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RESEARCH PAPER

Microsatellite markers for population studies of the ascomycete *Phyllosticta ampelicida*, the pathogen causing grape black rot

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Summary. Grape black rot, caused by the homothallic ascomycete *Phyllosticta ampelicida*, is a disease originating from North America and is widespread in Europe. To investigate population structure and epidemics of this pathogen, we developed 11 microsatellite markers. A multiplex PCR assay was used to amplify genomic DNA from environmental samples including mummified berries and foliar lesions, and from fungal cultures. Environmental samples were collected from five countries (Switzerland, France, Germany, Luxembourg and the USA), and consisted of 64 different genotypes. Five additional genotypes were identified from pure cultures isolated in Switzerland and Germany. The allele rarefaction approach indicated that French vineyards in the region of Bordeaux displayed the greatest mean allelic richness, probably related to the fact that France is the country where the disease was first reported outside North America, in 1885. Our results also suggest the absence of links between the species/cultivar of *Vitis* hosts and the infecting *P. ampelicida* genotypes. This is the first report of development of microsatellite markers and their deployment for population studies of *P. ampelicida*.

Key words: *Guignardia bidwellii*, SSR, population genetics, population structure.

Introduction

Grape black rot is caused by the ascomycete fungus *Phyllosticta ampelicida* (Engelm.) Aa (anamorph name; see Wikee *et al*., 2013; teleomorph: *Guignardia bidwellii*), that originated in North America. The pathogen was introduced into Europe in the early 1800s (Reddick, 1911) and is currently widespread in several European wine growing areas in France (Ramsdell and Milholland, 1988), Germany (Maixner and Holz, 2003), Northern Italy, Southern Switzerland (Pezet and Jermini, 1989) and Luxembourg (Anonymous, 2008), especially where summer precipitation is high. If not properly managed, grape black rot can lead to substantial yield losses and is therefore considered among the most economically destructive diseases in viticulture (Harms *et al*., 2005). Primary hosts of *P. ampelicida* include most varieties of bunch grapes (*Vitis vinifera*, *Vitis labrusca* and *Vitis arizonica*) and muscadine grape (*Vitis rotundifolia*), while secondary hosts include ornamental Vitaceae such as *Ampelopsis* sp., *Cissus* sp. and *Parthenocissus* sp. (van der Aa, 1973). Luttrell (1946) proposed the teleomorph *G. bidwellii* to consist of three distinct pathovars (var. *euvitis*, *parthenocissi* and *muscadinii*), and recent molecular studies confirmed at least the differentiation in two distinct clades of isolates from *Vitis* and from *Parthenocissus* (Wicht *et al*., 2012). Identification of the pathogen, which is usually based only on black rot symptoms and mycelium macromorphology (Kong, 2009), is therefore not suitable to reveal this differentiation, which should be confirmed by molecular analyses.

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The life cycle of *P. ampelicida* has been described by several authors (e.g. Wilcox, 2003), but not all aspects of the disease have been elucidated (Ullrich *et al.*, 2009). The fungus overwinters in mummified berries within host plants and on the ground, but also in canes or spurs. Spring rains trigger the release of ascospores from mummified berries, and conidia from these and canes within the vine trellises. The contributions of sexual and asexual spores in the initiation and spread of black rot have not been clearly quantified. Moreover, the homothallic reproductive nature of the fungus does not allow distinguishing conidial infection from ascosporic infection by ascospores resulting from self-fertilization (Jailloux, 1992). Both spore types are able to infect green tissues, especially young leaves. Later in the growing season, they can infect berries and mummify them. Despite the economic importance of grape black rot, very few studies have been carried out to investigate the genetic structure of *P. ampelicida* populations. Such information may become crucial in estimating the evolutionary potential of the pathogen while developing new black rot control strategies.

Microsatellite markers are commonly used for population studies of grape pathogens (Gobbin *et al.*, 2005, 2006; Matasci *et al.*, 2010; Frenkel *et al.*, 2012), as well as for many other organisms. This is because the microsatellites have high length polymorphism, and the possibility of automated high-throughput genotyping using a capillary sequencer can be used for their indentification. However, their development involves laborious processes of isolation, starting from purified genomic material of the targeted species (Zane *et al.*, 2002). Such an approach is currently relieved by cheaper next-generation sequencing (Zalapa *et al.*, 2012).

The aim of the present study was to develop simple sequence repeat (SSR) markers suitable for genotyping *P. ampelicida* isolates, to investigate the genetic variability of pathogen populations from different European wine growing areas, by comparing their polymorphism.

Materials and methods

Fungal isolates and environmental samples

A subset of monohyphal cultures of *P. ampelicida* used by Wicht *et al.* (2012), provided by the fungal public collections of Mycoscope (Agroscope, Switzerland) and the DLR Rheinpfalz (Germany), were grown on PDA at 20°C. The isolates are listed in Table 1. DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Lyophilized mycelium of isolate 559 was used, as no living culture of this isolate was available.

Environmental samples (n = 1354) consisted of berries (n = 662) and leaves (n = 692) affected by grape black rot, collected from 17 European vineyards located in Switzerland, France, Germany and

Table 1. Cultures of *Phyllosticta* analyzed in this study. CH, Switzerland; D, Germany. *Lyophilized mycelium.

ID	Species	Year	Country	Vineyard	Host
9390	*P. ampelicida*	2010	D	Geilweilerhof	*V. vinifera*
9449	*P. ampelicida*	2010	D	Diedesfeld	*V. vinifera*
9450	*P. ampelicida*	2010	D	Deidesheim	*V. vinifera*
9494	*P. ampelicida*	2010	D	Mühlheim-Andel	*V. vinifera*
9495	*P. ampelicida*	2010	D	–	*Vitis* sp.
170	*P. ampelicida*	1996	CH	St. Prex	*Vitis* sp.
1003	*P. ampelicida*	2003	CH	–	*Vitis* sp.
559*	*P. ampelicida*	1981	CH	Belmont	*V. labrusca*
264	*Phyllosticta* sp.	1996	CH	–	*Parthenocissus* sp.
1002	*Phyllosticta* sp.	2003	CH	–	*Parthenocissus* sp.

Table 2. Environmental samples investigated in this study. CH: Switzerland; F: France; D: Germany; LUX: Luxembourg and USA: United States of America.

Year	Country	Vineyard	Plants (n)	Collected berries (n)	Collected foliar lesions (n)	Complete SSR profiles (n)	Genotypes (n)	Latitude	Longitude
2007	CH	Cugnasco	24	192	-	149	5	46.17499	8.918889
2010	CH	Castelrotto	16	62	47	18	2	45.98972	8.842783
2010	CH	Astano	16	-	46	0	0	46.012298	8.817383
2010	CH	Sessa	48	-	51	0	0	46.001225	8.816993
2010	CH	Rivera	55	-	55	0	0	46.09999	8.933333
2010	CH	Verscio	3	26	38	4	2	46.18333	8.733331
2010	CH	Minusio	1	4	-	1	1	46.18333	8.816672
2010	CH	Grancia	1	6	15	2	1	45.97027	8.927778
2011	CH	Rudolfingen	13	7	23	7	1	47.64275	8.67254
2011	CH	Trüllikon	-	7 (from soil)	-	2	1	47.63868	8.69103
2007	F	Salleboeuf	14	116	-	104	33	44.84333	-0.39707
2007	F	Blanquefort	10	80	-	68	19	44.91547	-0.64033
2010	F	Blanquefort	24	24	123	10	4	44.91547	-0.64033
2010	F	Colmar	15	46	51	17	1	48.08588	7.358379
2010	D	Geisenheim	16	48	80	18	2	49.98294	7.954738
2010	D	Wolf	24	23	96	9	3	49.97237	7.089218
2010	LUX	Grevenmacher	24	21	56	3	3	49.68507	6.43558
2010	USA	Geneva (NY)	1	-	11	2	2	42.86822	-77.04586

Luxembourg, and from one single plant located in a North American vineyard (Table 2). DNA was extracted from infected berries following the CTAB method (Aldrich and Cullis, 1993) and from leaves following a "Quick and Dirty" procedure with the extraction and dilution buffers of the Extract-N-Amp TM Plant PCR Kit (Fluka; see Frey *et al.*, 2004).

Construction of microsatellite library and primer design

Microsatellite markers were developed according to the protocol described by Brunner and Frey (2004), with the following modifications: genomic DNA was extracted from *P. ampelicida* cultures 170 and 1003 (reference cultures provided by Mycoscope Agroscope, see Table 1) following the CTAB procedure (Aldrich and Cullis, 1993) and then digested with restriction enzyme *Rsa*I (New England Biolab). Super SNX24 linkers (see Glenn and Schable, 2005) were ligated onto DNA fragments using T4 DNA ligase (Fermentas). Polymerase chain reaction (PCR) using one strand of the linker as primer was performed using Dream Taq PCR Mastermix kit (Fermentas). Fragments from 500 to 1000 bp were excised from agarose gels following electrophoresis. Linker-ligated DNA was denatured for 5 min at 95°C and hybridized separately to 0.1 mM 5´ biotin-labeled oligonucleotide probes $(AT)_{10}$, $(AG)_{10}$ and $(AGC)_8$ with hybridization buffer (12 × SSC, 0.2% SDS) by Touch Down PCR in 50 µL total volume. The cycle of amplification was the following: 95°C

for 5 min, 72°C to 50°C (Δ = 0.2°C) for 5 s, 50°C for 10 min, 50°C to 40°C (Δ = 0.5 °C) for 5 s, and conservation at 15°C. Hybridized DNA fragments were captured with streptavidin-coated magnetic beads (Dynabeads M-280; Invitrogen), eluted overnight in 25 µL Tris-Low-EDTA (TLE) and recovered by PCR with the following cycle: 95°C for 2 min, 25 × (95°C for 20 s, 60°C for 20 s, 72°C for 90 s), 72°C for 30 min, conservation at 15°C. Enriched DNA fragments were ligated into plasmid vectors using the CloneJet PCR Cloning Kit, following the "Sticky-End cloning" protocol (Fermentas). Transformation into chemically competent *Escherichia coli* TOP10 cells was carried out using a TOPO TA Cloning Kit, according to the "OneShot TOP10" protocol (Invitrogen). For each probe, 48 to 96 positive colonies were re-plated on LB agar containing 50 µg mL^{-1} ampicillin, then screened by PCR for microsatellite inserts using pJET1.2 primers (Fermentas). Amplicons were visualized by agarose gel electrophoresis. For each probe, fragments of different sizes were purified with NucleoFast 96 PCR plates (Macherey-Nagel) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in an automated ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

Primers were designed from the sequences containing microsatellites with the software Primer3 v. 0.4.0 (Rozen and Skaletsky, 1999), in order to obtain amplicons between 100 and 350 bp with a minimum difference in size of 20 nucleotides, allowing the visualization of clearly distinct peaks in the fragment analysis profiles. The melting temperature was set with an optimum of 60°C to enable a single-step amplification by multiplex PCR. Primer specificity for *P. ampelicida* was checked by BLAST search (Altschul *et al.*, 1990). All sequences virtually binding to non-target DNA, including *Vitis* spp., all *Phyllosticta*/*Guignardia* spp., and other grape pathogens, were not considered.

A specificity test was carried out using the SSR markers on two pure cultures of *Phyllosticta* isolated from *Parthenocissus*, provided by Mycoscope Agroscope (cultures 264 and 1002, see Wicht *et al.*, 2012), because we consider this to be the closest taxon to *P. ampelicida* derived from *Vitis* in terms of genic homology (Wicht *et al.*, 2012). Moreover, nine microsatellite markers developed for *Phyllosticta* cultures 264 and 1002 in the course of a previous study (data not shown) were tested on all *P. ampelicida* monohyphal cultures derived from *Vitis*.

SSR amplification

Microsatellites were amplified by multiplex PCR, using the Multiplex PCR kit (Qiagen) following the "Amplification of microsatellite loci using multiplex PCR" protocol. The cycle was set as follows: 95°C for 15 min, 35 × (94°C for 30 s, 55°C for 90 s, 72°C for 1 min), and 60°C for 30 min. Fragment analysis was performed loading 1 µL PCR product with 8.8 µL formamide and 0.2 µL size standard GeneScan 500 LIZ (Applied Biosystems) in an automated sequencer (ABI PRISM 3130xl; Applied Biosystems). Allelic profiles were analyzed and corrected using the software GeneMapper v. 3.2 (Applied Biosystems).

Data analyses

In a first step, only isolates were considered for which the allele content at all microsatellite loci could be determined, and that therefore had complete SSR profiles. This set was first used to count the number of different genotypes present in each population. After genotype correction, haploid genetic diversity was calculated using GENALEX (Peakall and Smouse, 2006) and the clustering of individuals was performed using STRUCTURE v. 2.3.3 (Pritchard *et al.*, 2000), using the no admixture model, with Allele frequencies correlated, Burning length: 100,000, Number of MCMC Reps after Burning: 10,000, five replicates per run, varying K from two to 14. Allelic richness was calculated using ADZE (Szpiech *et al.*, 2008), considering first only populations with complete SSR profiles (prior genotype correction) and showing population size n > 15. Neighbour-joining genetic trees of individuals were generated using POPULATIONS (Langella, 2002), calculating distances according to Nei *et al.* (1983), with a 500 bootstrap replicas. Samples were grouped in populations according to the region of sampling.

Results

Microsatellite marker identification and polymorphism

Forty-nine colonies were sequenced for inserts, 23 of which contained repeat motifs of appropriate length and suitable for designing specific primers. Sets of 23 primer pairs were designed, 11 of which were polymorphic in the reference cultures, and were therefore selected for the amplification of *P.*

ampelicida microsatellite markers. The sequence of the primer pairs, 5´- modification and allele size are shown in Table 3.

The specificity test carried out on monohyphal cultures of *Phyllosticta* isolated from *Parthenocissus* was negative, i.e. the primers designed for *P. ampelicida* did not amplify any microsatellites in strains 264 and 1002. Negative amplification was neither observed by testing nine SSR markers specifically developed for these *Phyllosticta* strains (data not shown) on *P. ampelicida* isolates derived from *Vitis*.

The 1354 environmental samples collected were screened with the 11 microsatellites in multiplex PCR, yielding 1454 genotypes (including complete and partial profiles), as multiple alleles per SSR locus were observed in 88 samples (two alleles in one locus in 73 cases, two alleles in two loci in 11 cases, two alleles in three loci in two cases, and three alleles in one locus in two cases). Investigating the genotypes with a maximum of two missing values, the most polymorphic marker was GBMS03 (see Table 3), with six alleles. Marker GBMS08 also showed six alleles, but closer examination of their sizes showed the presence of two groups of alleles differing by a single base (320–321 bp and 330–331 bp, respectively). For further analysis, alleles 320 and 321 were therefore considered to be the same (called "321"), as well as alleles 330 and 331 (called "331").

Pathogen populations

Four hundred and fourteen environmental samples (407 of mummified berries and seven of foliar lesions) showed a complete SSR profile, among which

Table 3. Primers amplifying microsatellite markers developed for the characterization of *Phyllosticta ampelicida*.

Locus ID	GenBank Accession Number	Primer sequence (5´–3´)	5´ modification	Repeat motif	Allele range (bp)	Alleles (n)	Mean haploid genetic diversity
GBMS01 -	KF730254	F: CCTTTGAGACCCCTCAACAT R: GCCTTCCTCCATGTGTAACG	FAM	$(GCT)_8$	131-143	2	0.145
GBMS02 -	KF730255	F: GCCAGTAACCAATCGTTCG R: CTGGTTCATGCGTTGGAAG	AT550	$(GA)_9$	173-177	2	0.139
GBMS03 -	KF730256	F: GGCTTCTGCGAATAGCAAAC R: CTTCCTCAATCCTTCCGATG	YYE	$(AG)_{16}$	189-203	6	0.195
GBMS04 -	KF730257	F: GTGGACGAAGACTCCCATGT R: GCAATTTGGCAATAGGTGGT	AT565	$(GA)_{10}$	211-227	3	0.174
GBMS05 -	KF730258	F: CAGCGGAACTGTAGTCGTCA R: TGGATTCGAGATTTGAAGCA	FAM	$(TC)_8$	244-246	2	0.072
GBMS06 -	KF730259	F: GAATGAGCGCATGACGAGTA R: ATTCAACGCACCATCTCCTC	AT550	$(GAC)_{14}$	257-278	4	0.373
GBMS07 -	KF730260	F: AAGCTTTGCAGGGACTTGAA R: TGCTGCTGTCTATCTTGGCTA	YYE	$(CAG)_{10}$	280-298	3	0.283
GBMS08 -	KF730261	F: CTCAATTGCCTGGCTTTCAC R: CCGACTCACCGTCTTTTTGT	AT565	$(GA)_{12}$	321-335	4	0.312
GBMS09 -	KF730262	F: TTGGACCAAGGTTGAAGGAC R: CGTTTCGTTGTAGCGTTCAG	FAM	$(GA)_{10}$	342-360	2	0.056
GBMS10 -	KF730263	F: GGATATCGTTCGGTTTGTGG R: GTCTGCATCTAGGCCAGCTC	AT550	$(GAC)_{10}$	378-390	3	0.243
GBMS11 -	KF730264	F: ACTAAGCCGCATTCTGCAAT R: GGGGAGATTTGGTGTTTTGA	AT565	$(CAT)_9$	387-396	2	0.247

64 distinct genotypes were retrieved. Samples were divided by country, region and year of collection. Samples collected in 2010 in Northern Switzerland were also grouped together (CH-DE-10, collected in Rudolfingen and Trüllikon). All complete genotypes from Northern Switzerland (CH-DE-10, n = 9) represented a single SSR profile. Samples from Southern Switzerland (Canton Ticino) collected in 2010 (Castelrotto-2010, n = 18; Grancia-2010, n = 2; Minusio-2010, n = 1; Verscio-2010, n = 4) were grouped into a population (CH-TI-10, n = 25) consisting of five genotypes. The population from Cugnasco (Southern Switzerland) collected in 2007 (n = 149) consisted of five genotypes, one of which was found 115 times, while the second most frequent genotype was found 27 times. In France, the population collected in Blanquefort in 2007 (F-BLQ-07, n = 68) consisted of 19 genotypes, while the population collected in 2010 (F-BLQ-10, n = 10) was only of eight. The population from Salleboeuf collected in 2007 (n = 104) consisted of 33 genotypes, and that from Colmar (n = 17) was found to be clonal. In Germany, the population from Wolf consisted of three genotypes; two were found only once and a third was found seven times. The population from Geisenheim consisted of two genotypes, one found 17 times, and one only once. The few samples from Luxembourg (Grevenmacher: LUX-GRE, n = 3) and the USA (New York: USA-NY, n = 2) consisted of different genotypes.

Twelve genotypes out of the 64 identified were found in more than a single location, usually only two or three, but a specific genotype (134-175-193-215-246-269-280-321-342-390-390) was observed in seven different populations. Genotype-corrected data (genotype-specific per population) have been used to generate the phylogenetic tree shown in Figure 1. Several clades were identified, but only the one consisting of seven isolates from Canton Ticino showed a correlation with the geographic origin of the samples. However, the remaining genotypes

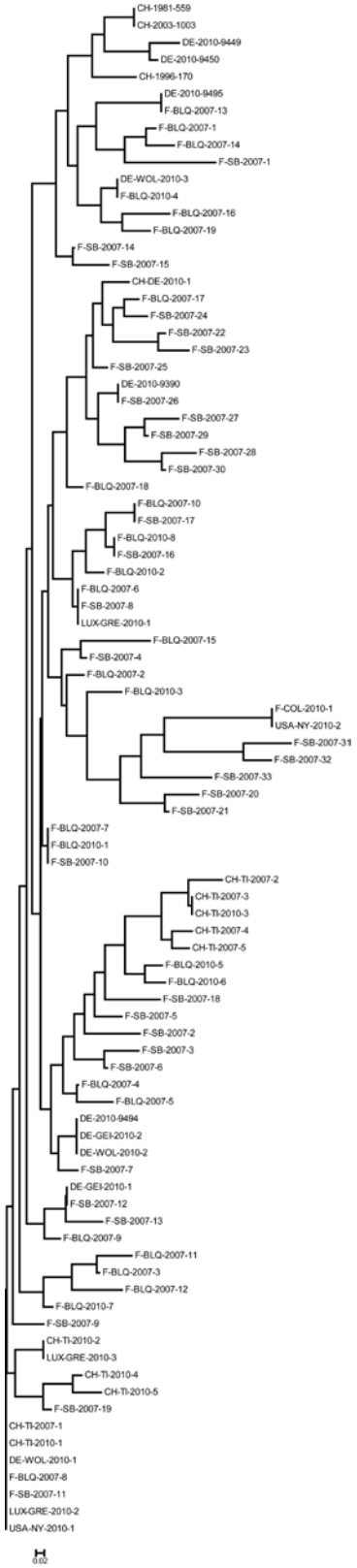

Figure 1. Neighbour-joining genetic tree of *Phyllosticta ampelicida* individuals generated using the POPULATIONS software, calculating distances according to Nei *et al.* (1983), with bootstrap values from 500 repetitions. Samples were grouped in populations according to region of sampling. Environmental samples are coded by country, region, collection year and a number assigned to the genotype. Isolates are coded by country and collection year, the isolate codes corresponding to those in Table 1. Countries: CH, Switzerland; F, France; DE, Germany; LUX, Luxembourg; USA, United States. Regions, TI, Southern Switzerland; DE, Northern Switzerland; BLQ, Blanquefort; COL, Colmar; SB, Salleboeuf; GRE, Grevenmacher; WOL, Wolf; NY, New York.

sampled in this region were spread over several different clusters. The same genotype-corrected population data were used for population clustering, and data resulting from the different numbers of cluster (K) were estimated by the statistic ΔK (Evanno *et al.*, 2005). This indicated that the samples could not be separated into subpopulations. Allelic richness was calculated for a sampling size up to 16 (Figure 2), considering complete SSR profiles from only five populations with n > 15. This indicated that for the two populations in France (2007) the allelic richness increased steadily with increasing sampling size. For the other populations this value increased much less. The population from Colmar could not be analyzed as this showed a single genotype, identical to a genotype identified from the USA. However, it was quite different from any other genotype identified in the other populations investigated.

Complete SSR profiles were retrieved for all monohyphal cultures and the lyophilized mycelium. Isolate 559 was identical to 1003, while isolate 9494 was found to be identical to two genotypes identified among the environmental samples (DE-GEI-10-2, DE-WOLF-10-2). Isolates 9450 and 9449 showed all common alleles, except one (marker GBMS06, alleles 268 and 277 respectively). Isolate 9390 was identical to F-SLB-07-26. In total, five new genotypes were identified from the reference strains of *P. ampelicida* provided by the Mycoscope (Agroscope).

Genotype distribution in vineyards from more than 50 collected berries

In order to study the distribution within a single vineyard, three locations were sampled extensively in 2007, collecting eight berries per bunch for each infected plant.

The vineyard of Cugnasco (Southern Switzerland) was made up of eight rows of 80 plants, and each row consisted of a different grape cultivar (Chambourcin, Gamaret, Bianca, Solaris, Müller-Thurgau, Isabella, Merlot and Regent). One hundred forty-nine berries from 24 different bunches were collected and a total of five genotypes (CH-TI-2007-1 to CH-TI-2007-5) were identified. The distribution of the genotypes (renamed A-E for simplicity) over the whole vineyard is shown in Figure 3. The number of genotypes identified from a single bunch ranged from one to four, with an average of 1.6 ± 0.8 genotypes/bunch. No particular subdivision of the genotypes was linked to the cultivar from which they had been sampled.

From the sampling performed in 2007 in Blanquefort (France), 19 genotypes were observed from 68 complete profiles of mummified berries out of ten bunches, with an average of 4.1 ± 1.5 genotypes/bunch. A similar situation was retrieved in Salleboeuf (France) in the same year: 33 genotypes were identified from 104 complete profiles from mum-

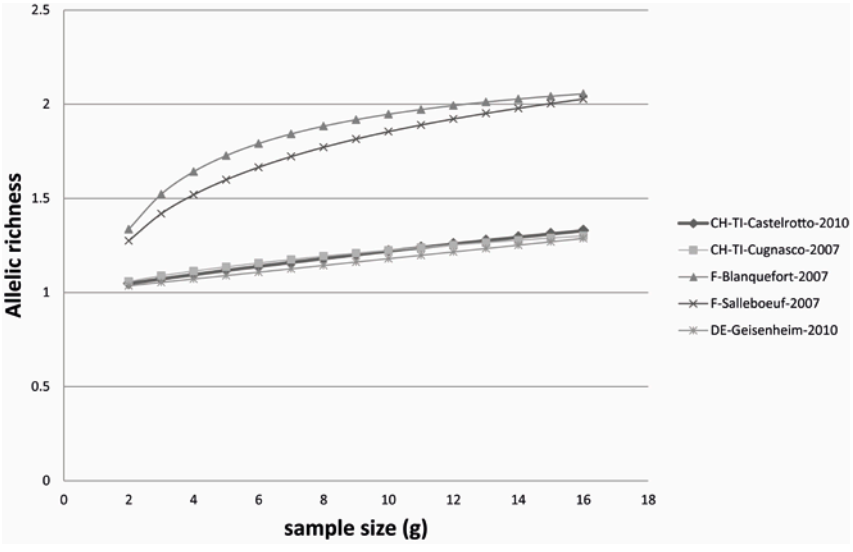

Figure 2. ADZE rarefaction results from 11 SSR loci of *Phyllosticta ampelicida* populations. The rarefied mean allelic richness is shown in relation to increasing sample size (g). Maximum g sample sizes are limited to 16.

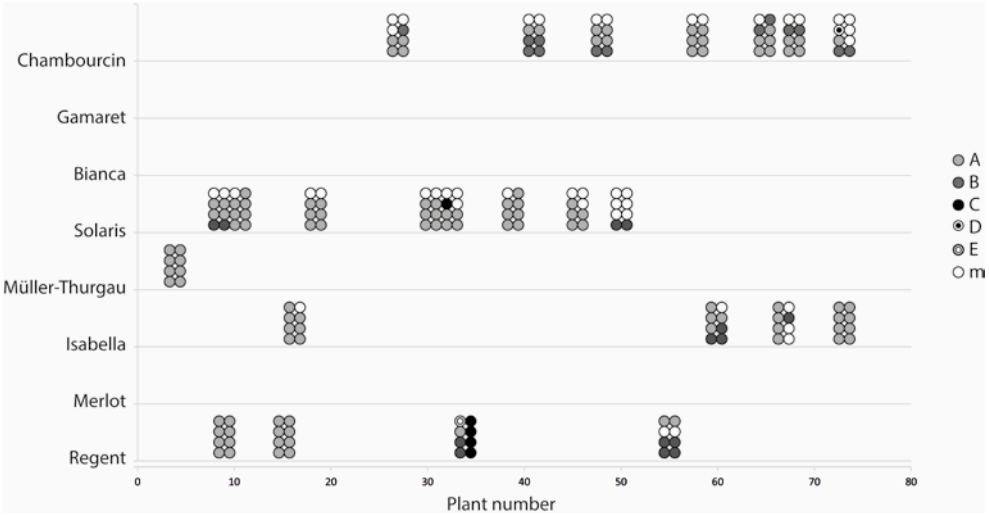

Figure 3. Graphical representation of the *Phyllosticta ampelicida* genotypes identified in the vineyard in Cugnasco (2007); Southern Switzerland; by investigating eight berries from a single bunch per plant showing black rot symptoms. The plot consisted of eight rows of 80 plants each; each row consisting of a single grape cultivar. The groups of eight circles indicate the positions where the bunches were sampled; and each colour or pattern corresponds to a genotype (A–E). Empty circles (m) indicate incomplete genotypes that were excluded from the analyses.

mified berries out of 14 bunches, with two to seven genotypes per bunch (average 4.4 ± 1.5 genotypes/bunch).

Discussion

We present for the first time the development of *P. ampelicida* specific SSR markers. They were used in multiplex PCR to provide knowledge of the biology of the causal agent of grape black rot, by using pure cultures as well as environmental samples from France, Germany, Switzerland, Luxembourg and few samples collected in New York state, USA. The 11 SSR primer pairs were designed in order to amplify regions differing by about 20 bp. This strategy, in combination with the use of different 5´-labelling fluorescent dyes, allowed the amplification of all samples in a single PCR reaction. However, the successful amplification of all loci was dependent on the quality of the template: DNA extracted from pure cultures using the Qiagen kit mostly amplified all loci successfully, while the CTAB-extraction method used for the mummified berries led in some cases to amplification failure of single loci. The "Quick and dirty" method used for the leaves instead led to a low number of complete SSR profiles. This problem can be ascribed to the fact that the DNA was not isolated from leaves but just "eluted", and still contained PCR-inhibiting substances. For two thirds of the samples three or more loci failed to amplify, which can also be ascribed to the large number of SSR combined in a single multiplex amplification, as well as the poor quality of the DNA. A kit-based DNA extraction protocol could reduce the amount of inhibitors, allowing better DNA amplification of all SSR loci in all samples. This tool, combined with the splitting of the 11 SSR markers into two separate multiplex PCRs, may increase the number of complete profiles.

The 1354 collected samples were subjected to multiplex SSR amplification, and 6.5% of these showed "mixed genotypes" (i.e. more than a single allele per SSR locus), indicating the presence of two different genotypes on individual berries. Seven monohyphal cultures and one lyophilized mycelium from public collections revealed that two Swiss isolates, i.e. isolate 1003 collected in 2003 on *Vitis* sp. (unknown location) and isolate 559 collected in 1981 (Belmont) from *V. labrusca*, showed an identical SSR profile. This indicates that this genotype has survived for over 22 years without undergoing any outcrossing, or that these two genotypes are identical in the SSR profile

by chance. Isolate 9494, collected in 2010 in Mulheim (Mosel, Germany) was also found to be identical with isolates collected near Colmar and Geisenheim, at distances of 60 to 200 km from each other. This could mean that a single genotype has spread clonally (asexually or sexually via self-mating) over this region, or that here identical genotypes have arisen by chance. Similar to the mentioned monohyphal cultures, we identified an identical clone from seven different regions over two collection years, even from overseas (the USA).

Due to the very limited number of foliar lesions providing a complete SSR profile (only seven compared to the 407 from mummified berries), it was not possible to draw a clear conclusion on whether isolates identified on berries also occur on leaves, and on which kind of tissue more genotypes occur. In Blanquefort (France, 2010) four genotypes were found from six berries, which were different from the four genotypes retrieved from foliar lesions. We tried to investigate and more generally compare samples showing up to two missing data, and to determine if the number of genotypes present on leaves was greater than that on berries. However, this was not possible due to the limited number of samples, and therefore we focused our research on mummified berries.

SSR data suggest the absence of relationships between the species/cultivar of *Vitis* host plants and the infecting genotype of *P. ampelicida*. In the vineyard of Colmar, only one genotype was found on 14 different cultivars of two *Vitis* species (*V. vinifera*: Chambourcin, Villard blanc, Regent, Merlot, Johanniter, Solaris, Bronner and six experimental varieties; and *Vitis amurensis*), while in Blanquefort and Salleboeuf 68 genotypes were isolated from a single variety (Merlot).

In general, the French populations collected in 2007 showed the greatest genetic diversity, as shown by the mean allelic richness plot (Figure 2), and the number of genotypes identified in these vineyards. France was the first country in which black rot was reported (already occurring in 1885), probably due to introduction of infected host material from the United States. From there, the disease probably spread undergoing both sexual reproduction (either by self-fertilization or by mating with another genotype), as well as overwintering as asexual spores.

The likelihood of this kind of mixed propagation is supported by the fact that identical clones can be found to be spread all over a vineyard (Figure 3), as well as at several hundreds of kilometers of distance (as result of self-fertilization or asexual reproduction), while the large numbers of genotypes found in some vineyards possibly result from sexual recombination of two or three genotypes (as in Blanquefort where all loci showed one to three alleles). As demonstrated for other plant pathogens (see Boehm *et al.*, 2003), it is possible that climatic conditions influence the sexual/asexual form of overwintering, while the proportion of self-fertilization to outcrossing must depend on the availability of different genotypes within a vineyard. These aspects should be further studied in *P. ampelicida*.

More information about other modes of overwintering of *P. ampelicida* could be obtained by investigating the primary lesions emerging in different locations under different climatic conditions, and following the progression of the disease. The number of clones occurring in spring could be estimated with assessment of how many are able to prevail and successfully disseminate by secondary infections. Similar research has already been performed studying the pathogen causing grape downy mildew (Gobbin *et al.*, 2005; Matasci *et al.*, 2010). All information from this research may allow the evolutionary potential of *P. ampelicida* to be estimated.

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Literature cited

Aldrich J. and C. Cullis, 1993. RAPD analysis in flax: optimization of yield and reproducibility using klenTaq 1 DNA polymerase, chelex 100, and gel purification of genomic DNA. *Plant Molecular Biology Reporter* 11, 128–141.

Altschul S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic Local Alignment Search Tool. *Journal of Molecular Biology* 215, 403–410.

Anonymous, 2008. *Das Weinjahr 2008*. Institute Viti-vinicole in Remich, Luxembourg, 27 pp.

Boehm E.W.A., S. Freeman, E. Shabi and T.J. Michailides, 2003. Microsatellite primers indicate the presence of asexual populations of *Venturia inaequalis* in coastal apple orchards. *Phytoparasitica* 31, 236–251.

Brunner P.C. and J.E. Frey, 2004. Isolation and characterization of six polymorphic microsatellite loci in the western flower thrips *Frankliniella occidentalis* (Insecta, Thysanoptera). *Molecular Ecology Notes* 4, 599–601.

Evanno G., S. Regnaut and J. Goudet, 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* 14, 2611–2620.

Frenkel O., I. Portillo, M. Brewer, J.-P. Peros, L. Cadle-Davidson and M. Milgroom, 2012. Development of microsatellite markers from the transcriptome of *Erysiphe necator* for analysing population structure in North America and Europe. *Plant Pathology* 61, 106–119.

Frey J.E., B. Frey, C. Sauer and M. Kellerhals, 2004. Efficient low-cost DNA extraction and multiplex fluorescent PCR method for marker-assisted selection in breeding. *Plant Breeding* 123, 554–557.

Glenn T.C. and N.A. Schable, 2005. Isolating microsatellite DNA loci. *Methods in Enzymology* 395, 202–222.

Gobbin D., M. Jermini, B. Loskill, I. Pertot, M. Raynal and C. Gessler, 2005. Importance of secondary inoculum of *Plasmopara viticola* to epidemics of grapevine downy mildew. *Plant Pathology* 54, 522–534.

Gobbin D., A. Rumbou, C.C. Linde and C. Gessler, 2006. Population genetic structure of *Plasmopara viticola* after 125 years of colonization in European vineyards. *Molecular Plant Pathology* 7, 519–531.

Harms M., B. Holz, C. Hoffmann, H. Lipps and W. Silvanus, 2005. Occurrence of *Guignardia bidwellii*, the causal fungus of black rot on grapevine, in the vine growing areas of Rhineland-Palatinate, Germany. In: *Plant Protection and Plant Health in Europe: Introduction and Spread of Invasive Species* (British Crop Production Council, ed.). Humboldt University, Berlin, Germany, 9–11 June. Symposium Proceedings No. 81, pp. 127–132.

Jailloux F., 1992. *In vitro* production of the teleomorph of *Guignardia bidwellii*, causal agent of black rot of grapevine. *Canadian Journal of Botany* 70, 254–257.

Kong G., 2009. *Diagnostic Methods for Black Rot of Grapes*. PaDIL Plant Biosecurity Toolbox, 28 pp.

Langella O., 2002. *POPULATIONS 1.2.32, Population Genetic Software (Individuals or Population Distances, Phylogenetic Trees)*, http://bioinformatics.org/~tryphon/populations.

Luttrell E., 1946. Black rot of muscadine grapes. *Phytopathology* 36, 905–924.

Maixner M. and B. Holz, 2003. Risiken durch invasive gebietsfremde Arten für den Weinbau. *Angewandte Wissenschaft* 498, 154–164.

Matasci C.L., M. Jermini, D. Gobbin and C. Gessler, 2010. Microsatellite based population structure of *Plasmopara viticola* at single vine scale. *European Journal of Plant Pathology* 127, 501–508.

Nei M., F. Tajima and Y. Tateno, 1983. Accuracy of estimated phylogenetic trees from molecular-data .2. Gene-Frequency Data. *Journal of Molecular Evolution* 19, 153–170.

Peakall R. and P.E. Smouse, 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6, 288–295.

Pezet R. and M. Jermini, 1989. Le Black-Rot de la vigne: symptômes, épidémiologie et lutte. *Revue Suisse de Viticulture, Arboriculture, Horticulture* 21, 27–34.

Pritchard J.K., M. Stephens and P. Donnelly, 2000. Inference of population structure using multilocus genotype data. *Genetics* 155, 945–959.

Ramsdell D. and R. Milholland, 1988. Black rot. *Compendium of Grape Diseases*. The American Phytopathological Society. St. Paul, MN, USA, 15–17.

Reddick D., 1911. The black-rot disease of grapes. *Cornell Agricultural Experiment Station Bulletin* 293, 287–364.

Rozen S. and H. Skaletsky, 1999. Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics Methods and Protocols*, 365–386.

Szpiech Z.A., M. Jakobsson and N.A. Rosenberg, 2008. ADZE: a rarefaction approach for counting alleles private to combinations of populations. *Bioinformatics* 24, 2498–2504.

Ullrich C.I., R.G. Kleespies, M. Enders and E. Koch, 2009. Biology of the black rot pathogen, *Guignardia bidwellii*, its development in susceptible leaves of grapevine *Vitis vinifera*. *Journal für Kulturpflanzen* 61, 82–90.

van der Aa H., 1973. Studies in *Phyllosticta* I. *Studies in Mycology* 5, 1–110.

Wicht B., O. Petrini, M. Jermini, C. Gessler and G.A.L. Broggini, 2012. Molecular, proteomic and morphological characterization of the ascomycete *Guignardia bidwellii*, agent of grape black rot: a polyphasic approach to fungal identification. *Mycologia* 104, 1036–1045.

Wikee S., L. Lombard, C. Nakashima, K. Motohashi, E. Chukeatirote, R. Cheewangkoon, E.H. McKenzie, K.D. Hyde and P.W. Crous, 2013. A phylogenetic re-evaluation of *Phyllosticta* (Botryosphaeriales). *Studies in Mycology* 76, 1–29.

Wilcox W.F., 2003. *Black rot*. Cornell Cooperative Extension, Disease identification sheet No. 102GFSG-D4.

Zalapa J.E., H. Cuevas, H.Y. Zhu, S. Steffan, D. Senalik, E. Zeldin, B. McCown, R. Harbut and P. Simon, 2012. Using next-generation sequencing approaches to isolate simple sequence repeat (ssr) loci in the plant sciences. *American Journal of Botany* 99, 193–208.

Zane L., L. Bargelloni and T. Patarnello, 2002. Strategies for microsatellite isolation: a review. *Molecular Ecology* 11, 1–16.

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