A First Pedigree-Based Analysis (PBA) Approach for the Dissection of Disease Resistance Traits in Grapevine Hybrids

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Abstract

To date, molecular markers are available for many economically important traits in grapevine (*Vitis vinifera* L.) as well as in other fruit crops. Unfortunately, lack of knowledge of the allelic variation of the related genes hampers their full exploitation in commercial breeding programs. These markers have usually been identified in a single cross. Consequently, only one or two favourable alleles of the related quantitative trait loci (QTL) are identified and exploitable for marker-assisted breeding (MAB), whereas a breeding program may include several alleles. Our novel employed approach, namely pedigree-based analysis (PBA), allows for the identification and exploitation of most alleles present in an ongoing breeding program. This is achieved by including breeding material itself in QTL detection, thus covering multiple generations and linking many crosses through their common ancestors in the pedigree. In this study we focus on the genetic characterization of numerous *Vitis* hybrids, donors of resistance to downy and/or powdery mildew, in order to identify selection signatures. In particular, 120 hybrids of American origin and 100 European hybrids, along with 44 related *V. vinifera* cultivars, have been genotyped at 190 SSR loci. These markers have been chosen based on their physical distance in order to have 10 SSRs well-scattered along each chromosome. Based on the SSR profiles, the historical pedigree records have been verified. Following the trueness-to-type validation for most of the studied hybrids, the identity-by-descent (IBD) analysis has been performed, tracing the allelic flow through the successive generations. Here we report results about the identification of non-*vinifera* genomic intervals retained into the *vinifera* background and the anchoring of the discovered breeding signatures to the grapevine genome. In addition, we report on the PBA-based QTL results for segregating populations coming from pedigree-supported parental genotypes donors of resistance to downy mildew in north-eastern Italy.

INTRODUCTION

Viticulture has been often plagued by encountering new parasites that still represent a major constraint. This is a particularly important issue because, even though some interspecific cultivars (hybrids) between *Vitis vinifera* L. and *Vitis* spp. are widely present, the majority of cultivated grapevines are pure *V. vinifera* cultivars, which are highly sensible to pathogen attack. The most severe pathogen include fungi or oomycetes, such as downy mildew, powdery mildew, gray mould, and bacteria, virus or other organisms transmitted by insects or nematodes, such as Pierce's disease, *fanleaf virus* and flavescence dorée. Concerning cryptogamic pathologies, investigations concentrate mostly on downy mildew (DM), caused by the oomycete *Plasmopara viticola*, and

powdery mildew (PM), caused by the ascomycete *Erysiphe necator*, which are considered of primary importance. Recently, the emergence of a DM strain adapted to overcome a newly employed resistant hybrid has been reported (Peressotti et al., 2010); this highlights the urgent need to identify different mechanisms of resistance through the screening of large populations of resistant wild grapes or particularly resistant hybrids as sources of novel resistances. The constitution of a "library" of resistant sources will boost the opportunity for gene pyramiding, which relies on the combination of multiple genes in a cultivar to reach a broad spectrum and therefore potential durable resistance (Joshi and Nayak, 2010). In fact, the development of a broad spectrum resistance can only be achieved through pyramiding different loci derived from a wide genetic background within the *Vitis* genus. This superior genotype will represent a pre-breeding product that will be employed as a donor of durable resistances in subsequent breeding programs taking into account also quality traits (Topfer et al., 2011).

Grapevine genetics and genomics information, and associated technologies, are developing rapidly, leading to numerous discoveries with potential application (Di Gaspero and Cattonaro, 2010). In 2012, not only genetic and physical maps are available, but also the genome of both a highly heterozygous cultivar (Pinot noir ENTAV 115) and a near-homozygous line (PN40024) has being sequenced (Velasco et al., 2007; Jaillon et al., 2007). In addition, several quantitative trait loci (QTL) and major genes have been identified, also concerning the most relevant disease resistances. Loci associated with DM and PM have been detected in different genetic backgrounds and most of them are particular for a specific genotype (VIVC 2014). Molecular alternatives include association studies and genomic selection (Jannink et al., 2010). Despite these advances in grapevine genetics and genomics, traditional breeding approaches are still dominant, while Marker-Assisted Breeding (MAB) implementation has been minimal. Traditional breeding is a time consuming process and requires intensive greenhouse and/or extensive field space. Application of genetic markers is a great opportunity to accelerate breeding decision making, but has been limited by significant technical, logistical and informational barriers.

Here we used a novel approach called pedigree genotyping or pedigree-based analysis (PBA) (Van de Weg et al., 2004; Bink et al., 2007) in order to dissect the genetic basis of DM and, in the near future, of PM resistance. The research efforts were directed to efficiently find allele-trait associations mainly in already existing breeding germplasm, enabling a cost- and time-effective implementation of MAB in grape breeding program.

MATERIALS AND METHODS

Grapevine Genetic Material

A total of 682 grapevine accessions were used in this study and included selected parents as donors of resistances against important pathogens (DM and/or PM), along with their putative ancestors (founders), and F_1 individuals obtained breeding several identified pedigree-supported parental genotypes.

1. Parents and Putative Ancestors. This 264 sample set consists of 120 *Vitis* hybrids of American origin, 100 *Vitis* hybrids of European origin, and 44 *V. vinifera* cultivars mainly employed in breeding programs and present in the hybrid pedigree. In order to reconstruct the pedigree of these studied genotypes, the historical information on these European and American hybrids was studied and collected into a database, which is constantly updated. Except for some American hybrids, this genetic plant material is mainly available and maintained at FEM (IT). In few cases it was necessary to recover some ancestral DNA to increase the depth of the phylogenetic tree and to enhance the power of the statistical analysis.

2. Segregating Populations. This 418 sample set refers to F_1 individuals belonging to nine segregating populations (*V. vinifera* × *Vitis* hybrid) obtained from the crossing combinations of 13 parental genotypes. These nine progenies were obtained during 2011 and 2012 by the FEM traditional breeding activities and sowed at the beginning of 2013.

DNA Extraction and Medium-Throughput Genotyping Analysis

Genomic DNA from the 682 overall studied accessions was isolated from young leaves using DNeasy Plant Mini Kit (Qiagen, The Netherlands).

1. Parents and Putative Ancestors. 264 genotypes were analysed at 190 SSR loci, in terms of an average of 10 SSRs well-scattered along each of the 19 grapevine chromosomes, in order to generate its backbone. The microsatellites were chosen based on their unique position and physical distance along the reference genome (http://www.genoscope.cns.fr). The applied medium-throughput genotyping strategy was as follows: i) per each SSR locus the allelic size range was identified by a simplex assay on 96 representative hybrids (Hot Start Taq, Qiagen); ii) the retrieved information was used to design a multiplexing protocol based on the principles reported by Migliaro et al. (2012) for the reduction of both time and costs; iii) all genotypes were analyzed based on the optimized multiplex reactions (4 SSRs/multiplex average) using the Type-it Microsatellite PCR Kit (Qiagen). Capillary electrophoresis was carried out in an ABI 3130xl Genetic Analyzer (Life Tech, Foster City, CA) and the fragments were sized with GeneMapper 4.0 in binning mode, using GeneScan 500LIZ size standard as an internal ladder (Life Tech).

2. Segregating Populations. 418 F_1 individuals were analysed at 80 (sub-set of the overall 190) SSR loci in order to screen an average of 10 well-scattered SSR loci along each of the 8 chromosomes, where the major QTLs for DM resistance (VIVC 2014) and clusters of Resistance Gene Analogs (RGAs) have been identified (Di Gaspero et al., 2007; Velasco et al., 2007; Malacarne et al., 2012). The adopted medium-throughput genotyping strategy was as described above.

High-Throughput Phenotyping Analysis

During 2013 a phenotyping analysis for DM resistance was carried out, but our goal is to extend this novel approach in order to also study PM resistance. Since the beginning of 2014 a high-throughput phenotyping method for PM symptoms assessment is under implementation and optimization.

1. Parents and Putative Ancestors. For all the genetic plant material grown and maintained at FEM, disease resistance records were available and strongly considered (Vecchione et al., in preparation). The DM resistance information for American hybrids present at Cornell University (NY) also taken into account (B.I. Reisch, 2011, pers. commun.). This general prior information was relevant to initially select genotypes, but was not included in the final genotype-phenotype association, given the difference in phenotyping methods and DM strains.

2. Segregating Populations. A total of 418 F_1 individuals, 13 parental and 4 control genotypes were phenotyped for DM resistance. Population size was rather variable, depending on germination rate and success of controlled pollinations. Controls, chosen based on literature where their resistance type has been thoroughly described, were two susceptible, one resistant and one mid-resistant genotype (‘Pinot Noir’, ‘Muscat Ottonel’, *V. riparia* ‘Gloire de Montpellier’ and *V. rupestris* ‘du Lot’, respectively). Parental genotypes, as wood cuttings, and progeny individuals, as seedlings, were grown in the same greenhouse at 25°C and natural light conditions. The adopted high-throughput phenotyping method was inspired from previous protocols on DM assessment (Peressotti et al., 2011; Miclot et al., 2012), and subsequently optimized based on FEM facilities and objectives. DM infection was performed with an inoculum of the same origins in a concentration of 100,000 sp/ml. These spores were collected in the summer 2012, sampling oil spots from natural infections on different cultivars of *V. vinifera*, in a non-treated experimental field at FEM. The inoculum, preserved at -20°C, was propagated on *vinifera* × *vinifera* seedlings one week before each planned bioassay. For each genotype leaf 4 and 5 (from the apex) were sampled for laboratory analysis and rinsed in distilled water. Two discs of 2 cm diameter were excised and put in petri dishes with the abaxial surface up until inoculation. The bottom of all dishes was covered in advance with filter paper damped with 5 ml sterile distilled water kept at 4°C. Discs were then sprayed with a

spore suspension of 100,000 sp/ml. Soon after infection samples were incubated at 21°C in a growth chamber and kept in total dark conditions for the first 3 days post infection (dpi). Afterward conditions switched in a 16/8 h (light/dark) photoperiod. Prior to plant topping, the whole experimental sampling was repeated once later in the season, to obtain a final scoring of 8 data point per genotype per observation. Visual observation of DM symptoms was carried out at 4 and 6 dpi. The degree of infection was evaluated according to two parameters: sporulation density and disease progress; this latter represents an adaptation of the OIV 452 for leaf disc bioassay. Evaluation of classes of resistance for each genotype plant was conducted on the basis of the survey of each single leaf disc and then averaged for data analysis. To represent more precisely a particular phenotype the two parameters were integrated into a third one. Finally, all three parameters were taken into account for the genotype-phenotype association analysis.

Pedigree-Based Analysis

Applied for the first time in grapevine and new also to several other fruit trees, pedigree-based analysis (PBA) is an existing statistical framework with associated software (FlexQTL; Bink, 2005) designed to identify, validate, and use QTL information from pedigree-linked individuals to inform breeding decision-making.

1. Pedigree Validation and Identity-by-Descent Probability. The pedigree validation and the identity-by-descent (IBD) analysis were performed with the first module of the FlexQTLTM software. The marker consistency check represented a basic step for the pedigree confirmation and relied on ten consistency codes (e.g., observed and consistent, missing and augmented) that were used to trace and solve errors. Thus, the IBD probabilities were estimated to identify the non-*vinifera* genomic intervals retained into the *vinifera* background and to detect those ones fixed by selection (breeding signatures). In particular, the principle of IBD was employed to express the identity of a modern selection allele in terms of the founding cultivars alleles, which was robustly used as factors in statistical analyses in case of a minimum number of three generations. Pedigree and allelic flow through successive generations were visualized with the Pedimap software (Voorrips, 2012). These analyses were carried out on the studied parents and putative ancestors (264 accessions) with the final aim to select all available pedigree-supported parental genotypes to be employed in the following PBA-based QTL analysis.

2. Genotype-Phenotype Association. The PBA-based QTL analysis was performed with the complete FlexQTLTM software which adopts a Bayesian statistics. The genotype-phenotype association was performed on the basis of the genotypic data of the nine segregating populations (418 individuals), their 13 pedigree-supported parental genotypes and their validated ancestors, along with the phenotypic data (DM resistance parameters) recorded for the nine progenies and their parents (the ancestors phenotypes were not necessary).

RESULTS AND DISCUSSION

SSR Analysis

Despite the fact that many SSR loci are available for grapevine, literature on SSR profile quality and allele range is deficient concerning *Vitis* spp. and hybrids. Therefore, we have decided to amplify all studied SSRs in a simplex assay on 96 genotypes considered representative on the basis of their geographical origin. Firstly, this has allowed us to discard or substitute SSRs that resulted in multilocus signatures or those with no clear amplification. Secondly, this preliminary genetic analysis enabled the retrieval of the characteristic allele range that was exploited to group markers in multiplex reactions (Fig. 1). Regarding the 264 sample set, final SSR profiles at 190 loci showed an average of 13 alleles per locus, highlighting the high informativeness of the selected SSR markers. Concerning the 418 F_1 individuals, they were genotyped at 80 SSR loci enabling the identification of few self- and out-crossed genotypes. In both datasets, SNP markers could be subsequently exploited to saturate specific regions of interest.

Downy Mildew Resistance Assessment

Our results showed that the studied segregating populations were differently allocated in the resistance classes. In some populations, progeny numbers were too low to establish a confident distribution tendency, as the weak number of individuals introduces a strong bias. However, even in the more numerous progenies (pop A and pop I) it was clear that there was no random dispersion, but a trend to follow a binomial or normal distribution (Fig. 2). This might depend on the different resistance sources inherited in the population and suggests that genes with different level of influence and interaction may lay underneath the studied resistance mechanisms. We aimed at developing a high-throughput method both to uniformly phenotype various segregating populations and to obtain a few targeted parameters, representing different important steps in the disease progression and resistance evaluation.

Pedigree-Based Analysis

With this study we intended to i) discover the genomic intervals fixed by the selection procedure, namely breeding signatures, by means of each founder allele contribution, ii) identify QTLs for DM resistance, exploiting a pool of pedigree-supported parental genotypes and linking several segregating populations through these common parents and their ancestors. Therefore, this research allowed for, firstly, the identification of the fixed haplotypes present in the studied germplasm (parents and putative ancestors, partially employed in the ongoing FEM breeding program) and, secondly, the proof-of-concept in grapevine of a novel and powerful genetic tool as the PBA-based QTL analysis (segregating populations).

1. Pedigree Confirmation and Breeding Signature Identification. Following the marker consistency check, SSRs with an error >10% and genotypes with an error >15% were deleted from the initial dataset of 190 loci and 264 accessions, respectively. The trueness-to-type of the remaining genotypes was verified with 9 reference SSRs thanks to the data available at the VIVC database (www.vivc.de) or, mainly, by the historical pedigree information. In doubtful cases, we took into account several marker positions and, finally, we validated the genetic profile of 178 genotypes. On these latter, a comprehensive IBD analysis was performed based on 185 robust SSR loci (Fig. 3). We then highlighted the allelic flow through successive generations identifying the ancestral (mainly non-*vinifera*) genomic intervals more frequently retained into the *vinifera* background (breeding signatures) (Fig. 4). Thanks to the uniquely positioned studied SSRs, the discovered breeding signatures were straightforwardly characterized by their anchoring to the published grapevine genomes (Jaillon et al., 2007; Velasco et al., 2007). The final confirmation of the selection signatures potential for MAB purpose will be reached by their validation on ad hoc segregating populations already available or produced worldwide.

2. PBA-Based QTL Identification. All pedigree-supported parental genotypes available at FEM were identified. Within this material, based on their DM resistance level and quality attributes 13 parental genotypes were selected to produce *V. vinifera* × *Vitis* hybrid crosses, leading to 418 F_1 individuals. The PBA-based QTL analysis with 80 SSR loci distributed along 8 selected chromosomes (as described above) resulted in the identification of 3 major QTLs on the overall DM resistance sources, with associated markers. These markers have usually been identified in one single cross. Consequently, only one or two favourable alleles of the related QTL are identified and exploitable for marker-assisted breeding, whereas a breeding program may include several alleles. Selection for just these alleles means that many favourable genotypes are ignored, which decreases efficiency and leads to genetic erosion.

CONCLUSIONS

Our results will boost implementation of marker-assisted pre-breeding applied to DM and, in the near future, also PM, increasing the efficiency of releasing grape cultivars with durable resistance and the successful adoption of interspecific cultivars. We trust that

the integration of modern genetics, genomics and biostatistics tools with pathology and traditional breeding approaches will transform crop improvement in grapevine, significantly increasing profitability and sustainability of grape industries.

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Figures

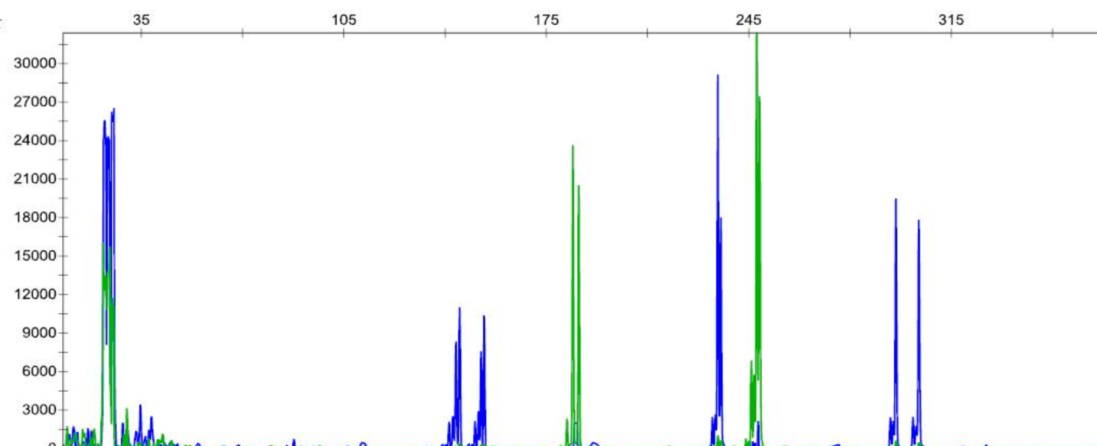

Fig. 1. Multiplex PCR assay including 5 different SSR loci.

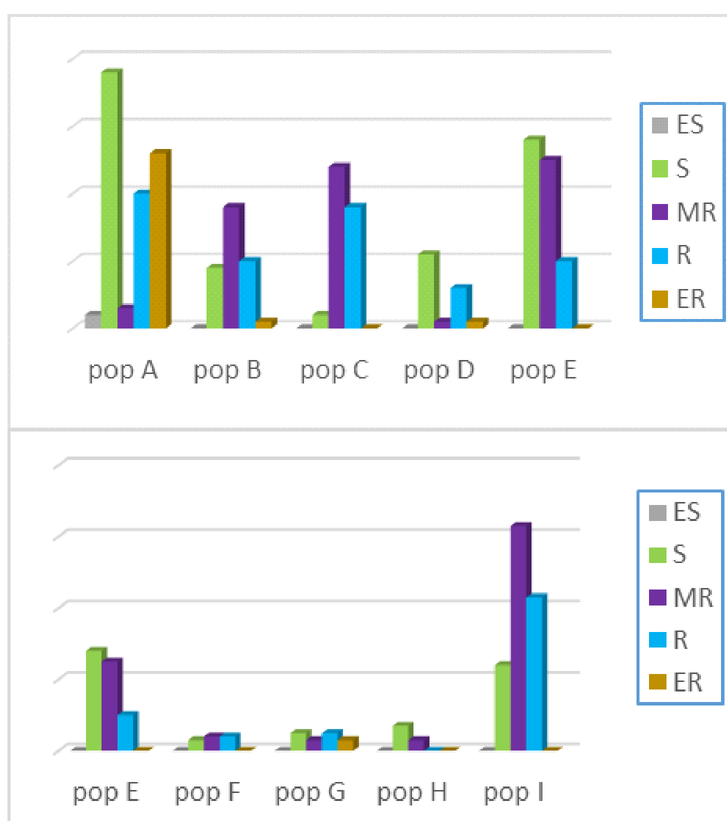

Fig. 2. Distribution of each studied population according to the integrated parameter at 6 dpi. All F_1 individuals were included in the following classes, ES: extremely susceptible, S: susceptible, MR: medium resistant, R: resistant, ER: extremely resistant.

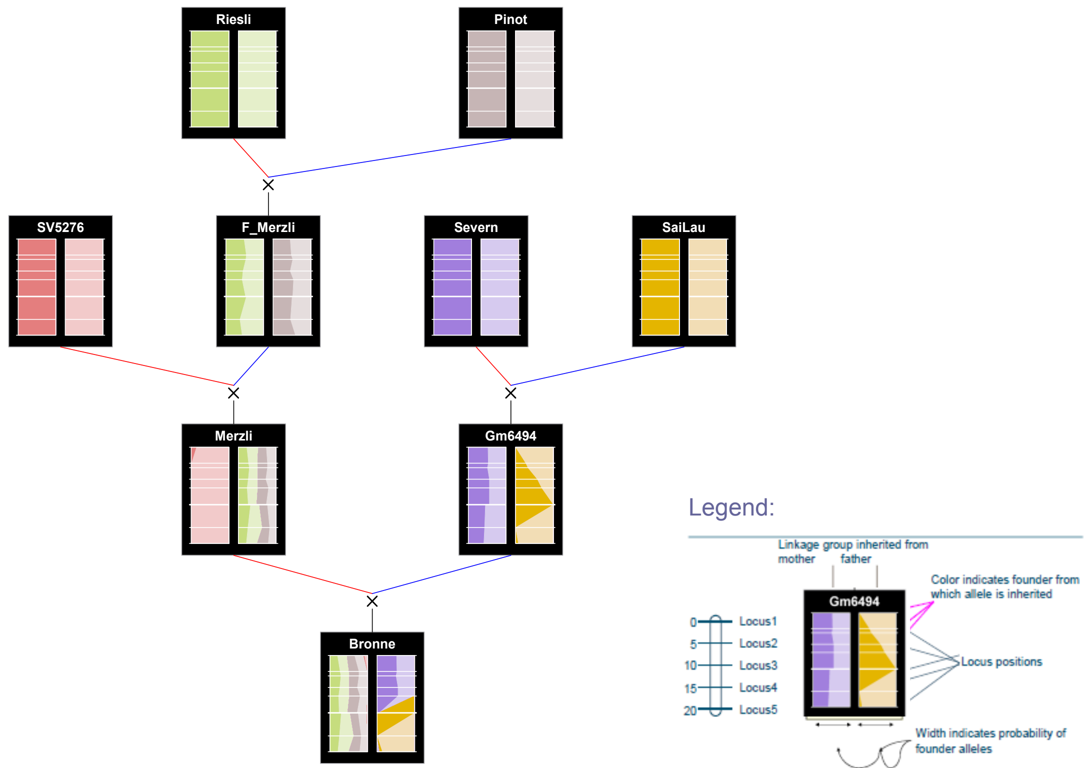

Fig. 3. The IBD analysis results at 8 SSR loci along chromosome 5 in a sub-set of related genotypes. The colour code reflects the allelic flow from the founders, through the gran-parents, to the parental Bronner. The names of hybrids/cultivars were shortened to 6 letters.

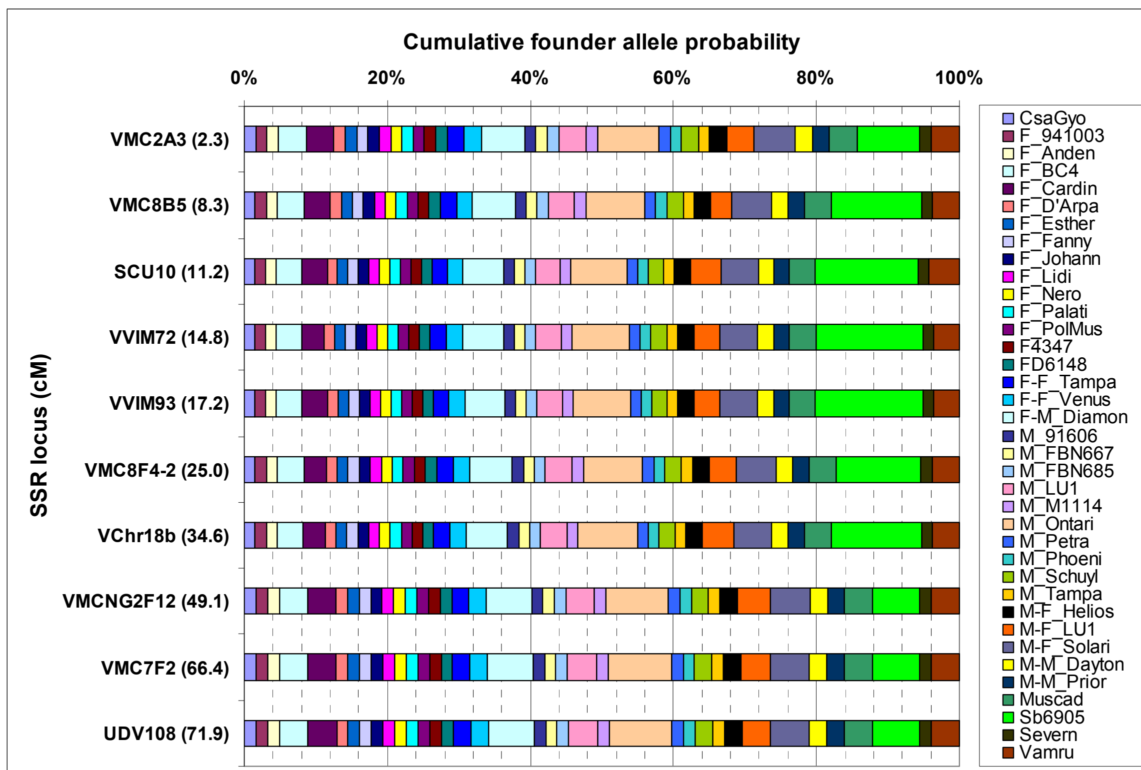

Fig. 4. Cumulative allele probability of each founder at 10 SSR loci along chromosome 18. The names of founders were shortened to 6 letters. “M_” means “mother of”, while “F_” means “father of”.