INFLUENCE OF ARTIFICIAL INTERSPECIES YEAST HYBRIDS AND THEIR F1 OFFSPRING ON THE AROMA PROFILE OF WINE

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In the present study we describe the fermentation characteristics of some novel, artificial yeast hybrids of three *Saccharomyces* species, two of them (*S. cerevisiae* and S. *uvarum*) adapted to high osmotic, high alcohol conditions. The non-commercial parental strains, hybrids (*S. cerevisiae* x *S. uvarum*, *S. cerevisiae* x *S. kudriavzevii*, *S. kudriavzevii x S. uvarum*) and meiotic segregants (F1 offspring) were utilized for fermentation of high- and low-osmotic grape juice, the dynamics of which was observed and the aroma profile of all resulting wines was compared to discover whether some of the hybrids or segregants have favourable properties concerning wine flavour and taste. The analyzed artificial hybrids of the *Saccharomyces* yeast strains had promising properties for production of high-quality wine: good glucose and fructose utilization even under high-alcohol conditions, high fermentation rates and generally a pronounced metabolic power. The latter is precondition to high-level aroma constituent generation during fermentation. Depending on osmotic stress aroma profiles were very diverse but hybrids outperformed their parental yeast strains under both conditions, probably because they could use more metabolic pathways and maybe produced even more enzymes and to a higher quantity. This may hold for some of the segregants, too, which reached a high quality level concerning wine production. Genetic similarity pattern of the hybrids was not resembled by the one of wine characters (fermentation dynamics and aroma profile), the main reason of the discrepancy is the higher metabolic power of hybrids.
Keywords: *Saccharomyces cerevisiae, Saccharomyces uvarum, Saccharomyces kudriavzevii*, artificial hybrids, segregants, hybrid evolution, juice, wine, aroma profile

Der Einfluss von *Saccharomyces-Interspecies-Hybriden* und ihrer F1 Nachkommen auf das Aromaprofil von Wein. In der vorliegenden Arbeit werden die Fermentationseigenschaften einiger neuer artifizieller Hybriden der Hefe-Arten *Saccharomyces cerevisiae, S. uvarum* und *S. kudriavzevi*i beschrieben. Zwei der Parentalspecien (*S. cerevisiae* und *S. uvarum*) sind relativ gut an hohe Zucker- und Alkoholkonzentrationen angepasst, wenngleich keiner der benützten Stämme kommerziell als Reinzuchthefe Verwendung findet. Die Parentalstämme, Hybriden (*S. cerevisiae x S. uvarum, S. cerevisiae x S. kudriavzevii, S. kudriavzevii x S. uvarum*) und meiotischen Segreganten (Nachkommen der F1-Generation) wurden dazu verwendet, Traubensaft sowohl ohne als auch mit Zuckerzusatz (Saccharose) zu vergären, Letzteres, um einen höheren osmotischen Stress zu verursachen. Die Fermentationsdynamik wurde beobachtet und die Aromaprofile aller resultierenden Weine verglichen. Die Hybriden der *Saccharomyces*-Hefestämme hatten vielversprechende Eigenschaften für die Produktion von Weinen mit hoher Qualität: gute Glucose- und Fructoseverwertung selbst unter sehr schwierigen Bedingungen (hohe Alkoholkonzentration und osmotische Belastung), hohe Gärraten und insgesamt eine höhere metabolische Leistungsfähigkeit. Die chemischen Eigenschaften (z. B. Aromarofil) der durch Hybridfermentation erzeugten Weine waren nicht einfach eine Kombination der Merkmale der von den Parentalstämmen erzeugten Weine. Es ließ sich zeigen, dass dies darauf zurückzuführen ist, dass die metabolische Leistungsfähigkeit der Hybriden gegenüber den Parentalstämmen deutlich gesteigert ist. In Abhängigkeit von der benutzten Hefe und auch vom osmotischen Stress im Gärmedium waren die beobachteten Aromaprofile sehr divers.
Schlagwörter: *Saccharomyces cerevisiae, Saccharomyces uvarum, Saccharomyces kudriavzevii*, artifizielle Hybriden, Segreganten, Hybrid-Entwicklung, Saft, Wein, Aromaprofil

Humans have consumed alcoholic drinks for at least nine thousand years to benefit from their exhilarating and germicidal impact (McGovern et al., 1996, 2004; McGovern and Hall, 2013). As a sugar source for fermentation, grapes have been used since the Neolithic as well as rice and other cereals and diverse sources like honey and different kinds of fruits even earlier.

Because of their tolerance to high sugar and alcohol concentrations species of the yeast genus *Saccharomyces* are of highest importance for the production of wine, beer, cider and spirits. Most economically relevant is *Saccharomyces cerevisiae*. Beside this species the cryotolerant yeast *S. uvarum* is also widely used for wine and cider fermentation. Recently it turned out (Almeida et al., 2014) that a Patagonian sub-population of *S. uvarum* gave rise to the Holarctic population through a bottleneck not so long ago. Holarctic strains display multiple introgressions from other *Saccharomyces* species, especially those from *S. eubayanus* are common in European strains associated with human-driven fermentations. In contrast, the cryotolerant *S. kudriavzevii*, discovered by Naumov et al. in 2000, and other *Saccharomyces* species that are not adapted to high sugar and alcohol concentrations are currently of no significance for spirit production.

Selmecki et al. (2015) showed that in yeast polyploidy alone is sufficient to speed up evolution under stressful environmental conditions. The same may be true for genome expansion by interspecific hybridisation

where the basis of genetic variability is much higher than in simple polyploids and this may explain why interspecies hybridisation in yeasts can be observed so frequently.

Exchange of genetic information between species is not only characteristic for *S. uvarum* but seems prevalent in all species of *Saccharomyces.* The lager beer yeast *S. pastorianus* has been identified as hybrid consisting sub-ge-

nomes of *S. cerevisiae* and *S. eubayanus* (Nakao et al., 2009; Libkind et al., 2011; Pérez-Través et al., 2014). Gonzales (2006; 2007) and Lopandic et al. (2007) discovered natural hybrids of *S. cerevisiae* and *S. kudriavzevii*. Other natural species hybrids of this genus are known too.

Interspecies hybrids between yeasts of *Saccharomyces* are now widely produced by rare mating under laboratory conditions (Antunovics et al., 2005; Bellon et al., 2011; Pfliegler et al., 2012; Bellon et al., 2013). The hybrid genomes are not stable and show a rapid development under stress conditions and hence they are an ideal tool for fundamental research concerning the influence of environment on the evolution of hybrid genomes. Since hybrids may become important for industrial fermentation, one question of special interest in this context is how hybrid genomes evolve under high-sugar and high-alcohol conditions, if one of the parental species is adapted to this environment but the other is not. In a parallel paper we set the focus of our research in this direction (Lopandic et al., 2016), whereas here we are more interested in physiological adaptations of hybrids and their F1 segregants.

In juice fermentation the yeast uses the juice constituents to produce hundreds of aroma compounds (Cole and Noble,1997; Lambrecht and Pretorius, 2000; Fleet, 2003). Thus the yeast is as important as the juice for the flavour and taste of the resulting wine. From the aroma profile of a wine even an artificial wine taster, an Artificial Neural Net (ANN), is with a high certainty able to find out not only which grape variety was used, but also which yeast strain fermented it (Tiefenbrunner et al., 2009).

Gangl et al. (2009) compared natural hybrids with *S. cerevisiae, S. uvarum* and *S. kudriavzevii* concerning their fermentation and aroma profile characteristics and found higher concentrations of distinct constituents in *S. cerevisiae x S. kudriavzevii* hybrids. Bellon et al. (2011) recognized that some artificial hybrids produce wines with concentrations of aromatic products that are different to what is found in wines made by their parents, commercial wine yeasts, and have a favourable impact on flavour and aroma profile. Parental strains were from the species *S. cerevisiae*, *S. paradoxus* and *S. kudriavzevii.*

Here we use three species, two of them adapted to high-osmotic, high-alcohol conditions for creation of hybrids (*S. cerevisiae x S. uvarum, S. cerevisiae x S. kudriavzevii, S. kudriavzevii x S. uvarum*). Parental strains, hybrids and meiotic segregants (F1 offspring) were utilized for juice fermentation. Fermentation dynamics was observed and the aroma profile of all wines was compared to find out whether some of the hybrids or segregants have favourable properties concerning wine flavour and taste and whether this depends on the level of adaptation of the parental strains to high alcohol and sugar concentrations.

MATERIALS AND METHODS

YEAST STRAINS

Interspecies hybrids and their F1 meiotic offspring were generated according to Pfliegler et al. (2014) by crossing parental strains of three species, a heterothallic *S. cerevisiae*, and two homothallic strains, *S. kudriavzevii* or *S. uvarum*, respectively. None of the strains was specially adapted to wine production. Three hybrids were created: *S. cerevisiae* x *S. kudriavzevii, S. kudriavzevii x S. uvarum* and *S. cerevisiae x S. uvarum*.

AFLP GENOTYPING

In order to determine the genetic difference of parental strains, hybrids and segregants, the amplified fragment length polymorphism (AFLP) technique was performed as described in Lopandic et al. (2016). Only one primer pair was used for selective amplification and thus only a small part of the genome was analysed. For any two yeast genomes Dice's similarity was computed.

In order to convert the resulting binary files into a graphic representation tree topologies are commonly used. Those are adequate for diverging processes like standard evolution but obviously do not fit for converging events like hybridisation. Net methods, developed for presentation of conflicting phylogenies (Huson et al., 2010) would be more appropriate, but nevertheless determine the topology, too. To be free of fixed topologies, we used a back propagation Artificial Neural Network (ANN) that performs a Principal Component Analysis (PCA)

if the data are appropriate, but can also go beyond this and is able to compute even if some restrictions of the PCA appear (Hartung and Elpelt, 1999), e. g. if there are many more characters than objects, like in the binary AFLP data. The necessary algorithms are realized by ViDaX software for visual data exploration (LMS-Data, Trofaiach, Austria). As expected, the resulting topology was a circle.

MICROVINIFICATION

A pasteurised grape juice of the Austrian vine variety 'Grüner Veltliner' with an initial sugar concentration of 81.1 g/l fructose and 76.7 g/l glucose, a pH value of 3.24 and a refractometrically measured gradation of 13.5 °KMW was inoculated on June, 2nd, 2014 in separate flasks with the parental strains S. *cerevisiae, S. kudriavzevii, S. uvarum*, the three hybrids and their twelve F1 segregants (18 samples). Microvinifications were carried out in 300 ml Erlenmeyer flasks filled with 250 ml grape juice at a standardised temperature of 20 °C. The fermentation progress was monitored by determining the weight loss caused by the production of CO_2. Fermentation lasted for 15 days before wine chemical analysis started.
In a second experiment 227.5 g/l sucrose were added to the juice which rose the gradation to 26.5 °KMW to enhance the osmotic stress. Microvinification took place in the same way and after 21 days wine samples were taken for chemical analyses.
The resulting wines were tested olfactorily. Odour and taste of all wines were acceptable.

WINE CHEMICAL ANALYSIS

BASIC ANALYSIS

Chemical analysis of organic compounds of juice and wine, like ethyl alcohol and glycerol, sugars (fructose, glucose) and acids (titratable and volatile acids, tartaric, malic, citric and lactic acid) was performed following the OIV Compendium of International Methods of Analysis of Wines and Musts Vol. 1 and 2 (OIV, 2014). Methods for density determination were taken from the ALVA-Methodenbuch für Weinanalysen in Österreich (ALVA, 1984).

AROMA PROFILE

For analysis of volatile aroma compounds of the wine, gas chromatography-mass spectrometry (GC-MS) was used as described by Gangl et al. (2009).

RESULTS AND DISCUSSION

GENETIC SIMILARITY OF PARENTAL STRAINS, THEIR HYBRIDS AND THE F1 SEGREGANTS

AFLP was performed before fermentation and the results are described in detail in Lopandic et al. (2016). However, the main events following hybridization and meiotic segregation are presented in Figure 1.
Since every combination of two parental strains was created, in the PCA representation the parental strains and hybrid yeasts arrange themselves in a circle (actually it is an ellipse). For a more detailed analysis Dice's similarity $D_{x,y}$ was computed for all combinations of two yeasts x and y (Appendix 1). The genetic resemblance of the hybrids to the parental strains is not equal to both in *S.c.* x *S.u.* but higher to *S. cerevisiae* (D = 0.65 vs. D = 0.53 for *S. uvarum*). For *S.k.* x *S.u.* the similarity is higher to *S. kudriavzevii* (D = 0.73 vs. D = 0.56 for *S. uvarum*). In *S.c.* x *S.k. S. cerevisiae* is more similar to the hybrid (D = 0.77 vs. D = 0.67 for *S. kudriavzevii*). This situation is reflected in Figure 1.
From each zygote four segregants result, but they are pairwise genetically identical as far as can be concluded from the AFLP data (D = 1 for all pairs). Thus each segregant symbol in Figure 1 represents two F1 hybrid offspring. In the case of the *S.c.* x *S.u.*, the segregants remain relatively similar to the hybrid (D = 0.8 and D = 0.81, respectively) and to one another (D = 0.86), the similarity to *S. cerevisiae* is high too (D = 0.71). In the other segregants that descend from *S. kudriavzevii* the distance to the genome of this species is remarkably higher than to the second parental strain.
In the hybrid offspring of *S.k.* x *S.u.* one pair remains more similar to the hybrid than to *S. uvarum* (D = 0.79 vs. D = 0.69) whereas the other one shows more genetic resemblance with *S. uvarum* (D = 0.75) than with the hybrid (D = 0.71). Bearing in mind the hybrids' higher similarity to *S. kudriavzevii* (D = 0.73) compared with the one to *S.uvarum* (D = 0.56), the low similarity of the

hybrids F1 offspring to *S. kudriavzevii* (D = 0.37 and D = 0.5, respectively) is surprising.
In *S.c.* x *S.k.* the genome of all F1 hybrid offspring is more similar to the hybrid than to *S. cerevisiae* but Dice's similarity is very high for both (for the hybrid D = 0.88 and D = 0.86, respectively, and for *S. cerevisiae* D = 0.85 and D = 0.81, respectively).

JUICE FERMENTATION WITH PARENTAL YEAST STRAINS, HYBRIDS AND THEIR SEGREGANTS

All 18 yeast strains were used to ferment 'Grüner Veltliner' juice into wine, either with sucrose added or not. The resulting 36 wines were analysed concerning concentration of basic chemical constituents like alcohol, sugars and acids, the physical property density, pH-value (Table 1) and of 32 important aroma compounds that have a significant influence on flavour and taste (Table 3).

BASIC CHEMICAL ANALYSIS OF THE WINES AND FERMENTATION PARAMETERS

Some basic parameters are physiologically connected via anaerobe glycolysis: ethyl alcohol and CO_2 are its products, glucose and fructose its educts. Thus it is not surprising that these parameters belong to the same correlation cluster (Table 1). Pro- and educts are negatively correlated whereas the correlation within the products is positive as well as within the educts (which is less self-evident though the yeasts are glucophilic).
Beside this, density is correlated negatively with ethyl alcohol and CO_2, but positively with glucose, fructose and malic acid. Furthermore in wine from sucrose added juice, malic acid and fermentation rate are negatively correlated.

Table 1: Correlation of basic chemical and fermentation parameters. Analysis was made separately for unmodified must (-) and must with sucrose added (+). Two parameters that correlate with a coefficient of more than 0.8 (regardless whether it is positive or negative) have both a grey field in the right column. Correlation between parameters where one is denoted by a "X" in the column and the other one is without, is negative.

	Saccharose	
	(-)	(+)
density		
ethyl alcohol	x	x
fructose		
glucose		
titratable acid		
pH		
volatile acids		
tartaric acid		
malic acid		
glycerol		
exp (fermentation rate)		x
CO_2 production	x	x

correlation cluster R > 0.8

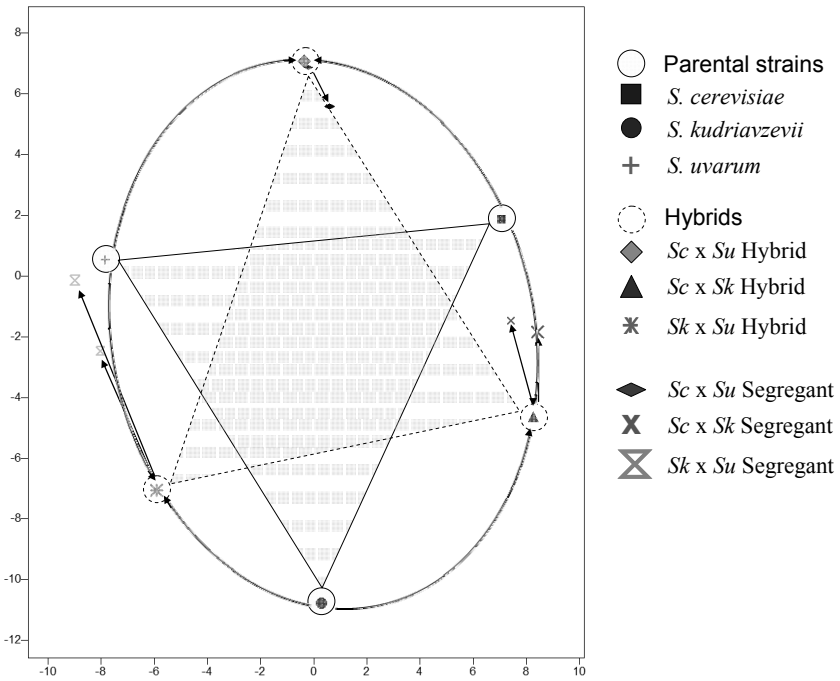


Fig. 1: Principal component analysis of AFLP data of the genomes of parental strains, hybrids and F1 hybrid offspring using an ANN. The first three principal components are shown, the third one is imaginable by the different size of the symbols. Arrows reflect the genealogy. Parental strains and hybrids, respectively, are connected to each other by a triangle to simplify comparison with Figure 2 and Figure 3 (tree presentation of genetic similarity turned out not to be useful for comparison of AFLP data and the ones of aroma constituents and basic chemical parameters). For a more conventional tree representation of the AFLP data see Figure 1 of Lopandic et al. (2016).

Table 2: Relative metabolic power (rMP) of the yeast strains. Hybrids are marked with "H", the F1 segregant pairs with "1" or "2", respectively.

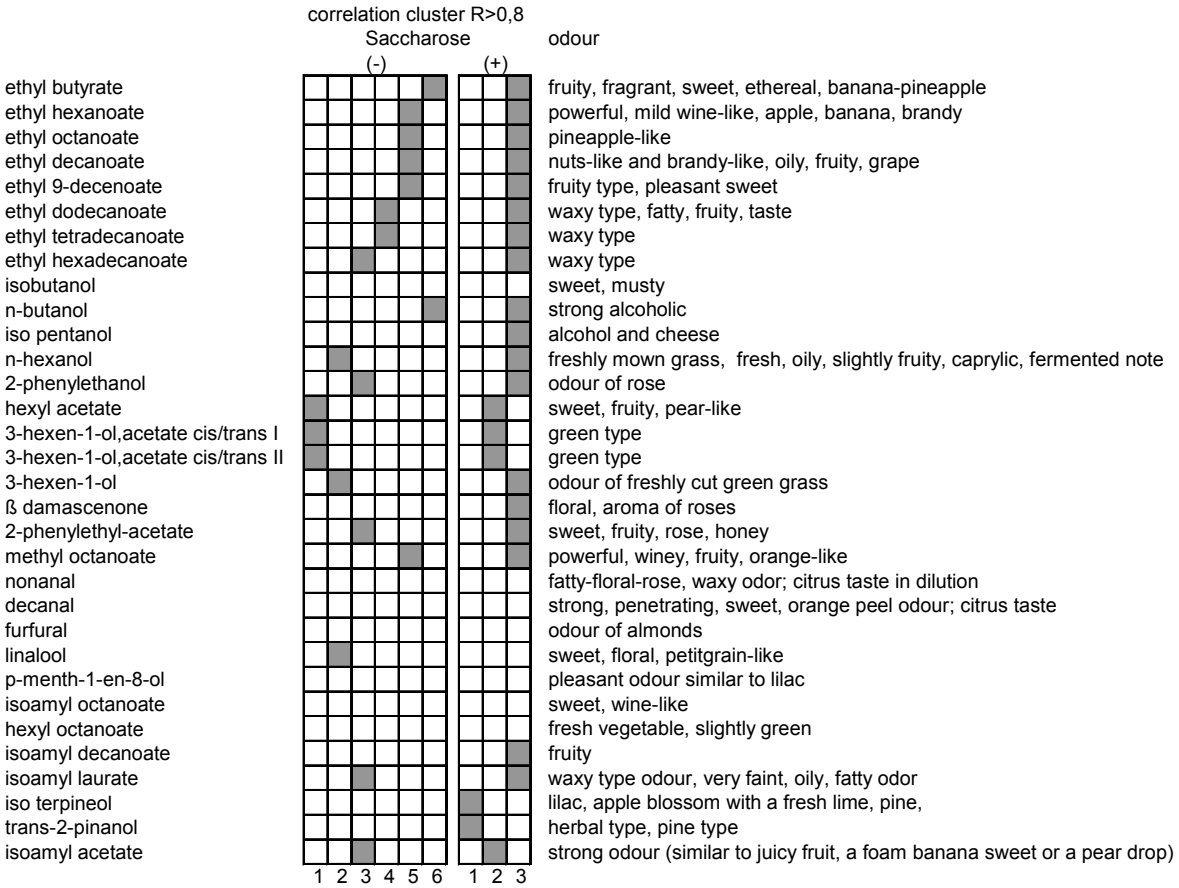
Yeast strains	Sucrose	
	(-)	(+)
cerevisiae	-0.33	-0.12
kudriavzevii	-0.54	-0.56
uvarum	-0.30	-0.33
kudriavzevii x uvarum H	0.37	0.07
kudriavzevii x uvarum 1a	-0.38	-0.33
kudriavzevii x uvarum 1b	-0.28	-0.29
kudriavzevii x uvarum 2a	0.17	-0.05
kudriavzevii x uvarum 2b	0.18	-0.06
cerevisiae x uvarum H	0.35	0.38
cerevisiae x uvarum 1a	0.33	0.23
cerevisiae x uvarum 1b	0.39	0.19
cerevisiae x uvarum 2a	-0.06	-0.22
cerevisiae x uvarum 2b	-0.09	-0.12
cerevisiae x kudriavzevii H	0.29	0.09
cerevisiae x kudriavzevii 1a	0.13	0.12
cerevisiae x kudriavzevii 1b	0.02	-0.16
cerevisiae x kudriavzevii 2a	-0.29	-0.31
cerevisiae x kudriavzevii 2b	-0.24	-0.13

Does the pattern of metabolic similarity – determined by the parameters listed in Table 1 – resemble those of the AFLP genetic similarity shown in Figure 1? To find out we used the same multivariate statistic method that was performed to create Figure 1: principal component analysis where the first three principal components are presented.

As can be seen from Figure 2, this is not the case. The hybridisation circle does not occur with these data, monospecies parental strains and hybrids are widely separated by PC1 instead, especially in the case of wine from juice without added sucrose. The F1 offspring generally lies between parental strains and hybrids. In most cases the yeast pair of genetically identical segregants behaves similar during fermentation. However, one of the *S.k.* x *S.u.* pairs of hybrid offspring depicts an important difference concerning the analyzed parameters in the wine from pure juice. This indicates that during fermentation evolution with a relevant influence of randomness occurred.

Which factor separates parental strains and hybrids? We supposed that relative metabolic power (rMP) makes the difference. Hence we designed an equation to compute it using the information included in Table 1. For calculation of rMP we used minimum-maximum scaled parameters (in the following equation indexed with an 's') with values between zero and one:

Table 3: Aroma constituents used in this paper to characterize the wine samples, their isolated odour impression and correlation. Correlation analysis was made separately for unmodified must (-) and must with sucrose added (+). Two parameters that correlate with a coefficient of more than 0.8 have both a grey field in the relevant column. No negative correlations with an absolute value > 0.8 were detected.



(1) $rMP = [ethyl\ alcohol_s + fermentation\ rate_s + CO_2\ production_s - (density_s + fructose_s + glucose_s + malic\ acid_s)] / 7,$

where, for instance, the fructoses of a special yeast is given by:

(2) $fructose_{s,yeast} = (fructose_{yeast} - fructose_{min}) / (fructose_{max} - fructose_{min}).$

rMP always lies between -1 and +1.

The relative metabolic power of the parental yeast strains is low, especially the one of *S. kudriavzevii* (-0.54 and -0.56, respectively). In untreated juice *S. cerevisiae* and *S. uvarum* are of similar quality whereas in treated one *S. cerevisiae* does better relatively. In contrast to parental strains, all hybrids have a high metabolic strength. In juice with sucrose added, the *S.c.* x *S.u.* hybrid is especially outstanding, whereas with untreated 'Grüner Veltliner' juice there is no pronounced difference.

In each of the three hybridisation groups, one pair of the segregants is of relatively high metabolic power, the other one not. In Figure 2, the pairs of F1 hybrid offspring with high metabolic strength lie around the hybrids, the other ones are nearer to the parental yeast strains, separated mainly by PC1. In untreated juice one of the segregant strains of *S.c.* x *S.u.* has even a bit more metabolic power than the hybrid (*S.c.* x *S.u.* 1b). The great distance of the two yeasts of one of the two pairs of *S.k.* x *S.u.* segregants, depicted in Figure 2 for untreated juice, concerns PC_2 and thus – as we shall see – is not a consequence of high differences in metabolic power (for PC_2 e. g. distinctions in the ratio of glucose to fructose usage are important).

Table 4: Relative metabolic power in aroma constituent production of the yeast strains. Hybrids are marked with "H", the F1 segregant pairs with "1" or "2", respectively.

Yeast strains	Sucrose	
	(-)	(+)
cerevisiae	0.11	0.18
kudriavzevii	0.12	0.11
uvarum	0.18	0.42
kudriavzevii x uvarum H	0.62	0.52
kudriavzevii x uvarum 1a	0.27	0.17
kudriavzevii x uvarum 1b	0.24	0.23
kudriavzevii x uvarum 2a	0.45	0.20
kudriavzevii x uvarum 2b	0.43	0.24
cerevisiae x uvarum H	0.38	0.66
cerevisiae x uvarum 1a	0.42	0.51
cerevisiae x uvarum 1b	0.49	0.34
cerevisiae x uvarum 2a	0.19	0.14
cerevisiae x uvarum 2b	0.17	0.20
cerevisiae x kudriavzevii H	0.46	0.21
cerevisiae x kudriavzevii 1a	0.29	0.29
cerevisiae x kudriavzevii 1b	0.39	0.17
cerevisiae x kudriavzevii 2a	0.11	0.09
cerevisiae x kudriavzevii 2b	0.21	0.14

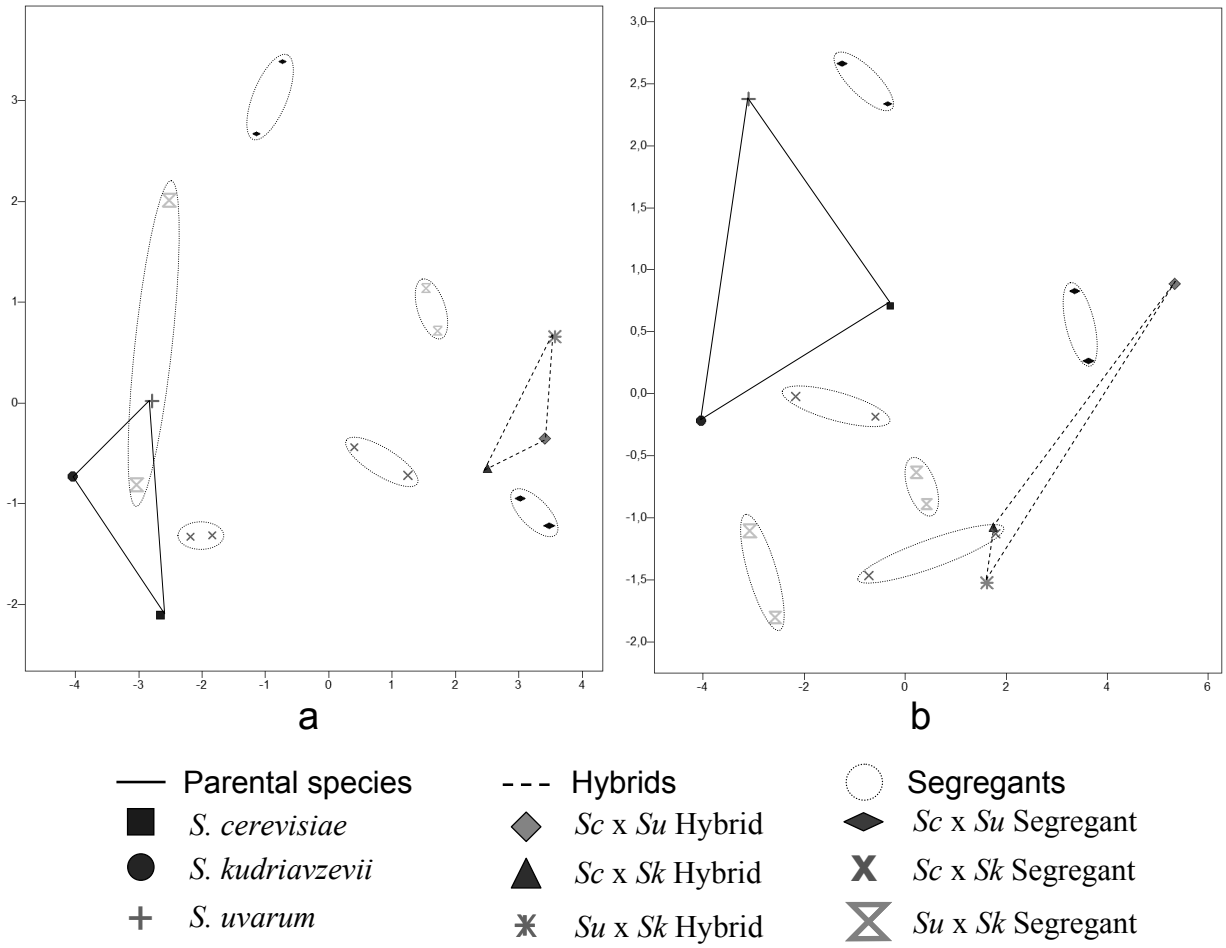


Fig. 2: Principal component analysis of basic chemical and fermentation parameters of the wines created by parental strains, hybrids and F1 hybrid offspring. a: Wine from must without sucrose added; b: Wine from must with sucrose added. The first three principal components are shown, the third one is imaginable by the different size of the symbols. Together they account for 86.6 % (untreated must) and 85.7 % (treated must), respectively, of the variability in the original data. Segregants that were genetically indistinguishable before fermentation are connected by an ellipse.

Our initial question concerning the reason why metabolic (Fig. 2) and genomic (Fig. 1) similarity are different thus has found a simple explanation. Hybrids can use more metabolic pathways and maybe produce even more enzymes and to a higher quantity. This can be an advantage in spirit and especially wine production since fast and continuous fermentation is desired as well as high alcohol concentrations, although, for instance, a too high fermentation rate may make expensive medium cooling necessary. Hybrids may achieve more metabolic flexibility too.

To proof that rMP is the main factor that separates parental yeast strains in Figure 2 we may compare equation 1 with the equation of the first principal component for untreated juice:

(3) PC1 = 0.37702 * density - 0.376621 * ethyl alcohol + 0.379913 * fructose + 0.353675 * glucose + 0.16584 * titratable acid - 0.0950516 * pH + 0.206915 * volatile acids + 0.130502 * tartaric acid + 0.350971 * malic acid - 0.0923039 * glycerol - 0.285122 * fermentation rate - 0.370425 * CO_2 production.

This becomes even more pronounced if we perform a factor analysis instead:

(4) CF1 = 0.982504 * density - 0.981462 * ethyl alcohol + 0.990042 * fructose + 0.921667 * glucose + 0.432174 * titratable acid - 0.247702 * pH + 0.539216 * volatile acids + 0.340084 * tartaric acid + 0.91462 * malic acid - 0.240541 * glycerol - 0.743021 * fermentation rate - 0.965317 * CO_2 production

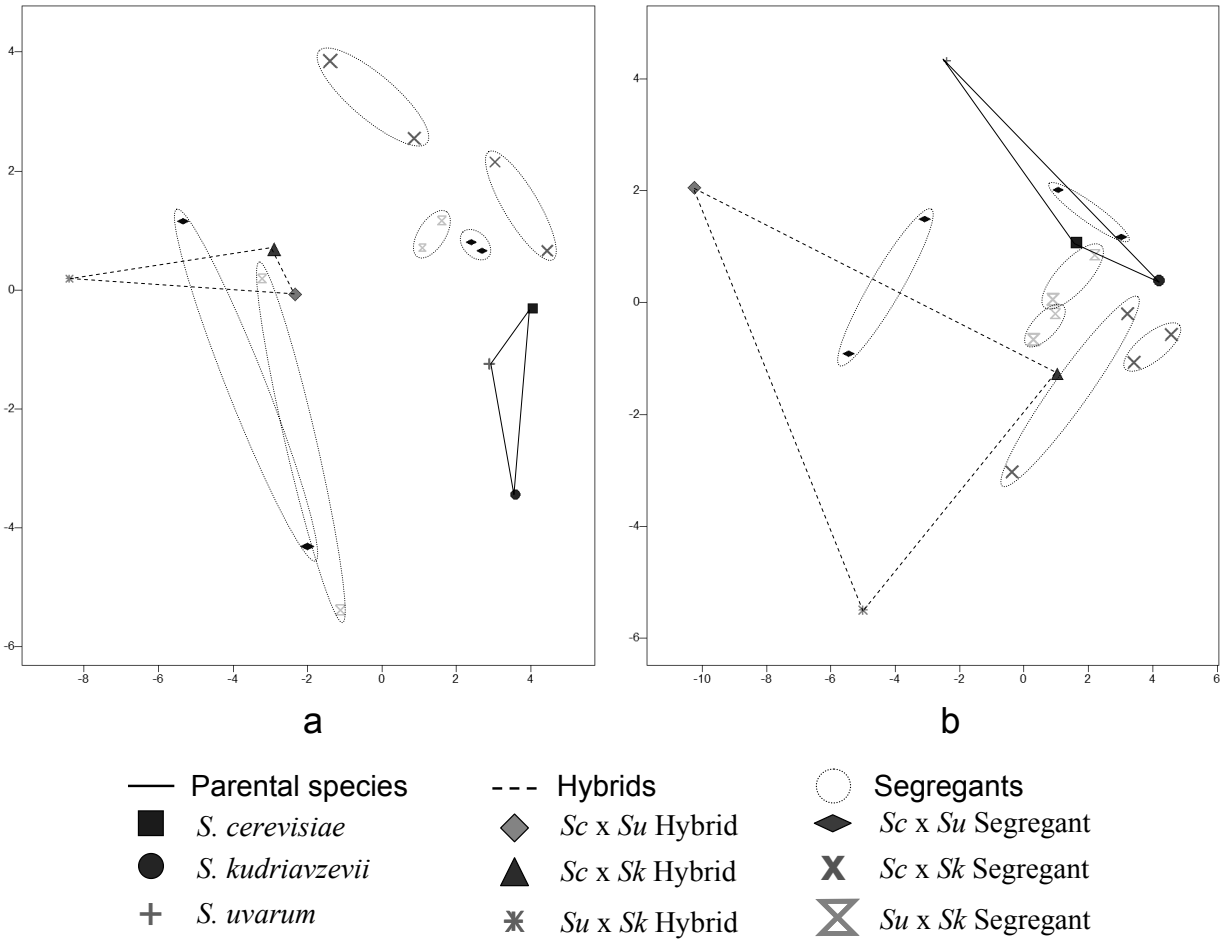


Fig. 3: Principal component analysis of the aroma constituents of the wines created by parental strains, hybrids and F1 hybrid offspring. a: Wine from must without sucrose added; b: Wine from must with sucrose added. The first three principal components are shown, the third one is imaginable by the different size of the symbols. Together they account for 68.9 % (untreated must) and 70.0 % (treated must), respectively, of the variability in the original data. Segregants that were genetically indistinguishable before fermentation are connected by an ellipse.

All basic parameters used in eq. 1 have a significant higher multiplication factor (weight) in eq. 3 (and 4, respectively) than the ones that are not utilized and the signs are the same if we multiply one of the equations with minus one. The same is true for the equation of the first principal component and first common factor for treated juice (not shown), although the pH has a relatively higher weight in that case.

ANALYSIS OF THE AROMA COMPOUNDS OF THE WINES

The different production of aroma-determining compounds is a very important distinction between yeasts, since it is significant for flavour and taste of the resulting wine. Flavour is a composite impression and thus it is not easy to attribute it to the single aroma constituents. Not all aroma compounds are of the same relevance and they are not independent of each other.

Gangl et al. (2009) concluded that, on average, hybrid yeast strains produce higher concentrations of aroma constituents than reference strains. Bellon et al. (2011) found that hybrid yeast strains produce wines with concentrations of aromatic products that are different to what is detected in wine made of commercial wine yeast parents.

Here 32 aroma components of 36 wines were determined as listed in Table 3.

According to a correlation analysis (Table 3) the constituents do not behave independently but concentrations differ between wines in a coherent way. More correlation clusters exist in untreated juice and thus the aromatic diversity – and likely the one of the flavour – is higher in this medium. There are no negative correlations at

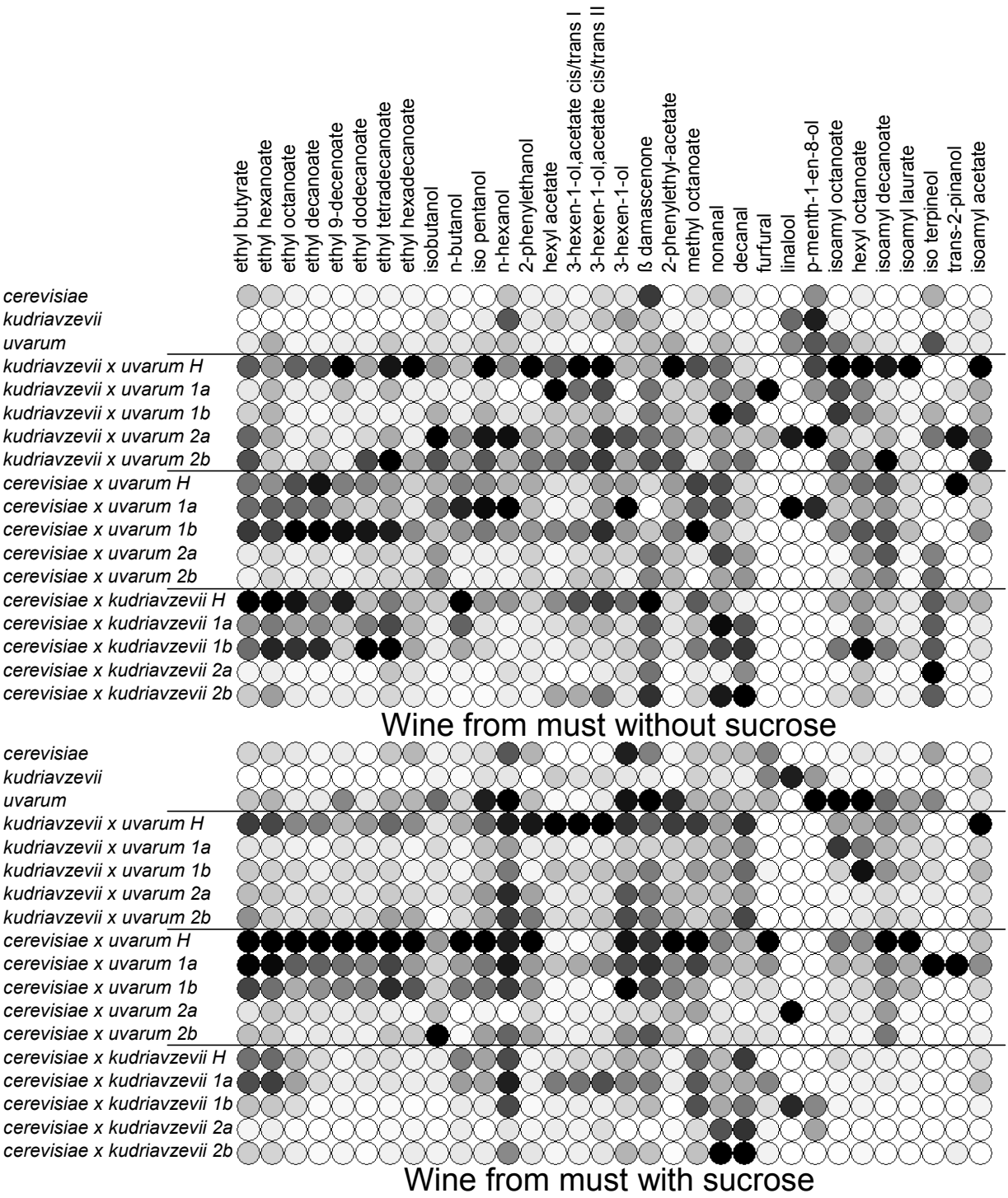


Fig. 4: Aromagram of wine from untreated and treated must. The rows represent 'Grüner Veltliner' wines fermented by different yeast strains, the columns single aroma constituents. The data (SIM areas that are proportional to the concentrations) are column-wise minimum-maximum scaled, where minimum is a white circle and maximum a black one. Scaling was done separately for treated and untreated must.

higher absolute values and hence more or less all aroma constituents are products of yeast activity.

Once again the (naïve) prime question is whether the aroma pattern similarity resembles those of the genetic one (Fig. 1 and Fig. 3).

Just as for the basic chemical and fermentation parameters this is not the case. Instead, Figure 3 somewhat resembles Figure 2, indicating a common cause. Aroma composition of wines created by parental yeast strains and their hybrids are very different, especially if produced with untreated juice. Some of the yeasts that belong to the same segregant pair develop very different aroma compositions although they were originally genetically very similar. Especially in untreated juice random-influenced evolution may have occurred in one pair of *S.c.* x *S.u.* and *S.u.* x *S.k.*, respectively. In treated juice aroma compositions are not as different (Table 3) and thus hybrids and parental strains are closer together in Figure 3. Nevertheless the aroma profiles of the hybrids are not similar to each other.

Since nearly all aroma constituents are positively correlated, the relative metabolic power of the yeasts concerning aroma production can be computed simply as the sum of all minimum-maximum scaled values of the single ion mode (SIM) areas of the aroma components (Table 4).

In wine of untreated juice the parental species produce relatively small amounts of aroma components. The hybrids do much better and surprisingly it is not the *S.c.* x *S.u.* hybrid that produces most, but the *S.k.* x *S.u.* one. Furthermore in the *S.c.* x *S.u.* group it is not the hybrid that produces most of the aroma constituents, but one of the segregant pairs. One of the F1 hybrid offspring pairs of *S.k.* x *S.u.* also has a very high relative metabolic power concerning aroma production. This result is resembled exactly by PC1 in Figure 3. Hybrids are not simple intermediates of their parental strains and segregants are very manifold.

In wine of treated juice the *S. uvarum* strain is ahead of the other parental yeast strains and only hybrids where this strain is one of the parents do even better. Most segregants clearly lie behind, but *S.c.* x *S.u.* 1a is a very effective producer of aroma constituents. The difference to *S.c.* x *S.u.* 1b is great so that we can assume that some kind of evolution has taken place during fermentation.

Differences between the wines can be seen in more detail in Figure 4.

In untreated juice the *S. cerevisiae* parental strain produces relatively high amounts of ß-damascenone, an acetone with the odour of roses. The *S. kudriavzevii* strain produces more p-menth-1-en-8-ol than others, a constituent that has a pleasant, lilac like smell. *S. uvarum* creates terpenes like linalool in higher than average amounts, a compound with sweet, floral odour and iso-terpineol with lilac flavour. Hybrids – and some of the segregants, too, especially the one with *S. uvarum* parent, *S.k.* x *S.u.* 2a and 2b and *S.c.* x *S.u.* 1a and 1b – are superior to the parental yeast strains and produce high levels of many esters, alcohols, aldehydes, ketones and terpenes and thus create very complex odours.

In treated juice the aroma composition of parental strains in comparison to hybrids remain very different (Fig. 4), although now *S. cerevisiae* and *S. uvarum* have a wider spectrum of constituents with high level production. The hybrids with an S. uvarum parent both produce high constituent concentrations but in a very different pattern. The *S.c.* x *S.u.* hybrid produces mainly ethyl esters and alcohols, the *S.k.* x *S.u.* hybrid prefers – beside some alcohols – ketones and esters which the former does not produce in high amounts. The *S.c.* x *S.k.* hybrid generates relatively low concentrations of most constituents and thus differs completely from the other hybrids. Only the aldehydes nonanal and decanal with strong and fatty odours are produced in comparably very high concentrations by *S.c.* x *S.k.* 2b in both analyzed media. In treated juice the *S.c.* x *S.u.* 1a F1 hybrid offspring seems more favourably with high production of some ethyl esters, ethyl butyrate and ethyl hexanoate, the alcohol n-hexanol and the terpenes iso-terpineol and trans-2-pinanol since all these constituents have a fresh or fruity note.

Antonelli et al. (1999) and Di Stefano et al. (1981) observed that a high concentration of the aroma component 2-phenylethanol (with a rose or honey smell) is characteristic for *S. bayanus* and *S. uvarum*. Gangl et al. (2009) confirmed this observation. However, in our stu-

dy 2-phenylethanol concentration is not especially high in the wine created by *S. uvarum* parental strain, but in the hybrids with an *S. uvarum* parent and some of its segregants. This is contrary to Gangl et al. (2009), who found low concentrations of 2-phenylethanol in hybrid produced 'Muskat Ottonell' and 'Blauburger' wines.
The same situation can be observed with 2-pheenylethyl-acetate (sweet, fruity, rose, honey aroma), a compound, Gangl et al. (2009) found typically in high concentration in *S. uvarum* wines. In our study this component appears in high concentrations in the *S. uvarum* hybrids and to a lesser degree in some of their segregants.
In the present study we have shown that artificial hybrids of *S. cerevisiae, S. uvarum* and *S. kudriavzevii* strains that were not specially adapted to spirit production have nevertheless promising properties for the generation of high quality wine: relatively good glucose and fructose utilization even under high-alcohol conditions, high fermentation rates and generally a pronounced metabolic power. Especially the latter influences aroma constituent production during fermentation of low- and high-osmotic stress grape juice. In dependence of osmotic stress aroma profiles are very different but hybrids outperform their parental yeast strains under both conditions. The same is valid for some of the F1 hybrid offspring. There are direct and indirect hints that these segregants evolve rapidly during fermentation and some of them reach a high quality level concerning juice fermentation. (Lopandic et al. 2016)

Conflict of Interest: The authors declare that they have no conflict of interest.
Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Appendix 1: Dice's similarity of yeasts using the binary AFLP data

	Sc	Sk	Su	Sk x Su H	Sk x Su 1a	Sk x Su 1b	Sk x Su 2a	Sk x Su 2b	Sc x Su H	Sc x Su 1a	Sc x Su 1b	Sc x Su 2a	Sc x Su 2b	Sc x Sk H	Sc x Sk 1a	Sc x Sk 1b	Sc x Sk 2a	Sc x Sk 2b
Sc																		
Sk	**0,34**																	
Su	**0,21**	**0,24**																
Sk x Su H	0,29	**0,73**	**0,56**															
Sk x Su 1a	0,23	**0,37**	**0,75**	**0,71**	**1**													
Sk x Su 1b																		
Sk x Su 2a	0,28	**0,5**	**0,69**	**0,79**	**0,79**		**1**											
Sk x Su 2b																		
Sc x Su H	**0,65**	0,37	**0,53**	0,57	0,61		0,57											
Sc x Su 1a	**0,71**	0,35	**0,6**	0,57	0,62		0,6		**0,81**	**1**								
Sc x Su 1b																		
Sc x Su 2a	**0,71**	0,36	**0,5**	0,53	0,57		0,57		**0,8**	**0,86**		**1**						
Sc x Su 2b																		
Sc x Sk H	**0,77**	**0,67**	0,19	0,55	0,27		0,39		0,6	0,61		0,61						
Sc x Sk 1a	**0,85**	**0,52**	0,19	0,4	0,25		0,35		0,6	0,65		0,63		**0,88**	**1**			
Sc x Sk 1b																		
Sc x Sk 2a	**0,81**	**0,53**	0,19	0,42	0,24		0,27		0,62	0,63		0,65		**0,86**	**0,73**		**1**	
Sc x Sk 2b																		

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