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**Research Note**

# New rapid PCR protocols to distinguish genetic groups in *Erysiphe necator*

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K e y w o r d s : powdery mildew, *Erysiphe necator*, *Uncinula necator*, SCARs, SSR.

**Introduction***:* Genetically distinct but morphologically similar groups of *Erysiphe necator* Schwein. (formally *Uncinula necator* (Schwein.) Burrill (Braun and Takamatsu 2000) have been identified in Europe, India (Délye *et al.* 1997) and in Australia (Stummer *et al.* 2000). One group reproduces only asexually and can thus provide well-adapted persistent clones, whereas the other group can also produce recombinant genotypes through sexual reproduction. The optimal choice for control strategies thus requires the description of the distribution of both groups in vineyards. One RFLP probe or one PCR primer would probably be sufficient to identify the genetic groups because of the marked differences between them. One drawback of these techniques is the need for several preparatory steps, *i.e.* isolation, production of conidia and DNA extraction. To circumvent this problem, Délye *et al.* (1999) developed nested allele-specific PCR assays based on nucleotide differences in the sequence of the gene encoding eburicol 14α-demethylase (*CYP51*) and in the ITS1 sequence of the rDNA. In our trials, these protocols needed to be replicated to obtain reliable results. We therefore experimented other strategies to obtain more simple and robust methods for group identification and designed specific PCR primers to target: (1) the sequences of RAPD fragments using the approach of characterized amplified region (SCAR) (Paran and Michelmore 1993), and (2) a sequence identified in a genomic library enriched in microsatellite motifs. These primers were evaluated using DNA templates obtained by different methods.

**Material and Methods:** A total of 83 isolates of *E. necator* collected in southern France were used in this study: 49 isolates from group A and 34 isolates from group B, these groups corresponding to group I and group III of Délye *et al.* (1997). DNA was extracted using three different methods: (1) A previously described CTAB method (Péros *et al.* 1996) modified as followed: 400 mg of glass beads (1.8 mm diameter) were added to the microtube containing the freeze-dried conidia (obtained after mass-propagation on detached leaves of cv. Cabernet-Sauvignon), 500 µl of extraction buffer was then added and the tube was vortexed for 1 min before incubation at 65 °C for 30 min, (2) Extraction from infected freeze-dried leaf disks (15 mm in diameter) using the DNA Easy Kit in 96 well-plates (Qiagen, Hilden, Germany), and (3) DNA recovery from a minute amount of fungal cells scraped from infected leaves after suspension in 20 µl of ultra-pure water and incubation at 95 °C for 1 min. DNA templates were quantified on 0.8 % agarose gel stained with ethidium bromide by visual comparison with different quantities of lambda DNA.

All PCR amplifications were performed in 25 µl reaction mixture including 1 U *Taq* (QBiogen, Illkirch, France), 1 x *Taq* buffer, dNTP at 200 µM, 10 pmol of each primer and 2 µl of template DNA. The PCR cycle was programmed in a PTC-100™ thermocycler (MJResearch, Watertown, USA) as follows: 94 °C for 4 min and then 35 cycles at 94 °C for 1 min, annealing temperature for 1 min (see Table), 72 °C for 1 min, then a final extension of 72 °C for 6 min. Amplified products were subjected to electrophoresis (TBE 0.5X) in 1.6 % agarose gel, detected with ethidium bromide and photographed under UV light.

Two RAPD fragments obtained each with a different primer and specific to isolates from group A were amplified, recovered from gels and cloned as described in Péros *et al.*

T a b l e

Oligonucleotides derived from SCAR fragments tested for identification of genetic groups in *Erysiphe necator*

| Primer pair (5'-3') | Original primer | Size (bp) | $T_a$(°C) | Amplification for A isolates | Amplification for B isolates |
|---|---|---|---|---|---|
| 1: GTGGGCTGACCTGGAGAATC<br>GTGGGCTGACATAGCGATAC | P06 | 1019 | 55 | + | - |
| 2: CCGTATGATTTTGATTTA<br>TCGTTTATATGGATGGAA | P06 | 739 | 49 | + | - |
| 3: GACCTGGAGAATCATAATCG<br>AAATGTGAGTGTGGGAAGTC | P06 | 646 | 55 | + | - |
| 4: AAGCGGCCTCGTTATTTATA<br>AAGCGGCCTCAATTGCTAAA | J20 | 464 | 55 | + | - |
| 5: TCGAGCTGACCTTTCCTGTG<br>CTTGATCACCCTGGTCCATA | J20 | 372 | 55 | + | + |

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(1997). Clones were sequenced (Genome Express, Meylan, France) and primers were designed either by adding 10 nucleotides to the original primer in order to amplify the full sequence or by using Oligo software (version 6.2, Molecular Biology Insights, USA) at default settings to determine internal primers.

DNA from a group B isolate was digested with RsaI. The fragments possessing SSR motifs were recovered and selected according to the methods described in Baquerizo *et al.* (2001). Primers were designed using Operon software for a total of 30 different sequences obtained from Genome Express.

**Results and Discussion**: We obtained a sequence of 464 bp length for the fragment amplified with RAPD primer J20 and a sequence of 1,019 bp for a fragment amplified with RAPD primer P06. Several primer pairs were designed for both fragments (Table) and tested with three isolates of each group. Primer pairs no. 1, 2, 3 and 4 yielded a single product only with group A isolates whereas primer pair 5 yielded a product for both groups (Table). The marker P06-1019 corresponded to a sequence that was probably absent in group B isolates since no PCR product was obtained in this group with any pairs. The absence of marker J20-464 for group B (Figure, a) probably resulted from a mismatch of the RAPD primer or a deletion in a sequence that is present in both groups since a shorter product was amplified in both groups.

Polymorphism between groups was observed after amplification by the primers mO3E11F: TTGGCTGGCTGTTGTGGT and mO3E11R: CCGCGTGAAGTTGAAGATTT (annealing temperature 50 °C). These primers were designed to amplify a sequence of 150 bp length that contained the SSR motif $(CA)_8(CT)_{17}$. Within the set of isolates tested, three PCR products with a strong signal were revealed (Figure, b) and directly sequenced. The products were also analyzed with an automated sequencing apparatus after the labeling (FAM) of the forward primer. All 49 isolates of group A showed a product sizing 131 bp that was an allele of the original sequence having a shorter SSR motif $(CA)_8(CT)_7$. This allele was found in two group B isolates (Figure, b, lane 7). The other group B isolates showed the 150 bp allele that was absent in group A. In addition, all 34 group B isolates showed a 221 bp band that was absent in group A and that did not possess any SSR motif. The primer pair mO3E11 was therefore able to identify groups unambiguously in one PCR reaction. Indeed, the presence of the 221 bp band identified isolates of group B whereas the absence of this band and the presence of the 131 bp allele of the SSR sequence characterized isolates of group A.

All primer pairs and polymorphism between groups were first evaluated using DNA templates (2-5 ng of DNA per reaction) extracted from conidia. Primer pairs 1, 4 and mO3E11 were also tested with DNA extracted from infected leaves and gave the expected results. This indicated that amplification was not affected either by DNA or other compounds from grapevine. DNA templates obtained after scraping the fungus had too low concentrations to be quantified on gels and gave the expected results with the SCAR primer pairs 1 and 4. However, several replications or even another DNA extraction had to be performed for some samples to obtain amplification. In contrast, the primer pair mO3E11 gave strong expected signals for all isolates tested (9 from group A and 18 from group B).

In conclusion, the primer pair mO3E11, primarily designed for the SSR sequence, allowed an automatized identification of the two genetic groups in *E. necator* in one PCR reaction using a very low concentration of DNA. For large samples, this new genotyping tool could be used with confidence to determine the distribution patterns and the temporal distribution of the genetic groups within one growing season.

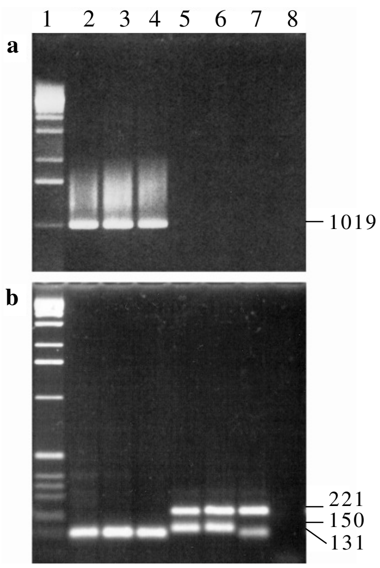


Figure: PCR amplification of DNA of *Erysiphe necator* using (a) SCAR primer pair 4 and (b) primer pair moE11. Lane 1: molecular weight marker (1 kb ladder); lanes 2-4: group A isolates; lanes 5-7: group B isolates; lane 8: control with no DNA template.

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