Endbericht für das Forschungsprojekt Nr. 1361

Monitoring der Hefepopulationsdynamik während der Spontanfermentation des Weines: Untersuchung der Sorten "Grüner Veltliner", "Welschriesling", "Sauvignon Blanc" und "Zweigelt" aus verschiedenen österreichischen Weinbaugebieten

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Einleitung

Die Weinfermentation ist ein komplexer mikrobiologischer Prozeß, der die Transformation von Most zum Wein mit Hilfe verschiedener auf Beeren und Kellerzubehör vorhandener Hefespezies darstellt. Sensorische Qualität und technische Eigenschaften des Weines variieren wesentlich mit der Qualität des Weinmosts und mit den Hefestämmen, die einen Fermentationsprozeß durchführen. Verschiedene Studien über die Hefebiodiversität haben gezeigt, dass eine große Vielfalt von Hefearten an den frühen Stadien der Gärung teilnehmen. Die wenig Ethanoltoleranten Hefearten, die laut Literaturdaten während der Weinfermentation oft vorkommen, sind Vertreter der Gattungen *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Torulaspora*, *Hansenula*, *Metschnikowia*, *Cryptococcus* und *Rhodotorula* (Fleet, 1993; Pretorius et al., 1999). In einer späteren Phase der Fermentation, die durch einen erhöhten Alkoholgehalt charakterisiert ist, werden diese Arten durch die alkoholtoleranten Hefen der Gattung *Saccharomyces* ersetzt. Es ist bekannt, dass verschiedene Stämme von *S. cerevisiae* und *S. bayanus* in der stürmischen Fermentationsphase dominieren, obwohl neulich eine andere Art, *S. paradoxus* auch aus dem Wein isoliert und charakterisiert wurde (z.B. Fleet and Heard, 1993, Querol et al., 1994; Redzepovic et al., 2002).

Beträchtliche Fortschritte in der Entwicklung molekularbiologischer Methoden mit erhöhten Auflösungsmöglichkeiten haben zu einer zuverlässigen Hefecharakterisierung geführt. Außerdem haben diese Techniken es ermöglicht, aufgrund neuer molekularen Merkmale genomische Polymorphismen in *S. cerevisiae* zu entdecken. Techniken wie zum Beispiel Karyotyping (Chromosomenverteilungsmuster), RFLP (Restriction Fragment Length Polymorphism), PCR Amplifizierung spezifischer Gene (z.B. rRNA codierende Gene), RAPD Analyse (Randomly Amplified Polymorphic DNA) und AFLP (Amplified Fragment Length Polymorphysm) werden häufig verwendet um die Hefen auf Art- oder Stammebene zu differenzieren (z.B. Querol et al., 1992, 1996; Baleiras Couto et al., 1996; de Barros Lopes et al., 1998; Guillamón et al., 1998; Montrochar et al., 1998, Kurtzman and Robnett, 1998; Azumi & Goto-Yamamoto, 2001).

Mehrere Studien haben auf eine Heterogenität von aus verschiedenen Weinbaugebieten isolierten *S. cerevisiae* Stämmen hingewiesen, so dass eine starke Korrelation zwischen genomischen Eigenschaften und geographischer Herkunft festgestellt wurden (Versavaud et

al., 1995; Nadal et al., 1996). Verschiedene Hefestämme beeinflussen die Weinqualität unterschiedlich, was andeutet, dass die Biodiversitätstudien von natürlichen Hefeisolaten aus diversen ökologischen Nischen und geographischen Regionen von großer Bedeutung für Grundlagen- und angewandte Forschung sind. Die autochthonen Hefen können neue molekulare und önologische Attribute enthalten, die den Weinen einen regionalspezifischen Charakter geben. Aus diesen Gründen haben sich viele Studien mit der Charakterisierung der Hefepopulationen und mit einem Monitoring des Wachstums während der Spontanfermentation der Weine in verschiedensten geographischen Regionen beschäftigt (Esteve-Zarzoso et al., 2000; Pramateftaki et al., 2000; Rementeria et al., 2003; Martínez et al., 2004; Combina et al., 2005; Raspor et al., 2006).

Ziele des Projekts

In dem vorliegenden Projekt wurde die Biodiversität von in die Weinfermentation involvierten Hefearten und -stämmen studiert. Innerhalb von vier österreichischen Weinbaugebieten (Wien, Donauland, Neusiedlersee-Hügelland, Südsteiermark) wurden sechs Standorte (Cobenzl, Stift Klosterneuburg, Agnes-Hof, Götzhof, St. Georgen, Silberberg) ausgewählt um die autochthonen Hefen zu isolieren und genotypisch zu charakterisieren. Auf Sequenz- und Fingerabdruckanalysen basierende molekularbiologische Techniken wurden angewandt, um ein Monitoring der Spontanfermentation an verschiedenen Standorten durchzuführen. Auf diese Weise wurde die Substitution der Hefearten während der Alkoholgärung verfolgt und die genomische Verwandtschaft zwischen den Stämmen verschiedener Herkunft studiert. Diese Studie hat die Möglichkeit eröffnet, autochthone Hefestämme zu selektieren, die als Starterkulturen in der Weinproduktion verwendet werden könnten.

Material und Methoden

Herkunft der Proben

Sechs Standorte (Donauland/Agnes-Hof, Donauland/Götzhof, Wien/Cobenzl, Wien/Stift Klosterneuburg, Neusiedlersee-Hügelland/St. Georgen, Südsteiermark/Silberberg) wurden von der Höheren Bundeslehranstalt und dem Bundesamt für Wein- und Obstbau (HBLABA) Klosterneuburg und vom Bundesamt für Weinbau Eisenstadt, ausgewählt, um Hefestämme aus Spontanfermentationen der regionalspezifischen Weine zu isolieren (Abbildung 1). Im Jahre

2004 wurden 284 Hefen aus Most bzw. Traubensaft der sechs Rebsorten "Grüner Veltliner", "Welschriesling", "Sauvignon blanc", "Zweigelt", "Blaufränkisch" und "Pinot Noir" isoliert. Die Probenahme erfolgte zu drei verschiedenen Zeitpunkten: am Beginn, in der Mitte und am Ende der Fermentation. Die Hefen wurden in mikrobiologischen Laboratorien der HBLABA Klosterneuburg und des Bundesamtes für Weinbau Eisenstadt isoliert und am Institut für Angewandte Mikrobiologie, Universität für Bodenkultur, Wien, genotypisch charakterisiert.

Abbildung 1. Lage der Probenahmestellen in den verschiedenen Weinbaugebieten

Charakterisierung von Hefearten

Die Identifizierung der Hefen auf Artebene wurde mittels Sequenzen der D1/D2 Domäne des 26S rRNA codierenden Gens und PCR-Fingerprinting nach Lopandic at al. (2006) durchgeführt. Die genomische DNA wurde nach dem Protokoll von MasterPureTM Yeast DNA Purification Kit (Epicentre, Madison, Wisconsin, USA) isoliert und gereinigt. Um

genomische Ähnlichkeiten festzustellen, wurde PCR-fingerprinting mit allen DNA Präparationen mit Hilfe des Primers M13 (5'-GAGGGTGGCGGTTCT-3') durchgeführt. Die Polymerase Ketten Reaktion (PCR) wurde in einem Thermocycler (MJ Research, PTC-200) mit folgendem Temperaturprogramm durchgeführt: 94°C/2 min., 38 Zyklen mit 94°C/40 sec., 37°C/90 sec., 72°C/100 sec. und 72°C/5 min. als Extensionszeit. 20 µl des PCR-Produktes wurden in einem 1,4 % Agarosegel aufgetrennt und die entstandenen Bandenmuster mit Hilfe eines UV-Transilluminators (BioRad) aufgenommen.

Aus jeder Gruppe von Hefen, die untereinander die gleichen PCR-Muster zeigten, wurden zwei Stämme zufällig ausgewählt und die Sequenzen der D1/D2 Region bestimmt. Zu diesem Zweck wurde ein Fragment von ca. 1600 bp mit den Primern ITS5w (GGAASTAAAAGTCGTAACAAG) und NL4 (GGTCCGTGTTTCAAGACGG) amplifiziert. Die PCR wurde mit folgendem Temperaturprogramm durchgeführt: 94°C/2 min.; 35 Zyklen mit 94°C/30 sec., 55°C/75 sec., 72°C/120 sec., und 72°C/10 min. als Extensionszeit. Mit den Primern NL1 (GGTCCGTGTTTCAAGACGG) und NL4 (GGTCCGTGTTTCAAGACGG) wurde ein Fragment von ca. 600 bp sequenziert. Die Sequenzen der D1/D2 Regionen wurden in die Genbank (http://www.ncbi.nlm.nih.gov) importiert, um die homologen Sequenzen mittels BLAST-Programm zu bestimmen. Die Arten, die einen IS Wert ("identity score") >99% zeigten, wurden als Typstämme selektiert und die Konspezifität mit den entsprechenden Hefeisolaten aus Traubenmost und Traubensaft mit PCR-fingerprinting untersucht. Die Hefestämme, deren DNA-Fingerabdrücke >80% Ähnlichkeit mit den DNA-Fingerabdrücken von Typstämmen zeigten, wurden als konspezifisch betrachtet. Die PCR-Fingerprints wurden mit Hilfe der Software GelCompare II v3.5. (Applied Maths, Kotrijk, Belgium) analysiert. Die Clusteranalyse wurde mittels UPGMA Methode durchgeführt.

Charakterisierung von Saccharomyces Stämmen

Die Variabilität von *S. cerevisiae* and *S. bayanus* Stämmen wurde mittels AFLP Technik untersucht. Die AFLP-Fingerprinting (amplified fragment length polymorphism) Methode wurde nach Lopandic et al. (2005) mit einigen Modifikationen durchgeführt. Restriktions- und Ligationsschritte wurden gleichzeitig mit 10 ng (5,5 µl) genomischer DNA, 1U MseI, 5U EcoRI und 3U T4 DNA Ligase (New England Biolabs) durchgeführt. Die Reaktion erfolgte in einem Gesamtvolumen von 11 µl mit 0,36 µM EcoRI Adapter und 3,64 µM MseI Adapter aus

dem Kit, 0,1 M NaCl, 0,91 mM Tris-HCl (pH 7,8), 0,18 mM $MgCl_2$, 0,18 mM Dithiothreitol, 18 µM ATP und 91,36 µg ml^{-1} BSA (Bovine Serum Albumin). Die Reaktion wurde bei 37°C für 3 Stunden durchgeführt. Die Restriktionsligationsmischung wurde mit 30 µl sterilisiertem bidestilliertem Wasser verdünnt. Die präselektive Amplifizierung erfolgte mit zwei Primern (Eco RI Kernsequenz und MseI Kernsequenz) und mit dem AFLP Amplification Core Mix aus dem AFLP Microbial Fingerprinting Kit unter folgenden Bedingungen: 72°C/ 2 min.; 20 Zyklen mit 94°C/ 20s, 56°C/ 30s und 72°C/ 2 min. Das PCR-Produkt wurde mit 40 µl sterilisiertem bidestilliertem Wasser verdünnt. Die selektive Amplifizierung erfolgte mit den Primerpaaren EcoRI-AC FAM/MseI-G, EcoRI-G JOE/MseI-CG und EcoRI-C NED/MseI-CG. Die Reaktion wurde unter den folgenden Bedingungen durchgeführt: 94°C/2 min; 10 Zyklen mit 94°C/20s, 66°C/30s mit 1°C Reduktion für jeden Zyklus, 72°C/2 min und 25 Zyklen von mit 94°C/20s, 56°C/30s, 72°C/2 min. Ein Aliquot von 1,5 µl jedes Produktes der selektiven Amplifizierung wurde mit 24 µl Formamid und 1 µl Genescan-500 (ROX) Größenstandard vermischt und bei 95°C für 3 min denaturiert. Die Kapillarelektrophorese wurde mit einem ABI 3100 Avant Sequencer (Applied Biosystems) durchgeführt. Die Ergebnisse wurden mit den Programmen Genescan 3.1, Genotyper (Applied Biosystems) und Excel®macro (Rinehart, 2004) ausgewertet. Aufgrund der resultierenden binary Matrix mit allen drei Primerpaaren wurde eine Clusteranalyse mit Hilfe der Software PAUP 4.0b10 durchgeführt (Swofford, 2002).

Ergebnisse und Diskussion

Charakterisierung von Hefearten

Um die Hefepopulationsdynamik während der Spontanfermentation der Weiß- und Rotweine aus sechs Weinbaustandorte zu studieren, wurde eine polyphasische Methode angewendet, die eine Sequenzanalyse der D1/D2 Region des 26S rRNA codierenden Gens und PCR-fingerprinting beinhaltete. Den Großteil (87%) der Hefen wurde auf Artebene charakterisiert (Abbildung 2, Tabellen 1, 2). Innerhalb der identifizierten Arten wurden *S. cerevisiae* (181 Stämme) und *Hanseniaspora uvarum* (44 Stämme) am häufigsten isoliert, während die Arten *Candida zemplinina*, *Issatchenkia occidentalis*, *Kregervanrija fluxuum*, *Lachancea thermotolerans*, *Metschnikowia viticola*, *Pichia kluyveri*, *S. bayanus* var. *uvarum* und *Zygoascus hellenicus* sporadisch in einzelnen Proben festgestellt wurden (Tabellen 1, 2). Aufgrund der Sequenzanalysen der D1/D2 Region zeigten einige Hefeisolate eine nahe

Verwandtschaft mit den Arten *Metschnikowia pulcherrima* und *Pichia membranifaciens*, aber die PCR-fingerprinting Analysen konnten die Konspezifität im Vergleich der resultierenden Muster mit denen von entsprechenden Typstämmen nicht bestätigen, die Ähnlichkeitswerte der DNA-Fingerabdrücke waren <80% (Abbildung 2). Da die Sequenzen der D1/D2 Domäne und die DNA-Fragmentprofile sehr heterogen waren, handelt es sich bei diesen Hefen mit hoher Wahrscheinlichkeit um neue Arten der Gattungen *Metschnikowia* und *Pichia*. Deren vollständige Identifizierung bedarf der Verwendung zusätzlicher molekularer Merkmale. Zu ähnlichen Ergebnissen sind Pallmann et al. (2001) gekommen, die eine große Divergenz der D1/D2 Sequenzen innerhalb der Pulcherrimin-produzierenden Hefen festgestellt haben, welche traditionell als *M. pulcherrima* klassifiziert wurden. Die nicht-*Saccharomyces* Arten wurden am Beginn und in der mittleren Gärungsphase identifiziert, wenn die Zucker- und Ethanolkonzentrationen noch niedrig waren (Tabelle 1, 2). Die *S. cerevisiae* Stämme wurden in der Anfangsphase nur in den Mostproben von "Pinot Noir" (Stift Klosterneuburg), "Zweigelt" (Agnes-Hof) und "Grüner Veltliner" (Cobenzl) festgestellt. Interessanterweise wurden die *S. bayanus* Stämme nur im Most und Traubensaft von dem Standort St. Georgen nachgewiesen (Tabelle 2).

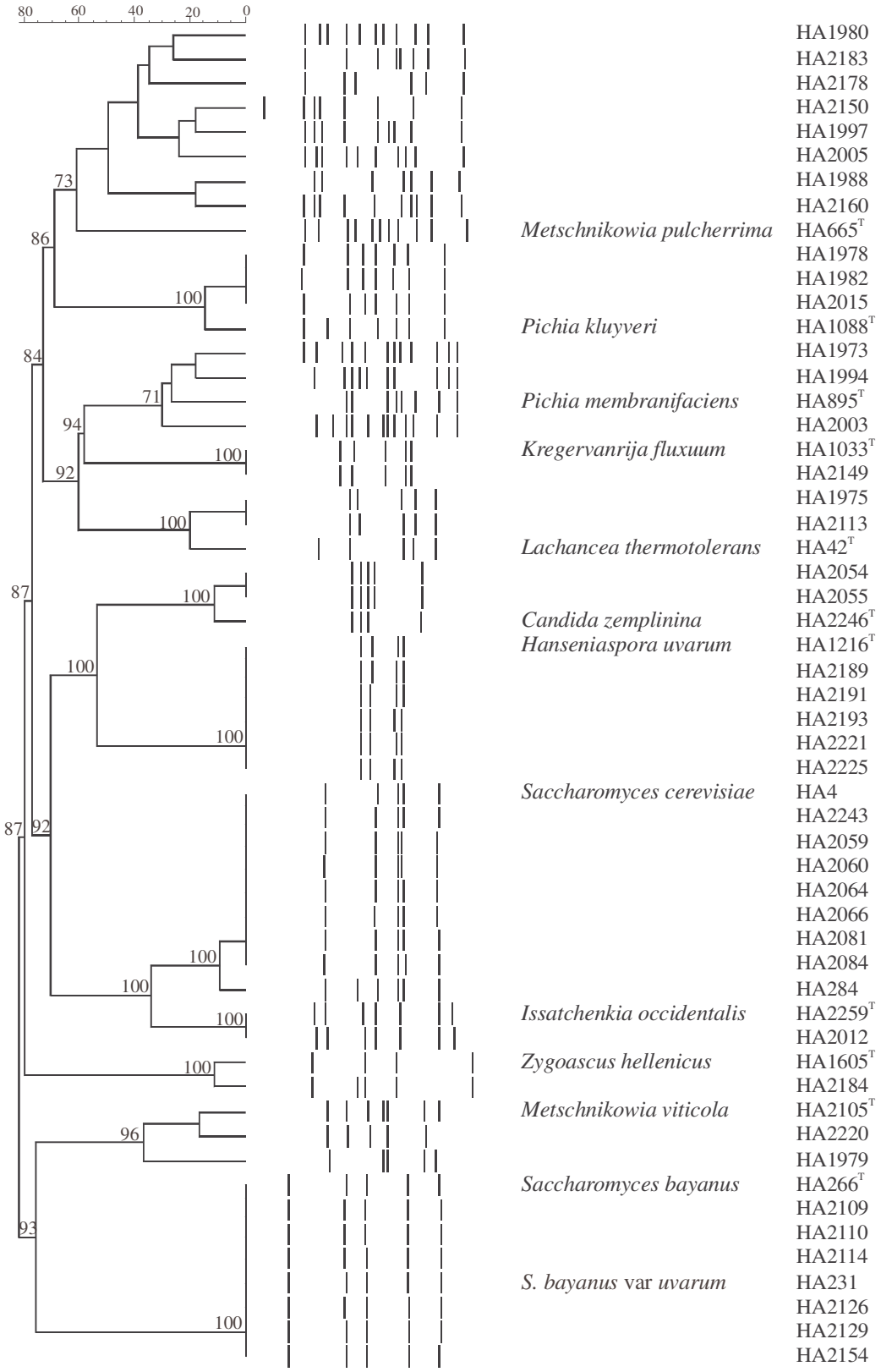

Abbildung 2. Clusteranalyse auf Basis von PCR-fingerprinting der Hefeisolate aus Traubenmost und Traubensaft

Tabelle 1. Zusammenstellung der aus drei Phasen der Spontanfermentation verschiedener Traubenmoste (BF=Beginn, MF=Mitte, EF=Ende) isolierten und charakterisierten Hefearten von den Standorten Stift Klosterneuburg, Agnes-Hof and Götzhof

Yeast species	No. of isolated colonies (Year 2004)														
	Wien, Stift Klosterneuburg			Donauland, Agnes-Hof						Donauland, Götzhof					
	Pinot Noir			Grüner Veltliner			Zweigelt			Grüner Veltliner			Blaufränkisch		
	BF	MF	EF	BF	MF	EF	BF	MF	EF	BF	MF	EF	BF	MF	EF
C. zemplinina		2													
H. uvarum				1			2			3			4		
I. occidentalis					1										
Metschnikowia sp.	2			7			1			2			2		
M. viticola										1					
P. kluyveri					1					2					
Pichia sp.	1						1						2		
S. cerevisiae	5	6	8		6	8	4	8	8		8	8		8	8
Sum of all investigated yeasts	24			24			24			24			24		

Tabelle 2. Zusammenstellung der aus drei Phasen der Spontanfermentation verschiedener Traubenmoste (BF=Beginn, MF=Mitte, EF=Ende) isolierten und charakterisierten Hefearten von den Standorten Cobenzl, St. Georgen and Silberberg

Yeast species	No. of isolated colonies (Year 2004)														
	Wien, Cobenzl			Neusiedlersee-Hügelland, St. Georgen						Südsteiermark, Silberberg					
	Grüner Veltliner			Welschriesling			Zweigelt			Zweigelt			Sauvignon blanc		
	BF	MF	EF	BF	MF	EF	BF	MF	EF	BF	MF	EF	BF	MF	EF
Candida sp.										2	1				
H. uvarum		2		6			5	6		2	6		7		
K. fluxuum				1											
L. thermotolerans	2						1								
Metschnikowia sp.		1		1	2					5			2		
M. viticola													1		
Pichia sp.	2							1							
S. bayanus				2			4	4	1						
S. cerevisiae	4	5	8		8	10		9	9		13	10		10	10
Z. hellenicus										1					
Sum of all investigated yeasts	24			30			40			40			30		

Charakterisierung von Saccharomyces Stämmen

Aufgrund der Ergebnisse der PCR-fingerprinting Analyse wurden 181 Hefeisolate als *S. cerevisiae* und 11 Hefen als *S. bayanus* identifiziert. Da PCR-fingerprinting die Hefen nur auf Artebene unterscheidet, war die Differenzierung der *S. cerevisiae* und *S. bayanus* Stämme nur mit Hilfe der AFLP Analyse möglich (Abbildung 2, 3, 4). Die AFLP Methode ist dadurch charakterisiert, eine höhere Anzahl an Loci als mit anderen PCR-basierenden Techniken analysieren zu können. Einige Autoren (De Barros Lopes et al., 1999; Azumi & Goto-Yamamoto, 2001) haben gezeigt, dass die AFLP-Analyse ein gutes Potential zur genetischen Abgrenzung von kommerziellen Stämmen besitzt. Um die Diversität der österreichischen *S. cerevisiae* Stämme aus spontangärendem Traubenmost mit den Hefestämmen aus anderen geographischen Regionen zu vergleichen, wurden auch drei aus Spanien (HA4), aus der Schweiz (HA284) und aus Westafrika (HA236) stammende *S. cerevisiae* Stämme, und zusätzlich einige direkt aus Trauben aus der Thermenregion (Perchtolsdorf und Tattendorf) isolierte Stämme analysiert (Abbildung 4). Die Ergebnisse zeigten, dass die österreichischen *S. cerevisiae* Stämme eine homogene Population darstellen, welche signifikante Unterschiede zu Weinhefen aus anderen Ländern aufweist. Andererseits waren bei den aus allen österreichischen Standorten isolierten *S. cerevisiae* Stämmen sieben bis 11 verschiedene AFLP-Profile feststellbar, dies weist auf eine relativ hohe genomische Heterogenität hin. Außerdem konnte man nachweisen, dass in Weinen aus verschiedenen Standorten einerseits viele Hefen mit gleichen AFLP-Mustern vorhanden sind, aber auch Stämme mit einzigartigen AFLP Mustern vorkommen (Abbildung 4). Da eine beschränkte Hefeanzahl analysiert wurde, ist es notwendig, die Standortspezifität der Stämme durch eine mehrjährige Probenahme zu überprüfen. Eine detaillierte Analyse der AFLP-Fragmentmuster zeigte, dass sich die österreichischen *S. cerevisiae* Stämme in 18 AFLP-Fragmenten voneinander unterscheiden (Tabelle 3A). Im Vergleich zu den Stämmen aus anderen Ländern konnte man vier informative AFLP-Fragmente feststellen (133, 240, 247, 378 bp), was darauf hinweist, dass diese DNA Segmente interessante genomische Eigenschaften enthalten könnten, welche die Stämme anderer geographischer Regionen nicht besitzen (siehe Anhang 1).

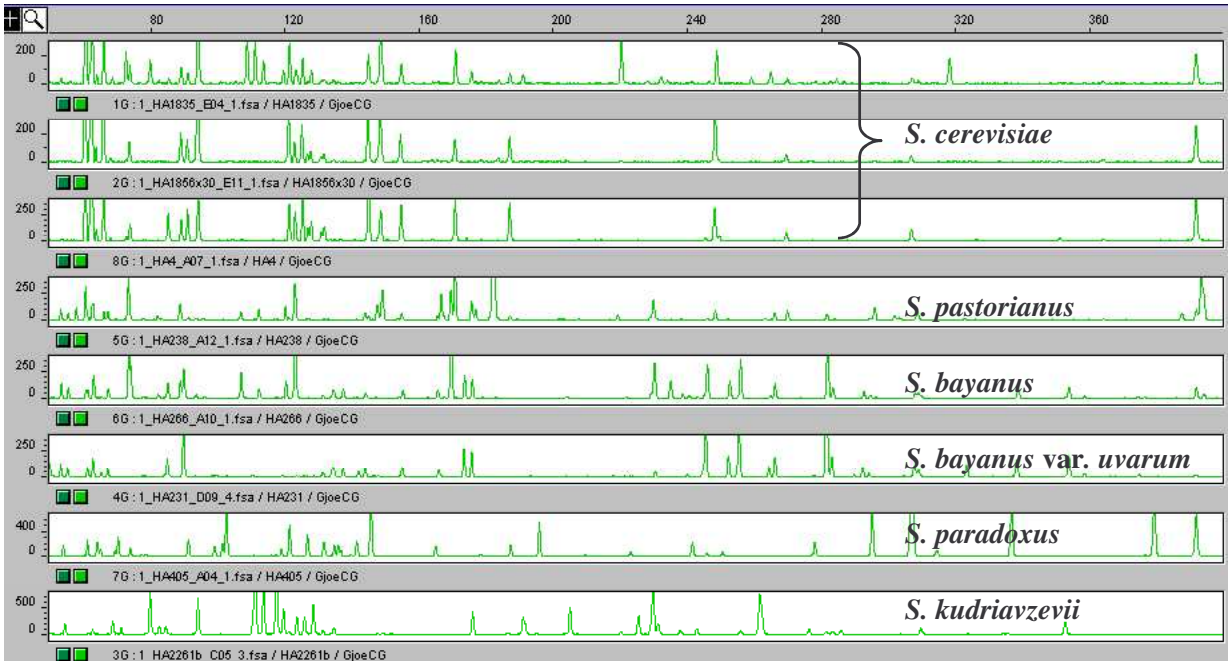

Abbildung 3. Genotypische Charakterisierung von *Saccharomyces* Arten und Stämmen mittels AFLP-Fingerabdruck Analyse

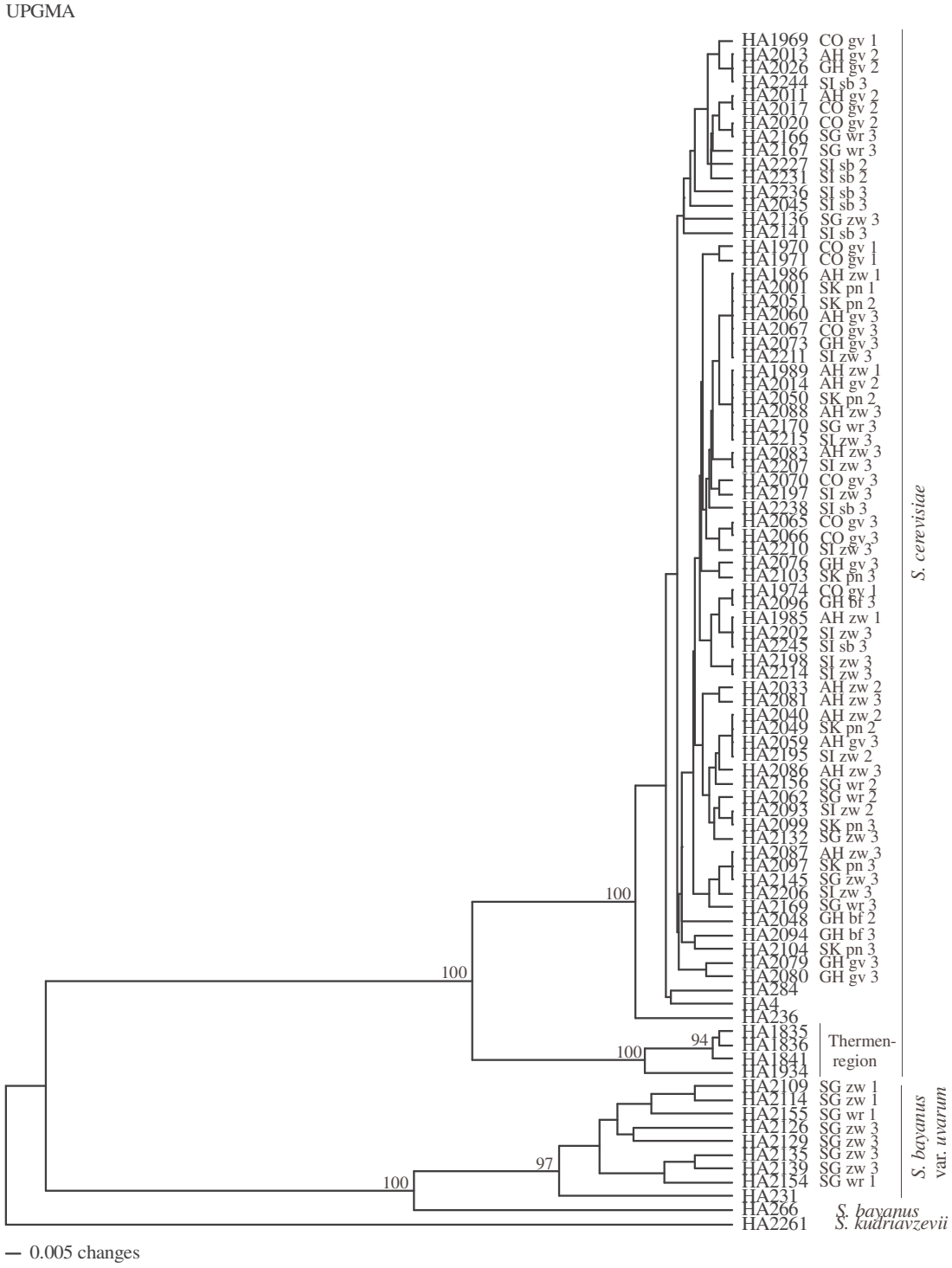

Abbildung 4. Clusteranalyse auf Basis von AFLP-Fingerprints, die die genomische Diversität der aus spontanfermentierten Mosten und Traubensäften isolierten *S. cerevisiae* and *S. bayanus* Stämme verschiedener österreichischer Weinbaugebieten zeigt. Die Standorte sind mit Großbuchstaben abgekürzt: AH-Agnes-Hof, GH-Götzhof, CO-Cobenzl, SK-Stift Klosterneuburg, SG-St. Georgen, SI-Silberberg. Weinsorten sind mit Kleinbuchstaben abgekürzt: gv-Grüner Veltliner, zw-Zweigelt, bf-Blaufränkisch, pn-Pinot Noir, wr-Welschriesling, sb-Sauvignon blanc. Die drei Fermentationsphasen (Beginn, Mitte, Ende), in denen die Probennahmen stattgefunden haben sind durch die Nummern 1, 2, 3, gekennzeichnet.

Erhebliche Unterschiede zu allen untersuchten aus Spontanfermentationen isolierten Hefen konnten bei einigen direkt aus Trauben von der Thermenregion isolierten Hefen festgestellt werden (Abbildung 4). Die AFLP-Analyse hat darauf hingedeutet, dass diese Stämme Hybride zwischen *S. cerevisiae* und *S. kudriavzevii* Arten sind. Von 34 insgesamt amplifizierten AFLP-Fragmenten teilen sich die aus der Thermenregion isolierten Stämme 11 Fragmente mit *S. kudriavzevii* (Tabelle 3A). Eine detaillierte Studie hat gezeigt (Anhang 2), dass diese Stämme zwei verschiedene Sequenzen der D1/D2 und ITS1/ITS2 Regionen beinhalten, davon ist eine Sequenz mit der von *S. cerevisae*, die zweite mit der von *S. kudriavzevii* homolog. Weitere Versuche haben gezeigt, dass es sich um aneuploide Stämme handelt, die keine wachstumsfähige Sporen bilden können, was eine typische Eigenschaft von Hybridstämmen ist. Erst in den letzten zwei Jahren wurden dank neuer hochsensitiver Methoden einige Hybridstämme innerhalb kommerziell angewandter Stämme entdeckt, bei denen *S. kudriavzevii* als ein Donor eines Teils des Genoms charakterisiert wurde (Naumova et al., 2005; Liti et al., 2005; Bradbury et al., 2006; González et al., 2006). Die Bedeutung dieser Hybride für die Qualität der Weine wurde noch nicht genauer untersucht, aber es ist bekannt, dass sich die genetischen Attribute solcher Hybridstämme auf die phänotypischen Eigenschaften auswirken (Masneuf et al., 1998). Erste Untersuchungen des Einflusses von Hybridstämmen zwischen *S. cerevisiae* und *S. kudriavzevii* auf die önologischen Eigenschaften des Weines haben gezeigt, dass diese Hybride eine höhere Konzentration von höheren Alkoholen im Vergleich mit einem kommerziellen *S. cerevisiae* produzieren (González et al., pers. comm.). Die Hybridstämme zeigen bei niedrigeren Temperaturen (14, 18, 22°C) außerdem ein besseres Fermentationsverhalten als *S. cerevisiae* Stämme. Die aus der Thermenregion (Standort Perchtoldsdorf) isolierten Hybridstämme konnten eine höhere Konzentration von Estern im Vergleich mit *S. cerevisiae* Stämmen aus den Weinbaugebieten Neusiedlersee-Hügelland und Neusiedlersee produzieren (siehe Anhang 2). Die von den Hybridstämmen produzierten Aromakomponenten waren jedoch den von *S. cerevisiae* Stämmen aus Pfaffstätten (Thermenregion) hergestellten ähnlich. Dies könnte darauf hinweisen, dass die Produktion von Aromakomponenten umweltbedingt ist, das heißt, Stämme aus dem gleichen Weinbaugebiet setzen während der Fermentation des gleichen Traubenmostes Aromakomponenten in ähnlicher Konzentration frei. Andererseits weisen ähnliche Profile von Aromakomponenten zwischen Hybridstämmen aus Perchtoldsdorf und *S. cerevisiae* Stämmen aus Pfaffstätten darauf hin, dass möglicherweise ein *S. cerevisiae* Elternteil aus Pfaffstätten zu dem Genom der Hybridstämme beigetragen hat. Diese Vermutungen muß man aber noch mit Sequenz- oder Restriktionsanalysen verschiedener

Gene untermauern. Alle diese Ergebnisse zeigen deutlich, dass weitere Untersuchungen der Hybridstämme für eine Grundlagen- und angewandte Forschung interessant sind und dass man die Erforschung der genomischen, technologischen und önologischen Eigenschaften dieser natürlichen Hefeisolate intensivieren muss.

Die AFLP-Analyse von *S. bayanus* Stämmen aus spontan fermentiertem Most und Traubensaft hat ergeben, dass alle Isolate der Art *S. bayanus* var. *uvarum* angehören (Abbildung 4). Elf untersuchte Stämme haben acht verschiedene AFLP-Muster gezeigt, was auf eine signifikante genomische Variabilität hindeutet. Im Vergleich mit dem *S. bayanus* Typstamm HA266 und dem *S. bayanus* var. *uvarum* Stamm HA231 können die österreichische Stämme aufgrund von neun Fragmenten (90, 138, 155, 219, 273, 284, 305, 310, 339 bp) unterschieden werden (Tabelle 3B). Die *S. bayanus* var. *uvarum* Stämme wurden nur vom Standort St. Georgen isoliert, dies kann man als eine Anpassung dieser Stämme an umweltbedingte Gegebenheiten dieses Areals deuten. Genauere Informationen über die genomischen und phänotypischen Eigenschaften, wie auch über das önologische Potential dieser Stämme können durch Vergleichsuntersuchungen mit natürlichen, aus Spontanfermentationen anderer geographischer Lokalitäten isolierten *S. bayanus* var. *uvarum* Stämmen erhalten werden.

Tabelle 3. Polymorphe AFLP-Fragmente in (A) *S. cerevisiae*, *S. kudriavzevii* und (B) S. bayanus, *S. bayanus* var. *uvarum* Stämmen

A

Hefestämme	AFLP Fragmente (bp)																																									
	68	80	82	85	86	90	98	106	109	112	115	118	128	129	133	136	137	141	156	177	183	187	191	195	200	201	221	230	232	237	240	242	246	247	265	277	281	284	288	307	318	356	378
österr. *S. cerevisiae* Stämme	0	0	0	1	0	V	V	V	0	0	0	0	1	0	V	V	V	V	0	V	V	V	0	0	0	0	0	0	0	0	V	0	V	V	0	V	0	V	0	V	0	V	V
HA284	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0
HA236	0	0	0	0	0	1	0	1	0	0	0	1	1	1	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	1	0
HA4	1	0	1	0	0	1	0	1	0	0	0	0	1	0	0	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0
Hybridstämme	0	1	0	1	1	1	V	1	1	1	1	0	1	0	0	1	V	1	1	1	1	V	1	1	1	V	1	1	V	0	0	1	1	0	1	1	1	V	1	V	1	V	0
S. kudriavzevii	0	1	0	1	1	0	1	1	0	1	1	0	1	0	0	0	0	0	1	0	1	0	1	1	1	0	0	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0

B

Hefestämme	AFLP Fragmente (bp)																																								
	61	67	74	75	77	87	89	90	92	103	106	107	110	111	114	121	123	129	135	138	146	148	153	155	171	182	189	208	218	219	231	235	273	278	282	284	305	310	327	330	339	392
österr. *S. bayanus* var. *uvarum* Stämme	V	0	V	V	0	0	V	V	V	V	0	0	0	V	0	0	0	0	0	V	V	0	V	V	0	V	0	V	V	V	V	V	V	V	V	V	V	V	0	0	V	V
HA231	1	1	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0
HA266	0	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	1	0	1	0	0	0	1	1	1	0	1	0	1	1	0	1	1	0	0	0	1	1	0	1

1: AFLP-Fragment vorhanden in allen Stämmen; 0: AFLP-Fragment nicht vorhanden; V: variabel vertretenes AFLP-Fragment

Schlussfolgerungen

In der vorliegenden Studie wurden aus verschiedenen regionalspezifischen Mosten und Traubensäften isolierte Hefen auf Art und Stammebene genotypisch charakterisiert. Die Ergebnisse zeigen eine große Heterogenität von in den Fermentationsprozessen von Weiß- und Rotweinen involvierten Hefearten und Stämmen. Die Stämme von *S. cerevisiae* und *S. bayanus* var. *uvarum* haben signifikante genomische Unterschiede aufgewiesen, die aber im Vergleich zu der limitierten Anzahl von Stämmen aus anderen geographischen Gebieten und ökologischen Nischen als eine homogene Population charakterisiert wurden. Diese Studie hat auch ergeben, dass österreichische Weinbaugebiete interessante Hefehybride enthalten, die durch natürliche Hybridisierungsprozesse zwischen *S. cerevisiae* und *S. kudriavzevii* entstanden sind. Da diese Stämme eine erhöhte, für die Weinqualität bedeutende Esterkonzentration während der Fermentation freisetzen können, muss man zusätzliche Untersuchungen ihrer Verbreitung und ihrer technologischen und önologischen Eigenschaften durchführen, um deren Potential als Starterkulturen evaluieren zu können. Diese Hefebiodiversitätsstudie, die zum ersten mal in österreichischen Weinbaugebieten durchgeführt wurde, ist eine Basis für weitere mikrobiologisch und genetisch orientierte Studien und stellt einen wichtigen Beitrag zur Erforschung und Erhaltung der genetischen Vielfalt der biotechnologisch relevanten Hefestämme dar.

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Anhang

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Diversity of yeasts involved in spontaneous fermentation of wine musts assessed by D1/D2 sequences of 26S rRNA encoding gene and AFLP fingerprinting. K. Lopandic, W. Tiefenbrunner, H. Gangl, G. Leitner, K. Mandl, G.A. AbdEllah, H. Prillinger. Journal of applied microbiology (**Anhang 1**)

Investigation of genomic variability of autochthonous *Saccharomyces cerevisiae* strains isolated from Austrian vine-growing regions and their influence on aroma composition of wines. K. Lopandic, H. Gangl, E. Wallner, G. Tscheik, G. Leitner, A. Querol, N. Borth, M. Breitenbach, H. Prillinger, W. Tiefenbrunner. FEMS Yeast Research (**Anhang 2**)

Molecular characterisation of yeasts during spontaneus fermentation of grape must. (2005) Lopandic, K., Mandl, K., Silhavy, K., Berger, S., Gangl, H., Tiefenbrunner, W., Leitner, G., Sterflinger, K., Prillinger, H. In: IAM, Huss, S.: Festsymposium 60-Jahre IAM, 28.-29. September 2005, Wien. Poster presentation.

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Molecular profiling of native yeasts from wine fermentation: a case study with yeast isolates from Austrian wine-producing areas. K. Lopandic
University of Valencia, Department of Valencia, Valencia, Spain, 20. 11. 2006-24. 11. 2006, SOKRATES-Lehrendenmobilitätsprogramm. Oral presentation.

Anhang 1

Diversity of yeasts involved in spontaneous fermentation of wine musts assessed by D1/D2 sequences of 26S rRNA encoding gene and DNA fingerprinting: a case study with yeast isolates from Austrian wine-producing areas

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Running title
Biodiversity of wine yeasts

ABSTRACT

Aims: The aims of this study was to evaluate the autochthonous yeast population during spontaneous fermentations of wine musts in Austrian wine-producing areas. This is the first step of a complex study with the ultimate goal to select the strains with valuable enological properties.

Methods and Results: An approach, combining sequences of D1/D2 domain of 26S rDNA and PCR fingerprinting was used for characterizing yeasts at the species level, whereas the

differentiation of *Saccharomyces* strains was accomplished by AFLP fingerprinting. At the beginning of fermentation, representatives of 11 genera were identified, whereby *Hanseniaspora* and *Metschnikowia* species were characterized most frequently. *S. cerevisiae* and *S. bayanus* var. *uvarum* strains, which were identified throughout entire fermentation process, showed a significant level of polymorphism.

Conclusions: Considerable genotypic diversity of wine yeasts at species and strain level was identified. A number of *Saccharomyces* strains were common in different wineries, but a wide range of strains with characteristic profiles were characterized at individual locations.

Significance and Impact of the Study: This biodiversity study, which was carried out for the first time in Austrian viticultural areas, represent a contribution to the investigation and preservation of genetic diversity of biotechnologically relevant yeasts. Application of sensitive tools enabled reliable genomic differentiation of strains, enological potential of which will be evaluated in further studies.

INTRODUCTION

Microbiology of wine fermentation has been extensively studied field due to different microorganisms (yeasts, bacteria, micro-fungi) involved in the process and their complex interactions, as well as because of the interesting genomic and genetic properties of *Saccharomyces cerevisiae*, that is the main generator of alcoholic fermentation and the most popular eukaryotic model organisms. Yeast biodiversity studies have shown that vast variety of species participate in the early stages of fermentation, which are substituted by *S. cerevisiae* species with increasing alcohol concentration in the latter stages (Fleet 2003). Considerable progress in developing molecular techniques with higher resolving power have led to a more reliable characterisation of yeasts, both at species and strain level. It is generally accepted that various representatives of the genera *Candida*, *Hanseniaspora*, *Issatchenkia*, *Metschnikowia*, *Pichia*, *Kluyveromyces* and *Zygosaccharomyces* are most frequently present in wine must. Extensive studies of genetic and genomic features of *S. cerevisiae* species elucidated, at least partially, the adaptive mechanisms of the yeast genome to stressful enological conditions (high sugar and low nitrogen content, high ethanol concentration, low pH, added antimicrobial compounds). Yeasts associated with alcoholic fermentation may exhibit genetic instability with gross chromosomal rearrangements and chromosome length polymorphisms (Miklos et al. 1997; Rachidi et al. 1999; Dunham et al. 2002), many strains may have trisomies or tetrasomies for some chromosomes (Bakalinsky and Snow 1990; Guijo

et al. 1997), they are often aneuploid or polyploid, and may form interspecieshybrids (Naumova et al. 2005; Bradbury et al. 2006; González et al. 2006; Lopandic et al. submitted). Reasons for the genomic changes may be manifold: spontaneous mutations, Ty-promoted chromosomal translocations (Rachidi et al. 1999), mitotic crossing-over (Aguilera et al. 2000), gene conversion (Puig et al. 2000). As these studies showed, genomic and genetic properties, as well as gene expression profiles (Hauser et al. 2001; Rossignol et al. 2003; Dunn et al. 2005) are strain dependent and due to the genome flexibility the strains are able to adapt to specific environmental conditions.

Various studies have pointed to molecular polymorphisms of the indigenous *S. cerevisiae* strains from different vine-growing regions and strong correlation between their genomic and phenotypic properties (Versavaud et al. 1995; Nadal *et al*., 1996; Esteve-Zarzoso *et al*., 2000; Lopandic et al. submitted). Different yeast strains contribute differently to the wine quality, suggesting that biodiversity studies of wine yeasts within various ecological niches are important not only for fundamental but for applied research too. Autochthonous yeasts may bear new molecular and enological attributes giving the wines a region-specific character. Wine quality is influenced by the wine juice composition and by microflora present during fermentation process. For that reason, characterisation of microbial population and monitoring of its growth attracted interests of many microbiologists, who studied biodiversity of indigenous wine yeasts in different geographical and ecological frameworks (Esteve-Zarzoso et al. 2000; Pramateftaki et al. 2000; Rementeria et al. 2003; Martínez et al. 2004; Combina et al. 2005; Schuller et al. 2005; González et al. 2006; Raspor et al. 2006).

The aim of the present study was to investigate yeast microbiota developed during spontaneous fermentations of wine musts in four wine-producing areas (Wien, Donauland, Neusiedlersee-Hügelland, Südsteiermark) of the eastern and south part of Austria. The selected areas are situated between 200 m and 560 m above sea level in a temperate climatic zone without great extremes and are characterized with different soil types (loess, slate, loam, gravel, marl, sandy soil). The climate is characterized with warm, sunny summers, long , mild autumn days and with the annual precipitation in the range from 400 mm to 800 mm. To gain a better insight into the yeast diversity of this geographical region, which was involved in the biodiversity study for the first time, we investigated yeast population dynamics in white and red wines produced in 2004 at six locations (Cobenzl, Stift Klosterneuburg, Agnes-Hof, Götzhof, St. Georgen, Silberberg). In order to assess genomic variability of non-

Saccharomyces and *Saccharomycs* species and strains, we used sequences of the D1/D2 domain of 26S rRNA encoding gene and information available from sequence databases, as well as two multi-locus techniques, PCR fingerprinting and amplified fragment length polymorphisms (AFLP).

MATERIALS AND METHODS

Sampling of yeast strains

Samples (500 ml) of wine must and juice were taken during the spontaneous fermentation carried out in 2004 with white (Grüner Veltliner, Welschriesling, Sauvignon blanc) and red (Zweigelt, Blaufränkisch, Pinot Noir) grape varieties in the wineries located in six wine-producing localities (Agnes-Hof, Götzhof, Cobenzl, Stift Klosterneuburg, St. Georgen, Silberberg) of the eastern and south part of Austria (Fig. 1). Sampling was accomplished at three stages, at the beginning, middle and end of fermentation, which were determined on the basis of sugar consumption. Aliquots of 0.1 ml from serially diluted samples in physiological solution were plated on lysine (Oxoid) and WL nutrient agar (Oxoid), which support the grow of non-*Saccharomyces* and *Saccharomyces* species, respectively. The media were supplemented with 10 mg l^{-1} of ampicilin and streptomycin to inhibit bacterial growth. After incubation at 25°C for 3 to 5 days, eight to twenty colonies from every fermentation stage (284 in total) were randomly selected, inoculated and maintained on GYP agar (2% glucose, 1% peptone, 0.5% yeast extract, 2% agar).

Characterisation of yeast at the species level

Identification of yeast isolates at the species level was carried out by sequencing of D1/D2 region of 26S rRNA encoding gene and PCR fingerprinting as already reported by Lopandic et al. (2006). Yeast DNA was isolated and purified according to the protocol of the MasterPureTM Yeast DNA Purification Kit (Epicentre, Madison, Wisconsin, USA). To assess genomic similarity, the yeasts were firstly analyzed by PCR fingerprinting with a bacteriophage M13 core sequence (5'-GAGGGTGGCGGTTCT-3'). Polymerase chain reaction was carried out in an MJ research thermal cycler (PTC200) programmed for the initial denaturation at 94°C for 2 min. and 38 cycles of 94°C/ 40 s, 43°C/ 70 s, 72°C/ 90 s, with a final extension of 72°C/ 10 min. From every group of yeasts showing the same PCR

patterns two strains were randomly selected and sequences of the D1/D2 domain of 26S rRNA encoding gene were determined. The D1/D2 sequences were used in a similarity search by means of the Blast program in Genbank (http://www.ncbi.nlm.nih.gov). The strains showing identity scores >99% were selected (Table 1) and the conspecificity with the corresponding yeast isolates from wine must and juice was examined by the PCR fingerprinting. The fingerprints were analyzed with GelCompare II software v.3.5 (Applied Maths, Kotrijk, Belgium). The similarity between the patterns was calculated using the Dice coefficient. The cluster analysis was performed by UPGMA method, with position tolerance (shift between two bands) of 1% and optimization (shift between two patterns) of 0.5%. Statistical significance of the clusters was tested with cophenetic correlation. The strains with PCR profiles showing more than 80% similarity were considered conspecific (Lopandic et al. 2005).

Characterisation of *Saccharomyces* yeasts at the strain level

Variability of *S. cerevisiae* and *S. bayanus* strains was investigated by the AFLP technique (amplified fragment length polymorphism) using the AFLP™ Microbial Fingerprinting kit of Applied Biosystems (Foster City, CA, USA). Restriction/ligation step, as well as the preselective and selective amplification were carried out as described by Lopandic et al. (submitted). Three primer pairs were used for selective amplification: EcoRI-AC-FAM/MseI-G, EcoRI-G-JOE/MseI-CG and EcoRI-C-NED/MseI-CG. Generated fragments were separated by electrophoresis on an ABI 3100 Avant Sequencer (Applied Biosystems). The Genscan 3.1 software was used for extraction of resulted electropherograms and the GeneMapper program (Rinehart 2004) for converting output into binary and nexus file. Cluster analysis was performed by the software PAUP 4.0b10 (Swofford 2002) using the UPGMA method and mean character difference as distance option. Bootstraping was performed with 100 replicates.

RESULTS

To characterize yeast population dynamics during the spontaneous fermentation of different white (Grüner Veltliner, Welschriesling, Sauvignon blanc) and red (Zweigelt, Blaufränkisch, Pinot Noir) wines carried out in six cellars of six wine-producing localities (Agnes-Hof, Götzhof, Cobenzl, Stift Klosterneuburg, St. Georgen, Silberberg) we applied a genotypic

approach comprising sequence determination of D1/D2 regions of 26S rRNA encoding gene and PCR fingerprinting. A predominant part (87%) of yeasts was characterized at the species level (Fig. 2, Table 2, 3). Among identified species, *Saccharomyces cerevisiae* (181 strains) and *Hanseniaspora uvarum* (44 strains) prevailed, whereas *Candida zemplinina*, *Issatchenkia occidentalis*, *Kregervanrija* (formerly *Pichia*) *fluxuum*, *Lachancea* (formerly *Kluyveromyces*) *thermotolerans*, *Metschnikowia viticola*, *Pichia kluyveri*, *Saccharomyces bayanus* and *Zygoascus hellenicus* appeared sporadically (Table 2,3). A number of yeast isolates was shown to be closely related to *Metschnikowia pulcherrima* and *Pichia membranifaciens* on the basis of the D1/D2 sequences (identity scores were <98%), but the PCR fingerprinting and comparison of the resulted patterns with those of the corresponding type strains did not support the conspecificity (Fig. 2). Obviously, different *Metschnikowia* and *Pichia* species grow in the wine musts and their complete identification requires characterisation of additional molecular markers. The non-*Saccharomyces* species were characterized at the beginning and in the middle of must fermentation, where amount of sugar was high and ethanol concentration low. *S. cerevisiae* strains were detected at the beginning of fermentation only in the samples of Pinot Noir (St. Klosterneuburg), Zweigelt (Agnes-Hof) and Grüner Veltliner (Cobenzl), whereas after increasing the ethanol concentration their domination was obvious in all wines. It is interesting that *S. bayanus* strains were detected only in the musts and wines of the locality St. Georgen (Table 3). Non appearance of *S. bayanus* in wines of the other regions may be a consequence of too small number of samples taken during fermentation process.

We employed the AFLP molecular markers for assessing genomic variability of the *S. cerevisiae* and *S. bayanus* strains isolated from different Austrian wine-producing areas. To compare diversity of Austrian wine yeasts with isolates from other countries, we included also three *S. cerevisiae* strains originating from wines produced in Spain (HA4), Switzerland (HA284) and West Africa (HA236), as well as the strains isolated from grapes in Thermenregion, Austria, which were recently described as hybrid strains between *S. cerevisiae* and *S. kudriavzevii* (Lopandic et al., submitted). A cluster analysis was performed with representative strains of every wine and locality which showed characteristic AFLP patterns. On the basis of the electropherograms generated by three primer pairs a phenogram was constructed depicting genotypic relatedness among the strains (Fig. 3). Austrian *S. cerevisiae* strains showed to be a homogenous population, different from wine yeasts isolated in Spain (HA4), Switzerland (HA284) and West Africa (HA236). Between 7 to 11 different

AFLP patterns were distinguished among yeast isolates in every of ten investigated wines, indicating a relatively high strain heterogeneity. If we suppose that the same AFLP patterns represent the same strains, then the cellars at different localities may contain a number of strains with the same genomic structure (Fig. 3). In addition, there are a number of strains with a unique patterns, suggesting that some strains may be typical for one locality (Fig. 3). Out of 69 amplified loci, 43 loci were polymorphic. Austrian *S. cerevisiae* strains could be distinguished among each other on the basis of 18 AFLP fragments (Table 4) and in comparison with the strains from the other countries, four AFLP fragments were found to be informative (133, 240, 247 and 378 bp). Two fragments of 68 and 82 bp are diagnostic for the *S. cerevisiae* isolate from Spain (HA4) and the 129 bp fragment is characteristic for the strain isolates from Switzerland (HA284) and West Africa (HA236). The latter ones are further distinguished by the presence and absence of the 137, 156 and 177 bp long fragments (Table 4). In addition, the West African strain could be diagnosed by two fragments of 118 and 237 bp. Several *S. cerevisiae* strains isolated from grapes in Thermenregion displayed significantly different AFLP profiles (Fig. 3). Out of 34 amplified fragments, 11 fragments were shared exclusively with *S. kudriavzevii* (Table 4), suggesting the hybrid nature of these strains (Lopandic et al., submitted).

Similar to *S. cerevisiae* strains, Austrian *S. bayanus* strains, displaying very different AFLP profiles, indicated a remarkable genomic variability. Eleven strains showed 8 different AFLP patterns. As Fig 3. shows Austrian *S. bayanus* strains seem to be more related to *S. bayanus* var. *uvarum* than to *S. bayanus* type strain. *S. bayanus* var. *uvarum* HA231 and *S. bayanus* HA266 strains displayed no differences by PCR fingerprinting with M13 primer (Fig. 2) and only after AFLP fingerprinting significant differences were observed and affiliation of the yeast isolates from wine juice to *S. bayanus* var. *uvarum* was possible (Fig. 3). Out of 62 amplified fragments within *S. bayanus* strains, 42 are polymorphic (Table 5). Within Austrian *S. bayanus* var. *uvarum* strains 26 fragments are informative. These strains can be distinguished from the HA231 and HA266 type strains by nine fragments (90, 138, 155, 219, 273, 284, 305, 310, 339 bp).

DISCUSSION

The purpose of this study was to evaluate yeast microflora present during spontaneous fermentation of wine musts and juices in several wine-producing areas of eastern part of

Austria. This investigation represents the first step of an extensive study with a final aim to select the strains with interesting enological potential typical for this geographical and climatic framework. The yeast-profiling was achieved by employing a combination of three methods, partial sequencing of the 26S rRNA encoding gene (D1/D2 region), and two multi-locus techniques, PCR- and AFLP fingerprinting. The D1/D2 domain of 26S rDNA is one of the most frequently used regions in species identification and prediction of phylogenetic relatedness among yeasts (Kurtzman and Robnet 1998; Fell et al. 2000). Although widely accepted as a standard procedure for yeast identification, Lopandic et al. (2006) indicated that differentiation based on 26 rDNA sequences may be hampered by high homology between closely related species. The authors suggested that an additional method should be employed to test reliability of the results based on the sequence information. To confirm conspecificity of the yeast isolates from wine with corresponding type strains, selected on the basis of sequence homology with sequences from Genebank, we performed PCR fingerprinting of genomic DNA with the primer M13. This approach enabled us to identify reliably 11 species belonging to the genera *Candida*, *Hanseniaspora*, *Issatchenkia*, *Kregervanrija*, *Lachancea*, *Metschnikowia*, *Pichia*, *Saccharomyces* and *Zygoascus* (Fig. 2, Table 2, 3). We observed that *Hanseniaspora uvarum* and different species of the genus *Metschnikowia* represent ubiquitous yeasts at the early fermentation stages. Furthermore, these yeasts, as well as *Candida zemplinina* and one unidentified *Candida* species were only non-*Saccharomyces* yeasts which persisted well into the middle stage of fermentation. A predominant part of *Metschnikowia* and *Pichia* isolates were characterized on the rank of genus only. They showed close relatedness to *M. pulcherrima* and *P. membranifaciens* species, but type strains of the corresponding species did not show conspecificity with the wine yeasts by PCR fingerprinting (Fig. 3). The sequences of D1/D2 domain of 26S rDNA were very divergent, as well as the PCR patterns (Fig. 3), suggesting that these yeasts might be a new candidates for the genera *Metschnikowia* and *Pichia*. To similar conclusions came Pallmann et al. (2001) who observed a high degree of divergence among sequences of D1/D2 region within the pulcherrimin producing yeasts, which were traditionally classified as a single species, *M. pulcherrima*. Importance of the non-*Saccharomyces* species for wine fermentation has been intensively discussed since recently due to their possible contribution to specific aroma properties of the wines from certain regions. Different yeast species and strains showed different ability of producing biogenic amines, extracellular hydrolytic enzymes, acids, esters and alcohols (Charoenchai et al. 1997; Soden et al. 2000, Caruso et al. 2002; Romano et al. 2003; Clemente-Jimenez et al. 2004). For that reason, a reliable identification of yeasts

followed by studies of the production of secondary metabolites and enological properties should be undertaken to evaluate real influence of the non-*Saccharomyces* species and strains on the final wine quality.

The AFLP fingerprinting is more polymorphic than the PCR fingerprinting technique, making this method more suitable for differentiation of yeasts at strain level (de Barros Lopes et al. 1999; Azumi and Goto-Yamamoto 2001; Lopandic et al. 2005). It is based on the specific restriction fragment analysis of the complete genome and selective amplification of the resulted fragments by high-stringency PCR (Zabeau and Vos 1993). *S. cerevisiae* strains from spontaneous fermentation of wine musts displayed very heterogeneous AFLP patterns (Fig. 3). A number of strains with the same AFLP profiles were shared between several wineries, although various strains with the unique patterns were identified too, suggesting that cellars of different localities may harbor domesticated strains. However, these observations are based on very small number of yeast isolates, hence, they should be looked at with some caution. Although this study did not involve a larger number of wine strains from other countries, a comparison of the AFLP profiles of the isolates from Spain, Switzerland and West Africa indicated that the Austrian *S. cerevisiae* strains is a homogenous population (Fig. 3). Four fragments (133, 240, 247 and 378 bp) were detected only within the Austrian strains, indicating that these loci contain interesting attributes specific for the strains of this geographical region. On the other hand, as already mentioned, the strains HA4, HA284 and HA236 can be distinguished from the Austrian yeasts by several specific fragments, suggesting that *S. cerevisiae* strains of different geographical origin may contain the genomic portions which designate their particularity. One of the crucial question is how these genomic differences influence production of aroma substances during wine fermentation and which impact they have on the quality of final product. Recently we investigated genotypic and phenotypic properties of *S. cerevisiae* strains originating from grape berries collected in four Austrian vine-growing regions (Lopandic et al. submitted). Results demonstrated that genomic properties may influence production of different alcohols, esters and acids, suggesting that screening programs carried out in different countries have importance in selecting strains with best attributes for alcoholic fermentation. In course of our studies we were able to isolate natural interspecies hybrid strains between *S. cerevisiae* and *S. kudriavzevii* from Thermenregion, which showed to have a mix of two sequences of D1/D2 and ITS1/ITS2 regions (Lopandic et al. submitted), as well as two alleles of the genes *CAT8*, *CYR1*, *GSY1*, *MET6* and *OPY1*, one allele similar to that from *S. cerevisiae* and the other to

that from *S. kudriavzevii* (A. Querol, pers. comm.). These hybrid strains showed some properties of enological interests. They acquired the ethanol tolerance and the ability to grow in media containing high levels of sugar from the *S. cerevisiae* and an improved growth at lower temperatures from the *S. kudriavzevii* parental part (Gonzáles et al. pers. comm.). Fig. 3 shows genotypic relatedness of the *S. cerevisiae* strains isolated from spontaneous fermented wines and the hybrids (strains from Thermenregion). Genotypic differences are significant due to the significant number of AFLP fragments shared between the *S. cerevisiae* and *S. kudriavzevii* in hybrid strains (Table 4). Unfortunately, we were not able to isolate and identify any hybrid strain during spontaneous fermentation and our further study would comprise more localities from Thermenregion and surrounding areas to study dissemination of the hybrids.

Beside *S. cerevisiae*, *S. bayanus* is considered as the principal yeast in wine fermentation. *S. bayanus* HA266^T (CBS380^T) was described as species with two varieties, "bayanus" and "uvarum" (Naumov 2000). Several authors have recently suggested that *S. bayanus* represents a hybrid species between *S. uvarum* and *S. cerevisiae* and therefore, *S. uvarum* should be considered as a distinct species within the *Saccharomyces sensu stricto* group (Nguyen et al. 2000; Pulvirenti et al. 2000; Nguyen and Gaillardin 2005). On the other hand, high DNA:DNA reassociation values (86-100%) and semi sterility of the hybrids between *S. bayanus* and *S. bayanus* var. *uvarum* clearly show that these varieties are only partially genetically isolated (Naumov 2000, Naumova et al. 2005). These results are in agreement with the estimation of genomic relatedness by AFLP fingerprinting performed in the present study. We have shown that the similarity level of the AFLP fingerprinting between *S. bayanus* HA266 and *S. bayanus* var. *uvarum* HA231 strains are 69% (Lopandic et al. submitted; present work). In our opinion, the strain HA231 should be considered as a variety of *S. bayanus*, and in this paper we keep using the name *S. bayanus* var. *uvarum*. The AFLP analysis has shown that Austrian yeast isolates from spontaneously fermented wines are more related to *S. bayanus* var. *uvarum* than to *S. bayanus* type strain (Fig. 3). Eleven strains displayed eight different AFLP profiles, indicating significant genomic heterogeneity of *S. bayanus* var. *uvarum* strains. Isolation from a winery in St. Georgen may indicate a well adaptation of *S. bayanus* var. *uvarum* strains to the environmental condition of this locality. This is an additional evidence that the wine-producing area is a specific framework of *S. bayanus* var. *uvarum*, which was already shown by Naumov et al. (1993, 2001) through

genetic and karyotypic analyses of a number of melibiose-fermenting yeasts isolated from wine, apple and cider juices.

In conclusion, results of the present study show a significant diversity of *Saccharomyces* and non-*Saccharomyces* yeasts originating from spontaneously fermented grape musts and juices. The Austrian wine-growing areas may harbor autochthonous wine yeasts with specific attributes, but this particularity should be verified with a higher number of wine yeasts originating from other countries. Enological and technological properties of selected strains should be examined in the further studies, which could elucidate their role and significance in formation of wine flavour.

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Table 1. Strains used in the comparison with wine yeasts by PCR- or AFLP fingerprinting

Species	ACBR No.	CBS No.	Status of the strain	Isolation source	Genebank Accession No. LSU D1/D2
Candida zemplinina	HA2246	CBS9494	T of *C. zemplinina*	botrytized wine, Tokaj wine region, Hungary	AY160761
Hanseniaspora uvarum	HA1216	CBS 314	T of *Kloeckeraspora uvarum*	muscatel grape, Russia	AF257273
Issatchenkia occidentalis	HA2259	CBS1910	T of *Candida sorbosa*	droppings of *Drosophila crucigera*	U76348
Kregervanrija fluxuum	HA1033	CBS639	T of *Candida mycoderma*	sour wine, Netherlands	U75719
Lachancea thermotolerans	HA42	CBS6340	T of *Zygosaccharomyces thermotolerans*	mirabelle plum conserve, Russia	U69581
Metschnikowia pulcherrima	HA665	CBS5833	T of *M. pulcherrima*	berries of *Vitis labrusca*, USA, Claifornia	U45736
M. viticola	HA2105	CBS9950	T of *M. viticola*	berries of grape *Vitis vinifera*, red cultivar, Hungary	AY626892
Pichia kluyveri	HA1088	CBS188	T of *P. kluyveri*	olives	U75727
P. membranifaciens	HA895	CBS107	T of *Saccharomyces membranifaciens*		U75725
Saccharomyces bayanus	HA266	CBS380	T of *S. bayanus*	turbid beer	AY130339
S. bayanus var. uvarum	HA231	CBS395	T of *S. uvarum*	juice of *Ribes nigrum*, Netherlands	
Saccharomyces cerevisiae	HA4	CBS4054	T of *S. aceti*	red wine, Spain	AY130346
S. cerevisiae	HA284	CBS423	T of *S. chodati*	wine, Switzerland, Riddes	
S. cerevisiae	HA236	CBS405	T of *S. mangini*	bili wine, West Africa	
Zygoascus hellenicus	HA1605	CBS6736	T of *Candida inositophila*	washings of ion exchange resin in guanine monophosphate plant, Japan	AJ508566

Table 2. Yeast species characterized at three stages (BF=begin, MF=middle, EF=end of fermentation) during the fermentation of different wines produced in the St. Klosterneuburg, Agnes-Hof and Götzhof localities

Yeast species	No. of isolated colonies (Year 2004)														
	Wien, Stift Klosterneuburg			Donauland, Agnes-Hof						Donauland, Götzhof					
	Pinot Noir			Grüner Veltliner			Zweigelt			Grüner Veltliner			Blaufränkisch		
	BF	MF	EF	BF	MF	EF	BF	MF	EF	BF	MF	EF	BF	MF	EF
C. zemplinina		2													
H. uvarum				1			2			3			4		
I. occidentalis					1										
Metschnikowia sp.	2			7			1			2			2		
M. viticola										1					
P. kluyveri					1					2					
Pichia sp.	1						1						2		
S. cerevisiae	5	6	8		6	8	4	8	8		8	8		8	8
Sum of all investigated yeasts	24			24			24			24			24		

Table 3. Yeast species characterized at three stages (BF=begin, MF=middle, EF=end of fermentation) during the fermentation of different wines produced in the Cobenzl, St. Georgen and Silberberg localities

Yeast species	No. of isolated colonies (Year 2004)														
	Wien, Cobenzl			Neusiedlersee-Hügelland, St. Georgen						Südsteiermark, Silberberg					
	Grüner Veltliner			Welschriesling			Zweigelt			Zweigelt			Sauvignon blanc		
	BF	MF	EF	BF	MF	EF	BF	MF	EF	BF	MF	EF	BF	MF	EF
Candida sp.										2	1				
H. uvarum		2		6			5	6		2	6		7		
K. fluxuum				1											
L. thermotolerans	2						1								
Metschnikowia sp.		1		1	2					5			2		
M. viticola													1		
Pichia sp.	2							1							
S. bayanus				2			4	4	1						
S. cerevisiae	4	5	8		8	10		9	9		13	10		10	10
Z. hellenicus										1					
Sum of all investigated yeasts	24			30			40			40			30		

Table 4. Polymorhic AFLP loci detected in *S. cerevisiae* and *S. kudriavzevii* strains

Yeast strains	AFLP fragments (bp)																																									
	68	80	82	85	86	90	98	106	109	112	115	118	128	129	133	136	137	141	156	177	183	187	191	195	200	201	221	230	232	237	240	242	246	247	265	277	281	284	288	307	318	356	378
Austrian S. cerevisiae strains	0	0	0	1	0	V	V	V	0	0	0	0	1	0	V	V	V	V	0	V	V	V	0	0	0	0	0	0	0	0	V	0	V	V	0	V	0	V	0	V	0	V	V
HA284	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0
HA236	0	0	0	0	0	1	0	1	0	0	0	1	1	1	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	1	0
HA4	1	0	1	0	0	1	0	1	0	0	0	0	1	0	0	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0
Hybrid strains	0	1	0	1	1	1	V	1	1	1	1	0	1	0	0	1	V	1	1	1	1	V	1	1	1	V	1	1	V	0	0	1	1	0	1	1	1	V	1	V	1	V	0
S. kudriavzevii	0	1	0	1	1	0	1	1	0	1	1	0	1	0	0	0	0	0	1	0	1	0	1	1	1	0	0	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0

Table 5. Polymorphic AFLP loci detected in *S. bayanus* strains

Yeast strains	AFLP fragments (bp)																																										
	61	67	74	75	77	87	89	90	92	103	106	107	110	111	114	121	123	129	135	138	146	148	153	155	171	182	189	208	218	219	231	235	273	278	282	284	305	310	327	330	339	392
Austrian *S. bayanus* var. *uvarum* strains	V	0	V	V	0	0	V	V	V	V	0	0	0	V	0	0	0	0	0	V	V	0	V	V	0	V	0	V	V	V	V	V	V	V	V	V	V	0	0	V	V
HA231	1	1	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0
HA266	0	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	1	0	1	0	0	0	1	1	1	0	1	0	1	1	0	1	1	0	0	0	1	1	0	1

Figure legends

Fig. 1. Wine-producing areas of the eastern and south part of Austria where the sampling of wine musts and juices was carried out

Fig. 2. Cluster analysis of the wine yeast isolates based on the PCR band patterns. The scale indicates percentages of similarity between patterns. The numbers on branches indicate co-phenetic correlation indexes.

3. Phenogram based on the AFLP fingerprints showing genomic diversity of *S. cerevisiae* and *S. bayanus* strains isolated from Austrian wine producing areas. Wine locations are indicated by capital letters: AH-Agnes-Hof, GH-Götzhof, CO-Cobenzl, SK-Stift Klosterneuburg, SG-St. Georgen, SI-Silberberg. Wine types are indicated by small letters: gv-Grüner Veltliner, zw-Zweigelt, bf-Blaufränkisch, pn-Pinot Noir, wr-Welschriesling, sb-Sauvignon blanc. Begin of fermentation, middle stage and end of fermentation, at which the sampling was carried out, are indicated by numerals 1, 2, 3, respectively.

Fig. 1


Donauland
Agnes-Hof
Götzhof
Wien
Cobenzl
Stift Klosterneuburg
Neusiedlersee-Hügelland
St. Georgen
Südsteiermark
Silberberg
Gmünd
NIEDERÖSTERREICH
Krems
Sankt Pölten
WIEN
VIENNA
Baden
Wiener Neustadt
Eisenstadt
Sopron
Bruck
BURGENLAND
Graz

Fig. 2

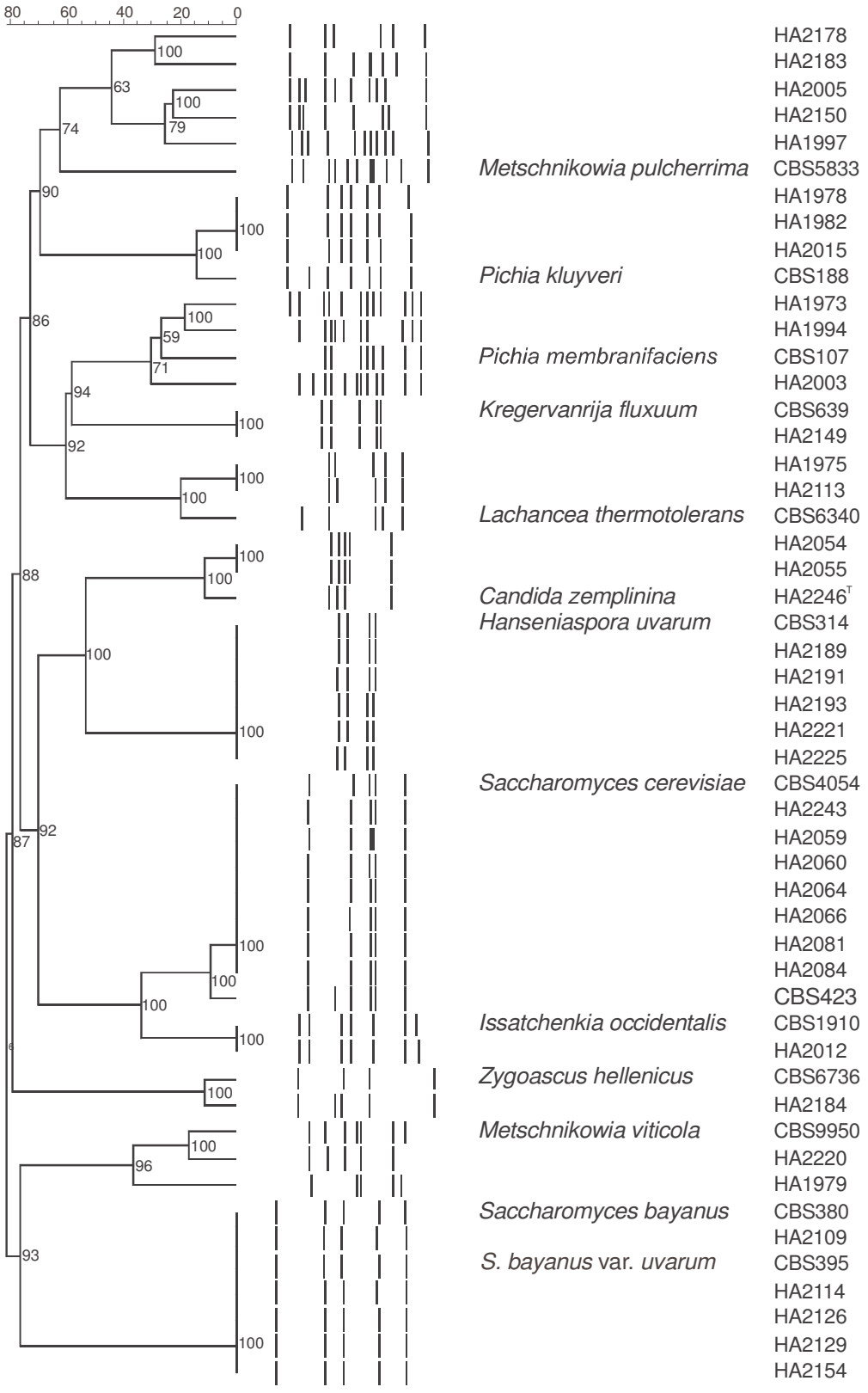
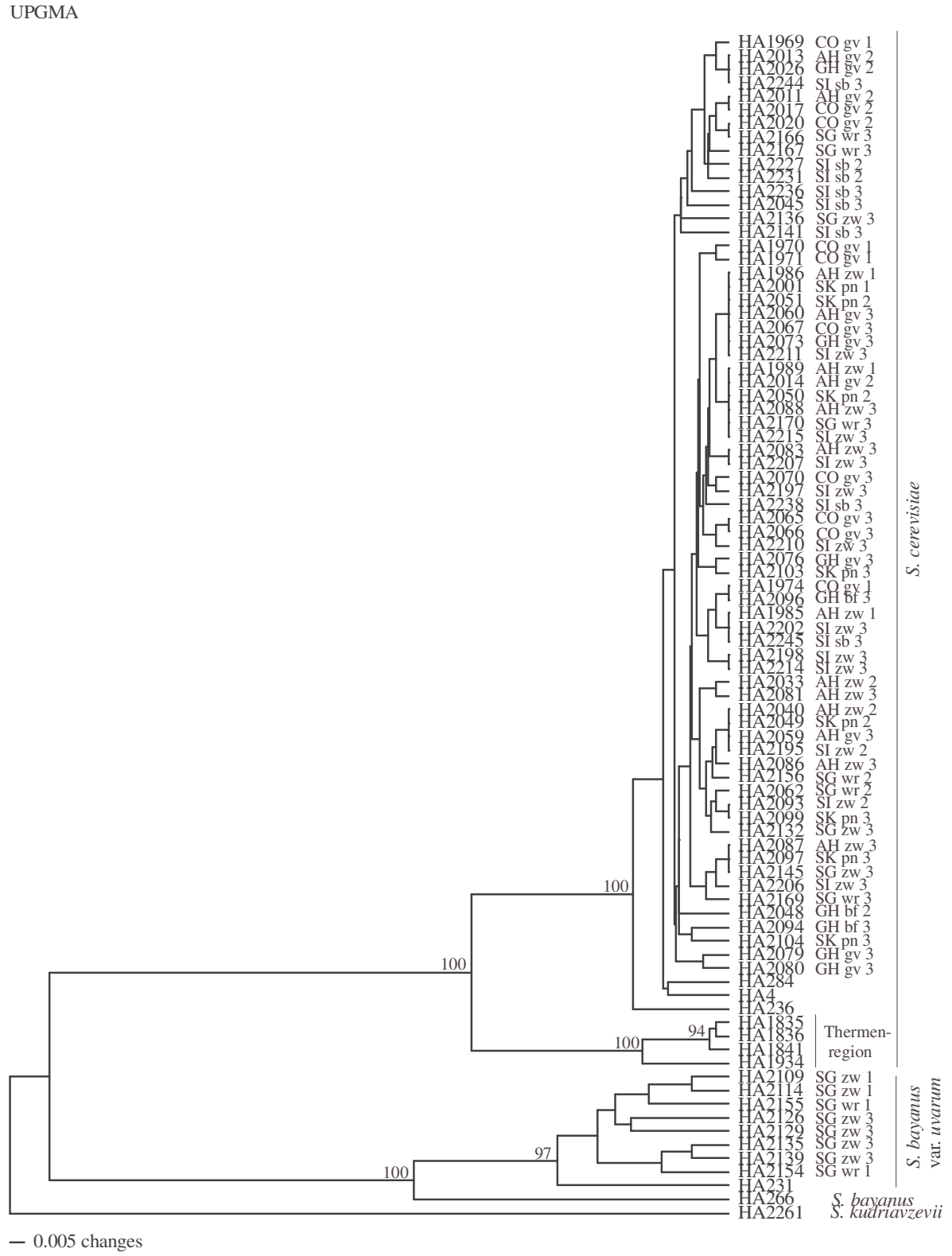

80
60
40
20
0
HA2178
HA2183
HA2005
HA2150
HA1997
Metschnikowia pulcherrima CBS5833
HA1978
HA1982
HA2015
Pichia kluyveri CBS188
HA1973
HA1994
Pichia membranifaciens CBS107
HA2003
Kregervanrija fluxuum CBS639
HA2149
HA1975
HA2113
Lachancea thermotolerans CBS6340
HA2054
HA2055
Candida zemplinina HA2246T
Hanseniaspora uvarum CBS314
HA2189
HA2191
HA2193
HA2221
HA2225
Saccharomyces cerevisiae CBS4054
HA2243
HA2059
HA2060
HA2064
HA2066
HA2081
HA2084
CBS423
Issatchenkia occidentalis CBS1910
HA2012
Zygoascus hellenicus CBS6736
HA2184
Metschnikowia viticola CBS9950
HA2220
HA1979
Saccharomyces bayanus CBS380
HA2109
S. bayanus var. uvarum CBS395
HA2114
HA2126
HA2129
HA2154

Fig. 3


UPGMA
HA1969 CO gv 1
HA2013 AH gv 2
HA2026 GH gv 2
HA2244 SI sb 3
HA2011 AH gv 2
HA2017 CO gv 2
HA2020 CO gv 2
HA2166 SG wr 3
HA2167 SG wr 3
HA2227 SI sb 2
HA2231 SI sb 2
HA2236 SI sb 3
HA2045 SI sb 3
HA2136 SG zw 3
HA2141 SI sb 3
HA1970 CO gv 1
HA1971 CO gv 1
HA1986 AH zw 1
HA2001 SK pn 1
HA2051 SK pn 2
HA2060 AH gv 3
HA2067 CO gv 3
HA2073 GH gv 3
HA2211 SI zw 3
HA1989 AH zw 1
HA2014 AH gv 2
HA2050 SK pn 2
HA2088 AH zw 3
HA2170 SG wr 3
HA2215 SI zw 3
HA2083 AH zw 3
HA2207 SI zw 3
HA2070 CO gv 3
HA2197 SI zw 3
HA2238 SI sb 3
HA2065 CO gv 3
HA2066 CO gv 3
HA2210 SI zw 3
HA2076 GH gv 3
HA2103 SK pn 3
HA1974 CO gv 1
HA2096 GH bf 3
HA1985 AH zw 1
HA2202 SI zw 3
HA2245 SI sb 3
HA2198 SI zw 3
HA2214 SI zw 3
HA2033 AH zw 2
HA2081 AH zw 3
HA2040 AH zw 2
HA2049 SK pn 2
HA2059 AH gv 3
HA2195 SI zw 2
HA2086 AH zw 3
HA2156 SG wr 2
HA2062 SG wr 2
HA2093 SI zw 2
HA2099 SK pn 3
HA2132 SG zw 3
HA2087 AH zw 3
HA2097 SK pn 3
HA2145 SG zw 3
HA2206 SI zw 3
HA2169 SG wr 3
HA2048 GH bf 2
HA2094 GH bf 3
HA2104 SK pn 3
HA2079 GH gv 3
HA2080 GH gv 3
HA284
HA4
HA236
HA1835
HA1836
HA1841
HA1934
HA2109 SG zw 1
HA2114 SG zw 1
HA2155 SG wr 1
HA2126 SG zw 3
HA2129 SG zw 3
HA2135 SG zw 3
HA2139 SG zw 3
HA2154 SG wr 1
HA231
HA266
HA2261
100
100
94
100
97
100
S. cerevisiae
Thermen-region
S. bayanus var. uvarum
S. bayanus
S. kudriavzevii
0.005 changes

Anhang 2

Investigation of genomic variability of autochthonous *Saccharomyces cerevisiae* strains isolated from Austrian vine-growing regions and their influence on aroma composition of wines

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Abstract

To evaluate the influence of the genomic properties of yeasts on the formation of wine flavour, genotypic diversity among natural *Saccharomyces cerevisiae* strains originating from grapes collected in four localities in three Austrian vine-growing areas (Thermenregion: locations Perchtoldsdorf and Pfaffstätten, Neusiedlersee-Hügelland: location Eisenstadt, Neusiedlersee: location Halbturn) was investigated and the aroma compounds produced during fermentation of the grape must of "Grüner Veltliner" were identified. Amplified fragment length polymorphism analysis (AFLP) was used to estimate genomic diversity among *S. cerevisiae* strains and gas-chromatography/mass spectrometry for identification of aroma compounds. The genotypic analysis showed that the yeast strains cluster in four groups corresponding to their geographical origin. The AFLP fingerprinting and sequencing of the D1/D2 domain of 26S rRNA encoding gene and ITS1/ITS2 regions demonstrated that the

Perchtoldsdorf strains are putative interspecies hybrids between *S. cerevisiae* and *S. kudriavzevii*. Analysis of the aroma compounds indicated a region-specific influence of the yeast strains on chemical composition of the wines. The aroma compound profiles generated by the strains from Perchtoldsdorf were more similar to those produced by the Pfaffstätten strains than by the Eisenstadt and Halbturn strains. It was assumed that a *S. cerevisiae* parental part from the Pfaffstätten locality might contribute to the genome of the Perchtoldsdorf strains.

Keywords: wine fermentation, *Saccharomyces cerevisiae*, *Saccharomyces kudriavzevii*, hybrids, AFLP fingerprinting, aroma compounds

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Running title: Genotypic and phenotypic diversity of *S. cerevisiae* strains

Introduction

Advances in molecular biology techniques and their higher resolving power contributed significantly to the studies of biodiversity and genomic properties of the wine yeasts. Apart from the various representatives of the genera *Candida*, *Hanseniaspora*, *Issatchenkia*, *Metschnikowia*, *Pichia*, *Kluyveromyces* and *Zygosaccharomyces* (Fleet, 2003), which dominate early stages of a fermentation process, *S. cerevisiae* is the principal species of an alcoholic fermentation. *S. cerevisiae* is able to grow in the substrate characterised by the high sugar and ethanol content, low pH and added antimicrobial compounds, demonstrating that its genome is well adapted to the stressful enological conditions (Querol *et al*., 2003; Barrio *et al*., 2006). Genetic studies have shown significant differences among commercial and laboratory *S. cerevisiae* strains (Benitez *et al*., 1996; Mortimer, 2000, Dunn *et al*., 2005). Whereas most strains used in the laboratory have defined set of chromosome lengths and are haploid or diploid, frequently investigated yeasts associated with alcoholic fermentation show chromosomal alterations and variations in ploidy (Versavaud *et al*., 1995; Miklos *et al*., 1997; Rachidi *et al*., 1999). Wine yeasts may exhibit genetic instability with gross chromosomal rearrangements, chromosome length polymorphisms and may be polyploid or aneuploid (Dunham *et al*., 2002; Naumova *et al*., 2005; Bradbury *et al*., 2006). In addition, a number of

interspecies hybrids have been described which have arisen through the hybridisation events between distinct species of the *Saccharomyces sensu stricto* complex (Nguyen *et al*., 2000; Naumova *et al*., 2005; Liti *et al*., 2005; Bradbury *et al*., 2006; González *et al.*, 2006).

Genetic properties of *S. cerevisiae* strains may be reflected in their phenotypic features (Nadal *et al*., 1996; Antonelli *et al*., 1999; Fleet, 2003; Romano *et al*., 2003). During an alcoholic fermentation a number of genetically distinct *S. cerevisiae* strains release various aroma compounds which influence organoleptic quality of wines. The impact of yeasts on wine flavour is determined by the production of more than 400 volatile compounds (Romano *et al*., 2006; Abbas, 2006). The aroma attributes of wines are determined by higher alcohols, organic acids, phenols, esters, carbonyl, nitrogen and sulphur compounds and to a lesser extent acetaldehyde (Antonelli *et al*., 1999; Lambrecht and Pretorius, 2000; Romano *et al*., 2003, 2006; Abbas, 2006). Various studies have shown significant molecular polymorphisms of the indigenous *S. cerevisiae* strains from different vine-growing regions and a strong correlation between their genomic and phenotypic properties (Nadal *et al*., 1996; Esteve-Zarzoso *et al*., 2000). Different yeast strains contribute differently to the wine quality, therefore, biodiversity studies of wine yeasts within an ecological framework are essential for discovering the strains with new molecular and enological attributes.

In order to investigate genomic variability and enological properties of the indigenous *S. cerevisiae* strains, we compared the yeast populations in four localities in three Austrian vine-growing areas (Thermenregion: locations Perchtoldsdorf and Pfaffstätten, Neusiedlersee-Hügelland: location Eisenstadt, Neusiedlersee: location Halbturn). Genomic diversity was studied by AFLP (amplified fragment length polymorphism) molecular markers, whereas the phenotypic properties of the natural yeast isolates and their impact on the formation of wine flavour were evaluated by analysis of the aroma compounds produced during the fermentation of the grape must of the typical Austrian grape variety "Grüner Veltliner".

Materials and methods

Yeast strains

Saccharomyces cerevisiae strains originated from grapes sampled directly from vine in 2003 in three vine-growing areas (Thermenregion: locations Perchtoldsdorf and Pfaffstätten, Neusiedlersee-Hügelland: location Eisenstadt, Neusiedlersee: location Halbturn) of the

eastern part of Austria (Fig. 1). Samples of four grape varieties were collected: "Weißer Burgunder" (Perchtoldsdorf), "Grüner Veltliner" (Pfaffstätten), "Welschriesling" (Eisenstadt) and "Chardonnay" (Halbturn). Sterilised bags were used to sample 1.5 kg physiologically ripen grapes with a sugar content of 20° Brix from each location. The grapes were crushed directly in the plastic bags and 200 ml grape juice from every location were spontaneously fermented in 300 ml Erlenmeyer flasks at 22°C until the ethanol concentration was 7% (v/v). Up to 10 yeast strains for each locality were isolated (10 from Pfaffstätten, 8 from Eisenstadt, 4 from Halbturn, 10 from Perchtholdsdorf) from the end products and inoculated on GYP agar (2% glucose, 1% peptone, 0.5% yeast extract). The grown yeast cultures were used in genomic characterisation. Table 1 shows all the *S. cerevisiae* strains isolated from Austrian vine-growing regions, as well as the reference strains used for comparison.

Wine fermentation and analysis

To analyse volatile compounds of the wines (higher alcohols, acids, esters, aldehydes), a standardised and pasteurised grape juice of "Grüner Veltliner" with an initial sugar concentration of 160 g L^{-1} and a pH value 3.6 was inoculated with the selected strains. Micro-fermentations were carried out in thirty two 300 mL Erlenmeyer flasks filled with 250 mL grape juice at 22°C until ethanol concentration was approx. 9% (v/v). The fermentation progress was monitored by determining the weight loss caused by the production of CO_2. The resulting wines were tested olfactorically by trained tasters. Odour and taste of all wines were acceptable.

DNA isolation

Yeast DNA was isolated and purified according to the protocol of the MasterPureTM Yeast DNA Purification Kit (Epicentre, Madison, Wisconsin, USA).

AFLP-genotyping with fluorescently labelled primer

The AFLP procedure was performed using the AFLP™ Microbial Fingerprinting kit of Applied Biosystems (Foster City, CA, USA). Restriction and ligation steps were performed simultaneously in a total volume of 11 µL with approx. 10 ng of genomic DNA, 1 x T4 DNA ligase buffer + ATP (New England Biolabs, Beverly, USA), 50 mM NaCl, 45 ng μL^{-1} bovine serum albumin, 1U of *Mse*I (NEB), 5U of *Eco*RI (NEB), 2U of T4 DNA ligase (NEB), 0.02

µM *Eco*RI adaptor and 0.2 µM *Mse*I adaptor. The restriction/ligation mixture was incubated for 3 hours at 37°C and diluted by adding 20 µl TE buffer. Preselective PCR-amplification was performed with 4 µL diluted restriction/ligation mixture, 0.5 µL (10 µM) of each *Eco*RI-, *Mse*I-core sequence and 15 µL Amplification Core Mix from the AFLP™ Microbial Fingerprinting kit under the following conditions: 72°C/2 min, followed by 20 cycles of 94°C/20 s, 56°C/45 s and 72°C/2 min. The PCR product was diluted by adding 20 µL TE buffer. Selective PCR-amplification was performed with 1.5 µL of the diluted pre-amplification mix, 0.5 µL (5 µM) *Mse*I-selective primer, 0.5 µL (1 µM) fluorescently labelled *Eco*RI-selective primer and 7.5 µL Amplification Core Mix. Three primer pairs were used for selective amplification: EcoRI-AC-FAM/MseI-G, EcoRI-G-JOE/MseI-CG and EcoRI-C-NED/MseI-CG. The reaction took place under the following conditions: 94°C/2 min, 10 cycles of 94°C/20 s, 66°C/45 s decreasing 1°C every step of the cycle, and 72°C/2 min, 25 cycles of 94°C/20 s, 56°C/45 s and 72°C/2 min. The products of the selective amplification were analysed by electrophoresis on a 36 cm capillary column on an ABI 3100 Avant Sequencer (Applied Biosystems). Aliquot of 1 µL was mixed with 24 µL formamide and 1 µL Genescan-500 [ROX] size standard (Applied Biosystems). The mix was heated for 3 min at 95°C and cooled on ice until electrophoresis was performed. The Genescan 3.1 software was used for extraction of generated chromatograms. AFLP fragment sizing and converting output into a binary file was performed by the GeneMapper software (Rinehart, 2004). This program introduces a novel Excel macro that creates a NEXUS format interleaved alignment compatible with software PAUP 4.0b10 (Swofford, 2002). A cluster analysis was performed using the UPGMA method and mean character difference as distance option. Bootstrapping was performed with 100 replicates.

Sequencing of the D1/D2 domain of 26S rRNA gene and ITS regions

A fragment of approx. 1500 bp was amplified with the primers ITS5w (GGAASTAAAAGTCGTAACAAG) (Molnar, pers. com.) and NL4 (GGTCCGTGTTTCAAGACGG) (White *et al*., 1990) in a 50 µL reaction mixture containing 10 µL of 10 x GoTaq®-buffer (Promega), 0.2 mM of each deoxynucleotide triphosphate (Peqlab, Erlangen, Germany), 100 nM concentration of each primer, 5-20 ng of DNA preparation and 1.25 U GoTaq®-DNA-Polymerase (Promega). The PCR was performed in a MJ Research thermal cycler (PTC200) programmed for the initial denaturation at 95°C for 2 min, and 36 cycles of 94°C/30 s; 56°C/75 s; 72°C/2 min, with a final extension of 72°C/10

min. To remove the remaining primers and nucleotides, the PCR products were purified with the QIAquick PCR Purification Kit (Qiagen). The LSU D1/D2 was sequenced with primers NL1 (GCATATCAATAAGCGGAGGAAAAG) and NL4 (GGTCCGTGTTTCAAGACGG) (White *et al*., 1990), whereas the ITS1-5.8S rDNA-ITS2 region with primers ITS5w (GGAASTAAAAGTCGTAACAAG) (Molnar, pers. com.) and ITS4p (TCCTCCGCTTATTGATATGC) (White *et al*., 1990). Sequencing was performed on an ABI 3100 Avant Sequencer (Applied Biosystems, Foster City, USA). Sequences are deposited in the EMBL data library with the accession numbers indicated in Table 1. Nucleotide sequences were aligned using the ClustalX program (Thompson *et al.*, 1997). Phylogenetic relationships were estimated by PAUP 4.0b10 (Swofford, 2002), treating all alignment gaps as missing. A maximum parsimony tree (MP) was inferred using heuristic search and stepwise-addition option. Confidence values for individual branches were determined by a bootstrap analysis with 100 repeated samplings of the data.

Cloning of PCR products

Due to the problems getting good sequences with the strains HA1835-HA1844 (yeasts isolates from Perchtoldsdorf region) with the above described method, the ITS5w-NL4 PCR product of a representative strain (HA1835) was cloned into the pDrive Cloning vector of the Qiagen PCR plus kit. Ligation mixes were used to transform Qiagen EZ competent cells from the Kit. Ten white recombinant colonies were randomly picked and used directly for PCR amplification and sequencing of the D1/D2 domain and ITS1/ITS2 regions.

Ploidy estimation

Yeast cells were treated as described by Bradbury *et al*. (2006) with minor modifications. Cultures were grown on YPD agar for 3 days at 25°C. Approximately $1x10^8$ cells were vortexed vigorously in 0.5 mL 0.9% NaCl solution to avoid clump formation. Cell fixation was performed overnight at 4°C after adding 0.5 mL of 70% ethanol in a drop by drop manner with strong mixing. The cells were washed once with 50 mM sodium citrate solution, pH 7.5, centrifuged at 3000*g* for 10 min and re-suspended in 0.5 mL of sodium citrate. Treatment with 2 µL RNAse (5 µg μl^{-1}) was carried out for 2 hours at 37°C. After addition of 10 µL propidium iodide (1 µg mL^{-1}), the samples were incubated for an additional hour and analysed by flow cytometer (FACS Calibur, Becton-Dickinson) in standard configuration. The propidium iodide signal was measured in FL2-H using a 575/26 BP filter. To estimate

genome size of the natural yeast isolates, *S. cerevisiae* strain ATCC 201388 was used as a haploid control.

Analysis of aroma components by gas chromatography-mass spectrometry (GC-MS)

Headspace solid-phase microextraction (SPME) coupled to capillary gas chromatography-mass spectrometry (GCD, Hewlett Packard, USA) was used for determination of 30 aroma compounds. The wine samples (6 mL) with 1.8 g sodium chloride and 50 µL 3-decanol (48.4 mg L^{-1}) as internal standard, were placed in a 10 mL headspace vial equipped with a magnetic bar and capped with a PTFE-coated silicone septum. For the headspace SPME process a fused silica fibre coated with 50/30µm DVB/Carboxen/PDMS was chosen to adsorb aroma compounds. The fibre was exposed in the headspace of sample vials for 30 min at 30°C. After extraction, the fibre was immediately inserted into the GC injector port for 2 min at 250°C for thermal desorption. For determination of different aroma compounds a CP-WAX 52 CB capillary column (50 m x 0.32 mm id, 0.40 µm ft; Chrompack, The Netherlands) was used. The oven temperature was held at 50°C for 6 min before being increased by 4°C per min to 230°C and then kept at this temperature for a further 10 min. The detector temperature was 250°C and helium was used as carrier gas with the constant flow rate of 1.12 mL min^{-1}.

Data analysis

The GC/MS data were exported to the Statgraphics plus 4.0 software (Manungistics, inc. USA, 1998). Biochemical profiles were evaluated using principal component analysis (Hartung & Elpelt, 1999). The component weights of the PCA were used to identify the aroma compounds that are relevant for the separation into aroma clusters.

Results

AFLP genotyping

The AFLP technique was used to identify genomic differences among *S. cerevisiae* strains isolated from vineyards of four vine-growing locations: Pfaffstätten, Eisenstadt, Halbturn and Perchtoldsdorf. To evaluate the usefulness and reliability of the AFLP fingerprinting under the applied conditions, additional strains of *S. cerevisiae* (HA284, HA4, HA236), *S. kudriavzevii* ($HA2261^{T}$), *S. pastorianus* ($HA238^{T}$, $HA452^{NT}$), *S. bayanus* ($HA266^{T}$), *S. bayanus* var. *uvarum* ($HA231^{T}$) and *S. paradoxus* ($HA405^{NT}$, $HA479^{A}$) were involved in the analysis. The AFLP fingerprinting generated 143 polymorphic loci with three primer pairs. A

combined phenogram constructed on the basis of three binary matrices is shown in Fig. 2. Generally, results demonstrate that the yeast representatives of every species generate similar AFLP profiles enforcing the argument that the clustering of these strains into a single species is valid. Within the *S. cerevisiae* strains, two clusters dominate, the first one comprising only the strains from Perchtoldsdorf, and the second one covering the yeast isolates from Pfaffstätten, Eisenstadt and Halbturn, as well as the three wine *S. cerevisiae* strains originated from Switzerland, Spain and West Africa, HA284 (type strain of *S. chodati*), HA4 (type strain of *S. aceti*) and HA236 (type strain of *S. mangini*). Although the strains of the latter cluster exhibit high similarity level of the AFLP profiles, the Austrian isolates split into three sub-clusters correlating to their geographical origin. The Pfaffstätten, Eisenstadt and Halbturn strains show up to 5% differences of the AFLP patterns, while remarkable differences of 20 % are demonstrated by the Perchtoldsdorf *S. cerevisiae* strains (Table 2). A detailed analysis of the AFLP fragments showed that within the *S. cerevisiae* strains 34 out of 67 amplified fragments are informative (Table 3). The Eisenstadt and Halbturn strains may be distinguished according to the presence of the fragments of 240 bp and 133 bp, respectively (Table 3). Two fragments of 68 and 82 bp are diagnostic for the *S. cerevisiae* isolate from Spain (HA4) and the 129 bp fragment is characteristic for the strain isolates from Switzerland (HA284) and West Africa (HA236). The latter ones are further distinguished by the presence and absence of the 137, 156 and 177 bp long fragments (Table 3). In addition, the West African strain could be differentiated by two fragments of 118 and 237 bp. On the other hand, the Perchtoldsdorf strains could be distinguished by 14 fragments from the investigated *S. cerevisiae* strains, 11 of which are shared with *S. kudriavzevii* type strain HA2261^{T}. A comparison of the AFLP patterns of the Perchtoldsdorf strains with those of *S. kudriavzevii* type strain showed that 15 fragments amplified in *S. kudriavzevii* type strain were not present in the Perchtoldsdorf strains (results not shown). This indicated that the yeast isolates from the Perchtoldsdorf area were not contaminated, but *S. kudriavzevii* species contributed to the formation of interspecies hybrids with *S. cerevisiae* species. The AFLP profiles of *S. kudriavzevii* species are (Fig. 2, Table 2) more similar to those of the Perchtoldsdorf strains (46%) than to the AFLP fingerprints of the other *S. cerevisiae* natural isolates (23%). The Perchtoldsdorf strains exhibit a slightly higher similarity value to the Pfaffstäten isolates (80.9%), in comparison to the Eisenstadt (80.1%) and Halbturn (78.3%) strains. The *S. pastorianus* strains, HA238^{T} and HA452^{NT} show a significant similarity to the *S. cerevisiae* strains, values are in the range from 46% to 48% (HA238^{T}) and 41% to 43% (HA452^{NT}). A remarkable similarity demonstrated the *S. pastorianus* strains also to *S. bayanus* (approx.

61%) and *S. bayanus* var. *uvarum* (approx. 39%) strains (Fig. 2, Table 2). The *S. bayanus* and *S. bayanus* var. *uvarum* strains share less AFLP fragments with *S. cerevisiae* strains, than the *S. pastorianus* strains. The similarity values of *S. bayanus* and *S. cerevisiae* strains (29% to 32%) are somewhat higher than those between *S. bayanus* var. *uvarum* and *S. cerevisiae* strains (24% to 27%) (Table 2). Two *S. paradoxus* strains, HA405^{NT} and HA479^{T} show the lowest genomic relatedness to the *S. cerevisiae* strains, values of similarity are in the range from 21% to 25% (Table 2).

Sequencing of D1/D2 domain and ITS1/ITS2 region

In order to investigate differences in the rDNA tandem repeat, the D1/D2 domain of the 26S rRNA encoding gene and ITS1-5.8S-ITS2 region of the representative yeast isolates from Pfaffstätten (HA1829), Eisenstadt (HA1856), Halbturn (HA1869) and Perchtoldsdorf (HA1835) was sequenced. Phylogenetic analysis based on both data sets confirmed identity of the yeast isolates with *S. cerevisiae* (Fig. 3). The cloned amplified fragments of the strain HA1835 revealed the sequence diversities in the D1/D2 domain and ITS1/ITS2 regions (Fig. 3). Four clones showed high homology with the *S. cerevisiae* sequences, whereas six clones, demonstrating a certain degree of variation, segregated from the rest of the strains. They showed identical sequences of D1/D2 domain and two nucleotide differences in ITS1 spacer region to those found in the type strain of *S. kudriavzevii* HA2261^{T} (NRRL Y-27339^{T}). These results brought additional evidences in favour of the hybrid nature of the HA1835 strain already indicated by the AFLP analysis.

Ploidy estimation

To estimate the DNA content of the studied strains we stained the cultures with propidium iodide and measured relative fluorescence of 10000 cells by flow cytometry. Fig. 4 shows the results for 6 strains. A haploid (ATCC 201388) *S. cerevisiae* strain assigned relative fluorescence values of approx. 122. If this value is compared with those of the yeast isolates from wine, significant differences in the DNA content are observed among *S. cerevisiae* strains originating from different vine-growing regions. Thus, the strain HA1869 originating from the Halbturn region showed to be a haploid, with a relative fluorescence value of 114. The strains HA1829 and HA1856 from the Pfaffstätten and Eisenstadt regions are diploids or close to diploids, with values of 224 and 198, respectively. Two Perchtoldsdorf strains, HA1835 and HA1844, showing the fluorescence values of 294 and 299, respectively, are most likely aneuploids.

Aroma compounds

Table 4 demonstrates the aroma compounds detected in 32 wine samples of "Grüner Veltliner" at the end of fermentation completed by *S. cerevisiae* strains of different geographical origin. The results show that notable differences exist among aroma profiles released by yeasts of different localities, suggesting that the inoculated yeasts differ in their fermentation performances. Differences were found in the concentrations of several alcohols and acids, but more notable variations were observed among identified esters. Generally, the yeasts isolated from two locations (Pfaffstätten and Perchtoldsdorf) of Thermenregion had the ability to produce much more ethyl esters (ethyldecanoate, ethylhexanoate, ethyloctanoate) and the esters citronellylpropionate, hexylacetate and isoamylacetate than the yeasts from the Eisenstadt and Halbturn locations. On the other hand, the yeast isolates from Thermenregion released less alcohols, especially α-terpineol and phenylethyl alcohol than the yeasts from Eisenstadt and Halbturn. Esters determine the fruity aroma of wines, indicating that the yeast isolates from Thermenregion may contribute to the aroma complexity to a larger extent than the yeasts from the other two regions. The Eisenstadt and Halbturn yeasts produced more alcohols (1-butanol, 3,7-dimethyl-2,6-octadien-1-ol, phenylethyl alcohol, n-propanol, α-terpineol) and organic acids (acetic, hexaonic and octaonic acid). On the other hand, the yeasts isolated from Pfaffstätten could be distinguished by the higher production of alcohols, i.e. isobutanol, isopentanol, n-propanol and the esters ethylacetate and isoamylacetate from the Perchtoldsdorf strains, which produce more decanoic acid. The Eisenstadt strains produced more alcohols (citronellol, isopentanol, nonanol) and esters than the Halbturn strains, which produced notable amount of n-propanol. The principal component analysis (Fig. 5) showed remarkable differences between aroma components of the wines fermented by yeasts isolated from Thermenregion (Perchtoldsdorf, Pfaffstätten), Neusiedlersee-Hügelland (Eisenstadt) and Neusiedlersee (Halbturn). The aroma profiles of the wines fermented by the Perchtoldsdorf strains are more similar to the profiles generated by the Pfaffstätten yeasts than to those produced by the yeasts originating from Eisenstadt and Halbturn localities.

Discussion

Azumi and Goto-Yamamoto (2001) and De Barros Lopes *et al.* (2002) used AFLP fingerprinting to examine DNA polymorphisms among different isolates of the genus

Saccharomyces. Similar to the results of these authors, the AFLP markers enabled us to discriminate among *Saccharomyces* yeasts at the species and strain level. Although identical AFLP fragments may not implicitly have the same sequences, in our opinion, a higher percentage of similarity among closely related strains indicates a higher similarity at genomic level. An overall analysis of Fig 2. demonstrates clear distinctions of the *Saccharomyces* species which make separate clusters statistically well supported. Generally, strains of the same species show more than 70% similarity of the AFLP patterns (Table 2). Considerable similarity was observed between the strains of *S. pastorianus* and *S. cerevisiae* (approx. 50%), as well as between *S. pastorianus* and *S. bayanus* (approx. 40% to 60%). The lager brewing yeast *S. pastorianus* HA238^T (CBS1513^T) is considered to be a hybrid between *S. cerevisiae* and *S. bayanus* species (Vaughan-Martini & Kurtzman, 1985; Casaregola *et al*. 2001; Naumova *et al*. 2005). A thorough examination of the AFLP fragments revealed that out of 55 amplified fragments, *S. pastorianus* HA238^T shares 9 fragments with *S. bayanus* HA266^T and 11 fragments with *S. cerevisiae* HA4 strain (data not shown), corroborating the idea that these species contributed to the hybrid genome of *S. pastorianus*. Only one fragment was found to be shared between *S. pastorianus* and *S. bayanus* var. *uvarum* HA231^T strains, what indicated that *S. pastorianus* contains a part of the *S. bayanus* var. *uvarum* genome too, as proposed by Nguyen *et al*. (2000) on the basis of molecular typing of nuclear and mitochondrial genomes. On the other hand, *S. bayanus* HA266^T (CBS380^T) has been described as a species with two varieties, "bayanus" and "uvarum" (Naumov, 2000). Based on the presence of several isomorphic chromosomes in *S. bayanus* and *S. uvarum*, as well as the presence of the *S. cerevisiae* Y' and *SUC* sequences in *S. bayanus*, several authors postulated that *S. bayanus* is a hybrid species between *S. uvarum* and *S. cerevisiae*, and moreover, *S. uvarum* was considered to be a proper species (Nguyen *et al*., 2000; Nguyen & Gaillardin, 2005). On the basis of the AFLP fingerprinting analysis, we could demonstrate that *S. bayanus* HA266^T includes an essential part of the *S. bayanus* var. *uvarum* genome, out of 47 amplified fragments these two species share 9 fragments (results not shown). Although a number of common fragments were detected between *S. bayanus* and *S. bayanus* var. *uvarum*, *S. pastorianus* and *S. cerevisiae*, we could not determine any fragment shared exclusively with *S. cerevisiae*. To a similar finding came Fernández-Espinar *et al*. (2003), who studying genetic variability within the *Saccharomyces sensu stricto* complex, were not able to detect any RAPD band shared between *S. bayanus* and *S. cerevisiae* species. Although *S. bayanus* var. *uvarum* was proposed to be a proper species within *Saccharomyces sensu stricto* (Nguyen *et al*., 2000; Pulvirenti *et al*., 2000; Nguyen and Gaillardin, 2005), in this paper we keep using the name *S. bayanus* var.

uvarum owing to the high similarity level (69.1%) of the AFLP patterns with *S. bayanus* type strain, high nDNA:nDNA reassociation values (86-100%) and semi-sterility of the *S. bayanus* x *S. bayanus* var. *uvarum* hybrids (Naumov, 2000, Naumova *et al*., 2005).

Recently Aa et al (2006) pointed out that *S. cerevisiae* strains collected in vineyards are genetically distinctive from those recovered from the grape must in fermentation vats. Moreover, the yeast strains found on grapes may differ significantly from vineyard to vineyard within a region. Results of the present study showed that autochtonous *S. cerevisiae* strains may be different not only at the genomic level, but also their metabolic profiles differ significantly under the same experimental conditions. Studying the DNA polymorphisms of *S. cerevisiae* strains isolated from four localities in three Austrian vine-growing regions we used the AFLP technique. The AFLP molecular markers allowed discrimination of the closely related strains and generated clusters which correlated well with geographical distribution of the yeasts (Fig. 2). Genotypic characterisation and ploidy estimation have shown that investigated natural *S. cerevisiae* strains represent closely related but genetically different populations. Flow cytometry has revealed significant differences in the ploidy of the yeast isolates from grapes, they may be haploids, diploids or aneuploids. The AFLP molecular markers revealed that the strains of the Perchtoldsdorf region exhibit remarkable differences to the other natural yeast isolates. Different from the Pfaffstätten, Eisenstadt and Halbturn strains, which showed up to 5% differences among each other, the AFLP profiles of the Perchtoldsdorf strains exhibited approx. 20% differences to the former groups (Table 2). The Perchtoldsdorf strains share 11 AFLP fragments with *S. kudriavzevii* HA2261^{T} strain (Table 3), suggesting a significant contribution of *S. kudriavzevii* to the formation of the present genome of these yeast isolates. Subsequent attempts to isolate viable spores from sporulated cultures of the HA1835 strain were unsuccessful. Although, the ascospores were abundantly formed, they were non-viable which is typically the case for aneuploids. Cloning and sequencing of the PCR products revealed that the HA1835 strain contained the LSU D1/D2 and ITS1/ITS2 sequences of *S. cerevisiae* and *S. kudriavzevii* (Fig. 3). Preliminary results of the restriction analysis of five nuclear genes have shown that another Perchtoldsdorf isolate (HA1841) contains two alleles of the genes *CAT8*, *CYR1*, *GSY1*, *MET6* and *OPY1*, one allele similar to that from *S. cerevisiae* and the other to that from *S. kudriavzevii* (in prep.) Results of all these experiments supported the conclusion that the Perchtoldsdorf strains represent interspecies hybrids between *S. cerevisiae* and *S. kudriavzevii* with sterile progeny. Biochemical and physiological tests (Yarrow, 1998), used to study assimilation and

fermentation capacity, as well as the growth ability at different temperatures, demonstrated that yeast representatives (HA1829, HA1835, HA1844, HA1856, HA1869) of the four geographical locations had the same assimilation and fermentation profile (results not shown). The only difference was observed in the growth ability at 40°C. Different from the strains HA1829, HA1856 and HA1869, the strains from Perchtoldsdorf area HA1835 and HA1844 were unable to grow at the increased temperature. The absence of the growth at 40°C was demonstrated by *S. kudriavzevii* too, suggesting that the Austrian hybrid yeasts acquired this trait from *S. kudriavzevii* parental part. Recently, several reports demonstrated that a number of commercial wine strains arose through the hybridisation event between *S. cerevisiae* and *S. kudriavzevii* (Liti *et al*., 2005; Bradbury *et al*., 2006, González et al., 2006), while the cider CID1 strain acquired its genome from *S. cerevisiae*, *S. kudriavzevii* and *S. bayanus* var. *uvarum* (Naumova *et al*., 2005). Bradbury *et al*. (2006) showed that different ITS sequences are present frequently in *Saccharomyces* hybrids and that the commercial wine yeasts are mainly *S. cerevisiae* with some putative interspecies hybrids with *S. kudriavzevii*. The authors suggested that these aneuploid strains were derived from hybridisation events followed by partial loss of one of the genomes. Since the members of the ribosomal tandem repeats are homogenised by concerted evolution, it seems that this evolutionary process has not been completed in the yeasts having mixtures of two D1/D2 and ITS1/ITS2 sequences.

To investigate whether the differences at the genomic level have impact on the enological performances of the investigated yeast strains we analysed aroma components in the final fermentation products of "Grüner Veltliner" by GC-MS. Significant differences were found out in metabolic outcome among wine samples (Fig. 5) indicating that *S. cerevisiae* strains show different metabolic activities during the fermentation of grape juice. As already discussed, the Perchtoldsdorf, Pfaffstätten, Eisenstadt and Halbturn strains showed to be different in the ploidy and genotypic properties (Table 2 and 3), and consequently these differences may influence aroma composition of wines significantly. We analysed 30 volatile compounds (Table 4) and our results showed significant heterogeneity among strains in the production of almost all investigated compounds. In general, *S. cerevisiae* strains isolated from Pfaffstätten and Perchtoldsdorf locations of the Thermenregion produced wines with higher concentrations of esters than the yeast isolates from Eisenstadt and Halbturn, but an overall difference in the production of alcohols and acids was also observed. The principal component analysis of the aroma components demonstrated four clusters correlating to the geographical origin of the yeast isolates (Fig. 5). The aroma profiles of the Perchtoldsdorf

strains are more related to those of the Pfaffstätten strains. It could be explained by the close proximity of these localities and by selective pressure influencing the production of aroma compounds. We did not find correlation between the genomic similarity of the *S. cerevisiae* strains from different localities and corresponding aroma profiles. Although the Pfaffstätten strains have AFLP profiles more related to the Eisenstadt (98%) and Halbturn yeasts (96%) than to the Perchtoldsdorf strains (81%), this higher genomic similarity does not correlate with the phenotypic properties (Table 2, Fig. 2). One of the reason might be a small number of informative fragments detected among studied strains by the AFLP technique. It must be emphasised that the complexity of the AFLP fingerprints depends on the selective primers used in the reactions and by employing more primer pairs raises the chance to discover new polymorphic loci. The AFLP markers are anonymous and there is no information about the amplified genes. More specific variations between commercial wine strains were found recently by Dunn *et al*. (2005), who using microarray karyotyping revealed that the genomic diversities are mostly in the form of depletions or amplifications of single genes. The inter-strain differences were detected in the transporter genes, especially hexose transporters (*HXT* genes), metal ion sensors/transporters (*CUPI*, *ZRTI*, *ENA* genes), members of the major facilitator superfamily, and in genes involved in drug response (*PDR3*, *SNQI*, *QDRI*, *RDSI*, *AYTI*, *YAR068W*). Rossignol *et al.* (2003) reported that strong transcriptional reprogramming take place during fermentation process, affecting more than 2000 genes, and genes involved in the key metabolic pathways of fermentation are strongly expressed.

It was already reported that both parental parts of the interspecies hybrids may contribute to the production of secondary compounds in wine, so that the concentration values are between those obtained using the parental strains (Zambonelli *et al*., 1993; Kishimoto, 1994). On the basis of the results in this report we cannot assume reliably whether, and in which extent, *S. kudriavzevii* influences metabolic profiles of the Perchtoldsdorf natural yeasts during alcoholic fermentation. Summing up results of the genotypic and phenotypic investigations, we can only speculate that the Perchtoldsdorf hybrid strains might acquire phenotypic properties mainly from the *S. cerevisiae* parental part originating from the Pfaffstätten area. The localities Pfaffstätten and Perchtoldsdorf are closely located to each other (Fig. 1) and from ecological point of view it would not be surprising that yeasts originating from these areas show similar genotypic and phenotypic features. Ecological barriers do not exist and appearance of the interspecies hybridisation only in the strains of Perchtoldsdorf area

indicates that the sampling program should be extended to several vineyards of the other regions to evaluate dissemination of the hybrid strains.

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Table 1. Yeast strains used in the present study with corresponding Genbank accession numbers

Species	Strain designation[a]		Isolation source	Country	Genbank Accession No.	
	ACBR[b]	Other[c]			ITS1/ITS2	26S D1/D2
Naumovia dairenensis		CBS421^{T}	dry fruit of *Diospyros sp.*		AY046181	AY048168
S. bayanus	HA266^{T}	CBS380^{T}	turbid beer	Unknown	AY046152	AY048156
S. bayanus var. *uvarum*	HA231^{T}	CBS395^{T}	juice of *Ribes nigrum*	Netherlands		
S. cerevisiae	HA1825-HA1834		wine	Austria, Thermenregion, Pfaffstätten		
	HA1829		wine	Austria, Thermenregion, Pfaffstätten	AM262826	AM262819
	HA1835-HA1844		wine	Austria, Thermenregion, Perchtoldsdorf		
	HA1835		wine	Austria, Thermenregion, Perchtoldsdorf	AM262824 (Sc[d]) AM262825 (Sk[e])	AM262822 (Sc[d]) AM262823 (Sk[e])
	HA1855-HA1862		wine	Austria, Neusiedlersee-Hügelland, Eisenstadt		
	HA1856		wine	Austria, Neusiedlersee-Hügelland, Eisenstadt	AM262827	AM262820
	HA1869-HA1872		wine	Austria, Neusiedlersee, Halbturn		
	HA1869		wine	Austria, Neusiedlersee, Halbturn	AM262828	AM262821
	HA284^{T}	CBS423^{T}	wine	Switzerland, Riddes	AM262829	
	HA236^{T}	CBS405^{T}	wine	West Africa	AM262830	
	HA4^{T}	CBS4054^{T}	wine	Spain	AM262831	
		CBS1171^{T}	beer	Netherlands, Rotterdam	AY046152	AY048154
S. kudriavzevii	HA2261^{T}	NRRL Y-27339^{T}	decayed leaf	Japan	AY046150	AF398480
S. paradoxus	HA405^{NT}	CBS432^{NT}		Unknown		
	HA479^{A}	CBS5829^{A}	moor soil	Denmark		
S. pastorianus	HA238^{T}	CBS1513^{T}	beer	Unknown		
	HA452^{NT}	CBS1538^{NT}		Unknown		

[a]T=type strain, NT=neotype strain, A=authentic strain
[b]ACBR: Austrian Center of Biological Resources and Applied Mycology, Muthgasse 18, 1190 Vienna, Austria
[c]CBS: Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands; NRRL: National Center for Agricultural Utilization Reasearch, Peoria, IL, USA
[d]*S. cerevisiae*-like allele
[e]*S. kudriavzevii*-like allele

Table 2. Similarity matrix (%) and standard deviations of the investigated strains (strains of *S. cerevisiae* are arranged according to their geographical origin)

	S. cerevisiae							*S. kudriavzevii*	*S. pastorianus*		*S. bayanus*	*S. bayanus* var. *uvarum*	*S. paradoxus*	
	Pfaffstätten	Perchtoldsdorf	Eisenstadt	Halbturn	HA236	HA284	HA4	HA2261	HA238	HA452	HA266	HA231	HA405	HA479
Pfaffstätten	99.8 ± 0.5	80.9 ± 0.9	98.3 ± 0.6	96.1 ± 0.9	93.7 ± 0.3	96.1 ± 0.4	94.9 ± 0.3	23.3 ± 0.1	48.4 ± 0.1	42.6 ± 0.1	32.1 ± 0.1	27.0 ± 0.1	25.3 ± 0.1	25.0 ± 0.1
Perchtoldsdorf	80.9 ± 0.9	98.1 ± 1.5	80.1 ± 0.8	78.3 ± 1.3	76.0 ± 0.9	77.6 ± 0.9	76.8 ± 0.9	46.6 ± 0.7	46.4 ± 0.7	41.5 ± 0.7	29.0 ± 0.4	24.3 ± 0.4	23.5 ± 1.3	23.2 ± 1.2
Eisenstadt	98.3 ± 0.6	80.1 ± 0.8	99.5 ± 0.6	95.5 ± 1.5	92.4 ± 0.6	94.7 ± 0.6	93.5 ± 0.6	23.2 ± 0.1	48.0 ± 0.2	42.3 ± 0.2	31.9 ± 0.1	26.8 ± 0.1	24.4 ± 1.1	24.0 ± 1.1
Halbturn	96.1 ± 0.9	78.3 ± 1.3	95.5 ± 1.5	98.1 ± 1.4	90.7 ± 0.6	92.3 ± 0.9	91.8 ± 0.6	23.5 ± 0.3	48.1 ± 0.6	42.9 ± 0.5	32.4 ± 0.4	27.2 ± 0.4	22.0 ± 1.2	21.7 ± 1.2
HA236	93.7 ± 0.3	76.0 ± 0.9	92.4 ± 0.6	90.7 ± 0.6	100.0 ± 0.0	92.5 ± 0.0	91.4 ± 0.0	23.1 ± 0.0	50.0 ± 0.0	44.4 ± 0.0	34.1 ± 0.0	26.7 ± 0.0	25.0 ± 0.0	24.7 ± 0.0
HA284	96.1 ± 0.3	77.6 ± 0.9	94.7 ± 0.6	92.3 ± 0.9	92.5 ± 0.0	100.0 ± 0.0	93.7 ± 0.0	18.4 ± 0.0	51.1 ± 0.0	45.5 ± 0.0	34.9 ± 0.0	27.4 ± 0.0	25.7 ± 0.0	25.4 ± 0.0
HA4	94.9 ± 0.3	76.8 ± 0.9	93.5 ± 0.6	91.8 ± 0.6	91.4 ± 0.0	93.7 ± 0.0	100.0 ± 0.0	20.8 ± 0.0	48.4 ± 0.0	42.7 ± 0.0	32.2 ± 0.0	27.0 ± 0.0	25.4 ± 0.0	25.0 ± 0.0
HA2261	23.3 ± 0.1	46.6 ± 0.7	23.2 ± 0.1	23.5 ± 0.3	23.1 ± 0.0	18.4 ± 0.0	20.8 ± 0.0	100.0 ± 0.0	17.4 ± 0.0	16.3 ± 0.0	11.9 ± 0.0	8.4 ± 0.0	11.8 ± 0.0	11.6 ± 0.0
HA238	48.4 ± 0.1	46.4 ± 0.7	48.0 ± 0.2	48.1 ± 0.6	50.0 ± 0.0	51.1 ± 0.0	48.4 ± 0.0	17.4 ± 0.0	100.0 ± 0.0	94.2 ± 0.0	60.8 ± 0.0	40.4 ± 0.0	14.0 ± 0.0	13.8 ± 0.0
HA452	42.6 ± 0.1	41.5 ± 0.7	42.3 ± 0.2	42.9 ± 0.5	44.4 ± 0.0	45.5 ± 0.0	42.7 ± 0.0	16.3 ± 0.0	94.2 ± 0.0	100.0 ± 0.0	62.5 ± 0.0	38.6 ± 0.0	12.5 ± 0.0	12.3 ± 0.0
HA266	32.1 ± 0.1	29.0 ± 0.4	31.9 ± 0.1	32.4 ± 0.4	34.1 ± 0.0	34.9 ± 0.0	32.2 ± 0.0	11.9 ± 0.0	60.8 ± 0.0	62.5 ± 0.0	100.0 ± 0.0	69.1 ± 0.0	20.5 ± 0.0	20.3 ± 0.0
HA231	27.0 ± 0.1	24.3 ± 0.4	26.8 ± 0.1	27.2 ± 0.4	26.7 ± 0.0	27.4 ± 0.0	27.0 ± 0.0	8.4 ± 0.0	40.4 ± 0.0	38.6 ± 0.0	69.1 ± 0.0	100.0 ± 0.0	15.4 ± 0.0	15.2 ± 0.0
HA405	25.3 ± 0.1	23.5 ± 1.3	24.4 ± 1.1	22.0 ± 1.2	25.0 ± 0.0	25.7 ± 0.0	25.4 ± 0.0	11.8 ± 0.0	14.0 ± 0.0	12.5 ± 0.0	20.5 ± 0.0	15.4 ± 0.0	100.0 ± 0.0	98.4 ± 0.0
HA479	25.0 ± 0.1	23.2 ± 1.2	24.0 ± 1.0	21.7 ± 1.2	24.7 ± 0.0	25.4 ± 0.0	25.0 ± 0.0	11.6 ± 0.0	13.8 ± 0.0	12.3 ± 0.0	20.3 ± 0.0	15.2 ± 0.0	98.4 ± 0.0	100.0 ± 0.0

Table 3. Polymorphic AFLP loci of *S. cerevisiae* strains compared with *S. kudriavzevii* (HA2261^{T}) type strain

Yeast strains	AFLP fragments (bp)																																
	68	80	82	85	86	98	109	112	115	118	128	129	133	137	156	177	187	191	195	200	221	230	232	237	240	242	265	277	281	285	288	307	318	378
Pfaffstätten	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	V
Perchtoldsdorf	0	1	0	1	1	1	1	1	1	0	1	0	0	1	1	1	V	V	1	1	1	V	V	0	0	1	1	V	1	V	1	V	V	0
Eisenstadt	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	V	0	0
Halbturn	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	V	0	0	1	0	0	0	V	0	0
HA236	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
HA284	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0
HA4	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0
HA2261	0	1	0	1	1	1	0	1	1	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0	0	1	0	0	1	0	1	0	0	0

1, presence of the AFLP fragment; 0, absence of the AFLP fragment; V, variable presence of the fragment within a group

Table 4. Volatile compounds in the wine "Grüner Veltliner" produced by the *S. cerevisiae* strains isolated from four vine-growing localities

Aroma compounds	Thermenregion Pfaffstätten	Thermenregion Perchtoldsdorf	Neusiedlersee - Hügelland Eisenstadt	Neusiedlersee Halbturn
Alcohols				
1-Butanol (mg L^{-1})	1.00±0.00	0.70±0.48	1.00±0.00	2.00±0.00
Citronellol	0.29±0.08	0.27±0.10	0.39±0.05	0.19±0.03
3,7-Dimethyl -2,6-octadien-1-ol	0.05±0.05	0.04±0.00	0.12±0.07	0.07±0.01
1-Hexanol	0.93±0.23	0.98±0.13	1.09±0.11	1.26±0.20
Isobutanol (mg L^{-1})	79.30±19.94	62.70±17.17	56.37±17.05	54.5±12.61
Isopentanol (mg L^{-1})	117.90±15.42	83.30±15.10	112±23.47	72.5±16.62
Linalool	1.95±0.12	1.91±0.16	1.44±0.54	1.34±0.18
Methanol (mg L^{-1})	41.90±1.29	43.20±1.32	38.37±1.30	37.00±1.15
2-Nonanol	0.07±0.01	0.09±0.05	0.11±0.04	0.05±0.01
Phenylethyl alcohol	0.91±0.14	0.86±0.10	1.58±0.37	1.38±0.36
n-Propanol (mg L^{-1})	54.1±4.68	39.60±3.86	54.75±13.72	102.25±18.01
1-Octanol	0.09±0.03	0.21±0.05	0.06±0.03	0.05±0.03
a-Terpineol	0.50±0.04	0.49±0.03	0.92±0.15	1.15±0.11
Esters				
Citronellylpropionate	0.79±0.10	0.52±0.08	0.25±0.05	0.02±0.01
Ethylacetate (mg L^{-1})	17.50±2.17	14.8±2.04	17.87±3.98	11.25±2.63
Ethylbutanoate	0.51±0.07	0.36±0.08	0.96±1.59	0.53±0.17
Ethyldecanoate	9.28±3.64	21.83±2.07	4.46±2.16	1.23±0.13
Ethyl 9-decenoate	3.05±0.66	2.97±0.46	2.64±2.72	0.95±0.26
Ethylhexanoate	9.07±0.85	7.69±0.92	2.67±0.64	1.84±0.45
Ethyloctanoate	28.06±3.97	32.31±2.27	13.75±3.58	5.68±1.37
Hexylacetate	3.25±0.54	3.01±0.99	0.54±0.10	0.36±0.13
Isoamylacetate	2.64±0.53	1.39±0.51	0.67±0.31	0.26±0.11
Octylacetate	0.17±0.06	0.26±0.08	0.32±0.14	0.42±0.07
Phenylethylacetate	0.49±0.16	0.58±0.11	0.67±0.20	0.40±0.04
Acids				
Acetic acid	0.25±0.05	0.32±0.13	0.68±0.23	0.44±0.14
Decanoic acid	1.62±0.75	4.77±0.64	3.19±1.58	3.25±0.52
2-Ethylhexanoic acid	0.15±0.02	0.16±0.10	0.12±0.04	0.20±0.07
Hexanoic acid	0.49±0.06	0.51±0.07	0.82±0.09	0.66±0.13
Octanoic acid	4.14±0.79	4.99±0.57	6.73±3.59	5.50±0.40
Aldehydes				
Decanal	0.07±0.05	0.06±0.02	0.14±0.07	0.29±0.25

Values are expressed either in mg L^{-1} or as relative peak area compared with the internal standard

Figure legends

Fig. 1. Eastern part of Austria with four localities from where the *Saccharomyces cerevisiae* strains used in the genotypic and phenotypic studies were collected

Fig. 2. Consensus phenogram based on the AFLP molecular markers depicting genomic similarity of *S. cerevisiae* strains from four Austrian locations and closely related species

Fig. 3. Phylogenetic trees constructed on the basis of the ITS1-5.8S-ITS2 and LSU D1/D2 sequences of the selected *S. cerevisiae* strains from four Austrian locations and closely related species. In the strain HA1835 two different sequences, *S. cerevisiae*-like and *S. kudriavzevii*-like allele were determined. Sequences of *Naumovia dairenensis* CBS421^{T} were used as an outgroup

Fig. 4. Flow cytometry analysis indicating the ploidy of five natural *S. cerevisiae* strains in comparison with the haploid strain ATCC201388. The wine yeast isolates were characterised as haploid, diploid or aneuploid

Fig. 5. Principal-component analysis of aroma products generated by native *S. cerevisiae* strains isolated from grapes originated from Pfaffstätten, Perchtoldsdorf, Eisenstadt and Halbturn

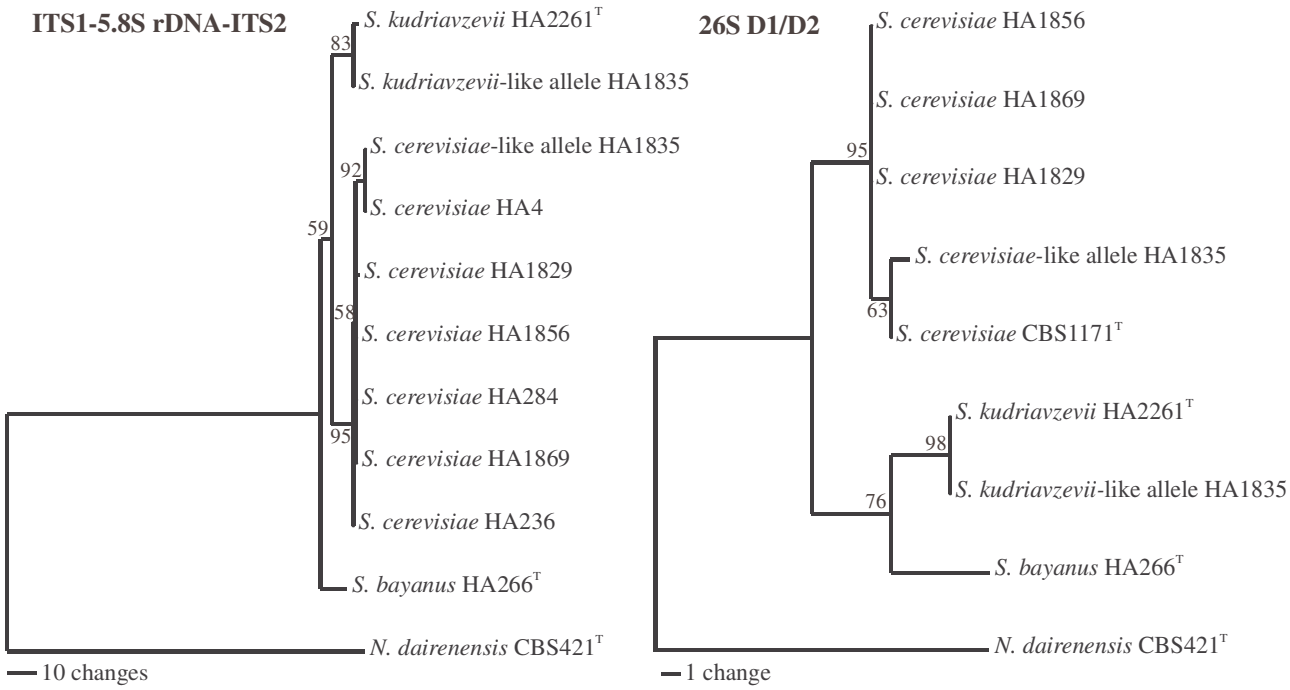
Fig. 1

● Perchtoldsdorf
▲ Pfaffstätten
■ Eisenstadt
▼ Halbturn

Fig. 2


UPGMA
HA1825
HA1826
HA1827
HA1828
HA1829
HA1830
HA1831
HA1833
HA1834
HA1832
Pfaffstätten
HA1855
HA1856
HA1857
HA1858
HA1859
HA1862
HA1860
HA1861
Eisenstadt
HA1869
HA1871
HA1870
HA1872
Halbturn
S. cerevisiae
100
HA284
HA4
HA236
HA1835
HA1838
HA1840
HA1843
HA1836
HA1841
HA1839
HA1844
HA1837
HA1842
Perchtoldsdorf
100
100
HA2261T
S. kudriavzevii
HA238T
HA452NT
S. pastorianus
100
89
99
HA266I
HA231T
S. bayanus
S. bayanus var. uvarum
HA405NT
HA479A
100
S. paradoxus
0.01 changes

Fig. 3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S. cerevisiae-like allele HA1835
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S. cerevisiae HA4
59
S. cerevisiae HA1829
58
S. cerevisiae HA1856
S. cerevisiae HA284
95
S. cerevisiae HA1869
S. cerevisiae HA236
S. bayanus HA266T
N. dairenensis CBS421T
10 changes
26S D1/D2
S. cerevisiae HA1856
S. cerevisiae HA1869
95
S. cerevisiae HA1829
S. cerevisiae-like allele HA1835
63
S. cerevisiae CBS1171T
S. kudriavzevii HA2261T
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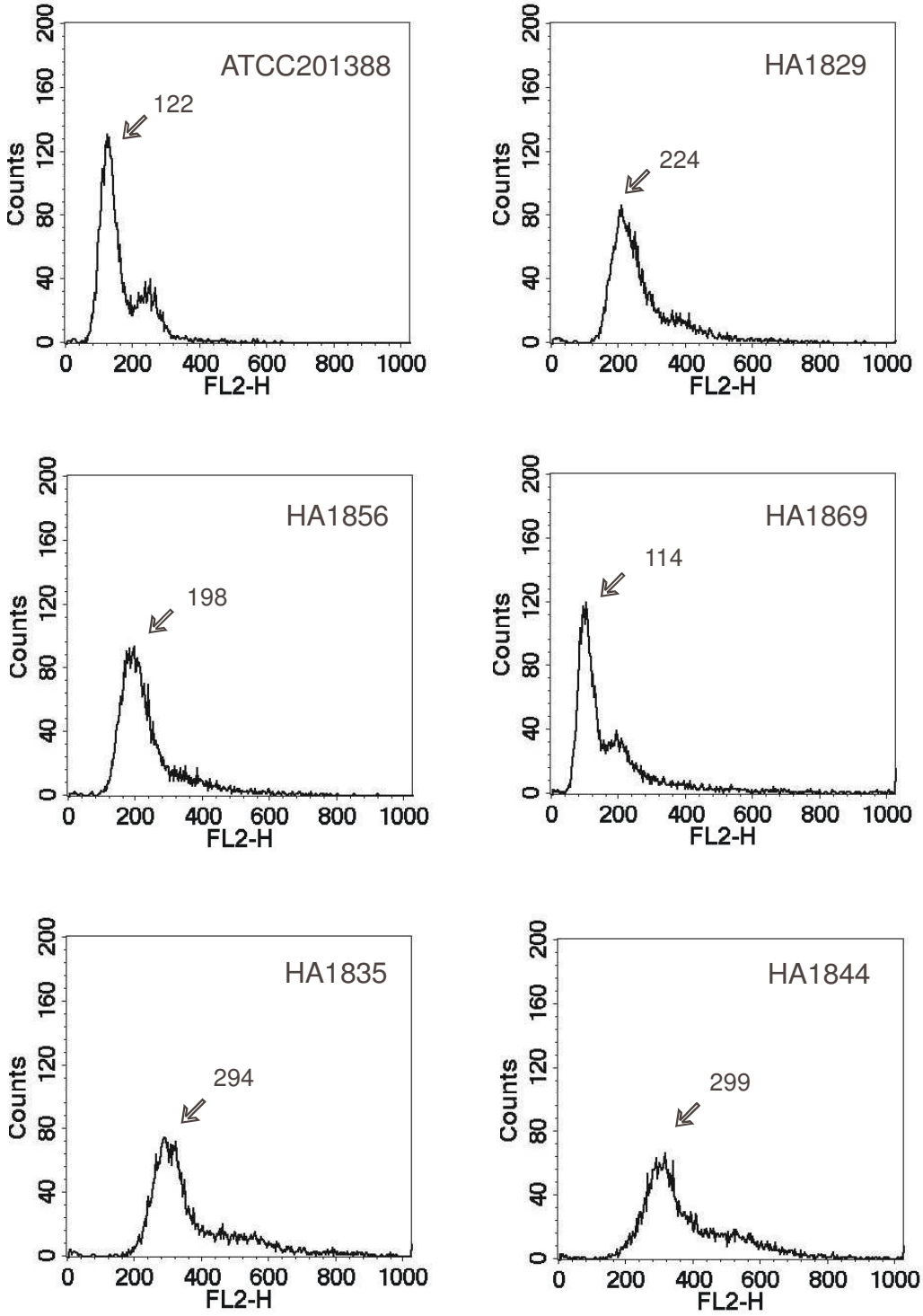
S. kudriavzevii-like allele HA1835
76
S. bayanus HA266T
N. dairenensis CBS421T
1 change

Fig. 4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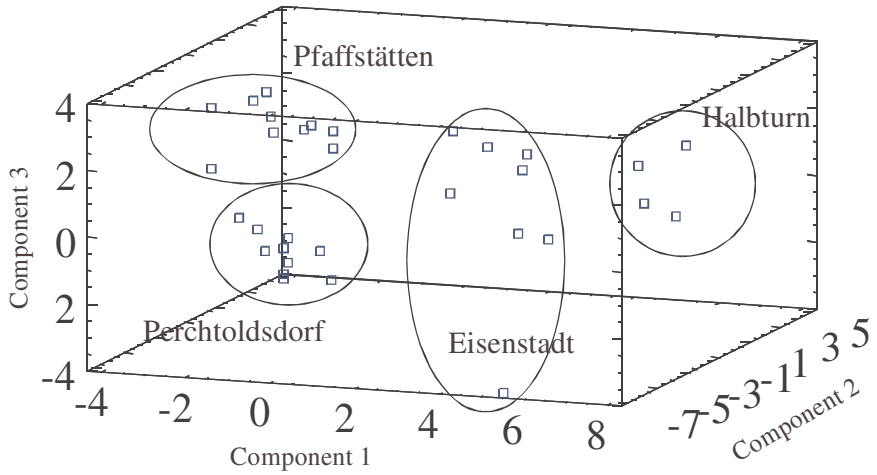
HA1829
224
HA1856
198
HA1869
114
HA1835
294
HA1844
299
Counts
FL2-H
0
40
80
120
160
200
400
600
800
1000

Fig. 5


Pfaffstätten
Halbturn
Perchtoldsdorf
Eisenstadt
Component 3
Component 1
Component 2
4
2
0
2
-4
-4
-2
0
2
4
6
8
-7
-5
-3
-1
1
3
5