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Research Paper

Improved DNA extraction and quantitative real-time PCR for genotyping *Erysiphe necator* and detecting the DMI fungicide resistance marker A495T, using single ascocarps

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Summary. DNA extraction from minute fungal samples is challenging in all genetic studies. Identification of genetic groups and population biology mostly rely on the laborious production of single conidium isolates or on field samples, including infected plant materials. This paper reports a simple and cost-effective protocol for DNA extraction from individual chasmothecia of *Erysiphe necator* for subsequent applications. It is a less laborious alternative for genotyping purposes than production and analysis of single conidium isolates or analysis of infected plant material from the field. Using the protocols described here for 186 *E. necator* samples tested, genetic groups A and B were assigned. Based on *CYP51* sequences, all the samples belonged to group B, while *TUB2* sequences exhibited SNPs also diagnostic for group A. Additionally, a quantitative real-time PCR detection method of single nucleotide polymorphism in the *CYP51* gene associated with DMI fungicide resistance was applied. The A495T marker, associated with DMI resistance, and here reported for the first time from Hungary, was detected by quantitative real-time PCR assays and direct sequencing of *CYP51*. The methods developed in this study can be applied as routine tests to monitor powdery mildew populations for fungicide resistance and other genetic characteristics.

Keywords. Y136F, azole fungicide sensitivity, *CYP51* gene, chasmothecium.

INTRODUCTION

Powdery mildews (PMs) are frequent diseases of many angiosperm hosts, and are caused by obligate biotrophic fungi in the Erysiphales (Glawe, 2008). These fungi are potential models in a wide range of studies, such as those that describe how coinfections are formed (Susi and Laine, 2017) or that explain disease dynamics (Jousimo *et al.*, 2014; Marçais *et al.*, 2017), pathogen host range expansions (Vági *et al.*, 2007; Beenken, 2017), host-pathogen co-evolution (Takamatsu *et al.*, 2013) or genome evolution (Frantzeskakis *et al.*, 2019). With very small uncultivable fungi, it is important to use the smallest discrete unit for DNA extraction, which can be identified based on morphology and is large enough to obtain sufficient genomic DNA for molecular genetic analyses. In this way, data can be obtained at the level of individuals, and the possibilities of contamination with other fungi (Sundberg *et al.*, 2018), or cross-contamination with another colony possibly belonging to a different genetic line of the same species, may be reduced.

Grapevine powdery mildew (GPM), caused by *Erysiphe necator*, is one of the most economically significant diseases of grape production (Gadoury *et al.*, 2012). In GPM, molecular characterizations are usually based on mycelium collected directly from the field (e.g. Frenkel *et al.*, 2012) or from single conidium isolates, i.e., GPM colonies each produced by a single conidium on the surface of a surface-sterilized leaf in laboratory conditions (e.g. Brewer and Milgroom, 2010).

Direct PCR methods without DNA purification have been successfully used to identify genetic groups in *E. necator* (Miazzi *et al.*, 2008), or to detect the pathogen in grapevine buds (Gindro *et al.*, 2014). Miazzi *et al.* (2008) obtained the best results with 4-10 conidia picked up from individual leaves using sterile eyelashes, and added directly to PCR mixtures. Gindro *et al.* (2014) used 10- and 100-fold diluted powdery mildew-infected plant samples crushed in polyvinylpolypyrrolidone, and added directly to PCR mixtures before amplification (Gindro *et al.*, 2014). Furthermore, PCRs and quantitative real-time PCRs (qPCRs) with DNA samples originating from individual conidia were also successful for two other powdery mildew species (Matsuda *et al.*, 2005).

Conidia are very small discrete units, and to obtain enough DNA for downstream applications, single conidium isolates should be produced, which is laborious and time consuming. It takes at least 1 month to obtain a clearly monoconidial GPM colony (Rallos and Baudoin, 2016). Ascocarps (chasmothecia) can be considered the smallest discrete unit of a powdery mildew fungus in nature that can still be easily handled, yet are large enough to contain sufficient DNA for multilocus analyses and qPCR.

Chasmothecia of *E. necator* are usually produced in large numbers at the end of the grapevine growing season (Gadoury and Pearson, 1988; Cortesi *et al.*, 1997). Young chasmothecia are hyaline spherical bodies that turn yellow, then brown during maturation. They finally become black with characteristic appendages, and are filled with asci containing ascospores (Gadoury and Pearson, 1988). Chasmothecia have been used for DNA extraction in molecular studies of other PMs (Hirata and Takamatsu, 1996), but not commonly for studies of GPM. Mougou *et al.* (2008) found that ten chasmothecia, collected directly from the surfaces of the host plant leaves, is the minimum number required for amplification of the nrDNA ITS region. However, to our knowledge, individual chasmothecia have not been extensively used for such purposes.

Two distinct genetic groups, A and B, have been described in *E. necator* using single conidium isolates (e.g. Brewer and Milgroom, 2010) or field samples (e.g. Frenkel *et al.*, 2012). The differentiation between these genetic groups is based on different molecular markers, such as sequences of the β-tubulin (*TUB2*) (Amrani and Corio-Costet, 2006) and the eburicol 14α-demethylase (*CYP51*) genes (Délye *et al.*, 1999), as well as sequences of the nrDNA internal transcribed spacer (ITS) region (Délye *et al.*, 1999) and intergenic spacer (IGS) region (Brewer and Milgroom, 2010).

Detection of fungicide resistance, in addition to population genetic studies, is an important task for characterisation of GPM. Demethylation inhibitor (DMI) fungicides inhibit CYP51, a key enzyme of the fungal sterol biosynthetic pathway. This enzyme catalyzes the biosynthesis of ergosterol, which is an important membrane component of fungi (Parker *et al.*, 2014). Because of their site-specific modes of action, the intensive use of DMIs may lead to the spread of the fungicide resistance in GPM populations (Frenkel *et al.*, 2015). A marker for DMI resistance is an A to T nucleotide substitution in position 495 (A495T) in the *CYP51* gene (Délye *et al.*, 1999). This results in amino acid substitution at position 136 (Y136F) and correlates with high levels of azole resistance (Frenkel *et al.*, 2015). Until recently, single conidium isolates (e.g. Miazzi and Hajjeh, 2011; Frenkel *et al.*, 2015) or field samples (Montarry *et al.*, 2009; Dufour *et al.*, 2011), but not chasmothecia, were used to detect marker A495T.

This paper describes a study which aimed to develop a protocol for DNA extraction from individual PM chasmothecia, to provide evidence that the samples are suitable for downstream applications. Here, we also present the application and evaluation of a qPCR assay for the detection the DMI fungicide resistance marker A495T in DNA extracts from individual *E. necator* chasmothecia.

MATERIALS AND METHODS

DNA extraction from individual chasmothecia

Powdery mildew-infected grapevine leaves were examined in the laboratory under a stereo microscope (Zeiss Stemi 2000C), to isolate chasmothecia. Single

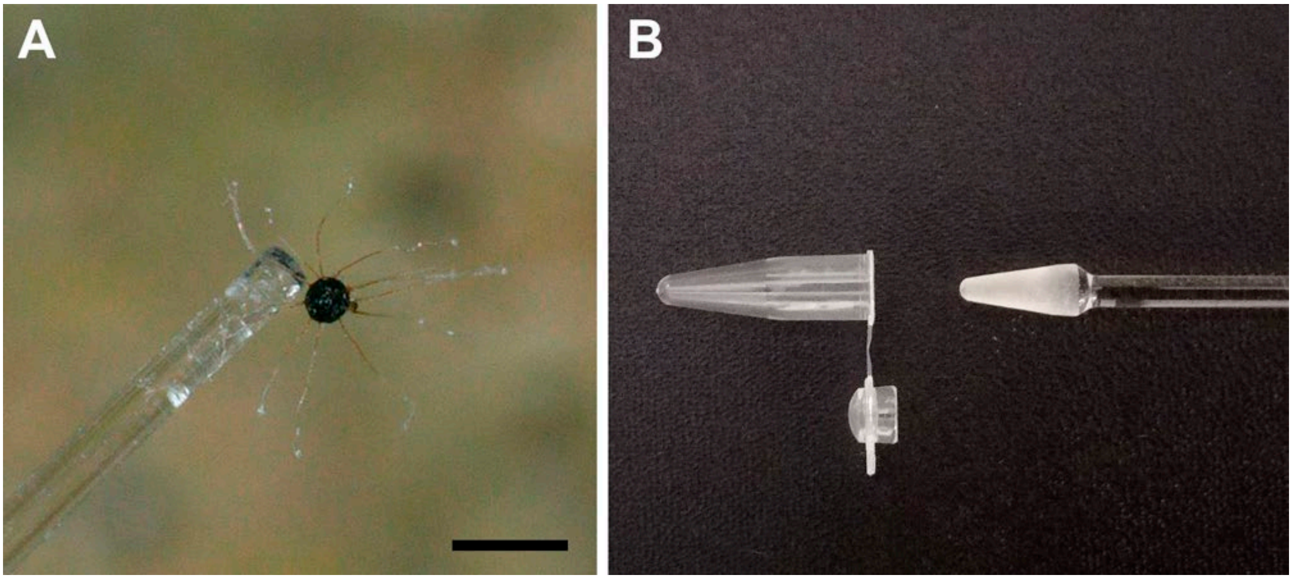

Figure 1. First steps of sample preparation. A: individual *Erysiphe necator* chasmothecium attached to the tip of a glass needle. Bar = 250 μm. B: plastic PCR tube (left) and conical glass micropestle (right).

immature (yellow, orange or brown) and mature (dark brown or black) chasmothecia were separated from the mycelium (without any visible hyphal fragments), using sterile glass needles (Figure 1A), and each placed in a 0.2 mL capacity PCR tube containing 20 μL of extraction medium. The following extraction media were tested: (a) Tris-EDTA (TE) buffer (pH 8; Lonza); (b) 1 M NaOH (Sigma-Aldrich) solution; (c) nuclease-free water (Thermo Fisher Scientific); or (d) dilution buffer of the Phire Plant Direct PCR Kit (Thermo Fisher Scientific). With each extraction medium, DNA was extracted from 15 chasmothecia as a preliminary experiment. To crush single chasmothecia in the extraction medium in PCR tubes, the following tools were tested; micropestles with either spherical or conical ends (Figure 1B), specifically manufactured for this purpose (Ranyák Üvegtechnika Anno 1967) or carved pipette tips. These tools were selected to fit into the PCR tubes. After disrupting individual chasmothecia under observation with a stereo microscope, the suspensions were vortexed for 1 min, centrifuged at 3000 × *g* for 1 min, and incubated at 97°C for 7 min. DNA samples obtained in this way were stored at -20°C. The most suitable extraction medium was selected based on the results of the PCR amplifications (see below).

DNA amplification and sequencing

Four loci were amplified in this study: ITS, IGS, and the *TUB2* and *CYP51* genes. The following primers were used in the amplifications: for ITS, ITSEnF/ITSEnR; for IGS, IGSEn1/NS1R; *TUB2*, Bt2c/Bt2d (Brewer and Milgroom, 2010); and for *EnCYP51*, EnCYP89F/EnCYP856R (Frenkel *et al*., 2015).

For PCR optimization, three ready-to-use PCR master mix solutions were evaluated. These were Red Taq 2× DNA Polymerase Master Mix (VWR), DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific) or Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific), which are hereafter referred to as, respectively, *Red Taq*, *DreamTaq* and *Phusion*.

All PCRs were performed in 20 μL final volumes. Reaction components included 1 μL of 10 μM forward and reverse primers (Sigma-Aldrich), 1 μL DNA template and 10 μL *Red Taq, DreamTaq* or *Phusion*. With the *Phusion* master mix solution, a range of target volumes (0.1 to 4 μL) was tested.

Samples previously resulting in successful amplifications were used as positive control samples, and nucleic acid-free water was used as the target in negative control reactions. To determine the optimal target DNA dilutions for the reactions, a series of dilutions was tested, i.e., 50-, 200-, 500-, 1000- and 2000-fold. This test was conducted twice with ten DNA samples for all loci described above using *Phusion*.

Amplification conditions for PCR with *Red Taq* and *DreamTaq* constituted an initial denaturation step for 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 55°C for ITS, at 56°C for IGS and *TUB2* and at 57°C for *CYP51* primers, and 60 s at 72°C, with a final extension step of 10 min at 72°C. For *Phusion* cycling, times and temperatures were as follows: 2 min at 98°C, followed by 36 cycles of 5 s at 98°C, 5 s at 60°C and 15 s at 72°C, and a final extension step of 5 min at 72°C, for all loci. PCR products were separated on 1.5% agarose gel containing GelRed (Biotium) in 0.5× TBE buffer, and were visualized under UV light.

Randomly selected PCR amplicons from ITS and IGS and all *TUB2* and *CYP51* products were sent for Sanger sequencing to a service laboratory (LGC Genomics GmbH). All PCR products were sequenced in both directions with the same primers used for amplifications. Electrophoregrams were processed and individually inspected using the Staden Program Package (Staden *et al.*, 2000). The SNP positions connected to the A and B genetic groups and the fungicide resistance marker A495T were checked based on Brewer and Milgroom (2010) and Délye *et al.* (1999). The following GenBank accessions were used as references: GQ255475 (*TUB2*), GQ255473 (ITS), GQ255476 (IGS; Brewer and Milgroom, 2010) and U83840 (*CYP51*; Délye *et al.*, 1997). Representative sequences, obtained in the present study were deposited in GenBank under accession numbers MT023360, MT023361 (ITS); MT023362, MT023363 (IGS); MT023364, MT023365 (*TUB2*) and MT023366, MT023367 (*CYP51*).

Molecular cloning of a fragment of CYP51

An approx. 1.8 kb fragment of the *E. necator CYP51* gene was amplified with primers EnCYP89F and EnCYP1752R (Frenkel *et al.*, 2015). In these amplifications *Phusion* was used as described above, with the elongation step in the PCR program set to 40 s. A single chasmothecium DNA extract of a sample was used as target, which harboured the A495T mutation. The product was purified from 1% agarose gel with a GenElute Gel Extraction Kit (Sigma-Aldrich), ligated into the plasmid pJET1.2/blunt using a CloneJET PCR Cloning Kit (Thermo Fisher Scientific), and transformed into One Shot TOP10 Chemically Competent *Escherichia coli* cells (Thermo Fisher Scientific). One clone was selected randomly from the positive clones, which was propagated and used for plasmid extraction with a GenElute Plasmid Miniprep Kit (Sigma-Aldrich). A whole insert sequence was obtained by sequencing with pJET1.2 forward and reverse sequencing primers. The molecular weight of the plasmid was calculated based on the obtained sequence; the concentration of the extracts was measured with a Nanodrop 2000c spectrophotometer. The copy number of plasmids present in the extracts was calculated as described by Whelan *et al.* (2003). A stock of 10^8 *CYP51* containing plasmids μL^{-1} was prepared by diluting in TE buffer, and was aliquoted and stored at -20°C.

Real-time PCR assay

To use qPCR for detection of A495T, the protocol described by Dufour *et al.* (2011) was modified. The allele specific reverse primer and qEN primer pair were the same as outlined in Dufour *et al.* (2011). The forward primer used along with the allele-specific primer was substituted to primer L502 (Rallos and Baudoin, 2016), resulting in slightly greater amplification efficiency (see below). Reactions were performed in a Bio-Rad CFX96 Touch C1000 qPCR machine in 10 μL final volumes, with 5 μL of iTaq Universal SYBR Green Supermix (Bio-Rad), primers (for final concentrations, see below) and 1 μL target. The reaction conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of 10 s at 95°C, 10 s at 58°C and 20 s at 72°C. Specificity of the qPCR amplifications was checked by registering a melt curve after cycling. Plasmid DNA extract was used as the target in the control reactions and single chasmothecium DNA extracts were used for allele-specific detection of A495T and for measurements. Negative controls (samples without target DNA) were always included. Each sample was assayed in triplicate, cycle threshold (Cq) data were averaged from the triplicates, and the averages were used in the subsequent data analyses.

A dilution series from 10^6 copies μL^{-1} to 10 copies μL^{-1} of the control plasmid DNA extract was prepared in TE buffer by serially diluting the extract 10 times in each step. This dilution series was used to assess the efficiency of primer pairs in the adapted qPCR method. Preparation of the dilution series and efficiency measurements were carried out at least three times in independent experiments for each of the three primer pairs (qEN forward and reverse; qEN136R forward and reverse; L502 paired with qEN136R reverse).

The optimal concentrations for L502 and qEN136R reverse used in allele-specific reactions were determined by the matrix method, in which 50 nM, 100 nM, 200 nM, 400 nM, 600 nM or 800 nM final concentrations of both primers were tested in all combinations. Plasmid extract diluted to 10^5 copies μL^{-1} was used as a target in these tests, and each test was repeated once. The qEN forward and reverse primers were used in a final concentration of 500 nM each, as indicated by Dufour *et al.* (2011).

To measure qPCR efficiency with *E. necator* DNA extracts, three single chasmothecium DNA extracts of samples harbouring the A495T were diluted 10-, 20- or

50-fold in TE buffer. These, along with the undiluted extract, were amplified with the qPCR method described above, and efficiencies were calculated from the Cq data obtained.

Determination of qPCR cut-off for detection of A495T SNP

A total of 186 single chasmothecium DNA samples were analyzed with the qPCR method described above. The known diagnoses of A495T presence, determined earlier with the direct sequencing method and coded as a binary variable, were paired to the corresponding Cq values. The dataset was used for determination of the qPCR cycle threshold cut-off following the epidemiologic approach (Caraguel *et al*., 2011). Determination of the cut-off was achieved by conducting a receiver operating characteristic (ROC) plot analysis with the MedCalc for Windows software, version 12.2.1.0 (MedCalc Software; Stephan *et al*., 2003). The prevalence of A495T among the analyzed samples (25.3%) was assumed to reflect the overall prevalence of the mutation in the sample populations. All other options were used as defaults in the software. The cycle threshold corresponding to the greatest Youden index (J) was selected, because using this Cq as a cut-off minimizes the probability of overall misclassification (Caraguel *et al*., 2011) and represents a balance between sensitivity and specificity (Nutz *et al*., 2011). The calculated cut-off was used for discrimination of positive and negative samples (i.e., diagnosis) based on Cq values. Samples having Cq values equal to or less than the cut-off were considered positive (Nutz *et al*., 2011).

False positive and false negative rates (Kralik and Ricchi, 2017) of the tested qPCR method were calculated based on MedCalc output data.

In 20 samples where incongruences between the results of genotyping by direct sequencing and qPCR analyses were found (see below), the relative amounts of mutant allele present in the samples were quantified using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). Plasmid DNA was used as an internal reference; this contained the A495T allele in a 1:1 ratio relative to the whole amount of *CYP51* DNA, which was reflected by the qEN amplifications.

RESULTS

DNA extractions and PCR amplifications

During the preliminary experiments, DNA was extracted from 15 *E. necator* chasmothecia, with each extraction medium and crushing tool. Because the carved pipette tips and spherical end glass micropestles were not effective for disrupting the chasmothecia, only the conical end glass micropestles were used further. Based on poor PCR amplifications, 90% of the DNA extraction did not result in extracts suitable for PCR amplification when NaOH or water were used as extraction media. However, DNA extractions were successful with 14 of 15 samples when TE buffer or the Phire Plant Direct kit buffer were applied, as reflected by PCR amplifications. For further work, TE buffer was selected, based on durability and the usability of the DNA extracts in molecular work.

All the target loci (ITS, IGS, *TUB2* and *CYP51*) were successfully amplified with all three master mix solutions tested from individual chasmothecium DNA extracts using TE buffer during the extraction protocol. However, the PCR performances of *Phusion* were more consistent with those of other master mixes (Supplementary Figure S1). Because of the low error rate of this high-fidelity enzyme, it was selected for all other comparisons and assessments.

To test the optimal target volume, different target amounts (0.1 to 4 μL) and a series of diluted DNA samples were used as templates for PCR amplifications using *Phusion*. The optimal target volume for *TUB2* was 1 μL from the undiluted DNA extract and 1 μL for the other loci from genomic DNA diluted as described below. Using this target volume, the optimal dilution was 50-fold for ITS and IGS, and 10-fold for *CYP51*. The greatest dilutions resulting in visible PCR amplicons were 2000-fold for ITS and IGS, and 100-fold for *CYP51*. PCRs targeting the *TUB2* locus using diluted DNA did not result in visible amplicons.

DNA was extracted from a total of 190 chasmothecia with TE buffer (including preliminary experiments), and sequences were obtained from 98% of all the samples after successful PCR amplifications.

No prominent differences were observed in PCR amplifications between DNA extracts from mature and immature chasmothecia, for the different master mix solutions, target volumes or dilution assays.

Sequence analysis of ITS, IGS, TUB2 *and* CYP51

All directly sequenced PCR products obtained from individual chasmothecia showed 99% similarity or were identical to the respective reference sequences. Based on *TUB2*, both A and B genotypes were detected in our samples, according to nucleotide site 79 in the referenced GenBank accession (GQ255475). All of the samples belonged to group B, according to two SNP positions (nucleotide sites 110 and 575 in the coding region of GeneBank accession U83840) in the *CYP51* sequences. Similarly, based on one SNP in ITS (nucleotide site 48 in GQ255473) and one in IGS (nucleotide site 108 in the GQ255476), all the samples belonged to group B.

The mutant allele bearing the A495T point mutation in *CYP51*, which confers DMI-resistance in *E. necator*, was detected from approximately one third of the DNA extracts originating from chasmothecia. This is the first report describing the mutant allele in GPM in Hungary.

Overlapping peaks at three polymorphic positions were found in one fifth of the samples during examination of electrophoregrams. Double peaks were present at position 79 and 368 in *TUB2* and 495 in *CYP51*. Thymine (T) or cytosine (C) at position 79 in *TUB2* is characteristic for groups A and B. In *CYP51*, the overlapping adenine (A) and thymine (T) peaks were present at the nucleotide position corresponding to the A495T mutation (Supplementary Figure S2).

Optimized qPCR protocol for detection of A495T

Regression curves resulting from qPCR efficiency tests using plasmid controls fit with correlation coefficients $R^2 \geq 0.96$. Measured amplification efficiencies were (mean ± standard deviation) 94 ± 2% for the qEN primer pair and 87 ± 1% for the L502-qEN136R reverse primer pair. Primer qEN136R reverse paired with qEN136R forward resulted in lesser amplification efficiencies (79 ± 7%) and less conclusive measurements than L502-qEN136R reverse. Therefore, for allele specific reactions, L502 was used with qEN136R reverse during this study. Primer concentration assay showed that the best combination with the greatest reaction efficiency and low Cq, relative to other combinations, was found at a final concentration of 400 nM for L502 and 600 nM for qEN136R reverse primer.

In the qPCR efficiency tests using single-chasmothecium DNA extracts, 90 ± 1% efficiency was measured for qEN amplifications. Allele-specific amplifications reached an efficiency of 93 ± 8%. All curves fit with regression coefficients $R^2 \geq 0.974$ (Supplementary Figure S3). Based on reaction coefficients, all the tested dilutions were considered to fit in the log-linear range of the applied qPCR method.

Detection of the A495T marker with qPCR

In the ROC analysis that used samples with known diagnoses based on direct sequencing, the resulting Cq cut-off value with the greatest Youden index was 31.3 (Supplementary Figure S4). The applied method has 4% false negative and 12% false positive rates using this Cq cut-off (Supplementary Figure S4). Twenty samples (10.8%) of the 186 tested were differently genotyped by qPCR than by direct sequencing. Seventeen of these samples were positive in qPCR (harbouring the A495T mutation) but were genotyped as wild-type by direct sequencing. Based on qPCR mea-surements, the ratio of the mutant allele was 20 ± 13% (mean ± standard deviation) in these samples.

The electrophoregrams showed that 39 samples contained double peaks at position 495 of *CYP51*. All but three of these samples were positive in qPCR for the presence of the A495T mutation exhibiting the presence of the specific allele involved in DMI resistance.

DISCUSSION

This paper describes an improved DNA extraction and qPCR protocol for genotyping *E. necator* samples, and for identifying the A495T mutation in this pathogen, which is involved in DMI fungicide resistance. This study demonstrates that only a single chasmothecium of starting material is sufficient, and that a crude DNA extract can be used to obtain nucleotide sequences and perform qPCR.

In powdery mildew research, molecular protocols without any purification steps, or with only a minor DNA purification step, such as direct PCR methods, have occasionally been used (Hirata and Takamatsu, 1996; Matsuda *et al.*, 2005; Miazzi *et al.*, 2008; Gindro *et al.*, 2014). Conidia and chasmothecia have been used in these protocols with different amounts of starting material. Miazzi *et al.* (2008) harvested 10 to 20 conidia from infected samples. Matsuda *et al.* (2005) picked only individual conidia from each powdery mildew colony, using a glass needle and a manipulator and transferred these directly to the PCR mixture. With these protocols, multiplex PCR amplifications are possible, but there is no remaining target DNA for further assessment. Gindro *et al.* (2014) extracted DNA from grapevine buds infected with *E. necator*, diluted the extracts and used them directly for PCR. The detection limit with this method, as for earlier reports (Matsuda *et al.*, 2005; Falacy *et al.*, 2007), was one conidium per microliter (Gindro *et al.*, 2014). Obtaining DNA from individual conidia for downstream applications, like qPCR and LAMP (Thiessen *et al.*, 2016), is also possible, but for this it is necessary to use either a DNA extraction kit (Thiessen *et al.*, 2016) or Chelex 100 reagent (Thiessen *et al.*, 2018). To obtain more DNA, with less purification steps, extracting from larger structures (such as chasmothecia), can be a solution. Based on our findings, only one chasmothecium is enough to obtain sufficient DNA for multiple PCR amplifications. In contrast to earlier protocols using 20 (Hirata and Takamatsu, 1996) or ten (Mougou *et al.*, 2008) chasmothecia, we were able to amplify and sequence four loci and perform qPCR from only 20 μL DNA extracts of individual chasmothecia.

During DNA extraction from one chasmothecium, different extracting media were tested. Sodium hydroxide can be used for DNA extraction from plant material, with the reasoning that the alkaline pH suppresses nucleases and does not affect the subsequent PCR amplifications (Wang *et al.*, 1993). However, our results showed that water and NaOH were of reduced extraction efficiency. TE buffer and Phire Plant Direct PCR kit buffer were superior as extraction media, demonstrated by the high success rate of PCR amplifications using these solutions. For further extractions, TE buffer was selected because the shelf life of the DNA extract was prolonged compared with other extraction media (Yagi *et al.*, 1996). DNA extractions from mature and immature chasmothecia gave equal success rates of PCR amplifications (98%), which demonstrates that this method can be implemented for small structures, such as yellow, immature ascocarps.

The method described here enabled genotyping of *E. necator* from individual chasmothecia, using multilocus sequencing to determine if each sampled belonged to genetic group A or B of the pathogen. Based on direct sequencing of *TUB2*, group A was detected several times. In contrast, all of our samples belonged to group B based on *CYP51* sequences, without any polymorphism in two marker nucleotide positions, which have been shown to be diverse in other samples (Délye *et al.*, 1999). Amrani and Corio-Costet (2006) and Araya *et al.* (2014) found no contradiction between the results obtained based on genotyping of *TUB2* and *CYP51*; that is, all the markers were congruent and assigned to one genetic group. Further work is needed to study the linkages between the loci used for the description of groups A and B.

Délye and Corio-Costet (1998) and Délye *et al.* (1997; 1999) stated that group A only reproduces clonally. However, samplings from some populations have suggested that sexual reproduction may be possible (Miazzi *et al.*, 2003; Cortesi *et al.*, 2004). Mating type assays revealed that group A can produce chasmothecia and viable ascospores in laboratory conditions (Miazzi *et al.*, 2003; Cortesi *et al.*, 2004). We detected markers of group A in chasmothecia, which indicates that sexual reproduction is also possible in field conditions. Moreover, co-occurrence of groups A and B was detected in DNA extracts from individual chasmothecia, indicating that mating between these two groups happens in vineyards. This phenomenon was demonstrated in *in vitro* experiments (Miazzi *et al.*, 2008), but has not been reported from field samples. If only anamorph structures are examined, it is not possible to determine if any of the genotypes reproduces sexually. Extended sampling of many chasmothecia and application of the genotyping method described here would address this question. Using only individual conidia or mixtures of chasmothecia for DNA extraction may hide the presence of group A, described previously as only clonal, if it were present at low ratios. This also emphasizes the importance using individual chasmothecia for DNA extractions. Double peaks were present in about a fifth of the electropherograms obtained in the present study, indicating the presence of a mixture of different genotypes in some DNA extracts from individual chasmothecia (Lesemann *et al.*, 2006; Kovács *et al.*, 2011). Possible explanations could be sexual processes between two colonies harbouring two different genotypes (for *TUB2* and *CYP51*), or increased copy number (for *CYP51*) under pressure from DMI fungicide applications (Jones *et al.*, 2014; Rallos and Baudoin, 2016). For DNA extractions, we only sampled chasmothecia, and despite great care was taken, it cannot be ruled out that in some cases mycelium around each chasmothecium was also sampled. This may have resulted in mixed genotypes in the DNA extracts, which were not present in the chasmothecia.

The qPCR and DNA extraction protocol described here is suitable for high-throughput genotyping of *E. necator* samples to determine the presence of the A495T mutation. The method is more rapid and cost-effective for this purpose than genotyping based on direct sequencing. From our samples, 20 were differentially diagnosed by qPCR and direct sequencing, and most (17) were diagnosed positive for the mutation only by qPCR. This implies that the method used for reference (e.g. direct sequencing) has less sensitivity than the developed qPCR-based diagnostic method (Kralik and Ricchi, 2017). Consequently, we conducted quantification of the resistant allele in these samples. The samples genotyped as resistant based on qPCR had a low ratio of the mutant allele (20 ± 13%), and these were identified as harbouring the wild-type allele only by direct sequencing. The protocol involving PCR followed by direct sequencing is possibly not sensitive enough for accurate characterization if the targeted allele is present at low ratios.

For cut-offs differentiating positive and negative samples based on measured Cq values, we selected that with the greatest Youden index. This method sets the cut-off so that the assay has the least probability of misclassification (Caraguel *et al.*, 2011), when the calculated cut-off of Cq = 31.3 is used for diagnostics. However, the cut-off can be set to prioritize other characteristics of the assay, for example, to minimize the probability of false-negatives or false-positives. The most suitable selection should be based on the purpose of each diagnostic test (Caraguel *et al.*, 2011).

An individual chasmothecium can be a discrete unit for gaining an adequate amount of DNA, and for providing information on inter- and intra-specific variation, species complexes and population biology. Conidia are also discrete units. However, to obtain enough DNA for downstream processes (e.g., multilocus analyses), labour intensive methods (e.g. Thiessen *et al.*, 2016) or time consuming production of single conidial colonies, is currently necessary (Erickson and Wilcox, 1997; Miazzi *et al.*, 2008). In contrast to single conidia, chasmothecia can be collected from herbarium material (e.g. pressed leaves) without any visible hyphal fragments from the adjacent colonies.

We have developed a rapid DNA extraction method for very small fungal samples to obtain DNA for downstream sequencing and qPCR purposes. As fruiting bodies are morphologically and taxonomically identifiable units, and are large enough to genotype fungal samples, this method may be applicable to any chasmothecia-producing powdery mildew species, as well as for fruiting bodies of other plant colonizing fungi. The qPCR protocol presented here for detection of the SNP linked to DMI fungicide resistance can be implemented in plant disease diagnostics.

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