

## Identification of grapevine rootstock cv. Börner and differentiation of 125 AA from 5 BB and SO 4

by

F. BAUER\*) and EVA ZYPRIAN

Bundesanstalt für Züchtungsforschung an Kulturpflanzen, Institut für Rebenzüchtung Geilweilerhof, Siebeldingen, Deutschland

**S u m m a r y :** Grapevine rootstock cultivars were investigated for polymorphic RAPD-PCR amplification products. Variety-specific RAPD markers were cloned and partially sequenced. Based on this information longer, sequence-specific primer pairs for PCR under highly stringent conditions were designed and tested. The primer pair Börner-1 yielded a reproducible and specific product of 589 bp from cv. Börner DNA. The second primer pair, 5C-1, derived from an originally cv. 5 C-specific RAPD band, yielded a more complex, but still polymorphic amplification pattern within the five rootstock varieties studied.

**K e y w o r d s :** rootstock, variety identification, specific PCR primers.

### Introduction

Modern wine marketing and trade of planting material require clear criteria for varietal identification in grapevine. Concerning rootstock varieties, ampelographic criteria are hard to apply as only the basal part (root and basal cane) of the plant remains, once they have been used for grafting. For these reasons, development of molecular markers easily scorable and independent of the physiological state and environment are highly desirable.

In recent years, the development of molecular markers as a supplementing or alternative method to ampelography has entered viticulture and will help to clarify identity and parentage of estimated 9,000 different grapevine cultivars world-wide (E. DETTWEILER, pers. comm.). Different strategies to obtain molecular markers have already been applied to rootstock varieties, too. However they still require large amounts of genomic DNA and rather laborious hybridization techniques such as RFLP (BOURQUIN *et al.* 1991; GUERRA and MEREDITH 1995), or highly specialized equipment like semi-automated sequencing machinery for the analysis of polymorphic microsatellite length variants (THOMAS and SCOTT 1993). The RAPD-PCR method has also been applied to rootstocks due to its simplicity of analy-

sis (REGNER and MESSNER 1993). However, this technique suffers from problems in reproducibility due to the sensitivity of the basal biochemical reactions to various experimental parameters (BÜSCHER *et al.* 1993). Usually, RAPD-PCR patterns can not securely be reproduced from one laboratory to another. For this reason we started to develop experimentally stable markers based on sequences obtained from polymorphic RAPD bands for rootstock cultivars frequently used in Germany. Cultivar-characteristic markers produced by longer, sequence-specific primer pairs are easier to obtain than the combination of specific PCR with RFLP (BOURQUIN *et al.* 1995) and bear the potential to be developed into easily manageable diagnosis systems.

### Materials and methods

**P l a n t m a t e r i a l :** The grapevine rootstock cultivars investigated represent 5 frequently used varieties in Germany and are listed in Tab. 1. For DNA preparation field-grown plants from the grapevine collection of the Federal Institute for Grapevine Breeding Geilweilerhof, greenhouse-grown plants or plants propagated *in vitro* under conditions described by ALLEWELDT (1991) were used.

Table 1  
Rootstock varieties investigated

Rootstock	(Abbr.)	Species origin
Geisenheim 5 C	(5 C)	<i>V. Berlandieri</i> x <i>V. riparia</i>
Kober 125 AA	(125 AA)	<i>V. Berlandieri</i> x <i>V. riparia</i>
Kober 5 BB	(5 BB)	<i>V. Berlandieri</i> x <i>V. riparia</i>
Selection Oppenheim No. 4	(SO 4)	<i>V. Berlandieri</i> x <i>V. riparia</i>
Börner		<i>V. riparia</i> 183 G x <i>V. cinerea</i>

Correspondence to: Dr. E. ZYPRIAN, Bundesanstalt für Züchtungsforschung an Kulturpflanzen, Institut für Rebenzüchtung Geilweilerhof, D-76833 Siebeldingen, Germany. Fax: +49-6345-919050. E-mail: e.zyprian@geilweilerhof.suew.shuttle.de

\*) Present address: F. BAUER, Universität Hohenheim, Institut für Lebensmitteltechnologie, D-70593 Stuttgart, Germany.

**DNA extraction:** Regular DNA extraction was carried out on leaves of field- or greenhouse-grown plants. In the case of *in vitro* plants, complete shoots were used. DNA extraction from scrapings of cambial layers of woody shoots and washed root apices from field-grown plants were also usable. The extraction procedure started with shock-freezing the plant material in liquid nitrogen and grinding the frozen material to powder with mortar and pestle. Further processing followed THOMAS *et al.* (1993). The DNA was analyzed for its quality and quantity by agarose gel electrophoresis in comparison to DNA standards derived from *Escherichia coli* phage lambda.

**Cloning of RAPD-PCR products:** RAPD-PCR followed our standard procedure (BÜSCHER *et al.* 1993). Polymorphic bands produced by dekamer primers from Operon Kit F (Operon, Alameda, USA) were cloned by ligating the mixture of amplification products after purification through QIAquick-spin PCR purification columns (Diagen, Hilden, Germany) to pCR Script™ SK(+) vector DNA (Stratagene, Heidelberg, Germany) in the presence of *SrfI* following the suppliers instructions. The ligation mixture was used to transform competent cells of *E. coli* XL1-blue (BULLOCK *et al.* 1987) according to HANAHAN (1983). Recombinant plasmids were selected on LB medium (SAMBROOK *et al.* 1989) supplemented with 100 µg ampicillin ° ml<sup>-1</sup> (Biomol, Hamburg, Germany) and 20 µl of 0.2 M 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Biomol) as well as 20 µl of 10 % isopropyl-β-D-thiogalactopyranosid (Serva, Heidelberg, Germany) in dimethylformamide spread per 9 cm diameter agar plate. White colonies were patched onto fresh selective plates and used for plasmid mini screens following essentially the procedure of BIRNBOIM and DOLY (1979). The structure of the recombinant plasmids was analyzed by restriction analysis. Plasmid DNA to be used for sequencing was purified through the QIA prep-spin plasmid preparation kit (Diagen).

**Labeling of cloned amplification products for use as hybridization probes:** Cloned amplification products were excised from the respective recombinant plasmid DNA by restriction with *SacI* and *SmaI* or *SmaI* only. The released fragments were purified by gelelectrophoretic resolution on 1 % agarose in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.8) and recovered by electroelution into dialysis bags (Medicell, London, UK) containing TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The fragments were subjected to a phenol/chloroform extraction and concentrated by precipitation in 70 % ethanol in the presence of 0.3 M sodium acetate (pH 6.0). The DNA pellets were dissolved in 20 µl of TE buffer.

Non-radioactive labeling used the DIG DNA labeling kit (Boehringer Mannheim, Mannheim, Germany) with random priming by hexanucleotides according to the supplier's instructions.

**Southern blot and hybridization:** Amplification products were resolved on standard agarose gels (1–2 %), stained with ethidium bromide (1 µg ° ml<sup>-1</sup> for 10 min) and photographed. The DNA was prepared for

capillary blot by soaking the gel for 20 min in 0.25 N HCl, followed by 30 min in 1.5 M NaCl, 0.5 N NaOH and neutralizing two times 15 min in 1.5 M NaCl, 0.5 M Tris-Cl, 1 mM EDTA, pH 7.2. Transfer onto nylon membranes (Hybond N, Amersham-Buchler, Braunschweig, Germany) was in 20x SSC as described by SAMBROOK *et al.* (1989). The DNA was fixed to the membrane by baking at 80 °C for 2 h. Hybridizations were carried out in DIG Easy Hyb solution (Boehringer Mannheim) as described by the supplier. Detection of hybridizing signals employed anti-digoxigenin-fab fragments coupled with alkaline phosphatase and CDP-Star™ (Boehringer Mannheim). Finally, chemiluminescence was recorded by exposing the membranes to X-ray film (AGFA CurixRP 1000G, AGFA-Gevaert, Leverkusen, Germany) for several seconds up to 1 min.

**DNA sequencing:** Sequence analysis of the inserted fragments was done extending the standard sequencing and reverse sequencing M13 primers in cycle sequencing reactions (Dig Taq DNA sequencing kit for standard and cycle sequencing, Boehringer Mannheim). Reaction products were analyzed on a GATC 1500 device for direct blotting from sequencing gels onto appropriate membranes (MWG Biotech, Ebersberg, Germany) according to the manual of this apparatus. Bands were detected basically as described in the hybridizations, but final visualization was with a colorimetric substrate for alkaline phosphatase (nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt; Boehringer Mannheim). Sequence records were analyzed with DNASIS™ (Hitachi, Olivet, France).

**Sequence-specific primers and PCR conditions:** PCR reactions with the specific primers outlined in Tab. 3 were carried out in 50 µl assay volumes containing 100 ng of genomic DNA, 100 pM of each oligonucleotide primer, 100 µM of each desoxynucleoside-triphosphate and 1 unit Taq DNA polymerase (Boehringer Mannheim) in PCR buffer (10 mM Tris-Cl, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, pH 8.3). The mixture was overlaid with 30 µl of mineral oil and denatured initially for 5 min at 94 °C. The following reaction cycles were repeated 30 times: 1 min denaturation at 94 °C, 1 min primer annealing at the temperature indicated in Tab. 3, 1.5 min. DNA synthesis at 72 °C with the ramping of the thermocycler (Hybaid Omni Gene TR3 CM, MWG Biotech) set at 2 s °C<sup>-1</sup>. PCR products were analyzed on 1.5 % agarose (FMC SeaKem LE, Biozym, Oldendorf, Germany) gels.

## Results

**Cloning of RAPD products:** Preliminary experiments testing the 20 dekamer primers of Operon Kit F had produced a total of 143 bands in the size range of 200–2000 bp, 33 % of which had revealed polymorphic RAPD PCR amplification products within the 5 rootstock varieties tested (ZYPRIAN, unpublished data and BÜSCHER *et al.* 1994). From this first screening we chose primers OP-F17 and OP-F18 for the amplification of variety-specific bands.

The following RAPD-PCR products were cloned into pCR Script™: A 620 bp OP-F17 product from cv. Börner (Fig. 1 a) and a 590 bp OP-F18 product obtained from cv. 5 C under reduced annealing (30 instead of 36 °C, Fig. 2 a).

The recombinant plasmids were characterized by restriction analysis using enzymes whose recognition sequences reside in the dekamer primer (*Sma*I in OP-F17 and OP-F18) and in the sequences directly flanking the insertion site in the vector.

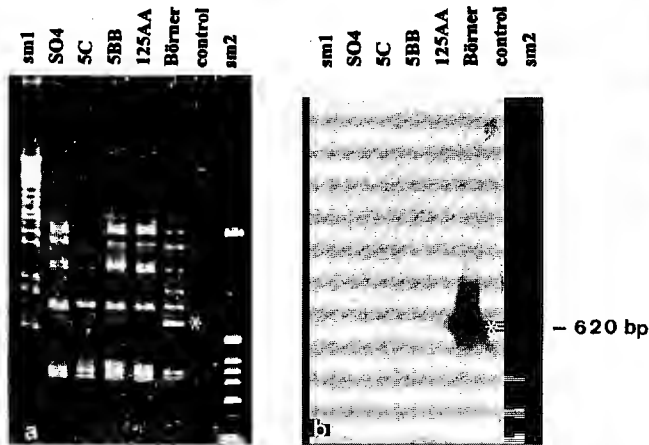


Fig. 1: RAPD-PCR products of OP-F17 resolved on 1.5 % agarose (a) and the blotted gel hybridized with the 611 bp cloned OP-F17 product from Börner (b). Detection was with the CDP™-Star (Boehringer Mannheim) substrate of alkaline phosphatase and luminescence recorded by 10 s exposition to X-ray film. Lane sm1 corresponds to a size marker obtained by digestion of phage lambda DNA with *Eco*RI and *Hind*III (fragment sizes 21226 bp, 5143 and 4973 bp, 2027 bp, 1904 bp, 1584 bp, 1375 bp, 947 bp, 831 bp and 564 bp; faint bands represent partial cleavage products); sm2 is a size marker obtained by *Hin*fI digestion of plasmid pBR322 DNA (fragment sizes 1629 bp, 517/507 bp, 396 bp, 344 bp, 298 bp, 221/220 bp, 154 bp). The cloned product from cv. Börner labeled as probe detected specifically its original RAPD band. This band and the hybridization signal are labeled by asterisks.

**Cultivar specificity of cloned RAPD-PCR products:** In order to recheck the cultivar specificity of the cloned RAPD-PCR products, the inserts were labeled and hybridized to blots of the RAPD-PCR products obtained with the corresponding dekamer oligonucleotides from all 5 varieties. When the cloned 620 bp product of OP-F17 from cv. Börner was hybridized to the OP-F17 amplifications of all varieties, a unique and strong signal of appropriate size was obtained only with cv. Börner amplicates (Fig. 1 b). In the same way the cloned 590 bp OP-F18 product from cv. 5 C hybridized exclusively to the corresponding amplicate of cv. 5 C (Fig. 2 b). This indicates that these inserts are highly specific.

**Sequencing and design of sequence-specific longer primers:** The cloned variety-specific RAPD-PCR products were partially sequenced from both ends. Between 320 and 360 bases from either binding site of the sequencing primers could be obtained. The small inserts could hence be matched to the complete sequence.

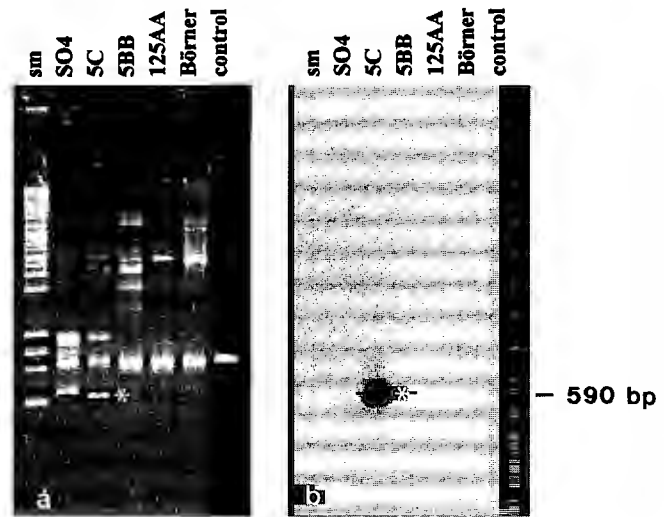


Fig. 2: RAPD-PCR products of OP-F18 resolved on 1.5 % agarose (a) and blotted gel hybridized with the cloned 590 bp product of OP-F18 from cv. 5 C (b). Its size is indicated. Detection of the hybridization signal was as in Fig.1b, exposition on X-ray film lasted 30 s. Lane sm corresponds to sm1 in Fig.1a. Hybridization was specific for the 590 bp product of cv. 5 C (labeled by an asterisk) obtained under reduced annealing (30 °C) during the cycling reactions of the RAPD-PCR.

The originally employed RAPD dekamer primers appeared unchanged in sequence in the cloned products.

The sequences have been deposited in the EBI (EMBO) database and are available by accession numbers (Y14787 and Y14788). The exact size of the cloned bands and their GC content was determined (cf. Tab. 2). Analysis with the program DNASIS™ did not indicate any long open reading frames nor a strong potential for the formation of secondary structures (on RNA level). Searches with “blast” (ALTSCHUL *et al.* 1990) at the NIH database did not provide significant matches to known sequences. The sequences were used to design longer, specific primer pairs (Tab. 3) enabling variety-specific marker synthesis under stringent PCR conditions.

Table 2

Length and GC content of sequenced RAPD-PCR products		
Product	GC content %	Length bp
620 bp OP-F17 from cv. Börner	42	611
590 bp OP-F18 from cv. 5 C	38	584

**Test of sequence-specific primers:** The specific primer pairs were tested in highly stringent PCR protocols (cf. Tab. 3) with the genomic DNA of the 5 rootstock varieties. Primer pair Börner-1 produced a 580 bp product specific for cv. Börner (Fig. 3 a). Control hybridization with the cloned insert resulted in a single specific signal (Fig. 3 b). The same product was obtained by amplification with *Taq* DNA polymerase from a different supplier (Eurogentec Red Goldstar, Seraing, Belgium; data not shown).

Table 3  
Specific oligonucleotide primers designed

Oligo designation	Rootstock cultivar	GC content %	Melting temperature, °C	Annealing temperature, °C	Product size, bp
Börner-1U: 5'CCCGGGAACATAGGAAGGAAC3' Börner-1O: 4'TCTCCACACCGGGGAAATCAG3' from cv. Börner OP OP-F17 611 bp product	Börner	57	66	70	589
5C-1U: 5'CCCGGGTTCATAGGAAGCATCAGG3' 5C-1O: 5'CCCGGGTTATGCAGGGGAGGTG3' from cv. 5 C OP-F18 584 bp product	5 C	54	74	67	584
		68	74	67	

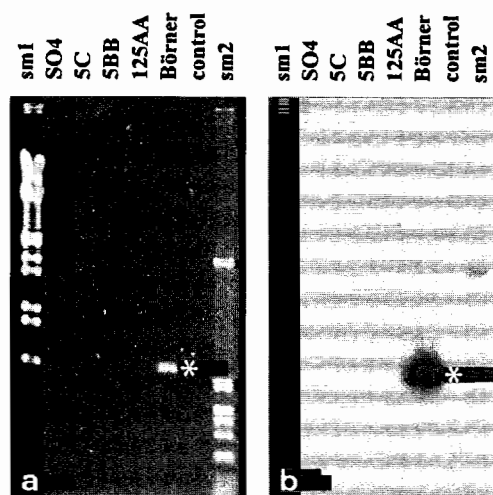


Fig. 3: PCR product of the specific primer pair Börner-1 derived from sequence analysis of the OP-F17 RAPD product of 611 bp from the cv. Börner (1.5 % agarose gels, sm1 and sm2 as in Fig. 1). PCR was carried out. (a): Specific amplification product; (b) shows the blot and hybridization of the cloned OP-F17 Börner product (X-ray exposition in the final detection step was 30 s). Note that the product of PCR with the longer, sequence-specific primer pair Börner-1 is only 589 bp in size due to the position of the binding sites of these primers within the sequence analysed.

However, the primer pair 5C-1 produced unexpected results. A monomorphic product of 580 bp was not only obtained from 5 C, but also from the other 4 varieties. The rootstock cvs 125 AA and Börner exhibited both a smaller, and 125 AA an even slightly larger additional band (Fig. 4 a). This pattern could not be improved by raising the annealing temperature in the cycling reactions. The 5C-1 product is thus not cultivar-specific but still can provide some informative polymorphism between the rootstocks. Hybridizations with the cloned original product showed homology between all the bands around 580 bp, while the smaller one did not light up (Fig. 4 b).

### Discussion

Within the 5 different rootstock varieties analysed, a sequence-specific molecular marker indicative of the cv. Börner has been identified. Amplification with primer pair Börner-1 under stringent conditions led to a unique and reproducible band of 589 bp.

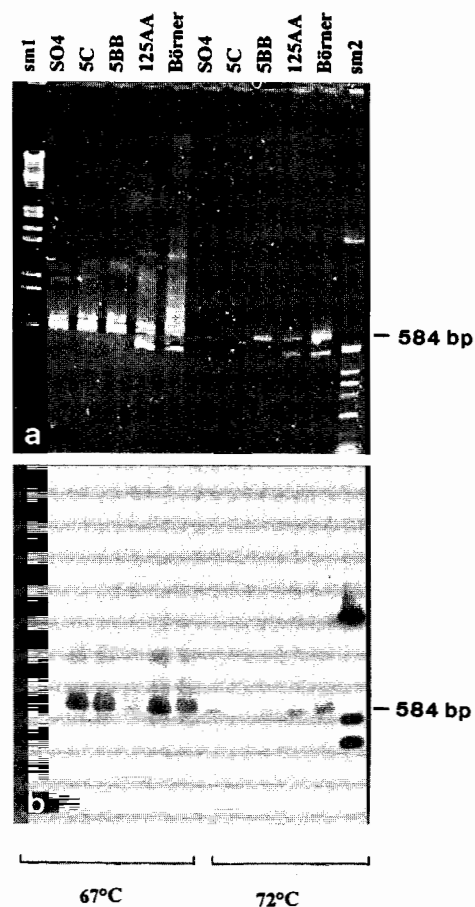


Fig 4: PCR products of the sequence-specific primer pair 5C-1 at two different annealing temperatures (a) as indicated on the bottom. The PCR products were resolved on 1.5 % agarose; sm1 and sm2 correspond to the size markers in Fig. 1. (b) shows the results of hybridization analysis using the originally cloned OP-F18 product from cv. 5 C as probe (detection as in Fig.1 b, X-ray exposition was for 30 s). The major PCR product appears as double band of 584 bp at 67 °C annealing; cvs 125 AA and Börner exhibit additional bands. At elevated annealing temperatures basically the same pattern is obtained with the exception of cv. 5 C, where any product is missing.

Börner differs from the other rootstock cultivars analyzed here by its descentance (cf. Tab. 2). This may explain the ease by which a specific marker could be obtained for this cultivar. In preliminary studies to differentiate the rootstocks by RAPD-PCR as mentioned above, Börner exhibited a rather high level of polymorphism. 15 %

of the polymorphic bands were exclusively polymorphic for Börner, and all of the polymorphic bands observed yielded polymorphisms with Börner in addition to one or two of the other varieties. The other varieties (all derived from *V. berlandieri* x *V. riparia*) showed a rather low level of polymorphisms, despite of their long selection (own unpubl. data).

As *V. riparia* is a parent shared by all the 5 rootstocks and Börner differs only by its ancestor *V. cinerea*, it is tempting to speculate, that the Börner-specific marker represents genetic material from *V. cinerea*. Future studies on the distribution of this marker and possible polymorphisms between *Vitis* wild species are needed to confirm this assumption.

The hybridization assays with the cloned cv. Börner OP-F17 611 bp and the cloned cv. 5 C OP-F18 584 bp markers to total RAPD gels of the corresponding dekamers indicated cultivar specificity. PARAN and MICHELMORE (1993) described a very similar strategy as used here the first time for lettuce. They performed hybridizations of cloned RAPD bands to original RAPD products in a segregating population claiming that multiple DNA species may be "hidden" within one RAPD band. This way a "minor species" not necessarily corresponding to the major visible product, may be cloned. In our case, we had no appropriate segregating populations for this purpose available yet.

The originally cv. 5 C-specific amplification product of 584 bp synthesized from OP-F18 at reduced annealing temperature (30 °C) caused other problems, when longer primers based on its sequence (5C-1) were tested. Under regular stringency of PCR (5-10 °C under TM), it produced a monomorphic double band with homology to the originally cloned marker. In addition, smaller, non-homologous products were obtained for cvs 125 AA and Börner. When stringency was raised by increasing the annealing temperature, this pattern remained basically unchanged with one exception: The cv. 5 C, where the marker had been derived from, did no longer show a visible product nor a hybridization signal. This fact may be explained by only partial genomic complementarity of cv. 5 C to 5C-1, possibly provoked by the reduced annealing originally used in this case. Loss of polymorphism has also been described in a similar approach on American rootstock varieties by XU *et al.* (1995).

In conclusion, two sequence-specific markers useful for the identification and differentiation of grapevine rootstock varieties have been developed. Their discriminative potential is presented as follows:

	Börner	SO 4	5 C	5 BB	125 AA
Börner-1 70 °C 589 bp	+	-	-	-	-
5 C-1 67 °C 500 bp	+	-	-	-	+
5 C-1 72 °C 584 bp	+	+	-	+	+

## Acknowledgements

We thank CLAUDIA WELSCH and DORIS SCHNEIDER for help in the photographic reproduction of the figures.

## References

- ALLEWELDT, G.; 1991: Die *in vitro*-Kultur der Rebe. BL-Journal 1 (2), 35-38.
- ALTSCHUL, S.F.; GISH, W.; MILLER, W.; MYERS, E. W.; LIPMAN, D.J.; 1990: Basic local alignment search tool. J. Mol. Biol. 215, 403-410.
- BIRNBOIM, H.C.; DOLY, J.; 1979: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. 7, 1513-1523.
- BÖHM, A.; ZYPRIAN, E.; 1997: A RAPD marker in grapevine (*Vitis* sp.) similar to plant retrotransposons. Plant Cell Rep. (in press)
- BOURQUIN, J.C.; OTTEN, L.; WALTER, B.; 1991: Identification of grapevine rootstocks by RFLP. C.R. Acad. Sci. Paris 312, Sér. III, 593-598.
- — — — —; 1995: PCR-RFLP analysis of *Vitis*, *Ampelopsis* and *Parthenocissus* and its application to the identification of rootstocks. Vitis 34, 103 - 108.
- BÜSCHER, N.; TSCHAMMER, J.; ZYPRIAN, E.; 1994: Search for molecular markers and their application in cultivar identification as well as marker-assisted selection. Proc. VIth Intern. Symp. on Grape Breeding, Yalta, Crimea, Ukraine, Sep. 4-10, 1994, 99-105.
- — — — —; ZYPRIAN, E.; BLAICH, R.; 1993: Identification of grapevine cultivars by DNA analyses: Pitfalls of random amplified polymorphic DNA techniques using 10mer primers. Vitis 32, 187-188.
- BULLOCK, W.O.; FERNANDEZ, J.M.; SHORT, J.M.; 1987: A high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques 5, 376
- GUERRA B.; MEREDITH, C.P.; 1995: Comparison of *Vitis Berlandieri* x *Vitis riparia* rootstock cultivars by restriction fragment length polymorphism analysis. Vitis 34, 109 - 112.
- HANAHAN, D.; 1983: Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166, 557-580
- MULLINS, M.G.; BOUQUET, A.; WILLIAMS, L.E.; 1992: Biology of the Grapevine. Cambridge University Press, Cambridge, UK.
- PARAN, I.; MICHELMORE, R.W.; 1993: Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genet. 85, 985-993.
- REGNER, F.; MESSNER, R.; 1993: Molekulare Differenzierung von Rebsorten mittels RAPD-Analyse. Mitt. Klosterneuburg 43, 160-164.
- SAMBROOK, J.; FRITSCH, E.F.; MANIATIS, T.; 1989: Molecular Cloning. A Laboratory Manual. 2nd edition. Cold Spring Harbor Laboratory Press, New York.
- STAUB, J.E.; SERQUEN, F.C.; GUPTA, M.; 1996: Genetic markers, map construction, and their application in plant breeding. HortScience 31, 729-740.
- THOMAS, M.R.; MATSUMOTO, S.; CAIN, P.; SCOTT, N.S.; 1993: Repetitive DNA of grapevine: Classes present and sequences suitable for cultivar identification. Theor. Appl. Genet. 86, 173-180.
- — — — —; SCOTT, N.S.; 1993: Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs). Theor. Appl. Genet. 86, 985-990.
- XU, H.; WILSON, D.J.; ARULSEKAR, S.; BAKALINSKY, A.T.; 1995: Sequence-specific polymerase chain-reaction markers derived from randomly amplified polymorphic DNA markers for fingerprinting grape (*Vitis*) rootstocks. J. Amer. Soc. Hort. Sci. 120, 714-720.

Received September 17, 1997