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Mycobiota of Barbera grapes from the Piedmont region from a single vintage year

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Abstract

In this paper a description of the yeast populations present on grapes of the Barbera cultivar is reported. Fifteen vineyards in the Monferrato area, in the Piedmont region – North West of Italy, were sampled at maturation during the vintage 2012.

Grapes were subjected to culture dependent and independent analysis. By plate count on WLN medium a yeast load of about 10^2 colony forming units (cfu)/mL was determined on the grapes. A total of 206 isolates were identified by using two different molecular approaches. The results obtained through the two identification techniques, PCR-RFLP analysis of the 5.8S-ITS rRNA region and PCR-DGGE coupled with sequencing of the 26S rRNA gene, resulted highly reproducible and comparable, confirming these methods as two reliable identification tools in the study of wine yeast ecology. Through both techniques, a mycobiota mainly constituted by *Aureobasidium pullulans* (73% of the isolates), followed by *Rhodotorula glutinis* (12%), *Hanseniaspora* spp. (8%), *Issatchenkia terricola* (5%), *Torulaspora delbruekii* (1%) and *Cryptococcus carnescens* (1%) was described. Moreover, DNA and RNA extracted directly from grapes were subjected to PCR-DGGE confirming the presence of *A. pullulans*. *H. uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) were also detected.

Keywords: grape mycobiota, ITS, DGGE, culture independent methods

Introduction

Microorganisms present on the surface of grape berries play an important role in winemaking, where different species of yeasts, bacteria and moulds coexist. The metabolic activities of these microorganisms lead to the transformation of grape juice into wine and in this context the knowledge of their behaviour during fermentation is fundamental to obtain final products with the desired organoleptic characteristics.

Grapes are the primary source of yeasts in wine production, especially non-*Saccharomyces* which, during the first stages of fermentation, can produce enzymes capable of developing aroma compounds and of intensifying the final wine characteristics (Arevalo Villena et al. 2007). At the same time, however, part of the yeast community may also harm the quality of the resulting wine. Recently it has been described that the main vector for *Brettanomyces* spp., a detrimental yeast for wine flavour, due to the high production of acetic

acid and volatile phenols, is represented by grapes (Renouf et al. 2007). In order to comprehend the impact of grape yeast communities on the final characteristics of the wine, a detailed inventory is deemed necessary. The population of non-*Saccharomyces* yeasts varies and may include the genera *Kloeckera*, *Candida*, *Brettanomyces, Cryptococcus*, *Pichia* and *Rhodotorula* (Fleet, 2003), which follow the grapes into the fermentation vats. As already known, the presence of these populations on grape berries are linked to different factors such as: geographical location, climatic conditions, grape variety and maturity and viticulture practices (Barata et al. 2012, Combina et al. 2005).

Accurate identification is crucial for ecology studies. Classical identification techniques, based on morphological, biochemical and physiological criteria, may prove incorrect due to heterogeneous phenotypic results. With the development of molecular methods, information regarding the microbial ecology can be rapidly achieved (Barata et al. 2012). Different authors adopted molecular methods in the study of indigenous yeast populations. Furthermore, in order to detect populations that are numerically less abundant or in stressed condition, also culture independent methods provide an important contribution to the study of grape ecology (Cocolin et al. 2011).

In this study, the attention was focused on the yeast populations on Barbera grapes at maturation in vintage 2012, coming from the Piedmont region, North West Italy, where Barbera wine represents a product of superior quality. A complete picture of the yeast ecology was obtained for grapes coming from different vineyards located in the Monferrato area of the Piedmont region through an experimental approach, which included both isolation and identification of yeasts, using RFLP analysis of the of 5.8S-Internal Transcribed Spacers (ITS) region and Denaturing Gradient Gel Electrophoresis (DGGE) analysis followed by 26S rRNA gene sequencing, and direct extraction of DNA and RNA from grapes followed by DGGE.

Materials and methods

Sample collection

Vitis vinifera L. cv Barbera grape samples were harvested from fifteen vineyards in the period ranging from September 12 to September 24, 2012, when the technological maturity was optimal for the production of Barbera wines. The grapes were in excellent phytosanitary condition.

Table 1 reports the different vineyards, labeled according to the name of the village they are located in. About 200 randomly picked grape berries were selected from each vineyard, placed in sterile stomacher bags and transported at refrigeration temperature to the laboratory.

Cultural microbiological analysis

The grapes, after being crushed in a sterile bag using a stomacher machine, were serially diluted in Ringer solution (Oxoid Milan, Italy) until dilution 1:10000 and plated on Wallerstein Laboratory Nutrient (WLN) medium (Oxoid), which is able to differentiate yeast populations on the basis of the colour and morphology of the colony. The plates were incubated at 25°C for 4 days. From plates of each vineyard at least 10 yeast colonies were selected and isolated. Streaking was carried out on WLN and pure cultures were stored at -80°C in YPD broth (2% [wt/vol] glucose, 2% [wt/vol] peptone and 1% [wt/vol] yeast extract, all coming from Oxoid) after the addition of glycerol (30%) (Sigma-Aldrich, Milan, Italy).

Molecular identification by RFLP of the region ITS1-5.8S rRNA-ITS2 and PCR-DGGE

For the molecular identification, two different approaches were carried out. Prior to identification, genomic DNA of each isolate was extracted from 1 millilitre of an overnight culture in YPD broth, according to Cocolin et al. (2000), quantified by using a Nanodrop ND-1000 spectrophotometer (Celbio, Milan, Italy) and standardized at 100 ng/µl. All isolates were analyzed by restriction fragment length polymorphism (RFLP) of the region ITS1-5.8S rRNA-ITS2. The DNA fragments were amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG -3') and ITS4 (5'-TCCTCCGCTTATTGATATGC 3') (White et al. 1990) in a PCR reaction of a final volume of 50 µL, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of deoxynucleoside triphospates (dNTPs), 1.25 U of *Taq* Polymerase (Applied Biosystems, Milan Italy), 0.2 µM of each primer and 100 ng of template DNA. Amplifications were carried out in a PTC-200 DNA Engine MJ Research thermal cycler (Biorad, Milan, Italy), as described by Esteve-Zarzoso et al. (1999). The amplified fragments were digested with endonucleases *HinfI*, *HaeIII*, *CfoI* (Promega, Milan, Italy), according to the supplier's instructions. The *DdeI* enzyme was adopted in order to discriminate species of the genus *Hanseniaspora*, as suggested by Esteve-Zarzoso et al. (1999). Both PCR products and restriction fragments were separated by electrophoresis in 1.5 and 3% agarose gels respectively and stained

with ethidium bromide (Sigma). The approximate lengths were determined by the use of molecular weight ladder (100 bp, Promega). PCR and RFLP fragment lengths were used for identification of yeasts by the comparison of the restriction bands with those available in literature (Esteve-Zarzoso et al. 1999, Granchi et al. 1999, Guillamon et al. 1998, Sabate et al. 2002).

At the same time, DNA extracted from all the pure cultures, was also amplified with NL1 and LS2 primers, as reported by Cocolin et al. (2000). Amplifications were carried out in a PTC-200 DNA Engine MJ Research thermal cycler (Biorad), as previously described (Cocolin et al. 2000) and subjected to DGGE, as described later. The DGGE profiles of the isolates were grouped and representatives of each group were amplified with NL1/NL4 primers (Kurtzman and Robnett, 1997) in order to amplify the D1-D2 domain of the 26S rRNA gene. The PCR products were sent to a commercial facility (MWG Biotech, Edersberg, Germany) for sequencing and the resulting sequences were aligned in Gene Bank using the Blast program (Altschul et al. 1990), for identification purposes.

Nucleic acids extraction from grapes juice

The DNA and RNA were extracted as described in Mills et al. (2002) by using the DNeasy Plant Mini Kit (Qiagen, Milano, Italy) and the Concert Plant RNA reagent (Invitrogen, Monza, Italy), respectively. DNA was standardized to 100 ng/µl. RNA was treated with Turbo DNase (Ambion, Monza, Italy), in order to eliminate co-extracted DNA. Complete DNA digestion was confirmed using 1 µl in PCR amplifications. In case of positive amplifications, the treatment was prolonged until a negative PCR reaction was obtained from all the RNA samples.

Direct PCR and RT-PCR amplification

The DNA extracted directly from grapes juice was amplified with primers NL1/LS2 as described above for the pure cultures, while the RNA was subjected to reverse transcription (RT) reactions using the M-MLV reverse transcriptase (Promega) as described in Alessandria et al. (2013).

DGGE analysis

The PCR products were analyzed by means of DGGE, as described by Cocolin et al. (2000). Amplicons

were applied to a 8% (wt/vol) polyacrilamide gel (acrylamide-bis acrylamide 37:5:1) with a denaturing gradient of 30 to 50% (Cocolin et al., 2000) in a 1X TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 M EDTA [pH 8]). The gels were subjected to a voltage of 130 volt for 4 h at 60°C, stained for 20 min in 1X TAE containing 1x SYBR Green I (Sigma) and then analysed under UV using UVI pro platinum 1.1 Gel Software (Eppendorf, Hamburg, Germany).

Sequencing of the DGGE bands obtained from the DNA and RNA analysis of the musts

Selected DGGE bands were extracted from the gels, using sterile pipette tips, transferred into 50 µl sterile water and incubated overnight at 4°C. Two µl of the eluted DNA were re-amplified using the conditions described above and checked by means of DGGE. The PCR products that gave a single band, co-migrating with the DNA/RNA controls, were then amplified with the same primers without a GC clamp and sequenced by MWG Biotech. The sequences were aligned in Gene Bank using the Blast Program.

Results and discussion

The grape microbial ecosystem is composed of highly diverse microorganisms, however most studies have focused on yeasts or lactic bacteria because of their involvement in the transformation of grape must into wine (as reviewed by Barata et al. (2012)). Within yeasts, grapes can also be an important source for non-*Saccharomyces* species. These species are of interest for the wine industry for their potential use as mixed starter together with *S. cerevisiae* (Rantsiou et al. 2012) and for their contribution to the organoleptic characteristics of wine (Fleet, 2003, Fleet et al. 2008).

This paper describes the yeast ecology of Barbera grapes coming from fifteen vineyards in the Monferrato area (Piedmont, Italy) during vintage 2012. Yeasts counts in the samples collected from the different analyzed vineyards, resulted to be very homogeneous, showing values of about 2 $\log_{10}$ cfu/mL (data not shown) independently of the geographical location, age of the vineyards and their agricultural conduction (Table 1). The counts resulted lower than those reported in other studies for mature grapes, usually of about 4 $\log_{10}$ cfu/mL (Fleet, 2003,, Renouf et al. 2005). Climatic conditions (temperature, humidity, UV and radiation), and viticultural practices are factors which influence the berry micro environment and, consequently, the microbial ecosystem on the surface (Renouf et al. 2005), conditioning the diversity and the

quantity of microbial populations (Barata et al. 2012). As stated on the Arpa Piemonte website [http://www.arpa.piemonte.it/rischinaturali/tematismi/clima/rapporti-di-analisi/annuale_pdf/clima2012.pdf], the rain percentage detected in the period from July to the end of September was 23% lower than the seasonal average and this could explain the lower microbial counts. As reported by Combina et al. (2005), rainfalls, near or during harvest, when grapes are mature, can explain the increase in yeast cell density on the grapes because of a major availability of nutrients on berry surface.

A total of 206 yeast isolates were identified using two different techniques. After restriction analysis of the 5.8S-ITS region, the isolates were clustered into 6 groups (Table 2). Four of these groups were directly identified by the comparison of restriction bands with those available in literature. One group could not be identified by RFLP analysis (group 6), while in the case of restriction profile 3, a genus identification was obtained without reaching the species level (*Hanseniaspora* spp.). Regarding DGGE, six groups were observed. Table 2 reports the number of isolates for each group and their identification.

The results obtained through the two mentioned methods showed a strong correlation: both techniques detected six groups, composed by the same number of isolates. Regarding the identification, the same results were obtained for four of the six groups and in particular for groups 1, 2, 4 and 5 (Table 2). In the case of group 3, using the RFLP analysis with the three enzymes adopted, identification at genus level was reached (*Hanseniaspora* spp.), while the DGGE with 26S rRNA gene sequencing identified the 17 isolates of the group as *H. uvarum.* According to results of Esteve-Zarzoso et al. (1999), *H. uvarum* and *Hanseniaspora guillermondi*, being closely related species, show the same restriction patterns using *AluI*, *CfoI*, *HaeIII*, *HinfI*, *ScrFI*, and *TaqI* restriction endonucleases. However their differentiation can be obtained with the *DdeI*. Using this enzyme, 5 strains of *H. guillermondi* and 12 of *H. uvarum* were identified showing a restriction fragments of 380+180+95+80 bp and 300+180+95+90+85 bp respectively. Group 6, composed by two isolates, could not be identified by the RFLP protocol applied, since an unknown pattern was obtained. The sequencing of the D1-D2 domain assigned those isolates to *Cryptococcus carnescens*.

PCR-RFLP analysis of the 5.8S-ITS rRNA region is a routine technique for the identification of wine yeast isolates. Restriction analysis has demonstrated that yeast species can be differentiated by combining the action of different restriction endonucleases (Baffi et al. 2011, Combina et al. 2005, Cordero-Bueso et al. 2011, Esteve-Zarzoso et al. 1999,). The results obtained through this approach are usually complemented

with other techniques, in order to confirm the species identification especially when the 5.8-ITS pattern has not been previously reported. The sequencing of D1/D2 domain of the 26S rRNA gene or the ITS1-5.8S rRNA gene-ITS2 region are most frequently carried out.

The first application of PCR-DGGE in wine microbiology dates back in 2000, when its use for the identification of yeast isolates from grapes, musts and wine was validated (Cocolin et al. 2000). After this study, the literature increased in the number of papers published exploiting this approach in the wine microbiology field (Alessandria et al. 2013, Mills et al. 2002, Rantsiou et al. 2013,). A limit of the PCR-DGGE approach for identification purposes is connected to the co-migration of bands. As stated in Kisand and Wikner (2003), rRNA genes in the ecological studies may cause a non specific taxonomic resolution from detection of species with very small nucleotide differences. Another issue may be connected to rRNA heterogeneity with the presence of multiple copies of ribosomal genes that produce several bands for each species as reported by Rantsiou et al. (2004) for bacteria. This phenomenon was observed in this study for *A. pullulans*, that showed a profile composed by different bands.

In this study, the application of two identification strategies allowed for the analysis of a high number of isolates in a short time and the results obtained by the two methods resulted highly reproducible and comparable. Through both techniques, six groups with a distinctive fingerprint were obtained. Analysis of RFLP profiles allowed to reach a direct identification for most of the isolates and only for one restriction patter it was necessary to proceed with sequencing. In one case the RFLP analysis resulted more discriminatory than DGGE. For *Hanseniaspora,* RFLP was able to differentiate between *H. uvarum* and *H. guillermondii* when an additional restriction endonuclease was used. Overall, both techniques showed a good discrimination power and confirmed to be suitable for the study of yeast biota in the enological field.

The distribution of yeast species among vineyards, is reported in Figure 1. *A. pullulans* was the species most frequently encountered on grapes, representing about 73% of the isolates, followed by *Rhodotorula glutinis* (12%), *Hanseniaspora uvarum* (6%)*, Issatchenkia terricola* (5%), *Hanseniaspora guillermondii* (2%), *Torulaspora delbrueckii* (1%) and *Cryptococcus carnescens* (1%). *A. pullulans* was detected in all the vineyards with different prevalence, except in the organic vineyard of Murisengo. In eight vineyards *A. pullulans* was the only species detected (vineyards located in Costigliole, Vinchio, Nizza Monferrato, Alice Bel Colle and Acqui Terme) while, in Incisa Scapaccino and San Martino Alfieri, it reached shares higher

than 90%. In the other samples it represented about 50% of the isolates. In Murisengo vineyard most of the isolates were *Issatchenkia terricola* with values of about 67%, together with isolates belonging to *H. guilermondii* (33%). A high biodiversity was detected in the grapes from Ricaldone where, beside the 50% of *A. pullulans, C. carnescens, T. delbruecki*, *H. uvarum*, *R. glutinis* and *I. terricola* were detected.

R. glutinis and *C. carnescens* were isolated together in two vineyards with *A. pullulans*. *R. glutinis* was detected in seven of the fifteen vineyards, with a prevalence on the grapes from Isola d'Asti (49%) and Montegrosso (43%).

As reported above, geographic location and climatic conditions have an impact on the population and diversity of yeasts on grapes. The vineyards analyzed in this research are situated in the Monferrato, a limited area where the environmental conditions of the vineyard (monitored using a Vantage PRO2 weather station, data not shown), did not present relevant differences among each other. This aspect could have a correlation with the results of the identification. As already reported, in almost all the sites, *A. pullulans* represented the predominant species. Different studies associate the presence of this ascomycete to immature berries (Lederer et al. 2013, Renouf et al. 2005), however a recent study on grape ecology during withering for the production of icewines (Alessandria et al. 2013) showed its presence until the end of withering, demonstrating how the maturity of the berries is not a significant parameter associated to the presence of *A. pullulans.*

The second species isolated in the vineyard, concerning the number of isolates, was *R. glutinis,* species known to be often present on grapes (Fleet, 2003), during all the ripening stages (Renouf et al. 2005), and responsible for the production of off-flavors in wine (Shinohara et al. 2000).

I. terricola is well known for its occurrence in grapes of several varieties throughout the word (Baffi et al. 2011), however in this study, it was detected only in the organic Murisengo vineyard. The connection between this species and the organic conduction of the vineyard has not being demonstrated. Tofalo et al. (2011) identified *I. terricola* in organic vineyards, while Milanovic et al. (2013) found this species in a similar percentage in conventional and organic vineyards. Different yeast species were observed in the grapes harvested from the organic vineyard. Loazzolo and San Martino Alfieri showed the presence of *A. pullulans* and *R. glutinis*, together with *H. uvarum* in Loazzolo, while the Murisengo vineyard was characterized by *I. terricola* and *H. guillermondii*. In Cordero-Bueso et al. (2011), the organic vineyard was

found to be an important reservoir for fermentative yeasts of interest, however in this study no *Saccharomyces* were detected.

The total DNA and RNA were extracted and analysed by means of PCR-DGGE. The DNA and RNA gels are shown in Figures 2 while the results of the band identification are presented in Table 3.

From the DNA analysis, three different species of yeast were detected (*A. pullulans, H. uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*)) and three different moulds (*Botryotinia fuckeliana*, *Chalastospora ellipsoidea e Cladosporium delicatulum). A. pullulans* was detected in all the samples. *H. uvarum* was detected in 4 vineyards (San Martino Alfieri, Murisengo, Nizza Monferrato and Ricaldone) while *Starm. bacillaris* was visible in the vineyards of Incisa Scapaccino and Isola d'Asti.

Regarding the RNA, the presence of *A. pullulans* was confirmed in the majority of the samples, and *Saccharomyces cerevisiae* (bands N and P) was detected in 5 vineyards. A band (O) related to *Vitis vinifera* was also visible.

Direct analysis through DGGE of the 26S rRNA amplicons contributed to outline the yeast community profiles in Barbera grapes. While DNA based DGGE analysis is useful to profile the microbial diversity, RNA allows the recognition of live and metabolically active microorganisms (Cocolin et al. 2011). In several food fermentation sectors, the DGGE technique has been able to highlight populations that may have an important impact on the final characteristics of the product, but which were not well described by means of culture-dependent methods (Cocolin et al. 2013). *A. pullulans* isolated in high percentage by the culture-dependent methods was also detected by DGGE in all the the vineyards. At the same time, other populations, namely *B. fuckeliana*, *Cl. delicatum*, *Ch. ellipsoidea* and *Starm. bacillaris* were detected only by DGGE at DNA level, underlining a probable presence of dead populations. As far as *Starm. bacillaris,* a known species able to resist in high osmotic stress conditions (Sipiczki, 2004) and able to control acetic acid production during alcoholic fermentation (Rantsiou et al. 2012), it was detected only in three vineyards, Incisa Scapaccino, Isola d'Asti and San Martino Alfieri, but it was never isolated in WLN plates reinforcing the hypothesis that the cells are dead.

Interestingly, analyzing the RNA, *S. cerevisiae* was observed in Incisa Scapaccino, Costigliole, Loazzolo and Ricaldone vineyards, supporting the role of grapes as possible source of *S. cerevisiae.* On the other hand, it is well known that this species is found in very low portions in healthy grapes (Combina et al. 2005). Presence

of the genus *Hanseniaspora* was confirmed also by DNA direct analysis, while *I. terricola* was not detected. In general, low incidence of apiculate yeasts was observed in the analyzed grapes.

Conclusion

The yeast ecology was studied concurrently by two techniques often used in wine ecology, that proved to be reproducible, confirming the possibility to choose any of these two techniques for identification purposes.

This study has provided the first information on the yeast biota present on the Barbera grapes in the Piedmont region. Since only 2012 vintage was investigated, which was characterized by a very specific climatic condition due to limited rainfall with respect to the seasonal average, further studies are necessary to confirm the yeast ecology described in this preliminary study.

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Figure legends:

Figure 1. Distribution of the isolated yeast species in the different vineyards (*organic vineyards).

Figure 2. DGGE profiles of the DNA (panel A) and RNA (panel B) extracted directly from grapes in the different vineyard sites**. Panel A:** Numbers indicate the vineyards: 1 Montegrosso, 2 Nizza Monferrato, 3 Incisa Scapaccino, 4 Acqui Terme Crocera South East, 5 Acqui Terme Dannona, 6 Agliano,7 Acqui Terme Crocera South West, 8 Vinchio, 9 Costigliole, 10 Murisengo, 11 Isola d'Asti, 12 San Martino Alfieri, 13 Loazzolo, 14 Ricaldone, 15 Alice Bel Colle**.** The bands indicated by letters were excised and after re-amplification (as described in Material and methods) subjected to sequencing. The identification of the bands is reported in Table 3. **Panel B:** Numbers indicate the vineyarsds: 1 Nizza Monferrato, 2 Acqui Terme Crocera South East, 3 Alice Bel Colle, 4 Vinchio, 5 Incisa Scapaccino, 6 Costigliole, 7 Murisengo, 8 Acqui Terme Crocera South West, 9 San Martino Alfieri, 10 Acqui Terme Dannona, 11 Loazzolo, 12 Ricaldone, 13 Isola d'Asti, 14 Agliano, 15 Montegrosso. The bands indicated by letters were excised and after re-amplification (as described in Material and methods) subjected to sequencing. The identification of the bands is reported in Table 3.

Table 1. The vineyards considered in this study labeled according to the name of the village where they are located, the age, the location and the agricultural practices.

Code	Vineyard	Age of the vineyard	Location of the vineyard	Agricultural practices
1	Isola d'Asti	8	Hill	Conventional
2	Costigliole d'Asti	32	Hill	Conventional
3	San Martino Alfieri	10	Hill	Organic
4	Loazzolo	9	Top hill	Organic
5	Murisengo	12	Hill	Organic
6	Alice Bel Colle	42	Hill	Conventional
7	Incisa Scapaccino	45	Hill	Conventional
8	Nizza Monferrato	25	Hill	Conventional
9	Vinchio	37	Hill	Conventional
10	Montegrosso d'Asti	75	Hill	Conventional
11	Agliano Terme	12	Hill	Conventional
12	Ricaldone	30	Hill	Conventional
13	Acqui Terme Crocera south east	9	Top hill	Conventional
14	Acqui Terme Crocera south west	9	Top hill	Conventional
15	Acqui Terme Dannona	9	Top hill	Conventional

Table 2. Results of the groups obtained by RFLP, with the size in bp of the PCR products and the restriction fragments of the isolates, and groups obtained by DGGE and identification by 26S sequencing.

		RFLP results						DGGE results		
Group	**Number of isolates**	**5.8-ITS PCR (bp)**	***CfoI* (bp)**	***Hinf* (bp)**	***HaeIII* (bp)**	**Species**	**Reference**	**Species**	**Identity%**	**Accession number**
1	150	600	190 + 180 + 100	290 + 180 + 130	450+150	*Aureobasidium pullulans*	Sabate et al., 2002	*Aureobasidium pullulans*	100	gb|KF263940.1|
2	24	640	320+240	170+ 150	430+210	*Rhodotorula glutinis*	Guillamón et al., 1998	*Rhodotorula glutinis*	98	gb|KC847100.1|
3	17	750	320+ 310+105	350+200+180	750	*Hanseniaspora* spp.	Esteve-Zarzoso et al., 1999	*Hanseniaspora uvarum*	98	gb|KF263939.1|
4	11	416	120+ 95+ 78+ 71+ 58	225+ 100+ 84	290+120	*Issatchenkia terricola*	Granchi et al., 1999	*Issatchenkia terricola*	100	gb|KF263952.1|
5	2	803	330+ 215+ 140+ 100	420+ 380	803	*Torulaspora delbrueckii*	Granchi et al., 1999	*Torulaspora delbrueckii*	100	gb|KC510055.1|
6	2	450	200+120	230	450	N.I.*	-	*Cryptococcus carnescens*	99	gb|KC494732.1|

*N.I. Identification not reached

Table 3. Identification of the PCR-DGGE bands retrieved from GenBank

Band	Closest relative	% identity	Source
A	*Botryotinia fuckeliana*	95	gb|KC292909.1|
B	*Starmerella bacillaris* (synonym *Candida zemplinina*)	100	gb|EU386719.1|
C	*Fungal endophyte*	98	gb|KF436419.1|
D	*Cladosporium delicatulum*	99	gb|JQ807971.1|
E	*Chalastospora ellipsoidea*	100	gb|FJ839643.1|
F	*Aureobasidium pullulans*	100	gb|KC160613.1|
G	*Botryotinia fuckeliana*	99	gb|KC292909.1|
H	*Hanseniaspora uvarum*	100	gb|JQ678683.1|
I	*Aureobasidium pullulans*	100	gb|KC160612.1|
L	*Aureobasidium pullulans*	100	gb|JX129902.1|
M	*Aureobasidium pullulans*	100	gb|KC160607.1|
N	*Saccharomyces cerevisiae*	100	gb|JX423564.1|
O	*Vitis vinifera*	100	emb|AM474244.2|
P	*Saccharomyces cerevisiae*	96	gb|KF141638.1|

Figure 1

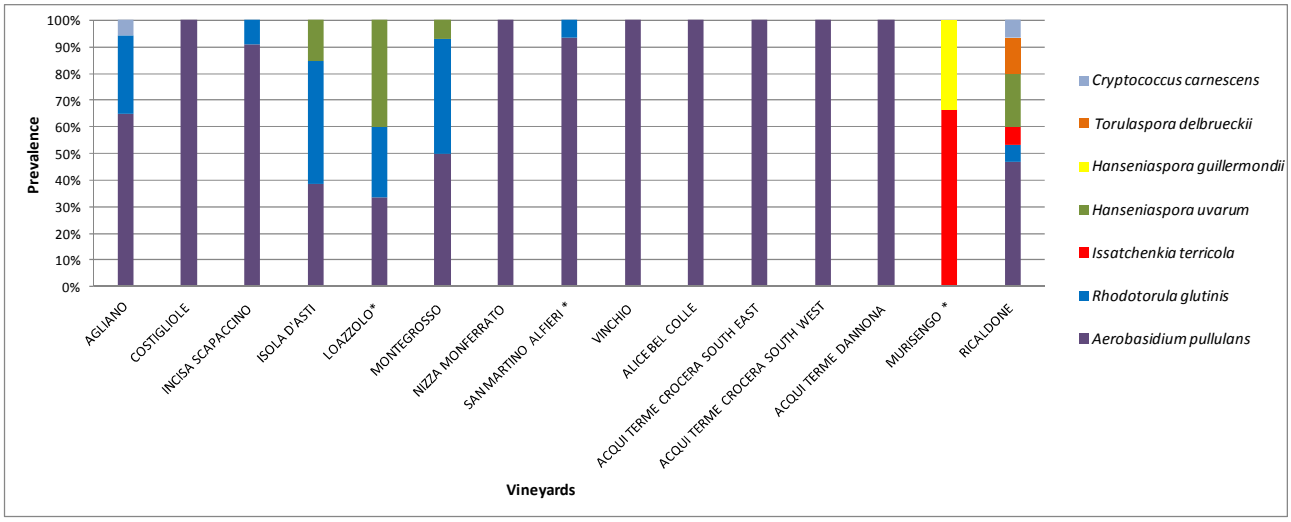
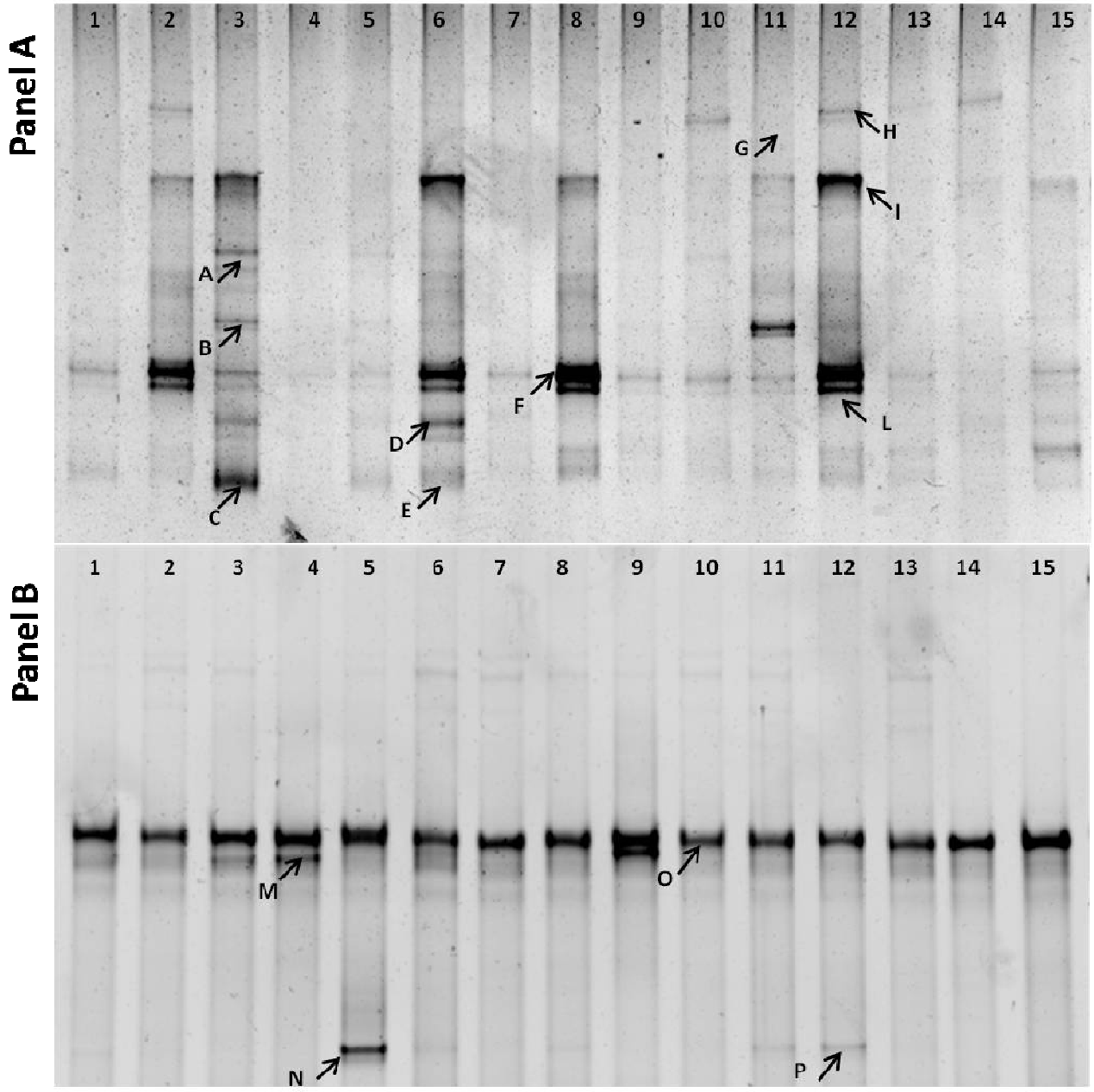

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Prevalence
AGLIANO
COSTIGLIOLE
INCISA SCAPACCINO
ISOLA D'ASTI
LOAZZOLO*
MONTEGROSSO
NIZZA MONFERRATO
SAN MARTINO ALFIERI*
VINCHIO
ALICE BEL COLLE
ACQUI TERME CROCERA SOUTH EAST
ACQUI TERME CROCERA SOUTH WEST
ACQUI TERME DANNONA
MURISENGO*
RICALDONE
Vineyards
Cryptococcus carnescens
Torulaspora delbrueckii
Hanseniaspora guillermondii
Hanseniaspora uvarum
Issatchenkia terricola
Rhodotorula glutinis
Aerobasidium pullulans

Figure 2


Panel A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
A
B
C
D
E
F
G
H
I
L
Panel B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
M
N
O
P