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Analysis of the hybridisation processes of yeasts
Élesztőgombák hibridizációs folyamatainak vizsgálata

Egyetemi doktori (PhD) értekezés

a szerző neve: Pfliegler Valter Péter
témavezető neve: Prof. Dr. Sipiczki Mátyás

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Pfliegler Valter Péter

Tanúsítom, hogy Pfliegler Valter Péter doktorjelölt 2010- 2013 között a fent megnevezett Doktori Iskola programjának keretében irányításommal végezte munkáját. Az értekezésben foglalt eredményekhez a jelölt önálló alkotó tevékenységével meghatározóan hozzájárult. Az értekezés elfogadását javasolom.

Debrecen, 2013.05.27.

Prof. Dr. Sipiczki Mátyás

ANALYSIS OF THE HYBRIDISATION PROCESSES OF YEASTS

ÉLESZTŐGOMBÁK HIBRIDIZÁCIÓS FOLYAMATAINAK VIZSGÁLATA

Értekezés a doktori (Ph.D.) fokozat megszerzése érdekében
a biológia tudományágban

Írta: Pfliegler Valter Péter okleveles molekuláris biológus

Készült a Debreceni Egyetem Juhász-Nagy Pál doktori iskolája
(biológia programja) keretében

Témavezető: Prof. Dr. Sipiczki Mátyás

A doktori szigorlati bizottság:

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Élesztőgombák hibridizációs folyamatainak vizsgálata.

1. Célkitűzés.

Munkánk során a *Saccharomyces* genuszba tartozó aszkuszos élesztőgombák három fajának segítségével (*S. cerevisiae* - pékélesztő, borélesztő; *S. uvarum*; *S. kudriavzevii*) vizsgáltuk a fajok közötti kereszteződés egyes aspektusait. Ezen élesztőgombák laboratóriumi körülmények között keresztezhetők, valamint tanulmányozásukhoz számos bevett vizsgálati módszer áll rendelkezésre, így kísérleteinkhez ideális modellszervezeteknek bizonyultak.

A *S. cerevisae* x *S. uvarum* fajpár esetében mesterséges hibridek előállításával és ezek utódnemzedékeinek izolálásával a hibrid genomok generációról generációra történő változásait, stabilizálódását kívántuk tanulmányozni, valamint célul tűztük ki a hibridek sterilitása ill. ritkán jelentkező fertilitása okának molekuláris módszerekkel történő tanulmányozását.

További célkitűzésünk volt, hogy mind a *S. cerevisae* x *S. uvarum*, mind a *S. cerevisae* x *S. kudriavzevii* fajpárok hibridjei segítségével tanulmányozzuk a hibridekben ill. ivaros utódnemzedékeikben végbemenő genetikai változások egyes jellemző fiziológiai tulajdonságokra gyakorolt hatását.

2. Irodalmi előzmények.

Az eukarióta szervezetek, különösen a növények esetében a fajok közötti hibridizáció fontos evolúciós szereppel bír, gyakran eredményezi új, poliploid fajok létrejöttét, melyek a szülőfajok közel teljes genomjával rendelkeznek (pl. Bento, 2008). Az élesztőgombák esetében ezzel szemben gyakori, hogy az interspecifikus hibridek aneuploidok, a szülőfajok genomjának csak egyes részeit tartalmazzák. Ezek között előfordulnak azonban külön fajnak tekintett, hibrid eredetű taxonok, mint pl. a lager-sörélesztő (*Saccharomyces pastorianus*) (Libkind et al., 2011). A részleges hibridek mellett a borászati *Saccharomyces*-élesztők között ismertek euploid, teljes hibridek is (pl. Sipiczki, 2008; Borneman et al., 2011), melyekhez hasonlók laboratóriumi körülmények közt is előállíthatók és tanulmányozhatók (pl. Antunovics et al., 2005). Így a *Saccharomyces*-fajok az interspecifikus hibridizáció jelenségének tanulmányozására különösen alkalmas modellszervezetek, mesterséges keresztezésük során pedig jelentős előnyt jelent, hogy a hibridek szülői törzsei ismertek, ellentétben a természetből izoláltakkal.

A fajok közötti hibridizációnak fontos szerepe van a iparilag jelentős élesztőtörzsek létrejöttében is - számos különböző steril hibridet izoláltak már fermentációs körülmények közül Európa-szerte (pl. González et al., 2006; Lopandic et al., 2007; Sipiczki, 2008; Peris et al. 2012a-b). Újabban pedig a mesterségesen keresztezett élesztők ipari felhasználásának kutatására is egyre több példa akad (pl. Bellon et al., 2013).

A fajok közötti hibridizáció folyamatainak vizsgálata során a hibridek szaporodóképessége, fertilitása fontos szereppel bír. Az élővilágban az egyes fajok között reproduktív izolációs barrierek működnek, általánosan megfigyelhető jelenség a pre- ill. posztzigotikus izoláció (pl. Wolf et al.,

2010; Maheshwari & Barbash, 2011). A posztzigotikus izoláció által elválasztott fajok hibridjei lehetnek életképesek, azonban ivaros szaporodásra képtelenek (emiatt evolúciós zsákutcát jelenthetnek). Ugyanakkor abban az esetben, ha a hibrid organizmus mitotikus szaporodásra képes, a sterilitás ellenére is életképes populációt hozhat létre, amint az pl. a *Saccharomyces* genuszba tartozó élesztőgombák esetében ismert (ez a jelenség leginkább a steril növények vegetatív szaporodásával analóg) (Sipiczki, 2008; Morales & Dujon, 2012).

A posztzigotikus izoláció jelentette akadály megkerülésének egyik módja a poliploidizáció: a poliploid (leggyakrabban tetraploid) hibrid eredetű fajokra számos példa ismert az élővilágban, elsősorban a növények között (pl. Hegarty & Hiscock, 2008; Schatlowski & Köhler, 2012). Ugyanakkor az azonos genuszba tartozó fajok kereszteződésével létrejött élesztőhibridekről ismert, hogy az allotetraploidizációt követően, bár képesek életképes F1 meiotikus generáció létrehozására, az utódjaik nagyrészt sterilek. Így - elsősorban az aszkuszos élesztők között - megkülönböztethető egy F1 sterilitási barrier, melynek mechanizmusa nem volt ismert (Greig, 2009). Ez a sterilitási barrier azonban ritka esetekben átléphető (pl. Antunovics, 2005). Az életképes és fertilis utódgenerációkkal rendelkező élesztőhibridek segítségével lehetőség nyílik olyan, evolúciós szempontból jelentős folyamatok tanulmányozására, mint pl. az alloploid genomok nagymérvű változásai.

Munkánk során a *Saccharomyces cerevisiae* (borélesztő v. pékélesztő) és a *Saccharmyces uvarum* (korábban *S. bayanus* var. *uvarum* (Nguyen & Gaillardin, 2005)) nevű, leginkább hidegen erjesztett borokból ismert élesztőfaj mesterségesen létrehozott hibridjei és azok ivaros utódnemzedékei segítségével vizsgáltuk az F1 sterilitási barrier átlépésének mechanizmusát,

valamint a hibrid genomokban bekövetkező változásokat, modellezve így a természetben is lezajló folyamatokat (Pfliegler et al., 2012).

Korábbi tanulmányok megállapították, hogy az ivaros szaporodásra képes hibridek utódaiban fokozatos genom-stabilizáció ill. redukció játszódhat le, mely az alloploid genetikai állomány egy részének elvesztésével jár (pl. Antunovics, 2005). A természetes módon létrejött hibrid eredetű törzsek esetén is megfigyelhető az alloploid genom redukciója ill. átrendeződése, valamint az introgresszió jelensége, ezeknek a folyamatoknak pedig - a genom újrarendezése által - jelentős hatásuk van az egyes élesztőtörzsek fiziológiai jellemzőire (pl. Naumova et al., 2005; Lopandic et al., 2007; Belloch et al., 2009). Emiatt a fajok közötti hibridizáció folyamatának vizsgálata nem csak az evolúciókutatás szempontjából, hanem az élelmiszer-mikrobiológia és biotechnológia számára is érdekes eredményekkel szolgálhat.

A hibrid élesztők ivaros generációiban fellépő genomszintű változások hatását a törzsek fiziológiai tulajdonságaira a már említett *S. cerevisiae* x *S. uvarum*, valamint az *S. cerevisiae* és a *S. kudriavzevii* nevű faj hibridjei és azok utódai segítségével vizsgáltuk, összehasonlítva őket a szülői törzsekkel, valamint a természetből izolált hibrid eredetű törzsekkel (Pfliegler et al., publikálás alatt).

Kísérleteink során így az élesztőfajok közötti hibridizáció jelenségét genetikai ill. evolúciós szempontból, valamint a fiziológia és az élelmiszer-mikrobiológia szempontjából is vizsgálatnak vetettük alá.

3. Módszerek.

3.1. Törzsek és hibridizáció.

Munkánk során a természetből izolált hibrideken kívül eltérő auxotróf mutációkkal rendelkező (azaz bizonyos aminosavakat vagy nukleotidokat igénylő) szülői törzsek keresztezésével állítottunk is elő hibrid törzseket. A hibridekben a szülői auxotróf mutációk nem jelennek meg (prototrófok), ami megkönnyíti izolálásukat minimál táptalajon. Törzseinket fiziológiai (szénforráshasznosítási képesség, hőmérsékletérzékenység) és PCR-RFLP módszerekkel is megvizsgáltuk, bizonyítandó hibrid mivoltukat, valamint kromoszómamintázatukat is analizáltuk. Az ivaros szaporodásra képes hibrid törzsek spóráit mikromanipulátor segítségével izoláltuk, az életképes spórákból kinőtt telepek jelentették az F1 nemzedéket (hasonlóan nyertük az F2 nemzedéket a spórázni képes F1 törzsek aszkuszaiból, és így tovább). A *Saccharomyces*-fajok aszkuszaiban általában 4-4 spóra található, ezeket együttesen tetrádként szokás említeni. A tetrádok tagjait betűkkel jelöltük (a-d). Valamennyi törzsünket -70°C-on, törzsgyűjteményünkben tároltuk és mindig csak a kísérletekhez szükséges ideig tartottuk fenn táptalajon, elkerülendő genetikai megváltozásukat.

3.2. Fiziológiai vizsgálatok.

Törzseink számos fiziológiai tulajdonságát (auxotrófia, hőmérséklet-érzékenység, spórázóképesség, spórák életképessége) megvizsgáltuk az életképesség ill. szaporodóképesség változásainak nyomonkövetésére. A fiziológiai tulajdonágokra vonatkozó tanulmányunk során (második tanulmány) a fenotipizáló microarray-tálcák segítségével nagyszámú különböző körülmény között teszteltük hibridjeinket, majd ezen előzetes adatok alapján a legjelentősebb különbségeket mutató szénhidrátokkal,

ozmotikus stresszorokkal és antibiotikumokkal végeztünk részletes teszteket négy-négy ismétlésben, összehasonlítva az egyes szülőfajok, hibridek és utódtörzsek növekedésének ütemét. A kísérletek során az élesztőkkel beoltott különböző tápfolyadékokban az optikai denzitás (OD) változását követtük nyomon mikrotálca-leolvasó készülék segítségével, 24 órás időközönként méréseket végezve 6 napon keresztül.

3.3. Molekuláris módszerek.

Törzseink molekuláris karakterizálására PCR-RFLP (restriction fragment lenght polymorphism), AFLP (amplified fragment lenght polymorphism) és RAPD-PCR (randomly amplified polymorphic DNA) módszereket, valamint interdelta-tipizálást használtunk.

Az RFLP-vizsgálatok során különböző kromoszómákon elhelyezkedő gének restrikciós profilját elemeztük törzseinkben (a profilok a különböző szülőfajokban eltérőek voltak). Emellett az ún. párosodási típust meghatározó lokusztól a kromoszómán mindkét irányban elhelyezkedő géneket is megvizsgáltunk ezzel a módszerrel, ezen lokusz sorsának a pontos nyomon követése érdekében.

A sterilitási barrierre vonatkozó tanulmányunk során (első tanulmány) két különböző, általánosan használt RAPD primer segítségével végeztük el a törzsek fingerprinting vizsgálatát, valamint a csak a *S. cerevisiae* szülőfajban megtalálható ún. delta-szekvenciák változásait is nyomon követtük a hibridek genomjaiban egy, az ezekre a genomban elszórtan előforduló szekvenciákra tervezett primerpár segítségével. A fiziológiai tulajdonságokra vonatkozó tanulmányunkban AFLP vizsgálatokat végeztünk, a fragmentek analíziséhez az automatizált kapilláris-elektroforézis módszerét használva.

A kromoszómák vizsgálatához CHEF-elektrokariotipizálást (contour-clamped homogeneous electric field) végeztünk, ez a módszer lehetővé teszi

a DNS-molekulák méretalapú elválasztását agaróz gélben. Az első tanulmányunkban a *S. cerevisiae*-eredetű kromoszómavégek beazonosításához az elektrokariotipizálással összekötve Southern blot eljárást is alkalmaztunk egy olyan próbával, mely ezen faj telomerjeihez kötődik (az ún. Y'-szekvenciához).

3.4. Élesztőtranszformáció.

A sterilitási barrierre vonatkozó tanulmányunkban egy újonnan tervezett primerpár segítségével a *S. uvarum* faj párosodási típust meghatározó lokuszát (*MAT*-lokusz) PCR-rel amplifikáltuk, majd a pEVP11 nevű élesztőplazmidba klónoztuk. Az így készült konstruktot használva egy *S. cerevisiae*-törzset transzformáltunk elektroporáció alkalmazásával.

4. Eredmények és értékelésük.

Munkánk során a *S. cerevisiae* és *S. uvarum* fajok hibridjeit ill. azok utódnemzedékeit vizsgálva elsősorban a *S. uvarum* szülőfajtól származó szubgenomot érintő változásokat mutattunk ki. A változások érintették a RAPD-markereket, valamint több kromoszomális gént is. Ezek a változások nem kötődtek kromoszomavesztésekhez. Az *S. cerevisiae* szubgenomban lényegesen kevesebb változást tapasztaltunk. Mindössze néhány hibrid F1 nemzedékében bukkant fel az *S. uvarum* faj auxotróf markere, ami az *S. cerevisiae*-eredetű allél elvesztére utal. Emellett a *cerevisiae*-transzpozonokhoz köthető (és törzsidentifikálásra is alkalmas) ún. delta-szekvenciákban mutatkoztak különbségek a hibridek utódaiban.

Az általunk vizsgált hibridek között előfordultak steril törzsek (feltételezhetően allodiploidok), valamint olyanok is, melyek életképes meiotikus utódokkal rendelkeztek (allotetraploidok). Ez utóbbiak között gyakori volt az utódok sterilitása, vagyis az F1 sterilitási barrier megfigyelhető volt. Egyes esetekben azonban az F1 nemzedékbe tartozó törzsek között akadtak olyanok, melyek életképes utód-tetrádokat hoztak létre. A hibridek sterilitásának ill. fertilitásának vizsgálata során felderítettük az F1 sterilitási barrier átlépésének okát ill. mechanizmusát. A kísérleteink során tett legfontosabb felfedezés, hogy a hibrid élesztőkben az egyik szülőfajtól származó, párosodási típust hordozó kromoszóma elvesztésével alakulhat ki az ivaros szaporodás öröklődő képessége, vagyis az F1-strerilitás által jelentett barrier áttörhető. Az élesztők párosodási típusai a magasabb rendű élőlények ivaraival analógok, a kétféle párosodási típust a *S. cerevisiae* fajnál a III. kromoszómán elhelyezkedő *MAT* lokusz kétféle allélje határozza meg. A diploid sejtekben mindkét allél jelen van, ezek a sejtek más sejtekkel nem párosodnak, meiózissal pedig spórákat hozhatnak létre. Az

eredményeink alapján felállított modellünk szerint a tetraploid hibridekben a meiózis során elveszhet az egyik *MAT*-lokuszt hordozó kromoszóma, így az a létrejövő aneuploid utódokba nem jut be. Emiatt ezekben a ritka esetekben a hibrid eredetű F1 nemzedék tagjaiban egyetlen *MAT*-példány marad meg. Így a kromoszómát vesztett törzsek sejtjei képesek a párosodásra, ezáltal pedig a közel-tetrapolid állapot visszaállítására is. Ennek következtében ezek a törzsek egyrészt képesek az ivaros szaporodására, másrészt utódaik is továbbszaporodhatnak. Tanszékünkön így elsőként írtunk le egy olyan folyamatot, melynek során fajok közötti hibrid élőlények kromoszómavesztés által válnak fertilissé.

A hibridek és utódaik fiziológiai változásaira, valamint a genomi és fiziológiai változások kapcsolatára irányuló vizsgálataink keretében a következő kulcskérdésekre kerestünk választ: (1) Hogyan változik a hibrid élesztők genomja az evolúciójuk során? Az AFLP-eredmények megmutatták, hogy egyik vagy mindkét szubgenomban diverz változások mehetnek végbe, illetve, hogy ezek a hibrid genomok néhány generáció alatt állandó körülmények között stabilizálódhatnak is. A természetes izolátumok nagyobb változékonyságot mutattak a mesterségesen létrehozott hibridekhez képest az AFLP-mintázatokat tekintve. (2) Hogyan változik a hibrid élesztők fenotípusa az evolúciójuk során? Az általunk létrehozott és vizsgált hibridek és azok utódai bizonyos tulajdonságokban felül-, másokban pedig alulmúlták a szülőknek használt törzseket. A természetes izolátumok kisebb változatossággal bírtak, ezek minden bizonnyal evolúciós nyomásra optimalizálták genomjukat az élőhelyükhöz. A cukrok felhasználásának képessége a szülőfajoktól függött, míg az ozmotikus stressztűrés és az antimikrobiális anyagok jelenlétében történő növekedés esetén nagyfokú variabilitást, új fenotípusok létrejöttét is megfigyeltük. (3) Milyen a vizsgált törzsek biotechnológiai felhasználhatósága? Egyes törzsek (*S. kudriavzevii* x

S. uvarum) jobban hasznosították a raffinózt és a melibiózt, mint a szülői törzsek, valamint ozmotikumok jelenlétében is előfordult, hogy a hibridek jobban nőttek a szülőknél. A kívánatos tulajdonságokkal bíró törzseket a későbbiekben érdemes lenne biotechnológiai szempontból egy következő kutatásban alaposabban megvizsgálni. Így genom- és expresszió-szinten tanulmányozható lenne a hibridek és azok ivaros szaporodással létrejött utódainak biotechnológiában való felhasználhatósága.

5. Köszönetnyilvánítás.

Szeretném köszönetemet kifejezni témavezetőmnek, Dr. Sipiczki Mátyásnak, hogy lehetővé tette számomra, hogy szakdolgozóként, majd doktoranduszként irányítása alatt a tanszéki munkába bakapcsolódjak. Köszönöm Gálné Dr. Miklós Idának, tanszékvezetőnknek támogatását, Dr. Ksenija Lopandicnak pedig (Bécs, BOKU), hogy irányításával részt vehettem az Universität für Bodenkultur-ban végzett, tanszékünkkel közös fiziológiai projektben. Köszönet illeti szerzőtársaimat, elsősorban Dr. Antunovics Zsuzsát és Karanyicz Edinát, továbbá a tanszékünk munkatársait, technikusait és PhD-hallgatóit és az Universität für Bodenkultur - Institut für angewandte Mikrobiologie munkatársait nélkülközhetetlen segítségükért.

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Analysis of the hybridisation processes of yeasts.

1. Aims.

During our study we investigated some aspects of the interspecific hybridisation using 3 species of the ascomycetous yeast genus *Saccharomyces* (*S. cerevisiae* - bakers' or wine yeast; *S. uvarum*; *S. kudriavzevii*. These yeasts can be hybridised in artificial conditions, furthermore, there are various established methods for the analysis of these organisms, thus they proved to be ideal model organisms for our experiments.

Using the *S. cerevisae* x *S. uvarum* species pair, we constructed hybrids and isolated their offspring generations, aiming to study the changes and stabilization of hybrid genomes that occur during the formation of sexual generations. Using molecular methods, our aim was also to study the reasons behind the sterility and the rarely observable fertility of hybrids.

We also designed experiments to investigate how the genetic changes in the hybrids of the species pairs *S. cerevisae* x *S. uvarum* and *S. cerevisae* x *S. kudriavzevii* and their meiotic offspring affect some key physiological properties.

2. Review of literature

Interspecific hybridisation is known to have an important role in the evolution of eukaryotic organisms (mostly in that of plants), often resulting in the formation of new, polyploid species that incorporate the nearly-complete genomes of the parent species (e.g. Bento, 2008). In contrast with this, aneuploid hybrids are common among yeasts - these contain only the partial genomes of the parents. Among these, there are hybridogenous taxa regarded as separate species, e.g. the lager brewing yeast (*Saccharomyces pastorianus*) (Libkind et al., 2011). Among the *Saccharomyces* wine yeasts, euploid complete hybrids are also known besides the partial ones (e.g. Sipiczki, 2008; Borneman et al., 2011), the like of which can also be produced and studied in laboratory environments (e.g. Antunovics et al., 2005). Thus, the *Saccharomyces* species are modell organisms particularly suitable for studying the processes of interspecific hybridisation. Their artificial crossing has the significant advantage that the parental strains of the hybrids are known, in contrast with the natural ones.

Interspecific hybridisation also has an important role in creating strains significant for the fermentation industry: several different hybrid strains have been isolated from fermentation environments across Europe (e.g. González et al., 2006; Lopandic et al., 2007; Sipiczki, 2008; Peris et al. 2012a-b). Additionally, more and more studies are focusing on the industrial use of artificially crossed yeasts (e.g. Bellon et al., 2013).

The reproductive capability, fertility of hybrids has a significant role in the analysis of the processes of interspecific hybridisation. Among all groups of organisms, reproductive isolation barriers act between species, the phenomena of pre- and postzygotic isolation are widely known (e.g. Wolf et al., 2010; Maheshwari & Barbash, 2011). The hybrids of species isolated by

postzygotic mechanisms may be viable, but are uncapable of sexual reproduction (generally, they mean an evolutionary dead-end). In cases when the organism is able to reproduce mitotically, it may establish a viable population despite its sterilty, as known, for example, among the yeasts of the genus *Saccharomyces* (this phenomenon is analogous to the vegetative reproduction of plants) (Sipiczki, 2008; Morales & Dujon, 2012).

One of the means of circumventing postzsgotic isolation is polyploidization: polyploid (mostly tetraploid) hybridogenous species are known from several examples, mainly from the plant kingdom (e.g. Hegarty & Hiscock, 2008; Schatlowski & Köhler, 2012). However, it is known that allotetraploid yeast hybrids that originate from the crossing of two species belonging to the same genus (even if they are capable of producing a meiotic F1 generation) behave else: their progenies are mostly sterile. Thus, mostly in ascomycetous yeasts, a second postzygotic sterility barrier, the F1 barrier is acting, the mechanism of which was unknown (Greig, 2009). In rare occasions, however, this barrier may be broken down (e.g. Antunovics, 2005). Yeast hybrids with viable and fertile offspring generations enable the study of evolutionarily significant processes, such as the large-scale changes in alloploid genomes.

During the course of our project, we examined the mechanism of the break-down of the F1 sterility barrier and the changes in the hybrid genomes using artificial hybrids of the species *Saccharomyces cerevisiae* (bakers' yeast) and *Saccharmyces uvarum* (which is mainly known from cold-fermented wine musts and was formerly known as *S. bayanus* var. *uvarum* (Nguyen & Gaillardin, 2005)), thus modelling the natural processes occuring in yeast hybrids (Pfliegler et al., 2012).

Earlier studies have concluded that in the filial generations of fertile hybrids, a gradual genome stabilization and reduction may take place,

resulting in the loss of some parts of the alloploid genome (e.g. Antunovics, 2005). In natural hybrids, the reduction and rearrangement of the alloploid genome is also observable, along with the phenomenon of introgression. By reshaping the genome, these processes have a significant effect on the physiological properties of certain yeasts strains (e.g. Naumova et al., 2005; Lopandic et al., 2007; Belloch et al., 2009). This means that studying the process of interspecific hybridisation may not only produce interesting results in the study of evolution, but also in the fields of food microbiology and biotechnology.

Using the aforementioned *S. cerevisiae* x *S. uvarum* hybrids and hybrids of *S. cerevisiae* and another species, *S. kudriavzevii*, we studied the effects of genome-scale changes on the physiological properties of the strains, comparing them to their parent species and to hybrid strains isolated from natural environments (Pfliegler et al., under publication).

Thus, during our work, we examined the phenomenon of interspecific hybridisation from an evolutionary and genetic, and also from a physiological and food-microbiological viewpoint.

3. Methods.

3.1. Strains and hybridisation.

During our experiments, along with natural hybrids, we also used artificial ones created by hybridising parental strains which possessed different auxotrophic mutations (these mutants require certain amino acids or nucleotides). In hybrids, these auxotrophies are not apparent in the phenotype (they are prototrophs), whic enables the quick selection of hybrids on minimal medium. Our strains were tested physiologically (utilization of carbon sources, temperature sensitivity) and with PCR-RFLP methods to prove their hybrid origins and their chromosomal composition was also analysed. We isolated the spores of the strains that were capable of sexual reproduction using a micromanipulator and the colonies grown from viable spores were used as F1 generations (similarly, we isolated the F2 generation from asci of F1 strains that were capable of producing spores, and so on). There are usually 4 spores in each ascus of *Saccharomyces* yeast species, which together are referred to as a tetrade. We designated the members of a given tetrade with letters (a-d). Every strain was conserved at -70°C in our strain collection and they were kept on solid medium only for as long as needed for the experiments, to prevent them from accumulating genetic changes.

3.2. Physiological tests.

Numerous physiological properties of our strains have been tested (auxotrophy, temperature-sensitivity, sporulation capability) to tract changes in viability and fertility during the meiotic generations. During our study about phenotype changes in meiotic generations of hybrids (second study), we used phenotyping microarray plates to test our strains under a large

number of different conditions, than we conducted detailed experiments (in quadruplicates) using the carbon sources and osmotic stressors that showed the most significant differences in the preliminary experiments. Thus we compared the growth rates of the parent species, hybrids and their offspring. For these analyses, the changes in the optical density (OD) were tracked in fluid media inoculated with the yeast strains using a microplate reader. Measurements were carried out every 24 hours for a period of 6 days.

3.3. Molecular methods.

For the molecular characterisation of our strains, PCR-RFLP (restriction fragment lenght polymorphism), AFLP (amplified fragment lenght polymorphism) and RAPD-PCR (randomly amplified polymorphic DNA) methods were used, as well as the so-called interdelta genotyping.

During the RFLP experiments, restriction profiles for several genes located on different chromosomes were analysed (the profiles were different in the parent species). This method was also used for testing genes located on both sides of the chromosome bearing the so-called mating type locus, to track the fate of this locus in our strains.

In the course of our study concerning the sterility barrier (first study) we used two different, widely used RAPD primers to conduct fingerprinting analysis on our strains. We also tracked the changes in the hybrid genomes affecting the so-called delta sequences (these sequence were only present in the *S. cerevisiae* parent species) by using a primer pair specific to these sequences. In our study about physiology of yeast hybrids (second study) we conducted AFLP-analysis on our strains, applying automated capillary electrophoresis to study the fragments produced.

Chromosomes were studied using CHEF-electrokaryotyping (contour-clamped homogeneous electric field). This method allows the separation of

DNA molecules based on thir size in agarose gel. In our first study, we used electrokaryotyping along with Southern blotting to identify *S. cerevisiae* chromosome ends using a probe specific to the so-called Y' telomeric sequences of this species.

3.4. Yeast transformation.

In our study about the sterility barrier, a newly designed primer pair was used to amplify the mating type locus (*MAT* locus) of the *S. uvarum* species with PCR, than this product was cloned into a yeast plasmid (pEVP11). The construct was used to transform a *S. cerevisiae* strain using electroporation.

4. Results and discussion.

In our study we detected changes in the *S. cerevisiae* x *S. uvarum* hybrids and their filial generations mainly affecting the *S. uvarum* subgenome. The genomic changes were observed in the RAPD-markers and also several chromosomal genes. These changes were not linked to chromosome-scale alterations. We detected much less changes in the *S. cerevisiae* subgenome. Only in a handful of hybrid F1 generations was the auxotrophic marker of *S. uvarum* found, suggesting the loss of the *S. cerevisiae* allele. In addition, differences in the offspring of hybrids were detectable in the so-called delta sequences, which are linked to transposons (and are used in strain fingerprinting).

Among the hybrids analysed in our study we could detect sterile ones (probably allodiploids) and strains that could produce viable meiotic offspring (allotetraploids). Among the latter, the sterility of the F1 strains was common, the F1 sterility barrier was observed. In same cases, however, F1 strains with viable offspring tetrades could also be detected. During the examination of hybrid sterility and fertility, we managed to uncover the cause and mechanism of the break-down of the F1 sterility barrier. The most interesting discovery of the experiments is that in the hybrid yeasts, the loss of a chromosome of one parent species that bears the mating-type determining locus results in the heritable ability for sexual reproduction, meaning that the F1 sterility barrier can be broken down. The mating types of yeasts are analogous to the genders of multicellular organisms, and the two different mating types are determined by the two alleles of the *MAT* locus on the chromosome III. in *S. cerevisiae*. In diploid cells, both alleles are present, these cells do not mate with others, but can produce meiotic spores. According to our model erected using our experimental results, in tetraploid

hybrids, the *MAT*-bearing chromosome of one parental species may be lost in meiosis, meaning that it will be missing in the aneuploid offspring. In these rare events, the strains of the hybrid F1 will possess only one *MAT*-copy. The result of this chromosome loss is that these strains will be capable of pairing (being hemizygous for *MAT*) and restoring the near-tetraploid state. This means that these strains and even their offspring are capable of sexual reproduction. Thus, according to our knowledge, we were the first to describe a process when interspecies hybrids become fertile by the loss of a chromosome.

In our project concerning the physiological changes of the hybrids and their filial generations, and the connections between genomic and physiological changes, we aimed to answer 3 key questions: (1) How does the genome of hybrid yeasts change during the course of their evolution? The results of the AFLP analysis have showed that in one or both subgenomes, diverse changes may happen. Also, these hybrid genomes may also be stabilized in a few generations under unvarying conditions. The natural isolates showed a higher level of diversity compared to the artificial hybrids in the AFLP-patterns. (2) How does the phenotype of hybrids change if they reproduce sexually? The artificial hybrids and their offspring exceeded their parent strains in some properties, whereas in others they showed a reduced growth capability. Interestingly, the natural isolates showed less diversity, these very probably have already optimised their genomes during their evolution to the environment they were isolated from. (3) What characterises the biotechnological usability of the strains analysed? The capability of assimilating different sugars depended on the parent species, whereas regarding the osmotic stress tolerance and growth rates in the presence of antimicrobial substances, a high degree of variability and novel phenotypes were detected. Some strains (e.g. *S. kudriavzevii* x *S. uvarum*) showed an

improved utilisation capability regarding raffinose and melibiose, and also some hybrids were found to grow better at osmotic stress conditions than their parents (*S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii*). It would be interesting to study the strains with potential biotechnological value more thoroughly in another project. Thus, the biotechnological usability of hybrid strains and their meiotic offspring could be studied on the level of genome and gene expression.

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Study I.: Double sterility barrier between *Saccharomyces* species and its breakdown in allopolyploid hybrids by chromosome loss

Pfliegler, W. P.; Antunovics, Zs. & Sipiczki, M. (2012) Double sterility barrier between *Saccharomyces* species and its breakdown in allopolyploid hybrids by chromosome loss. FEMS Yeast Research 12: 703-718.

Abstract.

The analysis of 57 synthetic interspecies hybrids revealed that *Saccharomyces cerevisiae* and *Saccharomyces uvarum* (*Saccharomyces bayanus* var. *uvarum*) are isolated by a double sterility barrier: by hybrid sterility (hybrid cells cannot produce viable spores) operating in allodiploids and by F1 sterility (F1 cells cannot produce viable spores) operating in allopolyploids. F1-sterility is caused by mating-type heterozygosity. It can be overcome by eliminating chromosome 2 of the *S. uvarum* subgenome that carries a *MAT* locus. The loss of this *MAT* gene abolishes the repression of mating activity. In cultures of the resulting fertile
alloaneuploid F1 segregants, the cells can conjugate with each other like haploids and form zygotes capable of performing meiotic divisions producing viable and fertile F2 spores. To the best of our knowledge, this is the first report on breaking down interspecies hybrid sterility by chromosome loss in eukaryotic organisms. The filial generations are genetically unstable and can undergo additional changes mainly in the *S. uvarum* subgenome (directional changes). It is proposed that regaining fertility and subsequent preferential reduction in one of the subgenomes may account for the formation of chimerical (‘natural hybrid’) genomes found among wine and brewery strains

and may also play roles in speciation of hybrid taxa in the *Saccharomyces* genus.

Introduction.

It is presumed that many fungi had whole-genome duplication events in their history and virtually all angiosperms are ancient polyploids (for recent reviews, see Scannel et al., 2007a, b; Van de Peer et al., 2009). Autodiploidization (genome duplication) and autopolyploidization facilitate evolution because the extra copies of certain genes can evolve to enrich the organism with novel functions. To mitigate the unfavourable consequences of drastic increase of genome size on fitness, large numbers of genes are usually sorted out after di- or polyploidization, resulting in smaller genomes containing extra copies of only those genes that enrich the organism with beneficial novel properties (Paterson et al., 2004; Scannel et al., 2007a, b). Since the retained genes take time to evolve and gain novel functions, the evolution leading to their functional divergence can be a long process.

Hybridisation with a different species (alloploidization) offers a faster way of obtaining genes with novel functions. An allodiploid hybrid contains all genes of both parental species and thus provides an excellent basis for reshaping one of the partner genomes with genes from the other partner (e.g. genetic introgression, recombination and gradual genome reduction) (reviewed in Baack & Rieseberg, 2007; Sipiczki, 2008). However, the interspecies hybrids are usually sterile (or infertile), unable to produce active gametes, and thus cannot propagate by sexual fertilization. This postzygotic reproductive isolation usually prevents the evolution of the alloploid genomes, making interspecies hybridisation an evolutionary dead end in most cases.

There are mechanisms, however, by which the problem of hybrid sterility can be circumvented. For example, duplication of the genome can make the alloploid hybrid fertile (Clausen & Goodspeed, 1925). This change in ploidy enables the correct pairing of chromosomes at meiosis and a diploid-like behaviour of the meiotic cell. In higher plants, allopolyploidization has become prominent mode of speciation, and many important crops have stabilized allopolyploid genomes (for recent reviews, see Rieseberg, 2001; Ma & Gustafson, 2005; Hegarty & Hiscock, 2008).

Much like the viable interspecies zygotes of higher organisms, the zygotes produced by closely related *Saccharomyces* species form vegetatively proliferating populations of alloploid cells (equivalents of somatic cells). As these yeast zygotes usually arise from conjugation of haploid cells (diploid *Saccharomyces* cells mate very rarely; Gunge & Nakatomi, 1972), their vegetative descendents have allodiploid genomes and are sterile insofar as they cannot produce viable haploid ascospores (ascospores are equivalents of gametes of higher organisms) (for reviews, see Sipiczki, 2008; Greig, 2009). If hybridisation is followed by genome doubling or the mating cells are diploid (rare occasion), the hybrid will be allotetraploid and able to form viable ascospores possessing allodiploid chromosomal sets (Naumov et al., 2000; Antunovics et al., 2005b). Thus, the allotetraploid *Saccharomyces* hybrids can perform reductional division producing cells analogous to allodiploid gametes of allotetraploid plants and animals.

However, there is an important difference between the allodiploid gametes and the allodiploid yeast spores. Unlike the gametes that fuse in the course of fertilization (restoring allotetraploidy) or die, the allodiploid yeast spores germinate and produce vegetatively propagating allodiploid cells instead of fusing. Later, when appropriate signals arrive from the environment (e.g. starvation), these cells can enter meiosis directly, without fertilizing each-

other beforehand. As they have only one set of chromosomes from each parental species, they cannot perform normal meiotic divisions and their spores will not be viable (Banno & Kaneko, 1989; Naumov et al., 2000; Sebastiani et al., 2002).

Thus, in spite of the viability of their first-generation (F1) spores, the allotetraploid *Saccharomyces* hybrids can also be considered sterile. This kind of sterility is referred to as F1 sterility to distinguish it from the sterility of the allodiploid hybrids (Sipiczki, 2008). Many *Saccharomyces* strains participating in beer and wine fermentation are alloploids or at least display traces of interspecies hybridisation event(s). Brewing yeasts usually have alloploid or alloaneuploid genomes composed of chromosomes and genes originating from at least two *Saccharomyces* species (Dunn & Sherlock, 2008; Nakao et al., 2009; Libkind et al., 2011; Nguyen et al., 2011).

Despite their diverse genome structures and low fertility, the lager brewery yeasts are regarded as separate species called *Saccharomyces pastorianus* (Martini & Martini, 1998). The other brewing yeast taxon, *Saccharomyces bayanus* (*S. bayanus* var. *bayanus*) is also an interspecies hybrid (Nguyen & Gaillardin, 2005; Libkind et al., 2011; Nguyen et al., 2011). Alloploids are far less common among wine yeasts. In their case, interspecies recombinants or chimeras (also referred to as 'natural hybrids') consisting of (nearly) complete *Saccharomyces cerevisiae* genomes and mosaics from *Saccharomyces uvarum* (*Saccharomyces bayanus* var. *uvarum*) and/or *Saccharomyces kudriavzevii* genomes seem to be more frequent (for a review, see Sipiczki, 2008). The genomes of the 'natural hybrids' of wine yeasts characterized so far are surprisingly diverse (Naumova et al., 2005; Le Jeune et al., 2007; Belloch et al., 2009; Bond, 2009), indicating that they must have undergone diverse series of postzygotic genome stabilization

events. As the progenitors are not known, it is practically impossible to reconstruct these events.

However, the examination of synthetic hybrids produced under laboratory conditions from genetically characterised parental strains may provide an insight into the postzygotic development of the alloploid genome. To test the applicability of this approach, we produced a synthetic *S. cerevisiae* x *S. uvarum* (*S. bayanus* var. *uvarum*) hybrid and monitored the changes of its genome (Antunovics et al., 2005b). We observed various structural interactions between the component genomes and gradual elimination of genes and chromosomes from the *S. uvarum* subgenome. These findings and the results of the molecular analysis of natural chimeras lead to a model for gradual stabilization of the *Saccharomyces* alloploid genomes (Sipiczki, 2008). Although consecutive meiotic divisions were proposed to be a major driving force for the stabilization process, the model did not address how the hybrid overcomes sterility.

In this work, we show by analysing a large number of *S. cerevisiae* x *S. uvarum* synthetic hybrids that the allotetraploid hybrid can overcome F1 sterility by eliminating its *S. uvarum* chromosome 2 that carries the counterpart of the *MAT* locus located on chromosome III of the *S. cerevisiae* subgenome. The resulting fertile alloaneuploid F1 segregants (nullisomic for Chr. 2 of *S. uvarum*) produce filial generations of viable and fertile spore clones in which additional, mostly directional changes can take place mainly in the *S. uvarum* subgenome.

To the best of our knowledge, this is the first report proposing a mechanism for breaking down postzygotic barrier between *Saccharomyces* species. The restoration of fertility and the subsequent genome reduction provide possibilities for genome rearrangements leading to alloaneuploids and mosaics whose genomes consist of (nearly) complete genomes from one

hybridising partner and certain genes from the other partner (horizontal gene transfer). In view of the estimates that up to 10% of the *Saccharomyces* strains may have composite genomes (Liti & Louis, 2005), and the observations indicating that the wine yeast genomes are subjects to frequent remodelling through the contribution of exogenous genes (Sipiczki, 2008; Novo et al., 2009), the exploration of the mechanisms of horizontal gene transfer may significantly contribute to our understanding of *Saccharomyces* genome evolution.

Materials and methods.

Strains and culture media.

Saccharomyces strains used in this study are listed in Table 1. The composition of culture media yeast extract peptone glucose agar (YPGA), YPGL (YPGA without agar), YPML (YPGL containing 2% melibiose instead of glucose), the acetate sporulation medium and the minimal medium agar (MMA) were described by Antunovics et al. (2005a, b) and references therein.

Hybridisation.

Synthetic interspecies hybrids were produced by mass mating of *S. cerevisiae* 10-170 and *S. uvarum* 10-522 cultures grown on sporulation medium as previously described (Antunovics et al., 2005b).

Table 1. Yeast strains used in this study

Strain	Other identifier	Species	Properties*	Origin†
10-126	S416B	*Saccharomyces cerevisiae*	MATa *gal7 met1 ura1 ade6 ade2*; for testing mating types	Jane Robinson, Pasadena, USA
10-166	ATTC 204884 (XJB-1C-1)	*Saccharomyces cerevisiae*	MATα *met1 ura1 ade2 trp2*; for testing mating types	ATCC (YGSC)
10-157	ATCC 204508 (S288c)	*Saccharomyces cerevisiae*	MATα *SUC2 mal mel gal2 CUP1 flo1 flo8-1 hap1*; reference strain for karyotyping	ATCC (YGSC)
10-170	ATCC 204891 (X4005-11A)	*Saccharomyces cerevisiae*	MATa *leu2* mel^{-}; parental strain for hybridisation	ATCC (YGSC)
10-174	ATCC 204955 (STX23-5B)	*Saccharomyces cerevisiae*	MATα *ade4 trp1 gal2*; for testing ploidy	ATCC (YGSC)
10-363	ATTC 204614 (X4037-14C)	*Sachharomyces cerevisiae*	MATa *gal1 leu2 arg9 ilv3 met14 lys7 pet17 trp1 gal2 his6*; for testing mating types	ATCC (YGSC)
10-368	ATCC 204985 (S2022D)	*Saccharomyces cerevisiae*	MATα *ura3 trp5 leu1 ade6 gal2*; for testing mating types	ATCC (YGSC)
10-522	m9	*Saccharomyces uvarum*	*ura3* mel^{+} ts, homothallic; parental strain for hybridisation	Antunovics *et al.*, 2005b

*mel^{+}, melibiose utilisation; ts, termosensitive (unable to grow at 37 °C).

†ATCC, American Type Culture Collection, Manassas, VA; YGSC, Yeast Genetic Stock Center, Berkeley.

Testing mating activity, sporulation and physiological properties.

Sporulation was examined microscopically in cultures grown on potassium acetate sporulation medium at 25 °C for 5 days. To test the mating activity of nonsporulating cultures, dense suspensions of cells were mixed with suspensions of mating-type tester strains and samples of the mixed suspensions were dropped on plates of sporulation medium. After 5 days of incubation, spore formation was checked microscopically. The mating activity of sporulation-proficient segregants was tested by mass-mating of their sporulating cultures with cells of the *S. cerevisiae* strains 10-368 (*MAT*a) and 10-126 (*MAT*a) as previously described (Antunovics et al., 2005b). As the segregants and the testers had different auxotrophic markers, the production of prototrophic colonies was taken as evidence of mating capability. Melibiose fermentation was examined in Durham tubes filled with YPML at 25 °C. Temperature sensitivity was tested on YPGA plates incubated at 37 °C for 3 days.

Random spore analysis and tetrad isolation.

For random spore analysis, samples were taken from the sporulating cultures, treated with zymolyase (the concentration of the enzyme and the duration of treatment varied according to the sensitivity of the ascus walls), sonicated (to separate the ascospores) and plated out on YPGA plates. After 5 days of incubation at 25 °C, the colonies were replica-plated onto MMA plates supplemented with uracil or leucine and onto YPGA plates. The MMA plates were incubated at 25 °C and used for the determination of the auxotrophic markers of the colonies. The YPGA plates were incubated at 37 °C to distinguish between temperature tolerant and temperature sensitive colonies. Ascus dissection by micromanipulation, isolation of tetrads of spores and the

analysis of the phenotypes of the spore clones were performed as previously described (Antunovics et al., 2005b).

PCR-RFLP, RAPD and interdelta analysis.

The primers used for RAPD, PCR-RFLP of nuclear genes or in PCR amplification of interdelta sequences, as well as the restriction endonucleases used for PCR-RFLP are listed in Table 2. PCR parameters are described in the references given in Table 2.

Electrophoresis.

The amplified DNA fragments and the subfragments generated by endonuclease treatments were identified and separated by electrophoresis in agarose gel. The method of electrophoretic karyotype analysis was described by Antunovics et al. (2005b).

Southern hybridisation.

The hybridisation probes were labelled with the DIGHigh Prime system (Roche) and used for hybridisation to the membranes of the karyotypes according to the manufacturer's instructions. The primers and the PCR parameters used for amplification of chromosomal sequences (probes) for Southern hybridisation are described in Table 2.

Cloning and transformation.

The *S. uvarum MAT* cassettes were amplified from genomic DNA prepared from strain 10-522 with the primers MAT-3 and MAT-5 (Table 2). The amplified DNA was cut with BamHI to generate sticky ends, ligated into the vector pEVP11 (Russell & Hall, 1983), linearised with the same enzyme and transformed into competent *Escherichia coli* cells. Plasmids were isolated

from 20 transformed bacterial colonies and used to transform the leucine auxotrophic *S. cerevisiae* strain 10-170 by electroporation. As pEVP11 carried the LEU2 marker gene, the yeast transformants could be identified as prototrophic colonies growing on the minimal medium MMA. The identity of the inserted fragments was checked by sequencing their ends. The primers used for sequencing were MAT-3 and MAT-5. The stability of the transformants was tested by culturing their cells in the nonselective complete medium YPGL. Samples of overnight cultures were plated out on YPGA, and the colonies produced were replica-plated onto MMA. The colonies that had lost the transforming plasmid did not grow on MMA.

Table 2. List of primers and restriction endonucleases used for amplification of sequences, RAPD analysis and PCR-RFLP

Gene/region			RFLP			
			Restr.	Fragments		
S. cerevisiae	*S. uvarum**	Primer pair	endonuclease	*S. c.*	*S. u.*	References
CAT8	c753_19463	CAT8-5: 5′-TCC AAT ATT AGT ATC AAC AAC TTT CTA TAC CAA AAT GA-3′ CAT8-3: 5′-CTA CTT GGC GTT TTG CCA TTG GAA-3′	MspI	2	3	González *et al.* (2006)
CYR1	c342_13338	CYR1-5: 5′-CTA CGA AGG AAA GTG TCC TCT TTG GTT CGT GG-3′ CYR1-3: 5′-CCG TGT GTA GAA TTT AGT GTA GAA TTG ACA GC-3′	MspI	2	1	González *et al.* (2006)
GSY1	c36_7403	GSY1-5: 5′-ATT GGA AAA AGA ATT TTC GAG CAT ACG ATG AG-3′ GSY1-3: 5′-AAT TTC TTG CCA CCG GCA AGG GTA TTC ATA TT-3′	MspI	2	3	González *et al.* (2006)
HCM1	c91_2876	HCM1-5: 5′-CCA AGA GAA CAC TAG AAG ACG AAA AGG AAA-3′ HCM1-3: 5′-GTC GTG ATA TAC CTG ATT GGA GTC TCT AT-3′	HaeIII	4	3	This study
HIS4	c95_2497	HIS4-5: 5′-ACT CTA ATA GTG ACT CCG-3′ HIS4-3: 5′-AAC TTG GGA GTC AAT ACC-3′	HindIII	3	1	Casaregola *et al.* (2001)
ITS1-5, 8S rDNA-ITS2† (GenBank: AB018043)	ITS1-5, 8S rDNA-ITS2† (GenBank: Z95946)	ITS1: 5′-TCC GTA GGT GAA CCT GCG G-3′ ITS4: 5′-TCC TCC GCT TAT TGA TAT GC-3′	HaeIII	4	3	McCullogh *et al.* (1998); Antunovics *et al.* (2005a)
KIN82	c609_3013	KIN82-5: 5′-GCCCTGAAAGTTTTGAGTAAACATGAGATGAT-3′ KIN82-3: 5′-TCGTCATCATTTGCAACTTTCTCACAAAACAT-3′	MspI	1	2	González *et al.* (2006)
LEU2	–	5′-ATG TCT GCC CCT AAG AAG AT-3′ 5′-CTT AAC TTC TTC GGC GAC AG-3′	–	–	–	Antunovics *et al.* (2005b)
	MAT	MAT-5: 5′-TTC TGG ATC CCT TGT TAC TAT TTG GCC CTG AAT AGC GAA G-3′ MAT-3: 5′-TCC TGG ATC CTA CTC GAA AGA TAA ACA ACC TCC GCC AC-3′				This study
MET2	c203_19897	MET2-U: 5′-CGA AAA CGC TCC AAG AGC TGG-3′ MET2-L: 5′-GAC CAC GAT ATG CAC CAG GCA G-3′	EcoRI, PstI	2 1	1 2	Hansen & Kielland-Brandt (1994); Masneuf *et al.* (1996)
MET6	c371_6890	MET6-5: 5′-CTA GAC CTG TCC TAT TGG GTC CAG TTT CTT ACT T-3′ MET6-3: 5′-TTA GCT TCT AGG GCA GCA GCA ACG TCT TGA CC-3′	HaeIII	1	2	González *et al.* (2006)
OPY1	c7_1855	OPY1-5: 5′-CCT CGG ACA ACA GAC CAT CAA TAT TGG TGT GT-3′ OPY1-3: 5′-CTC TTG AAA TTT ATT ATC CAA TCC ACC ATA TCT TG-3′	HaeIII	1	2	González *et al.* (2006)
STP22	c94_2585	YCL008c-U: 5′-TTC GTT GGA TGT GCC ACT G-3′ YCL008c-L: 5′-GGA GCC ACC AAG GGA TGG-3′	EcoRV PstI	2 1	1 3	Casaregola *et al.* (2001)
delta	–	Delta-1: 5′-CAA AAT TCA CCT ATA/T TCT CA-3′ Delta-2: 5′-GTG GAT TTT TAT TCC AAC A-3′	–	–	–	Le Jeune *et al.* (2007)
Subtelomeric sequence	–	5′-GAC GAG TTT GAG TCG GCT C-3′ 5′-GCA AAA TAT CAC CCA ATC GGT C-3′	–	–	–	This study
Genomic RAPD	Genomic RAPD	1283: 5′-GCG ATC CCC A-3′	–	–	–	Akopyanz *et al.*, 1992;
Genomic RAPD	Genomic RAPD	24: 5′-GCG TGA CTT G-3′	–	–	–	Baleiras Couto *et al.*, 1996

S. c., *Saccharomyces cerevisiae*; *S. u.*, *Saccharomyces uvarum*.

*Identification number of *Saccharomyces bayanus* locus in *Saccharomyces* Genome Database (http://www.yeastgenome.org).

†not listed in *Saccharomyces* Genome Database.

Results.

Production of synthetic hybrids.

Saccharomyces cerevisiae and *S. uvarum* frequently occur together in wine-making environment, where their cells propagate vegetatively by mitotic divisions during fermentation and sporulate upon the completion of fermentation when certain nutrients are no longer available (reviewed in Sipiczki, 2011). In mixed populations, their cells and spores can interact in many ways including sexual mating resulting in interspecies hybrids. As homothallic *Saccharomyces* strains are usually diploid and incapable of sexual mating, conjugation usually takes place between haploid spores at germination or soon thereafter (reviewed in Nasmyth, 1982) but 'rare mating' between vegetative cells is also possible (Gunge & Nakatomi, 1972). To simulate natural situations, we mixed cultures of *S. cerevisiae* 10-170 leu- and *S. uvarum* 10-522 ura- grown on acetate sporulation medium and incubated the mixed population of cells and spores in rich medium (YPGL), where spore germination and sexual mating could take place. Then we plated out samples on a selective minimal medium to identify prototrophic hybrids. Fifty-seven prototrophic colonies were isolated from the plates and deposited in a freezer to prevent genetic changes that might occur during vegetative propagation of the hybrid cells. The isolates were then tested for temperature sensitivity and melibiose fermentation (*S. cerevisiae* 10-170 was mel-, whereas *S. uvarum* 10-522 was sensitive to 37 °C). All isolates could ferment melibiose and grew at 37 °C, confirming that they were hybrids. To verify their hybrid nature, we subjected randomly selected hybrids and the parental strains to electrophoretic karyotyping. As shown in Fig. 1, the hybrids possessed all chromosomal bands of both parental strains.

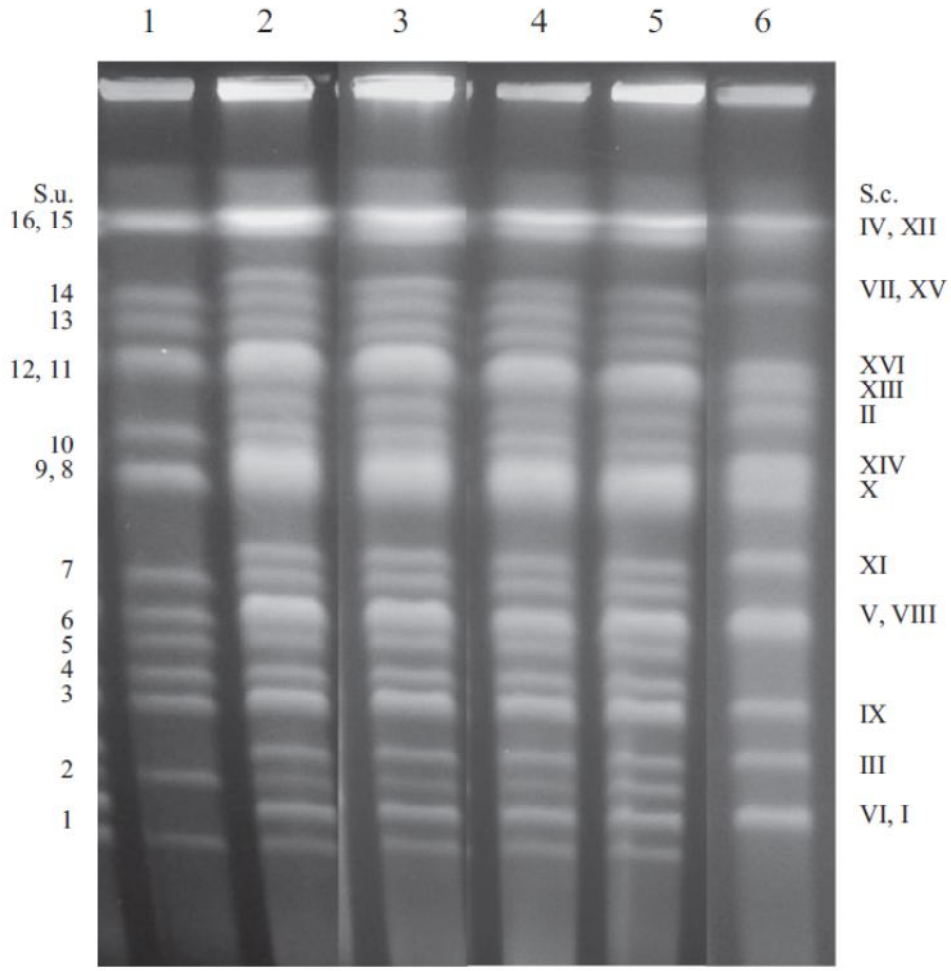


Fig. 1. Electrophoretic karyotypes of the parental strains and four synthetic hybrids. (1) *Saccharomyces uvarum* 10-522; (2) hybrid H10; (3) hybrid H21; (4) hybrid H29; (5) hybrid H35; and (6) *Saccharomyces cerevisiae* 10-170. Chromosome numbering is shown on the left size for S. uvarum and on the right side for *S. cerevisiae*. The numbering is based on the reference Antunovics et al., 2005b.

Sporulation and spore viability in synthetic hybrids.

Each hybrid was tested for sporulation by culturing on sporulation medium and for spore viability by random spore analysis (Table 3). Segregation of auxotrophic markers in random spore analysis was taken as evidence of production of viable spores. Eight hybrids (14%) either did not sporulate or showed very low sporulation efficiency (< 3%) on the sporulation medium. The rest of the hybrids produced more spores and could thus be subjected to random spore analysis. Nine sporulating hybrids (16%) did not produce auxotrophic segregants and their spores isolated by micromanipulation did not form colonies, indicating that these hybrids were sterile. None of them could conjugate with the mating type testers listed in Table 1. Seventy per cent of the hybrids were not sterile because they produced auxotrophic segregants. Remarkably, most of them formed leu- spores; ura- segregants were detected only in three hybrids. For four segregating hybrids (H10, H21, H29 and H35), we isolated tetrads of spores by micromanipulation from dissected asci and found that most isolated spores (80–98%) produced colonies (Fig. 2 and Table 3). We hereafter call the viable spores of the hybrids F1 spores and refer to the clones of vegetative cells produced by them as F1 clones.

Table 3. Sporulation of hybrids and segregation of auxotrophic markers in random spore analysis

	Description				
		Marker segregation			
Class	Sporulation	leu	ura	leu ura	List of hybrids (% sporulation)
I	–	n.d.			H44, H54
II	(+) < 3.0%	n.d.			H2 (1.1), H6 (1.0), H18 (2.4), H25 (2.3), H26 (0.9), H39 (2.1)
III	+	–	–	–	H9 (18), H34 (15.8), H43 (33.9), H45 (23.5), H47 (8.7), H48 (9.4), H49 (41.3), H51 (24.1), H57 (36.1)
IV	+	+	–	–	H3 (83.0), H5 (11.0), H8 (48.0), H10 (38.1), H11 (40.9), H12 (32.4), H13 (18.5), H14 (40.8), H15 (11.4), H16 (6.2), H17 (20.5), H19 (3.6), H21 (7.9), H22 (20.2), H23 (12.4), H24 (11.6), H29 (5.6), H30 (11.2), H33 (5.9), H35 (5.8), H36 (7.1), H37 (7.8), H40 (17.2), H41 (17.8), H42 (13.1), H46 (22.1), H50 (23.8), H52 (15.4), H53 (17.9), H55 (32.8), H56 (39.9)
V	+	–	+	–	H31 (10.7), H32 (21.4), H38 (7.7)
VI	+	+	+/–	+/–	H7 (3.5) 40 : 3 = leu : leu ura H1 (11.1) 25 : 1 = leu : leu ura H4 (98.8) 9 : 20 = leu : ura H27 (12.8) 1 : 26 = leu : ura H20 (51.5) 1 : 3 = leu : leu ura
VII	+	–	+	+	H28 (4.0), 1 : 2 = ura : leu ura

+: spores with parental auxotrophy marker(s) are produced.

–: spores with parental auxotrophy marker(s) are not produced.

Sporulation and spore viability in spore clones of synthetic hybrids.

The F1 clones of the hybrids H10, H21, H29 and H35 were also tested for sporulation. All of them formed spores with frequencies ranging from 38% to 85%. However, the spores of the H21 and H29 F1 clones were all dead. Thus, these hybrids were F1-sterile. In contrast, certain F1 clones of the hybrids H10 and H35 produced viable F2 spores, indicating that these hybrids were not fully F1-sterile. From one of the F2 spore clones, we isolated tetrads of F3 spores and found high levels of spore viability. Then, we isolated spores from two F3 clones to produce F4 generation. The F4 clones obtained also sporulated efficiently. The genealogy of the spore clones and the spore viability in selected clones are shown in Fig. 2.

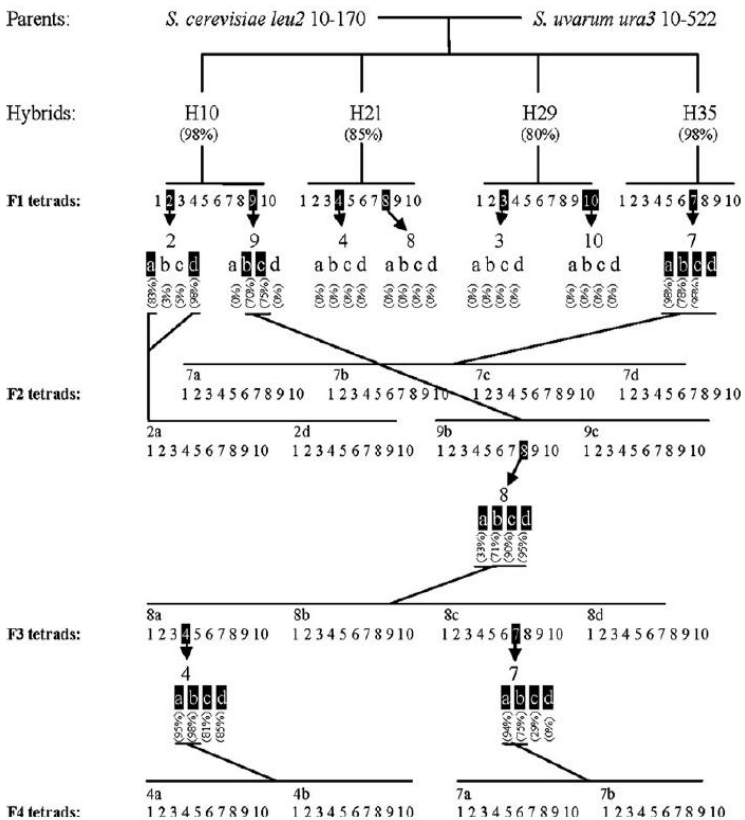


Fig. 2. Hybrids and their offspring analysed in detail. Tetrads and spore clones with black background were used for production of filial generations. Percentages of viable spores are shown in brackets.

Segregation of genetic and molecular markers.

To explore the genomes of the hybrids and their descendants, we tested the spore clones for the presence of parental markers and chromosomes. The marker analysis involved phenotypic testing for the genetic markers of the parents (leu2/LEU2, URA3/ura3 and mel/MEL) and the examination of eight pairs of orthologous chromosomal loci (Table 4) distinguishable by PCR-RFLP (molecular markers; Table 2). The eleven markers covered eight chromosomes of the *S. cerevisiae* genome. All F1 clones of H21 and H29 were prototrophic and mel+. In contrast, 74% and 46% of the H10 and H35 F1 clones, respectively, were auxotrophic for leucine, indicating that these hybrids were prone to loose the wild-type *S. uvarum* orthologue (c94_2554) of the mutant *S. cerevisiae* leu2 gene. The proportion of the leu+ and leu- spores (clones) in the tetrads was 2:2, 4:0 or 0:4. As no 1:3 and 3:1 tetrads were found, we presumed that the wildtype *S. uvarum* orthologue had been lost during meiosis I (or at a mitotic division preceding meiosis).

Remarkably, all F1 clones producing viable spores were leu-, and all sterile clones were prototrophic. Both categories were tested for mating activity with *S. cerevisiae MAT*a and *MAT*a testers. The leu- clones conjugated with both testers, whereas the sterile clones conjugated with neither. The four hybrids analysed were heterozygous for all molecular markers (Table 4). Their descendants also possessed both parental orthologues of most genes. In a few spore clones, the *S. uvarum* counterparts of the *S. cerevisiae* genes OPY1, SPT22, CYR1 and CAT8 were missing. The leu- clones that lacked the wild-type *S. uvarum* orthologue of LEU2 also lacked the *S. uvarum* orthologue (c94_2585) of STP22. As the LEU2 and SPT22 orthologues are assigned to the same contig (c94) of scaffold s10 in the *S. uvarum* genome sequence (available at http://www.yeastgenome.org/cgi-bin/FUNGI/FungiMap), their simultaneous absence in the leu- F1 clones indicated that a complete *S.*

uvarum chromosome, rather than individual genes, had been eliminated at meiosis I.

As LEU2 and SPT22 are located on the same arm of Chr. 2, the lack of their *S. uvarum* alleles only indicated but did not prove that the whole chromosome was missing in the leu- segregants. To check the strains for the presence of the other arm, we selected the genes HCM1, HIS4 and KIN82 whose PCR-RFP patterns differed in the parental strains. The hybrids and the sterile filial clones were heterozygous, whereas the leu- clones showed the patterns of the *S. cerevisiae* parent. These results confirmed that the entire Chr. 2 was missing in the leu- segregants.

In principle, the high proportion of leu- F1 clones could also be due to unequal copy numbers of the parental genes in the hybrid genomes. The copy number of the leu2 allele can be higher in the hybrid when the *S. cerevisiae* parent has more than one copy of the relevant chromosome (disomy for chromosome III). To test this possibility, we crossed the parental strain 10-170 with the haploid leu+ strain 10-174 of opposite mating type and isolated tetrads of spores. The proportion of the leu2- and leu2+ spores in 27 tetrads was nearly 1 : 1 (54% and 46%, respectively). This result makes it unlikely that the high frequency of leu- segregants was attributed to a higher copy number of Chr. III of the *S. cerevisiae* parent in the hybrids H10 and H35.

Table 4. Marker segregation in selected tetrads

Strains					Phenotypic markers			Molecular markers*				
								Orthologues† of				
Parental	Hybrid	F1	F2	F3	auxotrophy	Sporulation	Spore viability %	*OPY1* (II)	*STP22* (III)	*MET2* (IV)	*MET6* (V)	*GSY1* (VI)
S. cerevisiae					leu⁻	–	n.d	c	c	c	c	c
S. uvarum					ura⁻	+	40	u	u	u	u	u
	H10				–	+	98	h	h	h	h	h
		10/2a			leu⁻	+	83	h	c	h	h	h
		b			–	+	2.5	h	h	h	h	h
		c			–	+	5.0	h	h	h	h	h
		d			leu⁻	+	98	h	c	h	h	h
		10/9a			–	–	n.d.	h	h	h	h	h
		b			leu⁻	+	70	h	c	h	h	h
		c			leu⁻	+	75	h	c	h	h	h
		d			–	–	n.d.	h	h	h	h	h
			10/9b/8a		leu⁻	+	33	h	c	h	h	h
			b		leu⁻	+	71	h	c	h	h	h
			c		leu⁻	+	90	h	c	h	h	h
			d		leu⁻	+	95	h	c	h	h	h
				10/9b/8b/4a	leu⁻	+	95	h	c	h	h	h
				b	leu⁻	+	98	h	c	h	h	h
				c	leu⁻	+	81	h	c	h	h	h
				d	leu⁻	+	85	h	c	h	h	h
				10/9b/8c/7a	leu⁻	+	94	h	c	h	h	h
				b	leu⁻	+	75	h	c	h	h	h
				c	leu⁻	+	29	h	c	h	h	h
				d	leu⁻	+	0	h	c	h	h	h
	H21				–	+	85	h	h	h	h	h
		21/4a			–	–	n.d.	h	h	h	h	h
		b			–	–	n.d.	c	h	h	h	h
		c			–	+	0	h	h	h	h	h
		d			–	+	0	h	h	h	h	h
		21/8a			–	+	0	h	h	h	h	h
		b			–	+	0	h	h	h	h	h
		c			–	+	0	h	h	h	h	h
		d			–	+	0	h	h	h	h	h
	H29				–	+	80	h	h	h	h	h
		29/3a			–	–	n.d.	h	h	h	h	h
		b			–	+	0	h	h	h	h	h
		c			–	+	0	h	h	h	h	h
		d			–	+	0	h	h	h	h	h
		29/10a			–	–	n.d.	h	h	h	h	h
		b			–	–	n.d.	h	h	h	h	h
		c			–	+	0	h	h	h	h	h
		d			–	–	n.d.	h	h	h	h	h
	H35				–	+	98	h	h	h	h	h
		35/7a			leu⁻	+	98	h	c	h	h	h
		b			leu⁻	+	78	h	c	h	h	h
		c			leu⁻	+	98	h	c	h	h	h
		d			leu⁻	+	64	h	c	h	h	h

n.d., not determined.

*In brackets: the chromosomal location of the gene in the *Saccharomyces cerevisiae* genome.

†c: *S. cerevisiae* orthologue; u: *Saccharomyces uvarum* orthologue; h: both orthologues (heterozygous genome).

‡Fragment size. 1: ~ 800 nt; 2: ~ 520 nt; 3: ~ 450 nt; 4: ~ 350 nt.

§Fragment size. 1: ~ 2000 nt; 2: ~ 1500 nt; 3: ~ 1460 nt; 4: ~ 1330 nt; 5: ~ 1000 nt; 6: ~ 960 nt; 7: ~ 750 nt; 8: ~ 570 nt; 9: ~ 440 nt.

¶Fragment size. 1: ~ 2350 nt; 2: ~ 2280 nt; 3: ~ 1920 nt; 4: ~ 1400 nt; 5: ~ 1320 nt; 6: ~ 1250 nt; 7: ~ 950 nt; 8: ~ 870 nt; 9: ~ 685 nt; 10: ~ 615 nt; 11: ~ 590 nt; 12: ~ 530 nt; 13: ~400 nt.

CYR1 (X)	ITS (XII)	CAT8 (XIII)	Inter-delta fragments[‡]				RAPD fragments: Primer 24[§]									Primer 1283[¶]											
c	c	c	1	2	3	4	.	2	.	.	.	6	.	8	.	1	.	3	4	.	6	7	8	9	.	11	12
u	u	u	.	.	.	.	1	.	3	4	5	.	7	.	9	.	2	.	4	5	6	7	8	.	10	11	12
h	h	h	1	2	3	4	1	2	3	4	5	6	7	8	9	1	.	.	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	4	1	.	3	4	5	6	7	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	.	1	.	3	4	5	6	7	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	.	1	.	3	4	5	6	7	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	4	1	.	3	4	5	6	7	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	.	2	3	4	1	.	3	4	5	6	7	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	.	2	3	.	1	.	3	4	5	6	7	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	.	2	3	.	1	.	3	4	5	6	7	8	9	1	.	3	4	.	6	7	8	9	.	11	12
c	h	h	.	2	3	4	1	.	3	4	5	6	7	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	.	2	3	.	.	.	3	4	.	6	7	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	.	.	.	3	4	.	6	7	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	4	.	.	3	4	.	6	7	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	c	.	2	3	.	.	.	3	4	.	6	7	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	.	3	.	1	.	3	4	5	6	7	8	9	1	.	.	4	.	6	7	8	9	.	11	12
h	h	h	1	.	3	.	1	.	3	4	5	6	7	8	9	1	.	.	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	.	1	.	3	4	5	6	7	8	9	1	.	.	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	.	1	.	3	4	5	6	7	8	9	1	.	.	4	.	6	7	8	9	.	11	12
h	h	h	.	2	3	4	1	2	3	4	5	6	7	8	9	1	.	.	4	.	6	7	8	9	.	11	12
h	h	h	.	.	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
c	h	h	.	.	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
c	h	h	.	2	3	4	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	4	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
c	h	h	.	2	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
c	h	h	1	2	3	4	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
c	h	c	.	2	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	c	.	2	3	4	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	.	2	3	4	1	2	3	4	5	6	7	8	9	1	.	.	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	4	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	4	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
c	h	h	1	2	3	4	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	.	.	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	.	.	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
c	h	h	.	.	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	4	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	4	1	2	3	4	5	6	7	8	9	1	.	.	4	.	6	7	8	9	.	11	12
c	h	h	.	.	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	.	.	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	1	2	3	4	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12
h	h	h	.	.	3	.	1	.	3	4	5	6	.	8	9	1	.	3	4	.	6	7	8	9	.	11	12

n.d., not determined.
*In brackets: the chromosomal location of the gene in the *Saccharomyces cerevisiae* genome.
[†]c: *S. cerevisiae* orthologue; u: *Saccharomyces uvarum* orthologue; h: both orthologues (heterozygous genome).
[‡]Fragment size. 1: ~ 800 nt; 2: ~ 520 nt; 3: ~ 450 nt; 4: ~ 350 nt.
[§]Fragment size. 1: ~ 2000 nt; 2: ~ 1500 nt; 3: ~ 1460 nt; 4: ~ 1330 nt; 5: ~ 1000 nt; 6: ~ 960 nt; 7: ~ 750 nt; 8: ~ 570 nt; 9: ~ 440 nt.
[¶]Fragment size. 1: ~ 2350 nt; 2: ~ 2280 nt; 3: ~ 1920 nt; 4: ~ 1400 nt; 5: ~ 1320 nt; 6: ~ 1250 nt; 7: ~ 950 nt; 8: ~ 870 nt; 9: ~ 685 nt; 10: ~ 615 nt; 11: ~ 590 nt; 12: ~ 530 nt; 13: ~400 nt.

Karyotype segregation.

To verify the absence of a *S. uvarum* chromosome in the leu- clones, we karyotyped the tetrads. The hybrids and the prototrophic F1 clones had identical karyotypes containing all chromosomal bands of both parents, whereas the karyotypes of the leu- clones lacked Chr. 2 of *S. uvarum* (examples are shown in Fig. 3). In our previous study on a similar synthetic hybrid (Antunovics et al., 2005b), we also observed spore clones that lacked this chromosome. In those clones, the *S. uvarum* orthologue (c95_2497) of HIS4 was also missing. Remarkably, the HIS4 orthologue is also located on scaffold s10 in the *S. uvarum* genome database. To ascertain whether this scaffold corresponded to the band that was missing in the karyotypes of the fertile F1 clones, we amplified the *S. cerevisiae* LEU2 gene from the wild-type S288c, labelled it with digoxigenin (DIG) and hybridised the DIG-labelled probe to the karyotypes (Fig. 4). As presumed, the probe reacted with Chr. 2 of the *S. uvarum* parent and with Chr. III of the *S. cerevisiae* parent and with both corresponding bands in the hybrid karyotype.

Probing the karyotypes with a labelled *S. cerevisiae*-specific subtelomeric probe clearly identified most chromosomes of the *S. cerevisiae* parent. The only exception was Chr. III which does not bind the probe (Antunovics et al., 2005b). However, certain spore clones differed in signal intensity at the poorly resolved group of the largest chromosomal bands (Fig. 3). By modifying the running parameters of electrophoresis, we managed to separate the bands and found no segregation (Fig. 5).

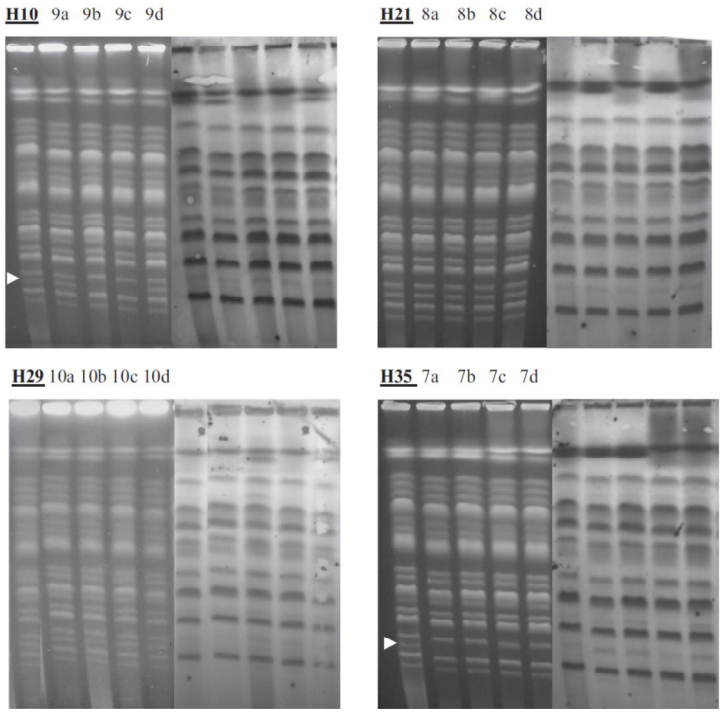


Fig. 3. Chromosomal patterns and hybridisation with *Saccharomyces cerevisiae*-specific subtelomeric probe in hybrids and F1 descendants. One example of tetrads is shown for each hybrid. Arrowheads mark *Saccharomyces uvarum* Chr. 2.

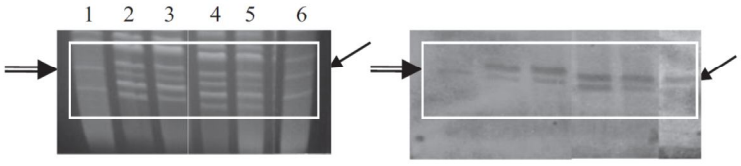


Fig. 4. Hybridisation of labelled LEU2 to chromosomal bands. Lane numbering is as for Fig. 1. Arrowhead with double line: *Saccharomyces uvarum* Chr. 2. Arrowhead with single line: *Saccharomyces cerevisiae* Chr. III.

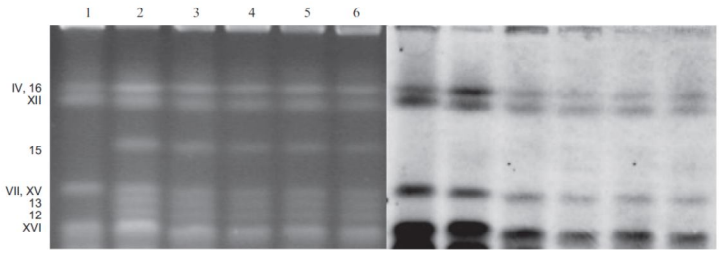


Fig. 5. Separation of large chromosomes and hybridisation with *Saccharomyces cerevisiae*-specific subtelomeric probe. (1) *S. cerevisiae* 10-170; (2) Hybrid H10; (3-6) spore clones of H10. Numbering of *S. cerevisiae* (Roman) and *Saccharomyces uvarum* (Arabic) chromosomes is shown on the left side.

Segregation of RAPD and interdelta patterns.

To reveal further differences between the genomes of the hybrids and their descendants, we performed RAPD analysis with two primers and amplified interdelta sequences (Table 4). The RAPD patterns of the hybrids contained most fragments of the parents, but numerous *S. uvarum* fragments were missing in the filial generations. There were only two *S. cerevisiae* fragments that were not present in all filial patterns. The amplification of interdelta sequences revealed higher diversity. No fragments were amplified from the *S. uvarum* parent, and many descendents lacked 1–3 *S. cerevisiae*-specific fragments.

Suppression of mating activity in *S. cerevisiae* by plasmid-borne *S. uvarum MAT*a cassette.

The inability of the *S. cerevisiae* x *S. uvarum* hybrids and their prototrophic spore clones to conjugate and the observed correlation between the loss of the *S. uvarum* Chr. 2 and the restoration of mating activity in the leu- segregants hinted at the involvement of Chr 2 in the repression of mating. As Chr. 2

carries the *MAT* locus of *S. uvarum*, we asked whether this repression might be attributed to the presence of the *S. uvarum MAT* in the interspecies hybrid cells. In *S. cerevisiae*, the diploid cells that are heterozygous at the *MAT* locus (*MAT*a/*MAT*a) do not mate because the concerted action of the *MAT*-encoded regulators blocks the activity of the mating-specific genes (Nasmyth, 1982). To test whether a similar interaction may also take place between the *MAT* loci of the hybridised species, we cloned the *MAT* cassettes of the *S. uvarum* partner in an episomal vector and transformed them into the *S. cerevisiae* hybridisation partner. We obtained both conjugating and nonconjugating transformants. End sequencing of the inserts of the plasmids that caused infertility revealed that they contained the *MAT*a cassette of *S. uvarum*. The sterility of the nonconjugating transformants was unstable. When they were cultured in a complete (nonselective) medium, segregants appeared in the cultures that were leu- and fertile. The sterility of the transformants and the simultaneous loss of their prototrophy and sterility demonstrated that a *S. uvarum MAT* cassette was sufficient to suppress the fertility of the *S. cerevisiae* cells.

Discussion.

Using the mass-mating method based on complementation of auxotrophic markers, we generated 57 novel synthetic hybrids of *S. cerevisiae* and *S. uvarum* (*S. bayanus* var. *uvarum*). As the mating mixture contained both spores and vegetative cells, interspecies hybrids could arise from all possible combinations of spores and vegetative cells of the partners. These combinations were unlikely to produce hybrids of identical ploidy because spores have smaller genomes than vegetative cells. Consistent with this, the

hybrids displayed differences in sporulation efficiency, spore viability and marker segregation.

Sterile (allodiploid) and F1-sterile (allopolyploid) hybrids.

Seventeen hybrids did not form viable spores. This property is reminiscent of the sterility of interspecies hybrids of higher organisms. It is well established that in allodiploids of higher organisms, the dissimilarities between the chromosomal sets of the parental species prevent correct pairing of chromosomes in meiosis and cause hybrid sterility or, at best, a severe reduction in gamete viability (Hegarty & Hiscock, 2008). Thus, these *S. cerevisiae* x *S. uvarum* hybrids were most probably allodiploids. This conclusion is consistent with our earlier observation that hybrids formed by *S. cerevisiae* and *S. uvarum* spores pulled together by micromanipulation were sterile (Naumov et al., 2002). The rest of the hybrids were not sterile because they produced spores that germinated and produced colonies. The viability of their F1 spores indicated that they had at least two sets of chromosomes from both progenitor species (allotetraploid genomes). They might have arisen from cell-to-cell fusion events ('rare mating' of diploid vegetative cells) or from diploid zygotes that duplicated their genomes soon after conjugation (e.g. by endomitosis, as suggested by Sebastiani et al., 2002). The viability of ascospores does not necessarily mean that the hybrid is fertile.

As the spores are yeast equivalents of gametes of animals and plants, a yeast hybrid can be regarded as fertile only if its spores or spore clones are able to produce zygotes by conjugation ('fertilization'). In *S. cerevisiae*, zygotes are formed by conjugation between cells or spores of opposite mating types determined by two alternate alleles of the mating type locus, *MAT*a or *MAT*a, located on chromosome III (for a review of sex determination, see Nasmyth,

1982). Homothallic haploid cells are able to convert their mating type almost as frequently as every cell division, so conjugation can take place between vegetative descendants of a homothallic spore. Presuming that the rules of sex determination apply also to interspecies hybrids (as suggested for example by Zill & Rine, 2008), the F1 spores of an allotetraploid hybrid are likely to be heterozygous for mating type (*MAT*a/*MAT*a) at birth. As heterozygosity at the *MAT* locus represses the genes of the conjugation pathway (Nasmyth, 1982), these spores and their vegetative descendents should not be able to conjugate. Instead, they should be able to sporulate because mating-type heterozygosity allows the activation of the meiosis/sporulation programme in response to signals generated by starvation conditions. However, if the hybrid is allotetraploid, its F1 spores are very likely allodiploid and unable to produce viable F2 spores.

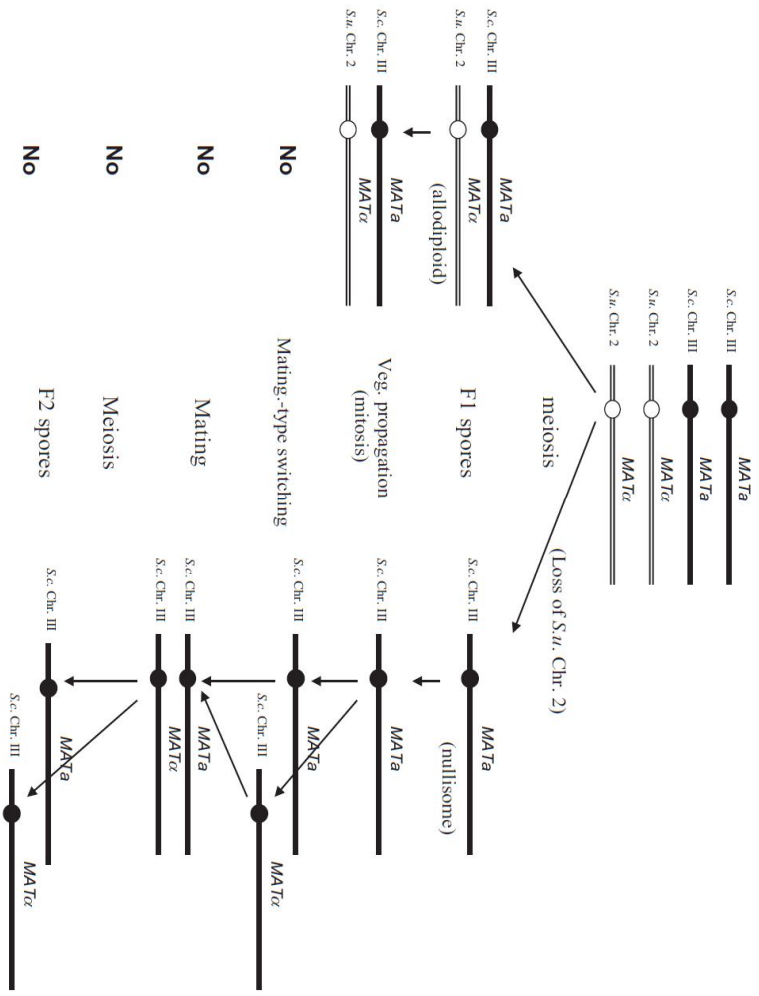
On the basis of these considerations, we presumed sterility in the F1 clones of our hybrids. Consistent with this, two of the four hybrids subjected to tetrad analysis did not form viable F2 spores. Thus, these hybrids were F1-sterile. Their F1 clones were most probably of *MAT*a/*MAT*a genotype because they did not conjugate with either *S. cerevisiae* mating-type tester. Correlation between breakdown of F1 sterility and loss of *S. uvarum* chromosome 2 In the other two hybrids analysed, many (but not all) F1 clones produced viable F2 descendants, demonstrating that F1 sterility can be broken down. When comparing the genotypes and fertility of the spore clones, we found perfect correlation between the loss of F1 sterility, the loss of the *S. uvarum* orthologues of LEU2, STP22, HCM1, HIS4 and KIN82 and the lack of the *S. uvarum* Chr. 2. This coincidence suggests that the abolishment of F1 sterility is attributable to the loss of Chr. 2.

How can the elimination of a chromosome from the genome make the alloploid hybrid fertile?

As the *S. uvarum* counterpart of the *S. cerevisiae MAT* locus is also located on scaffold s10, loosing of Chr. 2 during meiosis abolishes heterozygosity not only at LEU2, STP22, HCM1, HIS4 and KIN82 but also at the mating-type locus. We believe that this may account for the observed gain of fertility in the leucine auxotrophic alloploid F1 clones. Thus, the F1 sterility of the allotetraploid *S. cerevisiae* x *S. uvarum* hybrids is the consequence of mating-type heterozygosity and the hybrid becomes fertile if it abolishes this heterozygosity by eliminating the chromosome that carries the *S. uvarum MAT* locus (Fig. 6). Being nullisomic for the *S. uvarum* Chr. 2, the leu- clones are no longer *MAT*a/*MAT*a heterozygous because they have only one *MAT* allele (located on Chr. III in the *S. cerevisiae* subgenome). These nullisomes behave in sex determination as if they were haploid. They can switch their mating-type in haploid-like manner and can also activate the conjugation programme. The zygotes produced by the conjugating nullisomes of opposite mating types are also nullisomic for Chr. 2, as shown by karyotyping, but most probably heterozygous for mating-type in the *S. cerevisiae* subgenome. This heterozygosity prevents them from another mating and enables them to perform meiosis leading to viable alloaneuoploid F2 ascospores nullisomic for Chr. 2 (Fig. 6). A crucial element of the model is the assumption that the *MAT*-encoded regulators of the partner species interact in the hybrid cells essentially in the same way as they do in their own cells and this interaction makes the interspecies hybrids sterile or F1 sterile. The assumed regulatary interaction needs experimental verification, but the loss of mating activity in the *S. cerevisiae* parent upon transformation with a plasmid-borne *MAT* cassette of the *S. uvarum* parent clearly demonstrates that the *MAT* locus, without other genes of Chr 2, is sufficient to switch off

mating in *S. cerevisiae* cells and most probably also in the *S. cerevisiae* x *S. uvarum* hybrids.

Fig. 6. A model of breaking F1 sterility by chromosome loss. A *MAT*-carrying chromosome can be lost either during meiosis (as shown) or prior to it, during vegetative propagation of the hybrid cells.



Directional changes in the alloploid genomes.

The phenotypic and molecular characterization of the spore clones revealed several changes in the F1 to F4 genomes compared to the hybrid genome. Most changes were directional, affecting mainly the *S. uvarum* subgenome. Besides loosing of Chr. 2 characteristic of all fertile clones, certain clones also lost the *S. uvarum* orthologues of *S. cerevisiae* genes located on chromosomes II, X and XIII. Interestingly, the loss of these genes was not associated with karyotype alterations, indicating that mechanisms different from aneuploidization can also cause directional changes. These mechanisms may involve intergenomic (nonhomoeologous, homoeologous) recombination, such as conversion of the *S. uvarum* genes by their *S. cerevisiae* counterparts in a way similar or analogous to intergenomic gene-conversion-like events detected in synthetic *Brassica* alloploids (Song et al., 1995).

The lack of certain RAPD fragments of the *S. uvarum* parent in certain F clones could also be attributed to asymmetrical recombination events. Much fewer directional changes were detected in the *S. cerevisiae* subgenome. (1) Loss of a *S. cerevisiae* marker was observed in three hybrids (5% of all hybrids examined). These hybrids produced ura- F1 spores. As ura- was the marker of the *S. uvarum* parent, its phenotypic expression indicated that the *S. cerevisiae* URA3 gene was not transmitted into these spores during meiosis. (2) Loss of two RAPD fragments characteristic of the *S. cerevisiae* parent and variable numbers of interdelta fragments were also detected in numerous spore clones. As delta sequences were present only in the *S. cerevisiae* parent and the species *S. uvarum* is thought to be delta-free (LeJeune et al., 2007), the changes of the interdelta patterns were most probably due to internal rearrangements in the *S. cerevisiae* subgenome. Delta sequences are associated with mobile genetic elements and changes in

their location indicate chromosomal rearrangements (Argueso et al., 2008). Transposon activity has been recently impli- cated in genomic changes in synthetic *Arabidopsis* polyploids (Madlung et al., 2005). Preferential elimination of genomic loci of one of the hybridising partners was observed in our previous work in a similar yeast hybrid (Antunovics et al., 2005b), in hybrids of *Saccharomyces mikatae* and *Saccharomyces paradoxus* with *S. cerevisiae* strains reconfigured to make their genomes colinear with those of the hybridising partners (Delneri et al., 2003), as well as in numerous other groups of eukaryotes. For example, in synthetic *Triticale*, the predominant type of genomic changes was the elimination of rye-specific fragments (Ma & Gustafson, 2005; Bento et al., 2008). In wheat, DNA sequences that are genome and chromosome specific in established allopolyploids are deleted at high frequency from parental genomes in synthetic allopolyploids (Feldman et al., 1997; Liu et al., 2009). An analysis of *Aegilops sharonensis* x *Aegilops umbellata* hybrids showed that 14% of the DNA sequences from *A. sharonensis* were eliminated in the genome (Shaked et al., 2001). In synthetic hybrids of *Brassica nigra* and *Brassica rapa*, the former genome lost more genomic fragments (Song et al., 1995).

General implications.

Using a large number of synthetic alloploid hybrids, we have demonstrated that the nascent *S. cerevisiae* x *S. uvarum* (*S. bayanus* var. *uvarum)* hybrids are sterile or F1-sterile. The two types of infertility indicate that the postzygotic reproductive isolation of these species is ensured by double sterility barrier: by hybrid sterility (hybrid cells cannot produce viable spores) operating in allodiploids and by F1 sterility (F1 cells cannot produce viable spores) operating in allopolyploids. Hybrid sterility has been found to be due to the inability of homoeologous chromosomes to pair (recombine) in meiosis

(Greig, 2009). Here, we show that F1-sterility is ascribable to mating-type heterozygosity and can be circumvented by elimination of Chr. 2 of the *S. uvarum* subgenome. To the best of our knowledge, this is the first report on breaking down interspecies hybrid sterility by chromosome loss. As the fertility of the resulting aneuploids is heritable, series of filial generations can be produced by consecutive meiotic divisions at which additional, mostly directional genomic changes can take place. The observed predominance of directional changes may be the consequence of lethality of certain types of changes which remain undetectable in the few unviable spores found in almost all fertile spore clones. For example, loosing one of the parental alleles of a recessive 'speciation gene' or 'incompatibility gene' can have fatal consequences. A recent study reported on a gene (AEP2) of the *Saccharomyces bayanus* Chr. 13 that was incompatible with *S. cerevisiae* mitochondria (Lee et al., 2008). The replacement of a chromosome in the *S. cerevisiae* genome with this *S. bayanus* chromosome caused a sporulation defect and lethality in glycerol medium.

As spores are not only sexual products but also quiescent cells that ensure the survival of the yeast population under unfavourable conditions (for a review on wine yeasts, see Sipiczki, 2011), the inability to produce viable spores can severely reduce the chances of the interspecies hybrids to survive stressful environmental changes and persist in sympatry with populations of their progenitors. This handicap may account for the rare occurrence of allodiploid and allotetraploid *Saccharomyces* hybrids in nature. Abolishing mating-type heterozygosity by elimination of one of the homoeologous chromosomes carrying the *MAT* loci seems to be a solution to this problem because it restores fertility. The fertile nullisomic segregants remain hybrid for the rest of the genome but undergo further genomic changes during sexual propagation that can gradually lead to allo(aneu)ploid and/or mosaic genomes

identified in certain wine yeasts (reviewed in reference Sipiczki, 2008) and in brewing yeasts (Rainieri et al., 2006; Dunn & Sherlock, 2008; Nakao et al., 2009; Libkind et al., 2011; Nguyen et al., 2011). Our preliminary results indicate that similar mechanisms may also operate in other combinations of *Saccharomyces* species. Thus, the observed mechanism of gaining fertility and the ensuing directional genomic changes may also be implicated in speciation leading to hybrid species such as *S. pastorianus* and *S. bayanus* (Nguyen & Gaillardin, 2005; Bond, 2009; Libkind et al., 2011; Nguyen et al., 2011) whose strains are particularly adapted to large-scale fermentation of substrates of plant origin. *Saccharomyces pastorianus* (syn. *Saccharomyces carlsbergensis*) has a composite genome consisting of genomic components of *S. cerevisiae* and a non-*cerevisiae* species. Recent studies identified the source of the latter moiety as *Saccharomyces lagerae* (a hypothetical species; Nguyen et al., 2011) or *Saccharomyces eubayanus* (a species found in *Nothofagus* forest; Libkind et al., 2011). The genomes of the *S. bayanus* (*S. bayanus* var. *bayanus*) strains are composed of genomic sequences from *S. uvarum*, *S. eubayanus*/*lagerae* and *S. cerevisiae* (Libkind et al., 2011; Nguyen et al., 2011). Several models have been proposed for the formation and evolution of the *S. carlsbergensis* and *S. bayanus* genomes (Dunn & Sherlock, 2008; Libkind et al., 2011; Nguyen et al., 2011).

Essential elements of these models are interspecies hybridisations and horizontal transfer of chromosomes and/or large chromosomal fragments between different species and/or their hybrids. As the thick yeast cell wall is not penetrable for chromosomesize DNA molecules under natural conditions, it is reasonable to suppose that the horizontal transfer of genetic material also took place through interspecies hybridisations which were, however, followed by gradual reduction of one partner genome to smaller or larger mosaics favourable in brewing, as suggested for wine yeasts (Sipiczki, 2008).

However, the alloploid hybrids are usually sterile or F1-sterile and unable to produce viable spores that makes them less competitive in harsh environmental conditions and less efficient in adaptive evolution. The mode of fertility restoration described in this study may be one of the mechanisms that allow genomic evolution leading to domesticated yeast strains.

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Study II.: Generation of New Genotypic and Phenotypic Features in Artificial and Natural Yeast Hybrids

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Abstract.

Evolution and genome stabilization have mostly been studied on the *Saccharomyces* hybrids isolated from natural and alcoholic fermentation environments. Genetic and phenotypic properties have usually been compared to the laboratory and reference strains, as the true ancestors of the natural hybrid yeasts are unknown. In this way the exact impact of different parental fractions on the genome organization or metabolic activity of the hybrid yeasts is difficult to resolve completely. In the present work the evolution of geno- and phenotypic properties was studied in the interspecies hybrids created by the cross-breeding of *S. cerevisiae* with *S. uvarum* or *S. kudriavzevii* auxotrophic mutants. We hypothesized that the extent of genomic alterations in *S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii* should affect the physiology of their F1 offspring in different ways. Our results, obtained by AFLP (amplified fragment length polymorphism) genotyping and karyotyping analyses, showed that both the subgenomes of the *S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii* hybrids experienced various modifications. However, the *S. cerevisiae* x *S. kudriavzevii* F1 hybrids underwent more severe genomic

alterations than the *S. cerevisiae* x *S. uvarum* ones. Generation of the new genotypes also influenced the physiological performances of the hybrids and occurrence of the novel phenotypes. Significant differences in carbohydrate utilization and distinct growth dynamics at increasing concentrations of sodium chloride, urea and miconazole were observed within and between the *S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii* hybrids. Parental strains also demonstrated different contributions to the final metabolic outcomes of the hybrid yeasts. A comparison of the genotypic properties of the artificial hybrids with several hybrid isolates from the wine-related environments and wastewater demonstrated a greater genetic variability of the *S. cerevisiae* x *S. kudriavzevii* hybrids. *Saccharomyces cerevisiae* x *S. uvarum* artificial and natural hybrids showed considerable differences in osmolyte tolerance and sensitivity to miconazole, whereas the *S. cerevisiae* x *S. kudriavzevii* hybrids also exhibited differences in maltotriose utilization. The results of this study suggest that chromosomal rearrangements and genomic reorganizations as post-hybridisation processes may affect the phenotypic properties of the hybrid progeny substantially. Relative to their ancestors, the F1 segregants may generate different phenotypes, indicating novel routes of evolution in response to environmental growth conditions.
Key words: *S. cerevisiae*, *S. uvarum*, *S. kudriavzevii*, yeast interspecies hybrids, AFLP, karyotyping

Introduction.

Intensive development of molecular biology techniques and high-throughput sequencing technologies has improved the genetic characterization of yeasts immensely. The availability of different whole genome sequencing approaches has enabled us to study genome dynamics in a number of

different yeast species and to understand the ways they evolve and their speciation. Whole genome comparisons have demonstrated that the acquisition of foreign genes is not a rare event among yeasts. Moreover, genome duplication, introgression and interspecies hybridisation are considered to be one of the main molecular mechanisms of yeast genome evolution (Dujon, 2010; Morales & Dujon, 2012). Interspecies yeast hybrids have also become interesting in the study of cell adaptations to various environments in which the mode of genome stabilization and physiological cell responses are of particular interest. Hybridisation and introgression processes are recognized within the asco- and basidiomycetous yeast genera (*Debaryomyces*, *Millerozyma*, *Zygosaccharomyces*, *Cryptococcuss*), and the most comprehensive results come from the studies investigating hybridisation among species of the *Saccharomyces* sensu stricto complex (Boekhout et al., 2001; Bond et al., 2004; González et al., 2006; Gordon & Wolfe, 2006; Mallet et al., 2008; Peris et al., 2012a-b; Lopandic et al., 2013).

Saccharomyces sensu stricto is a group of phylogenetically closely related species (*S. arboricolus*, *S. bayanus*, *S. cariocanus*, *S. cerevisiae*, *S. eubayanus*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus*, *S. pastorianus*, *S. uvarum*) that show weak prezygotic barriers and under specific environmental conditions exchange genetic material, generating double or even triple hybrids (Pulvirenti et al., 2000; Nguyen et al., 2005; Rainieri et al., 2006). Interspecies hybridisation generates genomes of increased size, which evolve and stabilize over evolutionary time. These processes imply gross chromosomal rearrangements, translocations, inversions, and loss of DNA segments or entire chromosomes, resulting in aneuploid genomes (Bond et al., 2004; Peris et al., 2012a-b; Antunovics et al., 2005; Dunn & Sherlock, 2008; Belloch et al., 2009; Nakao et al., 2009). A high genetic diversity has been recognized among yeast hybrids isolated from

fermentations and natural environments (vineyards, oak trees, clinical material), indicating that genome reorganization and selective pressure have shaped the genomes in different ways. The majority of the characterized yeasts are hybrids between *S. cerevisiae* and *S. bayanus*, *S. eubayanus*, *S. uvarum*, *S. kudriavzevii* or *S. paradoxus* (Bradbury et al., 2006; González et al., 2006; Le Jeune et al., 2007; Lopandic et al., 2007; Wei et al., 2007; Zhang et al., 2010; Libkind et al., 2011). Among brewing, wine and cider yeast isolates triple hybrids have also been identified such as *S. cerevisiae* x *S. bayanus* x *S. kudriavzevii* and *S. cerevisiae* x *S. eubayanus* x *S. uvarum* (Masneuf et al, 1998; Naumova et al., 2005; González et al., 2006; Libkind et al., 2011; Nguyen et al., 2011). On the basis of the genome sequences it has recently been recognized that even the type strain of *S. bayanus* var. *bayanus* CBS380 is also a hybrid, containing a complex genome composed of *S. uvarum* and *S. eubayanus* sub-genomes, as well as some introgressed *S. cerevisiae* fragments (Nguyen et al., 2011). It can be assumed that many yeasts whose identification was based on rRNA coding sequences need to be re-examined by means of multigene sequences or even whole genome sequences. For a long time the taxonomy of yeasts as well as other microorganisms was based on the characterization of the conserved rRNA encoding genes, which in the majority of cases was unable to disclose the hybrid nature of a number of established species. One of the early recognized hybrid yeasts was *S. pastorianus*, well known as lager brewing yeast or *S. carlsbergensis*, which consists of *S. cerevisiae* and *S. bayanus*-like sub-genomes (Martini & Kurtzman, 1985). For a long time the non-*S. cerevisiae* genome portion was a subject of debate, since the various genetic and molecular markers suggested that it was closely related but not identical to *S. bayanus* species. An ecological study and comparative genomic analysis has recently identified *S. eubayanus*, which shows 99.5 % sequence identity with

the non-*S. cerevisiae* genome portion of *S. pastorianus* genome (Martini & Kurtzman, 1985). The complete sequenced genome of the commercial brewing yeast Weihenstephan 34/70 has shown that its genome contains three types of chromosomes, namely those which are characteristic for the parental species *S. cerevisiae* and *S. bayanus*, and eight chimera chromosomes (Nakao et al., 2009). These complex mosaic chromosomal structures have also been demonstrated by some *S. cerevisiae* x *S. kudriavzevii* hybrids isolated from beer, wine and vineyards (Belloch et al., 2009; Peris et al., 2012a-b). The whole genome sequence of the commercial strain VIN7 has shown that the strain is an allotriploid interspecies hybrid that contains a diploid *S. cerevisiae* and a haploid *S. kudriavzevii* genome (Borneman et al., 2011). The rearrangement of parental chromosomes with several reciprocal translocations and chromosomal substitutions, and with minimal loss of the *S. kudriavzevii* genome, indicated that the genome of VIN7 is undergoing consolidation.

Interspecies hybridisation and introgression increase genetic variations in natural yeast populations and have a great impact on their metabolic diversity. It is well known that the hybrids containing *S. uvarum* or *S. kudriavzevii* subgenome can better adapt to low-temperature fermentations, whereas the *S. cerevisiae* parental part accomplishes sugar fermentation more efficiently (Belloch et al., 2009, Querol & Bond, 2009). Different studies have also demonstrated the influence of environmental pressure on the acquisition of new advantageous attributes. Galeote et al. (2010) have recently identified the FSY1 gene in the EC1118 wine yeast that was acquired from another *Saccharomyces* species. The Fsy1p was characterized as a high-affinity fructose/H+ symporter with kinetic properties similar to those of *S. pastorianus* Fsy1p. This protein should play an important role in

alcoholic fermentations, especially at the end stages, as the FSY1 gene is highly expressed at high ethanol and low glucose and fructose concentrations. Brewery's yeasts may show different abilities to ferment maltose and maltotriose, a property which is extremely important for beer production. These different attributes have recently been explained by copy number differences of the MAL genes and the presence of the AGT1 permease gene in *S. pastorianus* strains that demonstrated an efficient utilization of maltotriose (Duval et al., 2010). Two commercial *S. cerevisiae* x *S. kudriavzevii* hybrid strains showed an increased production of higher alcohols in comparison to a commercial *S. cerevisiae* strain, suggesting the importance of hybrid strains for the enological properties of wines (González et al., 2007).

On the other hand, *S. cerevisiae* x *S. kudriavzevii* hybrids isolated from Austrian vineyards produced wines with increased ester concentrations, and the fermentation of sugars was more efficient when compared to the parental strains (Lopandic et al., 2007; Gangl et al., 2009). As a number of interspecies hybrids have been isolated in fermentation environments, it seems that these specific conditions (anaerobiosis, high alcohol and sugar concentration, high osmotic and hydrostatic pressure, and low temperature) induce their generation as a possible mode of adaptation (Bond, 2009). As the above-mentioned examples show, a number of yeast hybrids may show superior phenotypic properties in comparison to the parental strains, a fact that can be used in different biotechnological applications.

The impact of chromosomal rearrangements on the physiological properties of hybrids is difficult to monitor in natural yeast isolates, as their parents are unknown. We hypothesized that the extent of genomic changes after hybridisation event depends on the hybridisation partners and that the hybrid

offspring may develop quite different genetic and physiological properties compared to their ancestors. To evaluate the impact of the parental strains on the geno-and phenotype, we constructed a number of *S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii* hybrids, and examined the changes to their genomes in the first filial generation as well as the contribution of these changes to physiological alterations. Our results demonstrated that the F1 offspring show different genotyping patterns as well as different chromosomal make-up in comparison to the hybrid and parental cells, suggesting that the genomes underwent distinct alterations after the hybridisation event. The *S. cerevisiae* x *S. kudriavzevii* hybrid genome underwent more extensive modifications than the *S. cerevisiae* x *S. uvarum* genome, and these changes apparently influenced the phenotypic attributes of the first filial generation. Natural yeast hybrids demonstrated greater genotypic variability in comparison to artificial hybrid yeasts, and considerable physiological differences were also observed under some conditions.

Materials and Methods

Yeast strains.

The *Saccharomyces* strains examined in this study are listed in Table 1. The strains *S. cerevisiae* HA2796, *S. uvarum* HA2786 and *S. kudriavzevii* HA2787 were used to generate auxotrophic mutants, hybridisation experiments and hybrid isolation. The corresponding type strains were used to check their identity. The auxotrophic mutants were created using UV irradiation as described by Antunovics et al. (2005), and those of *S. kudriavzevii* HA2787 were generated using alpha-aminoadipate as described by Arroyo-López et al. (2011).

Table 1. Yeast strains used in the present study

Species	Strain designation[a]		Isolation source	Genotypic properties
	ACBR[b]	Other[c]		
S. cerevisiae	HA2764[T]	CBS8803[T]	S288C laboratory strain	
S. cerevisiae	HA2796	DEG 10-170	derivative of ATCC 204891	*MAT*a, leu$^-$, heterothallic
S. uvarum	HA231[T]	CBS395[T]	juice of Ribes nigrum, Netherlands	
S. uvarum	HA2786	DEG 10-522	wine must, Tokaj wine region	ura$^-$, homothallic
S. kudriavzevii	HA2261[T]	CBS8840[T]	decayed leaf, Japan	
S. kudriavzevii	HA2787	DEG 10-643	derivative of CBS8840[T]	lys$^-$, homothallic
S. cerevisiae x S. uvarum	HA2797	DEG H10		HA2796 x HA2786
S. cerevisiae x S. uvarum	HA2560		wastewater, Mongolia	
S. cerevisiae x S. kudriavzevii	HA2828			HA2796 x HA2787
S. cerevisiae x S. kudriavzevii	HA1836		grape, Thermenregion, Austria	
S. cerevisiae x S. kudriavzevii	HA1842		grape, Thermenregion, Austria	
S. cerevisiae x S. kudriavzevii	HA2654		Uvaferm CS2, Lallemand Inc.	

Table 1: [a]T=type strain, [b]ACBR: Austrian Center of Biological Resources and Applied Mycology, Muthgasse 11, 1190 Vienna, Austria, [c]CBS: Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands; DEG: Department of Genetics and Applied Microbiology, University of Debrecen, Hungary

Generation of interspecies hybrids.

The homothallic diploid strains (HA2786, HA2787) to be mated with the haploid *S. cerevisiae* HA2796 strain were grown on a sporulation medium (1 % potassium acetate, 0.1 % yeast extract, 0.05 % glucose, 2 % agar) at 26 °C for three days. A loop of the cell material was treated with 200 µg/ml Zymoliase 20T (MP Biomedical, Aurora, Ohio, USA) at 37 °C for 15 to 30 minutes depending on the sensitivity of the ascus walls. To obtain the interspecies hybrids, the strains with different auxotrophies were mixed in liquid GYP and incubated with shaking overnight. Samples of the hybridised strains were spread onto minimal medium plates (0.5 % (NH4)2SO4, 0.1 % KH2PO4, 0.05 % MgSO4 x 7H2O, 1 % glucose, 1 % vitamin mix and 2 % agar) and incubated at 25°C for 3 to 5 days. The hybrid nature of the grown yeast cultures was verified by means of the amplified fragment length polymorphism (AFLP) technique and pulsed-field gel electrophoresis.

Sporulation and ascus dissection of hybrid yeasts.

In order to obtain F1 offspring tetrads, cultures of the hybrid strains were inoculated onto potassium acetate sporulation agar medium and incubated at 22 °C for 5 days. Ascospore formation was monitored in a drop of water on a microscope slide at 400x magnification. Strains unable to sporulate at these conditions were also tested at 12 °C on the same medium and on sodium acetate sporulation medium (0.82 % sodium acetate, 0.19 % KCl, 0.25 % yeast extract, 0.1 % glucose, 2 % agar) at both temperatures. Strains unable to produce spores in any of these conditions were crossed with *MAT*a and *MAT*α tester strains to test if they were heterothallic. Sporulated cultures were treated with Zymoliase 20T at 35 °C for 15 to 30 minutes and 20 µl was transferred onto a GYP agar plate. Tetrads were isolated and dissected by micromanipulator (Zeiss, Germany). The GYP plates were incubated at 25 °C

for a week. Spore viability was calculated from ten tetrads and from each hybrid a fully viable tetrad was chosen for further geno- and phenotypic characterization.

AFLP fingerprinting.

The genetic variability of the *Saccharomyces* hybrids was investigated by means of the amplified fragment length polymorphism (AFLP) technique using the AFLP^{TM} Microbial Fingerprinting kit of Applied Biosystems (Foster City, CA, USA). The restriction/ligation steps, as well as the preselective and selective amplifications, were carried out as described by Lopandic et al. (2007) with minor modifications. One primer pair, EcoRI-AC-FAM/MseI-C, was used for selective amplification. The fragments generated were analysed by electrophoresis on a 50 cm capillary column by an ABI 3130 genetic analyser (Applied Biosystems). GeneMapper v.4.0 software was used for extraction and comparison of the resulting electropherograms, and the Excel® macro program (Rinehart, 2004) was used to convert the output into binary files. The tree topology was inferred using the simple matching genetic distance estimation and the UPGMA clustering method of the software Treecon version 1.3b (van de Peer & de Watcher, 1994). Bootstrapping was performed with 1000 replicates.

Karyotype analysis by pulsed field gel electrophoresis.

Karyotype analysis was carried out according to Antunovics et al. (2005). Separation of chromosomes was performed in a 1 % agarose gel (chromosomal grade, BioRad, CA) by a counter-clamped homogenous electric field electrophoresis (CHEF-Mapper; Bio-Rad). The following running parameters were used: run time 24 hours, voltage 6 V/cm, angle 120°, temperature 14 °C and pulse parameters 60 to 120 seconds. Gels were

stained with ethidium bromide and washed in sterile water thereafter for 48 hours before photographing with UV-transillumination.

Phenotypic assays.

Ability of yeasts to grow on different carbohydrates and at altered concentrations of sodium chloride, urea and miconazole was tested with yeast nitrogen base medium (YNB, pH 5.6) containing 0.67 % yeast nitrogen base and 0.2 % amino acid mixture (ForMedium, Norfolk, UK). In order to test the yeast's ability to assimilate different carbohydrates, the YNB medium was supplemented with 2 % glucose, raffinose or maltrotriose. Yeast growth at different sodium chloride (2.5 %, 5 %, 10 %), urea (1 %, 2 %, 5 %) and miconazole (0.01 µg/mL, 0.1 µg/mL, 0.5 µg/mL) concentrations were carried out in YNB medium supplemented with 2 % glucose. Yeast cultures were pre-grown on a starvation medium (0.1 % glucose, 1 % peptone, 0.5 % yeast extract, 2 % agar) at 25 °C for 48 hours and diluted with YNB medium to achieve an absorbance A750 of 0.25. The media with the indicated test substances were inoculated with 1000 cells/mL and 200 µL were transferred into 96-well plates (Greiner, Germany) and incubated at 25 °C for several days. Yeast growth was measured by a microplate reader (Tecan Infinite M1000, Männedorf, Switzerland) at 750 nm using orbital shaking between measurements. All measurements were performed in quadruplicate and were reproducible. The mean values were calculated and the values at t0 were subtracted from the values measured at different times to minimize the influence of different cell concentrations on the final results.

Results and Discussion.

Genetic analyses of hybrid yeasts demonstrated that the alloploid genomes can undergo drastic changes during meiotic and mitotic divisions of the hybrid cells (Sipiczki, 2008; Sipiczki, 2011). Hybrid yeasts undergoing meiosis and producing viable offspring plausibly exhibit more genotypic and phenotypic changes from one generation to another than the clonally reproducing ones, although mitosis may also generate novel geno- and phenotypes over the course of many generations. Antunovics et al. (2005) demonstrated by means of different genetic and molecular markers that hybrid genomes undergo a gradual reduction over several meiotic divisions. Genomic analyses of a number of natural hybrids also demonstrated the loss of distinct portions of the parent genomes (Dunn et al., 2008; Belloch et al., 2009; Peris et al., 2012a-b). The genome stabilization processes affect the genome constitution, ploidy, fertility, spore viability and physiology (Sipiczki, 2008; Sipiczki, 2011). In the course of this study we constructed a number of *Saccharomyces* interspecies hybrids and selected one of each *S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii* strain in order to monitor and correlate the changes of geno- and phenotypes in the F1 generation relative to their ancestors.

Generation and fertility controlling of interspecies hybrids and their offspring.

Interspecies *S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii* hybrids were created by crossing auxotrophic parental strains (a heterothallic *S. cerevisiae* and a homothallic *S. uvarum*/ *S. kudriavzevii* strain) and selected on the bases of their prototrophy. During this study numerous hybrid strains were generated and their ability to produce four-spore tetrads was

simultaneously tested. Among the hybrids created many were unable to sporulate and many produced unviable spores or only partially viable tetrads. We selected the *S. cerevisiae* x *S. uvarum* HA2797 and *S. cerevisiae* x *S. kudriavzevii* HA2828 strains due to their ability to produce fully viable tetrads. Both of these hybrids produced a similar number of asci after 5 days on potassium acetate medium: 38 % of HA2797 and 36 % of HA2828 cells created asci. However, while the spore viability of the former hybrid was very high at 98 %, only 53 % of the spores of the latter hybrid were viable. In both the chosen tetrads, leu- auxotrophy of the *S. cerevisiae* parental strain segregated in a 2:2 manner (HA2797 F1b and F1c, HA2828 F1 c and F1d were auxotrophic). As previously described HA2797 F1b and F1c spore clones were fertile, capable of generating viable offspring tetrads, while the other two F1 strains were non-sporulating (Pfliegler et al., 2012). The *S. cerevisiae* x *S. kudriavzevii* HA2828 F1b, F1c and F1d spore clones were found to be non-sporulating under any tested conditions (they were also unable to mate with a or α mating tester strains), while F1a produced tetrads with fully viable F2 spores. Therefore, the segregation of fertility was 2:2 in the *S. cerevisiae* x *S. uvarum* and 1:3 in the *S. cerevisiae* x *S. kudriavzevii* hybrid. Highly viable F3 generations were also isolated from all viable F2 strains, showing that fertility and viability were stably inherited into the next generations in both hybrids. Sporulation and spore viability of natural hybrids were also tested. The *S. cerevisiae* x *S. uvarum* natural hybrid HA2560 showed no sporulation or mating with testers and the *S. cerevisiae* x *S. kudriavzevii* natural hybrid HA2654 produced only deformed asci with non-separable and unviable spores. The other *S. cerevisiae* x *S. kudriavzevii* natural hybrids (HA1836, HA1842) were able to sporulate, but the spores were inviable.

Estimation of genomic alterations in the hybrids and their meiotic segregants.

Using anonymous AFLP markers and karyotyping analysis, we estimated variations of both the parental genomes in the hybrids and their F1 segregants. The genome alterations were qualitatively very simply observable by AFLP markers, the amplification of which is directly affected by point mutations, deletions, insertions and duplications. Figure 1. shows a cluster analysis of the hybrid and parental yeast strains based on the AFLP genotyping analysis. Two main clusters were recognized that correlate with two hybrid types studied, namely *S. cerevisiae* x *S. kudriavzevii* and *S. cerevisiae* x *S. uvarum*. The resulting tetrads of both the hybrid types segregated in a 2:2 manner for the parental AFLP markers. Natural yeast hybrids were closely related to the corresponding experimental hybrids, but at the same time demonstrated significant genetic variations. The AFLP analysis of the *S. cerevisiae* x *S. uvarum* HA2797 and *S. cerevisiae* x *S. kudriavzevii* HA2828 hybrid strains generated 80 and 77 fragments respectively, between 60 and 450 bp. A pairwise comparison of the banding patterns revealed that the hybrid yeasts contained almost all AFLP markers that are diagnostic for their parental strains. As Tables 2. and 3. show, the interspecies hybridisation event was followed by a loss of several markers of *S. cerevisiae* and non-*S. cerevisiae* origins in both the hybrids. Additional fragments, designated as gained as they were not detected in the electropherograms of any of the parental strains, were also observed. The process of the genome changes was observed in all the spore clones and was characterized by losses and gains of some additional AFLP markers (Tables 2 and 3). We believe that the changes of the AFLP profiles are correlated mainly with the alterations in the DNA sequences that can be influenced by chromosomal rearrangements, translocations, inversions and gene mutations.

The spore clones lost many more AFLP markers than the corresponding hybrid strains, suggesting that the additional more serious genomic reorganizations followed meiosis. Interestingly, the meiotic segregants of *S. cerevisiae* x *S. kudriavzevii* HA2828 hybrid underwent more extensive alterations than those of *S. cerevisiae* x *S. uvarum* HA2797 hybrid (Table 2 and 3). Particularly the *S. kudriavzevii* subgenome underwent major changes, as could be judged from the greater number of lost AFLP markers. Similarly, the chromosomal changes in the *S. cerevisiae* x *S. uvarum* hybrid F1 segregants were minor, limited to the loss of the chromosome 2, while the *S. cerevisiae* x *S. kudriavzevii* hybrids exhibited major changes in the karyotypes of the F1 generation (Fig. 2a, b). Even the HA2828 hybrid itself seemed to lack chromosome XV of the *S. kudriavzevii* subgenome, or at least distinct rearrangements induced the loss of large DNA segments, changing the size and the mobility of the chromosomes in the agarose gel. Besides the loss or alteration of the *S. kudriavzevii* parental chromosomes XV, the genome stabilization of the *S. cerevisiae* x *S. kudriavzevii* HA2828 hybrid strain resulted in the loss of the chromosomes V and VIII in F1a and F1b, chromosome X in F1b, F1c and F1d, as well as chromosome XII in F1a, F1b and F1d spore clones (Fig. 2b). As already mentioned, the *S. cerevisiae* x *S. uvarum* hybrid HA2797 displayed 98 % spore viability, and the *S. cerevisiae* x *S. kudriavzevii* HA2828 only 53 %. This difference may be related to the fact that the former hybrid produced F1 strains with much more consistent AFLP patterns and karyotypes than the latter. These results also indicated that meiosis operated with a more precise outcome in the *S. cerevisiae* x *S. uvarum* HA2797 than in the *S. cerevisiae* x *S. kudriavzevii* HA2828 hybrid. This is also supported by the observations of the other examined tetrads of HA2797 (Pfliegler et al., 2012), as well as by the karyotype analyses of additional HA2828 F1 segregants (not shown). The lower spore viability of

HA2828 may in fact be the result of the inability of the chromosomes to pair, preventing normal meiotic division and generating incorrect meiotic products. It has recently been shown that the interspecies barrier of F1 sterility in the *S. cerevisiae* x *S. uvarum* artificial hybrids can be broken down by the loss of the chromosome of the *S. uvarum* parental species that bears the mating type *MAT*-allele. The resulting alloaneuploid strains, which only possess one *MAT*-allele from the *S. cerevisiae* parent, are capable of mating-type switch and zygote formation, enabling correct meiosis and the generation of F2 hybrid offspring, resulting in heritable fertility (Pfliegler et al., 2012).

	***S. cerevisiae* x *S. uvarum* HA2797 hybrid**	
***S. cerevisiae* alleles lost**	121 139	
***S. uvarum* alleles lost**	148 259	
AFLP alleles gained	77 81	
Tetrad F1	**F1a=F1d**	**F1b=F1c**
***S. cerevisiae* alleles lost**	81 121 139	81 121 139
***S. uvarum* alleles lost**	148 259	148 219 259
AFLP alleles gained	77 81	77 298 323 349
	***S. cerevisiae* x *S. uvarum* HA2560 native hybrid**	
***S. cerevisiae* alleles lost**	81 108 121 215 310 311	
***S. uvarum* alleles lost**	63 66 90 120 137 152 161 192 217 233 234 248 252 259 320 322 330 359 375 385 386 387 440	
AFLP alleles gained	77 81 91 107 163 176 201 299 233 323 332 349	

Table 2. Changes of the AFLP patterns of the *S. cerevisiae x S. uvarum* artificial hybrid strain HA2797 and its offspring. As a comparison one natural hybrid isolate (HA2560) was used. All numerals indicate the sizes (bp) of the amplified fragments.

	S. cerevisiae x *S. kudriavzevii* HA2828 hybrid	
***S. cerevisiae* alleles lost**	423 446	
***S. kudriavzevii* alleles lost**	102 183 204 272	
AFLP alleles gained	68 323 349	
Tetrad F1	**F1a=F1b**	**F1c=F1d**
***S. cerevisiae* alleles lost**	139 423 446	139 423 446
***S. kudriavzevii* alleles lost**	69 87 88 89 91 97 102 111 179 183	63 65 69 77 102 110 119 134 160 172 183
	202 204 210 270 272 297 370 376 399	199 204 222 269 272 290 304 340 337 365 367
AFLP alleles gained	323 349	68 172 323 349
	***S. cerevisiae* x *S. kudriavzevii* native hybrids**	
***S. cerevisiae* alleles lost**		
1836	83 187 310 311	
1842	83 187 310 311 446	
2654	187 310 311	
***S. kudriavzevii* alleles lost**		
1836	69 102 183 204 270 304 376	
1842	69 102 183 204 270 304 376	
2654	69 102 183 204 270 304 367 376	
AFLP alleles gained		
1836	68 82 95 146 172 186 201 262 314 336 359 374 402 441 442	
1842	68 82 84 95 129 146 172 186 201 262 314 323 336 349 359 374	
2654	68 95 146 172 186 201 314 323 349 359 374 441 442	

Table 3. Changes of the AFLP patterns of the *S. cerevisiae x S. kudriavzevii* artificial hybrid strain HA2828 and its offspring. As a comparison two natural hybrid isolates (HA1836, HA1842) and one commercial strain (HA2654) were used. All numerals indicate the sizes (bp) of the amplified fragments.

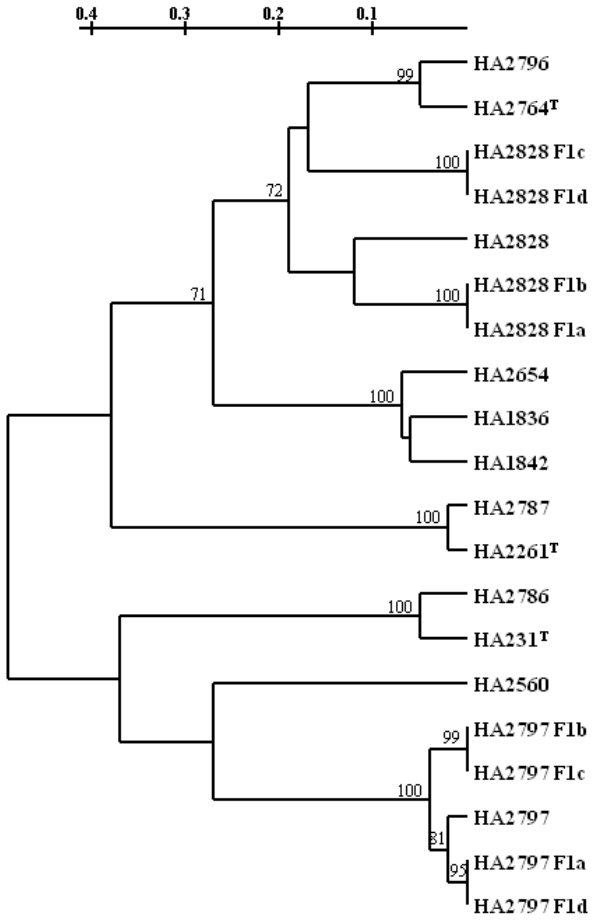

Fig. 1. Cluster analysis based on the AFLP molecular markers depicting genomic similarity between artificial and natural hybrid yeasts and parental strains. The scale bar indicates genetic distances. Bootstrap analysis was carried out with 1000 replicates, and values above 70% are given at nodes.

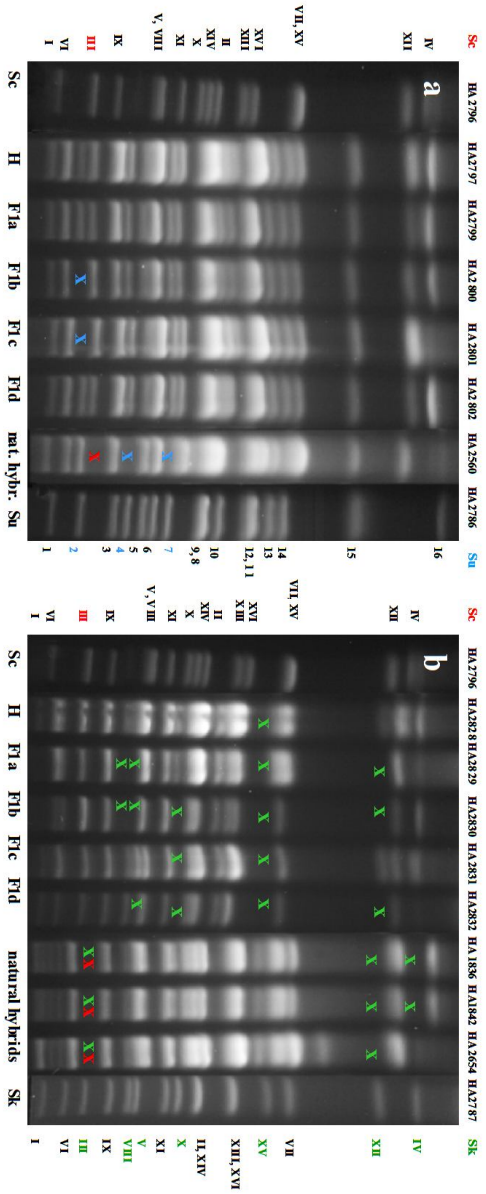

Fig. 2. Chromosomal patterns of the *S. cerevisiae* x *S. uvarum* (a) and the *S. cerevisiae* x *S. kudriavzevii* (b) artificial and natural hybrids. Chromosome bands of *S. cerevisiae* and *S. kudriavzevii* are indicated in Roman numerals, and those of *S. uvarum* in Arabic numerals. The missing chromosomes or those with supposed altered sizes are indicated with crosses.

In order to assess the extent of genomic variations in natural hybrids in comparison to the artificial ones, we assumed that their parental strains are different but closely related to *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* strains which were used for constructing the artificial hybrids (Fig. 1). The genome of *S. cerevisiae* x *S. uvarum* HA2560, isolated from wastewater, was characterized by a greater number of lost and gained AFLP markers in comparison to the artificial hybrid HA2797 and its progeny (Table 2). An increase in the number of lost *S. uvarum*-like AFLP alleles was particularly observed in the HA2560 strain. The *S. cerevisiae* x *S. uvarum* HA2560 isolate also showed a different chromosomal pattern when compared to those of the artificial hybrids (Fig. 2a). More chromosomes with altered size (*S. cerevisiae* chromosome III, and *S. uvarum* chromosomes 4 and 7) are identified in comparison to those of the artificial hybrids. The genomes of three wine-associated *S. cerevisiae* x *S. kudriavzevii* hybrid strains lost more AFLP markers from both parental genomes in comparison to the artificial hybrid HA2828, but the number of lost *S. kudriavzevii*-like AFLP markers was less when compared to those in the HA2828 F1 strains (Table 3). The most significant differences between the artificial and natural *S. cerevisiae* x *S. kudriavzevii* hybrids were observed in the form of a greater number of the new AFLP markers acquired by the natural strains. Natural hybrids also demonstrated a significant chromosomal polymorphism in comparison to the artificial ones (Fig. 2b). *Saccharomyces kudriavzevii*-like chromosomes III, IV and XII appear to be absent, or their size was changed by the genome modifications. An additional large chromosome between the chromosomes VII and XII was also observed in the commercially available *S. cerevisiae* x *S. kudriavzevii* HA2654 strain, suggesting chromosomal translocations or brakes.

All these results indicated that the genomes of the natural hybrid isolates underwent more severe reorganizations under environmental pressures. However, as in the case of the *S. cerevisiae* x *S. uvarum* HA2560 strain, it should be borne in mind that the true parental strains of the *S. cerevisiae* x *S. kudriavzevii* natural hybrids are unknown. Natural *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* strains may show rather heterogenous karyotypes (e.g. Csoma et al., 2010; Lopes et al., 2010), so that their direct comparison with the strains used in the present study should be interpreted carefully. Most likely the fertility is not a key factor for the survival of hybrids in natural, wine or brewing environments, as many hybrids from these habitats are sterile, as the four natural hybrids analysed in this study also are. These clonal (sterile) hybrids may loose some chromosomes of their alloploid chromosome sets in the course of their mitotical generations, but the rare fertile hybrids are probably much more prone to large-scale genomic changes that could be mediated by the recombination mechanism during meiosis (Belloch et al., 2009).

We used microarray-based comparative genomic hybridisation (CGH) to evaluate global changes in genomic DNA of the natural hybrid yeasts *S. cerevisiae* x *S. uvarum* HA2560 and *S. cerevisiae* x *S. kudriavzevii* HA1836 (not shown). Our preliminary results of CGH analysis, where the genome of the natural hybrid yeast was compared with the laboratory haploid *S. cerevisiae* strain S288C, identified a number of locations on chromosomes (III, V, VI, VII, VIII, XVI) where changes in ratio of hybridisation (ROH) were observed. Such changes in ROH are indicative of recombination sites between parental chromosomes or locations where gene deletion or amplification have occurred. Similar results were obtained by Bond et al (Bond et al., 2004), who used CGH analysis to elucidate DNA copy number

changes in two bottom-fermenting lager strains (*S. pastorianus*). The specific points with observed changes in ROH most likely represent intra- and/or inter-chromosomal recombinations between *S. cerevisiae* and *S. uvarum* homologous chromosomes. Our results also revealed chromosomes (III, VI, VIII, X, XI, XII, XV) with depleted DNA segments at subtelomeric regions. The low sequence homology with the control strain S288C indicated that the identified regions differ notably or are absent in natural hybrid yeasts. On the basis of these results we concluded that various chromosomal rearrangements may influence the karyotype of the HA2560 strain. It could be that the *S. cerevisiae* chromosomes with large depleted DNA segments changed their size and hence their position in agarose gel (Fig. 2a). The CGH analysis of the *S. cerevisiae* x *S. kudriavzevii* HA1836 strain against the *S. cerevisiae* S288C microarray did not reveal any large depleted DNA regions or changes in ROH compared to the *S. cerevisiae* x *S. uvarum* HA2560 strain (not shown). The investigated *S. cerevisiae* x *S. kudriavzevii* HA1836 natural strain contained a complete set of *S. cerevisiae* chromosomes, which appear most probably in two copies. Peris et al (2012a-b) recently analysed five additional hybrid yeasts isolated from Austrian vineyards and came to similar conclusions. The CGH analysis showed that one strain used in the present study (HA1842) contained all the *S. cerevisiae* and *S. kudriavzevii* chromosomes. This corroborated our assumption that the parental chromosomes of the natural hybrids may show a considerable polymorphism, and they should not align perfectly with the reference strains (Fig. 2a,b).

Phenotypic properties of the hybrid yeasts.

After remarkable genomic alterations were observed, we wondered how these genomic changes would affect the physiological properties of the hybrid yeasts and their offspring relative to parental strains. We monitored the

fitness of the hybrid yeasts under conditions with different carbohydrates and increasing concentrations of osmolytic and antifungal compounds. The first observation was that *S. cerevisiae* species determined the growth rates of all investigated hybrids at almost all tested conditions. The inhibited growth was detected only on raffinose, where *S. cerevisiae* achieved around 67 % of the growth rate of *S. uvarum* after 96 hours (Fig. 3). The *S. cerevisiae* x *S. uvarum* HA2797 and *S. cerevisiae* x *S. kudriavzevii* HA2828 hybrids exhibited similar fitness in the majority of investigated conditions. The two hybrid strains showed noteworthy differences only in the assimilation of raffinose and their sensitivity against miconazole. The HA2828 utilized raffinose less efficiently than the HA2797 strain (Fig. 3) and showed an increased resistance against miconazole (Fig. 4). The inhibited growth of the *S. cerevisiae* x *S. kudriavzevii* HA2828 hybrid may be explained by a decreased ability of the parental strains to assimilate raffinose. On the other hand, a somewhat greater growth rate in comparison to their ancestors was shown by the *S. cerevisiae* x *S. uvarum* HA2797 hybrid and its offspring, which could be explained by a synergistic activity of both the parental strains. The meiotic segregants of the two hybrid types displayed quite different behaviors. Whereas the *S. cerevisiae* x *S. uvarum* HA2797 spore clones as well as a natural isolate HA2560 strain exhibited the same growth rate as the hybrid HA2797 with all the tested sugars, the *S. cerevisiae* x *S. kudriavzevii* HA2828 F1 segregants showed significant variability in their carbohydrate consumption (Fig. 3). Sugar utilization was strain dependent, in which the HA2828 strain always showed the highest growth rate at the level of *S. cerevisiae*. A remarkable increase in growth of the HA2828 hybrid strain was observed on glucose at the beginning of incubation (t24). A shorter lag and accelerating growth phase were also observed at 0.5 % glucose (not shown), indicating that the better growth was not a random but intrinsic attribute of

the *S. cerevisiae* x *S. kudriavzevii* HA2828 strain. The spore clones demonstrated different growth abilities; for instance, HA2828 F1a grew slowly and could not reach the growth level on glucose and raffinose of its ancestors after 96 and 144 hours respectively. This and the HA2828 F1d strain also showed delayed growth on maltotriose. As *S. kudriavzevii* showed no growth on maltotriose, the delayed growth of the HA2828 F1 strains may indicate that some changes in the *S. cerevisiae* genome influenced expression of the genes involved in the maltotriose metabolism.

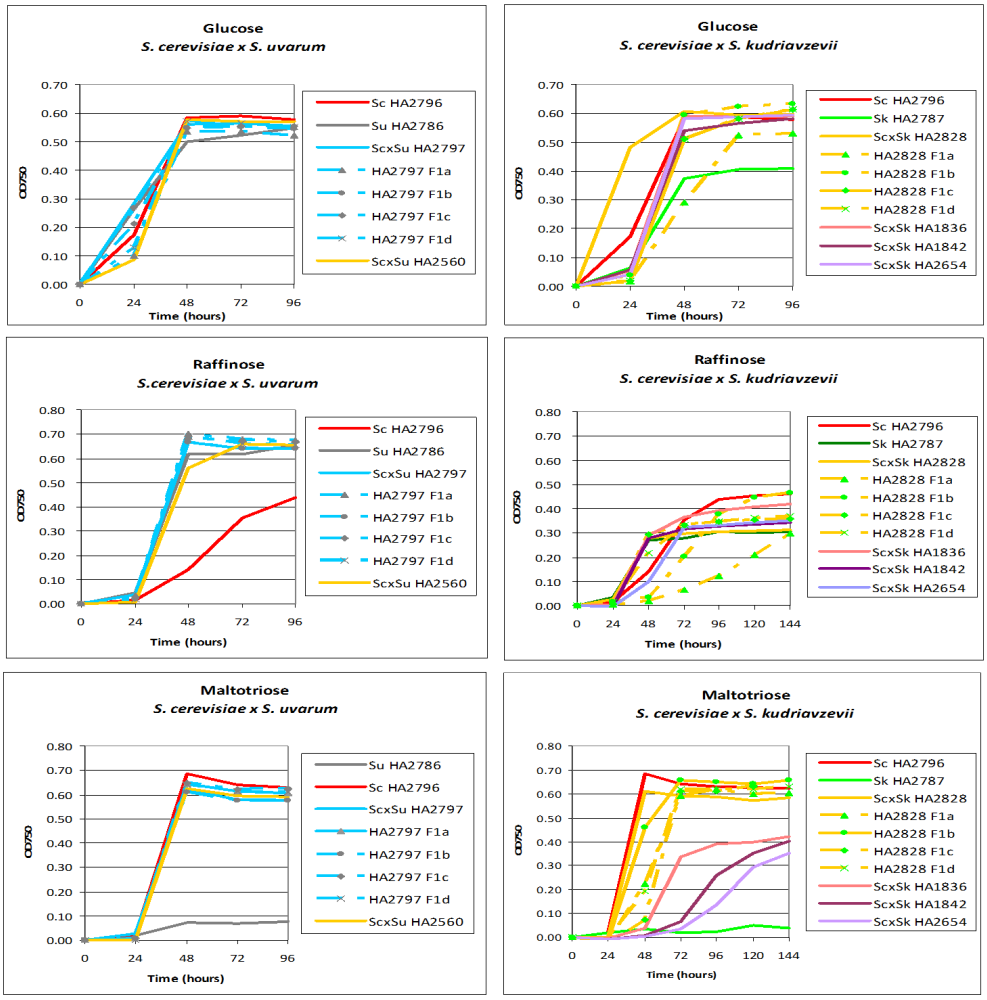

Fig. 3. Utilization of glucose, raffinose and maltotriose by *S. cerevisiae x S. uvarum* and *S. cerevisiae x S. kudriavzevii* strains.

Figure 4 shows the growth patterns of the interspecies hybrid yeasts at different concentrations of sodium chloride, urea and miconazole after 96 hours of incubation. In general, the growth of all investigated strains declined upon increasing the concentration of the osmolitic or antimicrobial substances in the medium, and the most significant differences among the strains were demonstrated at the highest concentrations. The artificial HA2797 and HA2828 hybrids demonstrated the ability to grow at 10 % sodium chloride and 5 % urea. The growth of HA2797 and HA2828 strains at an increased concentration of urea is also remarkable, because it was not inherited from any of the parental species, but was characterized as a novel phenotype. The *S. cerevisiae* x *S. uvarum* HA2797 F1 segregants exhibited a positive growth at 10 % sodium chloride, but the *S. cerevisiae* x *S. kudriavzevii* HA2828 F1 segregants showed an incomplete inheritance of the growth ability (only HA2828 F1d showed a positive growth). Similarly, the growth of all the *S. cerevisiae* x *S. kudriavzevii* HA2828 segregants was inhibited by 5 % urea. The *S. cerevisiae* x *S. uvarum* HA2797 F1 segregants demonstrated 2:2 segregation of urea phenotype. This phenotype correlated with the presence of the *S. uvarum* chromosome 2, the other two strains lacking this chromosome did not exhibit this phenotype (Fig. 2a).

Saccharomyces cerevisiae has frequently been used as a model organism for studying cellular and molecular mechanisms of osmolyte tolerance. Yeast cells accumulate glycerol to counteract high ion concentrations, and use proton gradients to control ion fluxes under stress (44-46). Less is known about the accumulation of glycerol and the maintenance of ion homeostasis in hybrid yeasts. As hybrid yeasts are exposed to similar stress in distinct natural, food and fermentation environments, it would be of interest to investigate how the non-*S. cerevisiae* parental fraction influences their adaptation to osmotic and ionic stress. In the stress conditions presented by

the high concentration of the antimicotic compound miconazole, the *S. cerevisiae* x *S. kudriavzevii* HA2828 hybrid demonstrated a considerable resistance comparable to its ancestors (Fig. 4). In the HA2828 F1 generation, growth capability varied greatly, from no growth to full tolerance, and an even somewhat better resistance was observed in the HA2828 F1a and F1d than in the parental strains. A comparison of these results with those generated by the *S. cerevisiae* x *S. uvarum* hybrids indicated that in addition to *S. cerevisiae*, *S. kudriavzevii* HA2787 played a significant role in the ability of the *S. cerevisiae* x *S. kudriavzevii* hybrids to develop resistance against miconazole (Fig. 4). By contrast, the *S. cerevisiae* x *S. uvarum* HA2797 and its offspring demonstrated a considerable sensitivity against miconazole, suggesting that one of the mechanisms regulating resistance against the antimicotic drug in this hybrid was affected by hybridisation (van den Bossche et al., 1978; Thevissen et al., 2007; Calahorra et al.; 2011).

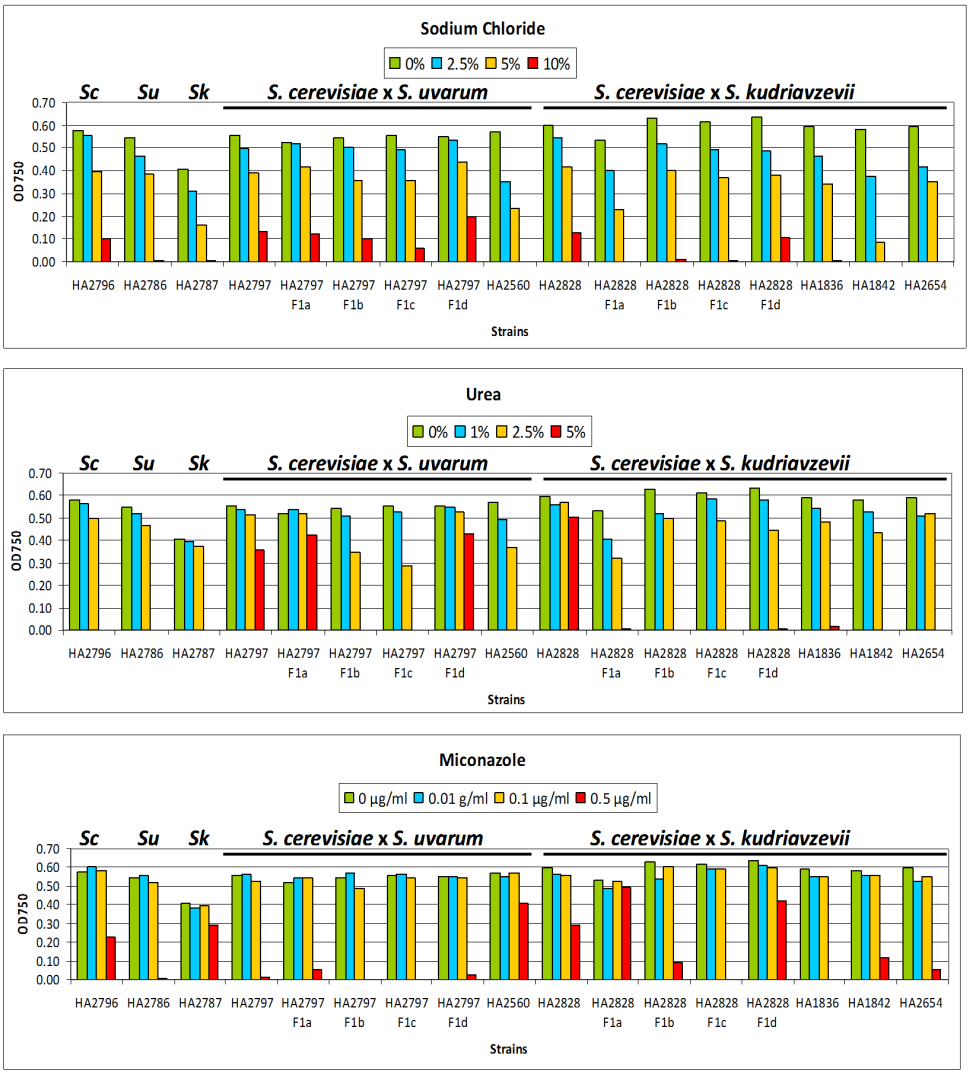

Fig. 4. Growth of the *S. cerevisiae x S. uvarum* and *S. cerevisiae x S. kudriavzevii* strains in the presence of increasing concentrations of sodium chloride, urea and miconazole.

Three wine associated strains HA1836, HA1842 and HA2654 displayed a very similar ability to consume glucose and raffinose as the artificial hybrid HA2828, but quite different profiles of maltotriose utilization (Fig. 3). These strains exhibited different growth rates compared to each other, and utilized maltotriose less efficiently than the artificial hybrids. As we already supposed above, the most reasonable explanation would be that the natural yeast isolates contain *S. cerevisiae* subgenomes of different origins that utilize maltotriose with different efficiency. Duval et al. (2010) have recently stressed the importance of the AGT1 gene for efficient maltotriose utilization in *Saccharomyces* strains. Our preliminary microarray-based CGH analysis indicated that the *S. cerevisiae* x *S. kudriavzevii* HA1836 hybrids had an increased copy number of MAL31 gene on chromosome II, encoding for maltose permease, and a depleted copy number of the AGT1 gene on chromosome VII in comparison to the control S288C *S. cerevisiae* strain, indicating that the AGT1 gene may be absent. This may explain a slow and delayed growth of the natural *S. cerevisiae* x *S. kudriavzevii* strains observed in the medium with maltotriose (Fig. 3). The same analysis showed that in addition to an increased copy number of MAL31 gene, *S. cerevisiae* x *S. uvarum* HA2560 strain had the permease encoding AGT1 gene too. Although these results need additional experimental verifications, they are in full agreement with the findings of Duval et al. (2010) and may likely also explain differences in maltotriose utilization between the natural and artificial *S. cerevisiae* x *S. kudriavzevii* strains.

The increased concentrations of sodium chloride and urea inhibited the growth of the natural *S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii* hybrid yeasts (Fig. 3). Differently from the artificial hybrids, the natural *S. cerevisiae* x *S. kudriavzevii* strains showed no or weak resistance to

0.5 µg/ml miconazole. On the other hand, the *S. cerevisiae* x *S. uvarum* HA2560 isolate from wastewater exhibited considerable growth in comparison to the corresponding artificial hybrids. As this strain was isolated from a specific ecological niche, which most likely also contains the azole antifungals among other antimicrobial substances, it could be assumed that this strain has developed resistance against miconazole over time.

Conclusions.

In the present study we used two *Saccharomyces* interspecies hybrids to correlate the evolution of their geno- and phenotypes. The results demonstrate that the hybridisation of different yeast genomes generate segregants with different viability, genomic and physiological properties. Relative to their ancestors, the hybrids stabilize their genomes and acquire similar or novel phenotypic attributes. The genomes of two species may recombine in such a way that the metabolic activity of the hybrid cells is influenced by one parental part, or both species may act synergistically, or even novel phenotypes may appear. Results of the present study suggest that the artificial *S. cerevisiae* x *S. kudriavzevii* HA2828 hybrid yeast underwent more extensive genome changes after meiotic segregation than the *S. cerevisiae* x *S. uvarum* HA2797, and the generation of the new genotypes obviously had a remarkable influence on phenotypic diversity. We believe that the observed phenotypic diversity may be attributed to genomic modifications of the former hybrids. Assuming that the hybrid strains examined here reached the environment, selective pressure would further modify their genomes. Distinct genotyping profiles and karyotypes in artificial and natural hybrid isolates, as well as an increase in miconazole resistance observed in the *S. cerevisiae* x *S. uvarum* HA2560 and decreased

maltotriose utilization ability identified in the *S. cerevisiae* x *S. kudriavzevii* natural hybrids, support the view that environmental growth conditions greatly determine genome constitution and gene expression. The importance and application of the studied yeasts in alcohol fermentations should be tested by additional experiments under circumstances characteristic for these processes. It can be assumed that the genomic alterations of the artificial hybrids will be more drastic in harsh fermentation than under laboratory conditions. This environment may increase the loss of genes and alter metabolic pathways which are important for fermenting yeasts. We suppose that the wine associated hybrids used in this study have lost important genes involved in maltotriose metabolism during their genome evolution. It was shown that the *S. cerevisiae* x *S. kudriavzevii* natural isolates from a vineyard (HA1836 and HA1842) and a commercial wine strain (HA2654) are less suitable for brewing, as deduced from their reduced ability to utilize maltotriose. Different behavior of the F1 segregants of the two hybrid types at increased concentrations of sodium chloride, urea and miconazole suggest that their tolerance mechanisms to osmolyte and antimicotic compounds should be investigated more comprehensively using the genome-wide screening approaches. In order to understand evolution of the phenotypic traits and genetic background of the differences which arise due to a hybridisation event in more detail, it would be of interest to explore the whole genome sequences in the hybrids and their progeny. Comparative analysis of the complete sequences can help link genetic changes to phenotypic modifications and better understand the role of specific genes in various metabolic and regulatory pathways.

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