MOLECULAR IDENTIFICATION OF *BOTRYTIS CINEREA*, *PENICILLIUM* SPP. AND *CLADOSPORIUM* SPP. IN LUXEMBOURG

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Abstract

Aim: Grapes are submitted to several fungal attacks, which can impair quantity and quality of the resulting wine. The aim of this paper is to describe sets of primers that are able to easily characterise the strains isolated on the grapes by DNA sequencing.

Methods and results: Four sets of primers targeting the beta-tubulin and Internal Transcribed Spacer (ITS) regions were used. Samples were isolated from grey or green moulds on mature berries during 5 years in vineyards located in the Moselle Valley of Luxembourg. Identifications were performed by comparing the obtained sequences with referenced sequences using several databases. The isolates obtained from the grey mould were identified as *Botrytis cinerea*, *Mucor fragilis* and *Chaetomium globosum*, whereas on green mould, *Penicillium expansum*, *Penicillium minioluteum*, *Davidiella tassiana* and *Cladosporium cladosporioides* were recovered. Identification may be impossible for two reasons: samples may not display a sequence of sufficient quality, which can tentatively be solved by cloning the PCR amplicon, or databases may not be exhaustive enough to unambiguously determine the species. We therefore suggest primer sets for each species according to these limiting factors.

Conclusion: The performances of the primers were species-dependent. Even though the ITS region is more highly represented in the databases than the beta-tubulin region, technical results were better for beta-tubulin sequences.

Significance and impact of the study: This work provides a basic methodology for the molecular characterisation of the fungal flora encountered on grapes.

Key words: fungal flora, grape, molecular identification

Résumé

Objectif: Les raisins sont sujets à plusieurs maladies fongiques qui peuvent altérer la qualité et diminuer la quantité de la récolte. Cet article a pour objectif de décrire des paires d'amorces qui sont capables de caractériser les souches isolées sur grappe.

Méthodes et résultats: Quatre paires d'amorces amplifiant les régions de la beta-tubuline ou de l'espaceur interne transcrit (ITS) ont été testées. Les souches ont été isolées sur cinq années à partir de grappes atteintes de pourriture grise ou verte dans des vignobles de la vallée de la Moselle luxembourgeoise. Les identifications ont été réalisées en comparant les séquences obtenues avec les souches de référence issues de plusieurs bases de données. Les isolats provenant de la pourriture grise ont été identifiés comme étant *Botrytis cinerea*, *Mucor fragilis* et *Chaetomium globosum* alors que sur la pourriture verte, nous avons identifié *Penicillium expansum*, *Penicillium minioluteum*, *Davidiella tassiana* et *Cladosporium cladosporioides*. L'identification peut être rendue impossible par deux facteurs: les séquences obtenues peuvent être de qualité insuffisante, ce qui est résolu en clonant les amplifiats de la PCR avant le séquençage, ou l'information contenue sur les bases de données n'est pas suffisante pour aboutir à une identification sans ambiguïté. Ainsi, nous proposons la/les paire(s) d'amorce(s) pour chaque espèce en fonction de ces facteurs limitants.

Conclusion: Les performances des amorces dépendent de l'espèce étudiée. Les bases de données contiennent davantage de séquences ITS que de séquences beta-tubuline. Néanmoins, les performances techniques des amorces beta-tubuline sont supérieures.

Signification et impact de l'étude: Cet article fournit une base méthodologique pour la caractérisation de la flore fongique des raisins par des méthodes moléculaires.

Mots clés: flore fongique, raisin, identification moléculaire

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INTRODUCTION

The fungal flora of grapes is susceptible to cause organoleptic deviations and to decrease the yield. Among these deviations, earthy-muddy aromas (mainly due to geosmin) are caused by the concomitant development of *Botrytis cinerea* and several species of *Penicillium* (La Guerche *et al*., 2005). The olfactory threshold of geosmin in wine is around 50 ng/L, thus, a few contaminated bunches are able to spoil the harvest. Fresh mushroom aroma can also be detected in grapes and wines; it is mainly due to 1-octen-3-one and 1-nonen-3-one, with an olfactory threshold around 20-50 ng/L in wine. Their biosynthesis is not yet elucidated. The occurrence of this problem has been high for some specific vintages in Luxembourg, with wines contaminated by geosmin or 1-octen-3-one. Grey rot, caused by *Botrytis cinerea*, is often the limiting factor for the maturation of grapes in Luxembourg. In order to characterise the diversity of fungi, molecular techniques have been extensively used on both grapes and other cultures (Dachoupakan *et al*., 2009; Martinez *et al*., 2008; Vega *et al*., 2006). Indeed, nowadays, the identification and phylogeny of fungi are assessed by molecular techniques and are no more exclusively based on phenotypical traits. Such a characterisation allows to easily distinguish several strains of a same species.

Grape fungal contamination with features of grey and/or green rots have been isolated and studied for 5 years in different locations in order to describe the fungal flora that is present and to get a better knowledge of the predisposition of grapes to produce wines with off-flavors. The paper describes two genomic DNA regions that are of particular interest for the identification of filamentous fungi: the Internal Transcribed Spacer (ITS) of the rDNA and the beta-tubulin (Glass and Donaldson, 1995). They are of interest because they combine the two mandatory conditions for species determination by PCR and sequencing: a highly conserved domain susceptible to anneal with the designed primers and a variable region to discriminate between the species or the strains. The primer pair ITS1-ITS4, designed by Glass and Donaldson (1995), amplifies the region between the 18S and the 26S rDNA units. Thus, this region includes ITS1, the 5.8S rDNA unit, ITS2, and part of the 18S and 26S rDNA unit. In order to minimize the problem of tandem-repeats, La Guerche *et al*. (2004) have designed a set of primers (ITS U5-R2) that targets a smaller sequence of the ITS region: ITS U5 anneals to the 18S rDNA unit, while ITS R2 anneals to the 5.8S rDNA unit, amplifying only the ITS1 and part of the 18S and 5.8S rDNA unit. Due to the potential problem of mutations within the multiple ITS copies, identification and phylogeny of fungi are nowadays preferentially performed with full or partial amplification of the beta-tubulin gene. As this gene is protein-coding, fewer mutations are expected (Einax and Voigt, 2003). In the present work, the sets of primers targeting the beta-tubulin gene in two distinct regions, designed by Glass and Donaldson (1995), are used. Overall, the four sets of primers used in this study have the advantage to be widely used for the identification of filamentous fungi. Thus, they have proved their interest for general, rapid identification, which is the aim of this paper. Each primer set was assessed on each strain and its suitability to identify and discriminate the strains was compared. This is the first report on the molecular characterisation of grey- and green rot-related species in Luxembourg.

MATERIALS AND METHODS

1. Isolation

Isolations were made on mature berries showing grey or green rot during the vintages 2007 to 2011 along the Luxembourgish and some French and German parts of the Moselle river (Table 1).

The mycelium was isolated from the grapes under sterile conditions and cultivated on Malt Extract Agar (Oxoid, Basingstoke, UK) for 48 hours at 25 °C. For further purification, a single colony was isolated and cultivated in the same conditions for 2 weeks. This way, 50 pure isolates of green mould and 19 pure isolates of grey mould were obtained.

2. DNA extraction

A sub-culture of each isolate was grown on Potato Dextrose Broth (Sigma-Aldrich, St Louis, MO) for one week on a rotary shaker at 120 rpm at room temperature. The biomass was filtered through Miracloth (Merck, Whitehouse Station, NJ) and recovered in a 2-mL Eppendorf with three metallic beads prior to lyophilisation for 48 hours and subsequent grinding. DNA from the lyophilised material was extracted using the DNeasy Plant Mini Kit (Qiagen), following the instructions of the manufacturer. DNA concentration was measured using the NanoDrop system (ND 1000, Thermo Scientific, Waltham, MA). The samples were stored at -20 °C.

3. PCR amplification

Primers specific to filamentous ascomycetes were used (Glass and Donaldson, 1995). To target the beta-tubulin gene, β-tubulin 1 (BT1) and β-tubulin 2 (BT2) primers were used, whereas to target the ITS region, ITS1-ITS4 and ITSU5-ITSR2 primers were used. The primers were purchased from Eurogentec (Liège,

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Table 1 - Identification of all strains using the primer pairs BT1, BT2, ITS1-4 and ITS U5-R2. [c] refers to cloned sequences; (L) and (F) refers to Luxembourgish and French sampling location, respectively. Fragment sizes (in bp) are indicated for the most relevant species.

Isolate ID	Town of isolation	Identification with primer set BT1	Identification with primer set BT2	Identification with primer set ITS1-4	Identification with primer set ITS U5-R2	Final identity
B9	Machtum (L)	*B. cinerea* (538 bp)	*B. cinerea* (467 bp)	*B. cinerea* (576 bp)	*B. cinerea* (252 bp)	*B. cinerea*
B10	Machtum (L)	*B. cinerea*	*B. cinerea*	*B. cinerea*	*B. cinerea*	*B. cinerea*
B11	Machtum (L)	*B. cinerea*	*B. cinerea*	*B. cinerea*	*B. cinerea*	*B. cinerea*
B15	Remich (L)	*B. cinerea*	*B. cinerea*	*B. cinerea*	*B. cinerea*	*B. cinerea*
B18	Ahn (L)	*B. cinerea*	*B. cinerea*	*B. cinerea*	*B. cinerea*	*B. cinerea*
B19	Ahn (L)	*B. cinerea*		*B. cinerea*	*B. cinerea*	*B. cinerea*
B21	Ahn (L)	*B. cinerea*	*B. cinerea*	*B. cinerea*	*B. cinerea*	*B. cinerea*
B22	Remich (L)	*Ellisomyces anomalus*		*Mucor fragilis* (742 bp)	*Mucor fragilis* [c] (314 bp)	*Mucor fragilis*
B23	Remich (L)	*Ellisomyces anomalus*		*Mucor fragilis*	*Mucor fragilis* [c]	*Mucor fragilis*
B24	Remich (L)	*B. cinerea* [c]	*B. cinerea*	*B. cinerea*	*B. cinerea*	*B. cinerea*
B25	Remich (L)			*Mucor fragilis*	*Mucor fragilis*	*Mucor fragilis*
B26	Wasserbillig (L)	*B. cinerea* [c]	*B. cinerea* [c]	*B. cinerea*	*B. cinerea*	*B. cinerea*
B27	Stadtbredimus (L)	*B. cinerea* [c]	*B. cinerea*	*B. cinerea*	*B. cinerea*	*B. cinerea*
B28	Niederdonven (L)	*B. cinerea* [c]	*B. cinerea* [c]	*B. cinerea*	*B. cinerea*	*B. cinerea*
B29	Grevenmacher (L)	*B. cinerea* [c]	*B. cinerea* [c]	*B. cinerea*	*B. cinerea*	*B. cinerea*
B30	Perl (G)	*B. cinerea* [c]	*B. cinerea* [c]	*B. cinerea*	*B. cinerea*	*B. cinerea*
B31	Bous (L)	*B. cinerea* [c]	*B. cinerea* [c]	*B. cinerea*	*B. cinerea*	*B. cinerea*
B33	Stadtbredimus (L)	*Chaetomium globosum* (512 bp)	*Chaetomium coarctatum* [c]		*Chaetomium globosum* (276 bp)	*Chaetomium globosum*
P1	Machtum (L)	*P. expansum* (484 bp)	*P. expansum* (477 bp)	*P. expansum* (716 bp)	*P. expansum* (280 bp)	*P. expansum*
P2	Remich (L)	*P. marneffei*	*P. minioluteum*	*P. minioluteum*	*P. minioluteum*	*P. minioluteum*
P3	Wellenstein (L)	*P. marneffei*	*P. minioluteum*	*P. minioluteum*	*P. minioluteum*	*P. minioluteum*
P4	Machtum (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P5	Mertert (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P6	Schwebsange (L)		*P. minioluteum*	*P. minioluteum*	*P. minioluteum*	*P. minioluteum*
P7	Remich (L)	*P. marneffei*	*P. minioluteum*	*P. minioluteum*	*P. minioluteum*	*P. minioluteum*
P8	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P9	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P10	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum* [c]	*P. expansum*
P11	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P12	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*

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Table 1 (suite) - Identification of all strains using the primer pairs BT1, BT2, ITS1-4 and ITS U5-R2. [c] refers to cloned sequences; (L) and (F) refers to Luxembourgish and French sampling location, respectively. Fragment sizes (in bp) are indicated for the most relevant species.

Isolate ID	Town of isolation	Identification with primer set BT1	Identification with primer set BT2	Identification with primer set ITS1-4	Identification with primer set ITS U5-R2	Final identity
P14	Remich (L)		*Cladosporium* sp.	*C. cladosporioides*	*C. cladosporioides*	*C. cladosporioides*
P16	Remich (L)		*Cladosporium* sp.	*C. cladosporioides*	*C. cladosporioides*	*C. cladosporioides*
P20	Schwebsange (L)			*C. cladosporioides*	*C. cladosporioides*	*C. cladosporioides*
P21	Remerschen (L)	*P. expansum*	*P. expansum* [c]	*P. expansum*	*P. expansum*	*P. expansum*
P22	Remerschen (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P23	Bous (L)	*P. expansum*	*P. expansum* [c]	*P. expansum*	*P. expansum*	*P. expansum*
P26	Erpeldange (L)	*P. expansum* [c]	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P27	Contz-les-Bains (F)		*Davidiella tassiana*	*Davidiella tassiana*	*Davidiella tassiana*	*Davidiella tassiana*
P28	Ahn (L)		*Cladosporium* sp.	*C. cladosporioides*	*C. cladosporioides*	*C. cladosporioides*
P29	Remich (L)			*C. cladosporioides*	*C. cladosporioides*	*C. cladosporioides*
P30	Contz-les-Bains (F)		*Davidiella tassiana*	*Davidiella tassiana*	*Davidiella tassiana*	*Davidiella tassiana*
P31	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P32	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P33	Remich (L)		*C. oxysporum* [c]	*C. cladosporioides*	*C. cladosporioides*	*C. cladosporioides*
P34	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P35	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P36	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P37	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P38	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P39	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P40	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P41	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P42	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P43	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P44	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P45	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P46	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P47	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P48	Remich (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P49	Grevenmacher (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P50	Wellenstein (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P51	Contz-les-Bains (F)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P52	Bous (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P53	Wormeldange (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P54	Schwebsange (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P55	Schwebsange (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P56	Wormeldange (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*
P57	Bous (L)	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*	*P. expansum*

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Belgium). The PCR reaction mixture contained 10 µL of Finnzymes Taq Phusion – buffer Mastermix HF (Thermo Scientific, Waltham, MA), 1 µL of each primer (10 µM), 1 µL of DNA extract (5-100 ng/µL) and 7 µL of ultrapure water for a final volume of 20 µL. Amplification was performed on a Biometra T-professional thermocycler (Biometra, Goettingen, Germany) as follows: initial denaturation at 98 °C (2 min); 30 cycles with denaturation at 98 °C (15 sec), annealing at 67 °C (β-tubulin), 63 °C (ITSU5-ITSR2) or 64 °C (ITS1-ITS4) (20 sec), and elongation at 72 °C for 20 sec (β-tubulin, ITS1-ITS4) or 10 sec (ITSU5-ITSR2); and final elongation at 72 °C (10 min). The size, quality and approximate quantity of the amplicons were checked on a 3 % (w/v) agarose gel stained with ethidium bromide (1 hour; 100 V) under UV transillumination.

4. Cloning of PCR products

Some products which did not display a sufficient quality after direct sequencing of the PCR products were cloned using Zero Blunt TOPO PCR Cloning Kit for sequencing (Invitrogen, Paisley, UK) and transformed into OneShot Top10 chemically competent cells (Invitrogen, Paisley, UK) following the manufacturer's instructions. Five colonies per PCR product were sequenced using the M13 primers. The strains that required cloning are labelled « c » in Table 1.

5. DNA sequencing and sequence analysis

PCR products were diluted to a concentration of 10-20 ng/µL. Sequencing of the PCR products was achieved using Big Dye products and following the manufacturer's instructions (Applied Biosystems, Carlsbad, CA). Both strands of each amplicon were sequenced. The PCR products were cleaned using the BigDye Xterminator Purification kit (Applied Biosystems, Carlsbad, CA). Sequencing was done on the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA).

Sequences were aligned using CLC Main Workbench 6 (CLC bio, Aarhus, Denmark). The parameters of the alignments were the following: gap open cost of 10, gap extension cost of 1 and end gap cost as any other. The consensus sequence of each strain was compared to nucleotide collections using BLASTn from NCBI set on MegaBLAST (http://blast.ncbi.nlm.nih.gov). An alignment was performed on CLC Main Workbench 6 (CLC bio, Aarhus, Denmark) with the sequenced strain and the referenced strain that had provided the best result in order to confirm the identification. An additional database was used to tentatively identify the *Penicillium* isolates that did not show good results through BLAST (http://www.cbs.knaw.nl/Penicillium/BioloMICSSequences.aspx).

RESULTS AND DISCUSSION

1. Identification of isolates

1.1. *Botrytis cinerea*-related species

Amplification with the BT1 primer set allowed to identify 14 strains (Table 1) of grey mold as *Botryotinia fuckeliana*, better known as and hereafter referred to its anamorph *Botrytis cinerea*, isolated in 2007, 2008 and 2009 in different locations. Two strains (B22 and B23) were tentatively identified as *Ellisomyces anomalus* (Zygomycota) but with only 93 % to 95 % of identity on the covered region and one strain (B33) was identified as *Chaetomium globosum* with 98 % of identity. The differences in homology between strains B22 and B23 (identified as *E. anomalus* by BT1 but hereafter identified as *Mucor fragilis* by ITS1-4 with 100 % sequence identity) and *Chaetomium globosum* as compared to *B. cinerea* are highlighted on a small sequence segment in Figure 1.

The identification of *B. cinerea*-related species by beta-tubulin 2 resulted in two groups. Thirteen isolates were identified as *B. cinerea*. Concerning the non-*B. cinerea* strains, B33 was ambiguously identified as a *Chaetomium*, confirming the result of the beta-tubulin 1 amplicon, or as *Chaetomium coarctatum*, with a percentage of identity in both cases of 93 % of the covered region. The absence of amplification for B22, B23 and B25 may be explained by their different phylum: they belong to the Zygomycota, while all the other strains are Ascomycota. The primers were not designed on this phylum (Glass and Donaldson, 1995).

The ITS1-4 sequences of the grey mould-related species were mainly identified as *B. cinerea*. Three strains (B22, B23 and B25) corresponded to *Mucor fragilis* (100 % of identity with a coverage of 92 %). The ITS1-ITS4 region is less conserved than the beta-tubulin one. Unlike the beta-tubulin primer sets, the ITS1-ITS4 primer set was not able to produce sequences of sufficient quality for *Chaetomium* spp.

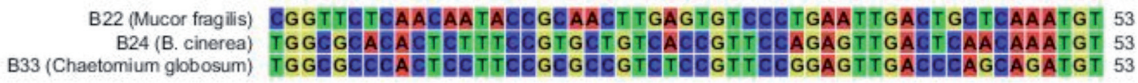


Figure 1 - Beta-tubulin 1 comparative fragment of an alignment of grey mould species.

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(B33), making its identification impossible. Nevertheless, despite the generally poor result of ITS1-based sequence, the ITS1-4 primer set was able to identify most of the species we have encountered in grey mould, due to the great diversity of ITS which has been previously described in fungi (Schubert *et al*., 2007; La Guerche *et al*., 2004; Skouboe *et al*., 1999; James *et al*., 2006).

The sequencing of the ITS U5-R2 amplification products for the *B. cinerea*-related species revealed one group of 14 strains, identified as *B. cinerea* (100 % of identity on the totality of the sequence). B22, B23 and B25 were identified as *Mucor fragilis*. *Mucor fragilis* was well differentiated from *B. cinerea* by three major insertions of 16, 48 and 9 bp. One isolate, B33, was identified as *Chaetomium globosum*.

1.2. *Penicillium* spp.

The beta-tubulin 1 amplicons of the *Penicillium* spp. strains were mainly assigned to *Penicillium expansum* (38 isolates) and another species which did not score high enough to allow its identification using BLAST. Thus, these sequences were analysed with the CBS *Penicillium* database, which tentatively identified them as *P. marneffei* with a homology of 89.5 %.

The sequences obtained with the beta-tubulin 2 primers were shown to belong to *P. expansum* isolates (38 strains). P2, P3, P6 and P7 showed a homology of 95 % (reference region: 332 nucleotides of the 466 nucleotides of the isolates) with *P. pinophilum*. However, comparison in the CBS database led to a better identification, with a homology of 98.6 % with *P. minioluteum*. In the present study, we can support that the sequencing of the beta-tubulin 2 region exhibited the most reliable information for the characterisation of *Penicillium* spp.: all the strains were successfully sequenced.

Thirty-eight sequences of the ITS1-4 region were identified as *P. expansum*. P2, P3, P6 and P7 were identified as *P. minioluteum*. A few insertions or deletions are observed between the *P. expansum* and *P. minioluteum* strains, as shown on a representative segment of the sequence (Figure 2). As compared to the beta-tubulin sequence, a lower polymorphism is present within the ITS1-ITS4 region. The amplicons of the ITS U5-R2 region mainly blasted to *P. expansum* isolates (38 strains). P2, P3, P6 and P7 were shown to be *P. minioluteum*. We observed insertions of 2, 3 and 5 bp and two deletions of 1 bp in *P. minioluteum*. This set of primer targets a region that is very highly conserved within species, enabling an easy identification. Thus, the sequencing of the ITS1 region can be recommended for the identification of *Penicillium* species. The ITS U5-R2 primer set provided stronger information than the ITS1-ITS4 set, due to sequences of better quality. Similar observations were done by La Guerche *et al*. (2004) on the ITS1 region, displaying a vast majority of *P. expansum* with strictly identical sequences.

1.3. *Cladosporium* spp.

Beta-tubulin 1 primers yielded exploitable sequences for 7 isolates, but BLAST searches were unsuccessful. These amplicons were divided in one group of 5 isolates (P14, P16, P20, P28 and P29) and 2 distinct sequences (P27 and P30). The separated strains presented a 95-96 % identity with the main group. They were characterised by several mutations, no deletion was observed. We may hypothesize that these 7 strains belong to closely related species.

The beta-tubulin 2 sequences of the *Cladosporium* genus did not exhibit a clear tendency. P14 and P28 were homolog to *Cladosporium oxysporum* (93 %, 372 positives on 400 nucleotides). P27 and P30 presented an amplicon of 412 bp, presumably identified as *Davidiella tassiana* (99 % of identity on 408 bp). The referent fragment for this BLAST was the beta-tubulin gene of *Davidiella tassiana*, which is considered as the teleomorph of *Cladosporium herbarum* by Zalar *et al*. (2007). P33 was quite well characterised, with a maximum homology of 99 % (coverage of 93 %) against *Cladosporium oxysporum*. The lowest homology was obtained for P16 against an unidentified *Cladosporium* species (90 % on 338 nucleotides). As a result, all the isolates could be clustered in three groups: *Cladosporium oxysporum* (P14, P28 and P33), *Cladosporium herbarum* / *Davidiella tassiana* (P27 and P30), and an unidentifiable *Cladosporium* species (P16). Thus, it may be concluded that we are in the presence of three different species of *Davidiella* / *Cladosporium*. Interestingly, *Davidiella* strains were isolated in Contz-les-Bains (Moselle, France), whereas the *Cladosporium* strains were isolated in Remich (Luxembourg), these two towns being distant from 10 kilometres. Such a variability within *Davidiella* / *Cladosporium* species has been described, using partial gene sequences of actin, calmodulin, EF1-alpha, histone H3 and ITS (Schubert *et al*., 2007), ITS, beta-tubulin and translation elongation factor (Briceño and Latorre, 2008), and ITS (Braun *et al*., 2003). Schubert *et al*. (2007) explained it by a low natural selection, resulting in a low level of recombination (Figure 3). Thus, many genotypes, differing by a few mutations, can cohabitate in the same location. All the beta-tubulin 2 sequences differed to some extent, indicating a strong

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polymorphism, which may cause problems to obtain clean sequences.

All the sequences related to the ITS1-4 amplicons were identified as *Cladosporium* or *Davidiella*. Six strains (P14, P16, P20, P28, P29 and P33) matched to *Cladosporium cladosporioides*. P27 and P30 were not clearly identified by BLAST; they could be considered both as *Cladosporium* or *Davidiella tassiana*. These two strains were identified by beta-tubulin 2 as *Davidiella tassiana*. Moreover, these strains shared only 96-97 % of identity with the main group, tiping the scales to the *Davidiella tassiana* side, and the ITS sequence corresponding to *Davidiella tassiana* has been published (Bukovska *et al*., 2010), contrarily to the *Cladosporium cladosporioides* one. ITS1-ITS4 succeeded in providing exploitable results on the totality of the *Davidiella* / *Cladosporium* strains, while beta-tubulin failed for three of them. However, some discrimination issues were encountered.

Finally, the ITS U5-R2 sequences were separated in two groups. The first one (P14, P16, P20, P28, P29 and P33) was identified as *Cladosporium cladosporioides*. The second group (P27 and P30) was tentatively identified as *Davidiella tassiana*. It showed 95 % of identity with the previous one and was tentatively identified as *Davidiella* / *Cladosporium*. It was not possible to determine the species, since several *Cladosporium* species, *Davidiella dianthi* or *Sphaerulina polyspora* have the same ITS sequence.

2. Merits and demerits of the different primers for the identification

BT1 primers allowed to successfully amplify all the strains isolated. Despite the quality of the amplification, some strains failed to be directly sequenced, mainly belonging to *B. cinerea*-related species (8/19 failed). Cloning was required for these strains to produce satisfactory sequences. These primers were however directly efficient for the sequencing of *P. expansum*, *Davidiella* / *Cladosporium* spp. and some strains of *B. cinerea*-related species.

BT2 primers were able to amplify most of the strains isolated, with however a few weaknesses: *Mucor fragilis* and one strain of *Davidiella* / *Cladosporium* spp. failed to produce an exploitable amplicon. This set of primer was of particular interest for *Penicillium* characterisation: we could observe a good polymorphism, useful for species discrimination. Nevertheless, databases for beta-tubulin sequences have to be enlarged. This set of primer was also efficient for the identification of *D. tassiana*.

Among all the tested primer pairs, ITS1-ITS4 has given the poorest satisfaction from a technical point of view. Satisfactory sequences in both forward and reverse direction could only be produced with *Davidiella* / *Cladosporium* strains, but this region did not always show a sufficient power of discrimination (*D. tassiana*). ITS4 was sufficient in itself to allow the identification of most of the isolates. A majority of *B. cinerea*- and *Penicillium*-related species failed to exhibit an exploitable ITS1-based sequence. This may be due to the nature itself of the targeted region: this gene is subjected to multiple tandem repeats, thus, the primer could anneal on several locations and produce distinct amplicons. Cloning was nevertheless a good option to produce exploitable sequences in both forward and reverse direction; however, the identification could be done with a simpler protocol by using the other sets of primers. The available database for ITS sequences of *Penicillium* species is more complete than the one for beta-tubulin (Samson *et al*., 2004), which allows a more powerful identification. Given the higher power of discrimination of the beta-tubulin region compared to the ITS region, it could be interesting to further sequence the beta-tubulin region of a high number of *Penicillium* species in order to ensure a better availability in genebanks.

ITS U5-R2 primers were designed in the ITS1 region by La Guerche *et al*. (2004) with the objective to identify several isolates picked on grape berries presenting an earthy-muddy aroma. The authors have selected it because of the relatively high proportion of variation between *Penicillium* species. By decreasing the size of the amplicon as compared to the ITS1-ITS4 set, a better result is expected: ITS1 is polymorphic enough to allow identification and the sequencing is partly freed of multiple tandem repeats, producing high-quality sequence. The U5 primer anneals to the 18S rDNA unit, a few nucleotides before the ITS1 primer; R2 anneals to the 5.8S rDNA unit. Thus, the ITS1 primer is recovered during the U5-R2 sequencing. This set of primer was the only one that succeeded in the identification of all the grey



Figure 2 - ITS 1-4 comparative fragment of an alignment of Penicillium spp.

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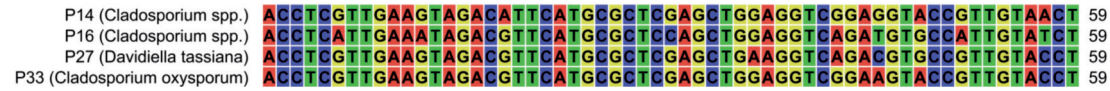


Figure 3 - Beta-tubulin 2 comparative fragment of an alignment of *Davidiella* / *Cladosporium* spp. strains.

rot-related species (*B. cinerea*, *Mucor fragilis* and *Chaetomium globosum*). The sequencing of the ITS1 region can be recommended for the identification of *Penicillium* species. It provides stronger information than the ITS1-ITS4 primer set, due to sequences of better quality that decrease the time of analysis and interpretation of the raw data. Characterisation of *Cladosporium* / *Davidiella* strains is not recommended with this set of primer because of the low polymorphism level of this region as compared to other species.

CONCLUSION

The strains isolated on grey mould were identified as *B. cinerea*, *Mucor fragilis* and *Chaetomium globosum*; on green mould, *Penicillium expansum*, *Penicillium minioluteum*, *Cladosporium cladosporioides* or *Cladosporium herbarum* (*Davidiella tassiana*) were identified. BT1 and BT2 primers were generally adapted to all these species with only few exceptions. ITS1-4 may not be recommended, as the technical quality of the amplification and sequencing results was not optimal. ITS U5-R2 can be recommended for the identification of *Penicillium* and grey rot-related species. This work provides a basic methodology for the molecular characterisation of the fungal flora encountered on grapes and can be used as a complementary technique to morphological identifications. Furthermore, large sets of populations from diverse growing area can be compared and used for phylogenic studies.

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