Molecular identification of bacteria on grapes and in must from Small Carpathian wine-producing region (Slovakia)

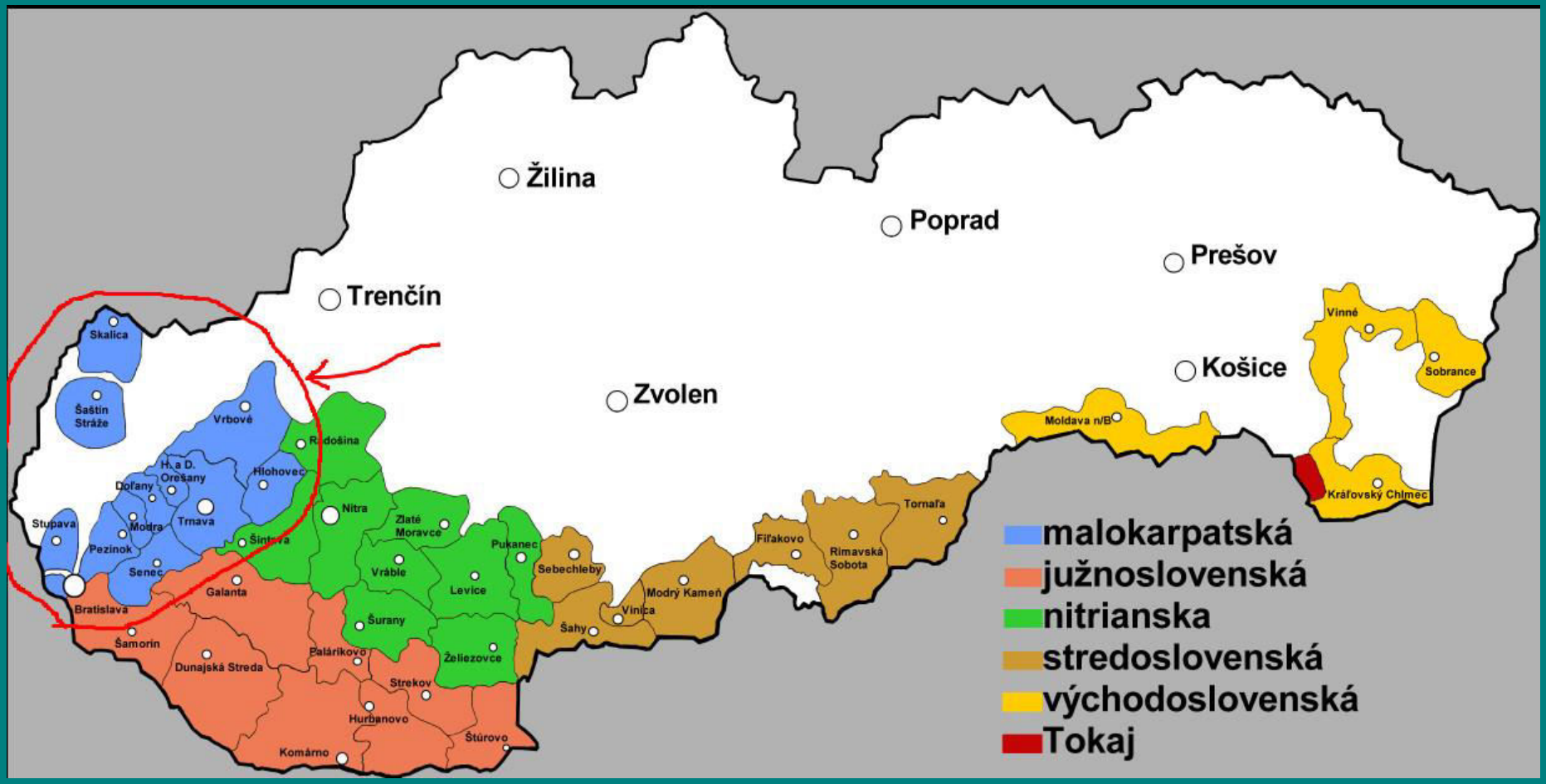
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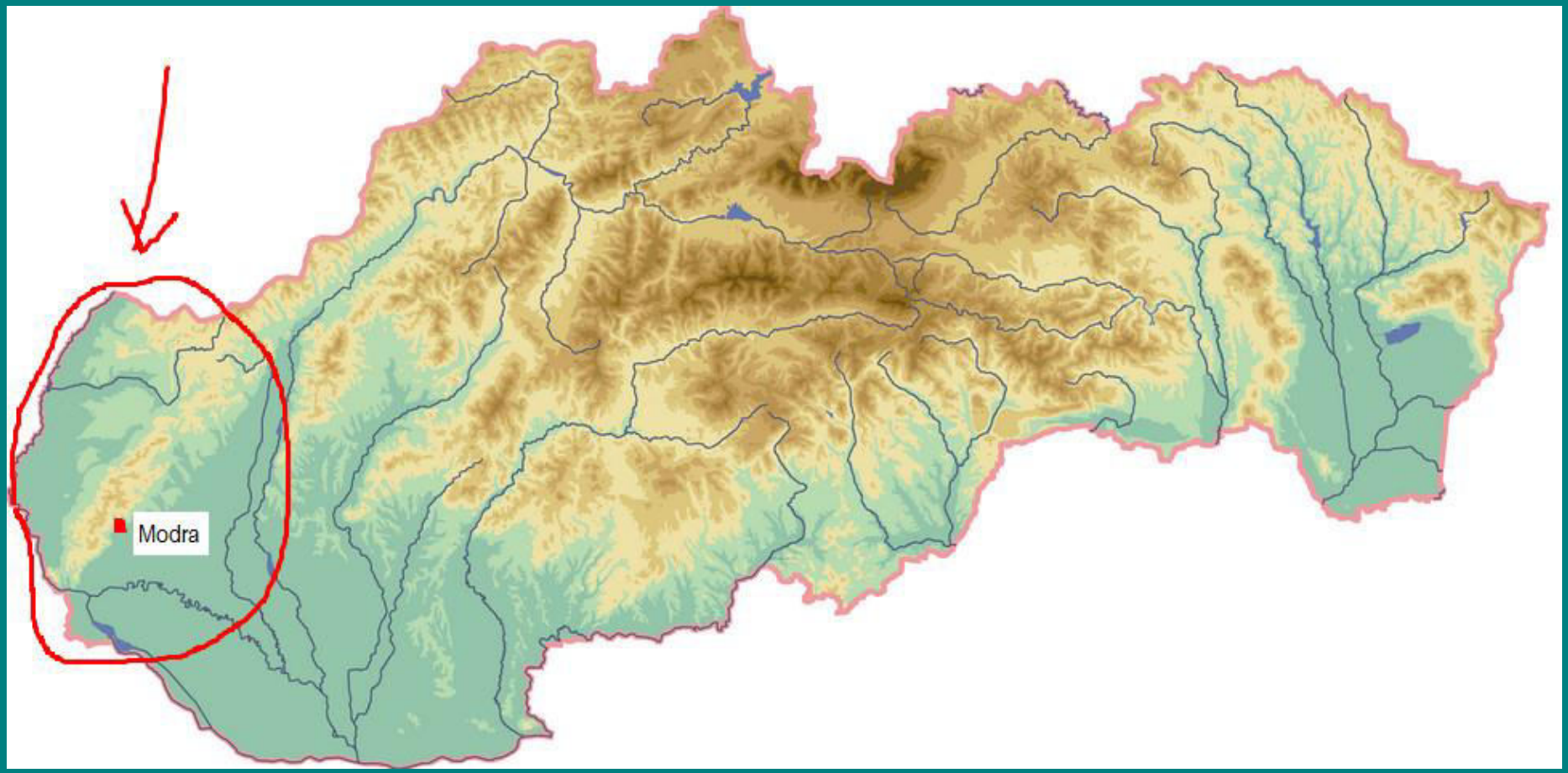
Slovakia


ICELAND
Norwegian Sea
North Atlantic Ocean
SWEDEN
FINLAND
NORWA
Gulf of Bothnia
RUSSIA
ESTONIA
LATVIA
North Sea
DENMARK
Baltic Sea
LITHUANIA
IRELAND
U. K.
BELARUS
POLAND
NETH.
GERMANY
English Channel
BELGIUM
UKRAINE
LUX.
CZECH REPUBLIC
SLOVAKIA
FRANCE
AUSTRIA
MOLDOVA
SWITZERLAND
HUNGARY
ROMANIA
Bay of Biscay
SLOVENIA
CROATIA
Black Sea
BOSNIA
SERBIA
BULGARIA
ANDORRA
PORTUGAL
ITALY
MONTENEGRO
KOSOVO
MACEDONIA
SPAIN
Adriatic Sea
ALBANIA
TURKEY
Tyrrhenian Sea
GREECE
Aegean Sea
Ionian Sea
Mediterranean Sea
CYPRUS
0
250 Km

Small Carpathian Wine-producing region



Small Carpathian Wine-producing region



Small Carpathians



Two typical grape varieties:

- Blaufränkisch
- Grüner Veltliner

Modra, vintage 2013



Samples:

- grapes, wash (G)
- initial phase must (M1)
- middle fermenting must, 7-10 days (M2)
- must at the end of fermentation, almost wine (M3)



Besides the quality of grapes,
microflora determines the quality of wine:

* yeasts are responsible for primary fermentation

** bacteria influence the quality of wine

- in a positive way

(malolactic fermentation, formation/transformation of volatile aroma-active compounds)

- or in a negative way

(formation of acetic acid, biogenic amines)

Culture-based approach for bacteria:

Decimal dilutions cultured on elective media

(supplemented with cycloheximide):

- R2A
- GYC (for cultivation of acetic acid bacteria)
- MRS (for cultivation of lactic acid bacteria)
- MRS-T (suppl. with tomato juice, cysteine and malic acid; for *Oenococcus* spp.)

Colonies → DNA → f-ITS PCR → capillary electrophoresis

Selected colonies → 16S rDNA sequences → BLAST

Culture-independent approach for bacteria:

- DNA extraction (DNeasy, Qiagen)
- semi-nested PCR for 16S rDNA amplification
- cloning in pGEM-T Easy vector (Promega)
- DGGE
- clones with different DGGE characteristics sequenced and identified by BLAST in GenBank

Culture-based quantitative data on bacteria isolated on different agar media during the production of wine

Cultivation media	Density [CFU/ml]							
	Frankovka modrá				Veltlínske zelené			
	G	M1	M2	M3	G	M1	M2	M3
R2A	4×10^8	10^7	3.6×10^7	n.g.	2.7×10^7	10^7	1.5×10^5	10^1
GYC	5×10^7	2.2×10^7	2×10^6	n.g.	2×10^6	2×10^7	2×10^6	n.g.
MRS	7×10^5	3.1×10^5	2×10^6	10^2	3×10^5	4×10^7	1.3×10^5	10^1
MRS-T	1.8×10^3	10^3	3.2×10^2	n.g.	10^3	2×10^4	10^1	n.g.

- decrease in bacterial isolates in M3

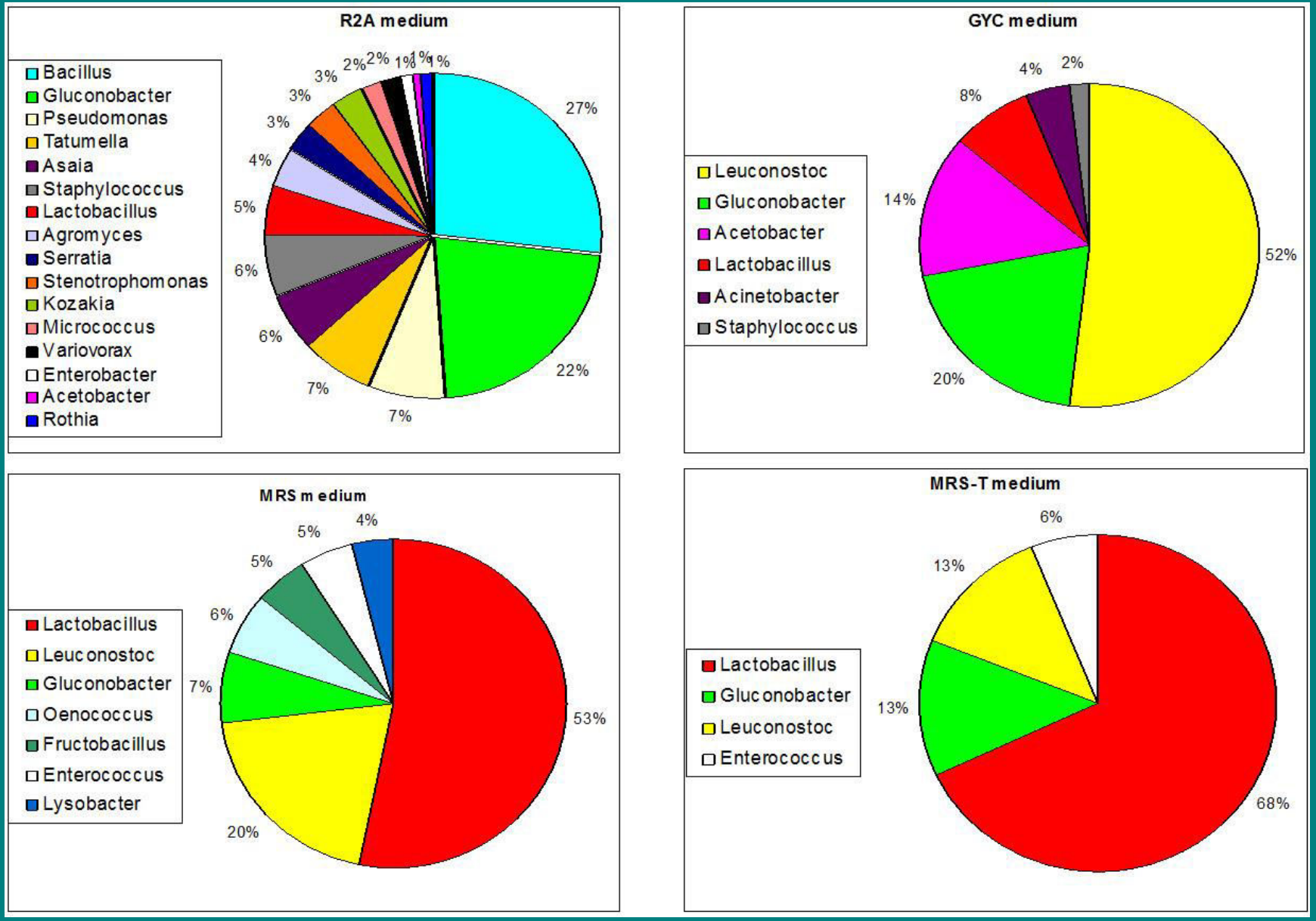
Culture-based identification of bacteria in wine

- large diversity (16 genera) detected on R2A medium (dominant: *Bacillus* and *Gluconobacter*)
- *Leuconostoc*, *Gluconobacter* and *Acetobacter* preferentially isolated on GYC medium
- *Lactobacillus, Leuconostoc* and *Gluconobacter* isolated on MRS and MRS-T
- larger diversity recovered on MRS than on MRS-T

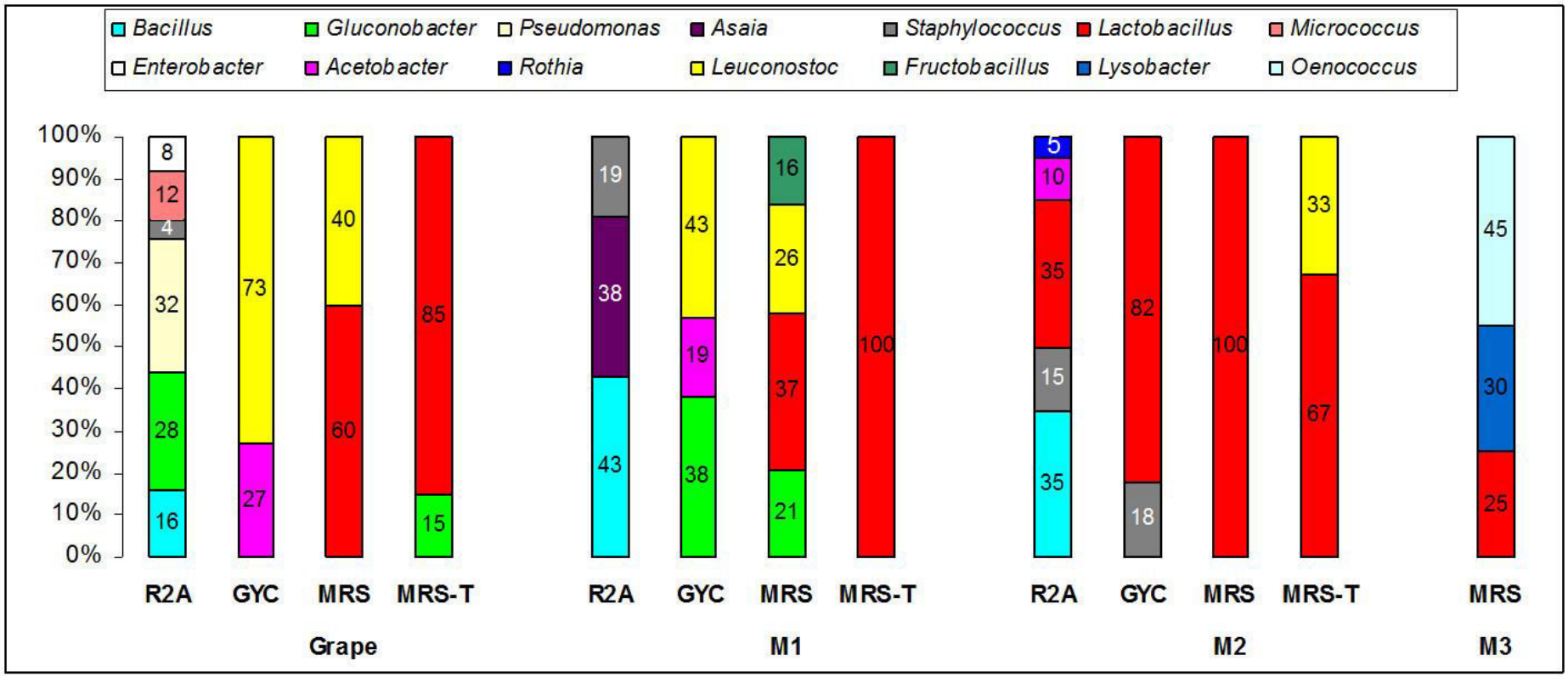
Clustering of isolates by f-ITS

- Each f-ITS profile contained 1 – 5 peaks
- 488 isolates were grouped into 45 clusters
- *Bacillus* was heterogenous – 36 isolates in 7 clusters
- *Gluconobacter* – 75 isolates in 4 clusters
- *Lactobacillus* – 161 in 5 clusters

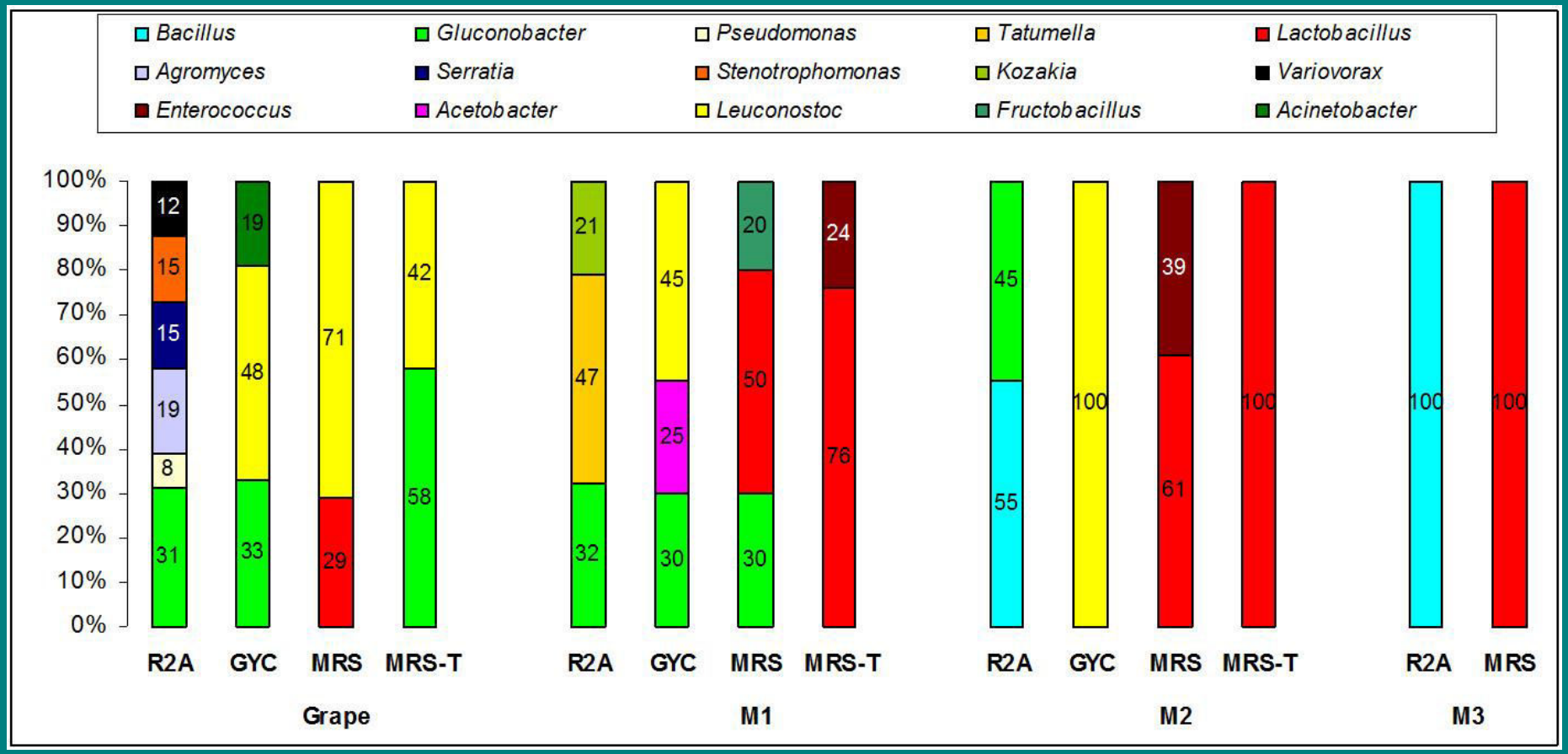
Comparison of the bacterial diversity on different cultivation media



Bacterial species isolated on different media in various wine production stages of Blaufränkisch



Bacterial species isolated on different media in various wine production stages of Grüner Veltliner



Identification of bacterial isolates

Taxonomic class	f-ITS profile bp	Species identification on the basis of the highest 16S rRNA similarity score	Frankovka modrá No. of isolates				Veltlínske zelené No. of isolates			
			G	M1	M2	M3	G	M1	M2	M3
Actinobacteria	377, 578	NR_116877 *Agromyces bauzanensis* 99%	-	-	-	-	5	-	-	-
	613	JX094953 *Micrococcus flavus* 99%	3	-	-	-	-	-	-	-
	198, 311, 385, 419	NR_074690 *Rothia mucilaginosa* 99%	-	-	1	-	-	-	-	-
Alphaproteobacteria	632	JQ314092 *Acetobacter* sp. 99%	-	-	2	-	-	-	-	-
	426, 643	AJ419840 *Acetobacter aceti* 99%	-	-	-	-	-	5	-	-
	394, 638	AB906405 *Acetobacter cibinongensis* 100%	-	4	-	-	-	-	-	-
	426, 657	GQ240641 *Acetobacter pasteurianus* 100%	6	-	-	-	-	-	-	-
	266, 285, 498	KP234012 *Asaia lannaensis* 99%	-	8	-	-	-	-	-	-
	693, 753	*Gluconobacter* sp. HM051360, JQ726620 100%	9	-	-	-	18	-	9	-
	285, 312, 747	AB178412 *Gluconobacter albidus* 100%	-	-	-	-	8	6	-	-
	686	*Gluconobacter cerinus* AB436556, KP234009 100%	1	8	-	-	-	12	-	-
	747	KP233998 *Gluconobacter oxydans* 99%	-	4	-	-	-	-	-	-
	286, 289, 312, 334, 337	NR_024773 *Kozakia baliensis* 99%	-	-	-	-	-	4	-	-
Betaproteobacteria	293, 511, 602	EU934231 *Variovorax* sp. 99%	-	-	-	-	3	-	-	-
Gammaproteobacteria	460, 552, 581	JN409466 *Acinetobacter johnsonii* 100%	-	-	-	-	4	-	-	-
	346, 371, 464,	FN357196 *Lysobacter capsici* 99%	-	-	-	6	-	-	-	-

Identification of bacterial isolates (continued)

Taxonomic class	f-ITS profile bp	Species identification on the basis of the highest 16S rRNA similarity score	Frankovka modrá No. of isolates				Veltlínske zelené No. of isolates			
	557									
	477, 530, 602	KM391405 *Pseudomonas* sp. 99%	1	-	-	-	-	-	-	-
	530, 572	KM030057 *Pseudomonas* sp. 99%	-	-	-	-	2	-	-	-
	533, 581	KC790322 *Pseudomonas gessardi* 100%	3	-	-	-	-	-	-	-
	529, 555	JF682514 *Pseudomonas putida* 100%	4	-	-	-	-	-	-	-
	458, 542, 738	EU627690 *Serratia proteamaculans* 100%	-	-	-	-	4	-	-	-
	211, 480, 522	AJ293462 *Stenotrophomonas rhizophila* 99%	-	-	-	-	4	-	-	-
	266, 282, 496	EU807749 *Tatumella* sp. 99%	-	-	-	-	-	9	-	-
Bacilli	263, 283, 311, 494	KC236572 *Bacillus* sp. 100%	-	4	-	-	-	-	-	-
	278, 453	AB970661 *Bacillus* sp. 99%	-	5	-	-	-	-	-	-
	285, 333, 366, 454	AB970685 *Bacillus* sp. 99%	-	-	4	-	-	-	-	-
	267, 290, 466	KC787602 *Bacillus* sp. 100%	-	-	-	-	-	-	4	-
	410, 440, 624	EU648109 *Bacillus* sp. 99%	-	-	-	-	-	-	-	5
	262, 282, 495	*Bacillus simplex* GU188928, KM817245 100%	3	-	3	-	-	-	7	-
	279, 282	HQ641336 *Bacillus licheniformis* 99%	1	-	-	-	-	-	-	-
	378, 450, 527	JF514549 *Enterobacter* sp. 100%	2	-	-	-	-	-	-	-
	366, 417	FJ917737 *Enterococcus durans* 100%	-	-	-	-	-	6	7	-
	425, 460, 476, 858	AB777209 *Fructobacillus fructosus* 99%	-	3	-	-	-	4	-	-
	300, 324, 518	*Lactobacillus brevis* KP165838, KM495920 100%	-	10	13	-	-	6	4	6
	288, 311, 324, 479, 518	KJ128234 *Lactobacillus hilgardii* 100%	-	-	4	-	-	-	-	-
	288, 311, 326, 495, 532	*Lactobacillus plantarum* HM462425, KP056258, KM507561, CP010528 100%	32	16	26	5	-	22	11	-
	415, 495	KM822614 *Lactobacillus rossiae* 99%	-	-	-	-	-	1	-	-

Identification of bacterial isolates (continued)

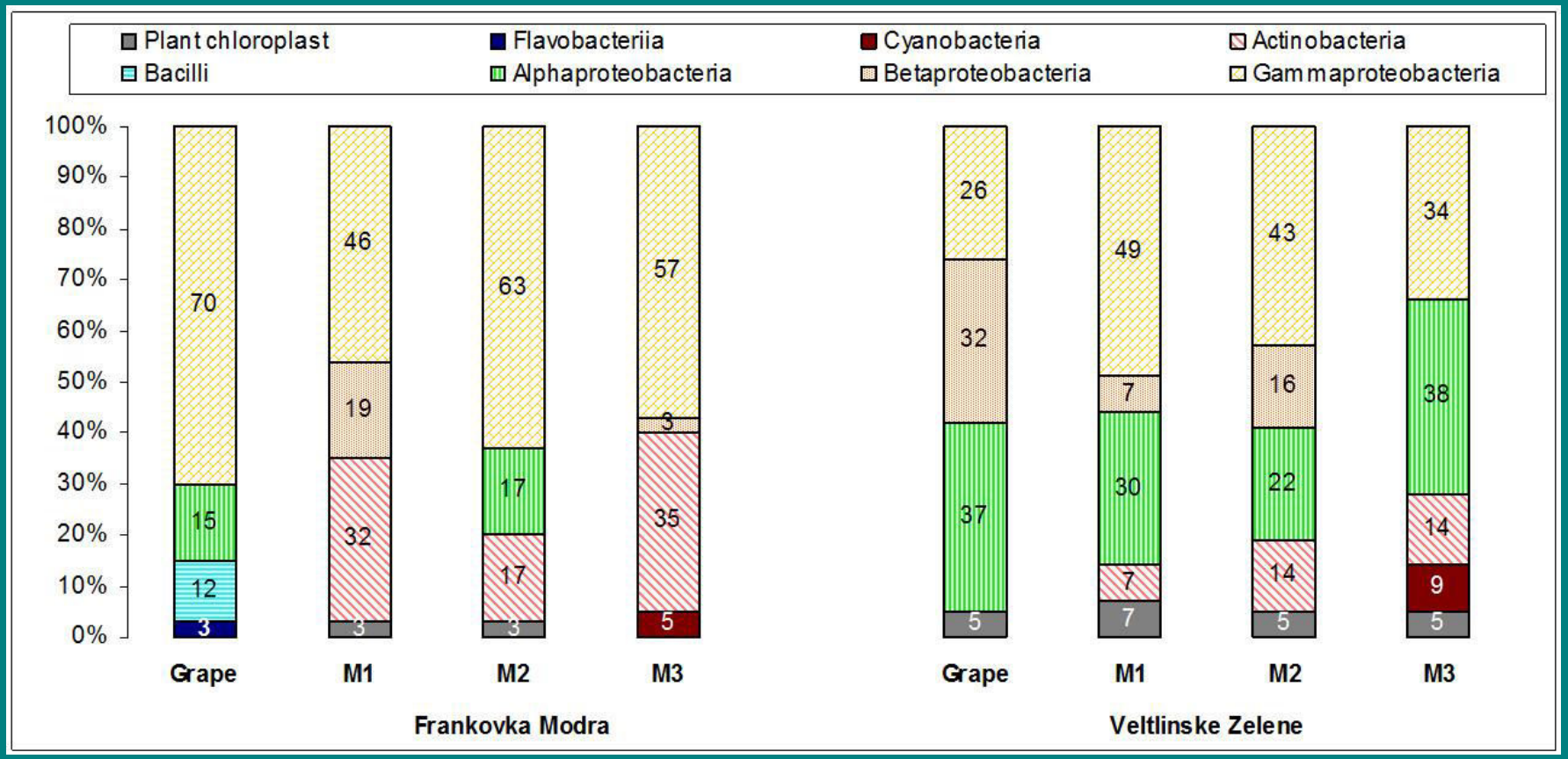
Taxonomic class	f-ITS profile bp	Species identification on the basis of the highest 16S rRNA similarity score	Frankovka modrá No. of isolates				Veltlínske zelené No. of isolates			
	309, 333, 543	EU794737 *Lactobacillus sakei* 99%	-	-	-	-	5	-	-	-
	417; 450	*Leuconostoc gasicomitatum* NR_074997, AF231131 100%	-	9	-	-	-	9	12	-
	444, 480	*Leuconostoc mesenteroides* EU621987, KM922578 100%	26	5	6	-	30	-	-	-
	453; 492	KP020117 *Oenococcus oeni* 100%	-	-	-	9	-	-	-	-
	349, 402, 438, 476, 716	KM253186 *Staphylococcus* sp. 99%	1	-	-	-	-	-	-	-
	407, 427	KF715393 *Staphylococcus* sp. 99%	-	4	-	-	-	-	-	-
	333, 348, 367, 410, 453	FJ957699 *Staphylococcus* sp. 100%	-	-	3	-	-	-	-	-
	694, 755	KP117257 *Staphylococcus warneri* 100%	-	-	2	-	-	-	-	-

Members of the same genus continuously identified in different vinification stages are described within grey squares.

Culture-independent analysis

- Blaufränkisch – 27 bacterial genera and 1 cyanobacterial
- Grüner Veltliner – 17 bacterial genera

Bacterial classes:



Culture-independent analysis

- Blaufränkisch – 27 bacterial genera and 1 cyanobacterial
- Grüner Veltliner – 17 bacterial genera
- The 16S rRNA sequences of bacterial clones were deposited in the GenBank database under the accession numbers KR704489 - KR704568.
- From different wine production steps, chloroplast 16S rDNA and mitochondrial 18S rDNA of *Vitis vinifera* were detected

Culture-independent identification of bacteria

Taxonomic class	Species identification on the basis of the highest 16S rRNA similarity score	Frankovka modrá No. of clones				Veltlínske zelené No. of clones			
		G	M1	M2	M3	G	M1	M2	M3
Actinobacteria	GU574109 *Amycolatopsis* sp. 99%	-	7	-	-	-	-	-	-
	NR_025424 *Arthrobacter nasiphocae* 98%	-	-	-	6	-	-	-	-
	KM021322 *Curtobacterium* sp. 100%	-	-	-	5	-	-	-	-
	LN774569 *Frigoribacterium faeni* 100%	-	-	7	-	-	-	-	-
	JX067686 *Kocuria* sp. 99%	-	8	-	-	-	-	-	8
	KF055020 *Microbacterium* sp. 100%	-	-	-	-	-	4	8	-
	KP860577 *Micrococcus* sp. 99%	-	-	-	3	-	-	-	-
	KP012225 *Propionibacterium* sp. 100%	-	1	-	2	-	-	-	-
	GQ046092 Uncultured bacterium clone 100% / NR_074675 *Propionibacterium acnes* 99% [a]	-	3	-	-	-	-	-	-
	HQ818199 Uncultured bacterium clone 100% / NR_074675 *Propionibacterium acnes* 99% [a]	-	-	3	-	-	-	-	-
	KF088615 Uncultured bacterium clone 99% / NR_074675 *Propionibacterium acnes* 99% [a]	-	-	-	3	-	-	-	-
	HM345484 Uncultured bacterium clone 100% / NR_118646 *Propionibacterium granulosum* 99% [a]	-	-	-	2	-	-	-	-
Alphaproteobacteria	NR_074285 *Agrobacterium vitis* 100%	-	-	-	-	-	8	13	11
	KP234007 *Gluconobacter albidus* 100%	-	-	-	-	9	-	-	11
	AB819118 *Gluconobacter frateurii* 100%	9	-	-	-	-	-	-	-
	Gluconobacter oxydans CP004373, JN004204 100%	-	-	10	-	7	-	-	-
	KP259730 *Sphingomonas* sp. 100%	-	-	-	-	-	9	-	-

Culture-independent identification of bacteria (cont.)

	JN167962 *Sphingomonas aurantiaca* 100%	-	-	-	-	6	-	-	-
Betaproteobacteria	KJ127965 *Acidovorax* sp. 99%	-	1	-	-	-	-	-	-
	KF896097 *Delftia* sp. 100%	-	4	-	-	-	-	-	-
	KC791004 *Hydrogenophilus* sp. 99%	-	-	-	2	-	-	-	-
	FR865952 *Massilia* sp. 99%	-	-	-	-	11	-	-	-
	KF093455 Uncultured bacterium clone 100% / NR_122055 *Snodgrassella alvi* 94% [a]	-	2	-	-	-	-	-	-
	JN590413 *Telluria* sp. 99%	-	-	-	-	-	4	-	-
	Variovorax sp. KP116945 100%; JQ034228 99%	-	4	-	-	8	-	9	-
Gammaproteobacteria	*Acinetobacter* sp. KJ726594, EU293155 100%	-	-	-	8	-	-	-	-
	HM317740 Uncultured bacterium clone 100% / NR_117624 *Acinetobacter johnsonii* 99% [a]	-	6	-	-	-	-	-	-
	GU356341 *Aeromonas* sp. 97%	-	-	9	-	-	-	-	-
	KC469704 *Aeromonas sharmana* 100%	-	-	-	11	-	-	-	-
	JN205049 *Cronobacter* sp. 99%	2	-	-	-	-	-	-	-
	Enterobacter sp. KJ950452, GQ179704, KJ534455, KP993213 99%; JF327476 96%	-	2	5	4	5	3	-	5
	KF999718 *Erwinia* sp. 99%	-	-	-	-	3	-	-	-
	NR_074869 *Erwinia tasmaniensis* 99%	6	-	-	-	-	-	-	-
	HM128172 Uncultured bacterium clone 96% / NR_121727 *Gilliamella apicola* 95% [a]	-	-	-	6	-	2	-	-
	KF914259 *Hafnia* sp. 100%	-	-	6	-	-	-	-	-
	KJ185017 *Klebsiella* sp. 99%	-	-	-	-	-	-	-	6
	AY601679 *Klebsiella oxytoca* 99%	-	-	-	-	7	-	-	-
	JN381005 *Legionella* sp. 96%	-	-	-	-	-	-	5	-
	KM458060 *Lelliottia* sp. 99%	-	-	-	-	-	-	9	-
	NR_118126 *Lonsdale quercina* 99%	-	-	-	-	-	5	-	-
	JF733375 *Moraxella* sp. 96%	-	5	-	-	-	-	-	-

Culture-independent identification of bacteria (cont.)

	Pantoea sp. KP325093 100%; HG315677, KM586992 99%	9	-	-	5	-	8	-	9
	KJ210675 *Pantoea agglomerans* 99%	3	-	-	-	-	-	-	-
	Pantoea vagans KP099965, JF460764 100%	10	-	-	-	-	-	11	-
	NR_126172 *Povalibacter uvarum* 99%	-	3	-	-	-	-	-	-
	KM203627 *Pseudomonas* sp. 100%	-	11	-	-	-	-	-	-
	EU877958 *Tatumella ptyseos* 99%	12	-	16	-	-	10	-	-
Bacilli	KM874426 *Bacillus* sp. 99%	7	-	-	-	-	-	-	-
Flavobacteriia	KF556703 *Flavobacterium* sp. 100%	2	-	-	-	-	-	-	-
Cyanobacteria	KF100515 Uncultured bacterium clone 100% / NR_117881 *Loriellopsis cavernicola* 86% [a]	-	-	-	3	-	-	-	-
	HQ781756 Uncultured bacterium clone 100% / NR_117881 *Loriellopsis cavernicola* 89% [a]	-	-	-	-	-	-	-	5
Plant chloroplast	KP308151 *Dunalia brachyacantha* chloroplast 16S ribosomal RNA gene 99%	-	-	-	-	-	-	-	3
	KP296185 *Iochroma loxense* chloroplast 16S ribosomal RNA gene 100%	-	2	2	-	3	4	3	-

Members of the same genus continuously identified in different vinification stages are described within grey squares.

- Detailed data on bacterial microflora of two typical wines of the Small Carpathian wine-producing regions were obtained

- Data and isolates provide the basis for improvement of the quality of regional wines

- The two scientific approaches produced complementary information on bacterial diversity

