros tipos de levaduras, tanto panaderas como cerveceras o de laboratorio, debido a que la utilización de sulfuroso como antiséptico sólo se da en la producción de vinos.

6.4. Formación de híbridos

En el caso del género *Saccharomyces*, uno de los mecanismos más interesantes observados en la adaptación de las levaduras a procesos industriales es la formación de híbridos entre especies de este grupo (de Barros Lopes *et al.* 2002).

Las especies del género *Saccharomyces* están presentes en el mismo nicho ecológico y pueden verse implicadas en la formación de híbridos. Las células haploides o las esporas de estas especies son capaces de cruzarse entre sí y formar

híbridos viables, aunque estériles, que se mantienen mediante reproducción asexual. La esterilidad de los híbridos puede ser superada mediante una duplicación del número cromosómico, lo que resulta en un alotetraploide (Naumov *et al.* 2000b), o al recuperar la euploidia por una diploidización homotálica de las pocas esporas viables, lo que resulta en un homoploide (Greig *et al.* 2002a)

Los híbridos están menos adaptados que sus parentales a condiciones ambientales específicas, pero pueden adaptarse mejor a condiciones fluctuantes intermedias, lo que les proporciona una ventaja selectiva (Greig *et al.* 2002b). Por otro lado, los híbridos adquieren propiedades fisiológicas de ambos parentales, por ejemplo, la tolerancia al alcohol y a la glucosa de *S. cerevisiae*, la tolerancia a bajas temperaturas de *S. kudriavzevii* o la mayor producción de compuestos aromáticos de *S. bayanus* (Masneuf *et al.* 1998).

Los híbridos interespecíficos del género *Saccharomyces* están presentes en diferentes procesos fermentativos. El ejemplo mejor descrito de una levadura híbrida es la levadura productora de cerveza lager *S. pastorianus* (sin. *S. carlsbergensis*). Esta levadura es un híbrido alotetraploide parcial entre una levadura de la especie *S. cerevisiae* y otra de la especie *S. eubayanus* (Hansen and Kielland-Brandt 1994; Nguyen et al. 2000; Casaregola et al. 2001; Nakao et al. 2009; Libkind et al. 2011). Prácticamente la totalidad de los cromosomas parentales se han preservado en *S. pastorianus* (Hansen and Kielland-Brandt 1994; Nakao et al. 2009), mientras que su genoma mitocondrial (mtDNA) procede del parental no-*S. cerevisiae* (Groth *et al.* 1999). También se han encontrado aneuploidías en diferentes aislados de *S. pastorianus* (Hansen and Kielland-Brandt 1994; Nguyen et al. 2000; Casaregola et al. 2001) y muchos de ellos presentan cromosomas quiméricos generados por recombinación entre cromosomas homeólogos de distinto parental (Bond *et al.* 2004).

Otros híbridos naturales identificados como tales han sido aislados de vino de Italia, como la cepa S6U, que presenta genes de *S. cerevisiae* y de *S. bayanus*; y el

triple híbrido aislado de sidra artesanal de la Bretaña francesa (cepa CID1), con dos copias del gen nuclear *MET2*, una de *S. cerevisiae* y la otra de *S. bayanus* (Masneuf *et al.* 1998), y el genoma mitocondrial de una tercera especie *S. kudriavzevii* (Groth *et al.* 1999). Recientemente se han descrito nuevos híbridos entre las especies *S. kudriavzevii* y *S. cerevisiae*, tanto en vinos de Suiza (González *et al.* 2006), Austria (Lopandic *et al.* 2007) y España (Peris *et al.* 2012) como entre cepas cerveceras de Bélgica, Inglaterra, Alemania y Nueva Zelanda (González *et al.* 2008), así como un triple hibrido *S. bayanus* x *S. cerevisiae* x *S. kudriavzevii* vínico suizo (González *et al.* 2006).

La cepa tipo de *S. bayanus* ha sido descrita como poseedora de genoma nuclear tanto de *S. cerevisiae* como de *S. uvarum* y *S. eubayanus* (Nguyen *et al.* 2000; de Barros Lopes *et al.* 2002; Libkind *et al.* 2011), aunque no está claro que se trate de un triple híbrido, ya que las secuencias de *S. cerevisiae* encontradas son subteloméricas y podrían estar allí debido a una introgresión (Naumova *et al.* 2011). Se postula que la introgresión se debe a un suceso de hibridación inestable entre dos cepas. Casos similares se han observado entre *S. cerevisiae* y *S. paradoxus* (Liti *et al.* 2006; Wei *et al.* 2007; Doniger *et al.* 2008).

La gran diversidad de híbridos dentro del género *Saccharomyces* encontrados en diversos orígenes y hábitats, indica que es más frecuente de lo esperado (Barrio *et al.* 2006). Los mecanismos propuestos para explicar la generación de híbridos interespecíficos entre las especies de levaduras del género *Saccharomyces* son, bien la conjugación de esporas de distintas especies en el tracto digestivo de invertebrados (insectos y gasterópodos) que se alimentan de levaduras, ya que producen enzimas que hidrolizan las ascas, lo que permitiría la liberación de las ascosporas y favorecería la hibridación intra- e interespecífica (Pulvirenti et al. 2002a); o bien un suceso de "rare mating" entre cepas diploides αα y aa de distintas especies del género *Saccharomyces* (Pulvirenti et al. 2002b).

7. El vino y las nuevas demandas en el sector enológico.

7.1. Elaboración del vino.

La fermentación del mosto es un proceso microbiológico complejo que implica interacciones entre levaduras, bacterias y hongos filamentosos. Tradicionalmente se produce por la fermentación natural causada por el desarrollo de levaduras procedentes de la uva y del ambiente de la bodega (maquinaria, fermentadores, etc.). Las levaduras apiculadas de los géneros *Kloeckera* y *Hanseniaspora* (especies mayoritarias sobre la superficie de la uva, en porcentajes del 50 al 75% del total de la población levaduriforme) y las levaduras oxidativas de los géneros *Candida*, *Cryptococcus*, *Kluyveromyces*, *Metchnikowia*, *Zygosaccharomyces*, *Pichia* y *Rhodotorula* crecen en las primeras fases de la fermentación. Sin embargo, las especies fermentativas del género *Saccharomyces* se encuentran en proporciones extremadamente bajas en estas fases por ser muy extraña su presencia en el suelo o en los racimos de uvas sanas (Frezier and Dubourdieu 1992; Martini et al. 1996). Debido al incremento de la concentración de alcohol y a las condiciones de anaerobiosis, *Saccharomyces* pasa a ser el género dominante y el responsable de la fermentación, ya que el resto de las especies posee una baja tolerancia al etanol y son incapaces de fermentar todos los azúcares presentes en el mosto (Beltran *et al.* 2002).

La microflora presente en la superficie de la uva se ve afectada por un gran número de factores que influyen en la proporción de las diferentes especies. Entre estos factores se incluyen la temperatura, la pluviosidad y otras influencias climáticas, el grado de madurez de la cosecha, el uso de funguicidas, el daño físico debido a hongos, insectos, etc. y la variedad de uva (Rosini *et al.* 1982; Bureau *et al.* 1982; Martínez *et al.* 1989; Querol *et al.* 1990; Longo *et al.* 1991). Esto es importante, ya que variaciones en la flora inicial pueden influir en la calidad del vino y dar lugar a

cambios en la acidez volátil y a sabores y olores desagradables (Querol and Ramón 1996).

Aunque pueden producirse diferencias en la diversidad microbiana del mosto inicial, ya no sólo entre regiones vitivinícolas distintas, sino también dentro de la misma bodega en diferentes vendimias, desde un punto de vista microbiológico, la variabilidad en la flora levaduriforme de los mostos puede solventarse adicionando en cada campaña de vendimia un inóculo microbiano que, al ser mayoritario, normalice la flora inicial y, de esta forma, dé lugar a una fermentación homogénea año tras año. Esta práctica fue establecida en la década de los 70.

La utilización de levaduras seleccionadas produce fermentaciones controladas y, como consecuencia de esta práctica, el vino mantiene sus características sensoriales año tras año (Lafon-Lafourcade 1983). La utilización de levaduras seleccionadas también puede evitar alteraciones químicas y microbiológicas en las primeras fases de la fermentación, evitar anomalías, como paradas espontáneas, o mejorar la composición química e influir en la calidad, tanto gustativa como aromática del vino (Cuinier 1986).

El mosto de uva es un medio en el cual todos los nutrientes se encuentran presentes desde un principio, y su concentración disminuye conforme son consumidos por la levadura, lo que puede provocar que el crecimiento pueda estar condicionado por la concentración de uno o varios nutrientes. Además de esto, la diferente composición de los mostos, además de ser crucial para las características del producto final, condiciona la evolución del crecimiento de las levaduras. En el mosto podemos encontrar azúcares, ácidos orgánicos, compuestos nitrogenados, polifenoles, sales minerales y también lípidos, todos ellos a concentraciones muy diferentes. El componente más abundante en el mosto, exceptuando el agua es el azúcar, más concretamente los monosacáridos. Los monosacáridos mayoritarios son la glucosa y fructosa que se encuentran en cantidades equimolares (Ough 1992) y su concentración total generalmente es de entre 170 y 220 g/l (Ribéreau-Gayon *et*

al. 2000). Con estos valores, la fermentación discurre con normalidad, aunque en mostos con más de 200 g/l se observa una ralentización pudiéndose producir la inhibición del crecimiento por encima de los 250 g/l (Nishino *et al.* 1985). Los ácidos orgánicos son el segundo grupo de compuestos en abundancia tras los azúcares, estando su concentración comprendida entre los 9 y los 27 g/l (Ough 1992). Los ácidos tartárico y málico son los mayoritarios, suponiendo el 90% de la acidez fija, mientras que el cítrico y el ascórbico se encuentran en menor cantidad. El contenido en nitrógeno es generalmente el factor más importante desde el punto de vista de la composición del mosto, ya que nutricionalmente, suele ser limitante para el crecimiento de *S. cerevisiae* (Schmidt et al. 2011; Gutiérrez et al. 2012).

La composición en fuentes nitrogenadas del mosto depende de numerosos factores, como la variedad de uva, la infección de la misma por *Botritis cinerea* (que elimina gran parte de los nutrientes asimilables por *S. cerevisiae*), el momento de la cosecha, los hábitos de fertilización, la suplementación o no en la bodega y la intensidad de la clarificación de los mostos, especialmente los blancos (Lagunas 1986). Esta variación en cuanto a la cantidad y forma en que se encuentra el nitrógeno en el mosto condiciona el crecimiento celular, la velocidad de fermentación y la tolerancia al etanol.

Los compuestos nitrogenados se pueden desglosar de la siguiente manera: amonio (3-10% del total), aminoácidos (25- 30%), polipéptidos (25-40%) y proteínas (5-10%). Es importante destacar que *S. cerevisiae* es incapaz de asimilar las fuentes de nitrógeno inorgánico del medio, como nitratos y nitritos, así como tampoco las proteínas y polipéptidos, ya que carece de sistemas de digestión extracelular de este tipo de compuestos, por lo que el crecimiento depende fundamentalmente de la cantidad de amonio y aminoácidos, las fuentes preferidas de nitrógeno (Ough 1992).

La fermentación completa de un mosto por *S. cerevisiae* conduce a la producción de 8 a 15% (v/v) de etanol, y otros co-productos fermentativos tales

como el glicerol (6-8 g/l), ácidos orgánicos como el acetato, el succinato y el piruvato en cantidades menores, y alcoholes superiores y ésteres (Barre *et al*., 2000). La fermentación vínica es distinta de la que tiene lugar en otros procesos industriales, como la elaboración de la cerveza, debido a que la elevada concentración de azúcares produce niveles de etanol inhibidores del crecimiento, capaces incluso de afectar a la viabilidad celular.

Al inocular un mosto con 10^6 células/ml, la fermentación comienza rápidamente. Tras un corto periodo de latencia se inicia el ciclo de crecimiento, que consta de tres etapas. La primera es una fase de crecimiento limitado que dura entre 2 y 5 días y produce un aumento de la población hasta 10^7-10^8 células/ml. En esta fase, la velocidad de crecimiento es máxima y suele consumirse entre un tercio y la mitad de la cantidad inicial de azúcares (Lafon-Lafourcade 1983; Ribéreau-Gayon *et al.* 2000). A continuación el crecimiento entra en una fase quasi-estacionaria que dura alrededor de 8 días y durante la cual no se produce aumento del número de células. Sin embargo, las células son metabólicamente activas y la velocidad de fermentación sigue manteniéndose en su valor máximo. Finalmente el cultivo entra en una fase de muerte de hasta varias semanas y durante la misma, el número de células viables decrece progresivamente hasta aproximadamente 10^5 células/ml. La pérdida de viabilidad va acompañada de una disminución de la velocidad de fermentación debida no únicamente a la disminución del número de células viables, sino también a la inhibición de la actividad metabólica de las células no proliferativas. Con la pérdida de viabilidad celular e inmediatamente a la muerte de las levaduras, se produce la autodegradación enzimática de los constituyentes celulares, fenómeno que se conoce como autolisis (Farrer 1946).

Como consecuencia de la autolisis de las levaduras, parte de los productos de la hidrólisis de las macromoléculas intracelulares y de la pared, generados por acción de los enzimas hidrolíticos de la propia célula, son liberados al vino modificando sus propiedades y otorgando algunas de las características deseadas al

producto final. Parte de esta liberación también se produce a lo largo de la fermentación. Entre los compuestos liberados encontramos proteínas, ésteres, polisacáridos y manoproteínas de la pared celular (Barre *et al*., 2000).

7.2. Demandas en el sector enológico.

Toda levadura comercial debe poseer una buena capacidad fermentativa y producir vinos con cierta calidad organoléptica. En la industria, cada vez más, se buscan aquellas levaduras que posean, además de estas, otras características fisiológicas que las hagan interesantes desde el punto de visa enológico, más aún si permiten resolver alguna de las exigencias actuales de las bodegas.

Una de las principales demandas del sector vitivinícola está asociada a resolver los problemas planteados con el cambio climático. Dicho cambio puede producir consecuencias negativas tales como un desfase temporal entre la madurez fenólica y madurez fisiológica de la uva. Para alcanzar la madurez fenólica y evitar una astringencia excesiva, se vendimia con uvas con alto contenido en azúcar y se obtienen vinos con elevado contenido en alcohol. Si se adelanta la vendimia para obtener una menor concentración de azúcares, los vinos tendrán mayor contenido en polifenoles produciendo una excesiva astringencia en los vinos. El disponer de levaduras con un menor rendimiento en etanol, o que incrementen el contenido en glicerol en los vinos pueden ser buenas alternativas para resolver este tipo de problemas.

Además de las características fisiológicas mencionadas, las levaduras también deben adaptarse a las actuales prácticas enológicas. Entre las prácticas más comunes encontramos las fermentaciones a bajas temperaturas, cuya finalidad es producir vinos altamente aromáticos. Otra de las prácticas en auge es la adición de manoproteínas al vino, debido a las numerosas propiedades beneficiosas que estas aportan.

7.2.1. Reducción del grado alcohólico e incremento de la concentración de glicerol

El alcohol más abundante en el vino es el etanol, este juega un papel importante en la estabilidad y envejecimiento del vino, así como en formar parte de las características sensoriales del mismo. El etanol actúa como disolvente de taninos y pigmentos, y puede influir en la concentración de ciertos antioxidantes (flavonoides) extraídos durante el proceso de vinificación (Soleas *et al.* 1997). Pese a este papel beneficioso, altas concentraciones de etanol pueden afectar negativamente a las propiedades sensoriales del vino (Pretorius and Hoj 2005).

En los últimos 15 años, el contenido en etanol de los vinos Españoles, Franceses e Italianos ha aumentado cerca del 3% (del 11-12% v/v al 13-14% de etanol); este aumento se debe, principalmente, al calentamiento global, al cultivo de variedades de uva más dulces y a la recogida tardía de las uvas. De forma opuesta, los consumidores demandan, cada vez más, vinos con un menor contenido en etanol.

Aunque la recogida temprana de la uva o el cultivo en regiones de clima frío pueden limitar el contenido en etanol del vino (Salamon 2006), se han desarrollado varias técnicas para disminuir el contenido de este (Michnick et al. 1997; Pickering 2000; de Barros Lopes 2002; Malherbe et al. 2003), adaptando el vino a las exigencias del mercado. Estas se pueden dividir en tres tendencias: reducción del etanol, reducción de los azúcares fermentables y utilización de cepas con un menor rendimiento en etanol.

La reducción del contenido en etanol de los vinos se ha abordado con diferentes técnicas, como son la destilación, la evaporación, los procesos a través de membrana (diálisis, ósmosis reversa y contactores de membrana) (Bui et al. 1986; Gómez-Plaza et al. 1999; Takács et al. 2007; Diban et al. 2008) (Massot et al. 2008; Labanda et al. 2009; Varavuth et al. 2009; Catarino and Mendes 2011; Diban et

al. 2013), la adsorción en resinas o geles y la extracción mediante solventes orgánicos o dióxido de carbono supercrítico (Pickering 2000; Ruiz-Rodriguez et al. 2010). De todas estas técnicas, la ósmosis reversa (Bui et al. 1986; Massot et al. 2008; Varavuth et al. 2009) y la destilación en vacio mediante columna de cono giratorio (destilación SCC) (Diban et al. 2008), son las más utilizadas. Pese a su utilización, son técnicas difíciles de llevar a cabo y costosas económicamente.

La segunda de las alternativas trata de eliminar parte de los azúcares fermentables presentes en el mosto utilizando enzimas exógenos como la glucosa oxidasa (GOX). El uso de este enzima fue introducido por Villettaz (1987; 1991) y Heresztyn (1986). Este enzima metaboliza la glucosa en ácido glucónico (Pickering 2000), liberando peróxido de hidrógeno, el cual tiene un efecto antimicrobiano (Malherbe et al. 2003), confiriendo mayores beneficios al uso de esta enzima. El uso de GOX ha sido ampliamente estudiado (Malherbe et al. 2003) y actualmente se presentan dos alternativas a su utilización, la adición del enzima a los mostos o su expresión, mediante ingeniería genética, en levaduras vínicas. También se pueden eliminar parte de los azúcares fermentables mediante la nanofiltración de los vinos (García-Martín et al. 2010).

Por último, la otra tendencia existente para reducir el contenido en etanol de los vinos se basa en la utilización de cepas con un menor rendimiento en etanol. Estas cepas pueden ser tanto cepas naturales que producen, por sí mismas, poca cantidad de etanol, como modificadas con técnicas de ingeniería genética para tal fin (Pickering 2000). La modificación genética de estas cepas ha llevado dos vías, desviar la síntesis de etanol hacia la producción de glicerol sobreexpresando genes como GPD1 (Nevoigt and Stahl 1997; Michnick et al. 1997; Remize et al. 1999; Nevoigt 2008), o convertir el piruvato en etanol y lactato, simultáneamente, al expresar el gen de la lactato deshidrogenasa en estas levaduras (Dequin and Barre 1994; Remize et al. 1999). Las cepas naturales que producen menor rendimiento en etanol suelen producir una mayor cantidad de glicerol, y son seleccionadas

atendiendo a ambas propiedades. En alguno de los casos, este bajo rendimiento en la producción de etanol puede deberse a que se trate de levaduras glicolíticamente deficientes (Loira et al. 2012). Recientemente, y siguiendo esta línea, se ha propuesto la utilización de levaduras no-Saccharomyces que reducirían los niveles de este alcohol mediante su respiración (Gonzalez et al. 2013; Quirós et al. 2014). Por ejemplo, cepas de la especie *Metschnikowia pulcherrima* y dos especies del género *Kluyveromyces* mostraron buenos resultados, mientras que cepas pertenecientes al género *Saccharomyces*, utilizadas de este modo, producían elevados niveles de ácido acético (Quirós et al. 2014). El uso secuencial de levaduras no-Saccharomyces y Saccharomyces ha dado buenos resultados en la producción de vinos chardonnay y shiraz (Contreras et al. 2014).

En una fermentación vínica, detrás del etanol y el dióxido de carbono, el glicerol es, cuantitativamente, el producto más importante de la fermentación. Debido a que no es una sustancia volátil, no contribuye al aroma del vino, pero sí que contribuye, de forma indirecta a su calidad sensorial. Su sobreproducción da lugar a vinos con una mayor suavidad en boca y una mayor complejidad. Debido al efecto favorable del glicerol en la calidad del vino, se ha enfatizado en los beneficios de un incremento en la producción de glicerol a la hora de mejorar las características sensoriales de vinos que carecen de cuerpo (Pretorius and Westhuizen 1991; Degré 1993; Barre et al. 1993).

La ruta de síntesis del glicerol implica un desvío del flujo glicolítico, que acabaría en la producción de etanol, hacia la producción de glicerol. Su síntesis implica la actuación secuencial de dos enzimas, llamadas GPD1 y GPD2, y la conversión del exceso de NADH en NAD^+; este proceso no produce ATP. El glicerol generado no es consumido, ya que los enzimas implicados en su asimilación están reprimidos en condiciones anaeróbicas (Pavlik et al 1993; Ronnow and Kielland-Brandt 1993). Como consecuencia de la sobreproducción de glicerol, la formación de etanol disminuye y aumenta la formación de otros metabolitos, como el acetato,

el piruvato, la acetoína…, alguno de ellos no deseado en el vino (Michnick *et al.* 1997; Pretorius 2000).

El contenido en glicerol de un vino se ve afectado tanto por parámetros que afecten al crecimiento de las levaduras como por factores ambientales, entre otros cabe destacar la influencia de la cepa, el nivel de inoculación, las concentraciones de sulfito, azúcar y nitrógeno, el pH, la aireación, la temperatura y la variedad de uva (Ribéreau-Gayon *et al.* 2000; Carrasco *et al.* 2001).

En los últimos años hay una demanda creciente de vinos con una mayor cantidad de glicerol, debido tanto a los efectos positivos de este sobre las propiedades sensoriales del vino como a que la producción de este metabolito está ligada a una reducción de la producción de etanol. Para este propósito, se han seguido tres estrategias distintas. La primera de ellas consiste en la modificación de las condiciones de fermentación, como la aireación, la temperatura, el pH, la concentración de azúcar y el contenido en sulfitos (Gardner et al. 1993; Remize et al. 2000; Yalcin and Ozbas 2008).

La segunda estrategia ha tratado de mejorar las cepas vínicas mediante técnicas clásicas de cruce, en condiciones de laboratorio (Rep et al. 1999), así como seleccionar las cepas que produzcan mayor cantidad de glicerol. La caracterización enológica de varias cepas híbridas *S. cerevisiae x S. kudriavzevii*, aisladas de fermentaciones vínicas, ha abierto nuevas alternativas, ya que poseen características interesantes que se aproximan a las nuevas tendencias en enología (González *et al.* 2007); de hecho, la especie *S. kudriavzevii* produce mayor concentración de glicerol y menor de etanol que las cepas de la especie *S. cerevisiae* (Arroyo-López *et al.* 2010). La especie *S. bayanus var uvarum*, que es la responsable de la fermentación de algunos vinos, también produce más glicerol y menos etanol (Gamero et al. 2013). Esto hace interesante la búsqueda y selección de nuevas cepas, tanto pertenecientes a *S. bayanus* como híbridas entre *S. cerevisiae* y alguna de estas dos especies. Incluso se ha utilizado la evolución adaptativa como

estrategia para desarrollar levaduras que produzcan mayores cantidades de glicerol (Kutyna et al. 2012; Tilloy et al. 2014).

La tercera aproximación ha sido la manipulación molecular de las levaduras, desviando la ruta de producción de etanol, como se ha comentado antes (Michnick et al. 1997; Remize et al. 1999; Remize et al. 2000; Remize et al. 2001; Remize et al. 2003).

7.2.2. Fermentaciones a bajas temperaturas.

La temperatura es uno de los parámetros más importantes para el desarrollo de la fermentación alcohólica. Esta puede afectar tanto a la cinética del proceso (duración, tasa y ecología de la fermentación) (Sharf and Margalith 1983; Torija et al. 2003a) como a la calidad final del vino (producción de metabolitos secundarios (Beltran *et al.* 2002; Novo *et al.* 2003; Torija *et al.* 2003b; Gamero *et al.* 2013).

Actualmente existe un interés creciente en fermentar a bajas temperaturas (10-15°C); principalmente en la producción de vinos blancos y rosados, sobre todo, en aquellos de gran potencial aromático, ya que estas temperaturas permiten que se retengan gran parte de los aromas primarios y secundarios, los cuales, al ser volátiles a temperatura ambiente, se escapan con facilidad durante la fermentación alcohólica a mayores temperaturas.

A pesar de las ventajas de este tipo de fermentación, las bajas temperaturas dificultan la realización de la fermentación alcohólica; sus efectos sobre esta son (Beltran *et al.* 2007):

- Se retrasa varios días el inicio de la fermentación (fase de latencia prolongada).
- Disminuye la velocidad del consumo de azúcares (fermentaciones más largas).

- Mayor riesgo de paradas de fermentación.

- Modificación de las poblaciones de microorganismos (a esas bajas temperaturas *Kloekera apiculata* domina la fermentación durante más tiempo, lo que dificulta la imposición de *Saccharomyces cerevisiae*); esto podría llevar a la producción de sustancias volátiles indeseables por parte de las levaduras no-*Saccharomyces* (Herraiz *et al.* 1990).

- Cambio en las actividades metabólicas de los microorganismos (lo que implica una modificación en la producción de metabolitos secundarios, debido a las necesidades adaptativas).

La utilización de levadura seca activa (LSA) asegura la reproducibilidad del producto final, reduce la fase de latencia y la duración de la fermentación, también reduce el número de cepas indígenas (tanto *Saccharomyces* como no-*Saccharomyces*), de modo que la selección de levaduras capaces de fermentar a bajas temperaturas y su producción como LSA, es de gran interés para la industria vitivinícola (Castellari *et al.* 1994; Giudici *et al.* 1998). Más aún teniendo en cuenta que sólo existe un pequeño número de estas levaduras criotolerantes, comercializadas.

7.2.3. Manoproteínas.

Las manoproteínas de la pared celular de *S. cerevisiae*, se han convertido en los últimos años en uno de los productos de mayor interés para la mejora de procesos tecnológicos y de las características sensoriales de los vinos, por lo que están ganando terreno en las bodegas de diversas maneras. Se les atribuyen diversas propiedades en enología, entre las que destacan su capacidad de evitar o minimizar algunas alteraciones que pueden sufrir los vinos, y que afectan negativamente a su calidad, reduciendo su valor comercial. Estas propiedades son:

Protección frente a la quiebra proteica. La quiebra proteica es una alteración producida en vinos blancos y rosados con una elevada concentración de proteínas de uva. Como consecuencia de las altas temperaturas, o el prolongado tiempo de almacenaje, se puede producir una precipitación de estas proteínas, formando agregados y dándole un aspecto turbio al vino. Esto puede ser especialmente problemático si se produce una vez que ha sido embotellado, dado que la presencia de partículas en suspensión puede ser rechazada por el consumidor al percibir que el vino puede estar microbiológicamente alterado (Waters *et al.* 2000). Las principales variedades de uva que dan lugar a vinos susceptibles de sufrir este tipo de alteración son *Sauvignon Blanc* y "Moscatel de grano menudo".

Los vinos criados sobre lías muestran una mayor estabilidad proteica (Dupin *et al.* 2000). Esta mejora, se debe al efecto protector de las manoproteínas liberadas de las paredes de las levaduras. De hecho, la adición de manoproteínas a un vino permite aumentar su estabilidad proteica y, por lo tanto, disminuir la dosis de bentonita necesaria para su estabilización (Ledoux *et al.* 1992), la cual puede llegar a ser muy alta.

Protección frente a la precipitación tartárica. La quiebra o precipitación tartárica es una alteración que se produce en vinos con una elevada concentración de sales de bitartrato potásico, y en ocasiones de tartrato cálcico. Como consecuencia de las bajas temperaturas y del aumento de la concentración de etanol durante la fermentación, estas sales son susceptibles de dar lugar a precipitados en forma de cristales. Al igual que en el caso anterior, puede ser problemático si se produce después del embotellado, por el rechazo que genera la presencia de cristales en las botellas por parte del consumidor.

Los vinos que han sufrido una crianza sobre lías tienen menos tendencia a presentar cristales de tartrato, y esto es debido al efecto protector que ejercen las manoproteínas liberadas de la pared de las levaduras durante la crianza (Feuillat *et al.* 1998).

Adsorción de Ocratoxina A. La Ocratoxina A (OTA) es una micotoxina encontrada frecuentemente en uvas, mosto y vino (Zimmerli and Dick 1996; Caridi 2006). Se ha demostrado que el uso de cepas seleccionadas de *S. cerevisiae* puede disminuir el contenido del vino en OTA durante la fermentación (Scott *et al.* 1995; Caridi 2006; Caridi 2007).

Retención de substancias aromáticas. Las manoproteínas y otros polisacáridos liberados por la levadura durante la fermentación alcohólica influyen en la volatilidad de las substancias del aroma, con el consiguiente efecto sobre las propiedades sensoriales del vino. La retención de compuestos como la β-ionona, el etil-hexanoato, y el octanal, por las manoproteínas, antes del embotellado, permitiría unos niveles superiores en el producto final, con el consiguiente efecto positivo sobre las propiedades organolépticas (Lubbers *et al.* 1994).

Disminución de la astringencia. La astringencia en los vinos tintos está ligada a la interacción entre los taninos del vino y las proteínas salivares. Se ha demostrado que la fracción proteica de las manoproteínas permite fijar los taninos, de modo que se limitan las reacciones con las proteínas salivares y se ve reducida así la astringencia (Saucier *et al.* 1999; Escot *et al.* 2001).

Estabilización del color. Los antocianos poliméricos (responsables del color en vinos tintos), en presencia de manoproteínas, son más estables y hacen al vino menos sensible a los cambios de color. La estabilización del color se produciría por la asociación entre las manoproteínas y estos antocianos (Escot *et al.* 2001).

Estimulación del crecimiento de las bacterias lácticas. La fermentación maloláctica juega un papel fundamental en la vinificación, ya que además de disminuir la acidez total, mejora la estabilidad del vino y sus propiedades organolépticas (Caridi 2006). Esta fermentación es llevada a cabo por las bacterias lácticas. Las manoproteínas han sido asociadas con la estimulación del crecimiento de las bacterias lácticas (Guilloux-Benatier *et al.* 1995; Rosi *et al.* 1999). La

estimulación de este crecimiento podría deberse a la unión de las manoproteínas con cadenas de ácidos grasos sintetizados por *Saccharomyces* que inhiben el crecimiento de las bacterias lácticas, de modo que su eliminación favorecería su crecimiento. Además, las bacterias lácticas son capaces de hidrolizar las manoproteínas, aumentando el contenido nutricional del medio y estimulando su actividad (Guilloux-Benatier and Chassagne 2002).

Estabilización de la espuma en vinos espumosos. Los vinos espumosos con una mayor concentración de manoproteínas de levadura, tienen una espuma más persistente (Feuillat 1987; Feuillat 2003).

Las numerosas propiedades de las manoproteínas, muestran el interés que existe por su utilización para la mejora de las características del vino. Actualmente el sistema más utilizado para el enriquecimiento del vino en manoproteínas es la crianza sobre lías, proceso en el que se prolonga el contacto del vino con los restos de levaduras, una vez que ha terminado la fermentación. Esta es una práctica complicada y trabajosa, que implica una importante dedicación de los recursos de la bodega. Así mismo, entraña cierto riesgo de aparición de alteraciones microbiológicas (Chatonnet et al. 1992). Por otro lado, también se comercializan preparados enzimáticos que ayudan a liberar manoproteínas, así como paredes de levadura, no purificadas, tratadas enzimáticamente. El uso de las manoproteínas como aditivos enológicos está sujeto a algunas limitaciones de tipo normativo.

Una alternativa interesante podría ser la utilización de levaduras superproductoras de manoproteínas, ya que el vino podría verse enriquecido al final de la fermentación. La utilización de este tipo de levaduras genera una ventaja económica con respecto a los otros métodos mencionados y elimina el riesgo de alteración microbiológica que se puede dar en la crianza sobre lias. Estas levaduras superproductoras podrían ser tanto levaduras *Saccharomyces* como no-*Saccharomyces* (González-Ramos *et al.* 2008; González-Ramos *et al.* 2010; Giovani *et al.* 2012).

8. Mejora genética de levaduras.

Desde los orígenes de la agricultura y la ganadería, la biotecnología ha sido una herramienta de mejora de las propiedades de los alimentos producidos. Sin embargo, la mejora de los microorganismos utilizados en la obtención de alimentos fermentados, como el vino, es algo relativamente reciente.

El aislamiento de cultivos puros de levaduras vínicas a finales del siglo XIX, junto con el aumento de los conocimientos genéticos abrió la posibilidad de hacer mejora de estos microorganismos aplicando técnicas a las que hoy se conoce con el nombre de técnicas de genética clásica. Estas estrategias son una vía rápida para la mejora de levaduras al inducir variaciones genéticas con una frecuencia superior a la natural, permitiendo seleccionar aquellas cepas que ofrecen las características deseadas. Los métodos de mejora genética por técnicas clásicas, aplicados a levaduras vínicas, implican la mutagénesis al azar, la hibridación sexual o la fusión de protoplastos (Spencer and Spencer 1983; Pretorius 2000; Bisson 2005), seguidas por la selección de aquellas cepas que adquieren el fenotipo deseado.

La irrupción de la tecnología del DNA recombinante junto con los avances en el campo de la biología molecular del organismo modelo *S. cerevisiae*, han permitido la aplicación de la ingeniería genética como estrategia más específica para la mejora genética de levaduras implicadas en procesos industriales.

El conocimiento de la naturaleza genética de las características deseadas es esencial para realizar la elección apropiada entre las diferentes aproximaciones de mejora. Normalmente, las características enológicas más importantes, como el vigor fermentativo, la producción y tolerancia al etanol, el perfil de temperaturas de crecimiento, entre otras, dependen de un gran número de loci (QTLs) (Marullo *et al.* 2004), los cuales están dispersos por todo el genoma y por lo tanto no están bien caracterizados. En estos casos, las técnicas de genética clásica son las más apropiadas. Por el contrario, cuando se conocen los genes o las mutaciones

concretas cuya expresión confieren el fenotipo deseado, las técnicas de ingeniería genética son las adecuadas.

8.1. Selección clonal.

Las fermentaciones vínicas pueden ser inoculadas con levaduras seleccionadas o se pueden dejar fermentar por la flora nativa presente en las uvas y en la bodega. El uso de levaduras seleccionadas como inóculo de las fermentaciones vínicas ha permitido la producción de vinos más consistentes, con características similares año tras año.

Desde hace años existe una nueva tendencia en las bodegas, basada en la utilización de levaduras autóctonas seleccionadas, específicamente, para cada área vinícola (Querol and Ramón 1996).

La aplicación de la selección clonal puede ser una buena técnica a la hora de buscar nuevas características. Sin embargo, es altamente improbable encontrar una levadura vínica con una combinación ideal de características enológicas. Esta situación ha hecho que la selección de levaduras se extienda, también, a las levaduras no-*Saccharomyces*.

8.2. Mutagénesis aleatoria.

La mutagénesis aleatoria, con agentes físicos o químicos, es la forma más simple de mejorar microorganismos industriales. Se ha utilizado de forma extensiva en la obtención de microorganismos productores de enzimas y antibióticos (Cebollero *et al.* 2007).

Como la frecuencia de mutación espontanea para cada locus es baja en la población de levaduras vínicas, el uso de mutágenos no es muy frecuente. La utilización de mutágenos como la radiación con UV o el tratamiento con agentes químicos aumenta de forma considerable la frecuencia de mutación en la población de levaduras vínicas. El principio básico de esta técnica consiste en poner en contacto la levadura con ese mutágeno, por un tiempo suficiente como para matar entre el 50% y el 95% de la población; después de ese tiempo el mutágeno es

eliminado. Generalmente está seguido por un proceso de selección mediante replica-plating o crecimiento en medio selectivo(Pretorius 2000).

En las levaduras se pueden inducir mutaciones de numerosos tipos. La mutagénesis tiene potencial a la hora de eliminar características indeseables y mejorar propiedades favorables de las levaduras. Sin embargo, el procedimiento de mutación, puede mejorar ciertas características y debilitar otras de forma simultánea (Akada 2002).

El uso de mutágenos para el desarrollo directo de cepas es limitado. Una de las principales limitaciones del uso de la mutagénesis aleatoria en levaduras vínicas radica en su estructura genómica: las levaduras vínicas son diploides o poliploides, no presentan auxotrofías y es difícil seleccionar mutaciones recesivas en estas levaduras, ya que presentan 2 o más copias de cada gen. Aunque la frecuencia de mutación es la misma en individuos haploides, diploides o poliploides, no son fácilmente detectables en células diploides y poliploides debido a la presencia de alelos no mutados. Sólo si la mutación es dominante y tiene efecto fenotípico puede ser seleccionada sin la necesidad de alteraciones adicionales. Se prefiere realizar mutagénesis en cepas haploides o en las esporas de las levaduras vínicas.

8.3. Evolución adaptativa.

La evolución adaptativa implica un conjunto de mutaciones que ocurren en respuesta a una situación y que son ventajosas para las células bajo esas condiciones (Foster 1999). Mediante esta técnica, un organismo está sujeto a un cultivo, continuo o secuencial, durante varias generaciones, en unas condiciones a las cuales no está adaptado (Brown *et al.* 1998; Ferea *et al.* 1999). En presencia de ese estrés ambiental, se genera un conjunto de variantes del individuo adaptadas de forma diferente a la condición en la que se encuentran; estas variantes se generan por selección natural (McBryde *et al.* 2006).

Como técnica de mejora de cepas, la evolución adaptativa tiene la ventaja de que no requiere modificaciones genéticas previas de la cepa, ni la aplicación de un

método complicado para la identificación de los derivados deseados, ni el conocimiento de los genes implicados en la característica a mejorar (McBryde *et al.* 2006).

8.4. Ingeniería genética.

La ingeniería genética ofrece claras ventajas sobre los sistemas de mejora basados en métodos clásicos. En primer lugar implica modificaciones genéticas dirigidas, de tal forma que se conoce en todo momento la naturaleza y la ubicación de la variación, pudiendo asignar el cambio fenotípico a una modificación genética concreta. Por otro lado, la tecnología del DNA recombinante permite la expresión en el huésped de genes heterólogos, lo que abre la posibilidad de conferir nuevos fenotipos que no se podrían obtener empleando la genética clásica como sistema de mejora.

Para la aplicación de la ingeniería genética sin embargo, resultó esencial el desarrollo de métodos eficaces que permitieran introducir una molécula de DNA exógeno en una célula (fenómeno conocido como transformación), el desarrollo de vectores para la clonación de genes y el desarrollo de marcadores para la selección de células transformadas con el DNA de interés.

Las levaduras vínicas y las de laboratorio presentan importantes diferencias de modo que la manipulación genética de estas últimas implica un abordaje diferente. La mayoría de las cepas de laboratorio son haploides, presentan una copia de cada cromosoma, por lo que no es difícil encontrar mutantes auxótrofos con un fenotipo recesivo, tanto naturales como inducidos. Las levaduras industriales sin embargo generalmente son aneuploides, con un número diferente de copias para cada cromosoma, o poliploides, con más de dos copias. La ploidía de las cepas industriales dificulta la construcción en el laboratorio de mutantes de este tipo.

El avance fundamental que ha permitido la aplicación de la ingeniería genética en levaduras industriales, ha sido el desarrollo de nuevos sistemas de selección basados en el empleo de marcadores dominantes que confieren fenotipos de resistencia. Estos sistemas permiten una selección directa de los transformantes y ofrecen la ventaja de que no requieren la modificación genética previa de las levaduras receptoras. Estos marcadores ofrecen algunas desventajas como son las menores frecuencias de transformación y las mayores frecuencias de falsos positivos por la aparición de mutantes resistentes espontáneos (Shimura *et al.* 1993; Park *et al.* 1999).

Los primeros sistemas de selección aplicados para la obtención de levaduras industriales recombinantes estaban basados en su mayoría en el uso de genes heterólogos de resistencia a antibióticos, valiéndose así del fenotipo de resistencia natural de otros organismos (Jimenez and Davies 1980). Sin embargo, existe un rechazo social generalizado en relación con el uso de este tipo de genes de resistencia para la obtención de organismos modificados genéticamente (OMGs) en el sector agroalimentario. Aunque no se ha demostrado científicamente que el empleo de estos marcadores implique algún riesgo medioambiental o de salud sobre los consumidores, se ha extendido el temor de que estos genes pueden transferirse horizontalmente a las bacterias residentes en el intestino, con la consiguiente adquisición de resistencias a antibióticos por parte de estos microorganismos. Esta presión social ha provocado que el uso OMGs esté estrictamente regulado. Las limitaciones a su uso han ocasionado que se desarrollen estrategias de modificación genética que puedan cumplir esas regulaciones y que hagan que los productos sean más aceptables para los consumidores. Estas estrategias se han centrado en dos objetivos principales: disminuir la cantidad de DNA no procedente de levaduras, en las cepas modificadas, y evitar el uso de marcadores de resistencia a antibióticos; de este modo se trataría de obtener levaduras modificadas genéticamente que sean estables en ausencia de presión

selectiva, en las cuales la modificación sea integrada en el genoma (Cebollero *et al.* 2007).

Alternativas biotecnológicas a la utilización de marcadores de resistencia a antibióticos son la aplicación de estrategias para la eliminación específica del marcador de selección (Puig *et al.* 1998; Akada *et al.* 1999), o bien la utilización de genes que confieran resistencia dominante a compuestos diferentes a los antibióticos (Petering et al. 1991; Bendoni et al. 1999; Cebollero and Gonzalez 2004).

Puig et al. (1998) propusieron una estrategia en la cual, tras interrumpir el gen diana mediante integración de DNA exógeno, el marcador de resistencia era eliminado por recombinación homóloga, aunque en el genoma quedaba un pequeño fragmento del DNA integrado, una pequeña huella del proceso. Akada et al. (1999) desarrollaron un sistema que permite la introducción de pequeñas modificaciones (incluyendo mutaciones puntuales) en un gen dado; con este sistema de transformación, cualquier otra modificación que se introduzca es eliminada al final, de modo que no queda huella del proceso sufrido.

Fundamentalmente la mejora genética de las levaduras vínicas se ha centrado en tres aspectos; la introducción de mejoras en el proceso de vinificación, la mejora de las características higiénico-sanitarias del producto y la mejora de la calidad organoléptica de los vinos obtenidos (Cebollero *et al.* 2007).Pese a las mejoras en el proceso, el uso de estos OMGs, en la industria, no está aceptado.

8.5. Hibridación.

Las características enológicas más apreciadas, como el vigor fermentativo, el perfil de temperaturas de crecimiento y el rendimiento y tolerancia al etanol, son fenotipos cuantitativos y de distribución continua, los cuales están determinados por la contribución acumulativa de múltiples loci polimórficos (QTLs) (Marullo *et al.* 2004). Por ejemplo, la tolerancia al etanol implica a más de 250 genes (Pretorius

2000). En este caso y en otros similares, el número de variables implicadas es muy alto y las interacciones entre ellas son difíciles de predecir. Por esta razón, la hibridación es el primer método a considerar cuando la propiedad está sujeta a un control multigénico o cuando esta se encuentra en otra cepa.

A la hora de obtener híbridos se han utilizado diferentes aproximaciones, como la conjugación de esporas, el "rare-mating" o la fusión de protoplastos.

8.5.1. Conjugación de esporas.

Se trata de la unión de dos células haploides de distinto tipo sexual, que se da tras la germinación de estas, para formar un individuo diploide. Las esporas o las células haploides que se pretende cruzar se deben colocar juntas sobre la superficie del agar; observando con el microscopio se puede observar los pares de células que han conjugado. Una variante de este procedimiento es el "mass-mating". Se trata de unir cultivos esporulados de ambos parentales en un medio líquido. Tras unos días de incubación se siembran las células en medio mínimo. Para realizar este proceso, los parentales deben llevar marcadores genéticos complementarios, como por ejemplo auxotrofías. De este modo, al sembrar el cultivo en un medio restrictivo para ambos parentales, sólo los híbridos serán capaces de crecer.

Un problema a la hora de aplicar esta técnica a las levaduras industriales es que esporulan mal, entre el 0 y el 75%, dependiendo de la ploidía de la célula. Además, la viabilidad de las esporas varía enormemente (entre el 0 y el 98%) y está inversamente correlacionada con heterozigosidad.

Las levaduras vínicas frecuentemente son aneuploides, con disomías, trisomías y, en ocasiones, tetrasomías. En algunos casos pueden llegar a ser casi triploides. Estas aneupolidías pueden llegar a conferir ventajas selectivas al aumentar el número de copias de genes beneficiosos o protegiendo a las levaduras contra mutaciones letales o deletereas. Algunos autores han descrito que las esporas derivadas de las cepas vínicas pueden perder las características de interés industrial de la levadura madre (Gimeno-Alcañiz and Matallana 2001).

8.5.2. Rare mating.

Se trata de una adaptación de las técnicas utilizadas en el cruce de esporas. Se utiliza con levaduras que se cruzan con baja frecuencia, con levaduras que producen esporas con baja viabilidad y con aquellas que no son capaces de cruzarse o de esporular. El método depende de que ocasionalmente se de un cambio de tipo sexual en las levaduras, las cuales son diploides o de ploidía mayor. Debido a esto se da hibridación, aunque con una frecuencia muy baja (Spencer and Spencer 1996).

Este método está basado en que, en una población de células diploides o poliploides, algún individuo se vuelva homocigoto para el tipo sexual, de modo que sea capaz de cruzarse con una célula de tipo sexual opuesto, tanto haploide como diploide (Barre *et al.* 1993). Este tipo de cruces son un suceso raro que sólo puede detectarse con un medio de selección muy efectivo. Lo más sencillo en este caso es cruzar cepas con dos marcadores de auxotrofías diferentes.

La ventaja de este método con respecto al cruce de esporas se encuentra en que no necesitas esporular los cultivos, de modo que se elimina el problema de la pérdida de características interesantes y de la baja viabilidad.

El "rare-mating" es posible tanto dentro de especie como entre las especies del género Saccharomyces; la frecuencia de estos cruces, aunque siempre es baja, depende del tipo sexual y de la ploidía de las células parentales (de Barros Lopes *et al.* 2002).

8.5.3. Fusión de protoplastos.

Se trata de un método hibridación en el que se elimina la pared de las células de ambos parentales por medios enzimáticos, los protoplastos se mezclan en presencia de polietilen glicol (PEG) e iones Ca^{2+} y se embeben en una capa de agar bajo condiciones selectivas (Spencer and Spencer 1996).

El PEG y los iones Ca^{2+} permiten que las paredes celulares se fusionen, generando heterocariontes. Aproximadamente el 5% de los heterocariotes generados sufren cariogamia y dan lugar a un híbrido real. El resto (95%) no

fusionará sus núcleos y uno de ellos será eliminado, el resultado es la transferencia de material citoplásmico de una célula a otra (Curran and Bugeja 1996).

La fusión de protoplastos puede utilizarse para obtener híbridos interespecíficos e incluso intergenéricos. Este método tiene la gran ventaja de que permite la hibridación entre cepas de diferente ploidía y evita la necesidad de que se dé la esporulación.

La eficiencia del proceso completo de fusión de protoplastos no es muy alta, aún así es mayor que la de rare-mating. Para que dos núcleos se fusionen, ambos deben encontrarse en un punto específico de la fase G1 del ciclo celular. Al contrario, la transferencia de orgánulos citoplásmicos (o citoducción) por este método es extremadamente eficiente. Estas eficiencias pueden cambiar utilizando cultivos sincronizados. (Curran and Bugeja 1996).

Sin embargo, el principal problema de este método es que, al igual que los métodos de ingeniería genética, genera OMGs; esto se debe a que es un tipo de cruce forzado que no ocurriría en la naturaleza.

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Justificación de la tesis

Uno de los mecanismos moleculares que han permitido la adaptación de las especies del género *Saccharomyces* a los procesos fermentativos es la formación de híbridos. Se han aislado una gran cantidad de híbridos interespecíficos de procesos industriales. Uno de estos casos es el taxón *Saccharomyces pastorianus*, originado por la hibridación entre *Saccharomyces cerevisiae* y *Saccharomyces bayanus** (Casaregola et al., 2001; Nakao et al., 2009), cuyas cepas se encargan de la producción de cerveza tipo 'lager'. En cerveza 'ale' también se han aislado híbridos entre *S. kudriavzevii* y *S. cerevisiae* (González et al., 2008; Peris et al., 2012a; Peris et al., 2012b). En vino se han encontramos híbridos entre *S. cerevisiae* y *S. bayanus** (Masneuf et al., 1998; González et al., 2006; Peris et al., 2012a; Peris et al., 2012b), entre *S. kudriavzevii* y *S. cerevisiae (González et al., 2006; Lopandic et al., 2007)* e incluso triples híbridos *S. bayanus** x *S. cerevisiae* x *S. kudriavzevii (González et al., 2006)*. En sidra también se han aislado triples híbridos *S. bayanus** x *S. cerevisiae* x *S. kudriavzevii* (Masneuf et al., 1998; Groth et al., 1999).

En todos estos casos las cepas que actúan como parentales de los híbridos implicados en procesos industriales son *S. bayanus**, *S. cerevisiae* y *S. kudriavzevii*. Al comenzar este trabajo nos planteamos estudiar en profundidad estas especies parentales. *S. cerevisiae* es un taxón bien definido, asociado a múltiples procesos fermentativos, como la producción de pan, vino, sidra, saké, cerveza tipo 'ale', bebidas y alimentos tradicionales entre otros productos. Es una de las especies más ampliamente estudiada, ya que es considerada como un organismo modelo; sin embargo poco se sabía de las otras especies, por lo que nos pareció necesario estudiar dichas especies.

Cuando se plantearon los objetivos sólo existían dos cepas pertenecientes a la especie *S. kudriavzevii*: IFO 1802 e IFO 1803, aisladas en Japón pocos años antes, descritas y caracterizadas (Naumov et al., 2000), de modo que un estudio de la especie no nos pareció relevante, aunque actualmente existen más cepas, aisladas en Portugal y en España (Sampaio and Gonçalves, 2008; Lopes et al., 2010). Posteriormente a este trabajo, se ha realizado en nuestro grupo, una

Nota: * Nomenclatura antigua

caracterización enológica de varios aislados de *S. kudriavzevii* (Peris et al., 2015, aceptado), en la cual he participado.

En cuanto a la tercera especie implicada en la generación de estos híbridos, *S. bayanus**, la situación era diferente. Al inicio del trabajo, esta especie contaba con muchos aislados descritos y muchos trabajos realizados sobre ella; aun así, su clasificación era motivo de controversia, estaba dividida en dos grupos (*uvarum** y *bayanus**) a los que no se sabía si considerar especies o variedades, y para uno de esos grupos (*S. bayanus var. bayanus**) no se había identificado ninguna cepa pura (Naumov, 2000; Pulvirenti et al., 2000; Nguyen and Gaillardin, 2005; Rainieri et al., 2006).

Dado que los híbridos están presentes en multitud de bebidas fermentadas (Masneuf et al., 1998; Groth et al., 1999; González et al., 2006; Lopandic et al., 2007; González et al., 2008), a que son capaces de adaptarse mejor que los parentales a situaciones fluctuantes o intermedias (Belloch et al., 2008), lo que les proporciona una ventaja selectiva (Masneuf et al., 1998), y a que adquieren propiedades fisiológicas de ambos parentales, por ejemplo, la tolerancia al alcohol y a la glucosa de *S. cerevisiae*, la tolerancia a bajas temperaturas de *S. kudriavzevii* o la mayor producción de compuestos aromáticos de *S. bayanus* , se pensó en la hibridación como un buen método de mejora de levaduras vínicas. Al pretender la comercialización de los híbridos mejorados, las técnicas con las que se debía llevar a cabo todo el proceso no debían incurrir en la generación de GMOs (microorganismos modificados genéticamente), puesto que la legislación vigente es estricta con el uso de estos (Schilter and Constable, 2002; Pretorius and Hoj, 2005; Cebollero et al., 2007).

De este modo tomamos como punto de inicio de este trabajo la caracterización y análisis de cepas pertenecientes a la especie *S. bayanus*. Seguidamente evaluamos distintos procedimientos para generar híbridos intra- e interespecíficos con interés en enología, desarrollamos un protocolo de estabilización y lo estudiamos en profundidad y finalmente caracterizamos los

híbridos estables del cruce intraespecífico con el fin de conocer si se había obtenido alguna mejora sobre sus cepas parentales.

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Objetivos

En el presente trabajo nos marcamos como objetivo global resolver algunas de las exigencias del sector vitivinícola, que cada vez demanda levaduras que posean características fisiológicas diferentes a las de *Saccharomyces cerevisiae.* Entre las nuevas exigencias de los enólogos cabe destacar algunas asociadas con el cambio climático tales como el disponer de levaduras con un menor rendimiento en etanol, que incrementen el contenido en glicerol en los vinos. Otra de las exigencias del sector es obtener vinos más aromáticos para lo cual se realizan fermentaciones a bajas temperaturas y por tanto se buscan levaduras adaptadas a fermentaciones a bajas temperaturas o levaduras que incrementen el contenido de manoproteínas en el vino. Para conseguir este objetivo global, se definieron una serie de objetivos parciales. Estos objetivos parciales y el capítulo en el que se abordan fueron los siguientes:

1. Descifrar la complejidad existente entre las cepas que forman el taxón *Saccharomyces bayanus* (actualmente dividido en las especies *S. uvarum* y *S. bayanus*). **– Capítulo 1 –**
2. Evaluar diferentes técnicas para la obtención de híbridos. **– Capítulo 2 –**
    a. Comparar este proceso tanto dentro de una misma especie (híbridos intraespecíficos) como entre especies (híbridos interespecíficos).
3. Analizar el proceso de estabilización, de híbridos recién obtenidos, en condiciones de vinificación. **– Capítulo 3 –**
    a. Comparar este proceso tanto dentro de una misma especie (híbridos intraespecíficos) como entre especies (híbridos interespecíficos).
4. Caracterizar fisiológicamente los híbridos estables obtenidos y seleccionar aquella/s cepa/s que mejore/n a sus cepas parentales. **– Capítulo 4 –**
5. Caracterizar genéticamente la/s cepa/s seleccionada/s y relacionar estas características con las características fisiológicas para las que ha/n mejorado. **– Capítulo 4 –**

Capítulo 1

Chapter 1. Analysis and study of the old *S. bayanus* taxon.

Part A - On the complexity of the *Saccharomyces bayanus* taxon: hybridization and potential hybrid speciation

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On the Complexity of the *Saccharomyces bayanus* Taxon: Hybridization and Potential Hybrid Speciation

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Abstract

Although the genus *Saccharomyces* has been thoroughly studied, some species in the genus has not yet been accurately resolved; an example is *S. bayanus*, a taxon that includes genetically diverse lineages of pure and hybrid strains. This diversity makes the assignation and classification of strains belonging to this species unclear and controversial. They have been subdivided by some authors into two varieties (*bayanus* and *uvarum*), which have been raised to the species level by others. In this work, we evaluate the complexity of 46 different strains included in the *S. bayanus* taxon by means of PCR-RFLP analysis and by sequencing of 34 gene regions and one mitochondrial gene. Using the sequence data, and based on the *S. bayanus var. bayanus* reference strain NBRC 1948, a hypothetical pure *S. bayanus* was reconstructed for these genes that showed alleles with similarity values lower than 97% with the *S. bayanus* var. *uvarum* strain CBS 7001, and of 99–100% with the non *S. cerevisiae* portion in *S. pastorianus* Weihenstephan 34/70 and with the new species *S. eubayanus*. Among the *S. bayanus* strains under study, different levels of homozygosity, hybridization and introgression were found; however, no pure *S. bayanus* var. *bayanus* strain was identified. These *S. bayanus* hybrids can be classified into two types: homozygous (type I) and heterozygous hybrids (type II), indicating that they have been originated by different hybridization processes. Therefore, a putative evolutionary scenario involving two different hybridization events between a *S. bayanus* var. *uvarum* and unknown European *S. eubayanus*-like strains can be postulated to explain the genomic diversity observed in our *S. bayanus var. bayanus* strains.

Citation: Pérez-Través L, Lopes CA, Querol A, Barrio E (2014) On the Complexity of the *Saccharomyces bayanus* Taxon: Hybridization and Potential Hybrid Speciation. PLoS ONE 9(4): e93729. doi:10.1371/journal.pone.0093729

Editor: Joseph Schacherer, University of Strasbourg, France

Received October 11, 2013; **Accepted** March 10, 2014; **Published** April 4, 2014

Funding: This work was supported by grants AGL2009-12673-CO2 (01 and 02) and AGL2012-39937-CO2 (01 and 02) from the Spanish Government to AQ and EB, respectively. LP-T and CAL acknowledge to CSIC and Fundación Carolina for the I3P and Formación Permanente fellowships respectively. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0093729

1.1. Introduction

The genus *Saccharomyces,* used worldwide to produce different fermented foods and beverages, encompasses the industrially most exploited species known to man. The complex diversity of the genus *Saccharomyces*, including pure, hybrid and introgressed strains, makes species definition difficult and classification controversial. According to the most recent edition of 'The Yeast, a taxonomic study' [1], the genus *Saccharomyces* is composed of eight species: *S. arboricolus, S. bayanus, S. cariocanus*, *S. cerevisiae*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus* and *S. pastorianus*. Although several studies have shown that *S. pastorianus* comprises a group of alloploid hybrid strains originated from *S. cerevisiae* and a cryotolerant species similar to *S. bayanus* [2,3], the last systematic revision maintained the species status for *S. pastorianus* [1].

In a recent study, Libkind et al. [4] isolated and characterized a new *Saccharomyces* species, named *S. eubayanus* and associated with *Nothofagus* spp. trees in Patagonia (Argentina). As the draft genome sequence of this species was closely related to the non *S. cerevisiae* portion of *S. pastorianus* (average divergence of 0.44%), the authors proposed *S. eubayanus* as the previously mentioned *S. bayanus*-like donor of this subgenome in *S. pastorianus* hybrids.

The other controversial *Saccharomyces* taxon is the species *S. bayanus* [1]. *S. bayanus* encompasses a group of cryotolerant strains with active fructose transport, including the former species *S. abuliensis*, *S. bayanus, S. globosus*, *S. heterogenicus*, *S. intermedius*, *S. inusitatus*, *S. tubiformis*, *S. uvarum* and *S. willianus*. Based on the quite diverse physiological [5] and genetic [6,7] traits found among different *S. bayanus* strains, some authors have proposed dividing this taxon into two different species, *S. bayanus* and *S. uvarum* [8,9]. However, the partial reproductive isolation between the strains of both groups has alternatively suggested the subdivision of the species into two varieties, *bayanus* and *uvarum* [10], which was maintained in the most recent taxonomical review of the genus *Saccharomyces* [1].

Rainieri *et al*. [11] evaluated the genetic variability of 35 yeast strains identified as *S. bayanus* or *S. pastorianus*, and observed a very complex picture. By means of PCR-RFLP and sequencing, the authors confirmed that the type strain of *S. bayanus* (CBS 380^T) was composed of clearly differentiated '*bayanus*' and '*uvarum*' subgenomes. The authors identified four

different genomic compositions among the studied strains: (i) a pure line named *S. uvarum* that included strains containing a single type of genome, with similar physiological and genetic characteristics to the type strain of the former species *S. abuliensis* CBS 7001; (ii) a pure line with a single type of genome named *S. bayanus* that included only strain NBRC 1948; (iii) a hybrid line including strains with portions of the genomes from the two pure lines, as well as alleles termed 'Lager' (representing a third genome present in lager brewing strains); iv) a group of *S. cerevisiae*/*S. bayanus*/ Lager and *S. cerevisiae*/*S. bayanus*/ *S. uvarum*/Lager hybrid strains (*S. pastorianus*). While the pure nature of strain CBS 7001 was confirmed by Libkind et al. [4], these authors together with Nguyen et al. [12] demonstrated that strain NBRC 1948 harbors a mosaic genome composed of a hybrid genetic background belonging to *S. uvarum* and a second unidentified species, which Nguyen et al. provisionally named *S. lagerae.* However, Libkind et al. identified it as belonging to the new species *S. eubayanus*, as well as some small introgressed regions from *S. cerevisiae*.

The main goal of the present study was to decipher the complexity of the *S. bayanus* taxon by performing PCR-RFLP analyses of 34 nuclear genes and by sequencing both nuclear and mitochondrial genes from the 46 different strains identified originally as *S. bayanus* or *S. uvarum*, including the type strains of the former species and the natural isolates from different sources (cider, wine, fruit fermentations, etc.) in the light of the discovery of the new taxon *S. eubayanus*. For this purpose, some *S. pastorianus* strains were also evaluated for comparative purposes. The putative hybridization events responsible for the genomic complexity found in the *S. bayanus* taxon are proposed and discussed.

1.2. Materials and methods

1.2.1. *Yeasts strains and media*

The yeast strains used in this study, together with their sources of isolation and geographical origins, are listed in Table 1.1. Strains were grown on YPD (w/v: 1% of yeast extract, 2% peptone, 2% glucose) at 28°C and were maintained on YPD supplemented with 2% w/v agar.

1.2.2. PCR amplification

The characterization of *S. bayanus* var. *bayanus, S. bayanus* var. *uvarum, S. cerevisiae* and *S. pastorianus* strains was performed by PCR amplification and the subsequent restriction analyses of 34 protein-coding genes distributed along the 16 chromosomes present in these yeasts (Figure S1.1). These genes were probed to be suitable to differentiate among the species of *Saccharomyces* genus [13]. The oligonucleotides used as primers for the PCR amplifications are provided in Supporting Information Table S1.1.

Although the *S. bayanus var. bayanus* and *S. bayanus* var. *uvarum* genomes are almost co-linear to that of *S. cerevisiae*, they differ in several reciprocal translocations, hence some gene regions are located in other linkage groups (Supporting Information Figure S1.1). In this way, *S. bayanus* var. *bayanus* differs from *S. cerevisiae* in two reciprocal translocations among chromosomes II and IV and VIII [3,14], while *S. bayanus* var. *uvarum* contains two other translocations between chromosomes VI and X, and between XIV and IItIV [15].

Total yeast DNA was isolated following standard procedures [16]. PCR reactions were performed in a final volume of 100 µl containing 10 µl of 10x *Taq* DNA polymerase buffer, 100 µM deoxynucleotides, 1 µM of each primer, 2 units of *Taq* DNA polymerase (BioTools, B&M Labs, Madrid, Spain) and 4 µl of DNA diluted to 1-50 ng/µl. PCR amplifications were carried out in Techgene and Touchgene thermocyclers (Techne, Cambridge, UK) as follows: initial denaturing at 95°C for 5 min, then 40 PCR cycles involving the following steps: denaturing at 95°C for 1 min, annealing at 55°C (for most genes), and extension at 72°C for 2 min, then a final extension at 72°C for 10 min. For genes *ATF1*, *DAL1*, *EGT2*, *KIN82*, *MNT2*, *MRC1*, *RRI2* and *UBP7*, annealing was performed at 50°C.

PCR products were run on 1.4% agarose (Pronadisa, Madrid, Spain) gels in 0.5x TBE buffer. After electrophoresis, gels were stained with 0.5 µg/ml of ethidium bromide solution (AppliChem, Darmstadt, Germany) and were visualized under UV light. A 100-bp DNA ladder marker (Roche Molecular Biochemicals, Mannheim, Germany) served as a size standard.

1.2.3. Restriction analysis of nuclear gene regions

Simple digestions with different endonuclases were performed with 15 µl of amplified DNA to a final volume of 20µl. Restriction endonucleases *Acc* I, *Asp* I, *Asp* 700I, *Cfo I*, *Dde* I, *Eco* RI, *Hae* III, *Hin*d III, *Hin*f I, *Msp* I, *Pst* I, *Rsa* I, *Sac* I, *Scr* FI, *Taq* I *and Xba* I (Roche Molecular Biochemicals, Mannheim, Germany) were used according to the supplier's instructions. Restriction fragments were separated on 3% agarose (Pronadisa, Madrid, Spain) gel in 0.5 x TBE buffer. A mixture of 50-bp and 100-bp DNA ladder markers (Roche Molecular Biochemicals, Mannheim, Germany) served as size standards.

1.2.4. Amplification, cloning, sequencing and phylogenetic analysis of nuclear genes.

The 34 gene regions used in this study were amplified and sequenced in NBRC 1948 strain for the g*enetic reconstruction of a hypothetical S. bayanus var. bayanus* genome. For genes *EPL1*, *GSY1*, *JIP5*, *KIN82*, *MRC1, PEX2, MAG2*, *NPR2* and *ORC1* additional sequences were obtained (sequences obtained from strains CECT 11186 and CBS 424). Additionally, new alleles were sequenced to confirm their nature ("*uvarum*", "*eubayanus*" or "*cerevisiae*" alleles). These sequences were deposited in the nucleotide databases under accession numbers KJ093508 to KJ093569.

Table 1.1. List of *Saccharomyces* strains analyzed in the present study.

Strain reference		Original epithet	Isolation source	Geographic origin	Present characterization
CECT	Other				
1189	CBS 6308		Ale beer	Yorkshire (England)	*S. uvarum*
1369			Unknown	Spain	*S. uvarum*
1884		*S. uvarum*	Wine fermentation	Mentrida (Spain)	*S. uvarum*
1941			Unknown		*S. eubayanus x S. uvarum*
1969^{T}	CBS 395^{T}	Type of *S.uvarum*	Juice of *Ribes nigrum*	Netherlands	*S. uvarum*
1991	DSMZ 70411		Turbid bottled beer		*S. eubayanus x S. uvarum*
10618			Alpechin	Spain	*S. uvarum*
10174			Unknown	Spain	*S. uvarum*
11035^{T}	CBS 380^{T}	Type of *S. bayanus*	Beer		*S. eubayanus x S. uvarum*
11036	CBS 381	Type of *S. willianus*	Spoiled beer		*S. uvarum*
11135	CBS375		Unknown		*S. eubayanus x S. uvarum*
11185	NBRC 1948	*S. bayanus*	Unknown		*S. eubayanus x S. uvarum*
11186	NCYC 115		Unknown		*S. eubayanus x S. uvarum*
12600		*S. ellipsoideus*	Sweet wine	Alicante (Spain)	*S. uvarum*
12627		*S. bailli*	Wine	Valladolid (Spain)	*S. uvarum*
12629		*S. uvarum*	Must	Zaragoza (Spain)	*S. uvarum*
12638		*S. uvarum*	Must	León (Spain)	*S. uvarum*
12669		*S. pastorianus*	Grapes	La Rioja (Spain)	*S. uvarum*
12922		*S. carlsbergensis*	Jerez grapes wine	Valladolid (Spain)	*S. uvarum*
12930		*S. bayanus*	Wine	Spain	*S. uvarum*
	CBS 377	Type of *S. intermedius*	Pear wine	Germany	*S. uvarum*
	CBS 378		Unknown	Unknown	*S. eubayanus x S. uvarum*
	CBS 424	Type of *S. globosus*	Pear juice	Meggen (Switzerland)	*S. eubayanus x S. uvarum*
	CBS 425	Type of *S. heterogenicus*	Fermenting apple juice	Tägerwilen (Switzerland)	*S. eubayanus x S. uvarum*
	CBS 431	Type of *S. tubiformis*	Fermenting pear juice		*S. uvarum*
	CBS 1546	Type of *S. inusitatus*	Beer	Rotterdam (Netherlands)	*S. eubayanus x S. uvarum*
	CBS 2898		Wine starter	Herrliberg (Switzerland)	*S. uvarum*

Strain reference		Original epithet	Isolation source	Geographic origin	Present characterization
CECT	Other				
	CBS 2946		Unknown	Unknown	*S. uvarum*
	CBS 2986		Wine	Salenegg (Switzerland)	*S. uvarum*
	CBS 3008		Must of soft fruit	Unknown	*S. eubayanus x S. uvarum*
	NCAIM676		Fermented drink	Hungary	*S. eubayanus x S. uvarum*
	NCAIM677		Fermented drink	Hungary	*S. eubayanus x S. uvarum*
	NCAIM789		*Carpinus betulus* exudate	Babat (Hungary)	*S. uvarum*
	NCAIM868		Slimy material on a stump	Dorog (Hungary)	*S. uvarum*
	S4		Cider	Clonmel (Ireland)	*S. uvarum*
	S10		Cider	Clonmel (Ireland)	*S. uvarum*
	S14		Cider	Clonmel (Ireland)	*S. uvarum*
	S20		Cider	Clonmel (Ireland)	*S. uvarum*
	ZIM 2113		Must of Kraljevina	Dolenjska (Slovenia)	*S. uvarum*
	ZIM 2122		Must of Žametna črnina	Dolenjska (Slovenia)	*S. uvarum*
1940^{NT}	CBS 1538^{NT}	Neotype of *S. pastorianus*	Lager beer	Denmark	*S. eubayanus* x *S. uvarum*
1970	CBS 1503	Type of *S. monacensis*	Lager beer	Denmark	*S. cerevisiae* x *S. eubayanus*
11037	CBS 1513	Type of *S. carlsbergensis*	Lager beer	Denmark	*S. cerevisiae* x *S. eubayanus*
11000	NCYC 2340		Lager beer	Unknown	*S. cerevisiae* x *S. eubayanus*
1885		*S. cerevisiae*	Wine	Valladolid (Spain)	*S. cerevisiae* x *S. eubayanus*
	S6U		Wine	Italy	*S. cerevisiae* x *S. uvarum*

^{NT}, neotype, ^{T}, type. Culture collection abbreviated as follows: CECT, Colección Española de Cultivos Tipo, Valencia, Spain; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; NBRC; NCAIM, National Collection of Agricultural and Industrial Microorganisms, Faculty of Food Sciences, Corvinus University of Budapest, Hungary; NCYC, National Collection of Yeast Cultures, Norwich, UK; ZIM, ZIM Culture Collection of Industrial Microorganisms, University of Ljubljana, Slovenia.

For gene *MNL1*, no diagnostic restriction patterns for the differentiation of the '*eubayanus*'- and '*uvarum*'-type alleles were found. Therefore, the *MNL1* PCR products were sequenced, in all the strains, for allele discrimination, and the corresponding sequences were deposited under accession numbers KJ093570 to KJ093618

The PCR products were purified using the Perfectprep Gel Cleanup Kit (Eppendorf, Hamburg, Germany) following the manufacturer's instructions, and were subsequently sequenced for allele discrimination. Sequencing was performed with the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. The sequencing reactions were run on a Techgene Thermal Cycler (Techne, Cambridge, UK), which was programmed as follows: an initial denaturation at 94°C for 3 min, followed by 99 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and polymerization at 60°C for 4 min. Sequences were obtained with an Applied Biosystems automatic sequencer model ABI 3730 (Applied Byosistems, Warrington, UK).

For the heterozygous strains exhibiting ambiguous nucleotide sequences, given the presence of more than one allele, the PCR amplifications were cloned and sequenced to obtain the nucleotide sequence of each allele. Cloning was carried out with the pGEM T Easy Vector System II kit (Promega, Madison, USA) by preparing a ligation reaction with a final volume at 3.3 µL and by incubating overnight at 4°C. The transformation reaction was performed with 20 µL of competent cells JM 109 (Promega, Madison, USA) and 2 µL of the ligation reaction, and the mix was incubated by shaking at 200 rpm for 1.5 h. A volume of 120 µL was plated in LB medium (1% tryptone, 0.5% yeast extract, 1% glucose, 1.5% agar) with 100 µg/mL ampicillin, 0.5 mM IPTG, and 80 µg/mL X-Gal. Plates were incubated for 24 h at 37°C and at least 12 positive colonies were isolated for the direct PCR amplification from colony, and the subsequent sequencing was done according to the conditions described above.

Alignments were done using the Clustal W algorithm as implemented in the MEGA 4.0 software [17]. Similarities between '*eubayanus*' and '*uvarum*' alleles were estimated as nucleotide identities per 100 sites (%).

The jModelTest program [18] was used to estimate the evolutionary model that best represents the nucleotide divergence data provided by the *MNL1* sequences by applying the Bayesian information criterion [19]. The best fitting model was the Kimura 2-paremetter model [20] with a gamma distribution (G) of substitution rates with a shape parameter of $\alpha = 0.099$. A maximum likelihood (ML) tree was obtained with PHYML 3.0 [21] by applying the corresponding K2-p +G model. The statistical support for the resulting topology was assessed using a nonparametric bootstrap with 100 pseudo-replicates [22].

1.2.5. Amplification, sequencing and phylogenetic analysis of COX2

To establish the *COX2* gene haplotypes present in the strains under study, this mitochondrial gene region was PCR-amplified and subsequently sequenced given the absence of diagnostic restriction sites. *COX2* was amplified using the primers and conditions described in Belloch *et al.* [23]. PCR products were cleaned with the Perfectprep Gel Cleanup kit (Eppendorf, Hamburg, Germany) and both DNA strands were sequenced directly using the BigDyeTM Terminator v3.0 Cycle Sequencing kit (Applied Biosystems, Warrington, UK) following the manufacturer's instructions, in an Applied Biosystems automatic DNA sequencer Model ABI 3730.

COX2 sequences (accesion numbers AF442212, AJ938046, AJ938045, AJ966729, and JN676768 to JN676813) were aligned and analyzed with the MEGA 4 program [17]. Due to low divergences and the presence of a putative recombination, phylogenetic trees were obtained by the Neighbor-Joining method using the p-distance (uncorrected nucleotide divergence). Tree reliability was assessed using a nonparametric bootstrap with 2000 pseudo-replicates.

1.3. Results

1.3.1. Genetic reconstruction of a hypothetical S. bayanus var. bayanus or 'bayanus' pure line.

By using a set of the 34 pairs of primers (Supporting Information Table S1.1) previously generated in our laboratory for *Saccharomyces* hybrids detection and characterization, the nuclear gene regions of NBRC 1948 strain were amplified and sequenced. This strain was selected as the most representative *S. bayanus* var. *bayanus* strain, because was defined by Rainieri et al. [11] as a "pure" *S. bayanus* var. *bayanus* strain. These sequences were then compared to the homologous regions of the genome sequence of strain CBS 7001(available at http://www.saccharomycessensustricto.org). This strain, also known as MCYC623, is considered as a pure *S. bayanus* var. *uvarum* strain [11].

Each pair of homologous sequences were aligned and the corresponding nucleotide similarities were estimated as shown in Table 1.2. From the 34 gene compared regions, identical sequence pairs for genes *EPL1*, *GSY1*, *JIP5*, *KIN82*, *MRC1* and *PEX2* (100% similarity), and almost identical sequences for genes *MAG2*, *NPR2* and *ORC1* (99.6 to 99.9% similarity), were observed. However, 25 homologous sequence pairs showed similarities lower than 97%, and between 86.0% for *CBP2* and 96.7% for *MET6* (Table 1.2).

To check if the nine identical or almost identical sequences found in both NBRC 1948 and CBS 7001 could be fixed characteristics of the *S. bayanus* species genome, sequences for those genes were obtained from two other *S. bayanus* var. *bayanus* strains, CECT 11186 (NCYC 115) and CBS 424. Three sequences from CECT 11186 (*JIP5*, *MAG2* and *PEX2*) and three others from CBS 424 (*KIN82*, *MRC1* and *ORC1*) were identical or almost identical to the sequences in CBS 7001 and NBRC 1948. However, all the remaining sequences analyzed in the two additional strains gave lower similarity values, around 89.5-95.4%, as compared to the reference sequences (Table 1.2).

By combining the sequence data from strains NBRC 1948, CECT 11186 and CBS 424, a complete set of '*bayanus*' alleles of a hypothetical *S. bayanus* var. *bayanus* pure line (alleles with similarity values lower than 97% as compared to strain CBS 7001) was obtained.

Using the sequences obtained in this study, we performed a genome BLAST search on the non *cerevisiae* sub-genome of the *S. pastorianus* strain Weihenstephan 30/70 available in NCBI. Two homologous sequences were obtained for all genes, each corresponding to one of the two subgenomes (the *S. eubayanus* and *S. cerevisiae* subgenomes according to Libkind et al. [4]). The only exceptions were genes *KIN82* and *GAL4,* for which only one highly similar sequence to *S. cerevisiae* was obtained. Sixteen gene sequences from the '*eubayanus*' fraction of *S. pastorianus* were 100% identical to the sequences comprising our hypothetical *S. bayanus* pure line, and 15 gene sequences were almost identical (between 99.2% and 99.9% of similarity)(Table 1.2).

The divergent genes between the '*uvarum*' pure line CBS 7001 and our hypothetical '*bayanus*' pure line (the alleles from NBRC 1948, CECT 11186 or CBS 424) were also divergent between CBS 7001 and the '*eubayanus*' Weihenstephan 34/70 gene sequences (similarities of 86.0-96.7%) (Table 1.2).

After considering the high similarity of the gene sequences between the '*eubayanus*' alleles in the *S. pastorianus* strain and our 'hypothetical *S. bayanus* var. *bayanus*', we used the name '*eubayanus*' , or simply 'E', to designate these alleles henceforth. Within this new framework, strain CBS 7001 contained only '*uvarum*' alleles (or simply 'U'), but strain NBRC 1948 contained both E and an important fraction of U alleles (26.5% of the genes under study). Our results indicate that the two alleles have an average divergence of 8.4% (between 3.3% and 14%) for the analyzed gene sequences.

Table 1.2. Sequence similarity for 34 protein-coding genes between the reference strain of *S. bayanus var. bayanus* (NBRC 1948) and the reference strains of *S. bayanus* var. *uvarum* (CBS 7001) and the '*eubayanus*' alleles of the *S. pastorianus* strain Weihenstephan 34/70 (W 34/70)

Gene	Similarity (%) between CBS 7001 and			Similarity (%) between W 34/70 '*eubayanus*' alleles and			Similarity (%) between CBS 7001 and W 34/70 '*eubayanus*' alleles
	NBRC 1948	CECT 11186	CBS 424	NBRC 1948	CECT 11186	CBS 424	
APM3	92.7	-	-	100	-	-	92.7
ATF1	91.2	-	-	99.2	-	-	91.8
BAS1	91.9	-	-	100	-	-	91.9
BRE5	86.7	-	-	100	-	-	86.7
BUD14	92.1	-	-	99.9	-	-	92.0
CAT8	91.9	-	-	99.6	-	-	92.1
CBP2	86.0	-	-	100	-	-	86.0
CBT1	91.4	-	-	99.4	-	-	91.6
CYC3	91.5	-	-	100	-	-	91.5
CYR1	93.2	-	-	100	-	-	93.2
DAL1	92.0	-	-	99.9	-	-	92.2
EGT2	88.6	-	-	99.7	-	-	88.9
EPL1	**100**	92.6	92.6	92.7	99.9	99.9	92.5
EUG1	90.3	-	-	99.5	-	-	90.4
GAL4	91.2	-	-	none	-	-	none
GSY1	**100**	95.4	95.4	95.4	99.7	99.7	95.4
JIP5	**100**	**100**	91.9	91.9	91.9	96.6	91.9
KEL2	87.7	-	-	99.9	-	-	87.8
KIN82	**100**	92.3	**99.7**	none	None	none	none
MAG2	**99.9**	**99.9**	93.9	94.0	94.0	100	93.9
MET6	96.7	-	-	100	-	-	96.7

Gene	Similarity (%) between CBS 7001 and			Similarity (%) between W 34/70 '*eubayanus*' alleles and			Similarity (%) between CBS 7001 and W 34/70 '*eubayanus*' alleles
	NBRC 1948	CECT 11186	CBS 424	NBRC 1948	CECT 11186	CBS 424	
MNL1	89.6	-	-	100	-	-	89.6
MNT2	91.0	-	-	100	-	-	91.0
MRC1	**100**	90.7	**99.8**	90.7	99.2	90.7	90.7
NPR2	**99.7**	93.0	93.0	93.2	99.9	99.9	92.9
OPY1	92.8	-	-	100	-	-	92.8
ORC1	**99.6**	89.5	**99.7**	89.7	100	89.7	89.5
PEX2	**100**	**100**	92.4	92.3	92.3	99.9	92.3
PKC1	91.9	-	-	100	-	-	91.9
PPR1	95.6	-	-	99.6	-	-	95.8
RPN4	90.5	-	-	100	-	-	90.5
RRI2	90.1	-	-	100	-	-	90.1
UBP7	92.5	-	-	100	-	-	92.5
UGA3	91.0	-	-	99.9	-	-	91.3

For those genes exhibited between the CBS 7001 and NBRC 1948 similarities ≥ 99.6% (in bold), additional sequences were obtained for *S. bayanus* strains CECT 11186 or CBS 424, exhibiting divergent alleles, and their similarities to CBS 7001 and W 34/70 are also provided. Those gene sequence comparisons for which *S. pastorianus* W 34/70 contains only '*cerevisiae*' alleles are indicated by 'none' (i.e., no *eubayanus* alleles are present to compare).

1.3.2. Characterization of the strains belonging to the S. bayanus taxon based on the presence of both alleles 'eubayanus, E' and 'uvarum, U'

To characterize the complex *S. bayanus* taxon and to find a putative pure *S. bayanus* var. *bayanus* strain, a PCR-RFLP analysis of the 34 gene regions was performed on a panel of 46 strains deposited in culture collections under species name *S. bayanus, S. uvarum* or *S. pastorianus* (Table 1.1). According to the sequence differences observed between alleles E and U, only the restriction endonucleases able to differentiate both alleles for each particular gene were chosen from those proposed by González et al.[13] (Supporting Information Table S1.2). New restriction endonucleases were used for gene sequences for which the enzymes proposed by González et al [13] did not differentiate between the two alleles (Supporting Information Table S1.2). In order to merely avoid wrong allele type assignation due to intra-type sequence variations, we used a single restrictase to assign U or E alleles only when more than two restriction site gains/losses were observed between both alleles (because small variant of the alleles can sometimes make one fragment get cutted into 2 fragments while the rest of the pattern remains the same). Whenever this condition was not achieved with a single restrictase, additional restriction enzymes were used.

Accordingly, the restriction patterns similar to those present in reference strain *S. bayanus* var. *uvarum* CBS 7001 were named 'U1', while those present in the reconstructed pure *S. bayanus* var. *bayanus* and in *S. pastorianus* strain Weihenstephan 34/70 (from *S. eubayanus*) were named 'E1'. The restriction patterns similar to those present in reference strain *S. cerevisiae* S288c were named 'C1'. As we were unable to find diagnostic restriction patterns to differentiate alleles E and U for the *MNL1* gene region, the analysis of the *MNL1* region was done by sequencing (Figure 1.1).

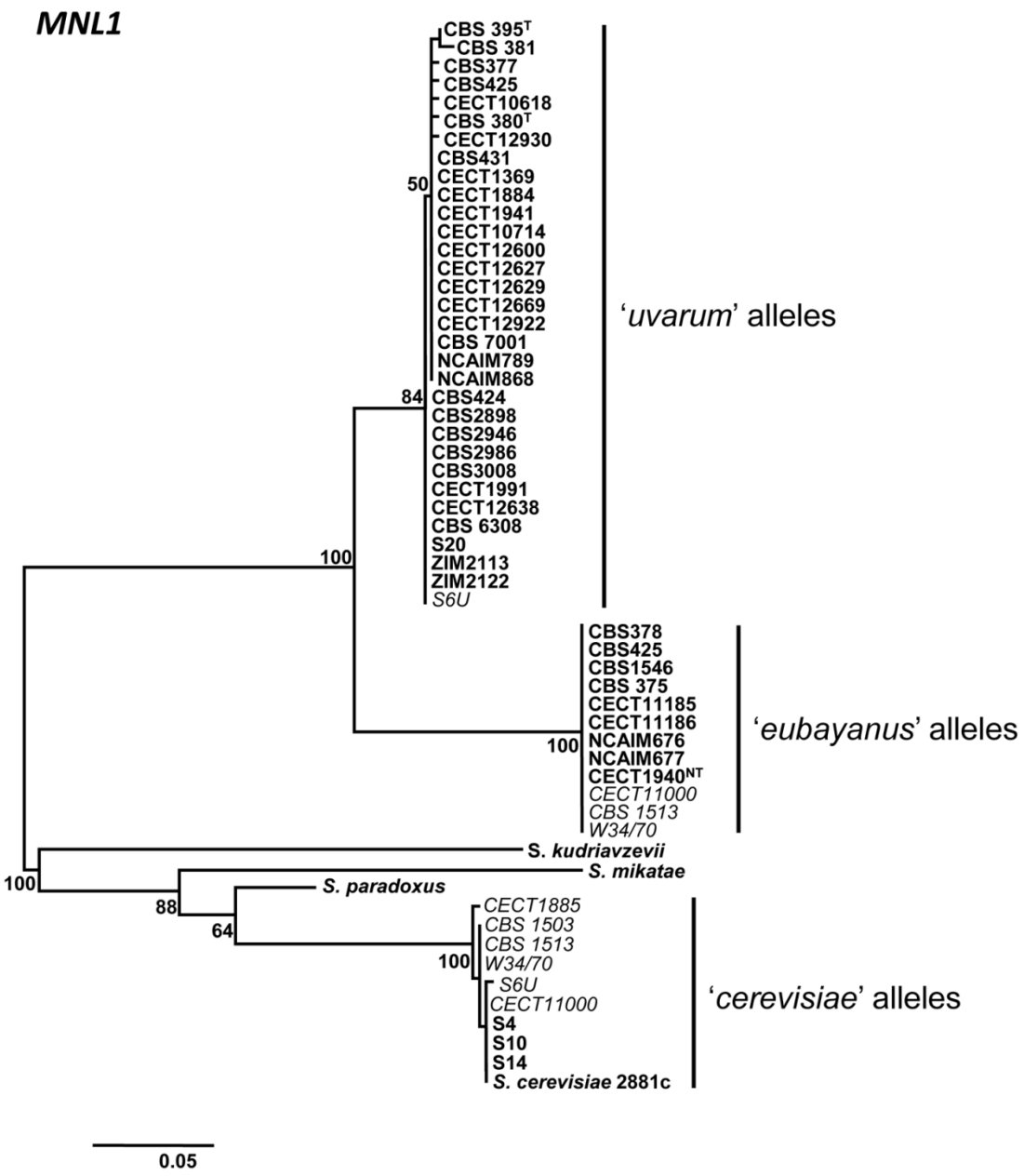


Figure 1.1. Phylogenetic tree obtained with the partial sequences of the nuclear *MNL1* gene. Numbers at nodes correspond to the bootstrap values based on 1000 pseudo-replicates. The scale is in nucleotide substitutions per site. All the strains are indicated in bold, except the *S. pastorianus* ones.

Following the procedures described before, we obtained a complete characterization of all the strains listed in Table 1.1. These results are summarized in Figure 1.2 and Supporting Information Tables S1.4 and S1.5. Some strains exhibited alternative restriction patterns, which differed by one restriction site gains/losses from the C1, E1 or U1 patterns present in the reference strains. These new alleles were sequenced and their similarities with the reference C, U and E alleles were tested. These new alleles were named C, E or U (depending on the closest allele), followed by an ordinal number from 2 onward, as shown in Supporting Information Table S1.3.

Twenty-seven of the 46 strains showed only U alleles for 33 of the 34 analyzed nuclear gene sequences. Seven of them showed U alleles for the 34 analyzed genes and twenty showed a C2 allele for *PEX2* gene region, being the most frequent alternative allele detected among the analyzed strains (20 of the 27 strains bearing only U alleles showed this C2 allele). Among them, 13 different nuclear genotypes were observed due to the presence of alternative U2 alleles for different gene regions (Supporting Information Table S1.4). These new allele variants were observed only for genes *MNT2*, *UBP7*, *BAS1*, *RRI2* and *BRE5.* Most of these strains exhibited only one allele for the 34 analyzed genes, except for strains ZIM 2122 and NCAIM 868, which were heterozygous U1/U2 for genes *RRI2* and *BAS1,* respectively. Finally, Irish cider strains S4, S10, and S14 contained a similar combination of alleles U1 and U2 to that found in strains CBS 2946 and NCAIM 789 for all genes analyzed, except for gene *MNL1* (Supporting Information Table S1.4), for which they showed a '*cerevisiae*' (C) allele, as observed after the sequence analysis (Figure 1.1).

Fourteen strains contained different combinations of alleles U and E (Supporting Information Table S1.5), indicating their '*uvarum*' x '*eubayanus*' hybrid nature. These included strains NBRC 1948, the type strain of *S. bayanus* CBS 380^{T} and the putative neotype strain of *S. pastorianus* CECT 1940^{NT}. It was possible to clearly differentiate these U x E hybrids into two groups (types I and II) according to their genetic constitution. To obtain a more illustrative picture of this situation, we represent the genetic constitution of these strains containing alleles U and E in Figure 1.2. The strains included in Type I (strains NBRC 1948, CECT 11186, CBS 424 and CBS 3008) appeared to be homozygous for all 34 genes under study (Figure 1.2); while the alloploid strains that presented some genes in heterozygosis (U/E alleles) were included in Type II (Figure 1.2 and

Supporting Information Table S1.5). The total number of heterozygous E/U loci varied from 9% in strain NCAIM 676 to 44% in strain CBS 1546. Alternative E2 alleles were observed for genes *DAL1* (strains CBS 424 and CBS 3008) and *BAS1* (strains CBS 424, CBS 3008, CBS 425 and CECT 1991), while alternative U2 alleles were observed for genes *MNT2*, *UBP7*, *BAS1*, *RRI1* and *BRE5* in different strains (Supporting Information Table S1.5).

Another group of strains included those identified as *S. pastorianus* and were, therefore, characterized by the additional presence of '*cerevisiae*' (C) alleles (Supporting Information Table S1.6). Among them, wine commercial strain S6U exhibited alleles U and C for 33 genes and alleles C1 and C2 for *PEX2* gene. Three strains, including the former type strains of *S. carlsbergensis* (CBS 1513 = CECT 11037) and *S. monacensis* (CBS 1503 = CECT 1970) and one wine strain (CECT 1885), contained different combinations of alleles E and C. Finally, lager brewing strain CECT 11000 contained the three types of alleles (E, C and U), although alleles U were found for only five gene regions, including *MRC1*, *NPR2*, *KEL2*, *GSY1* and *EGT2* (Supporting Information Table S1.6). Interestingly, all the previously mentioned yeasts (except S6U) exhibited alleles E2 for two genes: *BAS1* and *BRE5*.

According to our data, no pure strains bearing 100% E alleles were found among our *S. bayanus* strains. Based on the presence of alleles E and U, it was possible to divide the *S. bayanus* strains analyzed in this work into three groups: (i) a '*S. bayanus* var. *uvarum*' pure-line group that includes those strains containing only U, in which some limited *S. cerevisiae* introgressions may have occurred, as with strains S04, S10 and S14, showing a C allele in the subtelomeric gene *MNL1* or the 20 strains showing a C2 allele in the subtelomeric gene *PEX2*; (ii) a homozygous '*S. bayanus* var. *bayanus*' group including strains with both alleles E and U in homozygosis (Type I); (iii) an alloploid '*S. bayanus* var. *bayanus*' group containing strains with both alleles E and U in heterozygosis (Type II).

It was also possible to divide the *S. pastorianus* strains into three groups: (i) hybrids with alleles C, E and U (ii) hybrids with alleles C and E and (iii) hybrids with alleles C and U.

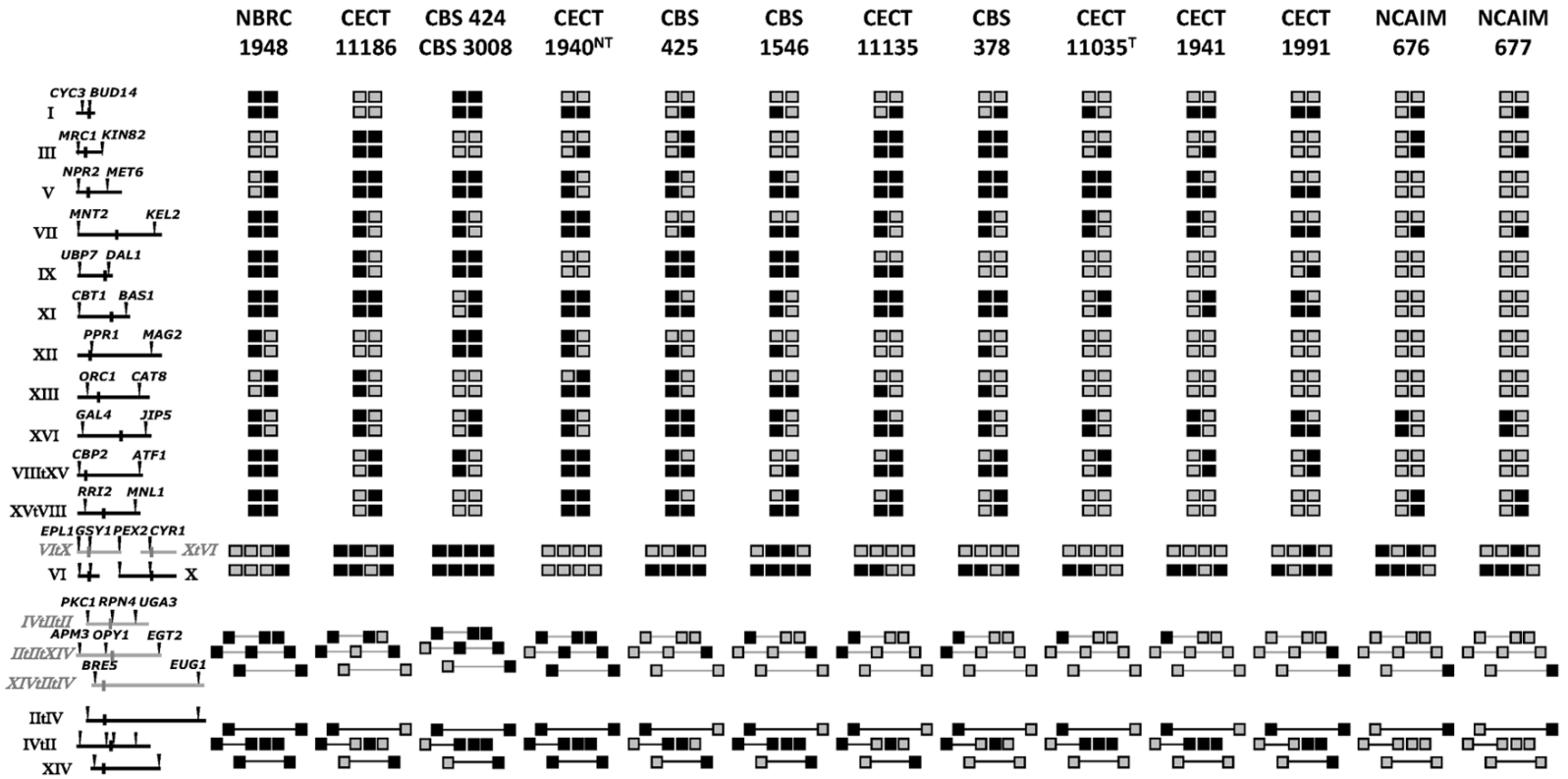


Figure 1.2. RFLPs of 34 nuclear genes from the *S. bayanus* strains analyzed in this work. Each square corresponds to a copy of each gene region according to its chromosome location, indicated in the map on the left. '*eubayanus*' alleles are indicated as black squares and '*uvarum*' alleles as gray squares. The genes order differs between *S. bayanus* var. *bayanus* and *S. bayanus* var. *uvarum,* as depicted, due to the presence of two translocations. The first involves chromosomes VI and X in *S. bayanus* var. *bayanus* and chromosomes VItX and XtVI in *S. bayanus* var. *uvarum*, while the second involves chromosomes IItIV, IVtII and XIV in *S. bayanus* var. *bayanus* and chromosomes IItIItIV, IVtIItII and XIVtIItIV in *S. bayanus* var. *uvarum*.

1.3.3.About the origin of mitochondrial DNA in S. bayanus

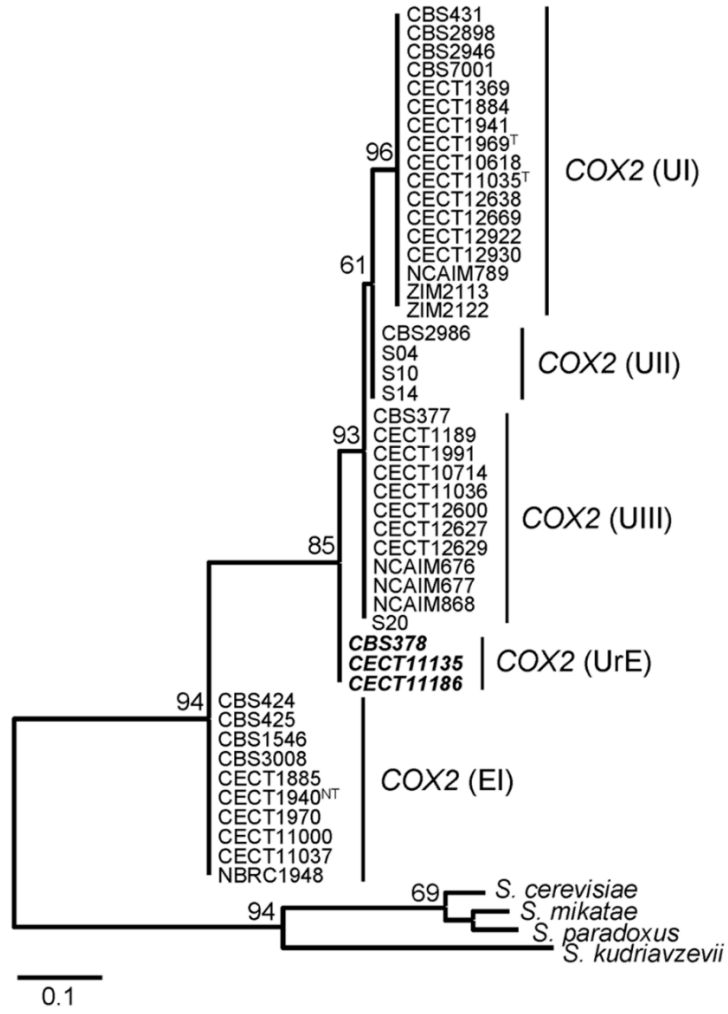
In order to obtain a more complete picture of the identity of the *S. bayanus* strains studied, we also analyzed the nature of their mtDNA. For this purpose, we evaluated mitochondrial gene *COX2* from all 46 strains. Due to the difficulties in unveiling *COX2* variability in *Saccharomyces* by restriction analyses, we performed direct sequencing. Five groups of strains were separated according to the *COX2* phylogenetic analysis (Figure 1.3A). The strains possessing only U haplotypes for the 34 analyzed genes (the '*uvarum*' pure line strains) were separated into three *COX2* haplotypes: U-I, U-II and U-III. Haplotype U-I, found in reference strain CBS 7001, was the most frequent among our strains. Haplotype U-II was shared by cider strains and wine strain CBS2986, and haplotype U-III was observed in nine strains of diverse origins (Figure 1.3A).

All the *S. pastorianus* strains CBS 1503, CBS 1513, CECT 1885 and CECT 11000 showed the same haplotype E-I, postulated as being received from the *S. eubayanus* progenitor according to the phylogenetic analysis of the sequences. The only exception was strain S6U, which exhibited an *S. cerevisiae COX2* haplotype (Figure 1.3A).

S. bayanus hybrids strains, with alleles U and E in their nuclear genes, exhibited four different *COX2* haplotypes which did not cluster together in the gene phylogeny. Some of their *COX2* sequences clustered with '*uvarum*' haplotypes U-I, U-II and U-III, and others did so with the '*eubayanus*' haplotype E-I present in *S. pastorianus* strains (Figure 1.3A). Interestingly, three *S. bayanus* hybrid strains, CECT 11186, CBS 375 and CBS 378, exhibited a *COX2* haplotype located in the phylogenetic tree at an intermediate position between the *uvarum* and *eubayanus* allele (E-I). A detailed analysis of the variable positions of the *COX2* sequences (Figure 1.3B) showed that the 5´region of this haplotype was identical to the '*uvarum*' haplotype sequences, but differed from the '*eubayanus*' sequence, while the 3´region was identical to the '*eubayanus*' sequence and differed from the '*uvarum*'. This result are indicative that these three hybrid strains may exhibit a putative recombinant *COX2* haplotype (called UrE), which could result from the recombination between the *uvarum* and *eubayanus COX2* genes. To check this putative recombination, we performed separate phylogenetic analyses of the 5´ and 3' *COX2* regions (Supporting Information

Figure S1.2) corresponding to nucleotide positions 1 to 525 and nucleotides 526 to 582, respectively. Accordingly in the 5´region phylogeny, the UrE haplotype clustered with the *uvarum* haplogroup and with the *eubayanus* E-I allele in the 3´ region phylogeny.

A



B

COX2 haplotype	Strains	Variable positions (in vertical) 1111122333334 555555 51444723467787 333444 77147124565871 456039
UI	17	TTCTTCGCTAATAC ATCTTG
UII	4	..... .AA.C.
UIII	12	..... .AA.A.
UrE	3	..... *TAACAA*
EI	10	*AATAATATATTCTT TAACAA*

Figure 1.3. Phylogenetic analysis of the partial sequences of the mitochondrial *COX2* gene.

A- NJ tree. Numbers at nodes correspond to the bootstrap values based on 1000 pseudo-replicates. The scale is in nucleotide substitutions per site. Strains with a controversial adscription are indicated in bold.

B- Variable positions on the different COX2 haplotypes. A dot indicates the presence of the same nucleotide at this position. Haplotype U-I is used as a reference.

1.4. Discussion

Most studies about complex species "*S. bayanus*" coincide on the existence of two well-differentiated groups of strains: the molecularly and physiologically heterogeneous group of strains belonging to *S. bayanus* var. *bayanus*, and the homogenous group of strains pertaining to *S. bayanus* var. *uvarum* [1] . These two varieties have even been considered to be two different species (*S. bayanus* and *S. uvarum*, respectively) by other authors because of their partial reproductive isolation [8,9]. However, the genetically heterogeneous nature of the '*bayanus*' variety, as several works have demonstrated [4,11,12], makes it difficult to obtain reliable information about hybridization data to evaluate the reproductive isolation between these two varieties. Together with the discovery of the pure species *S. eubayanus* and the association of this new taxon with the '*bayanus*-like' subgenome of *S. pastorianus*, Libkind et al. [4] proposed the use of *S. eubayanus* and *S. uvarum* as descriptors of species, but restricted the name *S. bayanus* to the hybrid lineages between pure species. *S. eubayanus* has not been detected in Europe; however, in order to explain its necessary contact with a *S. cerevisiae* ale strain to generate the hybrid *S. pastorianus*, it is feasible that this species inhabits a specific niche environment still to be sampled in this continent, as suggested by Gibson *et al.* [24].

For the purpose of finding a European strain of *S. eubayanus*, a set of 46 European strains obtained from different sources and annotated as *S. bayanus* in different culture collections have been genetically characterized. It is interesting to note that most analyzed strains (~ 85%) were diploid (preliminary results not shown). As expected, most of the gene alleles found in the *S. bayanus* var. *bayanus* reference strain NBRC 1948 were divergent (6-8% of nucleotide divergence)

as compared to the same ones in the *S. bayanus* var. *uvarum* reference strain CBS 7001. These divergence values were similar to those found between the pure lines of *S. eubayanus* and *S. bayanus* var. *uvarum* [4]. Contrarily, a significant fraction of identical or almost identical alleles was found between NBRC 1948 and CBS 7001 (27% of the genes under study). In a similar study, but with 35 *S. bayanus* and *S. pastorianus* strains (only nine strains coincide with our study), Rainieri et al., [11] have also identified alleles with high similarity between strains NBRC 1948 and CBS 7001. In their study, the authors considered that those alleles correspond to cases in which both the '*bayanus*' and '*uvarum*' varieties show same or similar allelic variants. In our work, these kinds of identical or almost identical alleles between the two varieties are considered '*uvarum*' (U) due to the divergence found between the genes common to NBRC 1948 and CBS 7001 and the non *S. cerevisiae* portion in *S. pastorianus* Weihenstephan 34/70. Accordingly, these genes also evidence the non-pure nature of strain NBRC 1948. According to our results, the alleles named '*bayanus*' by Rainieri et al.[11], which differed from '*uvarum*' alleles in only a few nucleotidic positions, must be reconsidered to be '*uvarum*' variants. Following the same argument, the 'lager' alleles in Rainieri et al. [11] must correspond to the real '*bayanus*' alleles because they demonstrate a homology percentage of around 89-94% between these *lager* and '*uvarum*' alleles, which are similar results to those observed in the present work between '*uvarum*' and 'eu*bayanus*' alleles.

The reconstructed *S. bayanus* var. *bayanus* pure line, which contains a combination of alleles present in different hybrid *S. bayanus* strains, shows a similarity of 99-100% with the non *S. cerevisiae* subgenome of the fully sequenced *S. pastorianus* lager strain Weihenstephan 34/70. After considering the genetic similarity demonstrated between *S. eubayanus* and the non *S. cerevisiae* portion of *S. pastorianus* [4], and as no complete database containing the whole *S. eubayanus* genome exists, we assigned the name '*eubayanus*' instead of '*bayanus*' to the non *uvarum* alleles in the *S. bayanus var. bayanus* strains (*S. eubayanus* x *S. uvarum* hybrids) analyzed in our work. Following the idea proposed by Gibson et al. [24], this hypothetical genotype may represent the genotype exhibited by a European pure line of *S. eubayanus*.

Of the 46 strains analyzed, 7 only exhibited U alleles for the 34 analyzed gene regions, 17 exhibited U alleles for 33 gene regions and a C allele for *PEX2*, and 3 exhibited U alleles for 32 gene regions and C alleles for *PEX2* and *MNL1*, and hence, they can be considered pure *S. bayanus* var. *uvarum* or *S. uvarum* strains. These strains were isolated mainly from grapes, grape must or wine, but also from pear or apple ciders, while a few were isolated from other sources; i.e., spoiled and ale beers, *alpechin* (olive mill waste), or tree exudates. Low variation in allele composition was observed among these strains in the *S. uvarum* group. This intraspecific homogeneity has also been evidenced in recent studies using microsatellite loci analyses [25,26]. Nevertheless, the presence of heterozygous strains in this group can be considered evidence for a certain degree of interbreeding among the strains of this variety. The sequence analysis of mitochondrial gene *COX2* is also in accordance with this homogeneity, which was detected in the nuclear DNA for all the *S. uvarum* strains. *COX2* is a highly variable gene that has proved most informative in determining the interspecies phylogenetic relationships in the *Saccharomyces-Kluyveromyces* complex [23,27] and different interspecific hybrids of the genus *Saccharomyces* [13,28,29].

Twenty strains from the *S. uvarum* group, isolated from Irish cider, wine, beer, as well as different unfermented musts and natural environments, exhibited a *S. cerevisiae* introgression in gene *PEX2*, located in a subtelomeric region of the translocated *S. uvarum* chromosome VItX; 3 of them, isolated from Irish cider, presented a second introgression in gene *MNL1*, also located in a subtelomeric region of the translocated *S. uvarum* chromosome XVtVIII. The presence of *S. cerevisiae* subtelomeric sequences has been previously reported for the *S. bayanus var. bayanus* [9,30] and *S. bayanus var. uvarum* [25] strains. According to the above-cited authors, the *S. cerevisiae* sequences in the *S. bayanus* genomes are the result of introgression following unstable interspecies hybridization [31,32]. Introgression may be particularly effective for regaining lost traits, which were functional in a common ancestor; in other words, introgression often serves as a repair or replacement strategy [33]. The two introgressed strains identified by Naumova *et al.* [25] as *S. bayanus* var. *uvarum* are also included in the present study where we demonstrate that they contain '*eubayanus*' alleles in nine different gene regions; hence they must be reclassified as hybrid *S. bayanus* var. *bayanus* strains. Introgressed *S. cerevisiae* telomeric Y' sequences have also

been described in three cider *S. bayanus* var. *uvarum* strains from Brittany and Normandy, France [34]. As Naumova et al. proposed [25], introgressed *S. bayanus* var. *uvarum* strains could be isolated if both, *S. cerevisiae* and *S. bayanus* var. *uvarum,* strains co-exist in the same environment, allowing hybridization [35], and, according our results, introgressions in subtelomeric regions seem to be quite frequent.

1.4.1. Origin of the S. bayanus var. bayanus genome complexity

The situation of the strains classified as *S. bayanus var. bayanus* is more complex due to the presence of different combinations of '*uvarum*' and '*eubayanus*' alleles in their nuclear genomes, as well as mtDNA of different origins, as indicated by the presence of the '*uvarum*' or '*eubayanus*' *COX2* haplotypes, as well as a rare possible recombinant haplotype.

The recombination between mitochondrial DNAs from different parental strains has already been described for *S. cerevisiae* in early studies into yeast mitochondrial genetics [36]. In *S. cerevisiae*, mitochondria from the two parental spores can fuse in the zygote after mating. In these fused mitochondria, parental mtDNAs mix and recombine to generate a recombinant lineage that can be established as homoplasmic during mitochondrial vegetative segregation [36]. *COX2* recombination was also postulated to occur in natural *S. cerevisiae* x *S. kudriavzevii* hybrids [37], and in this study, we showed that it could also have occurred in *S. bayanus* hybrids.

Based on the complexity observed in nuclear and mtDNA genes, we propose a scheme (Figure 1.4) to summarize the generation of all the different S. *bayanus* var. *bayanus* strains resulting from several hybridization events between pure strains from the '*uvarum*' group and a strain possessing only '*eubayanus*' alleles, which is related to the recently described Patagonian *S. eubayanus,* and is also similar to a European *S. eubayanus* strain.

Based on the fact that two different '*uvarum*' *COX2* alleles were detected among the strains in the '*bayanus*' hybrid group, we hypothesized that at least two '*uvarum*' pure strains were involved in the origin of the complete set of *S. bayanus* strains studied here. Regardless of the parental '*uvarum*' involved in hybridization, the first generation of hybrids would possess a

complete set of chromosomes from each parental species, and should show reduced fertility according to the low spore viability (7%) exhibited by the artificial hybrids between *S. eubayanus* and *S. uvarum* generated by Libkind et al. [4]. In addition, Liti et al. [38] had shown that a sequence divergence of over 5% between strains considerably reduces spore viability, and the average genome divergence between *S. eubayanus* and *S. uvarum* is ~6.9% [4].

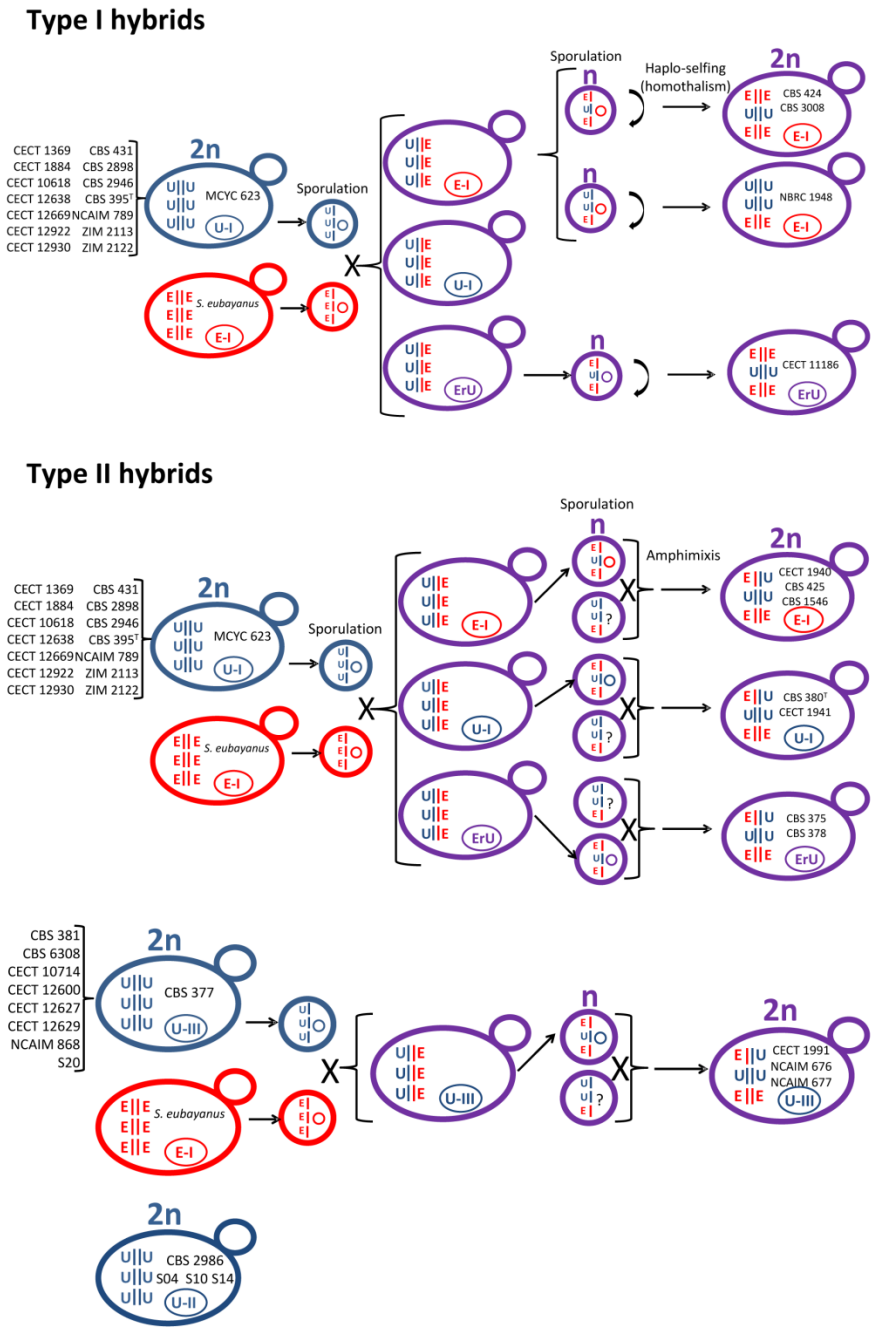


Figure 1.4. Possible origins of the *S. bayanus* (*S. uvarum x S. eubayanus*) hybrid strains.

After hybridization, and perhaps in a fermenting environment context where these hybrids are found and can reach extremely large yeast populations, some of these hybrids can sporulate to generate some viable spores (<7%) that can give rise to the two hybrid types I and II (Figure 1.4)

described in this work. Type I hybrids can arise through sporulation and haplo-selfing (homothallism), while Type II hybrids do so by sporulation and amphimixis, or by mating those spores carrying different a genetic background (heterothallism) (Figure 1.4). In this ancestral hybrid, recombination between "*uvarum*" and "*eubayanus*" chromosomes was also possible during sporulation.

Hybrid speciation has been performed under laboratory conditions from artificial *S. cerevisiae* x *S. paradoxus* hybrids [39] and, despite being uncommon, it has been described in plants [40]. This is partly due to the ability to self-fertilize, which produces identical homolog pairs for every chromosome (except at the mating-type locus on chromosome III), thus avoiding any incompatibility that might arise by fusion with other gametes, even from the same parent [39].

Although the initial hybrids obtained from *S. eubayanus* and *S. uvarum* mating show low spore viability [4], derived type I hybrids would probably recover a higher fertility due to their homozygosity. Contrarily, type II hybrids bearing an important proportion of genes in heterozygosis (E-U) should show lower fertility than the type I hybrids. According to this hypothesis, the larger number of heterozygous genes in hybrids, the lower their fertility. In this sense, spore viability for some of these strains was evaluated by Naumov [10]. In that study, Naumov observed 48% and 7% of spore viabilities for strains CBS 380 and CBS 425, respectively, corresponding to heterozygous type II strains in the present study, and 77% of spore viability for CBS 424, a homozygous type I strain.

It is important to note that, with the exception of the work by Libkind et al. [4], all previous studies evaluated the viability of the hybrids generated by mating a *S. bayanus* var. *uvarum* pure strain (i.e., CBS 7001) and different *S. bayanus* var. *bayanus* strains, such as NBRC 1948 or CBS 380, which correspond according to this study to *S. bayanus* hybrid lines. In this context, the analysis of hybrid fertility is confusing and extremely variable because they really correspond to backcrosses between hybrids and representative strains of the parental species, while the fertility of the progeny depends on the genome constitution of the hybrid.

1.4.2.Saccharomyces pastorianus

As previously mentioned, the most popular examples of hybrid yeasts are those involved in lager beer production included in the taxa *S. pastorianus* (syn. *S. carlsbergensis* and *S. monacensis*), which originated from natural hybridization between *S. cerevisiae* and *S. eubayanus* [4]. Although the different *S. pastorianus* strains possess chromosomes from both species [41,42], mitochondrial DNA was always acquired from the non *cerevisiae* parental [43]. Recent comparative genome hybridization studies have shown that *S. pastorianus* strains came about as a result of at least two independent hybridization events [12,44]. These hybridizations generated the two main groups of lager brewing strains, including Frohberg-type lager strains (i.e., Weihenstephan 34/70) and Saaz-type lager strains (i.e., *S. monacensis* CBS 1503 = CECT 1970).

Our study also agrees partly with these data as Saaz-type lager strains CECT 1885, CECT 11000, CBS 1503 (the type strain of *S. monacensis*) and CBS 1513 (the type strain of *S. carlsbergensis*) exhibit an E2 fixed allele for genes *BRE5* and *BAS1,* whilst strain Weihenstephan 34/70 (a Frohberg-type lager strain) possesses an E1 allele for the same gene regions.

No '*cerevisiae*' alleles were detected in strain CECT 1940, considered the type strain of *S. pastorianus*. This fact indicates that two different strains can be found under the same name, CECT 1940. The literature reports this confusion as to the use of the neotype of *S. pastorianus* several times, and it has been attributed to the misuse of two of its neotype strains: CBS1538^{NT} (from the CBS) and NRRLY-1551 (from the ARS collection in the past). A recent study by Nguyen et al. [12], together with the proteomic data reported by Joubert et al. [45], demonstrate that NRRLY-1551 has been misidentified and should be reclassified as *S. bayanus*. Our results coincide with the data reported by Nguyen et al [12], but we also observed that the strain CECT 1940 used in this work (probably originating from strain NRRLY-1551, and not from CBS 1538) is a *S. bayanus* hybrid strain that bears both the '*uvarum*' and '*eubayanus*' alleles.

Finally, while strain S6U seems to have been originated from the hybridization of a *S. bayanus* var. *uvarum* pure strain (such as CBS 381, CBS 431 and CECT 1884) and a *S. cerevisiae*, the remaining *S. pastorianus* strains evaluated in this work were obtained from the hybridization

between a *S. eubayanus* strain (strains Weihenstephan 34/70, CECT 1885, CBS 1503 and CBS 1513) or *S. bayanus* (bearing both '*eubayanus*' and '*uvarum*' alleles; i.e., CECT 11000) and a *S. cerevisiae* strain. The fact that wine strain *S. pastorianus* CECT 1885 shows '*eubayanus*' alleles does not fall in line with the hypothesis of Naumova et al. [30], which suggests that the non '*cerevisiae*' parental of *S. pastorianus* wine strains is always a *S. bayanus var. uvarum*, unless this strain is a contaminant strain from brewing environments.

In this work, the complexity of "*S. bayanus*" species group was deciphered using the restriction analysis of 34 genes used to differentiate 'uvarum' (U) and 'eubayanus' (E) alleles. From the 48 analyzed strains none was a pure *S. bayanus var. bayanus*/*S. eubayanus* strain. The '*uvarum*' group showed a high intraspecific homogeneity, although a certain degree of interbreeding among the strains of this variety was shown. The situation of the '*bayanus*' group is more complex, all these stains are hybrids between *S. uvarum* and *S. eubayanus* and can be divided in two subgroups: type I or homozygous strains and type II or heterozygous strains. A scheme summarizing the generation of all the different S. *bayanus* var. *bayanus* is proposed.

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Part B - Species definition and homoploid hybrid speciation in the *S. bayanus* complex.

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1.5. Introduction

Speciation, the splitting of one species into two, has been one of the most difficult evolutionary processes to decipher because several mechanisms can be contributing with a different relative importance. These speciation mechanisms and processes have long been studied in plants and animals (Ungerer et al. 1998; Rieseberg 2001; Rieseberg and Livingstone 2003; Lexer et al. 2005; Gross and Rieseberg 2005). However, the study of speciation in fungi is quite more recent (Greig et al. 2002a; Greig et al. 2002b; Greig et al. 2003; Liti et al. 2006; Kuehne et al. 2007; Chou and Leu 2010) in part, due to the complexity of their life cycles, which rises conflicts when fungal species have to be defined.

The Biological Species Concept (BSC), based on reproductive isolation (Mayr 1942), is the most popular and the most used species criterion. According to this concept, speciation mechanisms were classified into two types based on the form of reproductive isolation generated: prezygotic (fertilization is prevented) and postzygotic (a zygote, the product of fertilization, is produced).

The yeasts of the genus *Saccharomyces*, which includes the most useful microorganisms from a biotechnological point of view, are becoming a very interesting model to study the mechanisms of reproductive isolation (Fischer et al. 2000b; Liti et al. 2006; Maclean and Greig 2008; Greig 2008). At present, this genus comprises eight species according to the BSC: *S. arboricolus, S. cariocanus, S. cerevisiae, S. eubayanus, S. kudriavzevii, S. mikatae, S. paradoxus* and *S. uvarum* (Almeida et al. 2014; Boynton and Greig 2014).

Although there are some reports indicating the presence of prezygotic isolation in *Saccharomyces* species (Kuehne et al. 2007; Maclean and Greig 2008), postzygotic isolation is the most frequent situation in these species. In this way, *Saccharomyces* species easily hybridize and

their hybrids are viable but sterile, producing less than 1% viable spores (Naumov et al. 1995a; Naumov et al. 1995b; Greig et al. 1998; Naumov et al. 2000; Naumov 2009). Moreover, this rare viable progeny is postzygotically separated from their parents (Greig et al. 2002a), indicating that they belong to distinct species according to the BSC.

Saccharomyces interspecific hybrids are also found in fermentative environments (González et al. 2006; González et al. 2008), which is indicative of recent speciation in this complex. Different hybrid types have been described so far, such as lager yeasts belonging to the former *S. pastorianus* species (syn. *S. carlsbergensis*), which correspond to *S. cerevisiae* x *S. eubayanus* and *S. cerevisiae* x *S. uvarum* x *S. eubayanus* hybrids; those *S. uvarum* x *S. eubayanus* hybrids from the former taxon *S. bayanus;* cider and wine *S. cerevisiae* x *S. uvarum* hybrids; as well as *S. cerevisiae* x *S. kudriavzevii* and *S. cerevisiae* x *S. kudriavzevii* x *S. uvarum* hybrids (Peris et al. 2012).

Genetic incompatibility, as predicted by Dobzhansky (1936) and Muller (1942), is considered the primary cause of hybrid inviability and sterility in animals (Turelli and Orr 2000). However, it seems not to be clearly involved in the sterility of the *Saccharomyces* hybrids (Greig et al. 2002a; Greig 2007) or involve multiple incompatible loci, with weak individual effects (Kao et al. 2010). The only exception is the cyto-nuclear incompatibility involving the nuclear encoded gene *AEP2* from *S. uvarum* and the mitochondrial gene *OLI1* from *S. cerevisiae*, encoding the ATP synthase subunit 9, which is regulated by Aep2 (Lee et al. 2008).

Another of the possible causes driving speciation by postzygotic isolation are chromosomal rearrangements (Fischer et al. 2000a), due either to a reduction in fitness of the heterozygous hybrids or to a decrease of gene flow through the effects of chromosomal rearrangements on recombination rates. Although Fisher et al. (2000a) discarded chromosomal speciation as the cause of hybrid sterility, because the lack of correlation of the number of chromosomal rearrangements observed between *Saccharomyces* species with their genetic distances, Colson et al. (2004) demonstrated that the artificial restoring of genome collinearity between *S. mikatae* and *S. cerevisiae*, two species differing in a reciprocal translocation, substantially increased the ability of the interspecific hybrids to produce viable spores. This result indicates that postzygotic

reproductive isolation between these two species is not only due to incompatibility caused by genome divergence but also by the presence of chromosomal rearrangements.

Grieg et al. (2002a) proposed that an alternative mechanism, that can explain the postzygotic reproductive isolation in yeasts, is the mismatch repair-mediated genome incompatibility, which has been proven to be involved both in the reproductive isolation between *S. cerevisiae* and *S. paradoxus* (Hunter et al. 1996), and in the incipient speciation process between diverged wild isolates of *Saccharomyces paradoxus* (Greig et al. 2002b). In a sequence variation survey to measure the divergence necessary to produce the reproductive isolation in *Saccharomyces*, Liti et al. (2006) showed that, once the chromosomal rearrangement effects were eliminated, there is a smooth monotonic relationship between diversity and reproductive isolation. The only exception was *S. cariocanus*, which differs from the American populations of *S. paradoxus* by four translocations but not by sequence, a result that led the authors of this study to question the species status of *S. cariocanus*.

The *S. bayanus* taxon, as defined by Vaughan-Martini and Martini (Vaughan-Martini and Martini 1998), corresponded to a complex group of strains that included genetically diverse lineages of pure and hybrid strains (Nguyen and Gaillardin 1997; Rainieri et al. 2006; Nguyen et al. 2011). With the discovery of the new species *S. eubayanus*, Libkind et al. (2011) proposed the use of *S. eubayanus* and *S. uvarum* as descriptors of pure species, and restricted the name *S. bayanus* to the hybrid lineages between these species.

The differentiation of these sister species (Libkind et al. 2011), was based on their postzygotic reproductive isolation (F1 hybrids show 7.3% spore viability), genome nucleotide divergence (6.9%), geographic distribution and apparent niche differentiation.

In addition, *S. uvarum* and *S. eubayanus* also differ in three reciprocal translocations. These translocations (Pérez-Través *et al.*, 2014) were deduced from the genome sequence of *S. uvarum* (Kellis *et al.*, 2003) and the genome analysis or sequencing of the '*eubayanus*' subgenome of *S. bayanus* (Ryu *et al.*, 1996) and *S. pastorianus*/*S. calrbergensis* (Nakao *et al.*, 2009, Walther *et al.*, 2014). *S. eubayanus* exhibits 2 reciprocal translocations with respect to *S. cerevisiae*, involving *S.*

cerevisiae chromosomes II and IV (between YBR030w and YDR012w), and VIII and XV (between YOR018w and YHR015w), which resulted in chromosomes IItIV, IVtII, VIIItXV and XVtVIII in *S. eubayanus.* As mentioned, *S. uvarum* exhibits 3 reciprocal translocations with respect to *S. eubayanus*, the first concerning *S. eubayanus* chromosomes VI and X (YFR033c-YDR012w) and resulting in *S. uvarum* chromosomes VItX and XtVI; and two successive translocations involving *S. eubayanus* chromosomes IItIV, IVtII and XIV, in two possible orders: first between YBR084w and YBL028c, and second between YBR011c and YNL029c, or vice versa, which generated *S. uvarum* chromosomes IItIItXIV, IVtIItII, XIVtIItIV. Therefore, presence of these 3 translocation differences would explain an additional reduction of the hybrid fertility of 87.5%.

Hybridization between *S. eubayanus* and *S. uvarum* has also been very frequent in fermentative environments. These hybrids, included in the *S. bayanus* taxon, exhibit different fractions of the nuclear genomes of the parental species, as well as mtDNA of one or the other species. In a previous study (Pérez-Través *et al.*, 2014), we classified them according to their genomic composition in Type I hybrids, with different fractions of *S. eubayanus* and *S. uvarum* genes but all of them in homozygosis, and Type II hybrids, with different fractions, but many of them heterozygous. The presence of Type I hybrids, which likely originated by hybridization, sporulation and haplo-selfing, could be considered as evidence of homoploid hybrid speciation in yeasts. Homoploid hybrid speciation is a kind of speciation that occurs by hybridization, without a change in chromosome number, and is facilitated by adaptation to a novel or extreme habitats (Rieseberg 1997; Coyne and Orr 2004). This definition implicitly assumes that hybridisation is fundamental to speciation and reproductive isolation must arise during or after hybridization, by rapid chromosomal reorganization, ecological divergence, and/or spatial isolation (Rieseberg 1997). The definition also needs to be expanded to incorporate 'without changes in chromosome number and mating system'; in order to avoid the inclusion of asexual diploid putative hybrids (Mcfadden and Hutchinson 2004). Although homoploid hybrid speciation was historically considered as rare, a large number of recent studies have reinforced the notion that homoploid hybrid speciation may be common (Abbott et al. 2013).

Grant (1981) described two ways in which a homoploid hybrid species could originate without loss of sexual reproduction. The first of these required the hybrid species to be isolated from its parents by a chromosomal or genic sterility barrier and because of the genetic mechanisms involved was named "recombinational speciation". The second requires the hybrid to be isolated by an external isolating mechanism, such as habitat, ethological (behavioural) or geographical (spatial) isolation. Reviews of the incidence and process of homoploid hybrid speciation in plants (Rieseberg 1997; Gross and Rieseberg 2005) have made clear that ecological and spatial isolation are vital to the successful origin and establishment of a homoploid hybrid species and can enable the process to occur even in the absence of intrinsic isolation due to chromosomal or genic sterility barriers (Buerkle et al. 2000). Indeed, homoploid hybrid speciation is unlikely to occur unless there is at least some ecological and spatial isolation between the hybrid and its parent species (Buerkle et al. 2000).

In the present study, we analyzed *S. bayanus* genome, comparing with its parental strains genome, deciphering its chromosomal composition and evaluating the origin and formation of the two types of hybrids which comprises this specie. Speciation mechanisms between parental strains are also discussed.

1.6. Materials and methods

1.6.1. Yeasts strains and media.

The yeast strains used in this study and the sources of isolation, and geographical origins are listed in Table 1.3. Strains were grown on YPD (1% of yeast extract, 2% peptone, 2% glucose) at 24ºC and maintained on YPD supplemented with 2% agar.

Table 1.3. List of *Saccharomyces bayanus* strains analyzed in the present study. The characterization column is according to Pérez-Través et al. (2014) and Rodriguez et al. (2014 y chichas).

Strain reference		Original epithet	Isolation source	Origin	Characterization	
CECT	Other					
1189	CBS 6308		Ale beer	Yorkshire (England)	*S. uvarum*	
1369			Unknown	Spain	*S. uvarum*	
1884		*S. uvarum*	Wine fermentation	Mentrida (Spain)	*S. uvarum*	
1940	CBS 1538	*S. pastorianus*^{NT}	Lager beer	Denmark	*S. eubayanus x S. uvarum*	Type II
1941			Unknown		*S. eubayanus x S. uvarum*	Type II
1969	CBS 395	*S.uvarum*^{T}	Juice of *Ribes nigrum*	Netherlands	*S. uvarum*	
1991	DSMZ 70411		Turbid bottled beer		*S. eubayanus x S. uvarum*	Type II
10618			Alpechin	Spain	*S. uvarum*	
10714			Unknown	Spain	*S. uvarum*	
11035	CBS 380	*S. bayanus*^{T}	Beer		*S. eubayanus x S. uvarum*	Type II
11036	CBS 381	*S. willianus*^{T}	Spoiled beer		*S. uvarum*	
11135	CBS375		Unknown		*S. eubayanus x S. uvarum*	Type II
11185	NBRC 1948	*S. bayanus*	Unknown		*S. eubayanus x S. uvarum*	Type I
11186	NCYC 115		Unknown		*S. eubayanus x S. uvarum*	Type I
12600		*S. ellipsoideus*	Sweet wine	Alicante (Spain)	*S. uvarum*	
12627		*S. bailli*	Wine	Valladolid (Spain)	*S. uvarum*	
12629		*S. uvarum*	Must	Zaragoza (Spain)	*S. uvarum*	
12638		*S. uvarum*	Must	León (Spain)	*S. uvarum*	
12669		*S. pastorianus*	Grapes	La Rioja (Spain)	*S. uvarum*	
12922		*S. carlsbergensis*	Jerez grapes wine	Valladolid (Spain)	*S. uvarum*	
12930		*S. bayanus*	Wine	Spain	*S. uvarum*	
	CBS 377	*S. intermedius*^{T}	Pear wine	Germany	*S. uvarum*	
	CBS 378		Unknown	Unknown	*S. eubayanus x S. uvarum*	Type II
	CBS 424	*S. globosus*^{T}	Pear juice	Meggen (Switzerland)	*S. eubayanus x S. uvarum*	Type I

Strain reference		Original epithet	Isolation source	Origin	Characterization	
CECT	Other					
	CBS 425	*S. heterogenicus*^{T}	Fermenting apple juice	Tägerwilen (Switzerland)	*S. eubayanus x S. uvarum*	Type II
	CBS 431	*S. tubiformis*^{T}	Fermenting pear juice		*S. uvarum*	
	CBS 1546	*S. inusitatus*^{T}	Beer	Rotterdam(Netherlands)	*S. eubayanus x S. uvarum*	Type II
	CBS 2898		Wine starter	Herrliberg (Switzerland)	*S. uvarum*	
	CBS 2946		Unknown	Unknown	*S. uvarum*	
	CBS 2986		Wine	Salenegg (Switzerland)	*S. uvarum*	
	CBS 3008		Must of soft fruit	Unknown	*S. eubayanus x S. uvarum*	Type I
	CBS 7001	*S. abuliensis*^{T}			*S. uvarum*	
	NCAIM676		Fermented drink	Hungary	*S. eubayanus x S. uvarum*	Type II
	NCAIM677		Fermented drink	Hungary	*S. eubayanus x S. uvarum*	Type II
	NCAIM789		*Carpinus betulus* exudate	Babat (Hungary)	*S. uvarum*	
	NCAIM868		Slimy material on a stump	Dorog (Hungary)	*S. uvarum*	
	S4		Cider	Clonmel (Ireland)	*S. uvarum*	
	S10		Cider	Clonmel (Ireland)	*S. uvarum*	
	S14		Cider	Clonmel (Ireland)	*S. uvarum*	
	S20		Cider	Clonmel (Ireland)	*S. uvarum*	
	ZIM 2113		Must of Kraljevina	Dolenjska (Slovenia)	*S. uvarum*	
	ZIM 2122		Must of Žametna črnina	Dolenjska (Slovenia)	*S. uvarum*	
	NPCC 1289		*Araucaria araucana* bark	Argentina	*S. uvarum*	
	NPCC 1290		*A. araucana* seeds	Argentina	*S. uvarum*	
	NPCC 1288		*A. araucana* seeds	Argentina	*S. uvarum*	
	NPCC 1293		*A. araucana* bark	Argentina	*S. uvarum*	
	NPCC 1298		*A. araucana* bark	Argentina	*S. uvarum*	
	NPCC 1323		Chicha apple craft	Chile	*S. uvarum*	
	NPCC 1314		Chicha apple craft	Chile	*S. uvarum*	
	NPCC 1317		Chicha apple craft	Chile	*S. uvarum*	
	NPCC 1322		Chicha apple craft	Chile	*S. uvarum*	
	NPCC 1321		Chicha apple craft	Chile	*S. uvarum*	
	NPCC 1309		Chicha apple craft	Chile	*S. uvarum*	

Strain reference		Original epithet	Isolation source	Origin	Characterization
CECT	Other				
	NPCC 1282		*A. araucana* understory fruit	Argentina	*S. eubayanus*
	NPCC 1286		*A. araucana* understory fruit	Argentina	*S. eubayanus*
	NPCC 1292		*A. araucana* bark	Argentina	*S. eubayanus*
	NPCC 1302		*A. araucana* seeds	Argentina	*S. eubayanus*
	NPCC 1297		*A. araucana* seeds	Argentina	*S. eubayanus*
1940[NT]	CBS 1538[NT]	*S. pastorianus*[NT]	Lager beer	Denmark	*S. eubayanus* x *S. uvarum* Type II
1970	CBS 1503	*S. monacensis*[T]	Lager beer	Denmark	*S. cerevisiae* x *S. eubayanus*
11037	CBS 1513	*S. carlsbergensis*[T]	Lager beer	Denmark	*S. cerevisiae* x *S. eubayanus*
11000	NCYC 2340		Lager beer	Unknown	*S. cerevisiae* x *S. eubayanus x S. uvarum*
1885			Wine	Valladolid (Spain)	*S. cerevisiae* x *S. eubayanus*
	S6U		Wine	Italy	*S. cerevisiae* x *S. uvarum*

[NT], neotype, [T], type. Culture collection abbreviated as follows: CECT, Colección Española de Cultivos Tipo, Valencia, Spain; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; NBRC; NCAIM, National Collection of Agricultural and Industrial Microorganisms, Faculty of Food Sciences, Corvinus University of Budapest, Hungary; NCYC, National Collection of Yeast Cultures, Norwich, UK; ZIM, ZIM Culture Collection of Industrial Microorganisms, University of Ljubljana, Slovenia; NPCC, North Patagonian Culture Collection, National University of Comahue, Neuquén, Argentina.

1.6.2. Amplification, sequencing and phylogenetic analysis of nuclear genes.

Yeast DNA was isolated according to standard procedures (Querol *et al.* 1992). The nuclear genes sequenced for this study were *CAT*8, *CYC*3, *CYR*1, *GAL*4 and *MET*6. *BRE*5 and *EGT*2 were sequenced for the *S. bayanus* strains; *S. uvarum* and *S. eubayanus* sequences were obtained in a previous study (Rodriguez et al., send); these sequences were used in the divergence analysis. All the primers were described in Gonzalez et al. (2008).

The PCR reaction was performed in 100 µl final volume containing 10 µl of 10x *Taq* polymerase buffer, 100 µM deoxynucleotides, 1 µM of each primer, 2 units of Taq polymerase (BioTools, B&M Labs, Madrid, Spain), and 4 µl of DNA diluted to 1-50 ng/µl. PCR amplifications were carried out in Techgene or Touchgene thermocyclers (Techne, Cambridge, UK) as follows: initial denaturing at 95ºC for 5 min, then 40 PCR cycles with the following steps: denaturing at 95ºC for 1 min, annealing at 55ºC, and extension at 72ºC for 2 min; and a final extension at 72ºC for 10 min. In EGT2 case, annealing was performed at 50ºC.

PCR products were run on 1.4% agarose (Pronadisa, Laboratorios Conda S.A., Madrid, Spain) gels in 0.5 x TBE buffer. After electrophoresis, gels were stained with a dilution of 0.5 µg/ml ethidium bromide (AppliChem, Darmstadt, Germany) and visualized under UV light. A 100-bp DNA ladder marker (Roche Molecular Biochemicals, Mannheim, Germany) served as size standard.

In the case of hybrid strains with heterozygous positions on the sequenced genes, the PCR product was cloned with the pGEMs-T Easy vector system II (Promega, Madison, WI). Several clones were then sequenced to confirm the nucleotide sequence of each allele.

PCR products were cleaned with the Perfectpreps Gel Cleanup kit (Eppendorf, Hamburg, Germany) and both strands of the DNA were directly sequenced using the BigDyeTM Terminator V3.0 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK), following the manufacturer's instructions, in an Applied Biosystems automatic DNA sequencer Model ABI 3730 (Applied Biosystems).

Sequences from the reference or type strains of *S. bayanus* var. *uvarum* (CBS 7001), *S. cerevisiae* (S288C), and *S. kudriavzevii* (IFO 1802T) were retrieved from the fungal sequence alignment section (http://db.yeastgenome.org/cgi-bin/FUNGI/showAlignfrom) of the SGD.

Each set of homologous sequences was aligned with the ClustalW program (Thompson *et al.* 1994). Trees were obtained under the Neighbor-Joining method, according to the number of differences model. Tree reliability was assessed using nonparametric bootstrap resampling of 100 replicates. Distances were calculated with the number of differences model.

1.6.3. Pulsed-field gel electrophoresis.

DNA for electrophoretic karyotyping was carried out in agarose plugs (Carle and Olson 1985). Chromosomal profiles were determined by the contour-clamped homogenous electric field technique with DRIII equipment (Bio-Rad Laboratories), using as standard markers the chromosomes of the *S. uvarum* strain CBS 7001. Yeast chromosomes were separated on 1% agarose gels in two steps as follows: a 60-s pulse time for 16 h and then a 120-s pulse time for 11 h, both at 6 V cm–1 with an angle of 120°. The running buffer used was 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) cooled at 14°C.

1.6.4. Sporulation and viability analysis.

Sporulation was induced by incubating cells on acetate medium (1% potassium acetate, 0.125% yeast extract, 0.1% glucose, 2% agar) for 1 day at room temperature followed by 2-7 days at 30 °C. The ascus walls were preliminarily digested with 10µl of crude stomach enzyme complex from the snail *Helix pomatia* during 20 minuts at 28 °C, then 100 of sorbitol 1.2M (Sigma-Aldrich) was added. Between 13 and 18 ascus per strain were dissected. Ascospores were isolated in YPD medium using a Singer MSM Manual micromanipulator.

Yeast used in this part of the study were selected for be part of the '*bayanus*' group and to yield different number of heterozygous positions (Pérez-Través *et al.* 2014). The strains CECT 12930, CBS 7001, NPCC 1289, NPCC 1290, NPCC 1288, NPCC 1293, NPCC 1298, NPCC 1323, NPCC 1314, NPCC 1317, NPCC 1322, NPCC 1321 and NPCC 1309 were used as '*uvarum*' group representatives.

NPCC 1282, NPCC 1286, NPCC 1292, NPCC 1302 and NPCC 1297 were used as 'eubayanus' group representants. All the strains used were listed in table 1.

1.6.5. Flow cytometry.

For flow cytometry, cultures were grown in YPD at 24 ºC for 2 days to ensure that the cells reached stationary phase. Approximately $5x10^6$ cells were washed once 1X PBS buffer (pH 7.4), and fixed for 30min in 70% ethanol at -20ºC. The cells were washed once with 1 ml of 1X PBS buffer, resuspended in 250 µl of 1X PBS buffer containing 50 µl of RNAse A (0,5g/l) and incubated o/n at 37ºC. Cells were washed, resuspended in 495 µl of 1X PBS buffer and sonicated to disrupt aggregates. To stain cells, 5 µl of 50 mg/l propidium iodide was added and cells were incubated 15 min at 37ºC in the dark. DNA content of yeast cells was determined using a BD FACScan system (BD Biosciences). Propidium iodide fluorescence was recorded on F3 chanel in a linear scale, at 1039 V. After gating on the dominant cell population the median fluorescence of the main peak was recorded. Yeasts used in this part of the study were the same we used in viability analysis. Reference strain was CBS 7001, the sequenced *S. bayanus var. uvarum* strain.

1.6.6. Statistical data analysis.

The one-way ANOVA module of Statistica 7.0 software was used to check for significant differences between the species ploidy. The post-hoc comparison was carried out using the Tukey test.

Heterozigosity frequency was plotted against gamete viability on a linear scale with the non linear estimation module of Statistica 7.0 software. The data were fitted by lineal, exponential decay and sigmoid curves. The higher correlation (0,899) was found with a sigmoid adjustment:

$$y=(a+C*\exp(-\exp(b*(x-m))))$$

where **y** is gamete viability, **x** is the heterozigosity frequency, **a** is the lower point of the curve, **C** is the difference between the higher and the lower points, **b** is the slope and **m** is the heterozigosity frequency value in the turning point (Lambert and Pearson 2000).

1.7. Results

1.7.1. Phylogenetic analysis and nuclear gene divergence analysis.

Nucleotide sequences for genes *BRE*5, *CAT*8, *CYC*3, *CYR*1, *EGT*2, *GAL*4, *MET*6 and *MNL*1 were obtained for 15 *S. bayanus* hybrid strains as well as all European and Argentinian *S. uvarum* and *S. eubayanus* strains. Sequences were aligned and used to construct phylogenetic trees (data not shown), and trees were used for classified the sequences in groups. Once the groups were stablished, sequences were used to calculated genetic distances between and within groups.

Independently from the gene considered, two well differentiated groups (BV ≥99%) of sequences were obtained: the S. *uvarum* and S. *eubayanus* allele groups. For the "*uvarum*" group we considered all the sequences grouped with strain CBS 7001, the reference strain of the species *S. uvarum*, and for the "*eubayanus*" group the sequences grouped with the NPCC *S. eubayanus* strains (NPCC 1282, NPCC 1286, NPCC 1292, NPCC 1302, and NPCC 1297) obtained from natural habitats from Patagonia.

Acording with the strain origin, each allele group could also be divided into two subgroups: Ueu: European *S. uvarum*; Eeu: European *S. eubayanus*; Uarg: Argentinean *S. uvarum*; Earg: Argentinean *S. eubayanus*. *S. bayanus* hybrid strains contained alleles located in both European groups.

The divergence values within and between groups are shown in Table 1.4. The average divergences within groups range between 0.2% and 0.6%, being higher among the Argentinean groups than among the European groups (0.5-0.6% vs. 0.2-0.3%). The average divergence between both "*uvarum*" alleles groups was around 0.5% and between "*eubayanus*" alleles groups was 0.9% whereas the average divergence between "*uvarum*" and "*eubayanus*" alleles groups, regardless of origin, is placed around 8.6-8.7%. The less divergent genes in this study were *MET*6 and *CYR*1 (divergence around 3.3-6.9) and the most divergent ones were *EGT*2 and *BRE*5 (divergence around 11.4-13.3).

Table 1.4. Nucleotide divergence and standard error found within and between *S. uvarum* and *S. eubayanus* alleles. Nucleotide divergence was calculated as nucleotide substitutions per site (p-distance). Alleles grouping were done according to phylogenetic results.

Gen	Within allele groups				Between allele groups					
	Ueu	Eeu	Uarg	Earg	Ueu-Uarg	Eeu-Earg	Ueu-Eeu	Ueu-Earg	Eeu-Uarg	Uarg-Earg
BRE5	0.004±0.002	0.001±0.001	0.007±0.002	0.004±0.002	0.006±0.002	0.011±0.004	0.132±0.013	0.130±0.013	0.133±0.013	0.130±0.013
CAT8	0.002±0.001	0.003±0.001	0.009±0.002	0.006±0.002	0.008±0.002	0.008±0.003	0.077±0.010	0.075±0.009	0.078±0.010	0.076±0.009
CYC3	0.000±0.000	0.001±0.001	0.007±0.002	0.002±0.002	0.005±0.002	0.009±0.004	0.083±0.013	0.079±0.013	0.082±0.013	0.079±0.013
CYR1	0.002±0.002	0.002±0.001	0.000±0.000	0.000±0.000	0.001±0.001	0.001±0.000	0.069±0.014	0.069±0.014	0.068±0.014	0.068±0.014
EGT2	0.010±0.003	0.010±0.003	0.009±0.003	0.005±0.003	0.011±0.004	0.019±0.006	0.114±0.015	0.118±0.015	0.114±0.015	0.115±0.015
GAL4	0.002±0.001	0.001±0.000	0.004±0.002	0.009±0.003	0.003±0.001	0.007±0.001	0.090±0.012	0.094±0.012	0.090±0.012	0.094±0.012
MET6	0.000±0.000	0.000±0.000	0.003±0.002	0.005±0.003	0.002±0.001	0.004±0.002	0.033±0.008	0.035±0.008	0.034±0.008	0.037±0.008
MNL1	0.003±0.001	0.000±0.000	0.009±0.003	0.008±0.004	0.008±0.003	0.011±0.005	0.096±0.016	0.089±0.015	0.093±0.015	0.086±0.014
media	0.003	0.002	0.006	0.005	0.005	0.009	0.087	0.086	0.086	0.086

Ueu: European *S. uvarum* alleles; **Eeu**: European *S. eubayanus* alleles; **Uarg**: Argentinean *S. uvarum* alleles; **Earg**: Argentinean *S. eubayanus* alleles.

Table 1.5. Average nucleotide divergence and standard error between alleles and comparison with *S. cerevisiae* and *S. paradoxus*. Nucleotide divergence was calculated as nucleotide substitutions per site (p-distance). Alleles grouping were done according to phylogenetic results.

Gene	Between Ueu-Uarg	Between Eeu-Earg	Between Ueu-Eeu	Between Ueu-Earg	Between Eeu-Uarg	Between Uarg-Earg	Between *Sc and Spdx*	Between American and European *Spdx*	Between American and Far East *Spdx*	Between European and Far East *Spdx*
BRE5	0.006±0.002	0.011±0.004	0.132±0.013	0.130±0.013	0.133±0.013	0.130±0.013	0.183± 0.017	0.036± 0.007	-	-
CAT8	0.008±0.002	0.008±0.003	0.077±0.010	0.075±0.009	0.078±0.010	0.076±0.009	0.159± 0.015	0.022± 0.005	0.023± 0.005	0.008± 0.003
CYR1	0.001±0.001	0.001±0.000	0.069±0.014	0.069±0.014	0.068±0.014	0.068±0.014	0.115± 0.016	0.030± 0.008	0.032± 0.008	0.009± 0.004
EGT2	0.011±0.004	0.019±0.006	0.114±0.015	0.118±0.015	0.114±0.015	0.115±0.015	0.150± 0.021*	0.154± 0.022*	0.044± 0.012	0.142± 0.021*
GAL4	0.003±0.001	0.007±0.001	0.090±0.012	0.094±0.012	0.090±0.012	0.094±0.012	0.229± 0.019	0.047± 0.009	-	-
MET6	0.002±0.001	0.004±0.002	0.033±0.008	0.035±0.008	0.034±0.008	0.037±0.008	0.099± 0.012	0.032± 0.007	0.041± 0.009	0.012± 0.005
media	0.005	0.009	0.087	0.086	0.086	0.086	0.156	0.033[$]	0.035	0.010[$]

Ueu: European *S. uvarum* alleles; **Eeu**: European *S. eubayanus* alleles; **Uarg**: Argentinean *S. uvarum* alleles; **Earg**: Argentinean *S. eubayanus* alleles; **Sc**: *S. cerevisiae*; **Spdx**: *S. paradoxus.*

*****Divergence values between *S. cerevisiae* and the American and Far East populations of *S. paradoxus.* Divergence values with *S. cerevisiae* and the European population of *S. paradoxus* is 0.000. It is due to an introgression between *S. cerevisiae* and European *S. paradoxus* strains (Liti *et al.* 2006).

[$]. Calculated without *EGT2* data.

Table 1.5 summarizes the comparison among divergences between "*uvarum*" and "*eubayanus*" alleles and between *S. cerevisiae* and *S. paradoxus* strains or among *S. paradoxus* populations. Average divergences among "*eubayanus*" alleles were similar to the divergence found between the European and the Far East populations of *S. paradoxus*(0.9% vs 1%); divergences among "uvarum" alleles were lower. On the other hand, average nucleotide divergence between "*uvarum*" and "*eubayanus*" alleles is lower that that between *S. cerevisiae* and *S. paradoxus* but higher that the divergence between American and European-Far East S. paradoxus populations.

1.7.2. *S. bayanus* hybrid genetic characterization: viability, karyotype and ploidy analyses.

To know more about *S. bayanus* hybrids, analysis of viability, karyotype conformation and ploidy were carried out. With a comparative purpose, representative strains of the species *S. uvarum* and *S. eubayanus* were also included in the analyses.

Strains were sporulated in acetate agar media and, when it was possible, asci were dissected and spores viability was evaluated. Results obtained for the different strains are shown in Table 1.6. In both *S. uvarum* and *S. eubayanus* groups of strains, viability values were between 55% and 100%. According with a previous study carried out in our laboratory, the *S. bayanus* group of strains analyzed in this work belonged to two well differentiated types of strains: homozygous strains (type I) and heterozygous strains (type II). For type I strains we found two different situations in the spore viability analysis: in one hand hybrids NBRC 1948 and CECT 11186 presented spore viability values higher than 83% similar to those found in control strains; on the other hand, strains CBS 424 and CBS 3008 presented values similar or lower than 25%. For type II strains, spore viability values were lower than 55%, reaching values as low as 12% in strains bearing seven or more heterozygous positions. The *S. bayanus* type II strains also showed a reduction in the number of spores and their viability, when the number of heterozygous positions was equal or greater than 14.

Table 1.6. Viability and ploidy analysis of selected strains.

	Strain	Heterozigous positions$^{\Delta}$	Heterozigisity frequency$^{\Delta}$	Dissected asci	Viable spores	Viability	Translocations in heterozygosis$^{\Omega}$	Ploidity*	Corrected viability
S. uvarum	**CECT 12930**	0/33	0	14	43	76.80%	0	1,87±0,16^{a}	76.80%
	CBS 7001	0/33	0	13	47	90.40%	0	2±0,06^{a-c}	90.40%
	NPCC 1289	0/33	0	15	59	98.33%	0	1,99±0,03^{a-c}	98.00%
	NPCC 1290	0/33	0	15	60	100.00%	0	1,96±0,02^{a-c}	99.00%
	NPCC 1288	0/33	0	15	53	88.33%	0	1,88±0,03^{a,b}	89.00%
	NPCC 1293	0/33	0	15	45	75.00%	0	1,94±0,01^{a-c}	75.00%
	NPCC 1298	0/33	0	15	53	88.33%	0	1,92±0,04^{a-c}	89.00%
	NPCC 1323	0/33	0	15	56	93.33%	0	3,91±0,05^{e}	93.00%
	NPCC 1314	0/33	0	15	49	81.66%	0	1,95±0,02^{a-c}	81.00%
	NPCC 1317	0/33	0	15	60	100.00%	0	1,95±0,02^{a-c}	100.00%
	NPCC 1322	0/33	0	15	40	66.66%	0	2,01±0,04^{a-c}	66.00%
	NPCC 1321	0/33	0	15	58	96.66%	0	1,99±0,02^{a-c}	97.50%
	NPCC 1309	0/33	0	15	33	55.00%	0	2,08±0,01^{c}	55.00%
S. eubayanus	**NPCC 1282**	0/33	0	15	52	86.66%	0	1,95±0,02^{a-c}	86.66%
	NPCC 1286	0/33	0	15	53	88.33%	0	1,94±0,03^{a-c}	88.33%
	NPCC 1292	0/33	0	15	59	98.33%	0	1,98±0,03^{a-c}	98.33%
	NPCC 1302	0/33	0	15	33	55.00%	0	1,92±0,03^{a-c}	55.00%
	NPCC 1297	0/33	0	15	54	90.00%	0	1,86±0,03^{a}	90.00%
S. bayanus type I	**CBS 424**	0/33	0	14	2	3.60%	0	2.78±06^{d}	-
	CBS 3008	0/33	0	18	18	25.00%	0	2.78±09^{d}	-
	NBRC 1948	0/33	0	15	57	95.00%	0	2,1±0,07^{c}	95.00%
	CECT 11186	0/33	0	14	47	83.93%	0	2,01±0,02^{a-c}	83.93%

	Strain	Heterozigous positions[Δ]	Heterozigisity frequency[Δ]	Dissected asci	Viable spores	Viability	Translocations in heterozygosis[Ω]	Ploidity*	Corrected viability
S. bayanus type II	**NCAIM 676**	3/33	0.09	14	26	46.43%	1	1,95±0,02[a-c]	92.86%
	NCAIM 677	5/33	0.15	16	34	53.13%	1	2,02±0,03[a-c]	106.26%
	CBS 380	7/33	0.21	12	2	4.50%	1 and 2-3(1chr)¤	2,06±0,07[b,c]	18.00%
	CBS 378	8/33	0.24	12	2	4.15%	1	2,03±0,04[a-c]	8.30%
	CECT 1941	10/33	0.29	17	1	1.47%	1 **or** 1 and 2-3 (1chr)¤	1,94±0,05[a-c]	2.95-5.88%
	CBS 375	11/33	0.32	34[¥]	10	7.35%	1	1,99±0,02[a-c]	14.70%
	CBS 425	12/33	0.35	9[§]	1	2.77%	0	1,87±0,04[a,b]	2.77%
	CECT 1991	14/33	0.41	0[Φ]	0	0%	1 and 2-3(1chr)¤	1,96±0,03[a-c]	0.00%
	CBS 1546	15/33	0.44	0[Φ]	0	0%	1 and 2-3 (1chr)¤	1,98±0,01[a-c]	0.00%

[Δ]**-** Data extracted from RFLPs analysis of Pérez-Través et al.(2014), Rodriguez et al. (2014)

[¥]**-** We dissected an elevate asci number to allow a posterior analysis.

[§]**-** No more asci were detected.

[Φ]**-**The asci number were so low we coudn't dissect them.

[Ω]**-0** implies that no translocations were found in heterozygosis. **1** implies that chromosomes VItX and XtVI were found in heterozygosis with chromosomes VI and X.**2-3** implies that chromosomes IItIV, IVtII and XIV were found in heterozygosis with chromosomes IItIItIV, IVtIItII and XIVtIItIV. When more than one option is indicated, it means that both options are equally parsimonious; viability was corrected according the both options.

¤**-** When translocation **2-3** implies an only pair of chromosomes in heterozygosis, viability is only reduced in a 50%. When it happened, it was indicated in brackets.

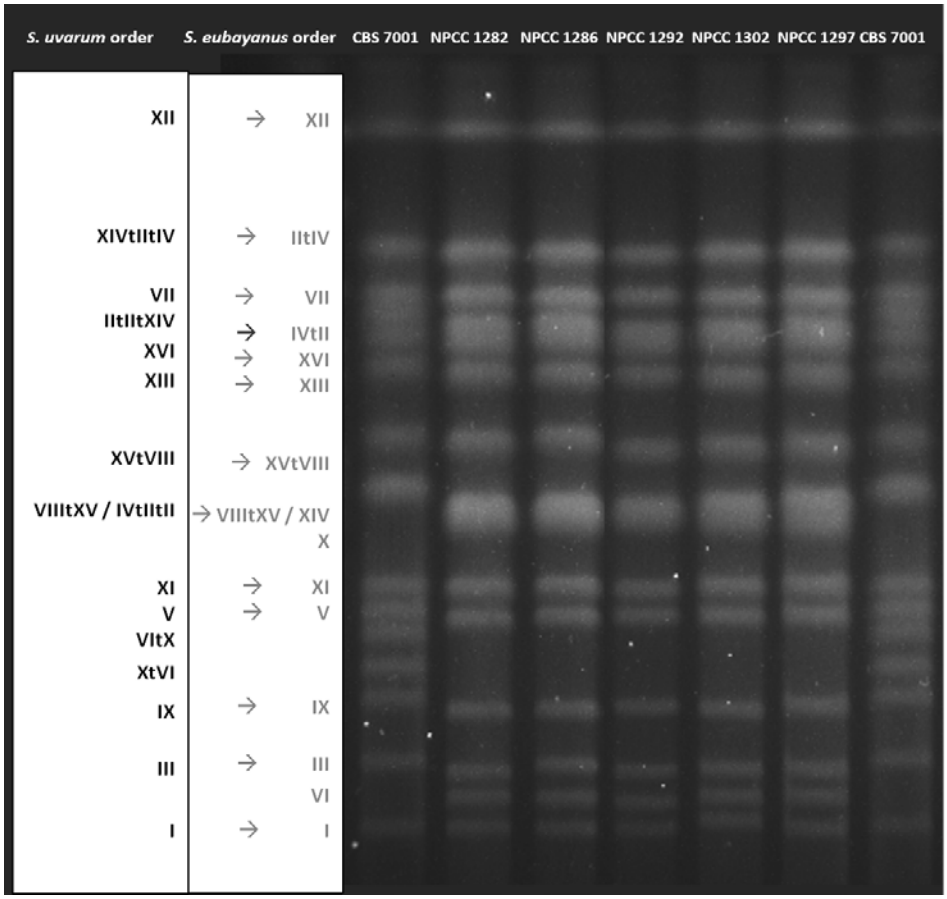
***-** Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, α=0.05, n=2).

Ploidy analysis (Table 1.6) revealed that all *S. bayanus* strains showed the same amount of DNA than the diploid strain *S. uvarum* CBS 7001, used as control, with the only exception of strains CBS 424 and CBS 3008. These two strains, corresponding to type I hybrids, are almost triploid with ploidy values ~2,8n (Table 1.6). Finally, all pure strains belonging to *S. eubayanus* and *S. uvarum* were perfect diploids, with the only exception of strain *S. uvarum* NPCC 1323 that resulted to be an almost tetraploid (3,91n; Table 1.6).

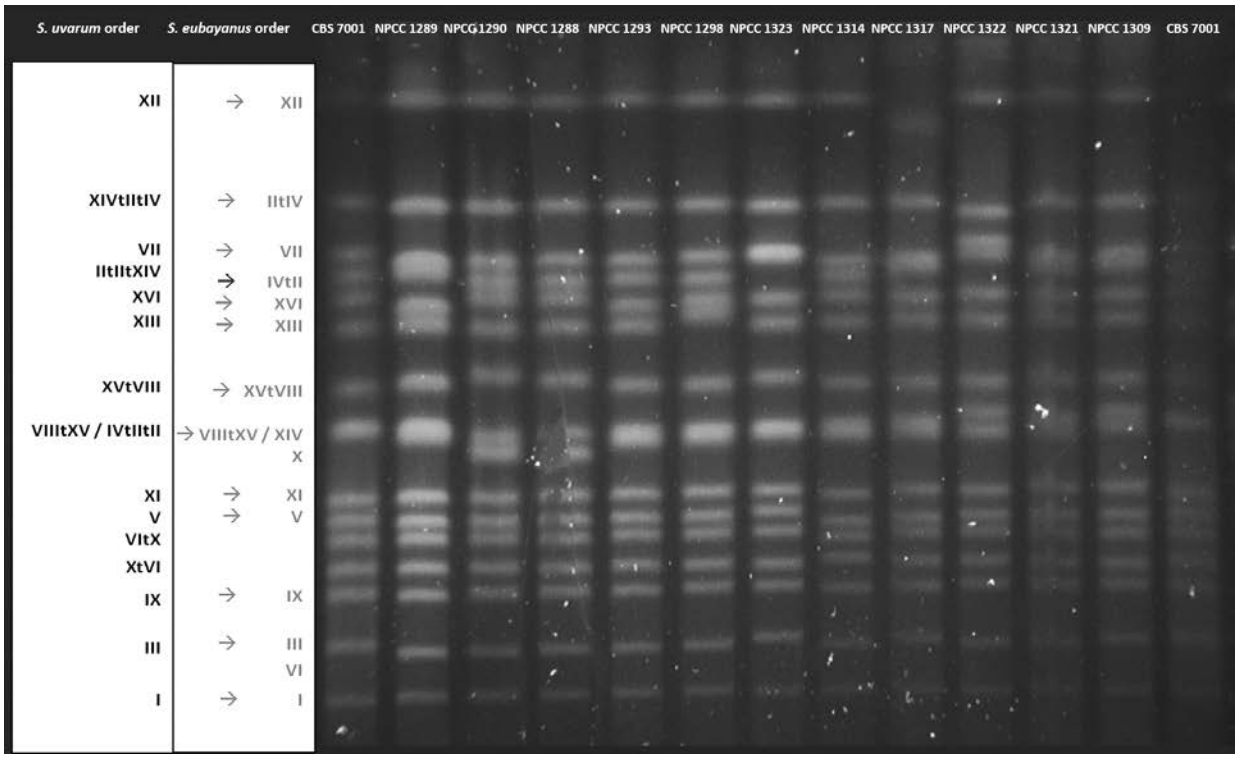
S. bayanus hybrids were also characterized for their chromosomal electrophoretic patterns. Figure 1A shows the karyotype obtained for a set of *S. eubayanus* strains. This is the first report of electrophoretic karyotyping for pure *S. eubayanus* strains. All these strains exhibited a similar chromosome profile, with only a very small number of differences in the band corresponding to chromosome XVtVIII. All the strains showed differentiated band for chromosomes VI and X. Chromosomes IItIV, IVtII and XIV co-migrate with XIVtIItIV, IItIItXIV and IVtIItII, respectively.

In the case of *S. uvarum* pure strains, karyotype variability was higher (Figure 1.5, B and C). These mobility differences were in the upper part of the Figures corresponding to the largest chromosomes. In all strains, bands for chromosomes VItX and XtVI are observed, but chromosomes IItIItIV, IVtIItII and XIVtIItIV were co-migrating with chromosomes IVtII, XIV and IItIV, respectively. When we analyzed *S. bayanus* hybrid strains (Figure 1.5, C and D), a more complex picture is observed. Karyotypes are more complex depending on the presence/absence of the parental chromosomes. It is the case for chromosomes VItX and XtVI that can migrate together or separately from chromosomes VI and X (translocation 1). According to Figure 1.5, CBS 424 and CBS 3008 presented chromosomes X and VI; NBRC1948, CECT 11186 and CBS 425 presented chromosomes VItX and XtVI. CBS 1546, CBS 380, CECT 1941, NCAIM 676, NCAIM 677, CBS 378, CBS 375 and CECT 1991 presented the translocated pair in heterozygosis with the non-translocated chromosomes.

Figure 1.5. *S. eubayanus* (A), *S. uvarum* (B and C) and *S. bayanus* (C and D) strains chromosomal profiles. In chromosome order column, big arrow shows similar bands with different chromosomes; in black, "*uvarum*" chromosomes; in grey, "eu*bayanus*" chromosomes. In the images C and D, ➔ VItX; ⇨ XtIV; ⊸ X; ⇄ VI.

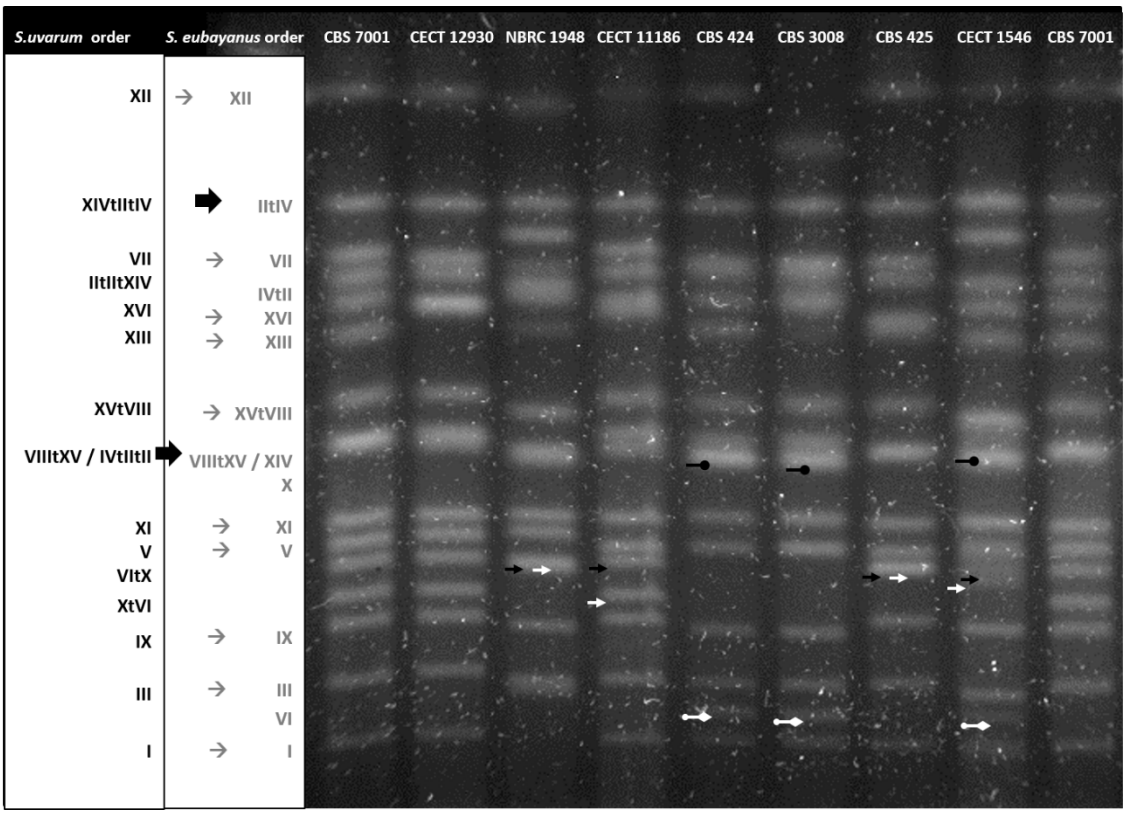

S. uvarum order
S. eubayanus order
CBS 7001
NPCC 1282
NPCC 1286
NPCC 1292
NPCC 1302
NPCC 1297
CBS 7001
XII → XII
XIVtIItIV → IItIV
VII → VII
IItIItXIV → IVtII
XVI → XVI
XIII → XIII
XVtVIII → XVtVIII
VIIItXV / IVtIItII → VIIItXV / XIV
X
XI → XI
V → V
VItX
XtVI
IX → IX
III → III
VI
I → I

A

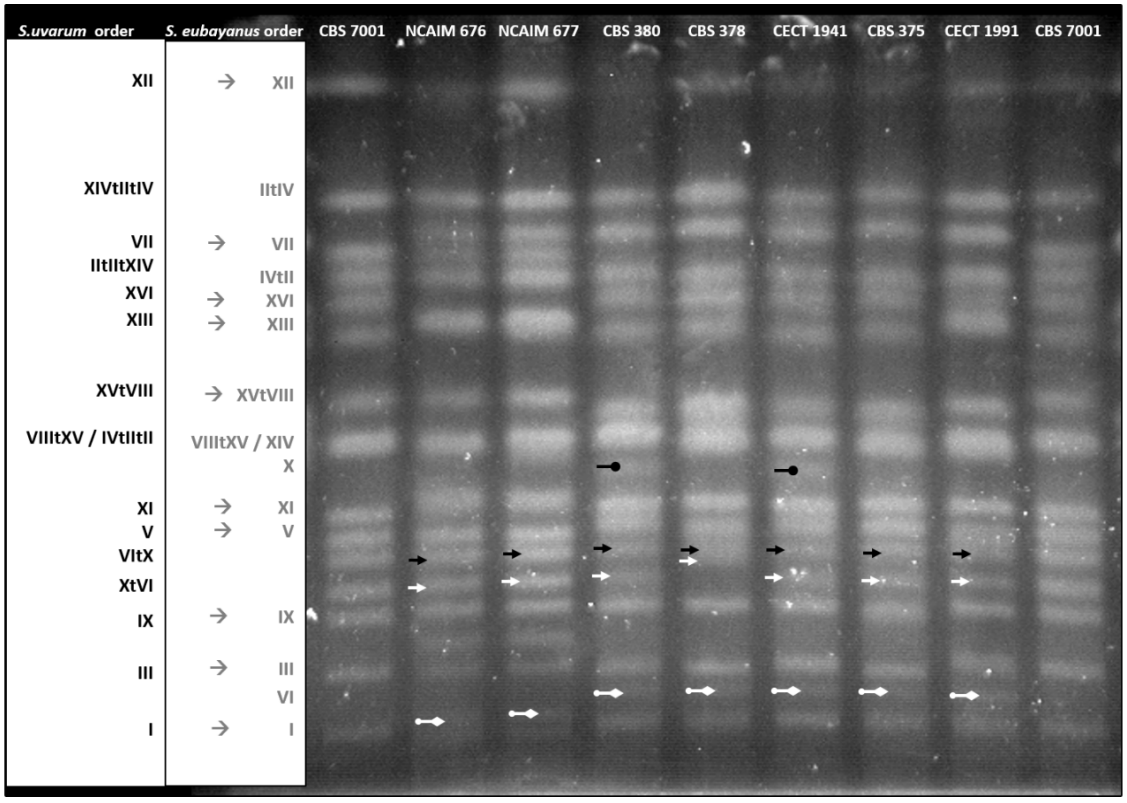

S. uvarum order
S. eubayanus order
CBS 7001
NPCC 1289
NPCG1290
NPCC 1288
NPCC 1293
NPCC 1298
NPCC 1323
NPCC 1314
NPCC 1317
NPCC 1322
NPCC 1321
NPCC 1309
CBS 7001
XII → XII
XIVtIItIV → IItIV
VII → VII
IItIItXIV → IVtII
XVI → XVI
XIII → XIII
XVtVIII → XVtVIII
VIIItXV / IVtIItII → VIIItXV / XIV
X
XI → XI
V → V
VItX
XtVI
IX → IX
III → III
VI
I → I

B

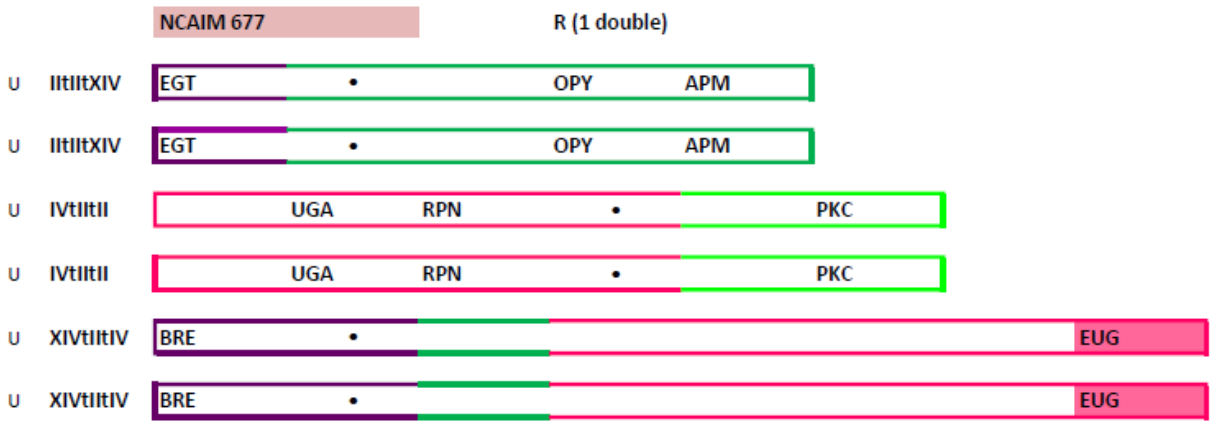
C

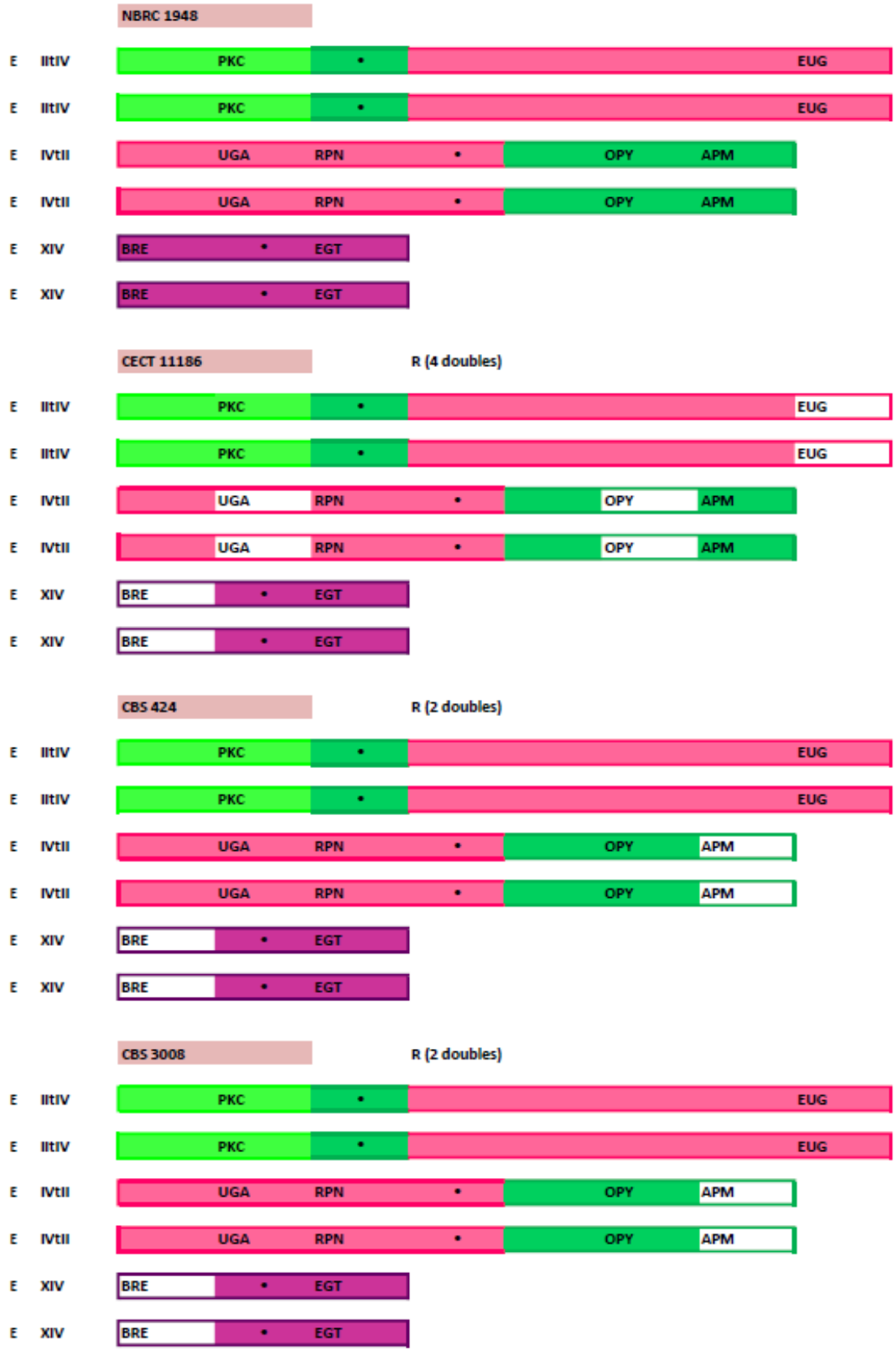

S.uvarum order
S. eubayanus order
CBS 7001
CECT 12930
NBRC 1948
CECT 11186
CBS 424
CBS 3008
CBS 425
CECT 1546
CBS 7001
XII
XII
XIVtIItIV
IItIV
VII
VII
IItIItXIV
IVtII
XVI
XVI
XIII
XIII
XVtVIII
XVtVIII
VIIItXV / IVtIItII
VIIItXV / XIV
X
XI
XI
V
V
VItX
XtVI
IX
IX
III
III
VI
I
I

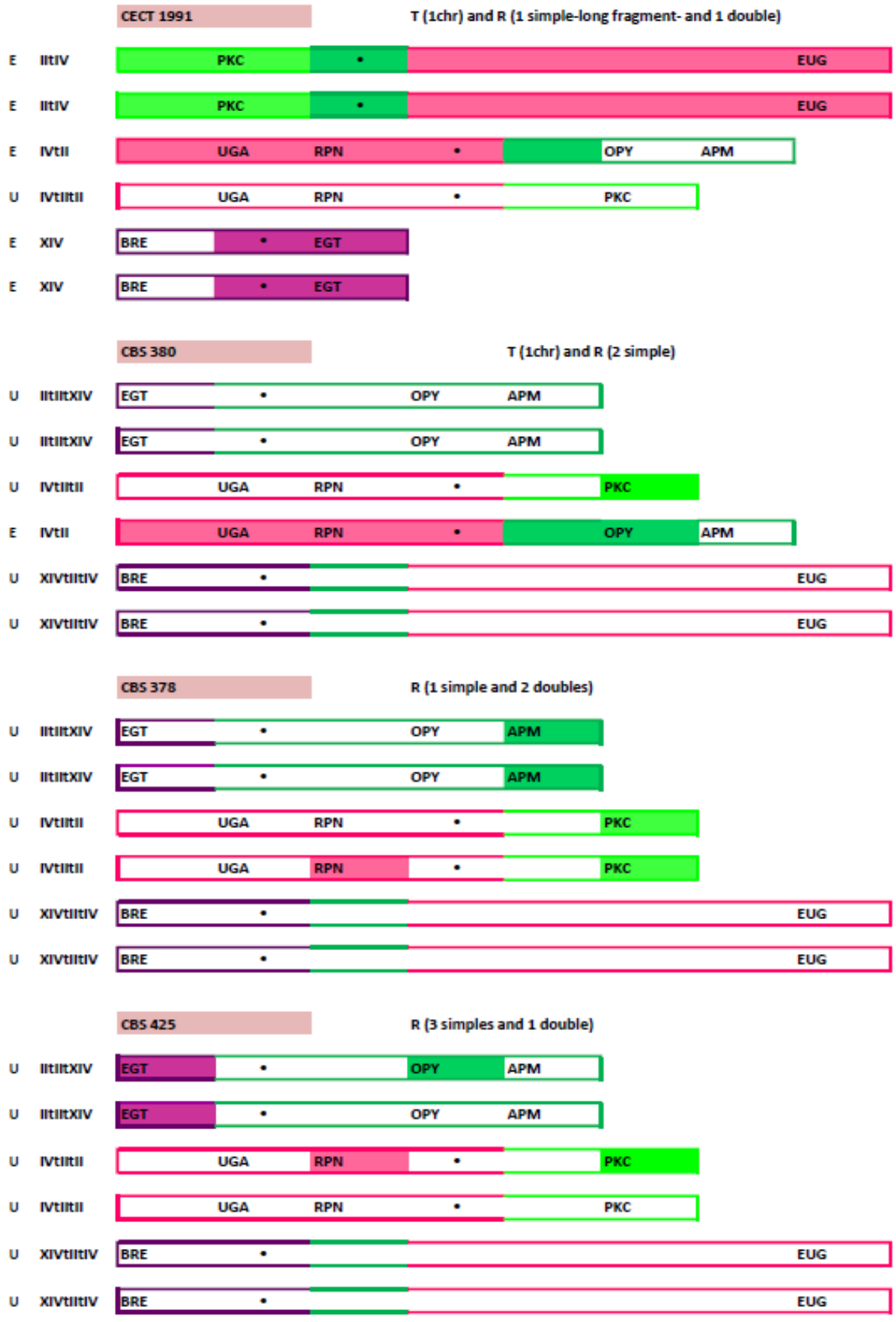
D


S.uvarum order
S. eubayanus order
CBS 7001
NCAIM 676
NCAIM 677
CBS 380
CBS 378
CECT 1941
CBS 375
CECT 1991
CBS 7001
XII
XII
XIVtIItIV
IItIV
VII
VII
IItIItXIV
IVtII
XVI
XVI
XIII
XIII
XVtVIII
XVtVIII
VIIItXV / IVtIItII
VIIItXV / XIV
X
XI
XI
V
V
VItX
XtVI
IX
IX
III
III
VI
I
I

Bands of chromosomes involved in the other two sequential translocations (translocation 2-3) cannot be distinguished in the karyotyping (Figure 1.5). In this case, RFLP data from Pérez-Través et al. (Pérez-Través et al. 2014) were used to deduce the presence/absence of the translocated and non-translocated chormosomes in the hybrids. In cases in which more than one solution was possible, the one requiring less chromosomal rearrangements, i.e. the most parsimonious solution, was chosen. The chromosome composition for the presence/absence of translocated chromosomes generated by the sequential translocations 2 and 3 is shown in Figure 1.6. According to this Figure, translocation 2-3, in heterozygosis, only was present in strains CBS 380, CECT 1991 and CBS1546. In both cases the translocation in heterozygosis implied only a chromosome pair (IVtII and IVtIItII). In the case of strain CECT 1941 we couldn't decipher if translocation was present in heterozygosis or not, because both solutions are plausible. If translocation was present in heterozygosis, it implied only the chromosome pair IVtII and IVtIItII. In order to explain this last situation, two recombination events are needed (in the region of *APM3*, which would have three copies and in the region of *PKC1*, which would have one copy); if translocation wasn't present in heterozygosis, it would imply that two copies of *S. uvarum* chromosome IVtIItII were present. In this case, two recombination events are needed, one for both copies of *PKC1* and another involving the segment *UGA3-RPN4*. In addition, it is postulated that the strains NBRC1948, CECT 11186, CBS 424 and CBS3008 possess the chromosomes from *S. eubayanus* whereas strains CBS375, CBS 378, CBS 425, NCAIM 676 and NCAIM 677 those from *S. uvarum*.




NBRC 1948
E IltIV PKC • EUG
E IltIV PKC • EUG
E IVtII UGA RPN • OPY APM
E IVtII UGA RPN • OPY APM
E XIV BRE • EGT
E XIV BRE • EGT
CECT 11186
R (4 doubles)
E IltIV PKC • EUG
E IltIV PKC • EUG
E IVtII UGA RPN • OPY APM
E IVtII UGA RPN • OPY APM
E XIV BRE • EGT
E XIV BRE • EGT
CBS 424
R (2 doubles)
E IltIV PKC • EUG
E IltIV PKC • EUG
E IVtII UGA RPN • OPY APM
E IVtII UGA RPN • OPY APM
E XIV BRE • EGT
E XIV BRE • EGT
CBS 3008
R (2 doubles)
E IltIV PKC • EUG
E IltIV PKC • EUG
E IVtII UGA RPN • OPY APM
E IVtII UGA RPN • OPY APM
E XIV BRE • EGT
E XIV BRE • EGT


CECT 1991
T (1chr) and R (1 simple-long fragment- and 1 double)
E IltIV PKC • EUG
E IltIV PKC • EUG
E IVtII UGA RPN • OPY APM
U IVtIltII UGA RPN • PKC
E XIV BRE • EGT
E XIV BRE • EGT
CBS 380
T (1chr) and R (2 simple)
U IltIltXIV EGT • OPY APM
U IltIltXIV EGT • OPY APM
U IVtIltII UGA RPN • PKC
E IVtII UGA RPN • OPY APM
U XIVtIltIV BRE • EUG
U XIVtIltIV BRE • EUG
CBS 378
R (1 simple and 2 doubles)
U IltIltXIV EGT • OPY APM
U IltIltXIV EGT • OPY APM
U IVtIltII UGA RPN • PKC
U IVtIltII UGA RPN • PKC
U XIVtIltIV BRE • EUG
U XIVtIltIV BRE • EUG
CBS 425
R (3 simples and 1 double)
U IltIltXIV EGT • OPY APM
U IltIltXIV EGT • OPY APM
U IVtIltII UGA RPN • PKC
U IVtIltII UGA RPN • PKC
U XIVtIltIV BRE • EUG
U XIVtIltIV BRE • EUG

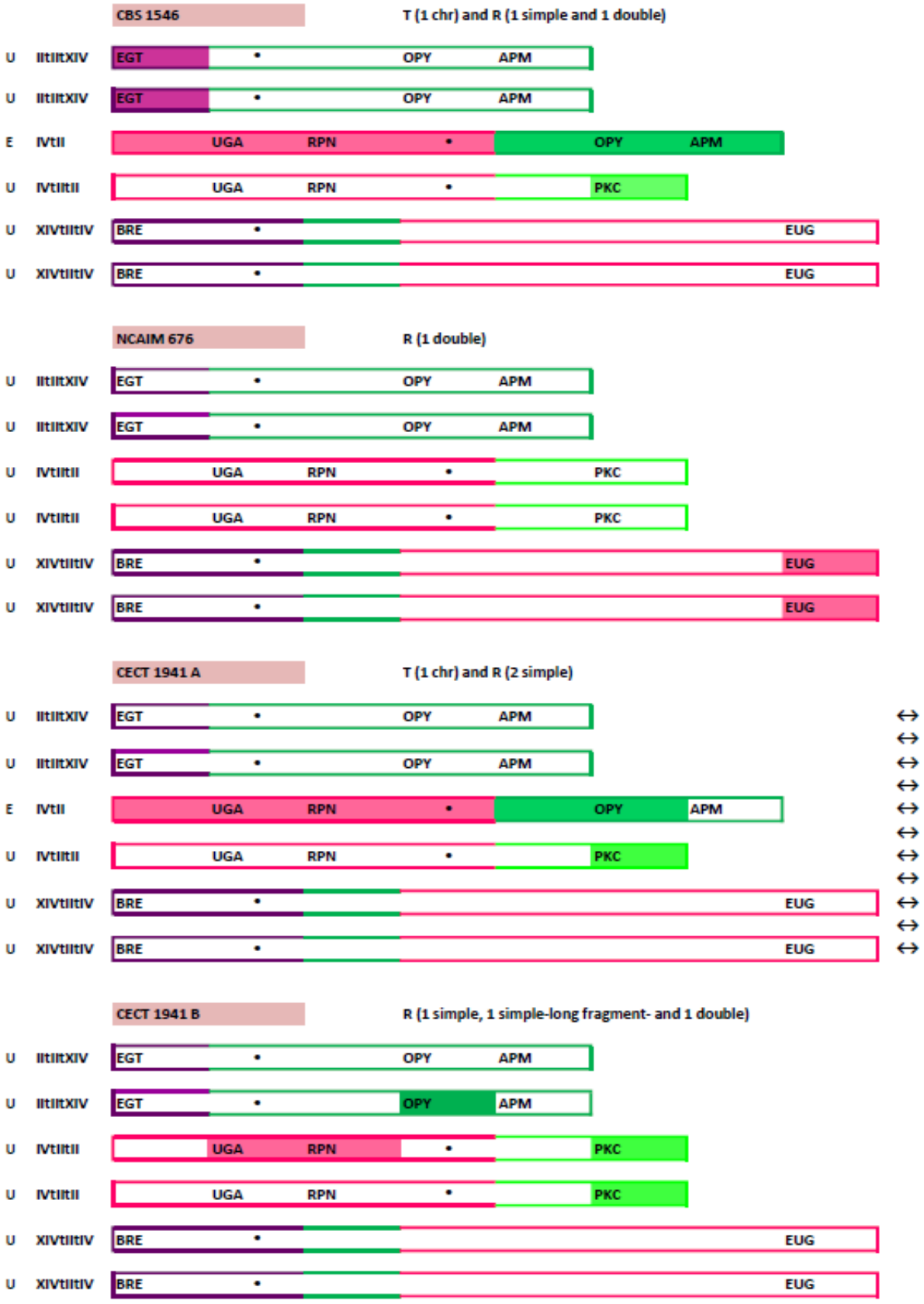

CBS 1546
T (1 chr) and R (1 simple and 1 double)
U IltIltXIV EGT • OPY APM
U IltIltXIV EGT • OPY APM
E IVtII UGA RPN • OPY APM
U IVtIltII UGA RPN • PKC
U XIVtIltIV BRE • EUG
U XIVtIltIV BRE • EUG
NCAIM 676
R (1 double)
U IltIltXIV EGT • OPY APM
U IltIltXIV EGT • OPY APM
U IVtIltII UGA RPN • PKC
U IVtIltII UGA RPN • PKC
U XIVtIltIV BRE • EUG
U XIVtIltIV BRE • EUG
CECT 1941 A
T (1 chr) and R (2 simple)
U IltIltXIV EGT • OPY APM
U IltIltXIV EGT • OPY APM
E IVtII UGA RPN • OPY APM
U IVtIltII UGA RPN • PKC
U XIVtIltIV BRE • EUG
U XIVtIltIV BRE • EUG
CECT 1941 B
R (1 simple, 1 simple-long fragment- and 1 double)
U IltIltXIV EGT • OPY APM
U IltIltXIV EGT • OPY APM
U IVtIltII UGA RPN • PKC
U IVtIltII UGA RPN • PKC
U XIVtIltIV BRE • EUG
U XIVtIltIV BRE • EUG

Figure 1.6. Chromosomal composition of S. bayanus strains for translocation 2-3. The most probable chromosomic composition is is indicated; if more than one solution is possible, both options are indicated.**Unfilled**: *S. uvarum* genes; **filled**: *S. eubayanus* genes. **Dot**: centromera; if unfilled, *S. uvarum* centromera, if filled, *S. eubayanus* one. **Green**: chromosome II (both green colors indicates the internal recombination of this chromosome). **Pink**: chromosome IV. **Purple**: chromosome XIV. Chromosome and procedence is indicated on the left of each chromosome. In each case, recombinations (**R**) or translocations (**T**) presents are indicated, as well as the number and the kind. The complete name of the genes is: *EGT2*, *OPY1*, *APM3*, *UGA3*, *RPN4*, *PKC1*, *BRE5* and *EUG1*.

Summarizing, *S. bayanus* type I hybrids can be subdivided in two groups according to their chromosomal composition: Ia) diploid strains NBRC 1948 and CECT 11186 with chromosomes from *S. uvarum* for translocation 1 and chromosomes from *S. eubayanus* for translocations 2-3, and Ib) triploid strains CBS 424 and CBS 3008 with *S. eubayanus* chromosomes for all translocations.

S. bayanus strains belonging to type II hybrids can also be subdivided in four groups: IIa) diploid strains CBS 375, CBS 378, NCAIM 676 and NCAIM 677 with translocation 1 in heterozygosis and *S. uvarum* chromosomes for translocations 2-3; IIb) diploid strains CBS 380 and CBS 1546 with all translocations in heterozygosis, with translocation 2-3 involving only chromosomes IVtII and IVtIItII and the rest of chromosomes inherited from *S. uvarum*; IIc) diploid strain CECT 1991, with all translocations in heterozygosis, translocation 2-3 involving only chromosomes IVtII and IVtIItII and the rest of chromosomes coming from the *S. eubayanus* parent; and IId) diploid strain CBS 425, with *S. uvarum* chromosomes for all translocations. Finally, strain CECT 1941 could be included in subgroups IIa or IIb.

1.7.3. Hybrid fertility and its relation with the level of heterozigosity.

We carried out a regression analysis to evaluate the possible correlation between hybrid fertility, measure through its spore viability, and hybrid heterozigosity. If there is a good correlation, we can estimate the heterozigosity level at which the fertility of the hybrids is

drastically reduced. This information could be useful to detect whether the genetic divergences are enough to consider *S. uvarum* and *S. eubayanus* as different species.

Hybrids strains CBS 424 and CBS 3008 presented spore viability lower than 25%, ploidy values close to 3n and didn't exhibit any translocation in heterozygosis (Table 1.6). The low spore viability values showed by these two strains could be due to their higher ploidy values rather than to the genomic divergences. For this reason, we did not include these strains in this analysis.

As translocations reduced the viability of a strain when they are found in heterozygosis, spore viabilities were corrected according to the reduction expected due to the presence of translocations in heterozygosis (50% reduction per translocation). Therefore, spore viabilities for *S. bayanus* type II strains were corrected with the exception of CBS 425 that exhibits no translocations (Figures 1.6 and S1.4). In the case of type II hybrid CECT 1941, as we couldn't decipher if translocation was present in heterozygosis or not, both options were considered to correct spore viability levels.

Figure 1.7 depicts the correlation between corrected spore viability of hybrids (Table 1.6) and their heterozygosity levels. When 3-5 heterozygous genes (U/E) were present, corrected spore viability was ~100%. This maximum values drastically drop to 3-18% when strains possess 7 or more heterozygous genes. Finally, hybrids with 14 or more heterozygous genes not only showed very low spore viability but also a reduced sporulation capability.

Data were adjusted to lineal, exponential decay and sigmoidal curves, but the higher correlation was found with a sigmoidal adjustment (Figure 1.7). In all cases, when heterozygosity frequency was 0.208 (m value), viability values drastically reduced.

The asymptotic value of the plot was situated to 0.0528 and 0.0479. Based on this result, a perfect collinear heterozygous hybrid between *S. uvarum* and *S. eubayanus* would exhibit a spore viability level of 5.3% or 4.8%. When these values were corrected by the three translocations present between both species, the spore viability level is reduced to 1.8-2%.

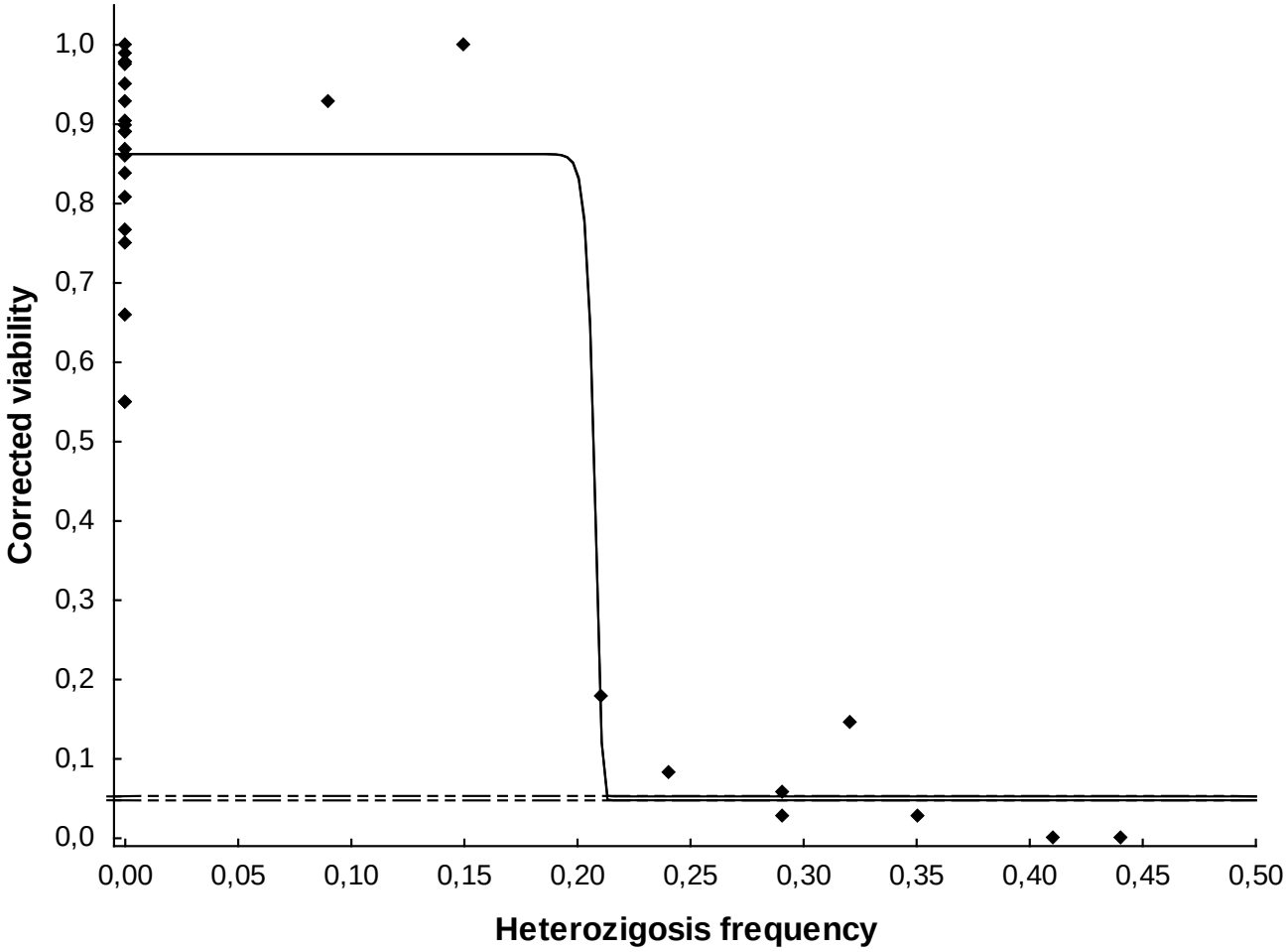


Figure 1.7. Regresion analysis of the corrected viability versus the heterozigosity frequence. Data were fitted with a sigmoid curve. The two curves fitted shared a R^2 value of 0. 895. Dotted lines indicated the asymptotic values to which both equations would approximate.

1.7.4. F1 segregants analysis.

Finally we selected two Type II hybrid strains, with different number of heterozygous positions, to analyze the fertility of their F1 progenies (Table 1.6). These strains were NCAIM 677 (5 heterozygous genes out of 33) and CBS 375 (11/33 heterozygous genes). Spore viability of their F1 was analyzed, for this reason, five F1 monosporic cultures were obtained for each hybrid, and sporulated on acetate media and their spores dissected. Spore viability values in the F1 progeny were higher than those for their parental strains (Table 1.7). In both cases, spore viability values

increased to values higher than 50% and in the range of those values exhibited by several *S. uvarum, S. eubayanus* or *S. bayanus* type I strains.

Table 1.7. Comparison between viability values of two type II '*bayanus*' and its descendence.

Strain	Viability	F1 viability
NCAIM 677	53,13%	86,50±9.71%
CECT 11135	7,35%	52,00±5.35%

1.8. Discussion

The existence of two groups within the species *S. bayanus* was always a known fact, but their separation into varieties (Vaughan-Martini and Kurtzman 1985; Naumov 2000) or species (Nguyen and Gaillardin 1997; Rainieri et al. 2006; Nguyen et al. 2011) was unclear until the discovery of *S. eubayanus* (Libkind et al. 2011). From this point *S. bayanus var uvarum* was considered as independent species (*S. uvarum*) and *S. bayanus var bayanus* as a group of hybrid strains between *S. uvarum* and *S. eubayanus*. *S. eubayanus* was initially isolated from the Argentinean Patagonia (Libkind et al. 2011), although now it has been isolated from other American regions, particularly other parts of Argentinean Patagonia and California, USA (Peris et al. 2014; Rodríguez et al. 2014), and Asia (Bing *et al.* 2014). The presence of hybrids between the two specie, isolated in European regions, give rise to two theories: that hybrids (*S. bayanus*) were originated from American *S. eubayanus* strains because this species "may have been absent in Europe until it was imported from overseas after the advent of trans-Atlantic trade"(Libkind et al. 2011) or that the search for this species in Europe has been unsuccessful, usually because of ignorance of its niche (Gibson *et al.* 2013; Pérez-Través *et al.* 2014). The discovery of this Asian *S. eubayanus* population agrees with the second hypothesis, but focused the hybrids origin in the Far East Asia (Naumov and Nikonenko 1988; Naumov et al. 2003; Bing et al. 2014).

1.8.1. Speciation between *S. uvarum* y *S. eubayanus*

Speciation in *Saccharomyces* genus through reproductive isolation could occur by premating or postmating barriers (Maclean and Greig 2008; Greig 2008). Premating ones seems to be weak, because these barriers may be caused by differences in the timing of spore germination and mating. Between the species there are, also, mate preferences, but hybrids could be formed when the nearest available mate is another specie. These weak barriers are evidenced by the large number of hybrids and introgressed strains in *Saccharomyces* genus (Liti et al. 2006). Ecological barriers may play an important role, because limit this contact and avoid hybrids formation. Postmating barriers are stronger than premating ones among species of this genus. Genetic distance, through the mismatch repair system (antirrecombination), seems to be the major factor that causes hybrid sterility (Maclean and Greig 2008; Greig 2008), but chromosomal rearrangements are important in speciation, as it was evidenced for *S. paradoxus* and *S. cariocanus* (Liti et al. 2006; Greig 2008) or for *S. cerevisiae* and *S. mikatae* (Delneri et al. 2003). No role in reproductive isolation was found for genetic incompatibilities (Coyne and Orr 2004; Greig 2008; Kao et al. 2010).

Doing away the translocations' effect, when we analyzed the heterozygosity level found in *S. bayanus* strains vs the corrected viability values, it fitted with a sigmoid adjustement (r^2 0.9) which inflexion point was situated in a heterozygosis frequency of 20.8%. At this point, viability values were reduced drastically in all the cases, and in a complete heterozygous hybrid, the viability values fell away to 5.3-4.8%, indicating that heterozygosity is responsible of part of the reproductive isolation between both species in these evolved hybrids. Nonetheless how this heterozygosity works allowing the reproductive isolation is unknown. As it was said, antirrecombination seems to be the major factor that causes hybrid sterility between collinear *Saccharomyces* species (Maclean and Greig 2008; Greig 2008), but, due to previous results, is hard to believe that it is the responsible for the speciation process between *S. uvarum* and *S. eubayanus* strains. In Pérez-Través et al. ((2014); Fig. 2) we can observe that recombination in the ancestral *S. uvarum*/*S. eubayanus* hybrid was allowed and no chromosomes were restricted to this success.

According to the genetic divergence, in one hand, *S. uvarum* and *S. eubayanus* are the closest sister species of the *Saccharomyces* genus, closer than *S. cerevisiae* and *S. paradoxus* (8.6% vs 15.6% of nucleotide differences between the studied genes). On the other hand, they are the species located on the base of the tree - the firsts ones to be differentiated from the rest of the genus - and the ones that had more time to separate, in a phylogenetically sense; but our data indicate that they haven't done it at the level of nucleotide divergence. This is another fact in favor of other mechanisms of speciation, different to the genetic distance, among *S. uvarum* and *S. eubayanus.*

The other factor that acts in S. *uvarum* and *S. eubayanus* speciation are translocations. The presence of these translocations, studied alone, would reduce the hybrid viability in an 87.5%, leaving only the 12.5% of viable spores. Translocation 1, with two chromosomes implied (*S. eubayanus* chromosomes VI and X and *S. uvarum* VItX and XtVI), would reduce the viability in a 50%, and translocation 2-3 with three chromosomes involved (*S. eubayanus* chromosomes IItIV, IVtII and XIV and *S. uvarum* IItIItIV, IVtIItII and XIVtIItIV), would reduce the viability in a 75%.

When crosses between wild populations of *S. uvarum* and *S. eubayanus* strains are made, we found that all the crosses presented diminished viability values, but, for the moment, only the West China *S. eubayanus* strain is completely isolated from Tibetan *S. uvarum* strains. In the rest of the cases the reproductive isolation is only partial (5-19% of spore viability), being stronger between *S. uvarum* and *S. eubayanus* Asian populations (5-10%) and between South American *S. eubayanus* and *S. uvarum* SA-A population (7.3-8.5%) (Bing et al. 2014; Almeida et al. 2014). These results indicate that it exist a higher postzygotic isolation between sympatric populations than between allopatric ones, diminishing the importance of ecological barriers between *S. uvarum* and *S. eubayanus* populations.

An important proportion of reproductive isolation between *S. uvarum* and *S. eubayanus* is due to the presence of translocations. Between the analyzed strains, differences in reproductive isolation can be seen, finding viability values around the 19%, in some crosses (Almeida et al. 2014). In other species, it has been seen population differences in the presence of chromosomal changes.

For these reasons the chromosome composition of the *S. eubayanus* strains should be explored further to explain the differences in reproductive isolation.

1.8.2. *Type I S. bayanus hybrids correspond to a homoploid hybrid species?*

Hybridization could have a range of outcomes, including the emergence of new species, the development and transfer of adaptations through introgressive hybridization, the formation of stable hybrid zones, the reinforcement of premating reproductive barriers and the origin of new homoploid hybrid species (Yakimowski and Rieseberg 2014). Even though homoploid hybrid speciation is infrequent it is particularly favored when hybrids invade novel or extreme habitats in which parental species are absent (Gompert et al. 2006). This is a speciation form wherein hybridization between two different species leads to a new species, without change in chromosome number and with partial or complete reproductive isolation from parental species (Rieseberg 1997).

Saccharomyces yeasts species are postzigotically isolated; even though hybrids form readily, they are sterile (they produce around 1% of viable spores). Because of yeasts populations can be very large, some viable gametes can be easily obtained. Moreover, the ability of *Saccharomyces* to switch mating type allows for self-fertilization. Despite their postzigotical isolation, these characteristics of *Saccharomyces* yeasts made them potentially susceptible for homoploid hybrid speciation.

Homoploid yeast hybrid speciation has been performed successfully under laboratory conditions from artificial *S. cerevisiae* x *S. paradoxus* hybrids (Greig et al. 2002a; Greig 2008). In nature, if given a choice to mate with, some species are able to avoid hybridization. When no mate choice was available, hybridization occurs (Maclean and Greig 2008). It occurs readily than in plants or animals and is partly due to the yeast ability to self-fertilize, which produces identical homolog pairs for every chromosome (except at the mating-type locus on chromosome III).

As a general rule, the strains classified as *S. bayanus* type I hybrids seemed to suffer this kind of speciation. Analyzing these hybrids to see if they meet the criteria suggested by Schumer

et al., (2014) for detect homoploid hybrid specie, we can observe the following. Criteria 1- Showing reproductive isolation from parental specie: although the reproductive isolation with their parental specie, at least with *S. uvarum* (Naumov 2000), is partial, there are ecological barriers (premating ones) between *S. bayanus* and *S. uvarum* or *S. eubayanus*, avoiding hybrids formation in nature (backcrosses). *S. eubayanus* is a species isolated only in nature (Libkind et al. 2011; Bing et al. 2014; Almeida et al. 2014; Peris et al. 2014; Rodríguez et al. 2014), *S. uvarum* can be found in natural environments, must, wine and cider and *S. bayanus* is mainly found in beer fermentations and in other musts; *S. bayanus* could be ecologically isolated from *S. uvarum* and *S. eubayanus.* Criteria 2- documenting past hybridization: RFLP data, sequences data and karyotype data are enough to document the hybridization between both parental strains. Criteria 3 - demonstrating that isolating mechanisms were derived from hybridization: The combination of translocations in the hybrid strains vs the parental ones could lead to a partial isolation between them.

Due to the isolation sources of *S. uvarum* and *S. eubayanus*, hybridization must take place in natural environments. These complete hybrids must colonize the fermenting environment of lager beer (and other musts) and thrive in it. During this process, changes in their genome were produced and *S. bayanus* type II hybrids appeared, reaching large population sizes. At this point, type II hybrids sporulate and the few viable spores autodiploidizate, becoming type I hybrids. These homozygous hybrids recovered the fertility and converted in a potential homoploid hybrid specie. *S. bayanus* type II hybrids are a reservoir of *S. bayanus* type I hybrids, being the previous step to potential homoploid hybrid speciation.

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Capítulo 2

Chapter 2. Evaluation of different genetic procedures for the generation of artificial hybrids in *Saccharomyces* genus for winemaking

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Evaluation of different genetic procedures for the generation of artificial hybrids in *Saccharomyces* genus for winemaking

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ARTICLE INFO

Article history:
Received 26 September 2011
Received in revised form 4 March 2012
Accepted 7 March 2012
Available online 14 March 2012

Keywords:
Saccharomyces cerevisiae
Saccharomyces kudriavzevii
Artificial hybrids
Rare-mating
Spore to spore mating
Genetic stabilization

ABSTRACT

Several methods based on recombinant DNA techniques have been proposed for yeast strain improvement; however, the most relevant oenological traits depend on a multitude of loci, making these techniques difficult to apply. In this way, hybridization techniques involving two complete genomes became interesting. Natural hybrid strains between different *Saccharomyces* species have been detected in diverse fermented beverages including wine, cider and beer. These hybrids seem to be better adapted to fluctuating situations typically observed in fermentations due to the acquisition of particular physiological properties of both parental strains. In this work we evaluated the usefulness of three different hybridization methods: spore to spore mating, rare-mating and protoplast fusion for the generation of intra- and inter-specific stable hybrids, being the first report about the comparison of different methods to obtain artificial hybrids to be used in fermentations. Spore to spore mating is an easy but time-consuming method; hybrids generated with this technique could lack some of the industrially relevant traits present in the parental strains because of the segregation occurred during meiosis and spore generation prior to hybridization. Hybrids obtained by protoplast fusion get the complete information of both parents but they are currently considered as genetically modified organisms (GMOs). Finally, hybrids obtained by rare-mating are easily obtained by the optimized methodology described in this work, they originally contain a complete set of chromosomes of both parents and they are not considered as GMOs. Hybrids obtained by means of the three methodological approaches showed a high genetic variability; however, a loss of genetic material was detected in most of them. Based on these results, it became evident that a last crucial aspect to be considered in every hybridization program is the genetic stabilization of recently generated hybrids that guarantee its invariability during future industrial utilization. In this work, a wine yeast genetic stabilization process was developed and vegetatively stable hybrids were obtained.

http://www.sciencedirect.com/science/article/pii/S0168160512001274

2.1. Introduction

Wine fermentation has been traditionally performed by *Saccharomyces cerevisiae* strains naturally present on grapes and wine equipment or artificially inoculated as the form of a starter culture. Although hundreds of starter cultures are commercially available in the market, producers and consumers are continuously pressing for new improved yeast strains able to produce distinctive and specific products (Cebollero et al., 2007; Pretorius, 2000).

The availability of natural yeast strains possessing an ideal combination of desirable industrial characteristics, according to the actual requirements in the market, is highly improbable. Additionally, the most important oenological traits such as fermentative vigour, ethanol production and tolerance, volatile acidity production and growth temperature profile among others, depend on a multitude of loci (QTLs) distributed throughout the genome and their unpredictable interactions (Giudici et al., 2005; Marullo et al., 2004). These facts, as well as the presence of a large number of allelic variants, the high heterozygosity degree and the presence of aneuploidies or polyploidies in wine yeasts (Barre et al., 1993; Codón and Benítez, 1995; Naumov et al., 2000), make whole-genomics blind approaches the most adequate methodologies to be used in the generation of new industrial strains (Giudici et al., 2005). In this context, hybridization of two complete genomes is one of the best methods to be taken into account. Hybridization among closely related species of *Saccharomyces* has been proposed as a natural mechanism involved in the adaptation of these yeasts to industrial processes (Barrio et al., 2006; Querol and Bond, 2009). A post-zygotic barrier usually prevents the production of viable spores; however, stable hybrids are generated among different species of this genus. Natural hybrids have been found in different fermentation processes (Masneuf et al., 1998; Groth et al., 1999; González et al., 2006), being the most studied examples *S. pastorianus* (Vaughan-Martini and Kurtzman, 1985) and the type strain of *S. bayanus* (Masneuf et al., 1998; Nguyen et al., 2000).

As a general rule, hybrids are better adapted to intermediate or fluctuating situations due to the acquisition of physiological properties of both parental strains (Belloch et al., 2008; González et al., 2006). In this way, several research laboratories in the world have made use of

diverse classic hybridization methods including spores to spores or spores to haploid cells mating, rare-mating or protoplasts fusion to generate hybrid strains with desirable features for different industrial processes or basic studies (Sipiczki, 2008 and Table S2.1).

The selection of the hybridization method to be used will be closely related with the final destination of the generated hybrid. The use of genetically modified organisms (GMOs) in food is limited by current legislations in different countries, as well as by public concern (Cebollero et al., 2007; Pretorius and Hoj, 2005; Schilter and Constable, 2002). As it was established in the Directive 2001/18/EC of the European Parliament and the Council of the European Union, a GMO is an organism whose genetic material has been altered in a way that does not occur naturally by mating or natural recombination. According to this definition, hybrids generated by mating of spores and rare-mating –based on the natural rare event of mating type switching in industrial yeasts- must not be considered as GMOs. Contrarily, protoplast fusion is an artificial hybridization method based in the fusion of yeast cells, previously subjected to enzymatic cell wall degradation, in the presence of a fussing agent as polyethylene-glycol (Curran and Bugeja, 1996). Consequently, hybrids generated by protoplast fusion are considered as GMOs according to the European Union legislations and, for that reason, its use has been generally limited to basic studies or industrial processes not involving foods (Kucsera et al, 1998; Law et al., 1993; Nakazawa and Iwano, 2004). On the other hand, hybrids obtained by spore to spore mating could lack some of the industrially relevant traits present in the parental strains because of the segregation occurred during meiosis and spores generation prior to hybridization (Caridi et al., 2002; Gimeno-Alcañiz and Matallana, 2001; Marullo et al., 2004).

In this work we evaluated the usefulness of two different non GMO producing hybridization methods (spore to spore mating and rare-mating) for intra and interspecific wine yeasts hybrids generation. The traditional technique of protoplast fusion was also done with comparative purposes. Finally, a genetic stabilization procedure was proposed and inter-specific vegetatively stable hybrids were obtained.

2.2. Materials and methods

2.2.1. *Yeasts*

Two selected *Saccharomyces cerevisiae* commercial strains provided by Lallemand Inc. (*Sc1* and *Sc2*) and the type strain of *S. kudriavzevii* NBRC 1802 (*Sk*) were used in the present study. *Sc1* parental was selected for its high polysaccharides release capacity inducing color stability. *Sc2* was selected for its excellent fermentative vigor and low volatile acidity production. *Sk* was selected for its cryotolerance and its capacity to produce high levels of glycerol. The three analyzed strains were homothallic.

2.2.2. *Generation of natural auxotrophic colonies from parental yeasts*

For the selection of natural auxotrophic parental strains, *Saccharomyces cerevisiae* (Sc1 and Sc2) and *S. kudriavzevii* (Sk) cells were grown on 15 mL of GPY medium (% w/v: yeast extract 0.5, peptone 0.5, glucose 2) during 5 days at 28ºC. Aliquots of each culture were seeded onto α-aminoadipic (α-AA) and fluoroorotic acid (5-FOA) agar plates in order to select *lys*$^-$ and *ura3*$^-$ natural mutant colonies respectively (Boeke et al., 1987; Zaret and Sherman, 1985). One milliliter of each culture was also seeded in 15 mL of fresh GPY medium and incubated again in the same conditions. This process was repeated over 4 times.

Colonies that were able to grow on α-AA or 5-FOA plates were picked again on a new α-AA or 5-FOA plate respectively. In order to confirm the presence of the auxotrophy, cells were grown on starvation medium (0,1% w/v of Yeast Nitrogen Base without amino acids supplemented with 0,1% w/v of glucose) during 4 h at 28ºC. Subsequently, cell suspensions were spotted onto GPY-A (GPY medium with 2% w/v agar-agar), Minimal medium (MM; 0,17% Yeast Nitrogen Base without aminoacids, 2% glucose and 2% agar) and MM supplemented with proline (1 g/L) and uracil (10 mg/L) or lysine (30 mg/L. Plates were incubated during 5 days at 28ºC.

2.2.3. Hybrid generation

2.2.3.1. Protoplast fusion

The method described by Curran and Bugeja (1996) was used with slight modifications. Strains carrying the auxotrophic markers were grown separately in GPY medium (25 mL) for 48 h at 28°C, recovered by centrifugation (3000 x g during 5 min at room temperature) and washed twice in sterile cold water. Cells were resuspended in 10 mL of protoplasting solution (sorbitol 1.2 M; Tris 0.1 M; EDTA 0.02 M; pH: 9.8) with 50 µL β-mercapto-ethanol, and incubated for 15 minutes at room temperature. The cells were washed with sorbitol 1.2 M, resuspended in 10 mL of sorbitol 1.2 M with 500 µg of zymolyase 20T (1µg/µl) (Seikagaku Corporation, Tokyo, Japan) and incubated for 60 minutes at 30°C. Protoplast formation was monitored by diluting the suspension in water and optical density decrease observation (600 nm) as a consequence of protoplast burst. Protoplasts were washed twice with sorbitol 1.2 M.

For protoplast fusion, 2 x 10^7 protoplasts from each parental strain were mixed and treated with 2 mL of 60% polyethylene-glycol (molecular weight 6000) and 100 mM $CaCl_2$ for 30 minutes. Cells were washed twice by centrifugation with sorbitol 1.2 M. Appropriate dilutions of cells (1/10, 1/50 y 1/100) in sorbitol 1.2 M were embedded into 5 mL of GPY-A molten agar medium containing sorbitol 1.2 M, overlayed onto pre-solidified MM and incubated at 26°C. After 3-7 days, the observed colonies were isolated and purified by restreaking on the same medium.

2.2.3.2. Rare-mating

Rare-mating assays were carried out according to the procedures proposed by Spencer and Spencer (1996) with some modifications. Strains carrying the auxotrophic markers were grown separately in 25 mL GPY broth for 48 h at 28°C. Cells were recovered by centrifugation (3000 x g for 5 min at room temperature) and resuspended in the residual supernatant. Pairs of yeast cultures to be hybridized were placed together in the same tube and aliquots of these mixed strains were inoculated in 2 mL of fresh GPY medium. After 5-10 days of static incubation in slanted position at 28°C, cells were recovered by centrifugation (3000 x g for 5 min at room temperature), washed in sterile water, resuspended in 1 mL of starvation medium and

incubated for 2 h. A heavy suspension of the mixed culture was spread on MM plates and incubated at 28°C. Prototrophic colonies usually appeared after 3-5 days. These colonies were isolated and purified by restreaking on the same medium.

2.2.3.3. Spore to spore mating

Yeast sporulation was induced by incubation on acetate medium (% w/v: CH_3COONa 1, Glucose 0.1, yeast extract 0.125 and agar 2) for 5-7 days at 28°C. Following preliminary digestion of the asci walls with 2 mg/mL glucuronidase (Sigma), pairs of different parental strains were seeded in the same GPY agar plate. Spores from both parental strains were dissected and placed together using a Singer MSM Manual micromanipulator. After incubation at 28°C for 3-5 days, all developed colonies were transferred to selection media plates. The colonies capable of growth in the selection media were isolated and purified by restreaking on the same media.

All protoplast, rare-mating and spore to spore generated hybrid strains were immediately placed in cryogenic vials containing glycerol 15% and conserved at -80°C in order to minimize possible genetic changes.

2.2.4. Molecular analyses

Yeast total genomic DNA was obtained according to standard procedures (Querol et al., 1992).

2.2.4.1. Restriction fragment length polymorphism of amplified DNA products (PCR-RFLP) analysis

The characterization of *Saccharomyces* hybrids obtained from interspecific crosses was performed by PCR amplification of *BRE5* and *PPR1* protein-encoding nuclear genes and subsequent RFLP analysis with the restriction enzyme *Hae* III (Takara Bio Inc., Shiga, Japan) following the methodology proposed by González et al. (2008).

2.2.4.2. *Microsatellite analysis*

Two microsatellite loci (named O and P and located in chromosomes XV and XVI in *S. cerevisiae*) were selected for the differentiation of the *Sc1* and *Sc2* parental strains used in intraspecific mating. Microsatellite analysis was carried out according to Bradbury et al. (2006) with modifications. Primer sets YOR267c-3 and YOR267c-5 for microsatellite O and YPL009c-3 and YPL009c-5 for microsatellite P, based on those previously described by Bradbury et al. (2006) and Legras et al. (2005), were enlarged according to the following sequences: YOR267c-3 5'-CTCTTTTCTTTGGATCTACTTGCAGTATACGG-3´, YOR267c-5 5'-AAGTTGATACTAACGTCAACACTGCTGCCAA-3´, YPL009c-3 5'-CGTATTTCTTTTGAATTTCTTCAATTTTCCTCTTTTACCAC-3´and YPL009-c 5'-CTGCTCAACTTGTGATGGGTTTTGGATTTTTATGGA-3´. Reverse primers were labeled with the fluorogenic compounds 6-carboxyfluorescein (FAM) or hexachlorofluorescein (HEX) (Applied Biosystem, Foster City, USA. PCR products were analyzed in an ABIprism 310 sequencer and the results were evaluated using the Peak Scanner software v1.1 (Applied Biosystems, Foster City, USA.

2.2.4.3. *Random Amplified polymorphic DNA (RAPD) analysis*

Eleven different primers (OPA2, 3, 7, 8, 9, 10, 11, 15 and 16; R1 and R3) previously reported by Fernández-Espinar et al. (2003), and Corte et al. (2005) were used to carry out RAPD analysis.

2.2.4.4. *Amplified inter-δ sequence DNA polymorphism analysis*

Primers delta 12 (5´-TCAACAATGGAATCCCAAC-3´) and delta 21 (5´-CATCTTAACACCGTATATGA-3´) as well as procedures proposed by Legras and Karst (2003) were used to amplify the yeast genomic DNA.

2.2.4.5. Mitochondrial DNA-restriction fragment length polymorphism (mtDNA-RFLP) analysis

Mitochondrial DNA restriction analysis was performed by the method of Querol et al. (1992) using the endonuclease *Hinf* I (Roche Molecular Biochemicals, Mannheim, Germany).

2.2.5. Flow cytometry

The DNA content of each parental and hybrid strain was assessed by flow cytometry using a FACScan cytometer (Becton Dickinson Inmunocytometry Systems, Palo Alto, California, United States) following the methodology described in Lopes et al (2010). DNA content values was scored on the basis of the fluorescence intensity compared with the *S. cerevisiae* haploid (S288c) and diploid (FY1679) reference strains. DNA content value reported for each strain is the result of two independent measures. Results were tested by one way ANOVA and Tukey HSD test (α=0.05, n=2).

2.2.6. Spores viability

Sporulation and ascus dissection in the hybrid strains were carried out as mentioned previously for spore to spore mating. Spores viability was calculated as the percentage of spores (from a total of 40 analyzed spores for each hybrid strain) that were able to form a colony on GPY agar after 48-72 h at 26°C.

2.2.7. Genetic stabilization

Four hybrid colonies (two obtained by rare-mating and two by spore to spore mating) from each cross (intraspecific and interspecific) were subjected to the same genetic stabilization procedure.

Each selected hybrid strain was individually inoculated into 25 ml screw cap tubes containing 20 mL of synthetic must (Rossignol et al., 2003) and incubated at 20°C without shaking. After fermentation (approximately 15-20 days), an aliquot was used to inoculate a new tube containing the same sterile medium and incubated in the same conditions. After five

successive fermentations, an aliquot of the fifth fermentation was seeded on GPY-agar plates and incubated at 20°C. Ten yeast colonies were randomly picked and characterized by mtDNA-RFLP, inter-δ sequences and RAPD (using primer R3) analyses, as well as DNA content, as previously mentioned. Simultaneously, the same colonies were inoculated in synthetic must and, after these individual fermentation, ten colonies from each one were analyzed by the same methods. We considered a genetically stable hybrid when the colonies recovered after individual fermentation maintained the same molecular pattern for the three markers and the same DNA content than the previously inoculated (original) culture.

2.3. Results

In order to generate artificial hybrids, both physiological and molecular differences among the parental strains must be established. Physiological differentiation became necessary to select an appropriate medium for hybrids recovery. Molecular characterization must be focused on finding markers to confirm the success of hybridization as well as the genetic variability among the generated hybrids.

2.3.1. Selection of hybridization markers in parental strains

α-aminoadipic (α-AA) and 5-fluoroorotic acid (5-FOA) containing agar plates were used to select natural *lys*$^-$ and *ura3*$^-$ auxotrophic colonies respectively from the three parents. Different numbers of colonies were obtained for each parental strain in both selection media. However, only 54% of them exhibited auxotrophic behavior, i.e. they were able to grow in GPY and minimal medium supplemented with lysine or uracil (depending on the origin of isolation; α-AA and 5-FOA agar plates respectively), but not in minimal medium without supplements. Some of these stable auxotrophic colonies (*Sc1 ura3*$^-$, *Sc2 lys*$^-$ and *Sk lys*$^-$) were used for hybrids generation.

Different molecular markers were evaluated to be used to confirm the success of hybridization as well as the genetic variability among the generated hybrids. Two microsatellite regions (O and P) were evaluated to be used in the confirmation of intraspecific (*Sc1* x *Sc2*)

hybrids generation. The length of alleles (bp) showed by each parental strain for both O and P microsatellite regions are shown in Table 2.1. Only one allele was observed for each microsatellite region in *Sc1*, while two different alleles were observed in *Sc2*. The different allele sizes in both *Sc1* and *Sc2* parents allowed us to use them for intraspecific hybrids confirmation.

Following the methodology proposed by González et al. (2008), PCR-RFLP analysis of genes *PPR1* and *BRE5* rendered *S. cerevisiae* and *S. kudriavzevii* specific patterns for *Sc1* and *Sk* parents respectively (data not shown). These differences were then used for the confirmation of interspecific hybrids generation.

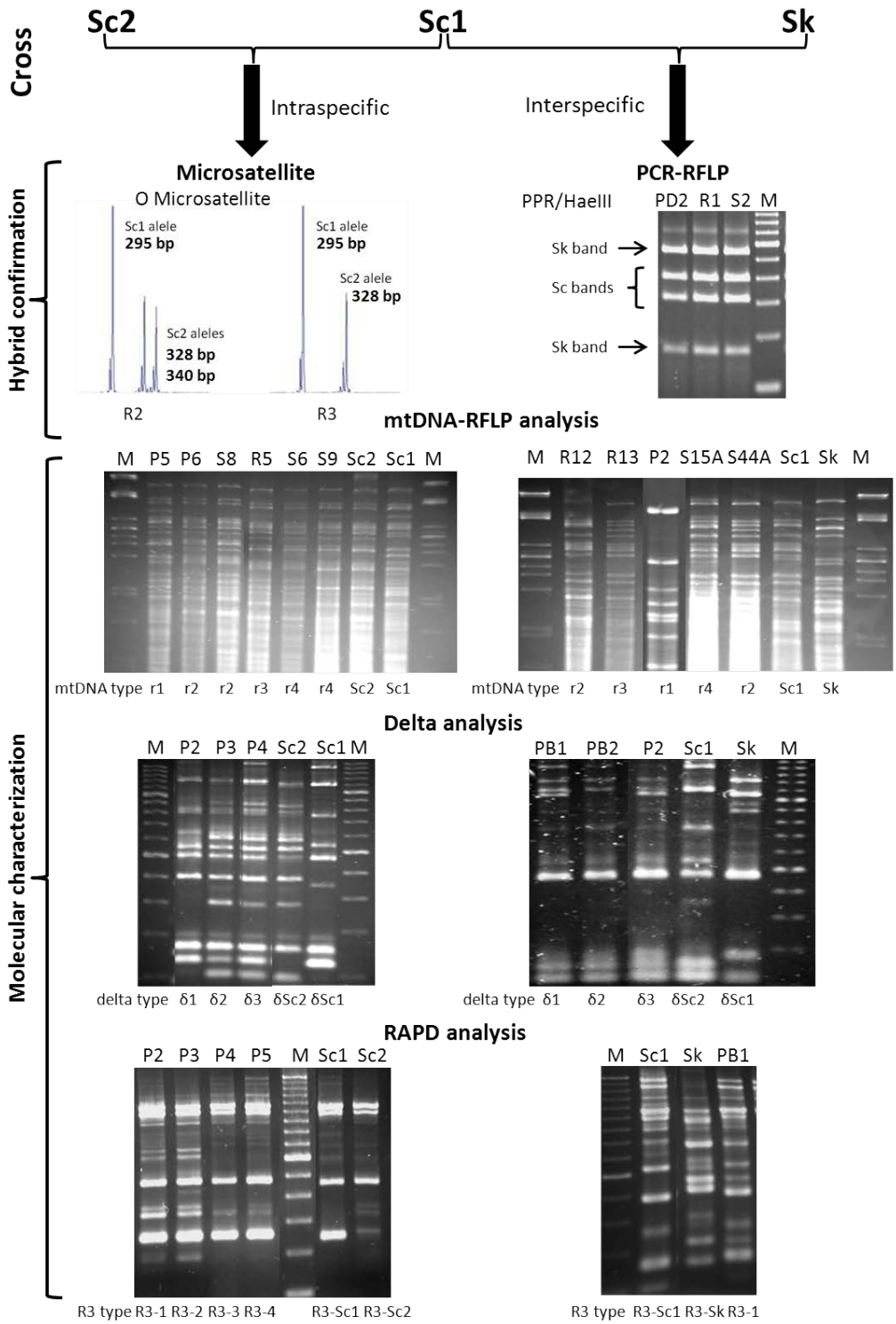

Fig. 2.1. Schematic representation of molecular markers used for both hybrids confirmation and molecular characterization. The codes at the top of the figures indicate the identity of some strains showed as illustrative examples and the codes at the bottom indicate their respective molecular patterns. M: molecular marker; 100-bp DNA ladder marker for PCRRFLP and RAPD analyses, mix of 100 bp and 50 bp DNA ladder market for interdelta analyses and λ-Pst I for mtDNA-RFLP analysis.

Additional molecular markers (mitochondrial DNA restriction analysis; inter δ-elements and RAPD analysis using 11 different primers) were evaluated in parental strains in order to better characterize and to study the genomic stability of the generated artificial hybrids. Differential mtDNA-RFLP and inter δ-elements patterns were obtained for each parental strain (Figure 2.1). From a total of 11 analyzed primers, only RAPD analysis with primer R3 allowed us to differentiate the three parental strains (Figure 2.1). The DNA content of the three parental strains was also analyzed: *Sk* showed a DNA content of 2.2 ± 0.1, while both *Sc1* and *Sc2* showed higher values (2.7 ± 0.2 and 2.5 ± 0.3 respectively).

2.3.2. Hybrids generation and characterization

Strains *Sc1* (*ura3*$^-$) and *Sc2* (*lys*$^-$) were used for the generation of intraespecific hybrids and *Sc1* (*ura3*$^-$) and *Sk* IFO1802 (*lys*$^-$) for interspecific hybrids.

2.3.2.1. Intraspecific hybrids

2.3.2.1.1. Protoplast fusion (P)

After applying a modification of Curran and Bugeja (1996) method we isolated a total of 30 putative hybrid colonies in minimal medium (MM). Molecular characterization and DNA content analyses were carried out on 12 colonies obtained by protoplast fusion (Table 2.1). Four colonies (named P1, P7, P10 and P11) presented the same microsatellite alleles combination present in one of the parental strains indicating that, despite growth in MM, they were not hybrids (data not shown). The hybrids P3, P4, P5, P8, P9 and P12 showed the complete set of the different alleles present in both *Sc1* and *Sc2* parental strains, while hybrids P2 and P6 only showed two alleles (each one coming from a different parental strain), indicating that they were hybrids that have already lost one allele (Table 2.1). None of the hybrids showed the same mtDNA-RFLP pattern present in the parental Sc1; 75% exhibited *Sc2* pattern and 25% showed different patterns (r1 and r2) probably obtained by a recombination between *Sc1* and *Sc2* mtDNAs (Table 2.1 and Figure 2.1). All inter δ-elements and RAPD-R3 patterns detected in hybrids generated by protoplast fusion corresponded to new patterns obtained by the combination of the respective parental patterns (Table 2.1). By combination of all applied

molecular techniques, we differentiated 7 molecular patterns out of 8 confirmed hybrids; hybrids P8 and P9 could not be differentiated (Table 2.1).

In general, DNA content values detected in hybrids (2.7 to 4.9) were significantly lower than the value expected by the addition of the parental values (5.2). Moreover, some hybrids showed DNA content values that were not significantly different than those exhibited by the parental strains, indicating a significant loss of genetic material (Table 2.1).

2.3.2.1.2. Rare-mating

The rare-mating hybrids were obtained applying the methodology described by Spencer and Spencer (1996) with some modifications. After mixing of parental strains, samples were taken at 3, 5, 7 and 10 days of incubation. In order to reduce the number of false positive colonies (those colonies growing onto MM by using their own reserves), samples were washed and maintained in starvation medium for 2 h before seeding on MM. The highest number of putative hybrid colonies was recovered from plates seeded after five and seven days of incubation. Twelve randomly selected colonies were reisolated in MM for further characterization (Table 2.1). The microsatellite analysis revealed that all colonies corresponded to *Sc1* x *Sc2* hybrids, i.e. they showed alleles coming from both parental strains for both O and P analyzed gene regions (Table 2.1). However, as happened after protoplast fusion, not all the strains showed the complete set of the alleles coming from both parental strains (Table 2.1). Regarding the mtDNA-RFLP patterns, 33.33% of the selected hybrids showed the profile presented in the parental *Sc1*, 41.67% Sc2, and the remaining 25% showed a new profile (r3) that could correspond to a recombinant pattern (Table 2.1). The analysis of inter δ-elements revealed 6 new profiles; the profile δ-4 was exhibited by 6 hybrids strains (R1, 3, 6, 8, 10 and 12), δ-9 by two hybrids (R9 and R11) and δ-5, δ-6, δ-7 and δ-8 by only one strain each. In the analysis of RAPD-R3 only two profiles (R3-7 and R3-8) were identified. Combining all the molecular markers was possible to differentiate the 12 different hybrids analyzed. The DNA content presented by the hybrids ranged from 3.5 to 5.0; these values were generally higher than those exhibited by hybrids generated by protoplast fusion (Table 2.1).

Table 2.1: Molecular and genetic characterization of the Sc1 x Sc2 intraspecific hybrids and parental strains.

METHODOLOGY	NAME	MICROSATELLITE ALLELES LENGHT (bp)		MOLECULAR PATTERNS[a]			DNA CONTENT[b]
		O	P	mtDNA	δ-PCR	RAPD-R3	
Parental	**Sc1**	295	460	Sc1	δ-Sc1	R3-Sc1	$2.7\pm0.2^{a-e}$
	Sc2	328, 340	446, 468	Sc2	δ-Sc2	R3-Sc2	2.5 ± 0.3^{a}
Protoplast fusion	**P2**	295, 340	460, 468	Sc2	δ-1	R3-1	$3.8\pm0.1^{d-j}$
	P3	295, 328, 340	446, 460, 468	Sc2	δ-2	R3-2	4.1 ± 0.3^{hij}
	P4	295, 328, 340	446, 460, 468	Sc2	δ-3	R3-3	4.9 ± 0.5^{j}
	P5	295, 328, 340	446, 460, 468	r1	δ-3	R3-4	$2.7\pm0.3^{a-e}$
	P6	295, 340	460, 468	r2	δ-1	R3-1	$3.9\pm0.5^{e-j}$
	P8	295, 328, 340	446, 460, 468	Sc2	δ-2	R3-5	$2.8\pm0.0^{a-f}$
	P9	295, 328, 340	446, 460, 468	Sc2	δ-2	R3-5	$3.1\pm0.6^{a-g}$
	P12	295, 328, 340	446, 460, 468	Sc2	δ-2	R3-6	$3.0\pm0.5^{a-g}$
Rare-mating	**R1**	295, 340	460, 468	r3	δ-4	R3-7	$3.7\pm0.3^{b-h}$
	R2	295, 328, 340	446, 460, 468	Sc2	δ-5	R3-8	5.0 ± 0.1^{j}
	R3	295, 340	460, 468	Sc2	δ-4	R3-8	$3.6\pm0.3^{a-h}$
	R4	295, 340	460, 468	Sc1	δ-6	R3-7	$4.0\pm0.4^{f-j}$
	R5	295, 340	460, 468	r3	δ-7	R3-7	$3.8\pm0.4^{c-i}$
	R6	295, 340	460, 468	Sc1	δ-4	R3-7	$3.7\pm0.2^{b-h}$
	R7	295, 328	460, 468	Sc2	δ-8	R3-7	$3.5\pm0.1^{a-h}$
	R8	295, 328, 340	446, 460, 468	Sc1	δ-4	R3-7	4.7 ± 0.3^{ij}
	R9	295, 328	446, 460	Sc1	δ-9	R3-7	$4.1\pm0.5^{g-j}$
	R10	295, 328, 340	446, 460, 468	r3	δ-4	R3-7	4.6 ± 0.1^{ij}
	R11	295, 328	446, 460	Sc2	δ-9	R3-7	$3.8\pm0.4^{c-i}$
	R12	295, 328, 340	446, 460, 468	Sc2	δ-4	R3-7	4.5 ± 0.4^{ij}
Spore to spore mating	**S1**	295, 340	460, 468	Sc2	δ-8	R3-8	$2.8\pm0.2^{a-f}$
	S2	295, 340	446, 460	Sc2	δ-10	R3-9	$2.7\pm0.1^{a-d}$
	S3	295, 340	446, 460	Sc2	δ-11	R3-9	2.6 ± 0.1^{ab}
	S4	295, 340	460, 468	Sc2	δ-12	R3-8	2.6 ± 0.2^{abc}
	S5	295, 340	460, 468	Sc2	δ-10	R3-8	$4.1\pm0.2^{g-j}$
	S6	295, 328	460, 468	r4	δ-13	R3-8	2.6 ± 0.3^{ab}
	S7	295, 340	446, 460	Sc1	δ-14	R3-10	$2.8\pm0.2^{a-e}$
	S8	295, 340	460, 468	r2	δ-15	R3-8	$2.9\pm0.0^{a-f}$
	S9	295, 328	460, 468	r4	δ-15	R3-8	2.7 ± 0.1^{abc}
	S10	295, 340	446, 460	Sc1	δ-16	R3-11	$2.6\pm0.3^{a-f}$
	S14	295, 328	446, 460	Sc1	δ-17	R3-10	4.1 ± 0.5^{ij}

a- Molecular patterns obtained by mtDNA-RFLP (**mtDNA**), interdelta sequence DNA polymorphisms (**δ-PCR**) and RAPD analysis using primer R3 (**RAPD-R3**).

b- Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, α=0.05, n=2).

2.3.2.1.3. Spore to spore mating

No colonies were obtained when spores dissection and mating were conducted on MM according to the standard methods. In order to improve the germination and consequently the hybridization process, asci dissection and crosses were repeated on complete medium (GPY). All colonies developed onto GPY agar plates were subsequently replica-plated in MM in order to select real hybrids bearing complementary auxotrophies. Sixty-four spore to spore crosses were made and the same number of colonies was obtained on GPY-agar plates. However, only 14 colonies were able to grow when transferred to MM. According to O and P microsatellites analysis, 11 colonies (17.2%) demonstrated to be hybrids; i.e. they presented one allele from each parent (Table 2.1). According to mtDNA, 45.4% showed a *Sc2* parental profile, 27.3% *Sc1* and the remaining 27.3% presented different mtDNA-RFLP patterns named r2 and r4 (Table 2.1). Interestingly, the pattern r2 was indistinguishable to pattern r2 found in one of the hybrids obtained by protoplast fusion (P6) (Table 2.1). As a result of inter δ-element analysis, nine profiles were identified including the pattern δ-8, that was also detected in hybrid R7 obtained by rare-mating (Table 2.1). Finally, 4 different profiles were found after RAPD-R3 analysis. Most colonies exhibited the profile R3-8, also observed in two isolates obtained after rare-mating (R2 and R3) (Table 2.1). Combining all molecular markers, we differentiated the 11 hybrid colonies recovered. Values of DNA content detected for most generated hybrids was around 2.7; however, the hybrid strains S5 and S14 showed values as high as 4.1 (Table 2.1).

2.3.2.2. Interspecific hybrids

2.3.2.2.1. Protoplasts fusion

Twelve colonies growing in MM after protoplast fusion were randomly selected and their hybrid nature of all of them was confirmed by PCR-RFLP. As a result of mtDNA-RFLP analysis, 10 colonies (83.3%) showed Sc1 parental pattern, the hybrid strain PC3 showed *Sk* parental pattern and P2 a new pattern (r1) (Table 2.2). Three different profiles were found after inter δ-elements polymorphism analysis (δ-1, δ-2 and δ-3) and, as in intraspecific mating, they were a combination of both parental strains patterns. All the hybrids showed the same RAPD-R3 pattern. Combining the three markers we differentiated 4 out of 12 generated hybrids. The

DNA content expected for these hybrids is 4.9, resulting from the addition of *Sc1* (2.7) and *Sk* (2.2) contents. However, the real values obtained for these hybrids ranged between 4 and 5 (Table 2.2).

2.3.2.2.2. *Rare-mating*

After rare-mating assays, fifteen colonies were isolated randomly from MM plates and only one of them (R8) was not confirmed as hybrid by PCR-RFLP of *BRE5* and *PPR1* genes. 42.9% of the hybrids had a *Sc1* mtDNA-RFLP pattern and 42.9% exhibited *Sk* pattern (Table 2.2). The remaining 14.2% of the colonies showed two different recombinant mtDNA-RFLP profiles (r2 and r3). Additionally, we identified 3 inter δ-element profiles (δ-4 to δ-6) and 7 RAPD-R3 patterns (Table 2.2). Combining the three markers we differentiated 10 out of 14 hybrids studied. The DNA content of these hybrids was quite diverse and ranged from 2.3 (R10) to 5.1 (R7 and R11) (Table 2.2).

2.3.2.2.3. *Spore to spore mating*

From a total of 40 spore to spore crosses performed on GPY agar plates, only 12 (30%) were confirmed to be hybrids after seeding on MM and PCR-RFLP evaluation. Among them, 66.7% presented *Sc1* mtDNA-RFLP pattern, 33.3% *Sk* and the remaining 33.3% showed new patterns (r3 and r4) (Table 2.2).

We identified three new inter δ-elements profiles, including δ-7 profile detected in only one strain (S1). RAPD analysis using primer R3 showed 8 new profiles. Combining the three markers we distinguished 9 out of 12 hybrid colonies evaluated. On the other hand, the mean DNA content value obtained in spore to spore hybrids was 3.2 (Table 2.2).

Table 2.2: Molecular and genetic characterization of the Sc1 x Sk interspecific hybrids and parental strains.

METHODOLOGY	NAME	MOLECULAR PATTERNS[a]			DNA CONTENT[b]
		mtDNA	δ-PCR	RAPD-R3	
Parental	**Sc1**	Sc1	δ-Sc1	R3-Sc1	2.7 ± 0.2^{abc}
	Sk	Sk	δ-Sk	R3-Sk	2.2 ± 0.1^{a}
Protoplast fusion	**PA1**	Sc1	δ-1	R3-1	$5.0\pm0.2^{j-l}$
	PB1	Sc1	δ-1	R3-1	$4.8\pm0.2^{i-l}$
	PB2	Sc1	δ-2	R3-1	$4.1\pm0.4^{d-l}$
	PB3	Sc1	δ-1	R3-1	$4.7\pm0.5^{h-l}$
	PC1	Sc1	δ-1	R3-1	$4.6\pm0.3^{h-l}$
	PC2	Sc1	δ-1	R3-1	$5.1\pm0.4^{j-l}$
	PC3	Sk	δ-1	R3-1	$4.2\pm0.3^{e-l}$
	PD1	Sc1	δ-1	R3-1	$4.5\pm0.3^{f-l}$
	PD2	Sc1	δ-1	R3-1	$4.8\pm0.1^{i-l}$
	PD3	Sc1	δ-1	R3-1	$4.8\pm0.1^{i-l}$
	PD4	Sc1	δ-1	R3-1	$4.7\pm0.1^{i-l}$
	P2	r1	δ-3	R3-1	5.1 ± 0.5^{l}
Rare-mating	**R1**	Sk	δ-4	R3-2	$3.2\pm0.2^{a-e}$
	R2	Sk	δ-4	R3-3	$4.9\pm0.1^{i-l}$
	R3	Sc1	δ-4	R3-4	$4.8\pm0.1^{i-l}$
	R4	Sk	δ-4	R3-2	$4.0\pm0.2^{d-j}$
	R5	Sc1	δ-5	R3-2	$3.1\pm0.6^{a-d}$
	R6	Sc1	δ-4	R3-4	$3.6\pm0.4^{c-h}$
	R7	Sk	δ-4	R3-5	5.1 ± 0.2^{l}
	R9	Sc1	δ-6	R3-6	$4.5\pm0.2^{g-l}$
	R10	Sc1	δ-6	R3-6	2.3 ± 0.2^{ab}
	R11	Sk	δ-6	R3-7	5.1 ± 0.1^{kl}
	R12	r2	δ-6	R3-6	$3.9\pm0.0^{d-i}$
	R13	r3	δ-6	R3-7	$4.6\pm0.3^{g-l}$
	R14	Sc1	δ-6	R3-6	$3.0\pm0.3^{a-d}$
	R15	Sk	δ-6	R3-8	$4.1\pm0.1^{d-k}$
Spore to spore mating	**S1**	Sc1	δ-7	R3-9	$3.1\pm0.4^{a-d}$
	S2	Sc1	δ-8	R3-10	$3.5\pm0.2^{c-g}$
	S3	Sc1	δ-8	R3-10	$3.0\pm0.2^{a-d}$
	S4	Sc1	δ-8	R3-10	$3.3\pm0.1^{b-e}$
	S5	Sk	δ-9	R3-11	$3.4\pm0.1^{c-f}$
	S6	Sc1	δ-8	R3-10	$3.2\pm0.0^{a-e}$
	S8	Sc1	δ-8	R3-12	$3.2\pm0.2^{a-d}$
	S15A	r4	δ-9	R3-13	$3.2\pm0.3^{a-e}$
	S15B	Sc1	δ-9	R3-14	$3.1\pm0.3^{a-d}$
	S44A	r3	δ-9	R3-15	$3.3\pm0.3^{b-e}$
	S44B	Sk	δ-9	R3-13	$3.6\pm0.4^{c-h}$
	S45	Sc1	δ-8	R3-16	$3.1\pm0.1^{a-d}$

a- Molecular patterns obtained by mtDNA-RFLP (**mtDNA**), interdelta sequence DNA polymorphisms (**δ-PCR**) and RAPD analysis using primer R3 (**RAPD-R3**).

b- Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, $\alpha=0.05$, n=2).

2.3.3. Genetic stabilization of hybrids during vegetative propagation

In order to obtain genetically stable hybrids to be used in future commercial fermentations, we developed a genetic stabilization procedure. This method was based on five consecutive fermentations on synthetic must at 20ºC (selective conditions). The stabilization process was evaluated by analyzing the variability of different molecular markers (mtDNA-RFLP, interdelta and RAPD) as well as the changes in total DNA content. A total of eight hybrid strains obtained by rare-mating and spore to spore mating of both intra and interspecific crosses were stabilized (Table 3). Rare-mating hybrid strains were selected among those with the highest values of DNA content. Significant differences were observed in the stabilization process of intraspecific and interspecific hybrids, particularly for those strains generated by rare-mating.

For interspecific hybrids, all colonies obtained after five fermentation steps showed the same molecular pattern detected in the original hybrid strain for interdelta and RAPD analysis, independently of the hybridization procedure used for generation (Table 2.3). Although no nuclear genetic variability was detected among original and derived colonies, significant differences in DNA content values, as well as changes in their mtDNA-RFLP profile, were observed in most cases. Colonies derived from R1, R3 and S5 showed values of DNA content significantly lower than those exhibited by the originals (Table 2.3), indicating that the DNA content is a good marker to analyse the genetic stabilization of the artificial hybrids, additionally to molecular markers. Colonies derived from R1 hybrid changed their mtDNA-RFLP profile (Fig 2.2A). All interspecific hybrids were able to sporulate in acetate medium; however, most of them showed spores unable to develop colonies in GPY. The only exception was vegetatively stable hybrid R3 that showed viability values of 50%.

Most hybrids obtained from intraspecific spore to spore mating exhibited the same behaviour during vegetative stabilization than interspecific spore to spore hybrids: no genetic variability and no changes in DNA content. Contrarily, colonies isolated after five consecutive fermentations of intraspecific hybrids obtained by rare-mating showed new molecular patterns (Table 3). Three new molecular patterns were detected among R2 derived colonies and four

patterns among R8 derived colonies. Only R8 derived colonies changed their mtDNA-RFLP profile (Fig. 2.2B). DNA content analysis evidenced that those derived colonies exhibiting the same molecular pattern detected in the original hybrid (R2-original and R8-original, as well as all spore to spore hybrids) showed similar DNA content to that present in the original hybrid. Contrarily, derived colonies exhibiting new molecular patterns (R2-A, R2-B, R2-C, R8-A, R8-B, R8-C and R8-D) showed significantly lower DNA content values (Table 2.3).

Finally, individual colonies representative of each derived hybrid detected after the complete set of five consecutive fermentations were used to inoculate fresh synthetic must in order to confirm their vegetatively genetic stability. After fermentation, ten colonies were isolated and characterized by mtDNA-RFLP, interdelta, RAPD and DNA content analysis. Only derived hybrids R2-original, R8-original and R8-A were shown to be unstable due to changes in DNA content (Table 2.3).

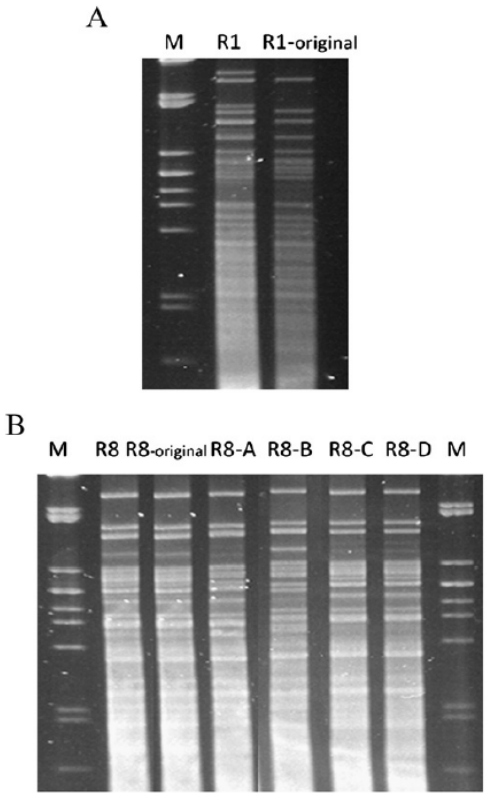

Figure 2.2. mtDNA-RFLP analysis comparing the initial hybrid and the derived ones.

A) R1 and **B)** R8. λ-PstI was used as molecular weight marker (M).

Table 2.3: Genetic stabilization process in selected intraspecific and interspecific hybrids.

CROSS, METHODOLOGY AND HYBRID NAME			ORIGINAL[a]			5° STEP OF FERMENTATION[b]					STABLE[e]
			MOLECULAR PATTERNS[c]		DNA CONTENT[d]	DERIVED HYBRID	MOLECULAR PATTERNS[c]		DNA CONTENT[d]	%	
			δ-PCR	RAPD-R3			δ-PCR	RAPD-R3			
Intraspecific	**Rare-mating**	**R2**	δ-5	R3-8	5,0±0,1[d]	R2-original	δ-5	R3-8	4,7±0,0[d]	20	NO
						R2-A	δ-18	R3-8	2,7±0,2[a]	60	YES
						R2-B	δ-19	R3-8	2,5±0,3[a]	10	YES
						R2-C	δ-20	R3-8	2,6±0,2[a]	10	YES
		R8	δ-4	R3-7	4,7±0,3[c]	R8-original	δ-4	R3-7	4,9±0,2[c]	40	NO
						R8-A	δ-21	R3-7	3,5±0,4[b]	20	NO
						R8-B	δ-22	R3-12	2,7±0,1[a]	10	YES
						R8-C	δ-23	R3-7	2,9±0,1[ab]	10	YES
						R8-D	δ-24	R3-7	2,6±0,1[a]	20	YES
	Spore to spore mating	**S2**	δ-10	R3-9	2,7±0,1[a]	S2-original	δ-10	R3-9	2,5±0,4[a]	100	YES
		S7	δ-14	R3-10	2,8±0,2[a]	S7-original	δ-14	R3-10	2,4±0,2[a]	100	YES
Interspecific	**Rare-mating**	**R1**	δ-4	R3-2	3,2±0,2[b]	R1-original	δ-4	R3-2	2,6±0,0[a]	100	YES
		R3	δ-4	R3-4	4,8±0,1[b]	R3-original	δ-4	R3-4	3,6±0,1[a]	100	YES
	Spore to spore mating	**S5**	δ-9	R3-11	3,4±0,1[b]	S5-original	δ-9	R3-11	2,4±0,2[a]	100	YES
		S8	δ-8	R3-12	3,2±0,2[a]	S8-original	δ-8	R3-12	2,8±0,2[a]	100	YES

a- Hybrid used to inoculate wine-like medium for the five-step stabilization procedure.

b- Hybrid colonies isolated after five fermentation steps. Percentages (%) were calculated from a total of 10 analyzed colonies.

c- Interdelta patterns (δ-PCR) are indicated as δ- followed by a Latin number. Patterns obtained by RAPD-PCR with primer R3 (RAPD-R3) are indicated as R- followed by a Latin number.

d- Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the columns of both original and derived hybrids are significantly different (ANOVA and Tukey HSD test, α=0.05, n=2).

e- Strains were considered as genetically stable when no changes in both molecular patterns and DNA content were detected after fermentation with a single derived hybrid colony (from a total of 10 colonies analyzed).

2.4. Discussion

Intraspecific hybrids -using two *S. cerevisiae* strains bearing interesting and complementary oenological features- as well as interspecific hybrids -using *S. cerevisiae* and *S. kudriavzevii*- were obtained by means of non GMO´s generating techniques: rare-mating (R) and spore to spore mating (S) and compared with hybrids generated by protoplast fusion (P), a commonly used GMOs producing technique.

Selection procedures of hybrids based on complementation of auxotrophic parental strains is difficult because industrial strains are prototrophic (Akada, 2002; Nakazawa and Iwano, 2004). For this reason, spontaneous *ura3*$^{-}$ and *lys2*$^{-}$ auxotrophic mutants were generated in this work by the use of 5-FOA and α-AA agar plates (Boeke et al., 1987; Zaret and Sherman, 1985). The generation of auxotrophic strains has been reported to be difficult for industrial *S. cerevisiae* strains, because of their generally polyploid or aneuploid nature (Bell et al., 1998). However, we succeeded in the recovery of these natural mutants for both *S. cerevisiae* industrial as well as for the wild *S. kudriavzevii* parental strains. The fact that both 5-FOA and α-AA allow the identification of naturally occurring mutants among the yeast populations, makes it possible to use these compounds in non-GMO producing protocols. Artificially generated mutants could not be used with the same purpose.

The need for a confirmatory method for the evaluation of hybrids generation was the second important step in this study. Confirmatory methods must be able to differentiate the parental strains involved in hybridization. Different methodologies have been described in scientific literature for the differentiation of *S. cerevisiae* strains including mtDNA-RFLP, RAPD, interdelta, karyotyping, microsatellites, among others (Baleiras Couto et al., 1996; de Barros Lopes et al., 1996; Hennequin et al., 2001; Pérez et al., 2001; Querol et al., 1992; Vezinhet et al., 1992). Some of them were also useful to discriminate strains belonging to other species of *Saccharomyces* genus as *S. kudriavzevii* and *S. bayanus* (Demuytier et al., 2004 ; Lopes et al., 2010 ; Masneuf-Pomarede et al., 2007). Due to the fact that mitochondial DNA could be inherited without changes from one of the parental strains involved in the hybridization event (Dujon et al., 1974), the use of mtDNA-RFLP analysis as a confirmatory method for hybrids

generation is not adequate. Both microsatellite and PCR-RFLP analyses were selected in this work because these methods are fast and simple to interpret. Using these methods, reliable molecular patterns able to discriminate between parental and hybrid strains in interspecific and intraspecific hybridizations respectively were obtained (Tables 2.1 and 2.2).

As a general rule, hybridization frequency obtained in this work after S was higher than the same for P and R (data not shown); however, a high number of hybrids was obtained in all cases even if R has been suggested as a difficult method to generate hybrids according to the literature. These results are in accordance with previous reports that showed a low hybridization frequency after P and R events (Bell et al., 1998; Gunge and Nakatomi, 1972; Spencer and Spencer, 1977).

Independently from the methodology used for hybridization, the frequency of hybrids was significantly improved when recently formed putative hybrid cultures were maintained in starvation medium for 1-2 h before seeding on MM agar plates. This procedure allowed us to diminishing the recovery of parental strains growing in MM by means of their own nutritional reserves.

In the particular case of S, probably due to the presence of individual spore auxotrophies, hybridization events were not observed when minimal medium was directly used for asci dissection and mating. To solve that problem, we proposed the use of a complete medium (GPY) to make the individual crosses prior to selection of hybrids in MM. Several S hybrids were obtained after using this additional step.

A high genetic variability in both nuclear and mitochondrial genomes was observed among hybrid colonies obtained from both intra- and interspecific crosses, according to their mtDNA-RFLP, interdelta and RAPD patterns. This variability indicates that different processes of lost and reorganization of genetic material from both parents are taking place during the early stages of hybrids generation.

With regards to mtDNA restriction patterns, most hybrid colonies showed mtDNA-RFLP patterns indistinguishable from those of the parental strains. This finding was previously

described by Dujon et al. (1974), who demonstrated that different mitochondrial genomes brought together by mating, are rapidly segregated through subsequent mitotic divisions and new generated buds inherit only one kind of mtDNA. This uniparental inheritance of mitochondrial genome have been detected in *S. cerevisiae* x *S. pastorianus* hybrids obtained by R (de Barros Lopes et al., 2002), in *S. cerevisiae* x *S. uvarum* hybrids obtained by mass mating (Antunovics et al., 2005) and in natural hybrids isolated from wine and beer (González et al., 2006; González et al., 2008; Rainieri et al., 2008). Interestingly, a minoritary number of hybrid colonies obtained in this work exhibited mtDNA-RFLP patterns originated by putative recombinant events among parental mtDNA molecules (Tables 2.1 and 2.2). These new patterns were named r1, r2, etc. (Tables 2.1 and 2.2 and Figure 2.1). In this sense, Berger and Yaffe (2000) showed that during the hybridization event, parental mtDNAs can recombine with high frequency and hybrid colonies could inherit either parental or recombinant mtDNA molecules. The fact that a same putative recombinant mtDNA pattern was detected in different hybrid colonies obtained by the same or different hybridization protocols (Tables 2.1 and 2.2) seem to indicate the potential presence of hot spots of recombination in the mtDNA, as it was suggested by other authors (Berger and Yaffe, 2000; Dujon et al., 1974; Piškur, 1994). The higher frequency of recombinant mtDNA molecules among intraspecific hybrids with regards to that in interspecific hybrids (26% and 13% on average respectively) can be explained by the higher genetic similarity among parental genomes in the former cross, which favours homologous recombination (Bernardi, 2005). Additionally, the possible incompatibility between the mitochondrion of one species and the nucleus of the other species –as it was demonstrated by Lee et al. (2008) for *S. cerevisiae* and *S. bayanus*- can be another explanation for the existence of recombinant mtDNA molecules. During genetic stabilization process, changes in mtDNA-RFLP patterns were observed, i.e. hybrids obtained after five fermentation steps inoculated with only one particular hybrid colony couldn't show the same mitochondrial pattern founded in the original (Table 2.3). This happened for the interspecific hybrid R1 and the intraspecific hybrid R8 (Figure 2.2). It may be due to the DNA repair machinery, active during the stabilization process and responsible for the chromosomal rearrangement, that could act on the mtDNA allowing recombination between sisters molecules. This process could be favored by the high homology between them (100% homology) (Bernardi, 2005).

Similar situations were also observed in nuclear genome of hybrid strains, these differences were detected with interdelta and RAPD analyses. For both molecular markers, most hybrids showed patterns basically originated for the addition of bands from the patterns generated with both parental strains involved in the mating (Figure 2.1); however, some hybrid strains also exhibited new bands probably originated from chromosomal rearrangements (Figure 2.1). The existence of these rearrangements was also evident during the vegetative stabilization process, particularly for colonies derived from the intraspecific hybrids R2 and R8, which showed different interdelta or RAPD patterns from a single pattern in the originally inoculated strain (Table 2.3).

Both interdelta and RAPD analyses demonstrated to be useful tools for the differentiation of recently generated hybrid strains; a total of 26 interdelta and 26 RAPD-R3 patterns were observed among the 69 confirmed hybrids analyzed; however, most molecular differences arising during stabilization process were only detected in interdelta analysis (Table 2.3).

As it was previously suggested, the genetic variability observed in nuclear molecular markers could be the consequence of chromosomal rearrangements or losses occurring among parental genomes during the early stages of hybrids generation. This hypothesis was partially confirmed with the results of microsatellite analysis in the recently generated intraspecific hybrids, where the loss of some parental alleles was evident (Table 2.1). The fact that parental *Sc1* was homocigous for both O and P microsatellite regions, made it difficult to totally interpret DNA losses; only the lost of alleles from parental *Sc2* were informative in this sense (Table 2.1). Loss of genetic material after hybridization was also demonstrated after DNA content evaluation. DNA content obtained for recently generated hybrids were generally lower than the values expected by the theoretical addition of DNA content from the respective parental strains in both inter and intraspecific crosses. This phenomenon was especially evident in hybrids generated by P and R (Tables 2.1 and 2.2), independently from the evaluated cross, and it seems to happen during the early stages immediately after hybrids generation. During the stabilization process, changes in DNA content were also evident particularly for intra- and interspecific R hybrids originally possessing a higher amount of DNA than S hybrids (Table 2.3).

In a similar way, a DNA content reduction process was also evidenced by Antunovics et al. (2005) after stabilization of *S. cerevisiae* x *S. uvarum* hybrids by successive sporulation events and Marinoni et al. (1999) after interspecific hybridization by mass-mating. The infertility found in most vegetatively stable interspecific hybrids indicated that, even after DNA losses, they still maintained DNA from each parental strain. Only interspecific hybrid R3 showed a 50% spores viability; this behavior could be related to the fact that this strain probably conserved a complete diploid set of chromosomes from one of the two parental, as can be inferred from the high DNA content (3.6)(Table 2.3).

Polyploid genomes, as those obtained after hybridization in this work after R and P, are known to be unstable in *Saccharomyces cerevisiae* (Gerstein *et al.*, 2008; Storchova *et al.*, 2006). Genome loss and rearrangement occurring during stabilization of newly formed hybrids have been reported (Gerstein *et al.*, 2006). These phenomena might lead to loss of industrially important traits in hybrids. For that reason, the final crucial step in this study was the careful selection of stabilization conditions. The stabilization process proposed in this work, based on five consecutive fermentation steps, was successful in the generation of some vegetatively stable colonies from all analyzed original hybrids (Table 2.3). However, some unstable hybrid colonies, generally maintaining the same molecular patterns and DNA content than the original hybrids, were still detected after the five fermentation steps. This behavior was particularly detected after stabilization of intraspecific hybrids generated by R (Table 2.3). Our results suggest that stabilization of hybrids obtained by S is faster than stabilization of hybrids obtained by R, although in general both kinds of hybrids finally stabilize with values of DNA content close to those presented by the parents (close to diploidy). Gerstein et al. (2006) also observed the same behavior, both triploid and tetraploid strains showed a reduction in their DNA content until a same value close to 2n. Additionally, the stabilization process in R hybrids generated by interspecific mating seems to have occurred faster than the same in intraspecific R hybrids (Table 2.3). In this case, genetic similarity among parental genomes in intraspecific mating -as it was mentioned for mtDNA- could favoured homologous recombination (Bernardi, 2005). In fact, differences in molecular patterns probably arisen from homologous recombination were also observed in intraspecific R hybrids during stabilization, as previously mentioned. A difference in the moment in which different chromosomes are lost during both

hybrids formation and stabilization in intra and interspecific hybrids can also be responsible for the differences detected in this work. It is important to remark that the particular vegetative stabilization process used in this work; however, does not guarantee that the hybrid will not change under different conditions. For that reason, as well as due to the differences detected among hybrids obtained from different methods and crosses, a more detailed study about the whole stabilization process in intra and interspecific hybrids are being carried out in our laboratory.

2.5. Conclusions

A high diversity of intra and interspecific hybrids was successfully obtained by three hybridization methodologies. Despite the low hybridization frequency obtained after protoplast fusion and rare-mating, hybrids generated by means of these methodologies have theoretically a more complete subset of genetic material inherited from each parental strain. Consequently, they possess an extremely high genetic plasticity which could render in a potentially better adaption to the environment. Due to the fact that a loss of genetic material occur during both hybrids generation and genetic stabilization, hybrids possessing a high amount of DNA became a better resource to obtain the best suitable hybrid strain for industrial purposes. The usefulness of rare-mating generated hybrids for industries in which GMO is a legal and public concern problem became evident. These hybrids were easily obtained and stabilized by the improved methodology described in this work.

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Capítulo 3

Chapter 3. Study of the stabilization process in *Saccharomyces* intra- and interspecific hybrids in fermentation conditions.

RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2014) 17:xxx-xxx
doi:10.2436/20.1501.01.xxx. ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

Stabilization process in *Saccharomyces* intra- and interspecific hybrids in fermentative conditions

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Received 8 September 2014 · Accepted 18 November 2014

Summary. We evaluated the genetic stabilization of artificial intra- (*Saccharomyces cerevisiae*) and interspecific (*S. cerevisiae* × *S. kudriavzevii*) hybrids under wine fermentative conditions. Large-scale transitions in genome size and genome reorganizations were observed during this process. Interspecific hybrids seem to need fewer generations to reach genetic stability than intraspecific hybrids. The largest number of molecular patterns recovered among the derived clones was observed for intraspecific hybrids, particularly for those obtained by rare-mating. Molecular marker analyses revealed that unstable clones could change during the industrial process to obtain active dry yeast. When no changes in molecular markers and ploidy were observed after this process, no changes in genetic composition were confirmed by comparative genome hybridization, considering the clone as a stable hybrid. According to our results, under these conditions, fermentation steps 3 and 5 (30–50 generations) would suffice to obtain genetically stable interspecific and intraspecific hybrids, respectively. [**Int Microbiol** 2014; 17(4):xxx-xxx]

Keywords: *Saccharomyces cerevisiae* · *Saccharomyces kudriavzevii* · rare-mating in yeast · molecular markers · DNA content evaluation · stabilization of genomes

3.1. Introduction

Detection of "natural" *Saccharomyces* hybrid strains in different fermentations [22,29,35], and among the starter cultures used for wine inoculation [9,22,23,33], led some research groups around the world to pay attention to the relevance of hybrids in these processes. These hybrids contain an almost complete set of chromosomes from partners in the form of allodiploid or allotetraploid genomes or only portions of the partner genomes resulting in alloaneuploids or strains with chimerical chromosomes [5,17,45,48]. The physiological advantage of hybrids has been proposed to be related with their better fitness under intermediate or fluctuating conditions than parental strains [44]. For this reason, the artificial generation of hybrids has become an interesting strategy in recent years to improve industrial yeast strains. Construction of hybrids in the *Saccharomyces* genus has been reported between wine strains of *Saccharomyces uvarum* and various strains of *Saccharomyces cerevisiae* (for a review, see [48]). The artificial hybrids between *S. cerevisiae* and other *Saccharomyces* species like *S. paradoxus* and *S. kudriavzevii* have also been recently reported by several authors [6,8,39]. Different procedures, including protoplast fusion, mass-mating, spore-to-spore mating and rare-mating, have been used for hybrids generation [48].However, only those methods occurring naturally by mating or natural recombination can be used for the generation of non-GMO (genetic modified organisms) microorganisms in accordance with Directive 2001/18/EC of the European Parliament and the European Council. Protoplast fusion is thus excluded from the group of non-GMOs-producing techniques [11].

Commercialized wine strains have been selected because of their fermentation qualities and stress adaptability during alcoholic fermentation and also because these strains ensures the production of consistent wines in successive vintages [42]. This means that the strains developed for industrial processes must to have stable genomes. In a previous work carried out in our laboratory, different inter- and intraspecific hybrid strains were obtained by employing several hybridization methodologies [39]. However we observed that instable hybrids showing high DNA content were generally obtained. In other works, polyploid genomes were known to be unstable in *S. cerevisiae* [20,50] or in hybrids of *Saccharomyces* genus [2, 26, 48]. Similarly, many newly formed polyploids in plants exhibit unstable genomes

that undergo rapid repatterning during first generations, which is particularly important for allopolyploids [49,52]. Because of this trend to the reorganization of the genome and the genetic heterogeneity of the new hybrids [26], the development of a method to ensure proper genetic stability of strains used in industrial applications, it is necessary.

Wine yeast should be adapted to several stresses, such as the low pH and high sugar content of grape must. The selectivity of fermenting must is further strengthened once anaerobic conditions are established; certain nutrients become depleted and the ethanol level increases [42]. During the process of active dry yeast (ADY) production and the posterior rehydration, yeast cells are exposed to a different group of stresses such as osmotic, oxidative, thermic, and/or starvation [3,4,14,36,38]. All these stresses exert a strong selective pressure on the microorganisms and could induce changes in unstable genomes. Loss of the type (i.e., parental origin) and content of DNA in the genetic stabilization process during hybrids formation can strongly influence future physiological characteristics and the adaptation of a hybrid to industrial processes. Several examples described in the bibliography correlate the influence of genome size differences with the phenotype variations such at the cell size[31], the generation time [41] and ecological tolerances [19]. Genomic changes, such as insertions, deletions and translocations, have also been related to yeasts adapting to novel environments [7,16,19].Variations in gene copy number occurring in polyploids or aneuploids have also been associated with altered gene expression patterns and metabolic activity [18,51]. Genome reduction and rearrangements occurring during the stabilization process might lead to loss of industrially important traits in hybrids, and can be avoided if a selective pressure, mimicking the desired industrial process, is applied during the stabilization. The knowledge of the stabilization process can help us to design the experimental conditions when a new lab made hybrid wants to be developed for industrial proposes.

By considering all the aforementioned aspects, this work aimed to validate a fast genetic stabilization method for newly generated *Saccharomyces* hybrids under selective oenological conditions, to know how many rounds (or generations) suffice to obtain stable hybrids and to study the changes that succeeded during the process. A comparison of the whole stabilization process in intra- and interspecific hybrids showing different ploidy levels, as

a result of using different hybridization methodologies, was also made. It should be noted that this is the first work that studies deeply the stabilization procedure under oenological conditions.

3.2. Materials and methods

3.2.1. Yeast strains

Four interspecific *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* hybrids, two obtained from rare-mating(R2 and R8) and two from spore-to-spore mating(S2 and S7) and four intraspecific *S. cerevisiae* hybrids, two obtained from rare-mating (R1 and R3) and two from spore-to-spore mating (S5 and S8) were selected from a previous work[39]to undergo a genetic stabilization procedure (see hybrid and parental characterization in Table 3.1).

3.2.2. Genetic stabilization procedure

A single colony of each hybrid strain was individually inoculated into 15 ml screw cap tubes containing 10 mL of synthetic must[46]with 50% glucose and 50% fructose, sterilized by filtration. The samples were incubated at 20ºC without shaking. After fermentation (approximately 15-20 days), an aliquot of approximately 10^7celswas used to inoculate a new tube containing the same sterile medium (synthetic must) and was incubated under the same conditions, while a second aliquot was seeded on GPY-agar plates and incubated at 20ºC. Ten yeast colonies were randomly picked and characterized by inter-δ sequences, RAPD-PCR analyses and mtDNA-RFLP patterns. The total DNA content was also measured for each colony showing a different molecular pattern.

All the yeast colonies displaying different molecular profiles, obtained in whatever fermentation step, were individually inoculated in the same synthetic must and, after these individual fermentations, ten colonies from each one were analysed by the same methods. When one pattern was recovered more than one time, we selected this pattern for the last round in which it appeared. We put the original pattern, selected in the fifth round, in an individual fermentation too. We considered that a clone was genetically stable when the colonies recovered after the individual fermentations maintained the same molecular profile (δ

elements, RAPD-PCR and mtDNA-RFLP patterns) and the same ploidy level as the previously inoculated culture.

3.2.3. DNA content evaluation

The DNA content of both hybrid and control strains was assessed by flow cytometry using a FACScan cytometer (Becton Dickinson Immunocytometry Systems, Palo Alto, California, United States) following the methodology described in Lopes *et al.*[30]. Previously, yeast cells were grown in GPY during 24h until stationary phase. DNA content values were scored on the basis of fluorescence intensity compared with haploid (S288c) and diploid (FY1679) reference strains. The value reported for each strain was the result of three independent measures. The results were tested by one-way ANOVA and a Tukey HSD test (α=0.05, n=2).

3.2.4. Active Dry Yeast (ADY) Production

Industrial cultivation and drying were performed according to the Laboratory of Research and Development standard protocols (Lallemand Inc. protocols; Lallemand S.A.S., Montreal, Canada) (not provided).A rehydration step, previous to the use of these yeasts in winemaking, is needed.

Table 3.1: Molecular and genetic characterization of hybrids and parental strains used in this study (extracted from Chap 2).

Cross	Methodology	Name	Molecular patterns[#]			DNA content[§]
			mtDNA	δ-PCR	RAPD-R3	
Parental strains		**Sc1**	Sc1	δ-Sc1	R3-Sc1	2.7±0.2[a-c]
		Sc2	Sc2	δ-Sc2	R3-Sc2	2.5±0.3[a]
		Sk	Sk	δ-Sk	R3-Sk	2.2±0.1[a]
Sc1xSc2	**Rare-mating**	**R2**	Sc2	δ-5	R3-8	5.0±0.1[j]
		R8	Sc1	δ-4a*	R3-7	4.7±0.3[ij]
	Spore to spore mating	**S2**	Sc2	δ-10	R3-9	2.7±0.[1a-d]
		S7	Sc1	δ-14	R3-10	2.8±0.2[a-e]
Sc1xSk	**Rare-mating**	**R1**	Sk	δ-4b*	R3-2	3.2±0.2[a-e]
		R3	Sc1	δ-4b*	R3-4	4.8±0.1[i-l]
	Spore to spore mating	**S5**	Sk	δ-9	R3-11	3.4±0.1[c-f]
		S8	Sc1	δ-8	R3-12	3.2±0.2[a-d]

#- Molecular patterns obtained by mtDNA-RFLP (mtDNA), interdelta sequence DNA polymorphisms (δ-PCR) and RAPD analysis using primer R3 (RAPD-R3). * Patterns δ4 in Pérez-Través et al., [Chap 2], both of them are different.

§- Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, α=0.05, n=2).

3.2.5. Comparative genome hybridization analysis

Array competitive genomic hybridization (aCGH) was performed using a hybrid clone before and after processing as ADY by following the methodology described in Peris *et al.[40]*. Experiments were carried out in duplicate and the Cy5-dCTP and Cy3-dCTP dye-swap assays were done to reduce the dye-specific bias. Microarray scanning was carried out using a GenePix Personal 4100A scanner (Axon Instruments/Molecular Devices Corp., USA). Microarray images and raw data were produced using the GenePix Pro 6.1 software (Axon Instruments/Molecular Devices Corp.) and the background was subtracted by applying the local feature background median option. M-A plots (M = Log2 ratios; A = log2 of the product of intensities) were represented in order to evaluate if the ratio data were intensity-dependent. The normalization process and filtering were done with Acuity 4.0 (Axon Instruments/Molecular Devices Corp.). Raw data were normalized by the ratio-based option. Features with artefacts or flagged as bad were removed from the analysis. Replicates were averaged after filtering. The data from this study are available from GEO (http://www.ncbi.nlm.nih.gov/geo/); the accession number is GSE46192.

3.2.6. Natural must fermentation, HPLC analysis of wines and kinetic analysis.

The must employed was Albariño. Fermentable sugars were measured using the HPLC (see below), that gave a value of 213.96 g L^{-1}. Yeast assimilable nitrogen was determined by the ammonia assay kit (Boehringer Mannheim; Mannehim, Germany), for the inorganic nitrogen (40% of the total nitrogen amount) and nitrogen content was adjusted to a total of 220mg L^{-1} by addition of a nitrogen supplement consisting in NH_4Cl.Prior to the fermentation, dimethyl dicarbamate (DMDC) at 1ml L^{-1} was added for sterilization purposes. Fermentations were carried out in 100ml bottles containing 80ml of Albariño must. Must was inoculated independently with the different yeast strains to reach an initial population of $2*10^6$ CFU/ml, and maintained at 22°C. Flasks were closed with Müller valves and monitored by weight loss until a constant weight was obtained. Immediately after the end of fermentation, yeast cells were removed by centrifugation and the supernatants analysed immediately or stored at -20°Cuntil use. Each fermentation system was carried out by duplicate.

Supernatants were analysed by HPLC in order to determine the amounts of residual sugars (glucose and fructose), glycerol, and ethanol. A Thermo chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector was used. The column was a HyperREZ^{TM} XP Carbohydrate H+ 8µm (Thermo Fisher Scientific) which was protected by a HyperREZ^{TM} XP Carbohydrate Guard (Thermo Fisher Scientific). The conditions used in the analysis were as follows: eluent, 1,5mM H_2SO_4; flux, 0.6 ml/min; and oven temperature, 50°C. Samples were diluted 5-fold, filtered through a 0.22-µm nylon filter (Symta, Madrid, España) and injected by duplicate.

Before curve fitting, weight loss data were corrected to % of consumed sugar according to the formula:

$$C=((m*[S-R])/(m_f*S))*100$$

Were C is the % of sugar consumed at each sample time, m is the weight loss value at this sampling time, S is the sugar concentration in the must at the beginning of experiment (g/L), R is the final sugar concentration in the fermented must (residual sugar, g/L) and m_f is the total weight loss value at the end of the fermentation (g).

Curve fitting was carried out using the reparametrized Gompertz equation proposed by Zwietering et al.[53]:

$$y = D* \exp\{ -\exp[((\mu max *e)/D)*(\lambda - t)+ 1]\}$$

where y is the % of consumed sugar; D is the maximum sugar consumption value reached (the asymptotic maximum, %), μ_{max} is the maximum sugar consumption rate (h^{-1}), and λ the lag phase period which sugar consumption was not observed (h). Data were fitted using the nonlinear regression module of Statistica 7.0 software package (StatSoft, Tulsa, OK, USA), minimizing the sum of squares of the difference between experimental data and the fitted model. Fit adequacy was checked by the proportion of variance explained by the model (R^2) respect to experimental data.

Kinetic parameters and HPLC data were analysed using Statistica 7.0 software package (StatSoft, Tulsa, OK, USA) by one way ANOVA and Tukey test for means comparison.

3.3. Results

We evaluated the genetic stabilization process of the intra- (*Saccharomyces cerevisiae* x *S. cerevisiae*) and interspecific (*S. cerevisiae* x *Saccharomyces kudriavzevii*) hybrids generated in a previous work[39] by both rare-mating and spore-to-spore mating. The stabilization method was based on consecutive fermentations on synthetic must at 20ºC and on the molecular variability analysis (δ elements, RAPD-PCR and mtDNA-RFLP) of hybrids after each fermentation step.

Significant differences were observed not only in the stabilization process of the intra- and interspecific hybrids, but also in the stabilization of those strains obtained by different procedures (rare-mating and spore-to-spore mating).

3.3.1. Stabilization of intraspecific hybrids

Different δ elements and RAPD-PCR patterns were detected in the colonies isolated during the successive fermentations inoculated with each particular hybrid strain. Table 3.2 provides the frequencies in which each particular combined δ elements-RAPD-PCR-mtDNA RFLP pattern appeared.

The genetic variability observed during the stabilization of hybrids generated by rare-mating (R2 and R8) was higher than that obtained by spore-to-spore mating (S2 and S7) for both nuclear and mitochondrial genomes. Six new δ elements patterns were found among the colonies derived from hybrid R2 (patterns I to VI), and eight patterns were obtained among the colonies derived from R8 (patterns I to VIII) (Table 3.2). Apart from the aforementioned patterns, the δ elements patterns exhibited by the original unstable hybrids R2 and R8 were recovered in the derived colonies isolated from all the successive fermentation steps (Table 3.2).

Low variation was detected among derived colonies by the RAPD-PCR method using primer R3. Only one different pattern was observed in one colony obtained in fermentation step 4 of hybrid R2 (named pattern a) and two (named patterns a and b) were obtained in the colonies derived from hybrid R8 after fermentation steps 4 and 5 (Table 3.2).

No variation in RAPD-PCR patterns was detected among the colonies isolated during the five successive fermentation steps inoculated with hybrids S2 and S7 generated by spore-to-spore mating. Only two δ elements patterns, which differed from that present in the original hybrid, were detected during the stabilization of S2 (patterns I and II) (Table 3.2).

Variations in the mtDNA-RFLP patterns were detected only during the stabilization of hybrid R8 obtained after rare-mating. Five different mtDNA-RFLP patterns were identified during the process.

Individual colonies (clones), representative of each hybrid and molecular pattern detected after the complete set of consecutive fermentations, were used to inoculate fresh synthetic must in order to confirm their genetic stability. Of those colonies showing a same molecular pattern, only those from the last fermentation steps were evaluated individually (i.e.,the R2ooo"original pattern" was taken from the fifth fermentation, R2Ioo, R2IIIao, R2IVoo and R2Voo from the fourth, and R2IIoo, R2IIIoo and R2VIoo from the fifth). We followed the same methodology used during the stabilization process: after fermentation, ten colonies were isolated and molecularly characterized. As a result of this evaluation, most clones conserved the same molecular patterns as before, except for clones R2Voo, R8ooA, R8ooB, R8ooC, R8IoB and R8IoD and the original R2 and R8 (data not shown).

Table 3.2: Molecular characterization of yeast colonies after successive fermentation steps of intraspecific hybrids and frequency.

Hybridization method	Hybrid[a]	Molecular patterns and frequency (%)[b]																							
		Original[c]				1° STEP				2° STEP				3° STEP				4° STEP				5° STEP			
		δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%
Rare-mating	**R2**	**δ-5 (o)**	**R-8 (o)**	**Sc2(o)**	**100**	**o**	**o**	**o**	**90**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**90**	**o**	**o**	**o**	**30**	**o**	**o**	**o**	**20**
		-	-	-	-	I	o	o	10	-	-	-	-	-	-	-	-	I	o	o	10	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	II	o	o	10	II	o	o	20	II	o	o	60
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	III	o	o	10	III	o	o	10
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	III	a	o	10	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IV	o	o	10	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	o	o	10	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	VI	o	o	10
	R8	**δ-4 (o)**	**R-7 (o)**	**Sc1(o)**	**100**	**o**	**o**	**o**	**20**	**o**	**o**	**o**	**40**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
		-	-	-	-	o	o	A	40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	o	o	B	30	o	o	B	60	o	o	B	90	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	o	o	C	10	o	o	C	40
		-	-	-	-	I	o	B	10	-	-	-	-	I	o	B	10	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	o	D	30	I	o	D	20
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	II	o	E	10	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	II	a	E	10	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	III	o	E	10	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IV	o	D	10	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	o	E	10	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	b	C	10
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	VI	o	C	10	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	VII	o	E	10
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	VIII	o	E	20
Spore to spore mating	**S2**	**δ-10 (o)**	**R-9 (o)**	**Sc2(o)**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**90**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**90**	**o**	**o**	**o**	**100**
		-	-	-	-	-	-	-	-	I	o	o	10	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	II	o	o	10	-	-	-	-
	S7	**δ-14 (o)**	**R-10 (o)**	**Sc1(o)**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**

a- Hybrid names R2, R8, S2 and S7 correspond to intraspecific hybrids in Pérez-Través et al. [39] (Chap 2).

b- **δ**: patterns obtained by δ elements characterization (identified with roman numbers, patterns exhibited by the original hybrids were designed as "o"); **R3**: patterns obtained by RAPD-PCR with primer R3 (identified with lowercase letters, patterns exhibited by the original hybrids was designed as "o"). **mt**: patterns obtained by mtDNA-RFLP analysis (identified with capital letters, patterns exhibited by the original hybrids were designed as "o"). **%:** percentage of detection of a particular combination of δ elements and RAPD-PCR patterns after a particular fermentation step.

c- Molecular patterns characterized by Pérez-Través et al. [39] (Chap 2). These patterns were identified as "original patterns (o)" in this work.

In order to evaluate if the changes detected between the molecular markers were also coincident with the changes in total DNA content, the clones possessing each different molecular pattern were subjected to measuring DNA content by flow cytometry (Table 3.3).Fig1 shows the evolution in the total DNA content values obtained for all analysed clones derived from each original hybrid strain during the stabilization process.

After this analysis, we observed that all the clones obtained after the consecutive fermentation steps of the spore-to-spore-generated hybrids conserved the same ploidy values found in original hybrids S2 and S7, including those showing different δ elements patterns (Fig1, Table 3.3).

Among the clones derived from rare-mating-generated hybrids R2 and R8, the DNA content values varied from 5n (n being the DNA content of a haploid laboratory strain) in the original inoculated hybrids to approximately 2.5n in the clones (Fig1, Table 3.3). Most of the clones derived from original hybrid R2 (obtained from fermentations steps 3, 4 and 5)had significantly different DNA content values from the value obtained in the original hybrid (close to 2.5n). An exception was observed for clone R2I00 and clone R2V00from fermentation steps 1 and 4, respectively, whose values came close to 5n (Fig1, Table 3.3). Finally, all the clones isolated from the different fermentation steps, but showing the original molecular pattern, also conserved the same ploidy value of around 5n (Fig1, Table 3.3). Three different situations were observed for the ploidy values shown by the clones derived from original hybrid R8. All the clones exhibiting an original molecular pattern in the nuclear genome (R8000, R800A, R800B and R800C) conserved high ploidy values ranging from 4.5n to 5n (Fig1, Table 3.3). The DNA content of clonesR8I0B and R8I0D, bearing δ elements pattern I, which emerged in fermentation step 1, was near 3.5n. The remaining clones, isolated from fermentations 4 and 5, exhibited ploidy values which came close to 2.5n (Fig1, Table 3.3).

$- Hybrid names R2, R8, S2 and S7 correspond to intraspecific hybrids in Pérez-Través et al. [39] (Chap 2).
&- Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, α=0.05, n=2).
#- Molecular patterns obtained by combination of interdelta, R3 and mtDNA-RFLP profiles.
¥- Colonies were considered as stable when both molecular patterns and DNA content did not change after individual colony fermentation.

Table 3.3: DNA content of hybrids showing different combined molecular patterns during the whole process of intraspecific hybrids stabilization.

Original hybrid[$]	DNA content[&]	1°STEP		2° STEP		3° STEP		4° STEP		5° STEP		Stable[¥]
		Pattern[#]	DNA content[&]	Pattern[#]	DNA content[&]	Pattern[#]	DNA content[&]	Pattern[#]	DNA content[&]	Pattern[#]	DNA content[&]	
R2	**5.0 ± 0.1[b]**	**ooo**	**5.0 ± 0.5[b]**	**ooo**	**4.8 ± 0.4[b]**	**ooo**	**4.9 ± 0.2[b]**	**ooo**	**5.0 ± 0.2[b]**	**ooo**	**4.7 ± 0.1[b]**	**NO**
		Ioo	5.0 ± 0.5[b]	-	-	-	-	Ioo	2.6 ± 0.1[a]	-	-	YES
		-	-	-	-	IIoo	2.8 ± 0.2[a]	IIoo	2.7 ± 0.1[a]	IIoo	2.7 ± 0.1[a]	YES
		-	-	-	-	-	-	IIIoo	2.7 ± 0.1[a]	IIIoo	2.5 ± 0.3[a]	YES
		-	-	-	-	-	-	IIIao	2.5 ± 0.1[a]	-	-	YES
		-	-	-	-	-	-	IVoo	2.3 ± 0.1[a]	-	-	YES
		-	-	-	-	-	-	Voo	5.1 ± 0.3[b]	-	-	NO
		-	-	-	-	-	-	-	-	VIoo	2.6 ± 0.2[a]	YES
R8	**4.7 ± 0.3[b]**	**ooo**	**4.8 ± 0.5[c]**	**ooo**	**4.7 ± 0.2[c]**	**-**	**-**	**-**	**-**	**-**	**-**	**NO**
		ooA	4.8 ± 0.3[c]	-	-	-	-	-	-	-	-	NO
		ooB	4.8 ± 0.5[c]	ooB	4.7 ± 0.3[c]	ooB	4.7 ± 0.2[c]	-	-	-	-	NO
		-	-	-	-	-	-	ooC	4.5 ± 0.2[c]	ooC	4.9 ± 0.6[c]	NO
		IoB	3.5 ± 0.5[b]	-	-	IoB	3.5 ± 0.1[b]	-	-	-	-	NO
		-	-	-	-	-	-	IoD	3.6 ± 0.2[b]	IoD	3.5 ± 0.4[b]	NO
		-	-	-	-	-	-	IIoE	2.3 ± 0.1[a]	-	-	YES
		-	-	-	-	-	-	IIaE	2.2 ± 0.1[a]	-	-	YES
		-	-	-	-	-	-	IIIoE	2.6 ± 0.1[a]	-	-	YES
		-	-	-	-	-	-	IVoD	2.5 ± 0.1[a]	-	-	YES
		-	-	-	-	-	-	VoE	2.6 ± 0.1[a]	-	-	YES
		-	-	-	-	-	-	-	-	VbC	2.7 ± 0.1[a]	YES
		-	-	-	-	-	-	VIoC	2.6 ± 0.3[a]	-	-	YES
		-	-	-	-	-	-	-	-	VIIoE	2.9 ± 0.1[a, b]	YES
		-	-	-	-	-	-	-	-	VIIIoE	2.6 ± 0.1[a]	YES
S2	**2.7 ± 0.1[a]**	**ooo**	**2.5 ± 0.2[a]**	**ooo**	**2.6 ± 0.1[a]**	**ooo**	**2.3 ± 0.2[a]**	**ooo**	**2.6 ± 0.2[a]**	**ooo**	**2.6 ± 0.2[a]**	YES
		-	-	Ioo	2.5 ± 0.2[a]	-	-	-	-	-	-	YES
		-	-	-	-	-	-	IIoo	2.4 ± 0.2[a]	-	-	YES
S7	**2.8 ± 0.2[a]**	**ooo**	**2.7 ± 0.1[a]**	**ooo**	**2,72 ± 0,17[a]**	**ooo**	**2.5 ± 0.2[a]**	**ooo**	**2.7 ± 0.2[a]**	**ooo**	**2.4 ± 0.2[a]**	YES

See foot notes in the page before.

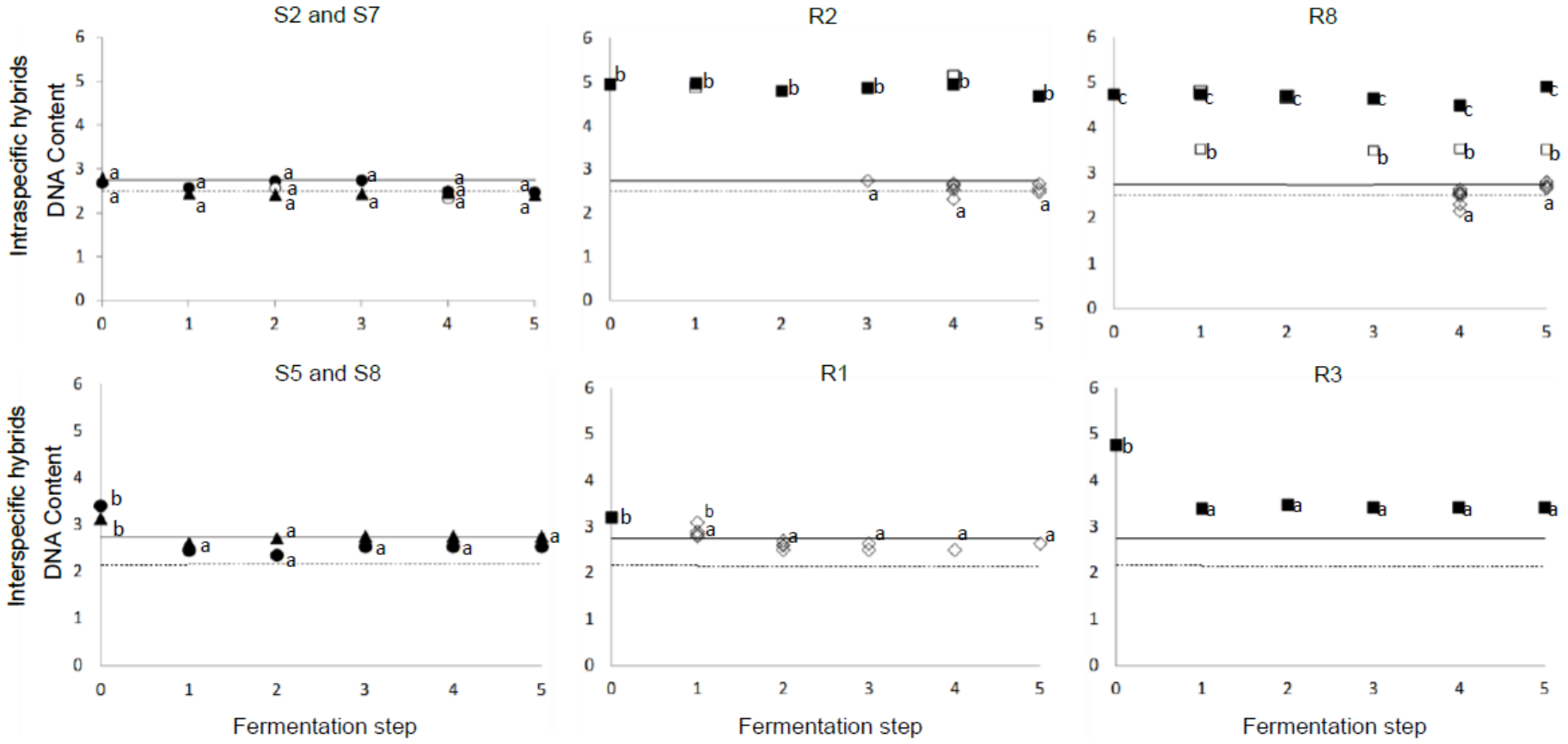

Figure 3.1: Changes in DNA content of hybrid cultures during stabilization process of intraspecific (spore to spore hybrids S2 and S7, and rare-mating hybrids R2 and R8) and interspecific (spore to spore hybrids S5 and S8, and rare-mating hybrids R1 and R3) hybrids. Circles: spore to spore hybrids S2 (intraspecific) and S8 (interspecific). Triangles: spore to spore hybrids S7 (intraspecific) and S8 (interspecific). Squares: Rare-mating hybrids. Diamonds: stable rare-mating hybrids. Dotted line indicate the ploidy value showed by the parental Sc2 (intraspecific hybrids stabilization) and parent Sk (interspecific hybrids stabilization). Filled symbols indicate cultures with the same molecular pattern found in the original hybrid inoculated in the first fermentation step. Empty symbols indicate cultures with molecular patterns different from the original. Symbols with different letters among cultures derived from a same original hybrid, indicate statistically significant differences (ANOVA and HSD Tukey test, α=0.05).

DNA content analysis carried out in those colonies obtained after individual clone fermentation revealed high ploidy variability among the colonies derived from the clones with high DNA contents (R2ooo, R2Voo, R8ooo, R8ooA, R8ooB, R8ooC, R8IoB and R8IoD). In their delta pattern, R8IoB and R8IoD also changed. The clones whose DNA content came close to 2.5n maintained the same values after individual fermentation. An example about the variation or the maintenance in the ploidy levels after individual clone fermentation is shown in Figure 3.2.

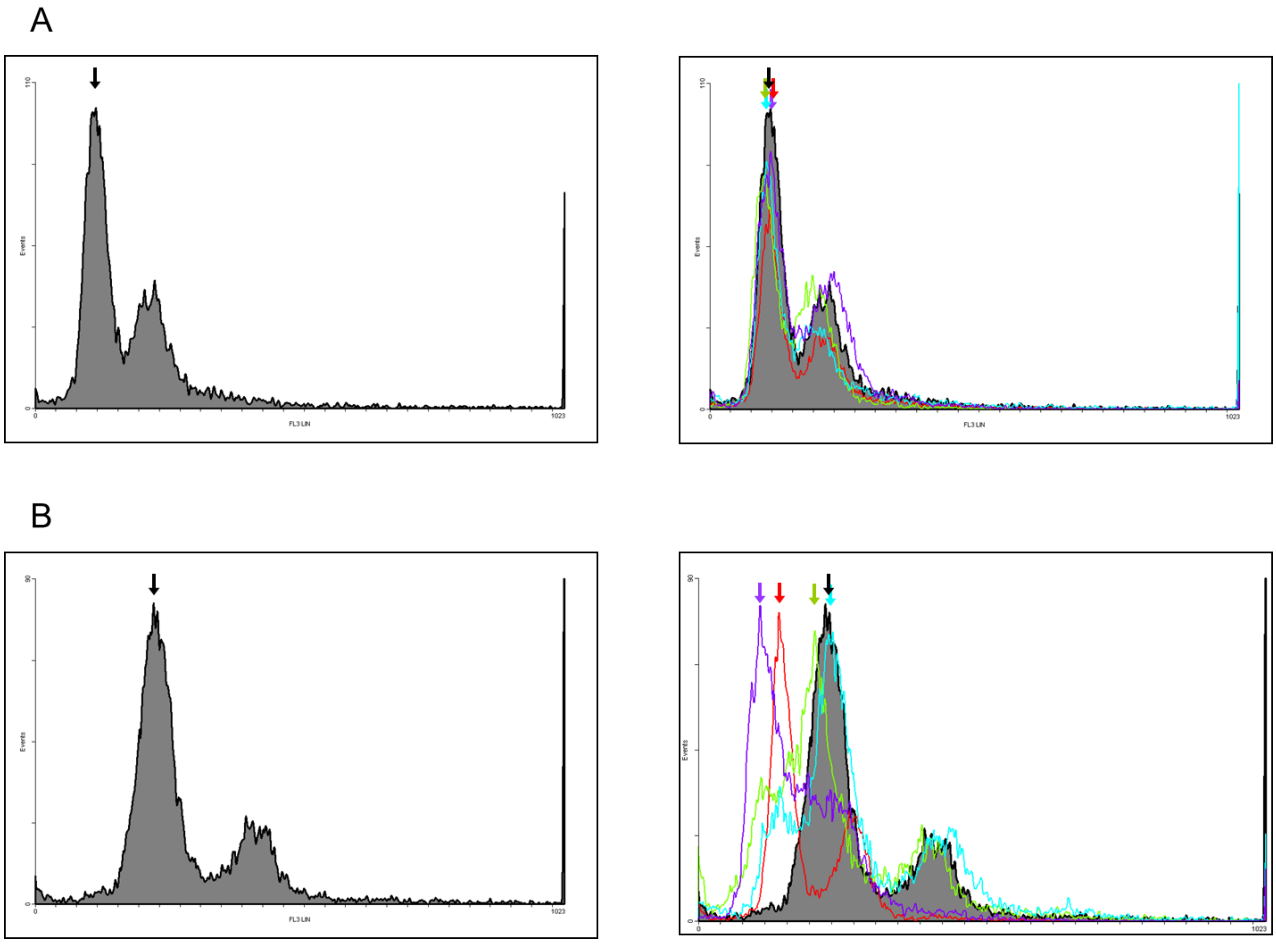

Figure 3.2: Analysis of total DNA content (as fluorescence intensity) carried out by flow cytometry in the stable hybrid **R2 IIIo (A)** and in the instable hybrid **R2 Vo (B)** before (left) and after (right) individual inoculation of synthetic must. Shadowed areas indicate the total DNA content of the cultures before inoculation and lines in color indicate fluorescence intensity of different colonies recovered after individual fermentations. Arrows indicate picks considered for DNA content determinations.

3.3.2. Stabilization of interspecific hybrids

For interspecific hybrid R3(generated by rare-mating), and S5 and S8 (generated by spore-to-spore mating), all the clones obtained during the five fermentation steps showed the same molecular pattern at both the nuclear and mitochondrial levels, as detected in the original hybrid strains (data not shown). The stabilization process of hybrid R1 evidenced no variation in genomic DNA patterns, nowadays new mtDNA patterns appeared, particularly in early process stages (fermentation step1 and 2; Table 3.4). The emergence of new mtDNA patterns could indicate that rearrangements have occurred. One of the new patterns was present until the end of the stabilization assay. In all cases, significantly different ploidy values were observed between the originally inoculated hybrid strains and all the clones recovered after each fermentation step, irrespectively of the hybridization method employed for hybrid generation (rare-mating or spore-to-spore mating). After the first step, clones maintained the same ploidy value until the end of the process (Figure 3.1, Table 3.5).

After inoculating fresh media with individual clones, no changes were observed in molecular patterns and ploidy levels (data not shown).

Table 3.4: Molecular characterization of yeast colonies after successive fermentation steps of interspecific hybrids and frequency.

Hybridization method	Hybrid[a]	Molecular patterns and frequency (%)[b]																							
		Original[c]				1° STEP				2° STEP				3° STEP				4° STEP				5° STEP			
		δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%
Rare-mating	**R1**	**δ-4 (o)**	**R-2 (o)**	**Sk (o)**	**100**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	o	o	A	20	o	o	A	40	o	o	A	20	o	o	A	100	o	o	A	100
		-	-	-	-	o	o	B	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	o	o	C	40	o	o	C	20	o	o	C	80	-	-	-	-	-	-	-	-
		-	-	-	-	o	o	D	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	o	o	E	20	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	o	o	F	20	-	-	-	-	-	-	-	-	-	-	-	-
	R3	**δ-4 (o)**	**R-4 (o)**	**Sc1(o)**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**
Spore to spore	**S5**	**δ-9 (o)**	**R-11 (o)**	**Sk (o)**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**
mating	**S8**	**δ-8 (o)**	**R-12 (o)**	**Sc1(o)**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**	**o**	**o**	**o**	**100**

a- Hybrid names R1, R3, S5 and S8 correspond to interspecific hybrids in Pérez-Través et al. [39] (Chap 2).

b- **δ:** patterns obtained by δ elements characterization (Identified with roman numbers, patterns exhibited by the original hybrids were designed as "o"); **R3**: patterns obtained by RAPD-PCR with primer R3 (identified with lowercase letters, patterns exhibited by the original hybrids was designed as "o"). **mt**: patterns obtained by mtDNA-RFLP analysis (identified with capital letters, patterns exhibited by the original hybrids were designed as "o"). **%:** percentage of detection of a particular combination of δ elements and RAPD-PCR patterns after a particular fermentation step.

c- Molecular patterns characterized by Pérez-Través et al. [39] (Chap 2). These patterns were identified as "original patterns (o)" in this work

Table 3.5: DNA content of hybrids showing different combined molecular patterns during the whole process of interspecific hybrids stabilization.

Original hybrid[$]	DNA content[&]	1°STEP		2° STEP		3° STEP		4° STEP		5° STEP		Stable[¥]
		Pattern[#]	DNA content[&]	Pattern[#]	DNA content[&]	Pattern[#]	DNA content[&]	Pattern[#]	DNA content[&]	Pattern[#]	DNA content[&]	
R1	**3.2 ± 0.2[b]**	ooA	2.8 ± 0.2[a]	ooA	2.7 ± 0.1[a]	ooA	2.65 ± 0.13[a]	ooA	2.50± 0.09[a]	ooA	2.64 ± 0.03[a]	YES
		ooB	3.1 ± 0.1[b]	-	-	-	-	-	-	-	-	YES
		ooC	2.8 ± 0.3[a]	ooC	2.6 ± 0.1[a]	ooC	2.52 ± 0.22[a]	-	-	-	-	YES
		ooD	2.9 ± 0.2[a]	-	-	-	-	-	-	-	-	YES
		-	-	ooE	2.5 ± 0.2[a]	-	-	-	-	-	-	YES
		-	-	ooF	2.6 ± 0.2[a]	-	-	-	-	-	-	YES
R3	**4.8 ± 0,1[b]**	**ooo**	**3.4 ± 0.1[a]**	**ooo**	**3.5 ± 0.1[a]**	**ooo**	**3.4 ± 0.3[a]**	**ooo**	**3.5 ± 0.1[a]**	**ooo**	**3.6 ± 0.1[a]**	YES
S5	**3.4 ± 0,1[b]**	**ooo**	**2.5 ± 0.2[a]**	**ooo**	**2.4 ± 0.2[a]**	**ooo**	**2.5 ± 0.3[a]**	**ooo**	**2.5 ± 0.1[a]**	**ooo**	**2.4 ± 0.2[a]**	YES
S8	**3.2 ± 0,2[b]**	**ooo**	**2.6 ± 0,2[a]**	**ooo**	**2.7 ± 0,1[a]**	**ooo**	**2.8 ± 0,2[a]**	**ooo**	**2.8 ± 0,3[a]**	**ooo**	**2.7 ± 0,2[a]**	YES

$- Hybrid names R1, R3, S5 and S8 correspond to interspecific hybrids in Pérez-Través et al. [39] (Chap 2).

&- Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, α=0.05, n=2).

#- Molecular patterns obtained by combination of interdelta, R3 and mtDNA-RFLP profiles.

¥- Colonies were considered as stable when both molecular patterns and DNA content did not change after individual colony fermentation.

3.3.3. Stability evaluation after active dry yeast (ADY) production

A decision was made to evaluate if clones, obtained by the methodology proposed in this work, maintained their genetic stability after undergoing the ADY production process (Lallemand Inc. protocols). For this purpose, stable intraspecific hybrid clones were selected to undergo the ADY preparation process. These clones were selected because intraspecific hybrids were more variable during the stabilization process than interspecific ones. Furthermore, our approach based on employing an *S. cerevisiae*-based microarray is not useful for detecting genes from *S. kudriavzevii* which greatly diverge with *S. cerevisiae*.

Stabilized clones R2IVoo and R8IIaEwere used for ADY production under the habitual conditions (Lallemand Inc. protocols). After the process, the produced ADY samples were rehydrated and seeded in the complete medium. Ten colonies of each sample, obtained after incubation were evaluated by the same genetic markers and ploidy previously employed during stabilization. No changes were observed in the evaluated parameters of the obtained colonies in relation to the clone R2IVoo before the dryness process, otherwise their happened for the clone R8IIaE, which changed in its delta profile (data not shown).

Additionally, in order to ensure that no changes in genomic constitution -including variation in genes copy number- occurred during ADY production for R2IVoo clone, the rehydrated culture was compared at a single gene resolution with the same strain without being subjected to the dryness process. For this comparison, genomic DNA isolated from the clone before dryness and labelled with one fluorescent dye was mixed with the DNA from the colonies obtained after ADY production and rehydration, which was labelled with a different dye. This mixture was then co-hybridized in a *S. cerevisiae* DNA microarray (see Materials and Methods).

Differences in the log_2 of the Cy5/Cy3 signal ratio obtained for each ORF possibly indicate variations in the relative copy number of *S. cerevisiae* genes present in the hybrid strain before and after the dryness process. Log_2 ratios close to zero for a particular ORF indicate the presence of the same number of DNA copies in both genomes, while higher or lower log_2 ratios than zero may indicate more or less copies, or even depleted genes (ORF deletions). Our results do not evidence changes in the gene copy numbers between the two analysed genomes, suggesting that no changes in the DNA composition of our clone R2IVoo occurred in the industrial dry yeast generation process (data not shown).

Finally we decided to carry out a fermentation in natural must with the hybrid clone before and after ADY production. No differences were found in residual sugars content, glycerol and ethanol production, neither in parameters analysis (latency, maximum fermentation rate and time necessary to consume 95% of fermentable sugars) (Table 3.6).

Table 3.6: Main kinetic parameters of the fermentations carried out with both parental and hybrid strains on albariño must at 22°C and chemical analysis of the final fermented products.

Strain	Kinetic parameters[$]			Chemical parameters[$]			
	K (hours^{-1})[&]	l (hours)	t_{95} (hours)[#]	Glucose (g/L)[¥]	Fructose (g/L)	Glycerol (g/L)	Ethanol (% v/v)
R2IVo	1.57±0.02[a]	19.38±0.92[a]	125.20±1.20[a]	bdl	1.09±0.11[a]	5.35±0.06[a]	11.81±0.01[a]
R2IVo LSA	1.54±0.01[a]	20.39±0.50[a]	125.97±0.95[a]	bdl	0.89±0.02[a]	5.38±0.03[a]	11.79±0.03[a]

$- Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, α=0.05, n=2).
&- K: kinetic constant.
#- t95: time necessary to consume 95% of fermented sugars.
¥- bdl: value below detection limit (0.05g/L)

3.4. Discussion

Interspecific hybrids have been isolated from different fermented beverages, including wine, cider and beer [45,48]. Even one of the most popular beverages, lager beer, is prepared by hybrid yeast *S. pastorianus* containing both the *S. cerevisiae* and *S. eubayanus* subgenomes[28]. In most cases, hybrids acquire interesting combinations of physiological features from parental strains, and prove to be promising tools for specific technological uses. For this reason, many artificial hybrid yeasts have been constructed in recent decades to improve different industrial processes such as winemaking [6,12,39], brewing [47]and bakery [25,47], and also for basic studies [13,34]. However, very few works mention and evaluate the necessary genetic stabilization process occurring immediately after hybridization [2,6,26,39], an important aspect when the strains are going to be used in industrial processes, where the product homogeneity is looked for because starters ensures the production of consistent products in successive vintages [42].

Genome reduction and rearrangements occurring during the stabilization of newly formed hybrids have been reported, and this process seemed to be different in unstressed or in a salt-stressed media [19,20]. These phenomena might lead to loss of industrially important traits in hybrids, and can be avoided if a selective pressure, mimicking the desired industrial process, is applied during the stabilization. For this reason, an important step in the hybrids study is the careful selection of stabilization conditions.

In a previous work carried out in our laboratory[39]*S. cerevisiae* x *S. cerevisiae* and *S. cerevisiae* x *S. kudriavzevii* hybrids were successfully obtained by means of different hybridization methods, which included protoplast fusion, rare-mating and spore-to-spore mating. Here we present the changes observed in some interspecific and intraspecific hybrid strains generated in that previous work throughout the genetic stabilization process carried out in selective media (in this case, by successive fermentation steps in synthetic must). We compared the stabilization process in the inter- and intraspecific hybrids showing high ploidy values (resulting from the rare-mating of two parental strains close to diploidy) and the stabilization of hybrids close to diploidy (most hybrids resulted from spore-to-spore mating).

Flow cytometry identifies large-scale (ploidy level) changes in genome size throughout the stabilization process in most hybrids. This reduction is significant, particularly for the hybrids generated by rare-mating, which originally possessed two diploid parental sets of chromosomes. Genome reduction in intraspecific rare-mating hybrids R2 and R8 seems to occur drastically in fermentation steps 3 and 4(Figure 3.1), although in hybrid R8 an intermediate reduction occurred in fermentation step 1.According to the results obtained after fermentation with the individual R2 and R8 derived colonies, stable clones correspond only to those exhibiting the same ploidy values found in parental strains Sc1 (2.7n) and Sc2 (2.3n).

The genome reduction process in interspecific hybrids is faster than that observed for intraspecific ones. This reduction occurs in fermentation step1; see Figure 3.1.All the colonies recovered in fermentation steps 2 to 5 show the same ploidy values. The ploidy values at which hybrids become stable are similar to the parental strains ploidy (in S5, S8 and R1) or to a higher one (R3). A similar genome reduction process was also evidenced by Antunovics *et al.*[2] after the stabilization of *S. cerevisiae* x *S. uvarum* hybrids by successive sporulation events, and also by Marinoni *et al.*[32] after interspecific hybridization by mass-mating. In experimental evolution studies, Gerstein *et al.*[19] observed that the DNA content of triploid and tetraploid cultures of *S. cerevisiae* diminished. This reduction occurs in the first generations and all the clones show a tendency to stabilize, with ploidy values close to 2n (historical ploidy values, the ploidy shown by the original strain). The authors also observed that cultures maintain a higher ploidy under stress conditions.

Chromosomal instability in artificial polyploid *S. cerevisiae* strains was previously observed by several authors [1,19,50], together with high mutation and recombination levels.

In this work, apart from a reduced ploidy, variation in nuclear (evidenced in new δ elements and RAPD-PCR profiles) and mitochondrial (evidenced in new mtDNA-RFLP patterns) genomes was observed during the stabilization process. As a result, all these changes gave us a large number of clones derived from an individual hybrid. Thus, the stabilization process generates a genetic variability among the recovered colonies. These new molecular patterns are observed mainly during the stabilization of the intraspecific hybrids obtained by rare-mating, which evidences the existence of extensive genetic rearrangements among genetically

similar genomes. This phenomenon is not observed for interspecific hybrids, irrespectively of the hybridization method used for their generation; only hybrid R1 shows mitochondrial genome variability after fermentation step 1, but only one pattern consecutively appears until the end of the process (R100A). Contrarily to our results, Bellón *et al.*[6] did not detect changes in DNA molecular patterns in recently generated interspecific hybrid strains after 50 generations in the model medium and grape juice; however, the authors reported neither changes in ploidy values nor monitoring these changes throughout the stabilization process.

To summarize, different situations emerge throughout the process after analysing hybrids: i) stabilization by gradual loss of genetic material with no detectable changes in nuclear or mitochondrial DNA patterns (interspecific hybrids R3, S5 and S8); ii) stabilization after nuclear genetic rearrangements and ploidy reduction until historical values in parental strains (rare-mating intraspecific hybrids) with (R8) or without (R2) mitochondrial genome changes; iii) stabilization after rapid loss of genetic material with no changes in genomic markers, but in the mtDNA-RFLP patterns (interspecific hybrid R1).

Our results confirm that both nuclear and mitochondrial genomes can undergo changes during the stabilization process of newly generated intra- and interspecific hybrids in the genus *Saccharomyces*. Intraspecific hybrids seem to require a larger number of generations to generate genetically stable cells, while interspecific hybrids undergo a faster stabilization process and are active mainly in early stages.

ADY production is an essential step to prepare a wine yeast starter culture during which yeast is affected by a number of different stresses[3,4,14,21,36]. As changes in the ploidy level, genes copy number and chromosomal rearrangements have been observed in *Saccharomyces* strains subjected to different stress[15,19,37] or culture conditions[10,16,24], the genomic stability of two representative hybrids strains were evaluated by molecular markers and ploidy analyses before and after ADY production. Two clones were selected, as representative of the set of hybrids obtained from intraspecific rare-mating, because the stabilization of such hybrids shows the highest variability in ploidy and molecular patterns. We observed no changes in DNA content of both strains, but molecular patterns changed in one of them (R8 IIaE hybrid strain). We observed no large amplification or deletion in the genome of R2 IVoo clone after the

process and no differences were found when we compared, in fermentation, the hybrid before and after ADY production.

These results evidence the success of both the stabilization protocol and the selection of stable hybrids proposed in this work. Our results suggest that molecular markers such as δ elements and mtDNA-RFLP patterns, as well as ploidy evaluation, allow the quick assessment of the genotypic stability of recently generated inter- and intraspecific *Saccharomyces* hybrid strains, and that the evaluation of these parameters should be done before and after ADY production. According to our results, and by considering that a stable hybrid must maintain the same molecular pattern and the same ploidy level during successive cell divisions, we find that fermentation steps 3 and 5 (30 and 50 generations) suffice to obtain genetically stable interspecific and intraspecific hybrids, respectively, irrespectively of the hybridization methodology used for their generation.

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Capítulo 4

Chapter 4. Physiological and genomic characterization of *Saccharomyces cerevisiae* hybrids with improved fermentation performance and mannoprotein release capacity.

International Journal of Food Microbiology 205 (2015) 30–40

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Physiological and genomic characterisation of Saccharomyces cerevisiae hybrids with improved fermentation performance and mannoprotein release capacity

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article info

Article history:
Received 18 July 2014
Received in revised form 14 March 2015
Accepted 2 April 2015
Available online 9 April 2015

Keywords:
Yeast hybridization
Rare-mating
Spore-to-spore mating
Wine yeast

abstract

Yeast mannoproteins contribute to several aspects of wine quality by protecting wine against protein haze, reducing astringency, retaining aroma compounds and stimulating lactic-acid bacteria growth. The selection of a yeast strain that simultaneously overproduces mannoproteins and presents good fermentative characteristics is a difficult task. In this work, a Saccharomyces cerevisiae × S. cerevisiae hybrid bearing the two oenologically relevant features was constructed. According to the genomic characterisation of the hybrids, different copy numbers of some genes probably related with these physiological features were detected. The hybrid shared not only a similar copy number of genes SPR1, SWP1, MNN10 and YPS7 related to cell wall integrity with parental Sc1, but also a similar copy number of some glycolytic genes with parental Sc2, such as GPM1 and HXK1, as well as the genes involved in hexose transport, such as HXT9, HXT11 and HXT12. This work demonstrates that hybridisation and stabilisation under winemaking conditions constitute an effective approach to obtain yeast strains with desirable physiological features, like mannoprotein overproducing capacity and improved fermentation performance, which genetically depend of the expression of numerous genes (multigenic characters).

http://www.sciencedirect.com/science/article/pii/S0168160515001853#

4.1. Introduction.

Since the inoculation concept of wine fermentations with pure yeast starter cultures by Mueller-Thurgau from Geisenheim was introduced in 1890 and the subsequent development of active dry yeasts in winemaking, several *Saccharomyces cerevisiae* starter cultures with particular features of enological interest have been developed (Pretorius, 2000).The use of these starters ensures the production of consistent wines that have particular desirable organoleptic characteristics in successive vintages.

The selection of *S. cerevisiae* strains as starter cultures for wine fermentation has been based on different physiological features. These features include good fermentative vigour and fermentation rate, low production of SH_2 and acetic acid, low foam production, resistance to SO_2, and the production of balanced levels of volatile aromatic compounds such as higher alcohols and esters, among others (Schuller and Casal, 2005).

In the last 15 years, the capacity of yeast strains to release mannoproteins has also been included among the selection criteria applied for wine yeast selection. These highly glycosylated proteins, which are mostly present in the yeast cell wall, have been associated with positive quality and technological traits of wines, including protection against protein and tartaric instability, retention of aroma compounds, reduced astringency, increased body and mouthfeel, stimulation of lactic acid bacteria growth and foam quality improvement(Caridi, 2006).

Wine ageing with yeast lees and addition of enzymatic preparations that enhance the mannoproteins released to wine are two possible ways to increase the mannoprotein content of wines. However, these practices are subjected to normative limitations and require careful management to avoid off-flavours and wine spoilage. In this context, the use of selected yeasts that overproduce mannoproteins and show good fermentative features seems an interesting alternative.

Despite the selection pressure exerted by the millennia of winemaking on wine yeasts, the combination of desired interesting oenological traits that matches the actual requirements of starter cultures is not easy to find in a single strain. In particular, mannoprotein release is a

difficult complex character to be used as a selection criterion, especially for screening large numbers of strains. For this reason, different strategies based on mutations of some specific genes or recombinant strains that have been improved for mannoprotein release have been developed(González-Ramos et al., 2008; González-Ramos et al., 2009; González-Ramos and González, 2006; Quirós et al., 2010). However, the practical usefulness of some of these approaches is limited since the use of GMOs (Genetic Modified Organisms) in food applications –particularly in wine- is strictly regulated in most countries and often faces consumer rejection. In order to solve this limitation, other non-GMO-producing methodologies must be used to generate wine strains that offer good fermentative features and high production and release of mannoproteins.

Additionally, given the multigenic character of mannoprotein production and release by yeast cells -just the synthesis and organisation of the cell wall directly or indirectly involves about 1,200 genes(Klis, 1994; Lesage et al., 2004)- and other oenologically relevant features like fermentative behaviour(Giudici et al., 2005; Marullo et al., 2004), wine strain improvement based on strategies such as the hybridisation of two genomes is one of the best methods to consider(Pérez-Través et al., 2012). Mating spores and rare-mating –based on the rare event of mating type switching in industrial yeasts- can be considered natural processes that can happen in nature without human intervention. Therefore, the obtained hybrid cells that make full use of these natural phenomena do not fall under GMO rules.

The objective of the present work is to improve the fermentation capability of a commercial strain (Sc1) that was been selected as a good mannoproteins producer. We develop an intraspecific hybrid between the two commercial strains Sc1 and Sc2 by rare mating that give rise to non-GMO strains. After the genomic stabilisation we obtain a strain that overproduce mannoprotein and show excellent fermentation capacities. The potential relationship between the copy number of specific genes and the improved features was also evaluated by a CGH analysis of the parental and hybrid strains.

4.2. Materials and methods.

4.2.1. Yeast strains and general culture conditions.

Nineteen stable intraspecific hybrids, obtained in a previous work (Perez-Través et al., 2015), were used. 15 from the R (rare-mating) hybrids and 3 from the S (spore-to-spore) hybrids (Table 4.1).

The two parental strains, two *Saccharomyces cerevisiae* industrial strains from Lallemand S.A.S., were used as a reference strains. According to producers' Sc1 was selected for its capacity to release large amounts of mannoproteins during industrial winemaking (Sc1 improves mid-palate mouthfeel, softens tannins, and enhances the varietal characteristics of the fruit; shows a good compatibility with malolactic fermentation and is a moderate rate fermenter, and for not to be an excellent fermenting yeast; Lallemand personal communication). Sc2 was chosen for its excellent fermentative behaviour (Sc2 is resistant to difficult fermentation conditions, such as low turbidity, low temperature and low fatty acid content, presents a fast fermentation speed and low relative nitrogen needs; Lallemand personal communication).

Strains were maintained in GPY-agar medium (% w/v: yeast extract 0.5, peptone 0.5, glucose 2, agar-agar 2).

4.2.2. Fermentation experiments.

4.2.2.1. Synthetic must fermentation.

All the strains were used in synthetic must fermentations. Fermentations were carried out in 100-ml bottles containing 80ml of synthetic must (Rossignol et al., 2003). The sugar concentration in the must (50% glucose + 50% fructose) was adjusted to 200 g/l. Must was inoculated independently with the different yeast strains to reach an initial population of $2*10^6$ CFU/ml and was maintained without aeration at 20°C. The fermentation process was monitored by the quantification of the total sugar concentration. For this purpose, 1-mL aliquots of must were taken every 2 days and the sugar concentration was determined enzymatically (the glucose-fructose determination kit, Symta, Madrid, Spain). Fermentations were considered as stopped when the sugar amount was the same during 3 measures. Each fermentation

experiment was done twice. The sugar consumption data obtained from each fermentation were fitted by the following exponential decay function: $Y = D + S * e^{(-K * t)}$ as previously used by Arroyo-López et al. (2009). In this function, "Y" is the total amount of sugar present in must, "t" is the time in days, "D" is the asymptotic value when $t \rightarrow \infty$, "S" is the estimated value of change, and "K" is the kinetic constant ($days^{-1}$) which defines the maximum fermentation rate. Equations were fitted by the linear and non-linear regression procedures with the Statistica 7.0 software package (StatSoft, Tulsa, OK, USA), and by minimizing the sum of the squares of the difference between the experimental data and the fitted model. Fit adequacy was checked by the proportion of variance explained by the model (R^2) in relation to the experimental data. The obtained equations were used to calculate the time required to consume 50% of the initial sugar content present in must (t_{50}) and the time needed to consume almost all the amount of sugars leaving a residual amount of 2g/L (t_2). t_2 wasn't obtained in the stuck fermentations.

4.2.2.2. Natural must fermentations.

Sauvignon Blanc must was used to perform the stabilisation tests and Verdejo must was used to perform mannoprotein determination. Grape berries were pressed and 1mL/L of dimetil dicarbamate (DMDC) was added in order to obtain microbiological stability. Before the fermentation, Verdejo must was supplemented with Lalvin nutritive supplements (0.3g/L). Fermentations were done with parental and selected hybrid strains (R2 IVo, R8 IIa and S7 in Sauvignon Blanc fermentation and R2 IVo in Verdejo fermentation), at 20°C in 250-mL flasks containing 175 mL of must and were inoculated with an initial population of $2*10^6$ CFU/ml. Flasks were closed with Müller valves and were monitored by weight loss until reaching a constant weight. Immediately after fermentations ended, yeast cells were removed by centrifugation and supernatants were stored at 4°C until use. All the fermentations were analysed by HPLC in order to determine the amounts of residual sugars, glycerol, and ethanol as is described in a previous section. Each fermentation experiment in Sauvignon Blanc must was done twice (due to problems of availability of natural must) as a better must variety to perform the stabilisation tests and each fermentation experiment in Verdejo must was done three times (is the most similar musts to Sauvignon Blanc).

Before curve fitting, weight loss data were corrected to % of consumed sugar according to the following formula:

$$C=((m*[S-R])/(mf*S))*100$$

Were C is the % of sugar consumed at each sample time, m is the weight loss value at this sampling time, S is the sugar concentration in the must at the beginning of experiment (g/L), R is the final sugar concentration in the fermented must (residual sugar, g/L) and mf is the total weight loss value at the end of the fermentation (g).

Curve fitting was carried out using the reparametized Gompertz equation proposed by Zwietering et al.(1990):

$$y = D* \exp\{ -\exp[((\mu max *e)/D)*(\lambda - t)+ 1]\}$$

where y is the % of consumed sugar; D is the maximum sugar consumption value reached (the asymptotic maximum, %), μmax is the maximum sugar consumption rate (h–1), and λ the lag phase period which sugar consumption was not observed (h). Data were fitted using the nonlinear regression module of Statistica 7.0 software package (StatSoft, Tulsa, OK, USA), minimizing the sum of squares of the difference between experimental data and the fitted model. Fit adequacy was checked by the proportion of variance explained by the model (R2) respect to experimental data.

4.2.3. HPLC analysis of wines.

The supernatants of the fermentation end points were analysed by HPLC in order to determine the amounts of residual sugars (glucose and fructose), glycerol, and ethanol. A Thermo chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector was used. The column employed was a HyperREZ^{TM} XP Carbohydrate H+ 8μm (Thermo Fisher Scientific) and it was protected by a HyperREZ^{TM} XP Carbohydrate Guard (Thermo Fisher Scientific). The conditions used in the analysis were as follows: eluent, 1.5 mM H_2SO_4; flux, 0.6 ml/min; and oven temperature, 50°C. Samples were diluted 5-fold, filtered through a 0.22-μm nylon filter (Symta, Madrid, Spain) and injected in duplicate.

4.2.4. Analysis of polysaccharides and mannoproteins.

4.2.4.1. Synthetic must.

Once fermentation finished, wines were centrifuged to remove yeast cells and monosaccharides were removed from the cultures' supernatants by one gel filtration in Econo-Pac columns (Bio-Rad, Alcobendas, Spain) following the manufacturer's recommendations.

The concentration of the total mannoproteins and polysaccharides in the eluted fraction was determined against a standard curve of commercial mannan (Sigma, Tres Cantos, Spain) according to the phenol-sulphuric acid method as described by Segarra et al. (1995). Five replicates were performed for each determination. Standard curve of commercial mannan was:

mannan (mg/L) = (A490nm – 0.0473) / 0.0106

For the specific detection of mannoproteins, supernatants were resolved by SDS-PAGE(Laemmli, 1970). Proteins were transferred to a nitrocellulose membrane using the Mini Protean transfer system (Bio-Rad) following the manufacturer's directions. The mannoproteins present in the membrane were detected by the use of peroxidase-conjugated concanavaline A (Sigma) as described by Klis et al. (1998): incubate the membrane during 1h in blocking solution (BSA 3% prepared in PBS-Tween20); wash, during 5 min, two times, with PBS-Tween20 (NaH_2PO_4 100mM, NaCl 100mM, Tween20 0.1% v/v, pH 6.8, adjusted with NaOH); incubate 1h with hybridization solution (2.5mM $CaCl_2$, 2.5mM $MgCl_2$, 1µg/ml Concanavaline A solved in blocking solution); wash, during 5 min, two times, with PBS-Tween20; wash, during 10 min, one time, with PBS-Tween20; remove all the PBS-Tween20 solution and incubate during 1min with 1ml/8cm^2 of ECL reactive (Amersham); expose and reveal the membrane. This method isn't a quantitative method, but allows us to establish differences in mannoprotein production. The analysis complements the polysaccharide quantification.

4.2.4.2. Natural must.

For mannoprotein analysis in Verdejo must, the methodology proposed by Quirós et al.(2012) was followed with few modifications. Wines were centrifuged to remove yeast cells. Samples were gel filtered twice through 30 × 10 mm Econo-Pac® 10 DG disposable

chromatography columns (Bio-Rad Laboratories, Hercules, CA). Two aliquots of 1.9 ml of the macromolecular fraction were concentrated in 2 ml screw-capped microtubes until complete evaporation. The dried material was carefully suspended in 100 µl of 1 M H_2SO_4. Tubes were tightly capped and incubated in a water bath at 100 °C for 5 h 30 min to undergo acid hydrolysis. After this treatment, tubes were briefly spun down, and 10-fold diluted with MilliQ water. Sulphuric acid was removed by solid-phase extraction (SPE) with a Strata NH_2 500 mg/3 ml column (Phenomenex, Torrance, CA, USA). After SPE, samples were filtered through 0.22 µm pore size nylon filters (Membrane Solutions) and analysed in duplicate in a Surveyor Plus chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector (Surveyor RI Plus Detector). The column employed was a 300 × 7.7 mm PL Hi-Plex Pb 8 µm (Varian, Inc., Shropshire, UK). MilliQ water was used as the mobile phase at a flux of 0.6 ml/min and a column temperature of 70 °C.

Mannoprotein amount was determined against a standard curve of commercial mannan (Sigma, Tres Cantos, Spain) processed in the same conditions.

mannan (mg/L)=(mannose (mg/L)+0.9296)/0.7205

4.2.5. Protein Haze Analysis (Heat Test).

For the bentonite fining assays, bentonite was previously suspended and hydrated in distilled water at 50 g/L. Different amounts of the homogenised suspension were added to 25 mL of wine to reach 0, 12, 24 36, 48, or 60 g/hL. Closed tubes were incubated at room temperature in a rocking shaker for 30 min. Wines were then clarified by centrifugation, 5 min at 3,000g, and were filtered through a 0.45 µm PVDF filter. The stability of the bentonite-treated wines was assayed by incubating 5-mL aliquots (5aliquots of 5ml were measured for each sample) at 85°C for 30 min and cooling on ice. The turbidity of wines was determined in a nephelometer (Hach, Loveland, CO, USA).

4.2.6. Statistical analyses.

The kinetic parameters, HPLC and polysaccharides data were analysed using the Statistica 7.0 software package (StatSoft, Tulsa, OK, USA) by one-way ANOVA and a Tukey test for the means comparison.

4.2.7. Comparative genomic hybridisation analysis (aCGH).

4.2.7.1. DNA labelling and microarray competitive genome hybridisation.

Parental and R2IVo cells were grown overnight (o/n) in 5mL of GPY medium at 25ºC. DNA was extracted following the methodology proposed by Querol et al.(1992), was resuspended in 50 µl of de-ionised water and was digested with endonuclease *Hinf* I (Roche Applied Science, Germany) according to the manufacturer's instructions to fragments of an average length of 250 bp to 8 kb. Each sample was purified using the High Pure PCR Product Purification Kit (Roche Applied Science) and 2µg were labelled in the BioPrime Array CGH Genomic Labelling System (Invitrogen, Carlsbad, CA, USA). The unincorporated label was removed using the MinElute PCR Purification Kit (Qiagen, Germany). Equal amounts of labelled DNA from the corresponding strains were used as probes for microarray hybridisation.

Array competitive genomic hybridisation (CGH) was performed as described in Peris et al. (2012). Experiments were carried out in duplicate and the Cy5-dCTP and Cy3-dCTP dye-swap assays were performed to reduce the dye-specific bias.

4.2.7.2. Microarray scanning and data normalisation.

Microarray scanning was done in a GenePix Personal 4100A scanner (Axon Instruments/Molecular Devices Corp., USA). Microarray images and raw data were produced with the GenePix Pro 6.1 software (Axon Instruments/Molecular Devices Corp.) and the background was subtracted by applying the local feature background median option. M-A plots (M = Log2 ratios; A = log2 of the product of the intensities) were represented to evaluate if the ratio data were intensity-dependent. The normalisation process and filtering were done using Acuity 4.0 (Axon Instruments/Molecular Devices Corp.). Raw data were normalised by the ratio-based option. Features with artifacts or those flagged as bad were removed from the analysis.

Replicates were averaged after filtering. The data from this study are available from GEO (http://www.ncbi.nlm.nih.gov/geo/); the accession number is GSE48117.

4.2.7.3. Gene Ontology (GO) analysis of overrepresented genes.

GO Term finder (available in the Saccharomyces Genome Database, SGD) was used to perform three different gene ontology (GO) analyses of the genes overrepresented in each particular strain based on the results obtained from the CGH analyses: i) Sc1 vs. Sc2, ii) R2 IVo vs. Sc1 and iii) R2 IVo vs. Sc2. In all cases, statistically significant GO term enrichments were shown by computing a *p*-value using the hypergeometric distribution (the background set of genes was 6241, the number of ORFs measured in the microarray experiments). GO terms showing significant values (z-score >2 and *p*-value <0.05) were sorted according to their corresponding GO category.

4.2.8. qRT-PCR analysis.

PCR primers for interesting genes (*MNN*10, *YPS*7, *HXT*9, *HXT*11 and *HXK*1) were designed according to the available genome sequences of *S. cerevisiae* (laboratory and wine) strains, using PrimerBlast software from NCBI web site. Specificity, efficiency, and accuracy of the primers were tested and optimized by standard PCRs. Primers showing specific amplification (*MNN*10, *YPS*7 and *HXK*1) were used in the subsequent quantitative real-time PCR (qRT-PCR) analysis. Primer sequences are listed in Suppl. Mat. Table 4.1.

4.2.8.1. Gene copy number estimation.

Parental and R2IVo cells were grown overnight (o/n) in 5mL of GPY medium at 25ºC. For every strain, DNA was extracted, in duplicate, from 10^6 CFU according to Querol et al. (1992). DNA was purified using phenol. qRT-PCR was performed with gene-specific primers (200 nM) in a 10-µl reaction mixture, using the LightCycler 480 SYBR Green I Master (Roche Applied Science, Germany) in a LightCycler 480 System (Roche Applied Science, Germany) device. All samples were processed for melting curve analysis, amplification efficiency, and DNA concentration determination using the LightCycler 480 1.5.0 software. For every strain, DNA extracted from 10^6 CFU and serial dilutions (10^{-1} to 10^{-5}) were used for a standard curve. The

copy number for each gene was estimated by comparing the DNA concentration for S288c (haploid *S. cerevisiae* strain).

4.2.8.2. Expression analysis.

Expression of selected genes was studied along a fermentation in synthetic must. Fermentations were carried out as in 2.2.2 and samples were taken at 24h (end latency-beginning of the exponential sugar consumption phase), 55h (middle of the exponential sugar consumption phase) and 120h (end of the exponential sugar consumption phase-beginning of the stationary consumption phase). When collected, samples were washed with cold DEPC water and frozen immediately until their use.

Frozen cells were lysed with zymolyase (Seikagaku corporation) and total RNA was extracted using the High Pure RNA Isolation Kit (Roche Applied Science, Germany). RNA was reversed transcripted to cDNA with Reverse Transcriptase Core kit (EuroGentec) following instructions from the manufacturer: 200 ng of RNA are used as template and oligo $d(T)_{15}VN$ at 2,5 µM as final concentration in a reaction volume of 10µl. The reverse transcription reaction was setup in a TECHNE PCR System: 10 min at 25ºC, 45 min at 48ºC and 5 min at 95ºC. mRNA level of the three genes, in different strains and conditions, was quantified by qRT-PCR with gene-specific primers (200 nM) in a 10 µl reaction, using the LightCycler 480 SYBR Green I Master (Roche Applied Science, Germany) in a LightCycler® 480 System (Roche Applied Science, Germany) device. All samples were processed for melting curve analysis, amplification efficiency and DNA concentration determination using LightCycler® 480 1.5.0 software. A mix of all samples and serial dilutions (10^{-1} to 10^{-5}) were used as standard curve. The mean of gene expression from constitutive genes *ACT*1 and *RDN*18 was used to normalize the amount of mRNA and absolute values are represented.

4.3. Results

4.3.1. Fermentation performance in synthetic must.

As a first selection step, all the stable hybrids along with the two parental strains were evaluated for fermentative features (see Table 4.1 and Suppl. Mat. F4.1). Fermentations were carried out at 20ºC and were monitored by measuring the sugar content until constant values

were reached for 3 consecutive days. Table 4.1 shows the fermentation parameters calculated for all the evaluated strains, including the maximum fermentation rate (K), the time required to consume 50% w/v of the total sugars (t_{50})and the time needed to reach 2% w/v of the residual sugars(t_2),as described in Materials and Methods section.

Although no differences between both parental strains were detected in both the K and t_{50} parameters, Sc1 parental was unable to complete fermentation and showed an estimated t_2 that was more than twice as high as Sc2 (Table 4.1).

As a general trend, no differences in the fermentation parameters were observed between the hybrids obtained by rare-mating and those obtained by spore-to-spore mating (Table 4.1). Strain R2 Io obtained the highest K value among the hybrids, higher than the values obtained for both parental strains. Hybrids R2 IIIa and R2 IVo gave a higher *K* value than parental Sc2,but no differences with parental Sc1were seen (Table 4.1).The same three hybrid strains (R2 Io, R2 IIIa and R2 IVo) achieved the lowest values for t_{50}, although only hybrid R2 Io exhibited significant differences for this value as compared to both parental strains (Table 4.1). Finally, strains R2 Io and R2 IVo also showed the lowest t_2 values.

Strains R2 IIIo and R8 IIIo displayed the same behaviour as Sc1, were unable to complete fermentation, and their estimated t_2 values were higher than 42 days (Table 4.1), according these data these strains suffered a stuck fermentation as was indicated in the Table 4.1.

By the end of fermentation, the concentration of some relevant metabolites (glucose, fructose, ethanol and glycerol) was analysed (Table 4.1). Even though all the hybrids and the two parental strains were able to consume almost all the glucose present in the medium, the amount of fructose remaining at the end of fermentations was variable. The fermentations carried out with strains R2 IIIa, R2 IIIo, R2 VIo, R8IIIo and parental strain Sc1showed significantly higher residual fructose values than the rest, including those fermentations carried out with parental strain Sc2 (Table 4.1).

Table 4.1: Main kinetic parameters of the fermentations carried out with both parental and hybrid strains on synthetic must at 20°C and chemical analysis of the final fermented products.

Strain[Ω]	Hybridization method[Ω]	Kinetic parameters[$]			Chemical parameters[$]			
		K (days^{-1})[&]	t_{50} (days)[*]	t_2 (days)[#]	Glucose (g/L)[¥]	Fructose (g/L)	Glycerol (g/L)	Ethanol (% v/v)
Sc1	**Parental**	**0.105±0.004^{d-f}**	**6.76±0.05^{b-f}**	**stuck**	**0.38±0.11^{a}**	**9.2±0.78^{f}**	**5.18±0.11^{a-d}**	**12.13±0.05^{d-f}**
Sc2	**Parental**	**0.082±0.004^{a-d}**	**7.15±0.08^{b-g}**	**22.58±0.69$^{a.b}$**	**bdl**	**2.18±0.25^{a}**	**5.83±0.11$^{d.e}$**	**12.48±0.05^{f}**
R2 Io	Rare-mating	0.144±0.003^{g}	4.93±0.11^{a}	23.48±0.15^{a-c}	bdl	2.03±0.32^{a}	5.55±0.07^{c-e}	11.78±0.11^{b-f}
R2 IIo	Rare-mating	0.100±0.005^{b-f}	6.5±0.38^{a-e}	27.11±1.03^{b-e}	bdl	2.40±0.50^{a}	5.45±0.07^{c-e}	12.29±0.08$^{e.f}$
R2 IIIa	Rare-mating	0.117±0.005^{e-g}	5.89±0.29^{a-c}	stuck	bdl	5.18±0.39$^{c.d}$	5.55±0.07^{c-e}	12.69±0.02^{f}
R2 IIIo	Rare-mating	0.102±0.020^{b-f}	6.25±0.51^{a-d}	stuck	bdl	7.00±0.42^{e}	5.20±0.00^{a-d}	12.03±0.06^{c-f}
R2 IVo	**Rare-mating**	**0.120±0.013$^{f.g}$**	**5.40±0.56$^{a.b}$**	**16.85±2.42^{a}**	**bdl**	**1.75±0.21^{a}**	**5.60±0.00^{c-e}**	**12.13±0.10^{d-f}**
R2 VIo	Rare-mating	0.104±0.006^{c-f}	6.81±0.19^{b-f}	stuck	0.3±0.42^{a}	3.18±0.47^{b}	4.65±0.14^{a}	10.56±0.24^{a}
R8 IIa	**Rare-mating**	**0.066±0.009^{a}**	**8.44±0.05^{f-h}**	**27.21±3.49^{b-e}**	**0.31±0.44^{a}**	**2.38±0.30^{a}**	**5.63±0.13^{c-e}**	**11.77±0.41^{b-f}**
R8 IIo	Rare-mating	0.080±0.008^{a-d}	8.06±0.59^{e-h}	32.89±2.42$^{d.e}$	bdl	2.37±0.68^{a}	5.67±0.07^{c-e}	12.36±0.18^{f}
R8 IIIo	Rare-mating	0.095±0.003^{a-f}	7.50±0.46^{c-h}	stuck	bdl	4.58±0.46$^{b.c}$	5.76±0.14^{c-e}	12.23±0.20^{d-f}
R8 IVo	Rare-mating	0.082±0.016^{a-d}	7.84±0.99^{d-h}	30.23±2.05^{c-e}	bdl	2.65±0.48^{a}	5.68±0.07^{c-e}	12.33±0.24^{f}
R8 Vo	Rare-mating	0.072±0.003^{a-c}	8.93±0.53^{h}	31.55±1.83$^{d.e}$	bdl	2.48±0.11^{a}	5.13±0.13^{a-c}	12.49±0.01^{f}
R8 Vb	Rare-mating	0.071±0.004$^{a.b}$	8.59±0.37$^{g.h}$	28.30±0.15^{b-e}	bdl	1.71±0.24^{a}	5.70±0.00^{c-e}	11.03±0.02^{a-c}
R8 VIo	Rare-mating	0.070±0.003$^{a.b}$	8.51±0.07^{f-h}	30.84±1.03^{c-e}	bdl	2.38±0.01^{a}	5.64±0.332^{c-e}	11.62±0.19^{a-f}
R8 VIIo	Rare-mating	0.071±0.006$^{a.b}$	8.33±0.39^{f-h}	30.22±1.04^{c-e}	bdl	2.00±0.11^{a}	6.09±0.19^{e}	12.23±0.16^{d-f}
R8 VIIIo	Rare-mating	0.086±0.001^{a-e}	7.55±0.16^{c-h}	33.18±3.89^{e}	bdl	2.58±0.62^{a}	5.36±0.24^{b-d}	11.19±0.29^{a-d}
S2 Io	Spore to spore	0.073±0.006^{a-d}	7.88±0.36^{d-h}	28.13±0.97^{b-e}	bdl	2.05±0.03^{a}	5.20±0.17^{a-d}	11.24±0.36^{a-e}
S2 IIo	Spore to spore	0.070±0.007$^{a.b}$	8.13±0.24^{e-h}	28.81±1.83^{b-e}	bdl	2.27±0.52^{a}	5.53±0.08^{c-e}	12.29±0.25$^{e.f}$
S7	**Spore to spore**	**0.091±0.008^{a-f}**	**6.87±0.31^{b-g}**	**25.35±0.38^{b-d}**	**bdl**	**2.27±0.06^{a}**	**5.55±0.07^{c-e}**	**11.78±0.11$^{a.b}$**

Ω- Extracted from Pérez-Través et al 2015

$- Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, α=0.05, n=2).

&- K: kinetic constant.

*- t50: time necessary to consume 50% w/v of the total sugars.

#- t2g/L: time necessary to reach 2 g/L of residual sugars.

¥- bdl: value below detection limit (0,05g/L).

In **bold** are indicated those strains chosen to be used in the following selection steps.

Regarding glycerol and ethanol production, no significant differences were observed among the fermentations conducted by the two parental strains and most hybrids. In particular, hybrids R2 VIo, R8 Vb and S7 produced significantly lower levels of ethanol than both the Sc1 and Sc2 parental strains.

Based on their fermentation performance (long t_2 and fructose amount higher than 2g/L, which indicates a stuck fermentation), hybrid strains R2 IIIa, R2 IIIo, R2 VIo and R8IIIo were not included in the second selection step (release polysaccharides and mannoproteins).

4.3.2. Release of total polysaccharides and mannoproteins in synthetic must.

The release of total polysaccharides for all the parental and hybrid strains showing good fermentative performance is shown in Figure 4.1. The aim of this selection step was to compare the production of mannoproteins (because the only polysaccharides presents in synthetic must are mannoproteins) by yeast strains under fermentation conditions at 20°C using a synthetic must that mimicked real grape must. Under these assay conditions, parental strain Sc2 produced a significantly larger amount of total polysaccharides (67.1mg/L) than strain Sc1 (56.8 mg/L), the last one selected in this work for its mannoprotein release capacity. Moreover, 12 of the 14 analysed hybrid strains released significantly bigger amounts of polysaccharides than both the parental strains (Figure 4.1). The remaining two hybrid strains, R8 VIo and R8 VIIo, released a similar amount of polysaccharides to parental Sc2. The maximum polysaccharides content was detected in the medium inoculated with hybrid R2 IVo (100 mg/L). This value represents an increase of around 1.5 times as compared to the values obtained with parental Sc2, and of around 2 times if compared to parental Sc1.

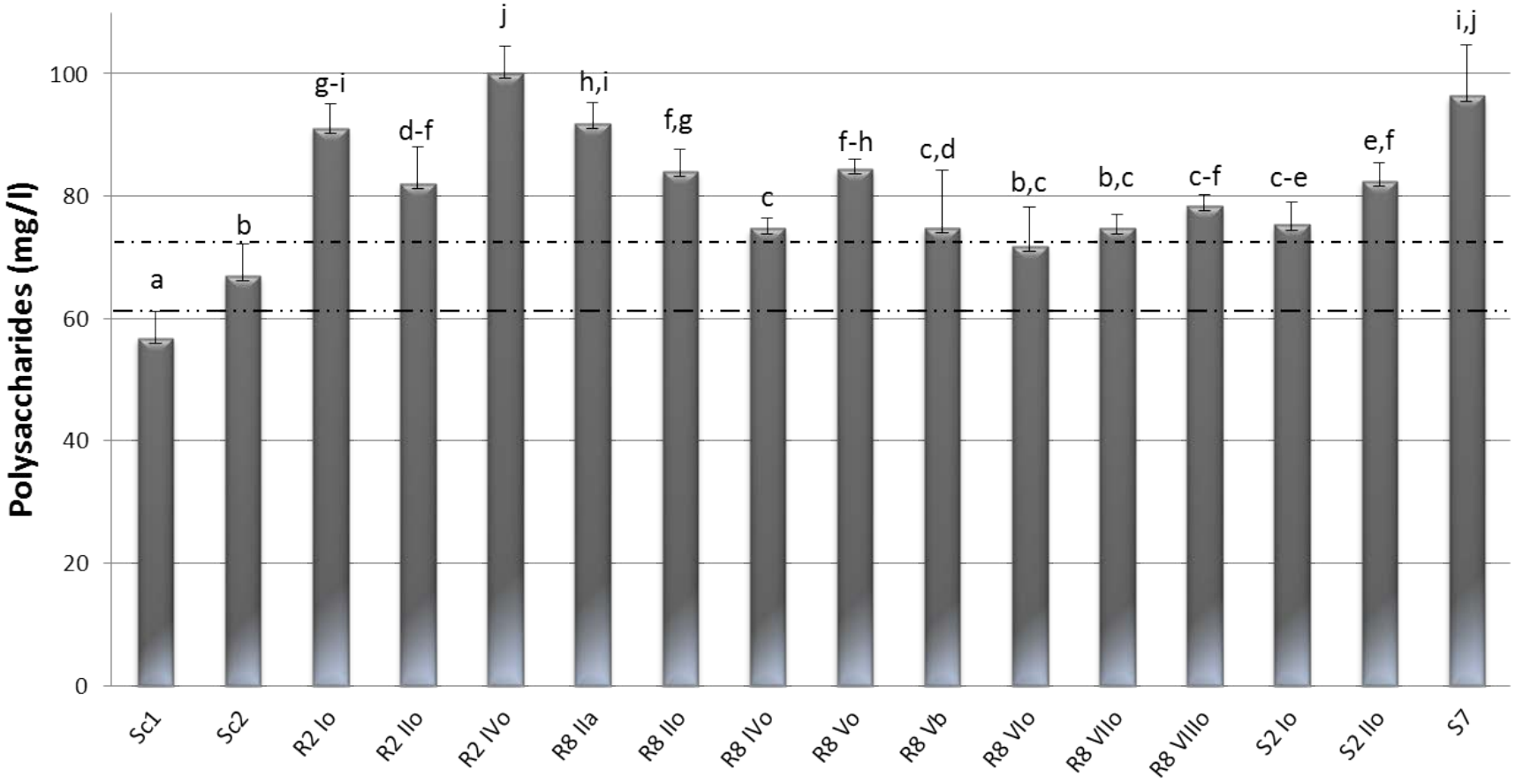


Figure 4.1: Final concentrations of the polysaccharides released by hybrids and parental strains in synthetic must. Bars not sharing the same letter were significantly different according to one way ANOVA and Tukey test (α=0.05). Dotted lines shown the parental polysaccharides value.

To confirm that the total amount of polysaccharides was in accordance with the presence of the mannoproteins in the medium, we carried out the specific detection of mannoproteins in fermented synthetic musts using peroxidase-conjugated concavalin A. As a general rule, the results obtained with this methodology confirmed those obtained by the quantification of total polysaccharides. Even though this is a qualitative detection method, our results clearly demonstrate that most hybrids released a larger amount of mannoproteins than the parental strains.

Comparing in each gel the intensity of the bands of the hybrids versus the parental strains, the fermentations carried out by hybrids R8IIa and S7 gave the largest amount of mannoproteins, followed by those obtained with hybrids R2 Io, R2 IIo and R2 IVo (Figure 4.2). Hybrids R2 Io and R2 IIo produced slightly different mannoprotein bands patterns from those produced by the parental strains and the remaining hybrids (Figure 2). Finally, the amount of mannoproteins released by hybrid strains R8 VIo and R8 VIIo was similar to that released by the other hybrids, which evidences similar mannoprotein profiles (Figure 2). Nonetheless, these two hybrids produced a smaller amount of total polysaccharides than the other hybrid strains (Figure 4.1).

Based on the results obtained from the total polysaccharides and mannoprotein release, we selected hybrid strains R2 IVo, R8 IIa and S7 to evaluate their capacity to increase the stabilisation of a white wine against protein haze.

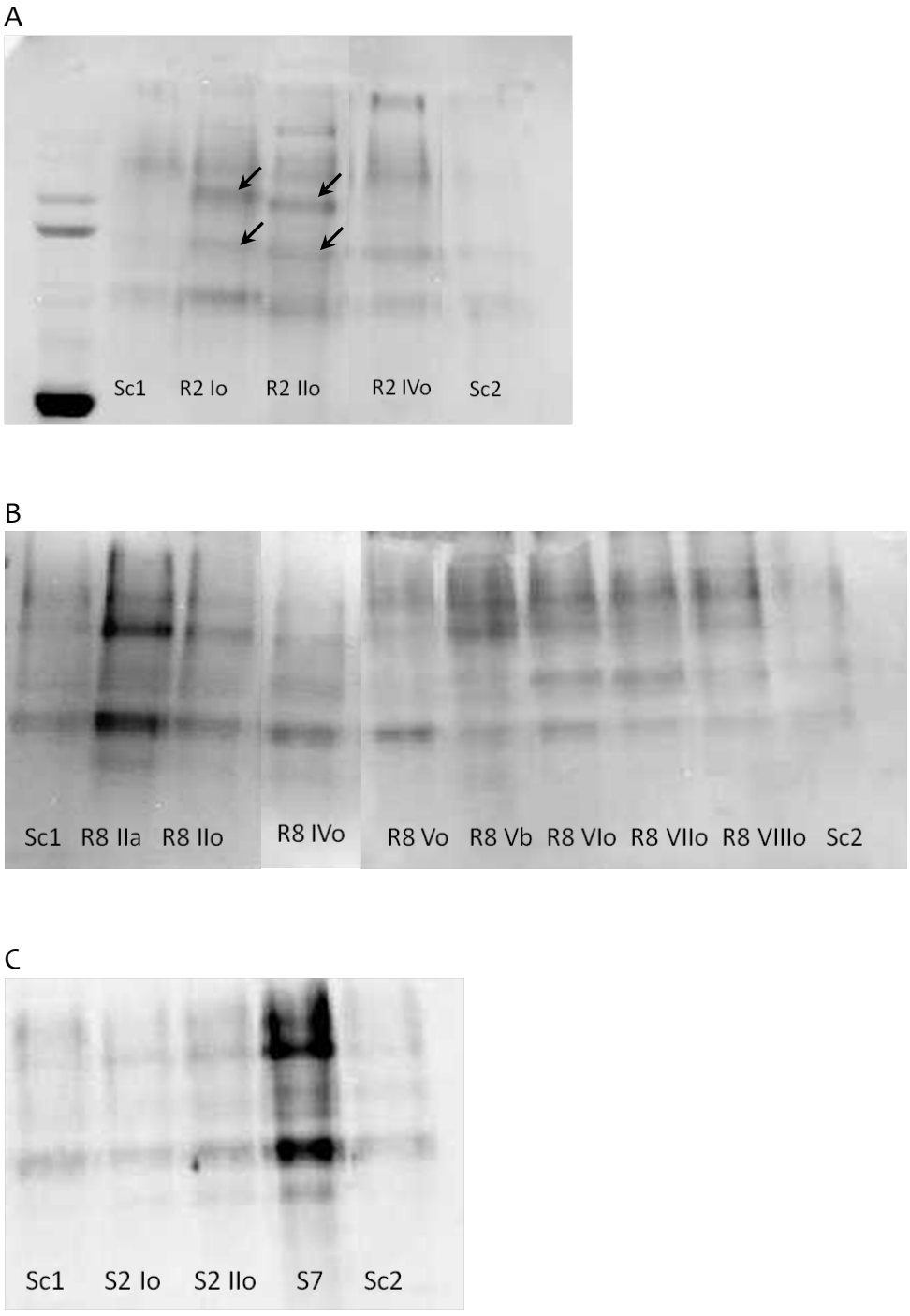


Figure 4.2: Mannoproteins released during fermentation of a synthetic must by the hybrid strains compared to their parental. The identities of the strains are indicated in each panel. A and B: hybrids obtained by rare-mating methodology; C: hybrids obtained by spore to spore mating. Arrows in A indicate mannoproteins bands present in hybrids and not observed in parental.

4.3.3. Protein haze stability of the wines fermented by the higher mannoprotein producer hybrids.

Fermentations of Sauvignon Blanc grape must were carried out with the three higher mannoprotein producer hybrids and the two parental strains to evaluate the effect on wine stability of the mannoproteins produced by each different strain. Chemical analyses of the wines evidenced that parental Sc1 and hybrid S7 were unable to consume all the fructose that was originally present in the must, and they left as much as 6.4 and 5.6 g/L of residual fructose, respectively (data not shown). After fermentation, wines were subjected to the heat test for protein stability before and after bentonite fining, as described in the Materials and Methods. Turbidity values close to 75 nefelos (nephelometric turbidity units) were obtained for the wines fermented with Sc1, Sc2 and R2 IVo without the addition of bentonite, while higher values were observed for hybrids R8 IIa and S7 (Figure 4.3). R2 IVo and the R8 IIa hybrid strains showed the best stabilisation profiles, with R2 IVo seemed to require slightly less bentonite for complete stabilisation. The results for R8 IIa and Sc1 reveal lack of correlation between protein instability before bentonite stabilisation and the response of the corresponding wine to bentonite fining. Although no clear differences were obtained with this approach, we can conclude that R2 IVo was the best in this test and was also the strain that produces maximum levels of polysaccharides, for this reason this hybrid was selected for further analysis.

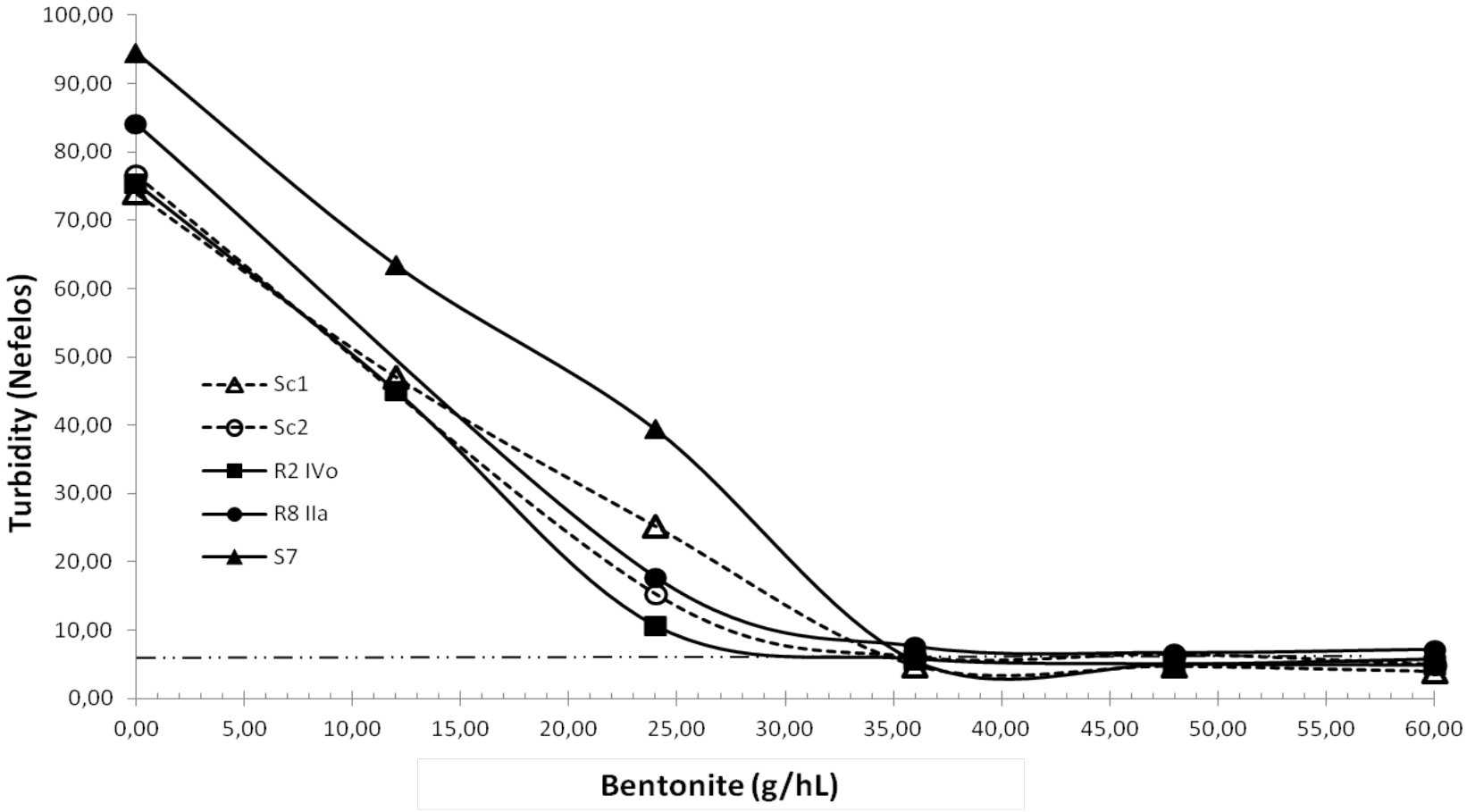


Figure 4.3: Effect of Bentonite finning on the heat-test results of Sauvignon Blanc wines fermented with selected hybrids compared to their parental strains. Horizontal dotted line indicates the asymptotic turbidity level representing wine stability. Error bars are included.

4.3.4. Measuring of the mannoprotein production in Verdejo fermentations.

To ensure that R2 IVo hybrid produce higher amounts of mannoproteins than its parental strains, we performed fermentation in Verdejo must. Fermentations were carried out at 20ºC and were monitored by measuring the sugar content until constant values were reached for 3 consecutive days. Table 4.2 shows the fermentation parameters calculated for all the evaluated strains, including the maximum fermentation rate (K), the latency (l) and the time required to consume 95% w/v of the total sugars (t_{95}), as well as the main chemical parameters (glucose, fructose, glycerol and ethanol). The three strains finished the fermentation. Although Sc2 was the strain that showed the higher Vmax and Sc1 and R2 IVo showed similar value of this parameter, the hybrid R2 IVo finished the process earlier than parental Sc1, indicating an improvement of the fermentative capability.

As the natural must contains other polysaccharides different to mannoproteins and the phenol sulphuric method detects polysaccharides in general, we used the methodology described by Quirós et al (2012) in order to analyse the amount of mannoproteins released by the selected strains. The results are shown in Figure 4.4. Sc2 was the strain that lower amount of mannoproteins produced (~123mg/L), followed by Sc1. The hybrid R2 IVo produced, statistically, more mannoproteins than both of its parental strains (~157mg/L).

As a resume, hybrid strain R2 IVo exhibited good fermentative behaviour in both synthetic and natural grape musts (Table 4.1 and 2; Suppl. Mat. F4.1), and released large amounts of mannoproteins and polysaccharides that seem related with protection of wine against protein haze (Figure 4.1, 4.2, 4.3 and 4.4). This strain seems to have inherited the positive physiological features from each parental strain. In order to characterize the potential genomic changes that may have occurred during hybrid generation and stabilisation, and which could be related with the improved physiological features of this strain, we performed array-comparative genomic hybridisation (CGH).

Table 4.2: Main kinetic parameters of the fermentations carried out on Verdejo must at 20°C and chemical analysis of the final fermented products.

Strain	Kinetic parameters[$]			Chemical parameters[$]			
	K (hours^{-1})[&]	*l*(hours)[*]	t_{95} (hours)[#]	Glucose (g/L)[¥]	Fructose (g/L)	Glycerol (g/L)	Ethanol (% v/v)
Sc1	1.24±0.01^{a}	21.50±0.22^{a}	164.57±2.69^{c}	bdl	1.01±0.08^{a}	5.90±0.10^{c}	13.27±0.11^{a}
Sc2	1.40±0.02^{b}	22.61±0.41^{a}	134.36±1.15^{a}	bdl	bdl	5.75±0.05^{b,c}	13.25±0.11^{a}
R2 IVo	1.20±0.00^{a}	21.97±0.12^{a}	152.44±0.44^{b}	bdl	bdl	5.45±0.06^{a}	13.16±0.06^{a}

$- Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test. α=0.05. n=2).

&- K: kinetic constant.

*- l: latency.

#- t_{95}: time necessary to consume 95% of residual sugars.

¥- bdl: value below detection limit (0.05g/L).

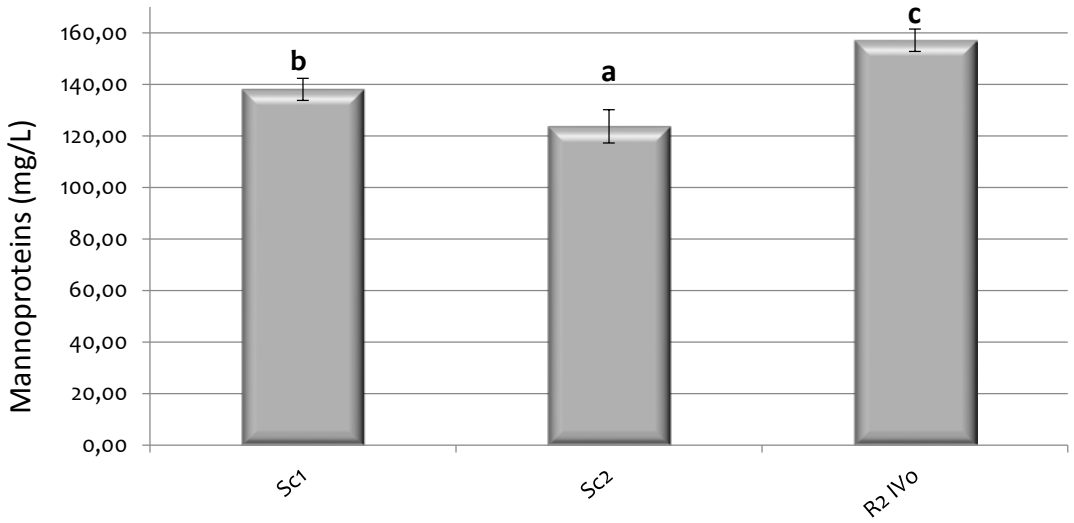


Figure 4.4: Final concentrations of released mannoproteins by hybrid, parental and control strains in verdejo must. Bars not sharing the same letter were significantly different according to one way ANOVA and Tukey test (α=0.05).

4.3.5. Comparative genomic hybridisation analysis of hybrid R2 IVo and the parental strains.

For the CGH analysis, genomic DNA from hybrid strain R2 IVo was competitively hybridised with genomic DNA from each parental strain. The DNA from the two parental strains was also competitively hybridised against each other to evaluate the genomic differences between them by following the methodology described in the Materials and Methods.

Of the 6000+ gene probes contained in the DNA microarray, only a few hundred showed a significant copy number variation among the three strains analysed (the hybrid and the two parental strains). An analysis of the data derived from the comparative hybridisation of the parental strains (Sc1 vs. Sc2) revealed significant differences in the copy number of some interesting genes. Ninety-four ORFs showed a significantly higher copy number in strain Sc2 and 41 ORFs had higher copy numbers in Sc1 (Figure 4.5 and Suppl. Mat. Table 4.2). A considerable number of these variable genes were located in the telomeric or subtelomeric regions, but only a few of them corresponded to the genes with an annotated function. Big groups of variable subtelomeric ORFs were identified as transposons and they were particularly overrepresented in parental Sc2. Another group of genes overrepresented in Sc2 corresponded to those belonging to the *HXT* family (Figure 4.5 and Suppl. Mat. Table 4.2). Interestingly, genes *GPM1* and *HXK1*, which codify for a phosphoglycerate mutase and hexokinase isoenzyme 1, respectively, seemed to be also overrepresented in parental Sc2 and displayed good fermentation performance.

Parental Sc1 was characterised by an overrepresentation of the genes typically found in wine yeast strains(Carreto et al., 2008), such as *MAL11*, *MAL13*, *CUP1*-1 and *CUP1*-2 (Figure 4.5 and Suppl. Mat. Table 4.2). This parental strain, characterised by its ability to produce and release mannoproteins also displayed an overrepresentation of some of the genes involved in oligosaccharides metabolism and processing (e.g., *SPR1),* which codify for a glucan 1,3-beta glycosidase), *SWP1* (dolichyl- diphosphooligosaccharide protein glycotransferase) and *IMA1* (a α-1,6-glucosidase).

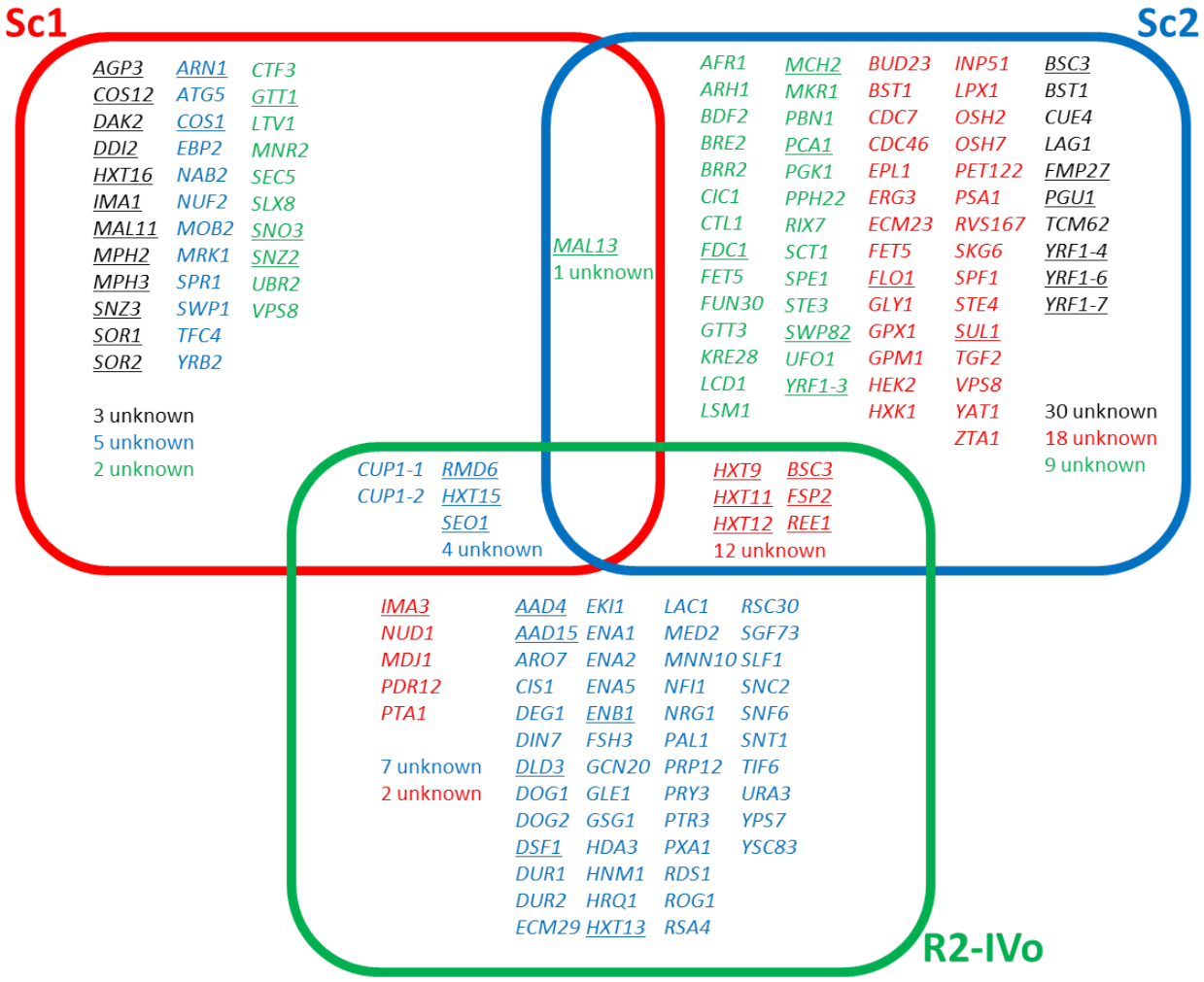


Figure 4.5: Schematic grouping of genes significantly overrepresented in each strain under study. Underlined: subtelomeric genes. In red: genes significantly overrepresented in Sc1. In blue: genes significantly overrepresented in Sc2. In green: genes significantly overrepresented in R2-Ivo. In black: genes significantly overrepresented in the two remaining strains.

Genes in the intersections are overrepresented genes in two strains with respect to the remaining one. Genes significantly overrepresented in the hybrid with respect to Sc1 (in red in R2-Ivo) that did not show copy number differences with Sc2, likely indicate that Sc2 has an intermediate copy number between Sc1 and R2-Ivo for these genes. Therefore, the hybrid should possess more copies of these genes than the two parentals. The same explanation applies to genes in blue in R2-Ivo, corresponding to genes significantly overrepresented in the hybrid with regards to Sc2.

The gene onthology (GO) analysis was carried out with the overrepresented genes detected in each particular parental strain and the significant GO terms obtained were sorted according to their corresponding GO categories (Suppl. Mat. Table 4.3). According to that analysis, the terms related to disaccharides and oligosaccharides metabolism were significantly overrepresented in parental strain Sc1,while terms related to transposition were associated with parental Sc2 (Suppl. Mat. Table 4.3).

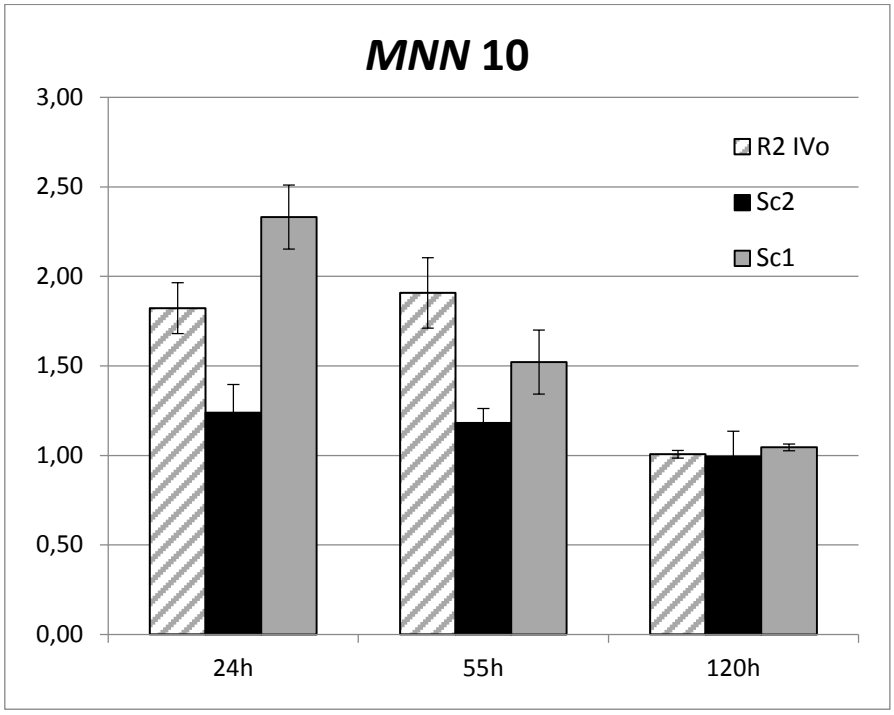
The comparative analysis, which derived from the competitive hybridisation of hybrid R2 IVo versus each parental strain, evidenced that the hybrid maintained the copy number of one parental strain or the other for several genes. The hybrid did not show genes significantly overrepresented in relation to the two parental strains. However we observed significantly overrepresented in the hybrid with regards to Sc1 (genes in red in R2-IVo in Figure 4.5) that do not show differences in copy numbers with Sc2, indicating that probably Sc2 has an intermediate copy number between Sc1 and R2-IVo for these genes. Then, the hybrid possesses more copies of these genes than the two parentals. The same explanation could be associated with genes in blue in R2-IVo in Figure 4.5, with genes significantly overrepresented in the hybrid with regards to Sc2. According to the data shown in Figure 4.5, the hybrid R2-IVo presented 25 overrepresented ORFs against Sc1 and 65 different genes overrepresented against Sc2 (Figure 4.5 and Suppl. Mat. Table 4.4). Both strains Sc1 and the hybrid shared nine overrepresented ORFs, which included five annotated genes (*CUP1*-1 and 2, *RMD6*, *HXT15* and *SEO1*). However, 18 ORFs, including six annotated subtelomeric genes (*HXT9*, *HXT11*, two ORFs of *HXT12*, *FSP2*, *REE1* and *BSC3*) and eight genes corresponding to transposons, were commonly overrepresented in both the hybrid and parental strain Sc2 (Figure 4.5 and Suppl. Mat. Tables 4.2 and 4.4).

Apart from the overrepresented ORFs shared between the hybrid and parental strains, the hybrid exhibited 7 and 56 genes in significantly higher copy numbers than Sc1 and Sc2, respectively (Figure 4.5 and Suppl. Mat. Table 4.4). In particular, those genes involved in cell wall organisation and maintenance, like the endopeptidase coding gene YPS7 and the gene coding for α-1,6-mannosyltransferase *MNN10,* had significantly higher copy numbers in the hybrid than in parental Sc2. No differences between hybrid and Sc1 were observed for these ORFs, indicating a similar copy number between these two mannoprotein higher producer strains.

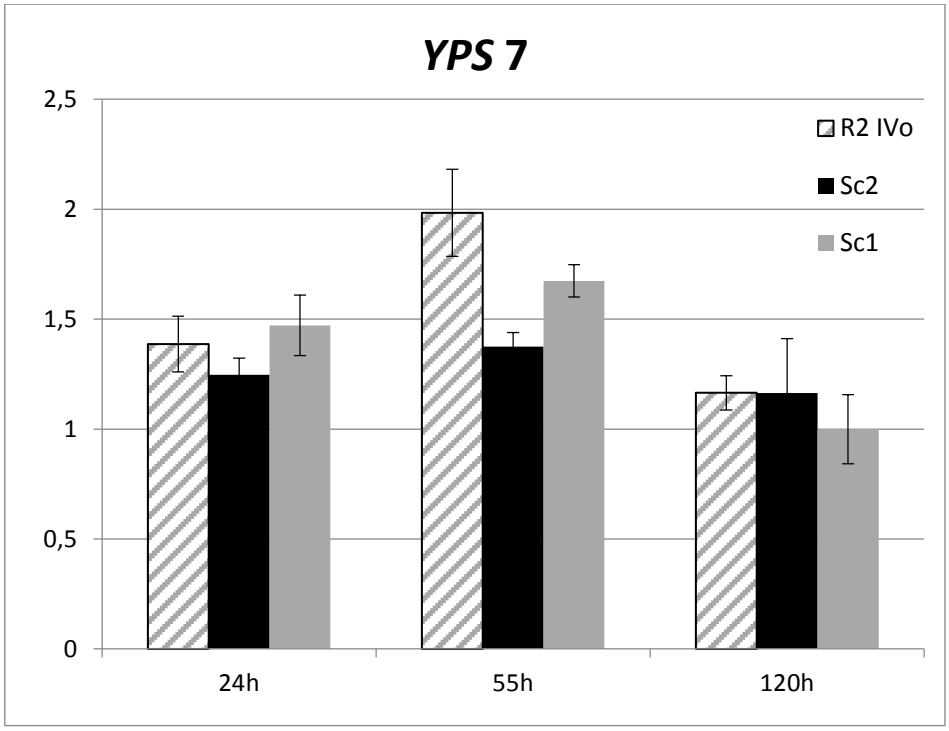
In this case, the GO analysis was separately performed with the ratio data obtained from the hybridisation of the hybrid versus parental Sc1 and parental Sc2 (Suppl. Mat. Table 4.3). According to this analysis, the terms related to transposition were also significantly overrepresented in the hybrid as compared to parental Sc1, as were some other terms related to carbohydrate metabolism and glycosidase activity (Suppl. Mat. Table 4.3). The GO analysis done with the over/underrepresented genes between the hybrid and Sc2 evidenced an overrepresentation of the terms related to detoxification in the hybrid genome.

If we consider its better fermentation performance, its greater mannoprotein release, and its effects on protein haze protection, the R2 IVo hybrid strain proved to be the most suitable strain for industrial purposes. These physiological properties may be related with the genes of the HXT family (HXT9, HXT11, HXT12), which showed significantly higher copy numbers in the hybrid and the strain Sc2. In addition, the genes associated with cell wall organisation were overrepresented in the hybrid genome and in parental Sc1, and may be responsible for the increase in polysaccharides produced by these two strains.

A)



B)



C)

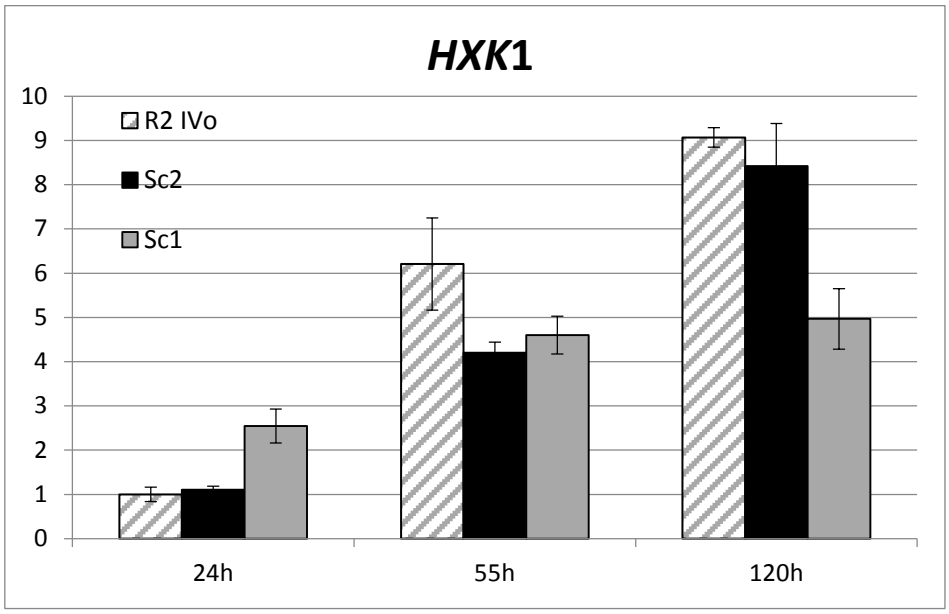


Figure 4.6: Relative expression of the genes A) *MNN10*, B) *YPS7* and C) *HXK1* during a fermentation. Expression of each one of the genes was related to the lower expression value obtained for this gene in all the experiment.

4.3.6. Validation of comparative genomic hybridisation analysis.

To validate the results observed in the CGH analysis, we perform qRT-PCR of several of the genes indicated above, as *MNN*10, *YPS*7, *HXT*9, *HXT*11 and *HXK*1, in order to confirm the gene copy number. As *HXT* genes are quite similar, were removed from the analysis. According the rest of the genes the hybrid R2 IVo should have more copies of *MNN*10 and *YPS*7 than Sc2 and should have more copies of *HXK*1 than Sc1, but less than Sc2. Using this approach the copy number differences were no conclusive (data not shown).

For this reason we decided studied the expression of these three genes during fermentation. Results are shown in Figure 4.6 and Table S5.

Comparing the relative expression of *MNN*10 gene, of the same strain at different time point (Suppl. Mat. Table 4.5B) the hybrid R2 IVo maintained a high relative expression value at 24h and 55h, diminishing at 120h; Sc1 diminished its expression at 55h and Sc2 maintained similar lower expression values at all fermentation points.

For *YPS*7 gene relative expression values of the same strain at different time point (Suppl. Mat. Table 4.5B), showed that the R2 IVo increased its expression values at 55h, Sc2 maintained it during all the experiment and Sc1decreassed its expression at 120h.

For *HXK*1 gene results (Figure 4.6C and Suppl. Mat. Table 4.5A and B) indicated that at 24h the strains showed the lower relative expression values of all the experiment, nowadays, relative expression values of Sc1 were higher than those showed by Sc2 and R2 IVo. At 55h the three strains increased their relative expression values, but all presented similar values. At 120h Sc1 maintained its expression value and Sc2 and R2 IVo increased their relative expression values; this increase was 7-10 folds the expression values showed at 24h.

This results indicated that the higher mannoprotein production of R2 IVo could be due to the maintenance of the increased expression of *MNN*10 during long time than Sc1 and to the higher expression values of *YPS*7 in the middle of the fermentation (55h point) as is shown in Figure 4.6A and B. Whilst the improvement in the fermentation kinetics could be due to the higher increment in the expression of *HXK*1, showed in Sc2 too, at the end of the fermentation (120h), see Figure 4.6C.

4.4. Discussion.

During the winemaking process, other than products and by-products of sugar metabolism, yeast cells release cell constituents, like proteins and polysaccharides, which also contribute to wine quality. A number of studies have been published in recent decades that have demonstrated the positive contribution of yeast mannoproteins to wine attributes (Caridi, 2006). Based on those reports, different experimental approaches have been proposed for the isolation and/or development of yeast strains that are able to secrete larger amounts of mannoproteins (González-Ramos et al., 2009; González-Ramos et al., 2010; Quirós et al., 2010). However, some of these methods are based on genetic engineering and could face regulatory constraints and consumer distrust. Others involve random mutagenesis and can face a risk of an unintended genetic modification of the desirable oenological features of the original wine yeast strain. In this work, we were able to combine by hybridisation techniques the desirable oenological features of two commercial *S. cerevisiae* strains in a single strain: Sc1, with a high capacity to release polysaccharides, including mannoproteins; Sc2, with excellent fermentative performance at industrial level. The strains obtained by making full use of these natural hybridisation processes do not face the regulatory and marketing restrictions that GMO microorganisms do.

The literature frequently mentions that hybrids can inherit particular physiological features in new combinations, which can be even higher than those of the parents. *S. cerevisiae* x *S. kudriavzevii* interspecific hybrids can retain the fermentation vigour of *S. cerevisiae* and the ability to produce particular aromatic compounds from *S. kudriavzevii*; while *S. cerevisiae* x *S. uvarum* hybrids can display the capacity to ferment at both low and high temperatures and to produce intermediate amounts of minor fermentative compounds (Sipiczki, 2008). Most of the stable hybrids analysed in this work give intermediate values between both parental strains for fermentation kinetics parameters K, t_{50} and t_2.In some cases, hybrids (particularly R2 I0, R2 IIIa and R2 IV0) gave even higher K values and lower t_{50} and t_2 values than parental Sc2, which was selected for its excellent fermentative behaviour (Table 4.1).

Strain Sc1, selected for its high mannoprotein release capacity, gave the lowest values of total polysaccharides produced (evaluated by the phenol/sulphuric method) when compared

with parental Sc2 and all the tested hybrids, in a synthetic must fermentation. However, mannoprotein specific staining indicated similar or bigger mannoprotein content for Sc1. These differences indicate that Sc2 could be releasing other polysaccharides different to mannoproteins being the total mannoprotein release or the mannoprotein/total polysaccharides ratio higher in Sc1, and that mannoproteins releasing –instead of the total polysaccharides release- are better related to the technological properties. It has been reported that not only the total amount of mannoproteins, but also their specific kind, has been associated with beneficial activity in wine(Moine-Ledoux and Dubourdieu, 1999; Waters et al., 1994). In this work, most hybrids exhibited similar mannoprotein patterns to the parental strains. As we wanted to improve parental traits, we selected for posterior analysis strains with similar bands but with higher intensity than the ones showed by the parental strains.

In this work, we chose protein haze stabilisation as a model application to detect interesting hybrid strains given its amenability to laboratory-scale experimentation. These methods are based on the haze susceptibility of Sauvignon Blanc(González-Ramos et al., 2009). Using this method we could see that the wine obtained with strain R2 IVo responded considerably better to bentonite-fining treatments, although the resolution of this method is not the best according our data. These results have been confirmed with a quantitative method (Quirós et al., 2012) in Verdejo must. When mannoproteins were quantified at the end of this fermentation, was revealed that Sc1 produced more mannoproteins than Sc2 (as was said by the producers). The selected hybrid R2 IVo released more mannoproteins than both of its parental strains, indicating that this trait was improved not only for the parental Sc2, but it was also improved with respect to the parental Sc1.

Many studies have shown that extensive genome rearrangements and gene duplication occur in organisms, particularly yeasts, during adaptation to changing environments. These changes can partially explain the hybrid improvement achieved in this work. It is well-known that microarrays data can be used to reflect such genome changes (Dunham et al., 2002; Dunn et al., 2005; Peris et al., 2012). The experiments carried out to detect specific alterations in the gene copy number in the selected hybrid, which might explain some of the inherited physiological properties and hybrid improvement, evidenced a number of overrepresented genes in the three strains compared (Sc1, Sc2 and R2 IVo).

The genes associated with cell wall organisation could be held responsible for the increased ability of strains to produce and release polysaccharides. In our study, gene *MNN10,* which codifies for a subunit of a Golgi mannosyltransferase complex, was overrepresented in the hybrid genome if compared to parental Sc2, while no differences in copy numbers were observed between R2 IVo and Sc1. The overrepresentation of *MNN10* might be associated with the better mannoprotein release in these strains. Indeed, deletion of either Mnn10p or its homologue Mnn11p results in defects in the mannan synthesis *in vivo*. An analysis of the enzymatic activity of the complexes isolated from mutant strains suggests that Mnn10p and Mnn11p are responsible for the majority of the complex's α-1,6-polymerizing activity(Jungmann et al., 1999). Additionally, the same behaviour was observed for gene *YPS7,* which codifies for a protease related to cell wall glucans incorporation and retention. *YPS7* also forms part of the transcriptional response to cell wall stress and is required during severe cell wall stress in *S. cerevisiae(Krysan et al., 2005)*. Finally, *SWP1,* which codifies for an oligosaccharyl transferase subunit required for N-linked glycosilation of proteins in the endoplasmic reticulum, was overrepresented in mannoprotein producer parental Sc1 if compared to Sc2, and Sc1 and hybrid R2 IVo present a similar copy number for this gene, which may also be related with the increased mannoproteins synthesis for hybrid R2 IVo. A combination of the genes associated with cell wall organisation obtained from parental Sc1 and the similar duplications in some genes like *SWP1* to parental Sc2 can justify that the hybrid is even better than both the parental ones for these properties.

An initial set of genes with an altered copy number has been associated with telomeric or subtelomeric regions in different chromosomes (Figure 4.5). Brown et al.(2010) suggested that these regions are "hotbeds for genomic evolution and innovation". Both telomeric and subtelomeric genes evolve faster than their internal counterparts, and they are frequently the sites of gene duplications(Ames et al., 2010). According to different authors, differences in the copy number of several telomeric genes are very important for adaptation and to overcome different environmental stresses(Carreto et al., 2008; Dunham et al., 2002). In our work, the subtelomeric genes belonging to the *HXT* family (*HXT9, HXT11, HXT12*) had significantly higher copy numbers in the hybrid. This set of subtelomeric genes was also overrepresented in strain Sc2. Although sugar utilisation *HXT* genes are virtually identical to each other, which allows the

possibility of cross-hybridisation and makes it impossible to know which particular gene(s) is(are)overrepresented in the pair Sc2 vs R2 IVo, this difference can be related to the best fermentation performance of both Sc2 and R2 IVo (Table 4.1). In this sense, Lin and Li (2011) found a strong correlation between the copy number of *HXT* genes and fermentative strain behaviour.

Furthermore, alterations in the copy number of glycolytic genes or the genes responsible for sugar transportation can be associated with the strains' improved fermentation performance. In this sense, parental strain Sc2, characterised for its good fermentative performance, had a significantly higher copy number of genes *GPM1* and *HXK1* than Sc1, but no differences with the hybrid R2 IVo (also showing good fermentation performance) were detected. In particular, the *HXK1* gene has been reported to be expressed when yeast cells are grown on a fermentable medium using glucose, fructose or mannose as a carbon source (Bisson and Fraenkel, 1983).

Our work demonstrates that hybridisation combined with stabilisation under winemaking conditions is an effective approach to obtain yeast strains with both improved mannoprotein producing capacity and fermentation performance, which are physiological features that genetically depend on the coordinated expression of numerous different genes (polygenic features). A hybrid with both features improved was selected and a number of genes potentially responsible for the improvement of the hybrid generated in this work have been postulated.

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Resumen de resultados y discusión

1. Análisis y estudio del taxón *S. bayanus.**

Utilizando un juego de 34 pares de cebadores, generados previamente en nuestro laboratorio para la detección y caracterización de híbridos del género *Saccharomyces* (González et al., 2008), caracterizamos la cepa NBRC1948, hasta el momento la cepa más representativa de *S. bayanus var. bayanus* (Rainieri et al., 2006), mediante la secuenciación de estas regiones. Estas secuencias las comparamos con las de la cepa CBS7001 (cepa pura de *S. bayanus var. uvarum*) y observamos que 9 de las regiones analizadas eran idénticas o casi idénticas entre ambas, mientras que el resto de secuencias presentaban valores de similaridad entre el 86 y el 97%. Estas 9 regiones se estudiaron en otras dos cepas (CECT1186 y CBS424) y en todos los casos se encontró que una, otra o ambas secuencias presentaban valores de similaridad con la cepa CBS7001 alrededor del 89-95%; de este modo se obtuvo un juego completo de alelos "*bayanus*" de una hipotética línea pura *S. bayanus var. bayanus.* Estas secuencias fueron idénticas o casi idénticas al subgenoma no-*cerevisiae* de la cepa de *S. pastorianus* Weihenstephan 30/70, de forma que nuestra línea pura *S. bayanus var. bayanus* correspondería a una reconstrucción de *S. eubayanus*. Siguiendo la idea propuesta por Gibson et al. (2013), este hipotético genotipo representaría al genotipo de una cepa de *S. eubayanus* Europea. Dado los resultados obtenidos, la cepa NBRC1948 pasaría de ser una cepa pura a ser un híbrido entre *S. uvarum* y *S. eubayanus*.

Con el objetivo de encontrar una cepa pura de *S. eubayanus* se analizaron 46 cepas Europeas obtenidas de diferentes fuentes y anotadas en las bases de datos como *S. bayanus, S. uvarum* o *S. pastorianus*. El análisis se llevó a cabo mediante la PCR-RFLP de 33 de las 34 regiones génicas analizadas anteriormente y la secuenciación del gen nuclear *MNL1* y del gen mitocondrial *COX2*. No se encontró ninguna cepa pura de *S. eubayanus*, pero se descifró la complejidad existente dentro de este grupo de cepas.

Nota: * Nomenclatura antigua

Atendiendo al análisis de genes nucleares, 27 de las 46 cepas resultaron pertenecer a *S. uvarum*, y de ellas, 20 mostraron una introgresión (un alelo perteneciente a *S. cerevisiae*) en el gen *PEX2*, cuatro mostraron una introgresión en el gen *MNL1* y dos de las 27 cepas mostraron alelos U1/U2 en heterocigosis. El análisis del gen mitocondrial COX2 de estas cepas también mostró una gran homogeneidad. Entre este grupo de cepas se observó que casi no existía variación en su composición alélica, como ya se había evidenciado anteriormente (Masneuf-Pomarède et al., 2007; Naumova et al., 2011), aún así, la presencia de cepas heterocigotas nos indican que existe cierto grado de entrecruzamiento entre las cepas de este grupo.

14 de las 46 cepas mostraron diferentes combinaciones de alelos U y E, lo que indicó su naturaleza híbrida *S.* eu*bayanus* x *S. uvarum,* también presentaron diferentes alelos del gen mitocondrial *COX2* entre ellos. Estas cepas se agrupan bajo la denominación *S. bayanus var. bayanus*. De acuerdo con su constitución genética nuclear, estos híbridos se pudieron dividir en dos grupos, los híbridos tipo I, homocigotos para todos los genes analizados y los híbridos tipo II, heterocigotos (U/E) para alguno de los genes analizados; el número de loci heterocigotos varió del 9 al 44%. Los alelos del gen mitocondrial *COX2* encontrados entre estas cepas se agruparon con los alelos '*uvarum*' o con los '*eubayanus*', e incluso se encontró una posible variable recombinante de este gen. Basado en la complejidad observada para este grupo, se propuso un esquema para resumir la generación de las cepas pertenecientes a *S. bayanus var. bayanus,* el cual implica varios eventos de hibridación entre cepas de *S. uvarum* y de *S.* eu*bayanus* Europeas; para explicar toda la variabilidad observada se necesitaron, al menos, dos cepas de *S. uvarum*.

Analizando el genoma nuclear, entre las cepas identificadas como *S. pastorianus* pudimos encontrar 3 grupos. i) híbridos con alelos '*cerevisiae*', '*eubayanus*' y '*uvarum*'; ii) híbridos con alelos '*cerevisiae*' y '*eubayanus*' e iii) híbridos con alelos '*cerevisiae*' y '*uvarum*', en este grupo se encontraría la cepa vínica S6U. De

acuerdo con el análisis del gen mitocondrial *COX2*, todas presentaron alelos 'eubayanus', a excepción de la cepa S6U que presentó un alelo 'cerevisiae'.

Posteriormente se decidió estudiar en profundidad las cepas englobadas dentro de *S. bayanus*, así como los mecanismos de especiación que se han dado entre las dos cepas parentales de este grupo (*S. eubayanus* y *S. uvarum*).

Para ello, analizando los alelos de varios genes presentes en las cepas híbridas *S. bayanus,* algunas cepas de *S. uvarum,* tanto Europeas como Argentinas y las cepas *S. eubayanus* Argentinas de las que disponíamos de secuencias, dividimos los alelos en grupos (**Ueu**: *S. uvarum* Europeas; **Eeu**: *S. eubayanus* Europeas; **Uarg**: *S. uvarum* Argentinas; **Earg**: *S. eubayanus* Argentinas) y calculamos las distancias genéticas dentro y entre ellos, y utilizamos los datos de divergencia de *S. cerevisiae* y *S. paradoxus* para comparar. *S. uvarum* y *S. eubayanus* resultaron ser las especies hermanas más próximas, situándose sus valores de divergencia entre los encontrados entre *S. cerevisiae* y *S. paradoxus* y los que existen entre las distintas poblaciones de *S. paradoxus*. La divergencia encontrada entre los alelos 'eubayanus' se situó en valores similares a la divergencia encontrada entre las poblaciones Europea y del 'Far East' de *S. paradoxus*.

También se analizó la viabilidad y la ploidía de las cepas. Las cepas de *S. uvarum* y *S. eubayanus* presentaron valores de viabilidad de esporas ente el 55 y el 100% y valores de ploidía de 2n, con la excepción de la cepa de *S. eubayanus* NPCC1323 que resultó ser casi tetraploide. Los híbridos de *S. bayanus* tipo I presentaron dos situaciones, cepas diploides con valores de viabilidad de esporas mayores al 83% y cepas casi triploides con valores de viabilidad de esporas menores al 25%. Los híbridos de *S. bayanus* tipo II resultaron ser todos diploides, y los valores de viabilidad que mostraron fueron menores del 55%, llegando a valores más bajos del 12% cuando las cepas presentaron 7 o más posiciones heterocigotas y perdiendo la capacidad para esporular cuando el número de posiciones heterozigotas fue mayor de 14.

De acuerdo con su composición cromosómica, las cepas de *S. eubayanus* mostraron un cariotipo muy homogéneo, con pequeñas diferencias de movilidad de la banda correspondiente al cromosoma XVtVIII; todas las cepas presentaron las bandas correspondientes a los cromosomas X y VI; la presencia o ausencia de la translocación 2-3 no se pudo confirmar.

Las cepas de *S. uvarum* mostraron mayor variabilidad en su cariotipo, las diferencias de movilidad se centraron en los cromosomas de mayor tamaño. Todas las cepas presentaron las bandas correspondientes a los cromosomas XtVI y VItX; la presencia o ausencia de la translocación 2-3 no se pudo confirmar.

Los híbridos de *S. bayanus* tipo I se pudieron dividir en dos grupos de acuerdo con su composición cromosómica: Ia) con cromosomas de *S. uvarum* para la translocación I y cromosomas de *S. eubayanus* para la translocación 2-3; estas cepas correspondieron a las cepas diploides con elevados valores de viabilidad. Ib) con cromosomas de *S. eubayanus* para las tres translocaciones; estas cepas correspondieron a las cepas triploides con viabilidades menores al 25%.

Los híbridos de *S. bayanus* tipo II se pudieron dividir en cuatro grupos: IIa) cepas diploides con la translocación 1 en heterocigosis y cromosomas de *S. uvarum* para la translocación 2-3. IIb) Cepas diploides con ambas translocaciones en heterocigosis, en la translocación 2-3 sólo los cromosomas IVtII y IVtIItII se encontraron en heterocigosis, mientras que el resto correspondieron a *S. uvarum*. IIc) Cepas diploides con ambas translocaciones en heterocigosis, en la translocación 2-3 sólo los cromosomas IVtII y IVtIItII se encontraron en heterocigosis, mientras que el resto correspondieron a *S. uvarum*. IIc) Cepas diploides con ambas translocaciones en heterocigosis, en la translocación 2-3 sólo los cromosomas IVtII y IVtIItII se encontraron en heterocigosis, mientras que el resto correspondieron a *S. eubayanus*. IId) cepas diploides sin translocaciones, los cromosomas fueron de *S. uvarum*.

Con los datos de viabilidad y número de posiciones heterocigotas se llevó a cabo un análisis de regresión para ver si existía una correlación entre fertilidad y heterocigosidad. Para llevarlo a cabo, los datos de viabilidad se corrigieron cuando fue necesario, por la por la reducción de viabilidad debida a la presencia de translocaciones en heterocigosis. Este análisis indicó que los valores de viabilidad se mantuvieron elevados cuando las cepas mostraron entre 3 y 5 posiciones heterocigotas, pero se redujeron drásticamente cuando la frecuencia de heterocigosidad fue del 20,8%. Según nuestros resultados un híbrido colinear, heterocigoto perfecto, presentaría valores de viabilidad del 4,8-5,3% (aislamiento reproductivo sólo debido a la divergencia nucleotídica). Si a estos valores les añadimos la presencia de las tres translocaciones que se encontrarían en este híbrido perfecto, la viabilidad estimada se reduciría hasta valores del 1,8-2%.

Al evaluar la fertilidad de la F1 de dos híbridos tipo II, con distintas posiciones heterocigotas, observamos que estos valores aumentaron hasta situarse por encima del 50%, valores similares a los encontrados en las cepas de *S. uvarum*, *S. eubayanus* y *S. bayanus* tipo I.

La especiación en el género *Saccharomyces* se puede dar por barreras pre-reproductivas o post-reproductivas. Las barreras pre-reproductivas son débiles y se dan por diferencias en el tiempo de germinación de las esporas, por la preferencia de cruce o por que el contacto entre las dos especies sea limitado (barreras ecológicas). Las barreras post-reproductivas son más fuertes, entre ellas se encuentra la distancia genética, que actúa a través del sistema de reparación de desapareamientos, impidiendo que se dé la recombinación, y la presencia de translocaciones. No se ha observado que las incompatibilidades genéticas tengan un papel en el aislamiento reproductivo de este género (Delneri et al., 2003; Coyne and Orr, 2004; Liti et al., 2006; Maclean and Greig, 2008; Greig, 2008).

Analizando el nivel de heterocigosidad presente en las cepas híbridas estudiadas (*S. bayanus*), así como la distancia genética entre los alelos 'uvarum' y 'eubayanus', podemos ver que ambas son responsables de una parte del

aislamiento reproductivo observado entre *S. uvarum* y *S. eubayanus*, pero la forma en la que estos factores actúan no se conoce. Entre *S. uvarum* y *S. eubayanus* la antirrecombinación (descrita como el principal factor que causa especiación entre otras especies del género) no parece estar implicada, ya que datos obtenidos en ente trabajo muestran que se produjeron eventos de recombinación en el híbrido ancestral y que no existía ningún cromosoma restringido a esta recombinación. Analizando más en profundidad la distancia genética, *S. uvarum* y *S. eubayanus* son las especies que primero se diferenciaron del género y las que más tiempo han tenido para separarse filogenéticamente, pero no se han separado tanto como se esperaría; se pueden considerar como las especies hermanas más cercanas dentro del género.

Las translocaciones son otro factor importante. La presencia de 3 translocaciones, debería de reducir la viabilidad de los híbridos entre *S. uvarum* y *S. eubayanus* en un 87,5%, pero datos obtenidos de cruces entre poblaciones naturales de ambas especies muestran valores que, en ocasiones son más elevados.

Los datos de viabilidad de las esporas de los cruces entre poblaciones naturales también nos indican que el aislamiento reproductivo es mayor entre poblaciones simpátricas que entre poblaciones alopátricas, disminuyendo la importancia de las barreras ecológicas en el proceso de especiación de estas dos especies.

El género *Saccharomyces*, debido a sus características (grandes poblaciones, capacidad de cambio de tipo sexual y autofertilización) hace a estas levaduras susceptibles de sufrir especiación híbrida homoploide. La especiación híbrida homoploide es un tipo de especiación donde la hibridación entre dos especies da lugar a una nueva especie, sin cambios en el número de cromosomas y aislada reproductivamente de forma parcial o completa con sus cepas parentales (Rieseberg, 1997). Las cepas clasificadas como *S. bayanus* tipo I se evaluaron para observar si habían sufrido este tipo de especiación. A primera vista cumplen todos los criterios descritos por Schumer et al.,(2014): muestra aislamiento reproductivo

parcial con las cepas parentales, reforzado por barreras ecológicas, que impiden la formación de híbridos en la naturaleza; los datos de RFLP y cariotipos nos sirven para documentar la hibridación, en un pasado, entre ambas especies parentales (*S. uvarum* y *S. eubayanus*); los mecanismos que las mantienen aisladas, por ejemplo, la presencia de translocaciones en heterocigosis, son consecuencia del proceso de hibridación. Por todo ello los híbridos tipo I de *S. bayanus* son una especie híbrida homoploide en potencia.

La generación de esta especie híbrida pudo darse de la siguiente forma: la hibridación entre *S. uvarum* y *S. eubayanus* se debió producir en ambientes naturales; el híbrido recién generado colonizó ambientes fermentativos y prosperó en el, sufriendo cambios en su genoma que dieron lugar a los híbridos tipo II. Estos híbridos alcanzaron grandes tamaños poblacionales, esporularon y las pocas esporas viables dieron lugar a los híbridos tipo I. Estos híbridos tipo I recuperaron fertilidad y se convirtieron en esta potencial especie híbrida homoploide. Los híbridos *S. bayanus* tipo II serían el reservorio de los híbridos tipo I, siendo el paso previo a la especiación híbrida homoploide.

2. Evaluación de distintas metodologías en la generación de híbridos artificiales del género *Saccharomyces*.

Durante el transcurso de este trabajo se obtuvieron 31 híbridos intraespecíficos (*S. cerevisiae* x *S. cerevisiae*) y 38 interespecíficos (*S. cerevisiae* x *S. kudriavzevii*) utilizando para ello cepas de *S. cerevisiae* con características enológicas interesantes y complementarias (Sc1 y Sc2) y la cepa tipo de *S. kudriavzevii* (Sk). Para ello se utilizaron tres metodologías: rare-mating entre células diploides (RM), cruce de esporas (S) y fusión de protoplastos (P), también entre células diploides de ambos parentales. De estas tres técnicas, sólo P es considerada como técnica que genera GMOs (Cebollero et al., 2007), mientras que RM y S estarían fuera de esta denominación de acuerdo con la Directiva 2001/18/EC del Parlamento Europeo y del

Consejo Europeo. Los productos resultantes al utilizar las técnicas RM y S podrían ser utilizados en procesos industriales.

Antes de iniciar los protocolos de hibridación se requiere que las cepas parentales dispongan de marcadores fisiológicos complementarios que permitan la selección de los posibles híbridos, y de marcadores moleculares diferenciales que confirmen la naturaleza híbrida de los aislados. Los procesos de selección de híbridos se suelen basar en el uso de marcadores tales como resistencias a antibióticos o auxotrofías. El uso de resistencias está principalmente asociado a la manipulación genética de las cepas, ya que se suelen introducir en plásmidos y la presencia de auxotrofías en cepas industriales no es común (Akada, 2002; Nakazawa and Iwano, 2004). La solución a esta encrucijada se basó en la selección de mutantes auxótrofos espontáneos de las cepas parentales por medio de medios como 5-FOA o α-AA (que seleccionan cepas *ura3*$^-$ y *lys2*$^-$ respectivamente) (Zaret and Sherman, 1985; Boeke et al., 1987). Debido a que estos compuestos seleccionan mutantes auxótrofos espontáneos, su uso no generaría GMOs y los híbridos obtenidos al cruzar parentales seleccionados con estas técnicas podrían utilizarse a nivel industrial. Aunque la obtención de auxótrofos espontáneos en cepas comerciales se dice que es complicada, dado el carácter poliploide de las levaduras industriales (Bell et al., 1998), en este trabajo se consiguieron obtener varios individuos auxótrofos de las tres levaduras que se pretendían utilizar como parentales. De todos los individuos obtenidos se seleccionó un auxótrofo *ura3*$^-$ para el parental Sc1 y un individuo auxótrofo *lys2*$^-$ para los parentales Sc2 y Sk. El uso de parentales auxótrofos en la obtención de híbridos hace que la recuperación de estos se pueda realizar en MM.

Encontrar métodos que confirmen la naturaleza híbrida de los aislados fue también un aspecto crucial a la hora de desarrollar este trabajo. Para discriminar cepas de una misma especie podemos encontrar métodos tales como el análisis de mtDNA-RFLP, RAPD, elementos delta, cariotipado o microsatélites entre otros (Querol et al., 1992; Vezinhet et al., 1992; Baleiras Couto et al., 1995; de Barros Lopes

et al., 1996; Pérez et al., 2001; Hennequin et al., 2001). Para discriminar entre cepas de diferentes especies, aunque también se pueden utilizar las técnicas anteriores, metodologías como la PCR-RFLP de genes nucleares son sencillas y rápidas (González et al., 2008). Dado el tipo de herencia del mtDNA (sólo se hereda una molécula de mtDNA, que puede ser parental o recombinante (Dujon et al., 1974; Berger and Yaffe, 2000), esta técnica se descartó a la hora de confirmar la hibridación, aunque se utilizó para analizar los híbridos. En este trabajo se optó por el análisis de microsatélites para diferenciar entre las dos cepas de *S. cerevisiae* (Sc1 y Sc2) y los híbridos intraespecíficos, y por el análisis de PCR-RFLP de genes nucleares para diferenciar entre los parentales de ambas especies (en este caso entre Sc1 y Sk) y los híbridos interespecíficos. Para las dos regiones microsatélite estudiadas, el parental Sc1 mostró un solo alelo, mientras que el parental Sc2 mostró dos alelos diferentes.

La mayor frecuencia de hibridación (tanto en cruces intra- como interespecíficos) se obtuvo mediante el cruce de esporas (S) – 17% en cruces intraespecíficos y 30% en cruces interespecíficos) –, lo que apoya otros trabajos que indican la dificultad (o la baja frecuencia) en la obtención de híbridos cuando se aplican las técnicas de P y RM (Gunge and Nakatomi, 1972; Spencer and Spencer, 1977; Bell et al., 1998). Aun así el número de híbridos obtenidos fue elevado, y la recuperación de estos mejoró cuando el cultivo se mantuvo en ayuno durante 1-2 horas antes de su siembra en el medio de selección, evitando así que crecieran las cepas parentales haciendo uso de sus reservas. En el caso de RM se observaron mayor número de colonias crecidas en MM cuando se incubaron los tubos durante 5-7 días. En el caso del cruce S, este se realizó en medio completo para permitir que las cepas parentales germinaran; posteriormente se sembraron en medio mínimo las colonias desarrolladas (sin olvidar el medio de ayuno).

El análisis de los híbridos obtenidos se realizó por medio de marcadores moleculares que se encuentran distribuidos por el genoma. Se analizaron los elementos delta, el RAPD R3 y el perfil de mtDNA, ya que se observaron diferencias, para estos marcadores, entre las cepas parentales (no sucedió así para otros 10

RAPD analizados). Los híbridos, obtenidos en ambos cruces, mostraron una elevada variabilidad genética tanto a nivel nuclear (análisis de elementos delta y RAPD) como mitocondrial (mtDNA), indicando que, desde el inicio del proceso de hibridación, estos aislados sufren reorganizaciones y pérdidas de material genético. Entre los 69 híbridos originados se detectaron 26 patrones de elementos delta diferentes y 26 patrones R3 y varios patrones de mtDNA. De acuerdo con el análisis del mtDNA, los híbridos mostraron tanto patrones equivalentes a los de sus cepas parentales, al igual que se había observado en otros casos (de Barros Lopes et al., 2002; Antunovics et al., 2005; González et al., 2006; Rainieri et al., 2008), como patrones nuevos. Estos nuevos patrones se presentaron sólo en una minoría de los híbridos recuperados y pudieron ser originados por una recombinación entre los mtDNA parentales, como ya describieron Berger y Yaffe (2000). Esta situación (presencia de mtDNA tanto de los parentales como recombinante) se dio en ambos tipos de híbridos, independientemente del tipo de metodología por la que se originaron (P, RM o S). También se detectó la presencia del mismo perfil de mtDNA recombinante en varios individuos del mismo cruce (obtenidos por igual o distinta metodología), lo que da pie a pensar en la posible presencia de puntos calientes de recombinación en el mtDNA, como sugirieron otros autores (Dujon et al., 1974; Piškur, 1994; Berger and Yaffe, 2000). La mayor frecuencia de moléculas recombinantes se dio en el cruce intraespecífico (26% frente al 13%); esto se puede explicar por la mayor similaridad de genomas entre las cepas parentales del cruce intraespecífico, lo que podría favorecer la recombinación homóloga (Bernardi, 2005). La elevada variabilidad genética, las reorganizaciones y las pérdidas de material genético también se observaron al analizar el genoma nuclear. En el análisis de R3 y secuencias delta muchos híbridos mostraron patrones originados por la adición de los patrones parentales, mientras que otros mostraron nuevas bandas, probablemente originadas por la recombinación de cepas parentales. Combinando el análisis de elementos delta, R3 y mtDNA se consiguieron diferenciar 7 de los 8 híbridos ScxSc obtenidos por P, todos los híbridos obtenidos por RM y todos los

híbridos obtenidos por S; en el cruce interespecífico se consiguieron diferencias 4 de los 12 híbridos obtenidos por P, 10 de los 14 híbridos RM y 9 de los 12 híbridos S.

La pérdida de material genético fue evidente al analizar los microsatélites en el cruce intraespecífico, donde se observó la pérdida de uno de los alelos del parental Sc2 en varios híbridos (el parental Sc1 fue homocigoto para los microsatélites analizados, por lo que fue imposible detectar la pérdida de alelos individuales), y quedó demostrada tras la evaluación del contenido en DNA. El contenido de DNA de las cepas parentales fue de 2,7 para Sc1, 2,5 para Sc2 y 2,2 para Sk. El contenido en DNA de los híbridos recién generados en ambos cruces fue menor al valor esperado si sumamos el contenido en DNA de ambos parentales, especialmente en aquellos generados por P y RM (5,2 para el cruce intraespecífico y 4,9 para el interespecífico). Esta pérdida se debió producir inmediatamente tras la generación de los híbridos.

Dado que los híbridos obtenidos mediante la técnica P son considerados GMOs, se dejó de trabajar con ellos.

Tras someter a 5 rondas de crecimiento a varios de los híbridos obtenidos, se observaron cambios en los marcadores nucleares, el marcador mtDNA y el contenido en DNA. Dos de los híbridos sufrieron cambios en sus perfiles de mtDNA-RFLP (R1, híbrido interespecífico, y R8, híbrido intraespecífico). Estos cambios pudieron suceder porque, durante el crecimiento, las cepas están sufriendo un proceso de estabilización, en el que la maquinaria de reparación del DNA, responsable de las reordenaciones cromosómicas, se encuentra activa, pudiendo actuar sobre las moléculas de mtDNA. Este proceso estaría favorecido debido a la elevada homología (100%) entre las moléculas de mtDNA (Bernardi, 2005). En el genoma nuclear de los híbridos se observó una situación similar para los marcadores RAPD-R3 y elementos delta, aunque la mayoría de cambios solo se detectaron en el análisis de este último. Estas reordenaciones fueron más frecuentes en las colonias derivadas de los híbridos intraespecíficos obtenidos por RM, que tras 5 rondas de crecimiento mostraron varios patrones R3 y delta

derivados del patrón que presentaba el híbrido original, indicando de nuevo que la recombinación se ve favorecida por la homología del genoma. Los híbridos también sufrieron una reducción de su contenido de DNA tras estas 5 rondas de crecimiento, esta reducción fue más evidente en aquellos híbridos que partían con un mayor contenido en DNA (aquellos originados por RM). La reducción del contenido en DNA también la observaron Antunovics et al., (2005) y Marinoni et al., (1999) tras obtener híbridos interespecíficos.

Estos hechos nos indican que los genomas poliploides son inestables, al igual que se observó en genomas poliploides de *S. cerevisiae* (Storchova et al., 2006; Gerstein et al., 2006). Esta inestabilidad podría ocasionar la pérdida de características importantes para el proceso en el que se pretenda utilizar estas cepas (en nuestro caso para vinificación), de modo que la estabilización es un punto esencial, y elección de las condiciones es crucial para el futuro uso de los híbridos en aplicaciones industriales.

3. Estudio del proceso de estabilización de híbridos intra- e interespecíficos del género *Saccharomyces* en condiciones fermentativas.

Pocos trabajos lo mencionan (Antunovics et al., 2005; Bellon et al., 2011; Kunicka-Styczynska and Rajkowska, 2011), pero la necesidad de llevar a cabo un proceso de estabilización de los híbridos recién obtenidos es un hecho, especialmente cuando estos se pretenden utilizar en procesos industriales. La mezcla de dos genomas, unida al incremento en la cantidad de DNA (que en este trabajo ocurrió tanto en híbridos obtenidos por RM como por S, ya que las cepas parentales Sc1 y Sc2 eran aneuploides) permite que se den reordenaciones y que se pierda el DNA no necesario para estas nuevas cepas, hecho que ya fue observado por Gerstein et al. (2006; 2008), siendo diferente en medios estresantes y no estresantes (Gerstein et al., 2008). Durante este proceso, la pérdida de material genético y las reorganizaciones podrían llevar a la pérdida de características

industriales importantes, pero esto se podría evitar si, para el proceso de estabilización, se utiliza un medio que imite el proceso industrial donde se pretende utilizar esta cepa.

Dado que la elección de las condiciones de estabilización parece ser un punto importante, definimos estas como fermentaciones sucesivas en 10 ml de mosto sintético a 20ºC, sin agitación y utilizando el sedimento de cada una de las fermentaciones para inocular la nueva fermentación. Como ocurre a nivel industrial, dejamos que las levaduras pasaran de un medio con elevada concentración de azúcar a un medio sin azúcares pero con etanol. De este modo estudiamos los cambios que ocurrieron a nivel genómico en estas cepas durante 5 fermentaciones sucesivas. Se analizaron tanto cambios en el contenido en DNA de las cepas como en los perfiles de mtDNA, elementos delta y RAPD R3. Se encontraron diferencias significativas entre el proceso de estabilización de híbridos intra e interespecíficos; también se encontraron diferencias en la estabilización de híbridos obtenidos por distinta metodología.

Durante este proceso observamos tanto la presencia de nuevos perfiles de mtDNA (híbrido intraespecífico R8 e interespecífico R1) como de nuevos patrones nucleares (elementos delta y RAPD en los híbridos R2, R8 y S2). La aparición de nuevos patrones nucleares se observó solamente en híbridos intraespecíficos, y fue más frecuente en aquellos obtenidos por RM. El mayor número de nuevos patrones moleculares se observó en las fermentaciones 4 y 5. El marcador R3 demostró ser muy poco variable durante el proceso y en todos los pases se recuperó el perfil original de la cepa y ningún perfil se impuso completamente sobre los demás. En cuanto a los híbridos interespecíficos, sólo el híbrido R1 mostró cambios, estos cambios fueron a nivel de su mtDNA y un único perfil logró imponerse al resto tras 40 generaciones. Este análisis muestra que la inestabilidad cromosómica va ligada a procesos como la recombinación de genomas (Andalis et al., 2004; Storchova et al., 2006); también evidenció que la mayor frecuencia de reordenaciones/recombinación se da entre genomas genéticamente similares (mtDNA o genoma nuclear de híbridos intraespecíficos).

Por norma general, los híbridos redujeron su ploidía hasta los valores encontrados en sus cepas parentales, al igual que ocurrió en el estudio de Gerstein (2006; 2008), con la excepción del híbrido interespecífico R3 que se estabilizó con valores cercanos a la triploidía (al igual que se observa en Gerstein 2008 en cepas que crecieron en medios estresantes). Dado el mayor contenido en DNA, la reducción de genoma fue mayor para los híbridos generados por RM, en los híbridos S no fue significativa. La reducción del contenido en DNA ocurrió drásticamente entre las fermentaciones 3 y 4 para los híbridos intraespecíficos R2 y R8, y en la primera fermentación para los híbridos interespecíficos R1 y R3, indicando una estabilización más rápida en estos últimos. Aun así, en los cultivos recuperados de los híbridos R2 y R8, varios aislados no redujeron sus valores de ploidía o los redujeron ligeramente. Estos aislados fueron los únicos que no se confirmaron como estables.

En un estudio realizado por Bellón et al (2011) tampoco se observaron cambios en el genoma de híbridos interespecíficos tras 50 generaciones, aunque ellos no analizaron los valores de ploidía y evaluaron el proceso sólo al final del mismo, pudiendo perder información en los puntos intermedios del proceso.

Dado que en condiciones estresantes los genomas pueden sufrir reordenaciones (Pérez-Ortín et al., 2002; Dhar et al., 2011), y que durante la producción de LSA (levadura seca activa, ADY) las levaduras se someten a múltiples estreses (Degré, 1993; Attfield, 1997; Bauer and Pretorius, 2000; Novo et al., 2006; Gomez-Pastor et al., 2011), se consideró que este sería un proceso adecuado para someter a los híbridos recién estabilizados y verificar así su estabilidad. Dos aislados de los híbridos R2 y R8 (R2 IVo y R8 IIa) -los híbridos intraespecíficos más variables- se sometieron a este proceso y fueron analizados posteriormente. Uno de los aislados (R8 IIa) sufrió cambios en su genoma nuclear, aunque no en su genoma mitocondrial ni en su ploidía; el otro híbrido (R2 IVo) se mostró estable. La estabilidad se confirmó tanto con un análisis del número de copias de los distintos genes (CNV) como con un análisis fisiológico de su capacidad fermentativa.

Para resumir este trabajo, podemos observar diferentes situaciones en el proceso de estabilización de híbridos recién generados: i) la estabilización se puede dar por una pérdida gradual de DNA, sin cambios detectables a nivel nuclear o mitocondrial (híbridos intraespecíficos R3, S5 y S8 e intraespecífico S7); ii) la estabilización se puede dar tras reordenaciones nucleares y reducción de los valores de ploidía, con o sin cambio en los perfiles de mtDNA (híbridos intraespecíficos R8 y S2 y R2 respectivamente) y iii) la estabilización puede darse tras una rápida pérdida de material genético, sin cambios aparentes en el DNA nuclear, pero con cambios a nivel mitocondrial (híbrido intraespecífico R1). El proceso es más rápido cuando se trata de híbridos interespecíficos (se estabilizaron en los primeros pases: 10-20 generaciones). Los híbridos intraespecíficos mostraron una mayor diversidad de perfiles derivados en los pases 4 y 5 (tras crecer durante 40 o 50 generaciones).

Estos resultados nos sugieren que el análisis de contenido en DNA, del perfil del mtDNA, y de marcadores nucleares como los elementos delta, permiten asegurar rápidamente la estabilidad de híbridos intra- e interespecíficos. Esta evaluación de la estabilidad debe realizarse, también, después de someter al híbrido a los puntos más estresantes del proceso para el que se han generado. Si consideremos como estable a aquel híbrido que mantenga su contenido en DNA, su perfil de mtDNA y su patrón de elementos delta tras divisiones celulares sucesivas, podemos decir que 30-50 generaciones (3-5 pases de fermentación) son suficientes para estabilizar los híbridos inter e intraespecíficos respectivamente. Estos pases de fermentación (o número de generaciones) no dependen del método de cruce empleado, sólo del tipo de híbrido.

4. Caracterización fisiológica y genómica de híbridos intraespecíficos de *Saccharomyces cerevisiae*.

Tras la hibridación y la estabilización de los híbridos obtenidos, el último punto antes de poder utilizar una cepa en condiciones industriales es su selección.

En esta selección se busca que el híbrido haya mejorado en las características en las que destacaban sus cepas parentales. Al principio de este trabajo, dos cepas vínicas de distintas características, pertenecientes a la especie *S. cerevisiae*, se cruzaron con el fin de obtener un híbrido con la capacidad fermentativa del parental Sc2 y la producción de manoproteínas del parental Sc1. Dado que las manoproteínas contribuyen positivamente a las características del vino (Caridi, 2006), se ha intentado mejorar su producción mediante técnicas de ingeniería genética y mediante mutagénesis (González-Ramos et al., 2009; Quirós et al., 2010; González-Ramos et al., 2010); en este trabajo se ha intentado mediante hibridación.

Como se ha mencionado en trabajos previos (Sipiczki, 2008), los híbridos pueden heredar las características parentales en nuevas combinaciones, pudiendo ser mejores que las encontradas en las cepas parentales. De un total de 19 híbridos estables incluidos en el proceso de selección las cepas R2 Io, R2 IIIa y R2 IVo presentaron valores de velocidad máxima (K) mayores que el parental Sc2, llevando a cabo la fermentación (t_{50} y t_2) en menor tiempo que este. En cuanto a la liberación de polisacáridos, parece que el parental Sc1 liberó la menor cantidad de polisacáridos, pero la tinción específica de manoproteínas reveló que este produjo mayor cantidad que el parental Sc2 (Sc1 presentó un mayor ratio manoproteínas/polisacáridos). Dado que no solo la cantidad de manoproteínas, sino también el tipo de estas, está implicado en las características tecnológicas de las cepas (Moine-Ledoux and Dubourdieu, 1999; Waters et al., 2000), se seleccionaron como buenos los híbridos que produjeron elevados niveles de polisacáridos y bandas de manoproteínas iguales a la de los parentales, en tamaño, pero más intensas. Los híbridos analizados produjeron mayor cantidad de polisicáridos que las cepas parentales, y los híbridos R8 IIa, S7, R2 Io, R2 IIo y R2 IVo presentaron las bandas más intensas. Cuando las cepas se utilizaron para estudiar la quiebra proteica de un vino Sauvignon Blanc (González-Ramos et al., 2008), la cepa R2 IVo fue la que necesitó menores valores de Bentonita para estabilizar el vino, mejorando al parental Sc1. Estos datos se confirmaron mediante un método cuantitativo (Quirós et al., 2012) en una fermentación en mosto Verdejo. Mediante

este método también se confirmó que Sc1 producía altos niveles de manoproteínas, mayores que Sc2.

De los 19 aislados analizados, el híbrido R2 IVo mejoró e incluso superó las características de sus parentales, tanto a nivel de cinética fermentativa como de liberación de manoproteínas, cumpliendo con las expectativas de un programa de mejora y confirmando lo mencionado en Sipiczki et al., (2008).

Para analizar lo que pasó a nivel genético, se realizó un estudio de CNV (variación en el número de copias), mediante chips de DNA, que nos permitiera observar si, durante la estabilización del genoma, habían sucedido cambios en el número de copias de algunos genes con respecto a los que presentaban las cepas parentales (Dunham et al., 2002; Dunn et al., 2005; Peris et al., 2012b). Estos cambios en el número de copias de algunos genes se intentaron relacionar con las características fisiológicas que se habían mejorado en el híbrido R2 IVo.

El análisis comparativo de R2 IVo frente a cada uno de sus parentales evidenció que el híbrido mantuvo el número de copias de uno u otro parental para varios genes. Se observó que el gen *MNN10* estaba sobrerrepresentado en el híbrido con respecto al parental Sc2, pero no se observaron diferencias entre el híbrido y Sc1; estudios enzimáticos sugieren que Mnn10p y Mnn11p son las responsables de la mayor parte de la actividad de polimerización de manoproteínas α-1,6 (Jungmann et al., 1999). Una situación similar se observó para el gen *YPS7*, que codifica una proteasa relacionada con la incorporación y retención de glucanos en la pared celular *(Krysan et al., 2005)*. El gen *SWP1*, que codifica para una subunidad de la oligosacaril transferasa, necesaria para la N-glicosilación de proteínas en el retículo endoplásmico, también se encontró sobrerrepresentado en el híbrido con respecto al parental Sc2 pero en el mismo número que en el parental Sc1. Por otra parte, el híbrido, al igual que el parental Sc2, presentaron un mayor número de copias que Sc1, de varios genes subteloméricos pertenecientes a la familia de las *HXT* (*HXT9, HXT11, HXT12*); Lin and Li (2011) encontraron una fuerte correlación entre el número de copias de las *HXT* y el carácter fermentativo de las cepas. También se ha visto

que alteraciones en el número de copias de genes glicolíticos están asociadas con la mejora de la capacidad fermentativa; en este sentido, tanto el parental Sc2 como el híbrido mostraron un mayor número de copias de los genes *GPM1* y *HXK1* que Sc1.

Para confirmar el número de copias de estos genes presentes en el híbrido se analizaron, mediante la técnica de qPCR, los genes *MNN*10, *YPS*7 y *HXK*1. De acuerdo con los datos de CNV, el híbrido debería presentar mayor número de copias que el parental Sc2 para los genes *MNN*10 e *YPS*7, y más copias que el parental Sc1 para el gen *HXK*1. Como esta aproximación no fue concluyente, se pasó a estudiar la expresión de estos tres genes durante la fermentación.

El análisis de expresión de los genes *MNN*10, *YPS*7 y *HXK*1 durante una fermentación en mosto sintético apoya los resultados obtenidos. Estos resultados nos indican que la mayor producción de manoproteínas en el híbrido puede deberse a que la expresión del gen *MNN*10 se mantiene elevada en el híbrido durante más tiempo que en el parental Sc1 y a que presenta unos mayores niveles de expresión del gen *YPS*7 en el medio de la fermentación (55h); por otra parte, la mejora en la cinética fermentativa puede deberse a un incremento en la expresión del gen *HXK*1 a partir de las 55h; este incremento en la expresión también se observa en el parental Sc2 al final de la fermentación (120h).

La caracterización fisiológica y la selección no se realizaron sobre los híbridos estables del cruce interespecífico porque la cepa de *Saccharomyces kudriavzevii* utilizada en este trabajo fue la cepa tipo (IFO 1802). Trabajos anteriores con híbridos naturales (González et al., 2008; Lopes et al., 2010; Peris et al., 2012a; Peris et al., 2012b; Peris et al., 2012c) habían puesto de manifiesto que el parental *S. kudriavzevii* de estas cepas era diferente a la cepa tipo, postulando así la presencia de esta especie en Europa, aunque todavía no hubiera sido encontrada. En 2008 Sampaio et al., encontraron cepas de *S. kudriavzevii* en Portugal; posteriormente Lopes et al., (2010) también encontraron aislados en España y confirmaron que presentaban alelos similares a aquellos presentes en los híbridos vínicos y cerveceros. Dado que el parental *S. kudriavzevii* utilizado en este trabajo era genéticamente diferente de

los aislados que parecían ser cercanos a los parentales de los híbridos naturales, decidimos dejar en este punto el trabajo realizado con este cruce. Aun así, los conocimientos adquiridos en la obtención y estabilización de híbridos interespecíficos *S. cerevisiae* x *S. kudriavzevii* han sido útiles para aplicar a otros cruces interespecíficos (*S. cerevisiae* x *S. uvarum*) e incluso nos han permitido realizar trabajos con varias empresas, datos que no se han incluido en el presente trabajo debido a la confidencialidad exigida por las empresas.

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Conclusiones

1. Se contribuyó al descifrado de la complejidad del grupo de especies anteriormente llamado *S. bayanus*, mediante el análisis de restricción (y en algún caso secuenciación) de 34 regiones génicas nucleares y una región mitocondrial. De las cepas analizadas, ninguna fue una cepa pura perteneciente a *S. eubayanus*.
2. Se observó una elevada homogeneidad intraespecífica en las cepas pertenecientes a *S. uvarum*, aunque la presencia de genes heterocigotos indica que se pueden producir cruces entre ellas. La presencia de introgresiones de fragmentos de *S. cerevisiae* en estas cepas también parece ser frecuente.
3. Todas las cepas pertenecientes a *S. bayanus* son híbridos entre *S. uvarum* y *S. eubayanus*, y se pueden dividir en dos subgrupos: híbridos de tipo I, homocigotos para todos los genes estudiados, e híbridos de tipo II, heterocigotos para alguno de los genes estudiados. Estas cepas probablemente aparecieron como consecuencia de varios sucesos de hibridación entre cepas pertenecientes a *S. uvarum* (al menos dos) y cepas europeas de *S. eubayanus*, similares a las cepas patagónicas descritas.
4. Las cepas pertenecientes a *S. pastorianus* también se pueden dividir en tres grupos, híbridos *S. cerevisiae* x *S. uvarum*, híbridos *S. cerevisiae* x *S. eubayanus* e híbridos *S. cerevisiae* x *S. uvarum* x *S. eubayanus*.
5. El linaje de *S. uvarum* y *S. eubayanus* es el más antiguo del género *Saccharomyces*, sin embargo, las especies *S. uvarum* y *S. eubayanus* son las más próximas entre sí.
6. Entre *S. uvarum* y *S. eubayanus* no está claro cuál es la contribución de la divergencia nucleotídica genómica en su aislamiento reproductivo postzigótico, ya que la antirrecombinación, descrita como el principal factor que causa especiación por esta vía en el género *Saccharomyces*, no parece ser suficiente para explicar dicho aislamiento.
7. Las translocaciones son otro factor importante en la especiación, pero datos obtenidos de cruces entre poblaciones naturales de ambas especies

muestran valores de viabilidad más elevados de lo esperado. Dado que alguna de las translocaciones que diferencian los cromosomas de ambas especies no son fácilmente detectables, debería llevarse a cabo un análisis más exhaustivo del cariotipo de las distintas cepas y poblaciones de *S. uvarum* y *S. eubayanus*.

8. El aislamiento reproductivo parece ser mayor entre poblaciones simpátricas que entre alopátricas, lo que disminuye la importancia de las barreras ecológicas en el proceso de especiación de estas dos especies.
9. Los híbridos de tipo I de *S. bayanus* podrían considerarse potenciales nuevas especies híbridas homoploides. Mantienen el mismo número de cromosomas que las especies parentales, pero están aisladas reproductivamente de ellas (completa o parcialmente) y cumplen los tres criterios descritos por Schumer et al. (2014) para que puedan considerarse como tales.
10. La hibridación entre *S. uvarum* y *S. eubayanus* se debió producir en ambientes naturales. Así, el híbrido recién generado colonizó ambientes fermentativos y prosperó en ellos, sufriendo cambios en su genoma que dieron lugar a los híbridos tipo II. Estos híbridos alcanzaron grandes tamaños poblacionales, esporularon y las pocas esporas viables dieron lugar a los híbridos tipo I. Estos híbridos tipo I recuperaron su fertilidad y se convirtieron en esta potencial especie híbrida homoploide.
11. Los híbridos *S. bayanus* tipo II podrían considerarse un reservorio de híbridos tipo I, siendo el paso previo a la especiación híbrida homoploide.
12. Se obtuvieron una gran diversidad de híbridos artificiales, tanto intra- como interespecíficos, utilizando las tres metodologías disponibles: cruce de esporas, 'rare-mating' y fusión de protoplastos.
13. A pesar de la baja frecuencia de hibridación obtenida en la fusión de protoplastos y el rare-mating, los híbridos generados por estas metodologías poseen, teóricamente, un juego completo de material genético de cada parental, lo que les proporciona una plasticidad genética extremadamente alta, que puede favorecer su mejor adaptación en ambientes fluctuantes.

14. Dado que la pérdida de material genético se da tanto durante la generación como durante la estabilización de los híbridos recién formados, los híbridos que posean una elevada cantidad de DNA serán los mejores candidatos para obtener la cepa híbrida más adecuada para fines industriales.
15. La técnica del rare-mating da lugar a híbridos, con las características descritas anteriormente, que no son considerados GMOs, por lo que es la técnica más útil para desarrollar cepas con un propósito industrial. Además, se obtienen y estabilizan fácilmente.
16. Durante el proceso de estabilización se pueden dar varias situaciones: i) estabilización mediante la pérdida rápida de material genético, sin cambios detectables en los patrones de DNA nuclear ni mitocondrial. ii) estabilización tras reordenaciones a nivel del genoma nuclear y pérdida gradual de material genético, con o sin cambios en el genoma mitocondrial. iii) estabilización mediante la pérdida rápida de material genético, sin cambios en los marcadores nuclear, pero con cambios en el genoma mitocondrial.
17. La estabilización de los híbridos intraespecíficos e interespecíficos es distinta; aunque el genoma nuclear y mitocondrial de ambos tipos de híbridos puede sufrir cambios (reordenaciones y/o pérdida de material genético), los híbridos intraespecíficos necesitan un mayor número de generaciones para dar lugar a células genéticamente estables, mientras que este proceso es mucho más rápido en los híbridos interespecíficos.
18. La reducción genética y la reorganización del genoma, que ocurre durante el proceso de estabilización, puede llevar a la pérdida de características industriales importantes, por lo que un punto importante en la generación de híbridos artificiales es la elección de esas condiciones de estabilización.
19. El análisis de marcadores moleclulares como los elementos delta o los patrones de RFLP del mtDNA, junto con el análisis de ploidía, permiten la rápida caracterización de la estabilidad genotípica de los híbridos.
20. Considerando que un híbrido estable debe mantener el mismo patrón molecular y la misma ploidía durante divisiones celulares sucesivas, se determinó que 3 y 5 pases fermentativos (30 y 50 generaciones) son

suficientes para obtener híbridos intra- e interespecíficos genéticamente estables, respectivamente, independientemente del método por el cual se hubieran obtenido.

21. En este trabajo fuimos capaces de combinar, mediante técnicas de hibridación, las características enológicas deseables de dos levaduras comerciales, en una única levadura. Muchos de los híbridos obtenidos presentaron características fermentativas intermedias a las presentadas por ambos parentales; y en algunos casos incluso superaron las características fermentativas del parental seleccionado por ello.
22. El híbrido seleccionado (R2 IV0) produjo mayor cantidad de manoproteínas que las producidas por ambos parentales. Esto se evidenció tanto de forma cualitativa, analizando la respuesta a la quiebra proteica de un vino Sauvignon Blanc tras un tratamiento con bentonita, como de forma cuantitativa analizando las manoproteínas liberadas tras una fermentación en mosto Verdejo.
23. Las mejoras observadas en el híbrido R2 IV0 pueden deberse a la duplicación de genes asociados con la reorganización de la pared celular, como son *MNN10* e *YPS7*, implicados en la mejora en la producción de manoproteínas, y a la duplicación de genes glicolíticos como HXK1 y transportadores de hexoxas (genes HXT), implicados en la capacidad fermentativa. Por tanto, la hibridación, combinada con la estabilización en condiciones de vinificación, es una aproximación efectiva a la hora de obtener levaduras mejoradas en cuanto a su capacidad de producción de manoproteínas y a su capacidad fermentativa.

Material suplementario

Table S1.1 Gene regions under restriction analysis and primers used for PCR amplification. Chromosome (Chr) positions of the genes correspond to *S. cerevisiae*, for other arrangements present in the other strains see Supporting Information Figure S1.1.

Chr	Gene (ORF)	Primer	Sequence
I	*BUD14* (YAR014c)	Forward	5'-TGAATTGTTGGAAAAATGARAAYATG
		Reverse	5'-CGAATAATTTCATCCAAYTGYTTCAT
I	*CYC3* (YAL039c)	Forward	5'-CTACAAAAAAATGGGTTGGTTTTGGGC
		Reverse	5'-GGGAACAGTAGGCCGCACARRTGCATCCA
II	*APM3* (YBR288c)	Forward	5'-AAGTATTTCTCAATTACAAATAAGCTCAAYTAYTGGTG
		Reverse	5'-CCTGTGGCAGTTGCTTTATCAAARATCCAYTG
II	*OPY1* (YBR129c)	Forward	5'-CCGCGGACAACAGACCAYCATTAYTGGTGYGT
		Reverse	5'-CTCTTGAAATTTATTATCCARTCCACCATRTCYTG
II	*PKC1* (YBL105c)	Forward	5'-GATTTCTGTGGCATGTCCATGGARATGG
		Reverse	5'-ACAGAAAGTCTTTGGTTTTGAACRTGCCACAT
III	*KIN82* (YCR091w)	Forward	5'-GCCCTGAAAGTTTTGAGTAAACAYGARATGAT
		Reverse	5'-TCGTCATCATTTGCAACTTTCTCRCARAACAT
III	*MRC1* (YCL061c)	Forward	5'-AAACGAAAGGTTTCAAGTTGGARGAYA
		Reverse	5'-TTGTTTATCATTTTGACATCCATYTCYTT
IV	*EUG1* (YDR518w)	Forward	5'-CTTGTCGAATTYTTYGCYCCATGG
		Reverse	5'-GTGGTAAACCATATTTCAGATTRTTRATCAT
IV	*RPN4* (YDL020c)	Forward	5'-GAAGATAATTATAATCCGAARATGCARTGG
		Reverse	5'-ACCACATGGTTCATTAGTGATRAGRTTCAT
IV	*UGA3* (YDL170w)	Forward	5'-GGCTCAGAGATATGTCAGGGTGATGTGAARTTYTGGAA
		Reverse	5'-TGCTCGCTGATGCCGTCAGCTTCRTTYTGCCA
V	*MET6* (YER091c)	Forward	5'-CTAGACCTGTCCTATTGGGTCCAGTTTCTTACTT
		Reverse	5'-TTAGCTTCTAGGGCAGCAGCAACRTCTTGACC
		K-Reverse	5'-GCGTTAGCTTCTAGAGCAGCAGTGACATTGC

Chr	Gene (ORF)	Primer	Sequence
V	*NPR2* (YEL062w)	Forward	5'-CACTAGACTGGGCAAAATGTTYAARGT
		Reverse	5'-AACAGGAGGTTTGTGAYTGYCTCCARTC
VI	*GSY1* (YFR015c)	Forward	5'-ATTGGAAAAAGAATTTTCGAGCAYACRATGAG
		Reverse	5'-AATTTCTTGCCACCGGCAAGGGTATTCATATT
VI	*EPL1* (YFL024c)	Forward	5'-GAGGATTGTTGTGGTACCAACTAYAAYATGGA
		Reverse	5'-CGTCTTCAATTTTCCGYTTYTCCATYTT
VII	*KEL2* (YGR238c)	Forward	5'-TATCTTTTCAATATTAATTCTTAYAARTGGAC
		Reverse	5'-GCAGTTTCAAGTTCYTTYTGYTG
VII	*MNT2* (YGL257c)	Forward	5'-ATACAGATCTATCTTTTTGGGARAAYTGG
		Reverse	5'-AGTCTCTGGCTATGCTCATARTCRTAYTCCCA
VIII	*CBP2* (YHL038c)	Forward	5'-GACTTCAAGCCATCTTTYGARTGGCARCA
		Reverse	5'-CATGGACTCGGTCTCTTGATAYTGYTTCCA
VIII	*MNL1* (YHR204w)	Forward	5'-TCGTTGAAAACTAARTGTAAAAAYGAYTGG
		Reverse	5'-ATCTCACCTCTTGGGRCAACCACAT
IX	*DAL1* (YIR027c)	Forward	5'-CAGGGCCAAATGTGGTGYGAYGT
		Reverse	5'-CTGCTTGGAAGTATTTTCRCARCACCA
IX	*UBP7* (YIL156w)	Forward	5'-CCTCTTAGGTGGGTATGAAAAATGGAARAARAC
		Reverse	5'-CCATTAACAATTACGTTTTTRTCAAACCARTG
X	*CYR1* (YJL005w)	Forward	5'-CTACGAAGGAAAGTGTCCTCTTTRGTTCGTGG
		Reverse	5'-CCGTGTGTAGAATTTAGTGTAGAATTGACRGC
X	*PEX2* (YJL210w)	Forward	5'-GATAAAGAACTGTATGGACARTTYTGG
		Reverse	5'-GCCTTTACAACGCACACATARCARTARTT
XI	*BAS1* (YKR099w)	Forward	5'-CATTCAACCCAGCATCTCTCGATGARCAYATGATG
		Reverse	5'-GTAGTAGCTAAGGATCTCAAGGTTTCCCARAARTC
XI	*CBT1* (YKL208w)	Forward	5'-TACAACACCTTCGTGCTTTAYGAYTGG
		Reverse	5'-TTCCAGTAGTTCAARTTGCGRTACAT

Chr	Gene (ORF)	Primer	Sequence
XII	*MAG2* (YLR427w)	Forward	5'-ATGGTAGAACCGGATATGCAGAARAARGC
		Reverse	5'-AGCTCCAAGGAATTAGATACACCRCAYTTCAT
XII	*PPR1* (YLR014c)	Forward	5'-CGGGTGTTTGGTAYACYATGGG
		Reverse	5'-TAAAGATAAGTCATACCTGTCATRAACAT
XIII	*CAT8* (YMR280c)	Forward	5'-TCCAATATTAGTATCAACAACTTTCTATAYCARAAYGA
		Reverse	5'-CTACTTGGCRTTTTGCCAYTGRAA
XIII	*ORC1* (YML065W)	Forward	5'-GGCTCTCACCTATTTGAGATGGTTYGAR
		Reverse	5'-GCTCTAGCGACATTTTTTTTTGTRAAYTT
XIV	*BRE5* (YNR051c)	Forward	5'-TGATTATAGCCACGGGTGARATGTTYTGG
		Reverse	5'-TTCATTCATCAACTTTGAGGCCCATGTCAT
XIV	*EGT2* (YNL327w)	Forward	5'-GTACGGACCCAGAYCAYTGGTT
		Reverse	5'-CATTGGTAAATCAACRGTRAACCA
XV	*ATF1* (YOR377w)	Forward	5'-TGGAAAAAATTTATATTTGTATCTAATCAYTGYATG
		Reverse	5'-CCAATGAAAATGCYTGRTGCCA
XV	*RRI2* (YOL117w)	Forward	5'-ATGTCTGATGAAGATGACAATTATGAYGACTTYATG
		Reverse	5'-AATATCTCTCTCCCGAAGTTTCCCCATYTCYTC
XVI	*GAL4* (YPL248c)	Forward	5'-TGTGCCAAGTGTCTGAAGAAYAAYTGGGA
		Reverse	5'-GCGATTTCAATCTGATTATTRTACARCATCAT
XVI	*JIP5* (YPR169w)	Forward	5'-GGCGTGGAAACACTTTGGAAGACYAARAG
		Reverse	5'-TATAGATGTTACCATTGGARCAACCRCACCA

Table S1.2 Composite restriction patterns deduced from the gene region sequences of the *eubayanus*-type alleles, present in the reference strains *S. bayanus* NBRC 1948, CECT 11186, CBS 424 or *S. pastorianus* Weihenstephan 34/70, the *uvarum* alleles exhibited by *S. uvarum* CBS 7001, and the *cerevisiae*-type alleles present in *S. cerevisiae* S288c. These composite patterns for each gene region have been named after the initial of the allele-type name followed by the order numeral 1. Chromosome (Chr) positions of the genes correspond to *S. cerevisiae*, for other arrangements present in the other strains see Figure S1.1.

Chr	Gene	Rest. enzyme	*uvarum*-type allele	Pat-tern	*eubayanus*-type allele	Pat-tern	*S.cerevisiae* S288C	Pat-tern
I	*CYC3*	*Cfo* I	490 30 15 5	U1	360 170 10	E1	540	C1
		*Hae*III	330 230 10		230 180 150 10		330 200 10	
		Hinf I	260 175 130		270 140 100		175 160 110 90	
	BUD14	*Msp* I	875 160 130	U1	560 315 160 130	E1	475 310 230 95 40	C1
		Taq I	340 280 240 210 70 25		560 340 230 30		420 300 290 100 55	
II	*PKC1*	*Asp* 700 I	460 440 280	U1	900 280	E1	480 470 230	C1
	OPY1	*Scr*F I	370 85	U1	250 120 85	E1	350 105	C1
		Taq I	235 125 95		220 155 80		420 300 290 100 55	
	APM3	*Msp* I	960 70	U1	690 340	E1	750 280	C1
		Hinf I	375 210 190 175 80		345 210 190 175 110		640 220 170	
III	*MRC1*	*Dde* I	590 180 80 75	U1	590 270 75	E1	470 240 100 75 40	C1
		Msp I	760 170		930		720 160 50	

Chr	Gene	Rest. enzyme	*uvarum*-type allele	Pat-tern	*eubayanus*-type allele	Pat-tern	*S.cerevisiae* S288C	Pat-tern
IV	***KIN82***	*Hae* III	430 330 170	U1	930	E1	500 430	C1
		Taq I	530 350 25 25		930		650 280	
	UGA3	*Hinf* I	260 210 165 140 80	U1	490 310 55	E1	305 295 225	C1
		Taq I	325 225 175 80 70		175 150 150 125 125 80 70		225 220 170 100 80 70	
	RPN4	*Taq* I	265 240 145 110 75	U1	325 220 180 110	E1	725 100 10	C1
	EUG1	*Hae* III	500 130 80 60 50	U1	710 110	E1	630 130 60	C1
V	***NPR2***	*Hinf* I	460 190 100 70 50 35 25	U1	460 245 190 35	E1	760 110 60	C1
	MET6	*Asp* I	680	U1	340 340	E1	500 180	C1
		Asp 700 I	440 240		680		440 240	
VI	***EPL1***	*Hae* III	435 375 320	U1	1130	E1	555 435 145	C1
	GSY1	*EcoR* I	770	U1	450 320	E1	500 270	C1
		Hae III	515 230 25		770		640 80 50	
VII	***MNT2***	*Msp* I	540 280 150	U1	540 250 150 50	E1	835 135	C1
		ScrF I	545 210 150 70		325 220 210 150 70		635 340	
	KEL2	*Hae* III	680 360 120 50	U1	360 350 330 120 50	E1	1010 120 50 20 10	C1
		Msp I	880 330		670 280 160 100		780 230 200	

Material suplementario

Chr	Gene	Rest. enzyme	*uvarum*-type allele	Pat-tern	*eubayanus*-type allele	Pat-tern	*S.cerevisiae* S288C	Pat-tern
VIII	***CBP2***	*Cfo* I	445 340	U1	610 175	E1	370 340 75	C1
		Hinf I	370 370 35		370 255 125 35		310 290 110 35	
	MNL1	Alleles differentiated by sequencing analysis						
IX	***UBP7***	*Hae* III	740 160 90	U1	545 445	E1	990	C1
		Hinf I	405 405 165 15		405 405 165 15		805 160 15 10	
	DAL1	*Hae* III	345 170 100 75 65 5	U1	470 210 80	E1	286 260 211 5	C1
X	***PEX2***	*Hae* III	230 215 125 95 45	U1	270 190 150 60 45	E1	345 260 110	C1
	CYR1	*Hind* III	395 155	U1	550	E1	405 155	C1
		Msp I	550		550		390 170	
		Sac I	550		340 210		560	
XI	***CBT1***	*Hae* III	300 180	U1	360 120	E1	480	C1
		Msp I	240 210 30		430 50		480	
	BAS1	*Hae* III	380 370 200 120	U1	690 380	E1	800 240	C1
		Msp I	930 150		1080		1080	
XII	***PPR1***	*Taq* I	260 185 140 70 55	U1	260 240 140 70	E1	230 185 165 125	C1
		Xba I	465 245		710		710	

Chr	Gene	Rest. enzyme	*uvarum*-type allele	Pat-tern	*eubayanus*-type allele	Pat-tern	*S.cerevisiae* S288C	Pat-tern
	MAG2	*Msp* I	835 175	U1	600 225 175	E1	480 400 110	C1
		Taq I	455 330 125 60 40		330 310 290 60 40		470 375 125 40	
XIII	***ORC1***	*Hae* III	530 350	U1	430 350 100	E1	670 180	C1
		Taq I	455 215 150 60		295 240 155 75 60 55		700 150	
	CAT8	*Msp* I	360 250 200	U1	360 330 120	E1	690 120	C1
	EGT2	*Hinf* I	270 110 55	U1	325 110	E1	435	C1
XIV		*ScrF* I	430 5		240 195		405 30	
	BRE5	*Hinf* I	335 300 145	U1	396 235 95 50	E1	290 230 100 65 60 40	C1
	RRI2	*Hinf* I	515 440 125 60	U1	515 235 215 165	E1	565 190 165 125 60 50	C1
XV		*Taq* I	575 490 40 35 15		420 300 240 170 20		585 380 145 45	
	ATF1	*Cfo* I	560 200 150	U1	560 350	E1	560 350	C1
		Hae III	435 275 200		435 235 200 40		635 235 40	
	GAL4	*ScrF* I	410 190 85 75	U1	760	E1	430 330	C1
XVI		*Taq* I	310 300 90 45 15		300 170 140 140		220 210 180 140	
	JIP5	*Msp* I	710	U1	580 130	E1	455 130 125	C1
		ScrF I	710		450 260		710	

Table S1.3 Alternative restriction patterns exhibited by *S. bayanus* or *S. uvarum* strains differing by one or two restriction site gains/losses (indicated in bold) from those found in the reference strains.

Gene	Restriction enzyme	Reference patterns		New derived patterns	
DAL1	*Hae* III	**470** 210 80	E1	**370** 210 **100** 80	E2
MNT2	*Msp* I	**540** 280 **150**	U1	**690** 280	U2
	ScrF I	**545** 210 **150** 70		**695** 210 70	
PEX2	*Hae* III	345 **260** 110	C1	345 **215** 110 **45**	C2
UBP7	*Hae* III	740 **160 95**	U1	740 **200 55**	U2
	Hinf I	410 410 160		410 410 160	
BRE5	*Hinf* I	335 300 **145**	U1	335 300 **95 50**	U2
	Hinf I	396 235 **95 50**	E1	396 235 **145**	E2
RRI2	*Hinf* I	515 440 125 60	U1	515 440 125 60	U2
	Taq I	**575** 490 40 35 15		490 **420 155** 40 35 15	
BAS1	*Hae* III	380 370 **200** 120	U1	380 370 **160** 120 **40**	U2
	Msp I	930 150		930 150	
	Hae III	690 380	E1	690 380	E2
	Msp I	**1080**		**930 150**	

Table S1.4 Conformation of the *S. uvarum* strains for each gene region according to the composite restriction patterns exhibited. For a description of the composite restriction patterns, see Supporting Information Tables S1.2 and S1.3. Mitochondrial *COX2* sequence haplotypes are described in Figure 1.2.

S. uvarum		MCYC623	CBS431				CECT12600; CECT12627		CECT12638; CECT12669
Chromosomes	Gene	CECT12922; CECT12930	CECT1884	CBS381	CBS2898	CECT10714	CECT12629	S20	CECT10618; CECT 1369
tDNA	*COX2*	UI	UI	UIII	UI	UIII	UIII	UIII	UI
I	*CYC3*	U1	U1	U1	U1	U1	U1	U1	U1
I	*BUD14*	U1	U1	U1	U1	U1	U1	U1	U1
III	*MRC1*	U1	U1	U1	U1	U1	U1	U1	U1
III	*KIN82*	U1	U1	U1	U1	U1	U1	U1	U1
V	*NPR2*	U1	U1	U1	U1	U1	U1	U1	U1
V	*MET6*	U1	U1	U1	U1	U1	U1	U1	U1
VII	*MNT2*	U1	U1	U1	U2	U2	U1	U1	U1
VII	*KEL2*	U1	U1	U1	U1	U1	U1	U1	U1
IX	*UBP7*	U1	U1	U1	U1	U1	U1	U1	U1
IX	*DAL1*	U1	U1	U1	U1	U1	U1	U1	U1
XI	*CBT1*	U1	U1	U1	U1	U1	U1	U1	U1
XI	*BAS1*	U1	U1	U1	U1	U1	U1	U1	U1
XII	*PPR1*	U1	U1	U1	U1	U1	U1	U1	U1
XII	*MAG2*	U1	U1	U1	U1	U1	U1	U1	U1
XIII	*ORC1*	U1	U1	U1	U1	U1	U1	U1	U1
XIII	*CAT8*	U1	U1	U1	U1	U1	U1	U1	U1
XVI	*GAL4*	U1	U1	U1	U1	U1	U1	U1	U1
XVI	*JIP5*	U1	U1	U1	U1	U1	U1	U1	U1
VIIItXV	*CBP2*	U1	U1	U1	U1	U1	U1	U1	U1
VIIItXV	*ATF1*	U1	U1	U1	U1	U1	U1	U1	U1
XVtVIII	*RRI2*	U1	U1	U1	U1	U1	U1	U1	U2
XVtVIII	*MNL1*	U1	U1	U1	U1	U1	U1	U1	U1
VItX	*EPL1*	U1	U1	U1	U1	U1	U1	U1	U1
VItX	*GSY1*	U1	U1	U1	U1	U1	U1	U1	U1
VItX	*PEX2*	U1	C2	C2	C2	C2	U1	C2	C2
XtVI	*CYR1*	U1	U1	U1	U1	U1	U1	U1	U1

S. uvarum		MCYC623	CBS431				CECT12600; CECT12627		CECT12638; CECT12669
Chromosomes	Gene	CECT12922; CECT12930	CECT1884	CBS381	CBS2898	CECT10714	CECT12629	S20	CECT10618; CECT 1369
IVtIItII	*UGA3*	U1	U1	U1	U1	U1	U1	U1	U1
IVtIItII	*RPN4*	U1	U1	U1	U1	U1	U1	U1	U1
IVtIItII	*PKC1*	U1	U1	U1	U1	U1	U1	U1	U1
IItXIV	*EGT2*	U1	U1	U1	U1	U1	U1	U1	U1
IItXIV	*OPY1*	U1	U1	U1	U1	U1	U1	U1	U1
IItXIV	*APM3*	U1	U1	U1	U1	U1	U1	U1	U1
XIVtIVtII	*EUG1*	U1	U1	U1	U1	U1	U1	U1	U1
XIVtIVtII	*BRE5*	U1	U1	U1	U1	U1	U2	U2	U2

Table S1.4. Cont.

S. uvarum		CBS2946								S04; S10
Chromosomes	Gene	CBS6308	NCAIM789	ZIM2113	ZIM2122	CBS2986	CBS395^{T}	CBS377	NCAIM868	S14
mtDNA	*COX2*	UIII	UI	UI	UI	UII	UI	UIII	UIII	UII
I	*CYC3*	U1	U1	U1	U1	U1	U1	U1	U1	U1
I	*BUD14*	U1	U1	U1	U1	U1	U1	U1	U1	U1
III	*MRC1*	U1	U1	U1	U1	U1	U1	U1	U1	U1
III	*KIN82*	U1	U1	U1	U1	U1	U1	U1	U1	U1
V	*NPR2*	U1	U1	U1	U1	U1	U1	U1	U1	U1
V	*MET6*	U1	U1	U1	U1	U1	U1	U1	U1	U1
VII	*MNT2*	U2	U1	U1	U1	U1	U2	U2	U1	U1
VII	*KEL2*	U1	U1	U1	U1	U1	U1	U1	U1	U1
IX	*UBP7*	U2	U1	U1	U1	U1	U1	U1	U1	U1
IX	*DAL1*	U1	U1	U1	U1	U1	U1	U1	U1	U1
XI	*CBT1*	U1	U1	U1	U1	U1	U1	U1	U1	U1
XI	*BAS1*	U1	U1	U2	U2	U2	U1	U1	U1 U2	U1
XII	*PPR1*	U1	U1	U1	U1	U1	U1	U1	U1	U1
XII	*MAG2*	U1	U1	U1	U1	U1	U1	U1	U1	U1
XIII	*ORC1*	U1	U1	U1	U1	U1	U1	U1	U1	U1
XIII	*CAT8*	U1	U1	U1	U1	U1	U1	U1	U1	U1

S. uvarum			CBS2946							S04; S10
Chromosomes	Gene	CBS6308	NCAIM789	ZIM2113	ZIM2122	CBS2986	CBS395^{T}	CBS377	NCAIM868	S14
XVI	*GAL4*	U1	U1	U1	U1	U1	U1	U1	U1	U1
XVI	*JIP5*	U1	U1	U1	U1	U1	U1	U1	U1	U1
VIIItXV	*CBP2*	U1	U1	U1	U1	U1	U1	U1	U1	U1
VIIItXV	*ATF1*	U1	U1	U1	U1	U1	U1	U1	U1	U1
XVtVIII	*RRI2*	U2	U2	U2	U1 U2	U2	U2	U2	U1	U2
XVtVIII	*MNL1*	U1	U1	U1	U1	U1	U1	U1	U1	C1
VItX	*EPL1*	U1	U1	U1	U1	U1	U1	U1	U1	U1
VItX	*GSY1*	U1	U1	U1	U1	U1	U1	U1	U1	U1
VItX	*PEX2*	U1	C2	C2	C2	C2	C2	U1	C2	C2
XtVI	*CYR1*	U1	U1	U1	U1	U1	U1	U1	U1	U1
IVtIItII	*UGA3*	U1	U1	U1	U1	U1	U1	U1	U1	U1
IVtIItII	*RPN4*	U1	U1	U1	U1	U1	U1	U1	U1	U1
IVtIItII	*PKC1*	U1	U1	U1	U1	U1	U1	U1	U1	U1
IItXIV	*EGT2*	U1	U1	U1	U1	U1	U1	U1	U1	U1
IItXIV	*APM3*	U1	U1	U1	U1	U1	U1	U1	U1	U1
IItXIV	*OPY1*	U1	U1	U1	U1	U1	U1	U1	U1	U1
XIVtIVtII	*EUG1*	U1	U1	U1	U1	U1	U1	U1	U1	U1
XIVtIVtII	*BRE5*	U2	U1	U1	U1	U1	U1	U1	U1	U1

Table S1.5 Conformation of the *S. bayanus* strains with *eubayanus*- and *uvarum*-type alleles according to the composite restriction patterns exhibited. For a description of the composite restriction patterns, see Supporting Information Tables S1.2 and S1.3. Mitochondrial *COX2* sequence haplotypes are described in Figure 1.2.

			Type I			Type II		
S. bayanus Chrom.	*S. uvarum* Chrom.	Gene	NBRC1948	CECT11186	CBS424 CBS3008	CECT1940^{NT}	CBS425	CBS1546
mtDNA	mtDNA	*COX2*	EI	UrE	EI	EI	EI	EI
I	I	*CYC3*	E1	U1	E1	U1 E1	U1	U1 E1
I	I	*BUD14*	E1	U1	E1	U1 E1	U1 E1	U1
III	III	*MRC1*	U1	E1	U1	U1	U1	U1
III	III	*KIN82*	U1	E1	U1	U1 E1	E1	U1
V	V	*NPR2*	U1	E1	E1	E1	E1	E1
V	V	*MET6*	E1	E1	E1	U1	U1	U1 E1
VII	VII	*MNT2*	E1	E1	E1	E1	U1	U1 E1
VII	VII	*KEL2*	E1	U1	U1	E1	U1 E1	U1 E1
IX	IX	*UBP7*	E1	E1	E1	U2	E1	E1
IX	IX	*DAL1*	E1	U1	E2	U1	E1	E1
XI	XI	*CBT1*	E1	E1	U1	E1	E1	E1
XI	XI	*BAS1*	E1	E1	E2	E1	U1 E2	U2
XII	XII	*PPR1*	E1	U1	E1	E1	U1 E1	U1 E1
XII	XII	*MAG2*	U1	U1	E1	U1	U1	U1
XIII	XIII	*ORC1*	U1	E1	U1	U1 E1	E1	U1 E1
XIII	XIII	*CAT8*	E1	U1	U1	E1	U1	U1 E1
XVI	XVI	*GAL4*	E1	E1	U1	E1	E1	E1
XVI	XVI	*JIP5*	U1	U1	E1	U1	E1	U1
VIIItXV	VIIItXV	*CBP2*	E1	U1	E1	E1	E1	U1
VIIItXV	VIIItXV	*ATF1*	E1	E1	U1	E1	U1 E1	U1 E1
XVtVIII	XVtVIII	*RRI2*	E1	U1	U2	E1	E1	U2 E1
XVtVIII	XVtVIII	*MNL1*	E1	E1	U1	E1	U1 E1	E1
VI	VItX	*EPL1*	U1	E1	E1	U1	U1 E1	U1 E1
VI	VItX	*GSY1*	U1	E1	E1	U1	U1 E1	E1

S. bayanus Chrom.	*S. uvarum* Chrom.	Gene	Type I			Type II		
			NBRC1948	CECT11186	CBS424 CBS3008	CECT1940^{NT}	CBS425	CBS1546
X	VItX	*PEX2*	U1	U1	E1	U1	E1	E1
X	XtVI	*CYR1*	E1	E1	E1	U1	U1 E1	U1 E1
IItIV	XIVtIItIV	*EUG1*	E1	U1	E1	E1	U1	U1
IItIV	IVtIItII	*PKC1*	E1	E1	E1	E1	U1 E1	E1
IVtII	IVtIItII	*RPN4*	E1	E1	E1	E1	U1 E1	U1 E1
IVtII	IVtIItII	*UGA3*	E1	U1	E1	E1	U1	U1 E1
IVtII	IItIItXIV	*APM3*	E1	E1	U1	U1 E1	U1	U1 E1
IVtII	IItIItXIV	*OPY1*	E1	U1	E1	E1	U1 E1	U1 E1
XIV	IItIItXIV	*EGT2*	E1	E1	E1	E1	E1	E1
XIV	XIVtIItIV	*BRE5*	E1	U1	U2	E1	U1	U2

Table S1.5. Cont.

S. bayanus Chrom.	*S. uvarum* Chrom.	Gene	Type II						
			CBS375	CBS378	CBS380^{T}	CECT1941	CECT1991	NCAIM676	NCAIM677
mtDNA	mtDNA	*COX2*	UrE	UrE	UI	UI	UIII	UIII	UIII
I	I	*CYC3*	U1	U1	U1 E1	U1 E1	U1 E1	U1	U1
I	I	*BUD14*	U1 E1	U1 E1	U1	U1 E1	U1 E1	U1 E1	U1 E1
III	III	*MRC1*	E1	E1	U1	U1	U1	U1	U1
III	III	*KIN82*	E1	E1	U1 E1	U1 E1	U1	E1	U1 E1
V	V	*NPR2*	E1	E1	E1	E1	U1 E1	U1	U1
V	V	*MET6*	E1	E1	E1	U1 E1	U1 E1	U1	U1
VII	VII	*MNT2*	E1	E1	E1	E1	U2 E1	U2	U1 U2
VII	VII	*KEL2*	U1	U1	U1	U1	U1 E1	U1 E1	U1 E1
IX	IX	*UBP7*	U1 E1	U1	U1	U1	U1	U1	U1
IX	IX	*DAL1*	U1 E1	U1	U1	U1	U1 E1	U1	U1
XI	XI	*CBT1*	E1	E1	U1	U1	E1	U1	U1
XI	XI	*BAS1*	E1	E1	E1	E1	U1 E2	U2	U2

S. bayanus Chrom.	*S. uvarum* Chrom.	Gene	Type II						
			CBS375	CBS378	CBS380^T	CECT1941	CECT1991	NCAIM676	NCAIM677
XII	XII	*PPR1*	U1	U1 E1	U1	U1	U1	U1	U1
XII	XII	*MAG2*	U1	U1	U1	U1	U1	U1	U1
XIII	XIII	*ORC1*	U1 E1	U1 E1	U1	U1	U1	U1	U1
XIII	XIII	*CAT8*	U1	U1	U1	U1	U1	U1	U1
XVI	XVI	*GAL4*	E1	E1	E1	E1	E1	E1	E1
XVI	XVI	*JIP5*	U1 E1	U1	U1	U1	U1 E1	U1	U1
VIIItXV	VIIItXV	*CBP2*	U1 E1	U1 E1	U1	U1	U1	U1	U1
VIIItXV	VIIItXV	*ATF1*	E1	E1	E1	E1	E1	U1	U1
XVtVIII	XVtVIII	*RRI2*	U1 E1	U1	U1	U1	U2	U1 U2	U1 U2
XVtVIII	XVtVIII	*MNL1*	E1	E1	U1	U1	U1	E1	E1
VI	VItX	*EPL1*	U1 E1	U1 E1	U1 E1	U1 E1	U1 E1	E1	U1 E1
VI	VItX	*GSY1*	U1 E1	U1 E1	U1 E1	U1 E1	U1	U1 E1	U1 E1
X	VItX	*PEX2*	U1	U1	U1	U1	E1	E1	E1
X	XtVI	*CYR1*	U1	U1 E1	U1	U1 E1	U1 E1	U1	U1
IItIV	XIVtIItIV	*EUG1*	U1	U1	U1	U1	E1	E1	E1
IItIV	IVtIItII	*PKC1*	E1	E1	E1	E1	U1 E1	U1	U1
IVtII	IVtIItII	*RPN4*	U1 E1	U1 E1	U1 E1	U1 E1	U1 E1	U1	U1
IVtII	IVtIItII	*UGA3*	U1	U1	U1 E1	U1 E1	U1 E1	U1	U1
IVtII	IItIItXIV	*APM3*	E1	E1	U1	U1	U1	U1	U1
IVtII	IItIItXIV	*OPY1*	U1	U1	U1 E1	U1 E1	U1	U1	U1
XIV	IItIItXIV	*EGT2*	U1 E1	U1	U1	U1	E1	U1	U1
XIV	XIVtIItIV	*BRE5*	U1	U1	U1	U1	U1	U2	U2

Table S1.6 Conformation of the *S. pastorianus* strains with *eubayanus*- *cerevisiae*- or *uvarum*-type alleles according to the composite restriction patterns exhibited. For a description of the composite restriction patterns, see Supporting Information Tables S1.2 and S1.3. Mitochondrial *COX2* sequence haplotypes are described in Fig 1.2, except S6U *COX2*, which is similar to *S. cerevisiae* (C) *COX2*.

S. bayanus Chrom.	*S. uvarum* Chrom.	*S. cerevisiae* Chrom.	Gene	W 34/70	S6U	CECT 1885	CBS1503	CECT 11000	CBS1513
mtDNA	mtDNA	mtDNA	*COX2*	EI	C	EI	EI	EI	EI
I	I	I	*CYC3*	E1 C1	U1 C1	E1 C1	E1 C1	C1	E1 C1
I	I	I	*BUD14*	E1 C1	U1 C1	E1 C1	C1	C1	C1
III	III	III	*MRC1*	E1 C1	U1 C1	E1 C1	E1	U1 C1	C1
III	III	III	*KIN82*	C1	U1 C1	E1	E1	C1	C1
V	V	V	*NPR2*	E1 C1	U1 C1	E1 C1	E1	U1 C1	E1 C1
V	V	V	*MET6*	E1 C1	U1 C1	E1	E1	E1 C1	E1 C1
VII	VII	VII	*MNT2*	E1 C1	U1 C1	C1	C1	E1 C1	E1 C1
VII	VII	VII	*KEL2*	E1 C1	U1 C1	E1	E1	U1 C1	E1 C1
IX	IX	IX	*UBP7*	E1 C1	U1 C1	E1 C1	E1 C1	E1 C1	E1 C1
IX	IX	IX	*DAL1*	E1 C1	U1 C1	E1 C1	E1 C1	E1 C1	E1 C1
XI	XI	XI	*CBT1*	E1 C1	U1 C1	E1 C1	E1 C1	E1 C1	E1 C1
XI	XI	XI	*BAS1*	E1 C1	U1 C1	E2	E2	E2 C1	E2
XII	XII	XII	*PPR1*	E1 C1	U1 C1	E1	E1	E1 C1	E1
XII	XII	XII	*MAG2*	E1 C1	U1 C1	E1	E1	E1 C1	E1
XIII	XIII	XIII	*ORC1*	E1 C1	U1 C1	E1 C1	E1	E1 C1	E1 C1
XIII	XIII	XIII	*CAT8*	E1 C1	U1 C1	E1 C1	E1 C1	E1 C1	E1 C1
XVI	XVI	XVI	*GAL4*	C1	U1 C1	E1 C1	C1	C1	C1
XVI	XVI	XVI	*JIP5*	E1 C1	U1 C1	E1 C1	E1	E1	E1
VIIItXV	VIIItXV	VIII	*CBP2*	E1 C1	U1 C1	E1 C1	E1 C1	E1 C1	E1 C1
VIIItXV	VIIItXV	XV	*ATF1*	E1 C1	U1 C1	E1 C1	E1 C1	E1 C1	E1 C1
XVtVIII	XVtVIII	XV	*RRI2*	E1 C1	U1 C1	E1 C1	E1 C1	E1 C1	E1 C1
XVtVIII	XVtVIII	VIII	*MNL1*	E1 C1	U1 C1	E1 C1	C1	E1 C1	E1 C1
VI	VItX	VI	*EPL1*	E1 C1	U1 C1	E1	E1	C1	E1
VI	VItX	VI	*GSY1*	E1 C1	U1 C1	E1	E1	U1 C1	E1
X	VItX	X	*PEX2*	E1 C1	C2 C1	E1 C1	E1 C1	E1 C1	E1 C1
X	XtVI	X	*CYR1*	E1 C1	U1 C1	E1 C1	E1 C1	E1 C1	E1 C1

S. bayanus Chrom.	*S. uvarum* Chrom.	*S. cerevisiae* Chrom.	Gene	W 34/70	S6U	CECT 1885	CBS1503	CECT 11000	CBS1513
IItIV	XIVtIItIV	IV	*EUG1*	E1 C1	U1 C1	E1	E1	E1	E1
IItIV	IVtIItII	II	*PKC1*	E1 C1	U1 C1	E1 C1	E1 C1	E1 C1	E1 C1
IVtII	IVtIItII	IV	*RPN4*	E1 C1	U1 C1	E1 C1	E1 C1	E1 C1	E1 C1
IVtII	IVtIItII	IV	*UGA3*	E1 C1	U1 C1	E1 C1	E1 C1	E1 C1	E1 C1
IVtII	IItIItXIV	II	*APM3*	E1 C1	U1 C1	E1	E1 C1	E1 C1	E1 C1
IVtII	IItIItXIV	II	*OPY1*	E1 C1	U1 C1	E1 C1	E1 C1	E1 C1	E1 C1
XIV	IItIItXIV	XIV	*EGT2*	E1 C1	U1 C1	E1	E1	U1 C1	E1 C1
XIV	XIVtIItIV	XIV	*BRE5*	E1 C1	U1 C1	E2 C1	E2	E2 C1	E2 C1

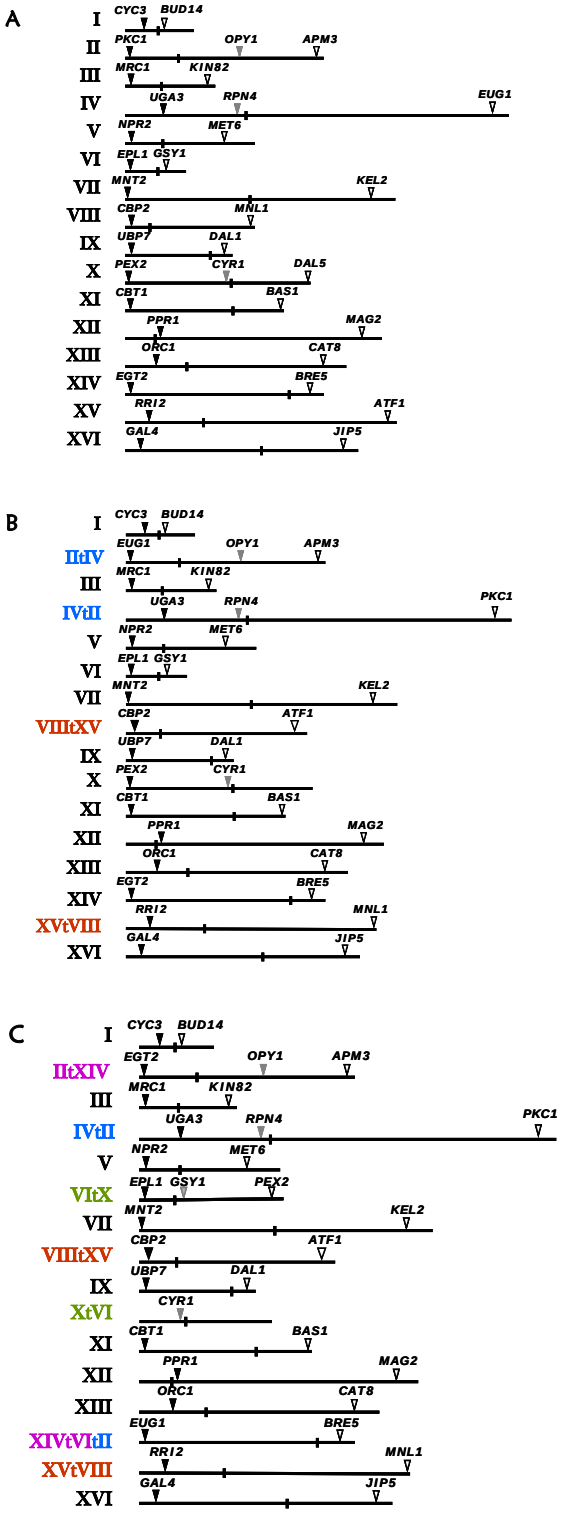


Fig S1.1. Relative position of the used genes in the different chromosomes. A) *S. cerevisiae*; **B)** *S. bayanus*; **C)** *S. uvarum.* Translocations are marked in colors.

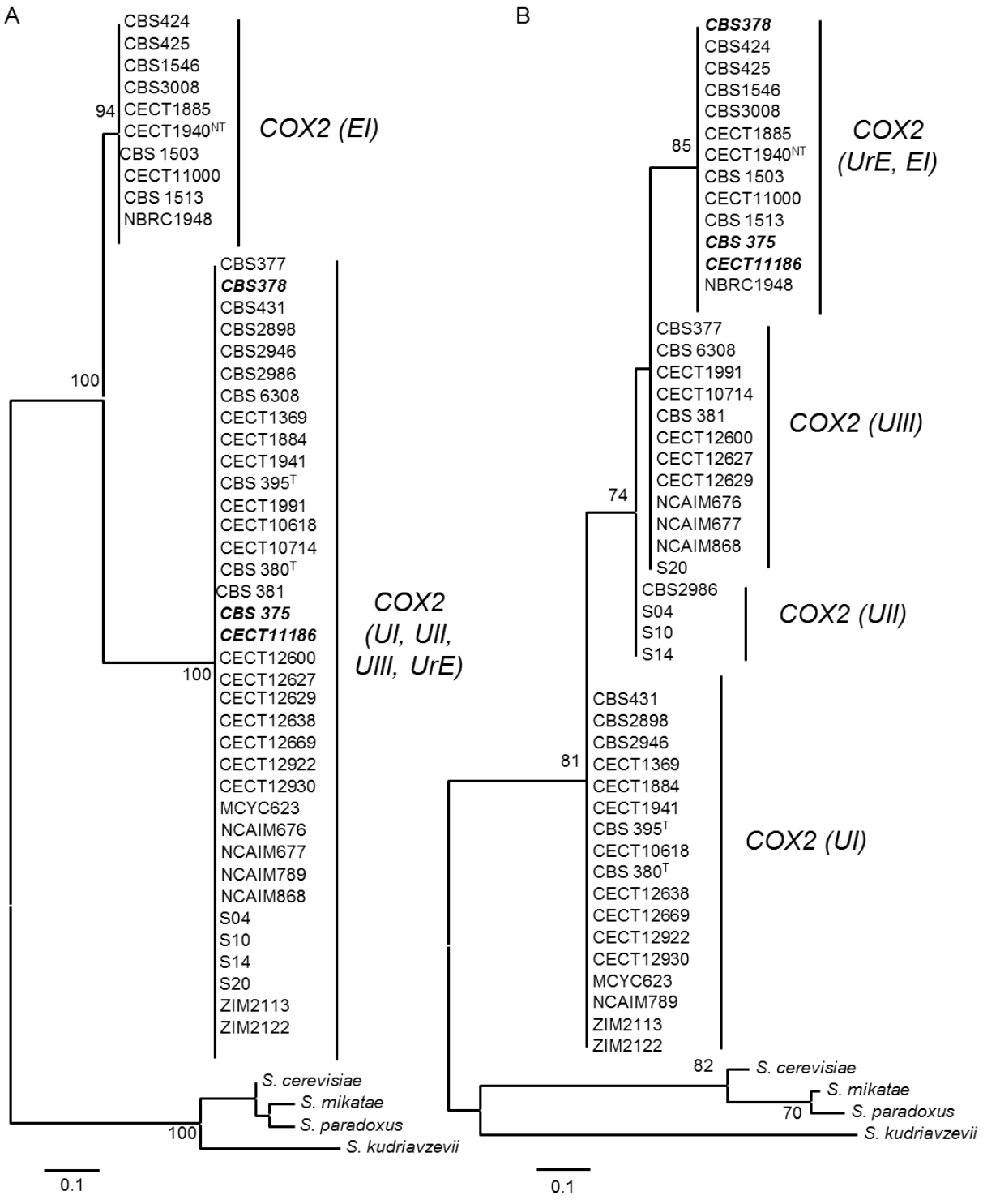


Figure S1.2 Phylogenetic analysis of the 5′ and 3′ regions of the mitochondrial *COX2* gene.

A- 5′ region. **B**- 3′ region.


NBRC1948
R (1 double)
U VItX EPL • GSY PEX
U VItX EPL • GSY PEX
U XtVI • CYR
U XtVI • CYR
CBS 424
E VI EPL • GSY
E VI EPL • GSY
E X • CYR PEX
E X • CYR PEX
CECT 1941
T and R (1 simple
E VI EPL • GSY
U VItX EPL • GSY PEX
E X • CYR PEX
U XtVI • CYR
CBS 3008
E VI EPL • GSY
E VI EPL • GSY
E X • CYR PEX
E X • CYR PEX
CBS 378
T and R (1 simple
E VI EPL • GSY
U VItX EPL • GSY PEX
E X • CYR PEX
U XtVI • CYR
CECT 1991
T and R (2 simple)
E VI EPL • GSY
U VItX EPL • GSY PEX
E X • CYR PEX
U XtVI • CYR
NCAIM 676
T and R (3 simple
E VI EPL • GSY
U VItX EPL • GSY PEX
E X • CYR PEX
U XtVI • CYR
CBS 375
T and R (1 simple-long fragment-)
E VI EPL • GSY
U VItX EPL • GSY PEX
E X • CYR PEX
U XtVI • CYR
NCAIM 677
T and R (2 simple
E VI EPL • GSY
U VItX EPL • GSY PEX
E X • CYR PEX
U XtVI • CYR
CBS 1546
T and R (2 simple)
E VI EPL • GSY
U VItX EPL • GSY PEX
E X • CYR PEX
U XtVI • CYR

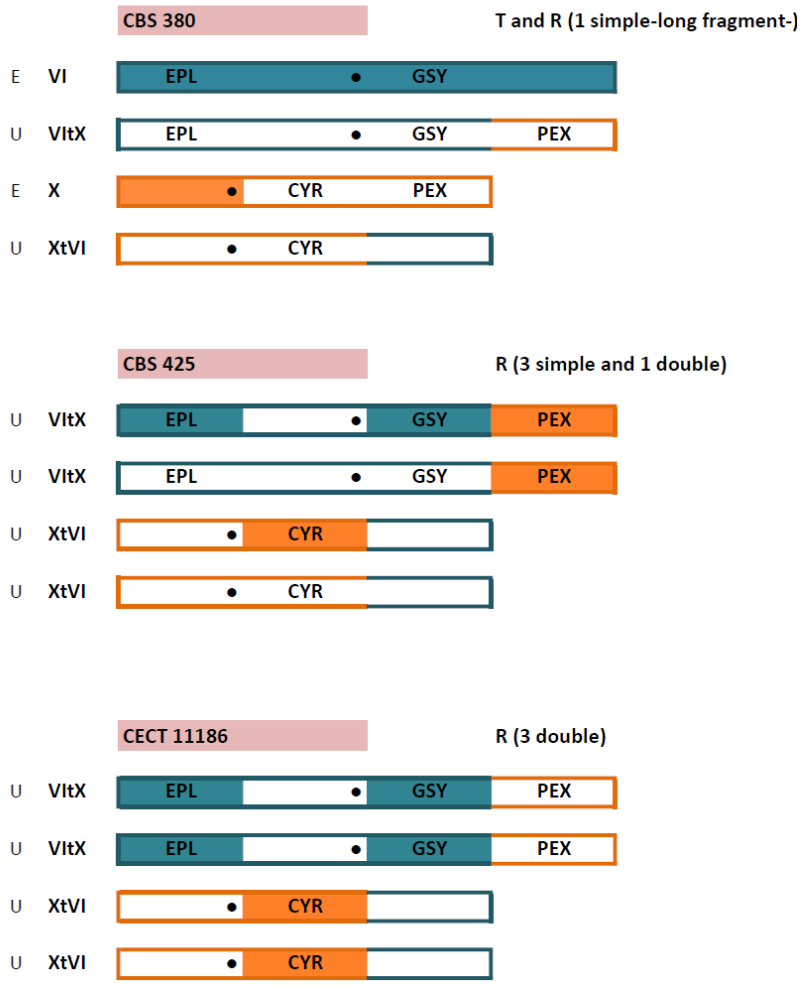


Figure S1.4. Chromosomal composition of S. bayanus strains for translocation 1. The most probable chromosomic composition is is indicated. Unfilled: S. uvarum genes; filled: S. eubayanus genes.Dot: centromera; if unfilled, S. uvarum centromera, if filled, S. eubayanus one. Blue: chromosome VI. Orange: chromosome X. Chromosome and procedence is indicated on the left of each chromosome. In each case, recombinations (R) or translocations (T) presents are indicated, as well as the number and the kind. The complete name of the genes is: *EPL1*, *GSY1*, *PEX2* and *CYR1*.

Table S2.1. List of some relevant publications reporting classic hybridization methods in *Saccharomyces*.

PARENTAL SPECIES	METHOD OF GENERATION	RESEARCH FIELD	REFERENCE
C. albicans	Protoplast fusion	Basic studies	Law *et al.(1993)*
S. cerevisiae	Protoplast fusion	Basic studies	Kucsera *et al.(1998)*
S. cerevisiae	Protoplast fusion	Basic studies	Nakazawa and Iwano(2004)
Candida boidinii and *C. tropicalis*	Protoplast fusion	Fermentations	Kobori *et al*.(1991)
S. cerevisiae and *K. lactis*	Protoplast fusion	Fermentations	Gera *et al.*(1997)
S. cerevisiae and *K. lactis*	Protoplast fusion	Fermentations	Taya *et al.*(1984)
S. cerevisiae and *Pachysolen tannophilus*	Protoplast fusion	Fermentations	Heluane *et al.*(1993)
S. diastaticus and *S. rouxii*	Protoplast fusion	Bakery	Spencer *et al.(1985)*
S. cerevisiae and *Saccharomycopsis fibuligera*	Protoplast fusion	Fermentations	Choi *et al.(1997)*
S. cerevisiae and *Torulaspora delbrueckii*	Protoplast fusion	Fermentations	Lucca *et al.(1999)*
S. cerevisiae and *Torulaspora delbrueckii*	Protoplast fusion	Wine	Santos *et al(2008)*
S. cerevisiae and *Zygosaccharomyces fermentati*	Protoplast fusion	Fermentations	Pina *et al(1986)*
Pichia stipitis and *Fusarium moniliforme*	Protoplast fusion	Fermentations	Vazquez *et al.(1997)*
Pichia stipitis and *Trichoderma reesi*	Protoplast fusion	Fermentations	Vazquez *et al.(1997)*
S. cerevisiae	Rare-mating	Bakery	Oda *et al.(1991)*
S. cerevisiae S. kudriavzevii and *S. paradoxus*	Rare-mating	Wine	Bellón et al. (2011)

PARENTAL SPECIES	METHOD OF GENERATION	RESEARCH FIELD	REFERENCE
S. cerevisiae and *S. paradoxus*	Rare-mating	Basic studies	de Barros Lopes *et al.(2002)*
S. cerevisiae and *S. diastaticus*	Rare-mating	Basic studies	Schillberg *et al.(1991)*
S. cerevisiae and *S. bayanus*	Spore to spore	Basic studies applied to wine	Sebastiani *et al.(2002)*
S. cerevisiae and *S. bayanus*	Spore to spore	Wine	Rainieri *et al.(1998)*
S. cerevisiae and *S. bayanus*	Spore to spore	Wine	Caridi *et al.(2002)*
S. cerevisiae and *S. bayanus*	Spore to spore	Wine	Kishimoto (1994)
S. cerevisiae and *S. bayanus*	Spore to spore	Wine	Zambonelli *et al.(1997)*
S. cerevisiae and *S. uvarum*	Spore to spore	Wine	Coloretti *et al.(2006)*
S. cerevisiae and *S. bayanus*	Spore to spore	Brewing	Sato *et al.*(2002)
S. cerevisiae and *S. bayanus*	Spore to spore	Basic studies	Giudici *et al.(1998)*
S. cerevisiae, *S. uvarum* and *S. douglasii*	Spore to spore	Basic studies	Hawthorne and Philippsen(1994)
S. cerevisiae	Mass mating	Wine	Ramírez *et al*.(1998)
S. cerevisiae	Mass mating	Bakery	Nakazawa *et al.*(1999)
S. cerevisiae	Mass mating	Bakery	Higgins *et al*.(2001)
S. cerevisiae and *S. uvarum*	Mass mating	Basic studies	Antunovics *et al*.(2005)
S. cerevisiae, *S. bayanus*, *S. exiguus* and *S. castelli*	Mass mating	Basic studies	Marinoni *et al.(1999)*

Table S4.1. Primers used for qRT-PCR analysis.

Gene		Primer
HXK1	Forward	TCCAATGATTCCCGGTTGGG
	Reverse	ACCGCTCAACTTGACCAACA
YPS7	Forward	GACTTTCTGAGCCCAGCCTT
	Reverse	TCCACATAAGTGGCCGCAAT
MNN10	Forward	GCCTATGCGAAGAGACATGGA
	Reverse	GGAAACTCCCTGAACGTCTG

Table S4.2. Genes upper represented in the parental strains Sc1 and Sc2 when a comparison between them is made.

Strain	Gene	Function	Process
Sc1	*AGP3*	amino acid transporter activity	amino acid transport
	ARN1	siderochrome-iron transporter activity	iron-siderochrome transport
	ATG5	unknown	protein-vacuolar targeting; autophagy
	COS1	unknown	unknown
	COS12	unknown	unknown
	CUP1-1	copper ion binding	response to copper ion
	CUP1-2	copper ion binding	response to copper ion
	DAK2	glycerone kinase activity	glycerol catabolism; response to stress
	DDI2	unknown	unknown
	EBP2	unknown	rRNA processing
	HXT15	mannose transporter activity; fructose transporter activity; glucose transporter activity	hexose transport
	HXT16	mannose transporter activity; fructose transporter activity; glucose transporter activity	hexose transport
	IMA1	hydrolase activity, hydrolyzing O-glycosyl compounds	unknown
	MAL11	alpha-glucoside:hydrogen symporter activity; maltose:hydrogen symporter activity; trehalose transporter activity	alpha-glucoside transport; trehalose transport
	MOB2	protein kinase activator activity	establishment and/or maintenance of cell polarity (sensu Saccharomyces); regulation of exit from mitosis; protein amino acid phosphorylation
	MPH2	carbohydrate transporter activity; maltose porter activity	carbohydrate transport
	MPH3	carbohydrate transporter activity; maltose porter activity	carbohydrate transport
	MRK1	glycogen synthase kinase 3 activity	proteolysis and peptidolysis; protein amino acid phosphorylation; response to stress
	NAB2	poly(A) binding	poly(A)+ mRNA-nucleus export; mRNA polyadenylation
	NUF2	structural constituent of cytoskeleton	microtubule nucleation; chromosome segregation
	RMD6	unknown	unknown
	SEO1	transporter activity	transport
	SNZ3	protein binding	pyridoxine metabolism; thiamin biosynthesis
	SOR1	L-iditol 2-dehydrogenase activity	mannose metabolism; fructose metabolism
	SOR2	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	hexose metabolism
	SPR1	glucan 1,3-beta-glucosidase activity	sporulation (sensu Saccharomyces)
	SWP1	dolichyl-diphosphooligosaccharide-protein glycotransferase activity	N-linked glycosylation
	TFC4	RNA polymerase III transcription factor activity	transcription initiation from Pol III promoter
	YRB2	structural molecule activity	protein-nucleus export; nuclear pore organization and biogenesis; ribosomal protein-nucleus import; mRNA-binding (hnRNP) protein-nucleus import; snRNP protein-nucleus import; NLS-bearing substrate-nucleus import; tRNA-nucleus export; snRNA-nucleus export;
	12 unknown		
Sc2	BSC3	unknown	unknown
	BST1	unknown	vesicle organization and biogenesis; ER-associated protein catabolism
	BUD23	S-adenosylmethionine-dependent methyltransferase activity	bud site selection
	CDC46	chromatin binding; ATP dependent DNA helicase activity	pre-replicative complex formation and maintenance; DNA replication initiation; DNA unwinding; establishment of chromatin silencing
	CDC7	protein serine/threonine kinase activity	protein amino acid phosphorylation; regulation of DNA replication; DNA replication initiation
	CUE4	unknown	unknown
	ECM23	unknown	cell wall organization and biogenesis; pseudohyphal growth
	EPL1	histone acetyltransferase activity	regulation of transcription from Pol II promoter; histone acetylation
	ERG3	C-5 sterol desaturase activity	ergosterol biosynthesis
	FET5	ferroxidase activity	iron ion transport
	FLO1	cell adhesion molecule activity	flocculation

Strain	Gene	Function	Process
Sc2	FMP27	unknown	unknown
	FSP2	alpha-glucosidase activity	
	GLY1	threonine aldolase activity	glycine biosynthesis; threonine catabolism
	GPM1	phosphoglycerate mutase activity	glycolysis; gluconeogenesis
	GPX1	glutathione peroxidase activity	response to oxidative stress
	HEK2	mRNA binding	telomerase-dependent telomere maintenance; mRNA localization, intracellular
	HXK1	hexokinase activity	fructose metabolism
	HXT11	galactose transporter activity; mannose transporter activity; fructose transporter activity; glucose transporter activity	hexose transport
	HXT12		
	HXT9		
	INP51	inositol-polyphosphate 5-phosphatase activity	dephosphorylation; endocytosis; cell wall organization and biogenesis; phosphatidylinositol biosynthesis
	LAG1	protein transporter activity	replicative cell aging; ceramide biosynthesis
	LPX1	lipase activity	peroxisome organization and biogenesis
	OSH2	oxysterol binding	steroid biosynthesis
	OSH7		
	PET122	translation regulator activity	protein biosynthesis
	PGU1	polygalacturonase activity	pectin catabolism; pseudohyphal growth
	PSA1	mannose-1-phosphate guanylyltransferase activity	GDP-mannose biosynthesis; protein amino acid glycosylation; cell wall mannoprotein biosynthesis
	REE1	unknown	unknown
	RVS167	cytoskeletal protein binding	polar budding; response to osmotic stress; endocytosis
	SKG6	unknown	unknown
	SPF1	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	calcium ion homeostasis; protein amino acid glycosylation
	STE4	heterotrimeric G-protein GTPase activity	signal transduction during conjugation with cellular fusion
	SUL1	sulfate transporter activity	sulfate transport
	TCM62	chaperone activity	protein complex assembly
	TGF2	general RNA polymerase II transcription factor activity	transcription initiation from Pol II promoter
	VPS8	unknown	late endosome to vacuole transport
	YAT1	carnitine O-acetyltransferase activity	alcohol metabolism; carnitine metabolism
	YRF1-4	DNA helicase activity	telomerase-independent telomere maintenance
	YRF1-6		
	YRF1-7		
	ZTA1	unknown	unknown
	60 unknown		

Table S4.3: Significantly overrepresented biological processes, functions and components obtained from Gene Ontology analysis for the three analyzed strains (Using Term Finder, p:0.05).

R2-IVo vs Sc1	**overrepresented genes in R2-IVo**	**Process**	GO:0032197	transposition, RNA-mediated	0.0000
			GO:0032196	transposition	0.0001
			GO:0008643	carbohydrate transport	0.0421
		Function	GO:0004574	oligo-1,6-glucosidase activity	0.0293
			GO:0005354	galactose transmembrane transporter activity	0.0438
		Component	GO:0000943	retrotransposon nucleocapsid	0.0001
	overrepresented genes in Sc1	**Process**	GO:0000023	maltose metabolic process	0.0000
			GO:0005984	disaccharide metabolic process	0.0004
			GO:0008643	carbohydrate transport	0.0006
			GO:0008614	pyridoxine metabolic process	0.0010
			GO:0008615	pyridoxine biosynthetic process	0.0010
			GO:0009311	oligosaccharide metabolic process	0.0024
			GO:0010273	detoxification of copper ion	0.0039
			GO:0071585	detoxification of cadmium ion	0.0039
			GO:0042816	vitamin B6 metabolic process	0.0042
			GO:0042819	vitamin B6 biosynthetic process	0.0042
			GO:0055085	transmembrane transport	0.0078
			GO:0009228	thiamine biosynthetic process	0.0128
			GO:0006772	thiamine metabolic process	0.0154
			GO:0042724	thiamine-containing compound biosynthetic process	0.0154
			GO:0042723	thiamine-containing compound metabolic process	0.0213
			GO:0046688	response to copper ion	0.0393
			GO:0044262	cellular carbohydrate metabolic process	0.0399
			GO:0071702	organic substance transport	0.0432
		Function	GO:0051119	sugar transmembrane transporter activity	0.0000
			GO:0015144	carbohydrate transmembrane transporter activity	0.0000
			GO:0005363	maltose transmembrane transporter activity	0.0000
			GO:0015154	disaccharide transmembrane transporter activity	0.0000
			GO:0015151	alpha-glucoside transmembrane transporter activity	0.0002
			GO:0042947	glucoside transmembrane transporter activity	0.0002
			GO:0003939	L-iditol 2-dehydrogenase activity	0.0014
			GO:0046870	cadmium ion binding	0.0014
			GO:0022891	substrate-specific transmembrane transporter activity	0.0085
			GO:0046914	transition metal ion binding	0.0113
			GO:0005215	transporter activity	0.0173
			GO:0022857	transmembrane transporter activity	0.0201
			GO:0016209	antioxidant activity	0.0295
			GO:0004784	superoxide dismutase activity	0.0299
			GO:0016721	oxidoreductase activity, acting on superoxide radicals as acceptor	0.0299
			GO:0022892	substrate-specific transporter activity	0.0332
			GO:0043167	ion binding	0.0439
			GO:0043169	cation binding	0.0439
		Component	No significant terms were found		

R2-IVo vs Sc2	**overrepresented genes in R2-IVo**	**Process**	GO:0010273	detoxification of copper ion	0.0249
			GO:0071585	detoxification of cadmium ion	0.02495
			GO:0006814	sodium ion transport	0.0343
		Function	GO:0008554	sodium-exporting ATPase activity, phosphorylative mechanism	0.0000
			GO:0015081	sodium ion transmembrane transporter activity	0.0050
			GO:0003850	2-deoxyglucose-6-phosphatase activity	0.0070
			GO:0008556	potassium-transporting ATPase activity	0.0070
			GO:0046870	cadmium ion binding	0.0070
			GO:0015662	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	0.0458
		Component	No significant terms were found		
	overrepresented genes in Sc2	**Process**	GO:0032197	transposition, RNA-mediated	0.0000
			GO:0032196	transposition	0.0000
			GO:0016032	viral reproduction	0.0024
			GO:0019058	viral infectious cycle	0.0024
			GO:0019067	viral assembly, maturation, egress, and release	0.0024
			GO:0019068	virion assembly	0.0024
			GO:0019069	viral capsid assembly	0.0024
			GO:0021700	developmental maturation	0.0024
			GO:0022415	viral reproductive process	0.0024
			GO:0046797	viral procapsid maturation	0.0024
			GO:0015074	DNA integration	0.0024
			GO:0000722	telomere maintenance via recombination	0.0180
			GO:0000723	telomere maintenance	0.0386
			GO:0060249	anatomical structure homeostasis	0.0386
			GO:0032200	telomere organization	0.0420
		Function	GO:0004386	helicase activity	0.0000
			GO:0008026	ATP-dependent helicase activity	0.0002
			GO:0070035	purine NTP-dependent helicase activity	0.0002
			GO:0017111	nucleoside-triphosphatase activity	0.0004
			GO:0016462	pyrophosphatase activity	0.0008
			GO:0016817	hydrolase activity, acting on acid anhydrides	0.0008
			GO:0016818	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	0.0008
			GO:0004523	ribonuclease H activity	0.0012
			GO:0005524	ATP binding	0.0012
			GO:0032559	adenyl ribonucleotide binding	0.0013
			GO:0030554	adenyl nucleotide binding	0.0013
			GO:0003964	RNA-directed DNA polymerase activity	0.0032
			GO:0004190	aspartic-type endopeptidase activity	0.0032
			GO:0070001	aspartic-type peptidase activity	0.0032
			GO:0003678	DNA helicase activity	0.0043
			GO:0016787	hydrolase activity	0.0056
			GO:0016891	endoribonuclease activity, producing 5'-phosphomonoesters	0.0087
			GO:0003887	DNA-directed DNA polymerase activity	0.0095
			GO:0035639	purine ribonucleoside triphosphate binding	0.0104
			GO:0032553	ribonucleotide binding	0.0108
			GO:0032555	purine ribonucleotide binding	0.0108
			GO:0017076	purine nucleotide binding	0.0117
			GO:0042623	ATPase activity, coupled	0.0140
			GO:0034061	DNA polymerase activity	0.0146
			GO:0016893	endonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5'-phosphomonoesters	0.0186
			GO:0004521	endoribonuclease activity	0.0233
			GO:0003676	nucleic acid binding	0.0355
			GO:0016887	ATPase activity	0.0372
		Component	GO:0000943	retrotransposon nucleocapsid	0.0000

Sc1 vs Sc2	**overrepresented genes in Sc1**	**Process**	GO:0000023	maltose metabolic process	0.0000
			GO:0005984	disaccharide metabolic process	0.0008
			GO:0008643	carbohydrate transport	0.0012
			GO:0009311	oligosaccharide metabolic process	0.0045
			GO:0010273	detoxification of copper ion	0.0051
			GO:0071585	detoxification of cadmium ion	0.0051
			GO:0005975	carbohydrate metabolic process	0.0064
			GO:0044262	cellular carbohydrate metabolic process	0.0176
		Function	GO:0051119	sugar transmembrane transporter activity	0.0000
			GO:0015144	carbohydrate transmembrane transporter activity	0.0000
			GO:0005363	maltose transmembrane transporter activity	0.0001
			GO:0015154	disaccharide transmembrane transporter activity	0.0001
			GO:0015151	alpha-glucoside transmembrane transporter activity	0.0003
			GO:0042947	glucoside transmembrane transporter activity	0.0003
			GO:0003939	L-iditol 2-dehydrogenase activity	0.0020
			GO:0046870	cadmium ion binding	0.0020
			GO:0022891	substrate-specific transmembrane transporter activity	0.0225
			GO:0004784	superoxide dismutase activity	0.0413
			GO:0016721	oxidoreductase activity, acting on superoxide radicals as acceptor	0.0413
			GO:0005215	transporter activity	0.0494
		Component	No significant terms were found		
	overrepresented genes in Sc2	**Process**	GO:0032197	transposition, RNA-mediated	0.0000
			GO:0032196	transposition	0.0000
			GO:0016032	viral reproduction	0.0085
			GO:0019058	viral infectious cycle	0.0085
			GO:0019067	viral assembly, maturation, egress, and release	0.0085
			GO:0019068	virion assembly	0.0085
			GO:0019069	viral capsid assembly	0.0085
			GO:0021700	developmental maturation	0.0085
			GO:0022415	viral reproductive process	0.0085
			GO:0046797	viral procapsid maturation	0.0085
			GO:0015074	DNA integration	0.0148
		Function	GO:0004386	helicase activity	0.0000
			GO:0004190	aspartic-type endopeptidase activity	0.0009
			GO:0070001	aspartic-type peptidase activity	0.0009
			GO:0004523	ribonuclease H activity	0.0038
			GO:0008026	ATP-dependent helicase activity	0.0097
			GO:0070035	purine NTP-dependent helicase activity	0.0097
			GO:0003964	RNA-directed DNA polymerase activity	0.0100
			GO:0005524	ATP binding	0.0250
			GO:0032559	adenyl ribonucleotide binding	0.0261
			GO:0016891	endoribonuclease activity, producing 5'-phosphomonoesters	0.0268
			GO:0030554	adenyl nucleotide binding	0.0278
			GO:0003887	DNA-directed DNA polymerase activity	0.0293
			GO:0034061	DNA polymerase activity	0.0444
		Component	GO:0000943	retrotransposon nucleocapsid	0.0000

Table S4.4. Genes overrepresented in the hybrid in CGH analysis.

Comparison	Gene	Function	Process
R2 vs Sc1	*BSC3*	unknown	unknown
	FSP2	alpha-glucosidase activity	
	HXT9	galactose transporter activity; mannose transporter activity; fructose transporter activity; glucose transporter activity	hexose transport
	HXT11		
	HXT12	unknown	
	IMA3	oligo-1,6-glucosidase activity	disaccharide catabolic process
	MDJ1	co-chaperone activity	proteolysis and peptidolysis; protein folding
	NUD1	structural constituent of cytoskeleton	microtubule nucleation
	PDR12	organic acid transporter activity; xenobiotic-transporting ATPase activity	organic acid transport; propionate metabolism; transport
	PTA1	cleavage/polyadenylation specificity factor activity	tRNA processing; mRNA polyadenylation; mRNA cleavage; transcription termination from Pol II promoter, poly(A) independent; transcription termination from Pol II promoter, poly(A) coupled
	REE1	unknown	unknown
	14 unknown		
R2 vs Sc2	*AAD4*	aryl-alcohol dehydrogenase activity	aldehyde metabolism
	AAD15		
	ARO7	chorismate mutase activity	aromatic amino acid family biosynthesis
	CIS1	unknown	regulation of CDK activity
	CUP1-1	copper ion binding	response to copper ion
	CUP1-2		
	DEG1	pseudouridylate synthase activity	RNA processing
	DIN7	nuclease activity	DNA repair
	DLD3	D-lactate dehydrogenase (cytochrome) activity	lactate metabolism
	DOG1	2-deoxyglucose-6-phosphatase activity	glucose metabolism
	DOG2	2-deoxyglucose-6-phosphatase activity	response to stress; glucose metabolism
	DSF1	unknown	unknown
	DUR1	allophanate hydrolase activity; urea carboxylase activity	urea metabolism; allantoin catabolism
	DUR2		
	ECM29	unknown	cell wall organization and biogenesis
	EKI1	choline kinase activity; ethanolamine kinase activity	phosphatidylethanolamine biosynthesis
	ENA1	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	sodium ion transport
	ENA2		
	ENA5		
	ENB1	ferric-enterobactin transporter activity	ferric-enterobactin transport
	FSH3	unknown	unknown
	GCN20	unknown	regulation of translational elongation
	GLE1	unknown	poly(A)+ mRNA-nucleus export
	GSG1	unknown	ER to Golgi transport; meiosis
	HDA3	histone deacetylase activity	regulation of transcription, DNA-dependent; histone deacetylation
	HNM1	choline transporter activity	choline transport
	HRQ1	ATP-dependent 3'-5' DNA helicase activity	DNA duplex unwinding; DNA strand renaturation
	HXT13	mannose transporter activity; fructose transporter activity; glucose transporter activity	hexose transport
	HXT15		
	LAC1	protein transporter activity	aging; ceramide biosynthesis
	MED2	RNA polymerase II transcription mediator activity	transcription from Pol II promoter
	MNN10	alpha-1,6-mannosyltransferase activity	N-glycan processing; mannan metabolism; cell wall

Comparison	Gene	Function	Process
			mannoprotein biosynthesis; actin filament organization
	NFI1	unknown	chromosome condensation
	NRG1	DNA binding; transcriptional repressor activity	regulation of transcription from Pol II promoter; glucose metabolism; invasive growth (sensu Saccharomyces); response to pH
	PAL1	unknown	unknown
	PRP12	exonuclease activity	rRNA processing; mitochondrial genome maintenance
	PRY3	unknown	unknown
	PTR3	amino acid binding	chemosensory perception
	PXA1	ATP-binding cassette (ABC) transporter activity	fatty acid transport
	RDS1	transcription factor activity	response to xenobiotic stimulus
	RMD6	unknown	unknown
	ROG1	lipase activity	lipid metabolism
	RSA4	unknown	ribosomal large subunit assembly
	RSC30	DNA binding	regulation of transcription, DNA-dependent
R2 vs Sc2	*SEO1*	transporter activity	transport
	SGF73	unknown	histone acetylation
	SLF1	RNA binding	regulation of translation; copper ion homeostasis
	SNC2	v-SNARE activity	vesicle fusion; endocytosis; Golgi to plasma membrane transport
	SNF6	general RNA polymerase II transcription factor activity	chromatin remodeling
	SNT1	NAD-dependent histone deacetylase activity; NAD-independent histone deacetylase activity	negative regulation of meiosis; histone deacetylation
	TIF6	unknown	processing of 27S pre-rRNA; ribosomal large subunit biogenesis
	URA3	orotidine-5'-phosphate decarboxylase activity	pyrimidine base biosynthesis
	YPS7	aspartic-type endopeptidase activity	unknown
	YSC83	unknown	
	11unknown		

Table S4.5. Homogeneous groups obtained in the expression analysis of *MNN10*, *YPS7* and *HXK1* genes.

A) Comparisons of the different strains at the same time point.

Gene	*MNN10*			*YPS7*			*HXK1*		
	R2IVo	**Sc2**	**Sc1**	**R2IVo**	**Sc2**	**Sc1**	**R2IVo**	**Sc2**	**Sc1**
24h	b	a	c	a	a	a	a	a	b
55h	b	a	a,b	b	a	a,b	a	a	a
120h	a	a	a	a	a	a	b	b	a

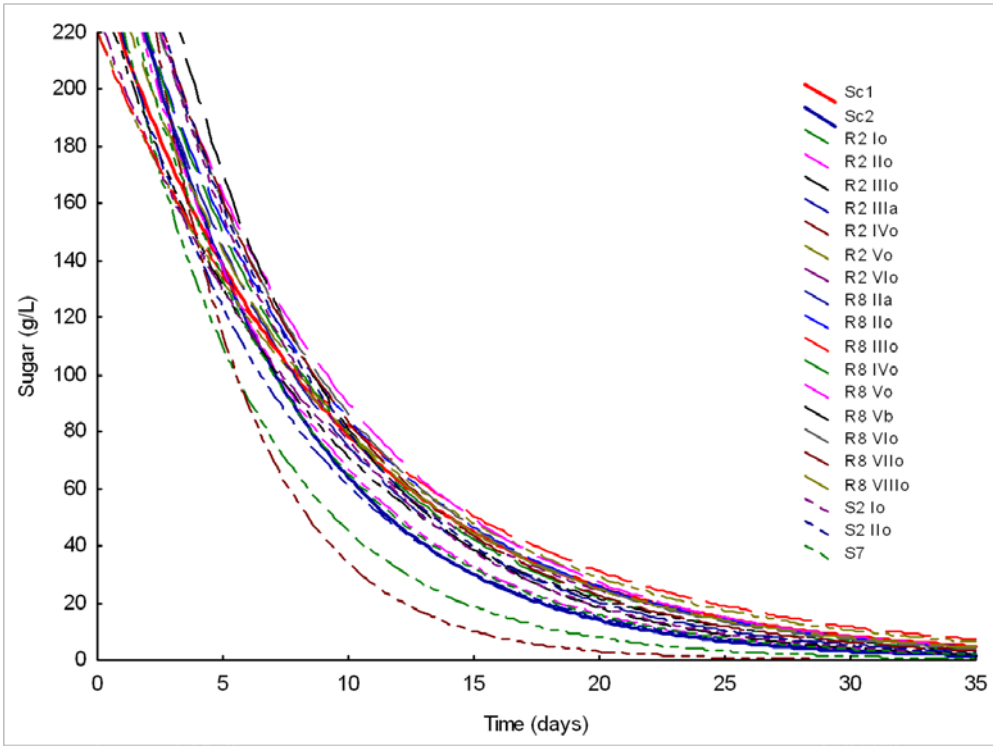
Homogeneous groups obtained by ANOVA and Tukey HSD test, α=0.05, n=3

B) Comparisons of the same strain at different time point.

Gene	*MNN10*			*YPS7*			*HXK1*		
	24h	**55h**	**120h**	**24h**	**55h**	**120h**	**24h**	**55h**	**120h**
R2IVo	b	b	a	a	b	a	a	b	b
Sc2	a	a	a	a	a	a	a	b	c
Sc1	b	a	a	b	b	a	a	b	b

Homogeneous groups obtained by ANOVA and Tukey HSD test, α=0.05, n=3

a)



b)

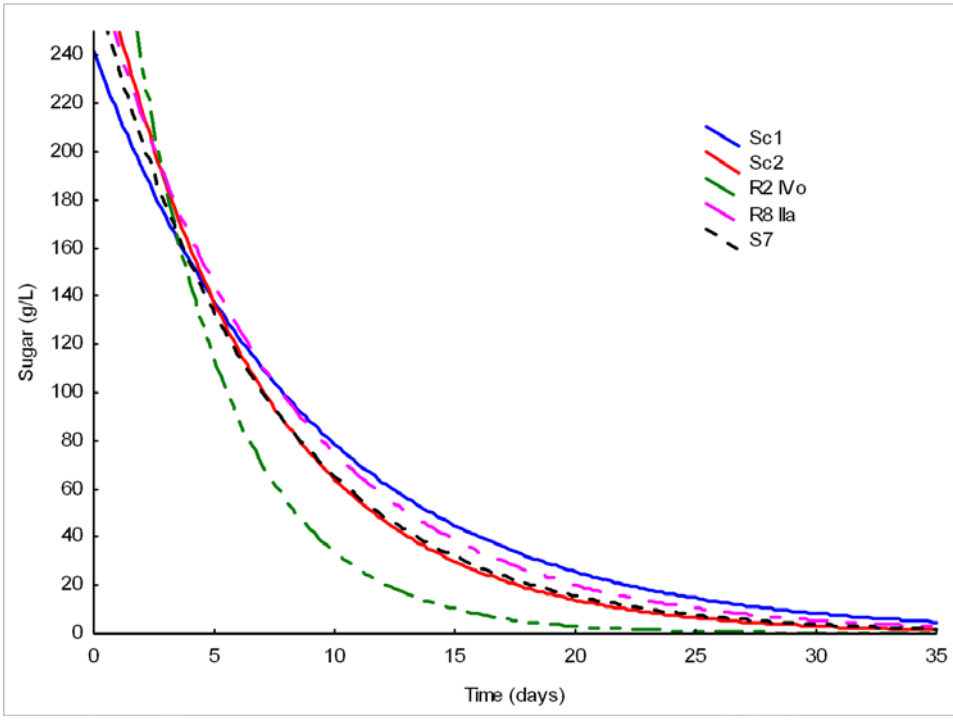


Fig S4.1. Sugar consumption in synthetic must. a) all the strains; b) selected and parental strains.

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