SHORT COMMUNICATION

SPECIFIC SCAR PRIMERS FOR THE "FLAG SHOOT" AND "ASCOSPORE" BIOTYPES OF THE GRAPE POWDERY MILDEW FUNGUS *ERYSIPHE NECATOR*

H. Hajjeh, M. Miazzi, M.A. De Guido and F. Faretra

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi di Bari, Via Amendola 165/A, 70126 Bari, Italy

SUMMARY

Eight RAPD amplicons selected previously as specific for isolates belonging to the "flag shoot" or the "ascospore" biotypes of *Erysiphe necator* were purified, cloned and sequenced. Thirty-seven SCAR (Sequence Characterised Amplified Region) primers were designed on the obtained sequences and tested for their specificity. Two pairs (UnE-UnF and F6-F6a) were specific for *E. necator* and effectively distinguished the two biotypes. Both primer pairs were tested on 374 *E. necator* isolates (83 "flag shoot" and 291 "ascospore"), already characterized by RAPD analysis, confirming their specificity and corroborating previous results. The primers are now being used to assess the biological significance and epidemiological role of the two biotypes in *E. necator* populations.

Key words: *Uncinula necator*, grapevine, SCAR, molecular markers, population genetics, fungal biotypes.

The biotrophic fungus *Erysiphe necator* Schw., formerly *Uncinula necator* (Schw.) Burr. (Braun and Takamatsu, 2000), is the causal agent of grape powdery mildew, a disease causing heavy yield losses worldwide. Nevertheless, basic questions on the pathogen biology and variation, and the disease's epidemiology still remain unanswered. The pathogen is known to overwinter in grapevine buds as mycelium or conidia (Sall and Wyrsinski, 1982), which infect young shoots soon after bud break (symptoms known as flag shoots), and as cleistothecia on leaves, on vine bark and in soil (Diehl and Heintz, 1987; Gadoury and Pearson, 1988, 1990; Pezet and Bolay, 1992; Munshi *et al.*, 1996; Cortesi *et al.*, 1997; Schneider *et al.*, 1998; Steinkellner, 1998).

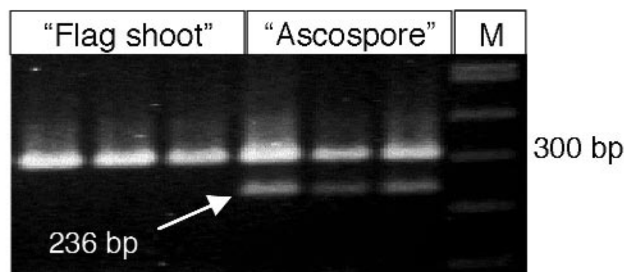
Recently, the usage of molecular markers, such as RAPD (Random Amplified Polymorphic DNA) (Délye *et al.*, 1997, 1998; Miazzi *et al.*, 2003), RFLP (Restriction Fragment Length Polymorphism) and microsatellites (Stummer *et al.*, 2000), suggested the existence of sub-specific groups in European and Australian populations of *E. necator*. These seem ascribable to the two overwintering forms and have been designed the "flag shoot" and the "ascospore" biotypes. A third genetic group has been detected in India (Délye *et al.*, 1997).

The "flag shoot" biotype seems responsible for the typical symptoms on young shoots early in the season; the "ascospore" biotype seems responsible for later infections mostly on bunches (Délye *et al.*, 1997, 1998; Miazzi *et al.*, 2003). Délye *et al.* (1997, 1998) believed the two biotypes genetically separated being intersterile in sexual crosses, but this was not confirmed with Italian isolates of the fungus (Miazzi *et al.*, 2003). Moreover, results of spatial and multilocus analysis showed that both sexual reproduction and clonal multiplication occurred in a "flag shoot" population of *E. necator* in a single vineyard in Italy (Cortesi *et al.*, 2004). Due to these conflicting data, the biological and epidemiological role of the two biotypes need to be clarified through exhaustive field monitoring of *E. necator* populations in different grapevine-growing areas.

Délye *et al.* (1999) developed a nested allele-specific (NAS) PCR assay distinguishing the two biotypes, based on single-point mutations in the rDNA internal transcribed spacers (ITS) or in the gene *CYP51*, encoding for eburicole 14α-demethylase. In preliminary investigations, the assay was only partially reproducible. This paper deals with the development of new molecular diagnostics.

Eight RAPD markers specific for the two biotypes were identified in previous work (Miazzi *et al.*, 2003): OPA-2_{700}, OPD-5_{900}, OPD-7_{550}, OPD-8_{450}, and OPU-12_{420} were specific for the "flag-shoot" biotype; OPD-5_{500}, OPD-7_{1450} and OPU-12_{1150} were specific for the "ascospore" biotype. These were used to derive SCAR (Sequence Characterised Amplified Region) primers. SCAR primers are 20-24-mer oligonucleotides designed on the 3' and 5' regions of the original sequence and have the advantage of higher specificity and reliability than RAPD primers in PCR reactions (Michelmore *et al.*, 1987).

Corresponding author: F. Faretra
Fax: +39.080.5443052
E-mail: faretra@agr.uniba.it




```
  1 gtgattacta ataacggggc gatctgcatt gtcaattggg gattgaaaag ggtcgtaggc
 61 gttgggtgat gcaggatcgt atagagtagg aagaggattt ttagaattca tattgttttt
121 tgtattgctt tatatgcttt gctgaataga caaaacaata ttaaattcag tggtaagtat
181 gtataatggc ttcgcatgta ataagtttgt atgtgtatag tttgacctgg gtggcc
```

Fig. 1. Electrophoretic patterns obtained by amplification of DNA of *E. necator* isolates belonging to the "flag shoot" and the "ascospore" biotypes with the primer pair F6-F6a, and sequence of the 236-bp SCAR marker specific for the latter (arrowed). M = Size marker (100-bp DNA ladder, New England Bio-Labs, Beverly, USA).

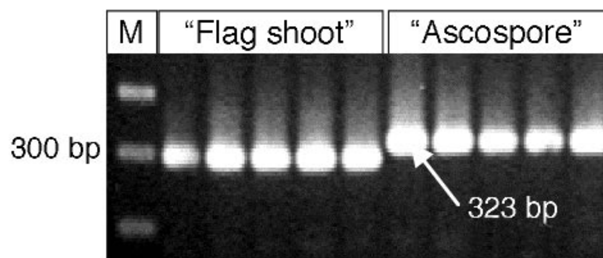
After DNA extraction and PCRs, carried out as described by Miazzi *et al.* (2003), RAPD markers were separated by electrophoresis in agarose gel and recovered by mechanical excision of small gel plugs. DNA was eluted and purified using the Qiaex II Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany). DNA fragments were inserted into an appropriate plasmid vector using pGEMT/Easy Vector System Kit (Promega, Madison, WI, USA), suitable for cloning PCR products. The construct was cloned in the strain DH5α of *Escherichia coli* (Migula) Castellani & Chalmers following standard procedures (Maniatis *et al.*, 1982). Plasmid DNA was extracted from bacteria and purified using the Wizard Plus SV Minipreps DNA Purification System Kit (Promega, Madison, WI, USA). The correct size of each insert was evaluated by digestion of plasmid DNA with *Eco*RI and *Pst*I (Promega Madison, WI, USA), followed by electrophoresis. Cloned RAPD markers were sequenced by MWG Biotech (Florence, Italy).

At first, the cloned sequences were analysed for nucleic acid similarity and homology against EMBL (European Molecular Biology Laboratory, Heidelberg, Germany) fungal nucleotide and protein databases using the FASTA software (version 3.3t09 - May 18, 2001; available at the web site http://www.ebi.ac.uk; Pearson and Lipman, 1988). No significant similarity was found for the RAPD markers except OPD-7_{1450} which was highly similar to the 18S ribosomal RNA gene of other fungi, such as *Microsphaera friestii* var. *dahurica*, *Blumeria graminis* f.sp. *hordei*, *Erysiphe orontii*, *Phyllactinia moricola*, *Uncinula mori*, *Sphaerotheca cucurbitae*, *Leveillula taurica*, *Bulgaria inquinans*, and *Guignardia citricarpa*, identity with the best 50 scores being 95-98%, and 95-99% as ungapped sequence. The 1414-bp sequence of OPD-7_{1450} was deposited in the EMBL-EBI GenBank under accession no. AJ876756.

SCAR primers were designed on the sequence of each RAPD amplicon, using the Primer3 software (Ver. 0.2; Whitehead Institute for Biomedical Research, Cambridge, MA, http://www.ebi.ac.uk; Steve and Helen, 2000) and synthesized by MWG Biotech (Florence, Italy).

Optimal PCR conditions and specificity for each selected SCAR primer pair were evaluated in amplification experiments with genomic DNA of *E. necator* isolates belonging to the two biotypes.

PCRs were performed in 25 ml reaction mixtures containing 2.5 µl 10x $MgCl_2$ Free Reaction Buffer (Sigma, St. Louis, Missouri, USA), 2 mM $MgCl_2$ (Sigma, St. Louis, Missouri, USA), 75 µM each of dATP, dCTP, dGTP and dTTP (Promega Madison, WI, USA), 0.25 µM of each primer, 1 U Red Taq Polymerase (Sigma, St. Louis, Missouri, USA), 2 µl of fungal DNA extract (around 100 ng template DNA), ultrapure water to 25 µl. The thermal cycler (Geneamp PCR System 9700; Perkin Elmer, Norwalk, USA) was programmed as follows: a denaturation phase of 4 min at 95°C; 30 cycles of 30 sec at 94°C, 60 sec at the annealing temperature suitable for each primer pairs, 60 sec at 72°C; and a final extension phase of 7 min at 72°C. Amplification products were loaded on 1.4% agarose gel and run in 0.5xTBE buffer (45 mM Tris-borate, 1 mM Na-EDTA; pH 8) at 110 V for 110 min (Sub-Cell TM, Bio-Rad Laboratories, Hercules, CA, USA). A 100-bp ladder (New England Bio-Labs, Beverly, USA) was used as a size marker. Gels were stained with 1 µg ml^{-1} ethidium bromide. A Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA, USA) and the software package Diversity Database™ (Ver. 2.1 for Windows; Bio-Rad Laboratories, Hercules, CA, USA) were used for manipulation of gel images and data elaboration.



1 gaagctaaaa atccccgtcg aaaatgacaa aggggatgcg tgtcggtcag ccttgcttgc
61 ctgcctgttc agttgagacc gccacac**tca tcatcatcca tatcatcatt a**tcatcatca
121 acgctggaag ggcggtacta atagccctta attacttgca ggtaacgtac tgaatcaagc
181 aatctcattc cttgccgtcg aagacatgga cggggttcat ttcgtggttt tacaagcaac
241 agcaacaacg acgaagtgct tcgctctagc agaggtcctg gcatccagtc tccattctcc
301 atggattggc tggcagatta gga

Fig. 2. Electrophoretic patterns obtained by amplification of DNA of *E. necator* isolates belonging to the “flag shoot” and the “ascospore” biotypes with the primer pair UnE-UnF and sequence of the 323-bp SCAR marker specific for the latter (arrowed). The 24-bp sequence in bold was present in “ascospore” isolates and deleted in the 299-bp marker of “flag shoot” isolates. M = Size marker (100-bp DNA ladder, New England Bio-Labs, Beverly, USA).

A total of 21 primer pairs from the markers OPA-2_{700}, OPD-7_{550}, OPD-7_{1450}, and OPD-8_{450}, were tested but none distinguished the two biotypes.

Among the four primer pairs from the RAPD markers OPU-12_{420} and OPU-12_{1150} only the pair F6-F6a (5’-GGCCACCCAGGTCAAACTAT-3’; 3’-ACTAATAAC GGGGCGATCTG-5’; optimal annealing temperature: 56°C), from OPU-12_{1150}, distinguished the biotypes producing 2 bands: one of 302 bp was common to both biotypes; a second of 236 bp was specific for isolates belonging to the “ascospore” biotype (Fig. 1). The latter amplification product was cloned and sequenced as described above, and proved identical to the expected region of the OPU-12_{1150} sequence. The 236-bp sequence specific for “ascospore” isolates was deposited in the EMBL-EBI GenBank under accession no. AJ876755.

For the RAPD marker OPD-5_{900}, the insert carried by the construct plasmid proved be 500 bp in size, instead of the expected 900 bp, and had a sequence identical to the OPD-5_{500} amplicon. Hence, the marker was not further considered.

Among the twelve primer pairs from OPD-5_{500}, only the pair UnE-UnF (5’-CCTAATCTGCCAGCCAATCC-3’; 3’-GAAGCTAAAAATCCCCGTCG-5’; optimal annealing temperature: 58°C) distinguished the biotypes yielding major bands of 299 bp and 323 bp, respectively specific for “flag shoot” and “ascospore” isolates (Fig. 2). Both amplicons were cloned and sequenced. They proved to differ only in a 24-bp deletion in the 299-bp amplicon of “flag shoot” isolates. The sequences were deposited in the EMBL-EBI GenBank under accession no. AJ876754 (“flag shoot” isolates) and AJ876753 (“ascospore” isolates).

The primer pairs F6-F6a and UnE-UnF gave no

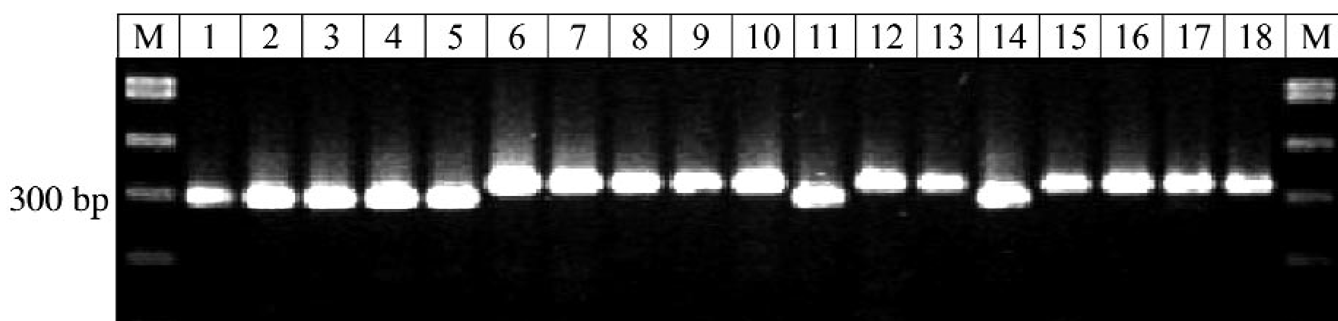


Fig. 3. Example of biotype-specific PCR with the primers UnE-UnF: 1-5 and 14, *E. necator* isolates sampled from typical flag shoots; 6-10, 13, 15-18, isolates likely deriving from ascospores; 11, “flag shoot” representative strain F1; 12, “ascospore” representative strain F3. M = Size marker (100-bp DNA ladder, New England Bio-Labs, Beverly, USA).

product when tested by PCR with DNA of other fungi commonly associated with grapevine [*Botryotinia fuckeliana* (de Bary) Whetz., *Fomitiporia mediterranea* (Fr. *ex* Karsten) M. Fisher, *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. *et* L. Mugnai) Crous *et* Gams, *Phomopsis viticola* (Sacc.) Sacc., *Penicillium* spp., and *Aspergillus* spp.].

Both primer pairs were tested by PCR with DNA extracted from 374 *E. necator* isolates, including 83 "flag shoot" and 291 "ascospore" isolates, previously characterised by RAPD analysis (Miazzi *et al.*, 2003). Both pairs distinguished isolates in agreement with the RAPD data and their origin (Fig. 3).

The role of the "flag shoot" and "ascospore" biotypes in the genetic structure and dynamics of *E. necator* populations and in the epidemiology of grapevine powdery mildew needs further clarification. The biotype-specific SCAR primers described here permit discrimination of the two hypothetic biotypes in a one-step PCR, making it more reliable, more rapid and less expensive than the (NAS) PCR based on single-point mutations in the rDNA ITS or *CYP51* gene sequences previously reported by Dèlye *et al.* (1999). The technique is based on analysis of a single locus associated with the two biotypes sampled in a geographically limited area, but it should prove useful for the large-scale screening of fungal sample now required.

ACKNOWLEDGEMENTS

Research granted by the University of Bari, project "Epidemiology and genetics of phytopathogenic microorganisms", and by CEGBA (Centro di Eccellenza in Genomica Comparata in Campo Biomedico e Agrario), Research Line n. 7. We thank Prof. M.F. Corio-Costet (National Institute of Agricultural Research, Bordeaux, France) who kindly provided us with DNA of representative strains of the two biotypes of *E. necator*.

REFERENCES

Braun U., Takamatsu S., 2000. Phylogeny of *Erysiphe*, *Microsphaera*, *Uncinula* (Erysipheae) and *Cystotheca*, *Podosphaera*, *Sphaerotheca* (Cystotheceae) inferred from rDNA ITS sequences – some taxonomic consequences. *Schlechtendalia* **4**: 1-33.

Cortesi P., Bisiach M., Ricciolini M., Gadoury D.M., 1997. Cleistothecia of *Uncinula necator* – an additional source of inoculum in Italian vineyards. *Plant Disease* **81**: 922-926.

Cortesi P., Ottaviani M.P., Milgroom M.G., 2004. Spatial and genetic analysis of a flag shoot subpopulation of *Erisiphe necator* in Italy. *Phytopathology* **94**: 544-550.

Délye C., Corio-Costet M.F., 1998. Origin of primary infections of grape by *Uncinula necator*: RAPD analysis discriminates two biotypes. *Mycological Research* **102**: 283-288.

Délye C., Corio-Costet M.F., Laigret F., 1997. RAPD analysis provides insight into the biology and epidemiology of *Uncinula necator*. *Phytopathology* **87**: 670-677.

Délye C., Ronchi V., Laigret F., Corio-Costet M.F., 1999. Nested allele specific PCR primers distinguish genetic groups of *Uncinula necator*. *Applied and Environmental Microbiology*, **65**: 3950-3954.

Diehl H.J., Heintz C., 1987. Studies on the generative reproduction of grapevine powdery mildew (*Uncinula necator* (Schw.) Burr.). *Vitis* **26**: 114-122.

Gadoury D.M., Pearson R.C., 1988. Initiation, development, dispersal and survival of cleistothecia of *Uncinula necator* in New York vineyards. *Phytopathology* **78**: 1413-1421.

Gadoury D.M., Pearson R.C., 1990. Germination of ascospores and infection of *Vitis* by *Uncinula necator*. *Phytopathology* **80**: 1198-1203.

Maniatis T., Fritsch E., Sambrook J., 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York, USA.

Miazzi M., Hajjeh H.R., Faretra F., 2003. Observations on the population biology of the grape powdery mildew fungus *Uncinula necator*. *Journal of Plant Pathology* **85**: 123-129.

Michelmore R.W., Hulbert S.H., 1987. Molecular markers for genetic analysis of phytopathogenic fungi. *Annual Review of Phytopathology* **25**: 383-404.

Munshi G.D., Thind T.S., Tajinder S., Chander M., Sokhi S.S., Singh T., Mohan C., 1996. Occurrence of cleistothecia of *Uncinula necator* on grapes. *Plant Disease Research*, **11**: 52-53.

Pearson W.R., Lipman D.J., 1988. Improved tools for biological sequence comparison. *Proceeding of the National Academy of Sciences* **85**: 2444-2448.

Pezet R., Bolay A., 1992. L'oidium de la vigne: situation actuelle et conséquences pour la lutte. *Revue Suisse of Viticulture, Arboriculture and Horticulture* **24**: 67-71.

Sall M.A., Wyrsinski J., 1982. Perennation of powdery mildew in bud of grapevines. *Plant Disease* **66**: 678.

Schneider S., Gadoury D.M., Kassemeyer H.H., 1998. The role of cleistothecia in the epidemiology of grape powdery mildew in Germany. In: Magarey P.A., Thiele S.A., Tschirpig K.L., Emmett R.W., Clarke K., Magarey R.D. (eds.). *Proceedings of the 3rd International Workshop on Grapevine Downy and Powdery Mildew, Loxton, South Australia* 1998, 13-17.

Steinkellner S., 1998. Overwintering of *U. necator* in Austrian vineyards. *Vitis* **37**: 193-194.

Steve R., Helen J.S., 2000. Primer3 on the www for general users and for biologist programmers. In: Krawetz S., Misener S. (eds.). Bioinformatics Methods and Protocols: Methods in Molecular Biology, pp. 365-386. Humana Press, Totowa, New Jersey, USA.

Stummer B.E., Zanker T., Scott E.S., Whisson D.L., 2000. Genetic diversity in populations of *Uncinula necator*: comparison of RFLP- and PCR-based approaches. *Mycological Research* **104**: 44-52.

Received 25 September 2004
Accepted 11 February 2005