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IDENTIFICATION STRATEGY USING RAPD AND AFLP MARKERS FOR BELGIAN POT AZALEA'S

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IDENTIFICATION STRATEGY USING RAPD AND AFLP MARKERS FOR BELGIAN POT AZALEA'S

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Introduction

Molecular markers can reveal (dis)similarities in the genome of related plant species or cultivars. They provide a DNA fingerprint, which can be complementary to the plant passport based on morphological traits. Both randomly amplified polymorphic DNA (RAPD) markers and amplified fragment length polymorphism's (AFLP) are generated by polymerase chain reaction. RAPD polymorphism's originate because short oligonucleotide PCR primers of random nucleotide sequence anneal differentially to several sites in the genomic DNA. The AFLP technique is based on the selective PCR amplification of primer ligated restriction fragments from a total digest of genomic DNA. Therefore, AFLP provides a hybrid technique between RFLP and RAPD (Vos et al., 1995).

In the present study, Belgian pot azalea (*Rhododendron simsii* hybrids), the most important Belgian flowering pot plant, was used as model species for vegetative propagated horticultural crops. The current assortment has been created from a relatively narrow basis of collectors material, from botanical gardens and private collections brought from the far east. Several closely related species are accepted to be the ancestors. Azalea's are propagated by cuttings, therefore a new variety can start from a single attractive plant. New varieties are obtained by two main strategies. Breeders make paircrosses within the existing collections and screen the offspring for desired genotypes. One new variety selected from 10.000 seedlings is a workable average. On the other hand, spontaneously variant plants occur during commercial production by bud sporting. These sports often have modified flower colours or colour patterns and are often more attractive plants than the original varieties. E.g. "Hellmut Vogel" gave rise to a bud sport series of 24 types with flower colours ranging from carmine red, red, pink white and patterned flowers. These bud sports are indistinguishable when they are not flowering. Most of phenomena induced by bud sporting are not heritable. However, regarding uniformity and stability of a variety, excessive bud sporting can be a problem.

In a first approach the similarity of DNA fingerprints was evaluated as identification protocol. For this purpose, different coefficients (Jaccard, Dice, Simple Matching Coefficient) for the calculation of the similarity between DNA patterns were applied using the GELCOMPAR software (Vauterin & Vauterin, 1992). In the experiments reported here six *Rhododendron* species were tested. Some of these were the ancestors of the current commercial Azalea assortment (*R. indicum*, *R. mucronatum*, *R. scabrum* and *R. simsii*), other are related species (*R. kiusianum* and *R. noriakianum*). Hereupon, the best primers (primer combinations) for RAPD (AFLP) fingerprinting were selected. We are currently testing a set of 70 genotypes of Belgian pot azalea's; some of them with common pedigree. Also bud sports are studied. Moreover, AFLP markers are mapped in offspring populations (approximately 200 individuals per population) to construct genetic maps.

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Materials and Methods

DNA isolation

Different DNA isolation protocols were tested. Suitable methods were based on Dellaporte et al. (1983), Doyle & Doyle (1987), Greenwood et al. (1989), Kobayashi et al. (1995) and Weising et al. (1991). In a first approach, genomic DNA was extracted from young leaves manually ground to powder (pestle and mortar). Common NTES and CTAB based protocols yielded an unusable sticky matrix of DNA with interfering compounds. Interference of phenolics and sugars was supposed. An extra ethanol or PEG precipitation (Rowland & Nuygen, 1993) could not solve this problem. The combination of a guanidine hydrochloride based lysis buffer and a DNA purification using Qiagen genomic DNA columns (Qiagen, 1995), yielded good quality DNA. However, if older leaf material was used, the Qiagen columns were liable for blocking.

To improve the milling of the plant material (and as a consequence the efficiency of the used buffers), young leaves were first lyophilised during 48h and then milled using a Culatti mechanical mill. To avoid interference of cytoplasmatic compounds, first the nuclei fraction was separated from the crude extract and pelleted by centrifugation. On the nuclei fraction, a modified CTAB protocol was applied. This protocol has until now proven to be effective for DNA isolation from a broad range of recalcitrant plant species and tissues.

DNA concentration and quality was always checked compared to a standard series of lambda-DNA on a 1.5% TAE buffered agarose gel.

RAPD

Sixty decamer primers (sets A, B and C; Operon Technologies Inc.) were evaluated for their capacity to regenerate RAPD polymorphism's using one *R. simsii* hybrid. Ten of them were selected for further experiments. PCR conditions were as described by De Loose et al. (1993). PCR products were separated on a TAE buffered 1.5% agarose gel and were stained with ethidium bromide.

AFLP

AFLP was performed using the commercially available kit from Perkin-Elmer Applied Biosystems for fluorescent fragment detection (Perkin-Elmer, 1995). *Eco*RI and *Mse*I were used for DNA digestion. Adapter ligation, preselective and selective amplification were as in the supplied protocols. Selective amplification was done using fluorescent labelled *Eco*RI-*Mse*I primer combinations with 6 selective bases. PCR amplifications were performed using a Perkin-Elmer 9600 or a MJ PTC-200 PCR machine. AFLP fragments were separated by PAGE on a ABI Prism 377 DNA Sequencer on 36 cm gels.

Results and Discussion

RAPD

In the experiments reported here six *Rhododendron* species were compared. Some of these were the ancestors of the current commercial *R. simsii* hybrids (*R. indicum*, *R. mucronatum*, *R.*

scabrum and *R. simsii*), other are related species (*R. kiusianum* and *R. noriakianum*). All RAPD primers allowed the generation of polymorphic DNA fragments). For the six most informative primers i.e. those generating most of the polymorphism's between the species, the DNA fingerprints were combined and analysed with the GELCOMPAR software (Vauterin & Vauterin, 1992). In this way, the DNA fingerprint for each species consisted of approximately 50 loci. Cluster analysis was performed on these data, using the UPGMA algorithm with the Jaccard coefficient. The six species were grouped in a dendrogram showing the correlation between distinct groups. The highest correlation, approximately 62%, was observed between *R. scabrum* and *R. mucronatum*. This value is still low enough to allow a distinct separation between both species. This shows that identification of *Rhododendron* species can be effectively performed using RAPD markers, resulting from a limited set of primers. However, the grouping based on a DNA fingerprint with a limited numbers of bands will seldom be indicative for phylogenetic relationships between species. The selected RAPD primers did not allow to distinguish the set of 70 genotypes of *R. simsii* hybrids (data not shown).

Fluorescent AFLP on the ABI Prism 377 DNA Sequencer

Reproducibility of the fluorescent system compared to radioactivity

The protocols prescribed in the manuals for fluorescent (Perkin-Elmer, 1995) and radioactive (Gibco BRL Life Technologies, 1995) AFLP show some slight differences. Also, for fluorescent labelling an other type of *Taq*-polymerase is used. However, after optimisation of *Taq*-concentrations and PCR programs, exactly the same DNA profiles were obtained using both detection systems. Due to the different gel system, on a sequencer the fragments run off the glass plates, with radioactive PAGE fragments are fixed over the gel before visualisation, the positions of the bands may be shifted.

Fragment analysis on the ABI Prism 377 DNA Sequencer

In contrast to radioactive AFLP using a phosphor-imager or a X-ray film, the ABI technology offers the advantage that to each sample an internal lane standard for interpolation of the size (in bp) can be added. This "size calling" is performed by the Genescan software module. In this way problems concerning normalisation of patterns from different lanes are avoided. This also enables band recognition, based on its size, also in samples were few non-polymorphic bands are present. The Keygene AFLP software for band recognition and scoring needs a more or less dense framework of constant (non-polymorphic) bands as is present in a F2 or backcross segregating offspring. The number of these bands can be to low for high polymorphic species, for varieties that are build up as populations or for genepool characterisation.

Perkin-Elmer supplies a GS-500 size standard with 15 bands ranging from 50 to 500 bp. We also tested a more dense Sequa Mark 10 bp ladder (Research Genetics Inc.) in the same range. Size calling of the AFLP fragments is done by interpolation to the standard according to different algorithms (Local Southern, Global Southern, 2^{nd} and 3^{th} order equations or cubic splines). Resolution of the system (i.e. the capacity to separate two subsequent bands in one lane) is 1 bp. However, due to lane to lane variation and differences in interpolation of the standard the same band position between different lanes can vary within 1 bp. This sometimes leads to problems for automated band recognition solely based on size.

Multiplexing AFLP reactions with different labelled primer combinations

The use of fluorescent dyes with a different emission spectrum combined with the data collection technology on the ABI Prism 377 enables the detection of four different dyes per sample lane. This technology has proven its power in DNA sequencing because the four sequencing reactions for detection of A, T, C and G ending fragments can be pooled in one gel lane (and in one sequencing reaction when using dye terminators). However, for AFLP there still are some drawbacks. Filter sets (detection channels) specific for the used dye combination can be selected. Spectral overlap between the four emission spectra (e.g. detection of some of the "blue" signal in the "yellow" detection channel) is corrected by applying a correction matrix on the raw sample data. However, until now the applied matrix correction algorithm can not completely subtract interference of pooled samples. This may lead to erroneous bands. However, if reactions can be pooled in fragment analysis, this enhances the capacity of a gel with a factor 3 (one of the dyes is reserved for an internal lane standard for fragment size calling). This could offers to AFLP advantages comparable to multiplexing of microsatellites.

Data management

At this moment, the standard software available on the ABI Prism 377 for fragment analysis (Genotyper), requires a good definition of band categories. An upper and a lower border must be defined for each marker position before fingerprints can be scored. For segregating populations where the set of the expected markers is defined from the fingerprints of the parents, this is feasible although laborious. For variety comparison or genepool studies, band categories are not fixed. For such cases an automated band recognition algorithm where the user can define a certain position tolerance (as in GelCompar) is needed. Until now, band scoring was performed by exporting the data to Microsoft Excel and Access.

Fingerprinting of the azalea genepool

This study is currently being undertaken. To illustrate the importance of an appropriate band scoring and the effect of selection to obtain a final data set used for computation of similarities, we tested arbitrarily some criteria for band scoring. The data set for this test consisted of 32 azalea varieties fingerprinted with one AFLP primer combination (*EcoRI* ACT-*Mse*I CTA). Based on the presence or the absence of a band, a band scoring table (1,0) was made. At first hand the complete data set of 255 unique band positions (i.e. detected at least once in the 32 varieties) was chosen (Fig. 1). Afterwards, those positions were selected whose peak surface was at least for one plant higher than an arbitrarily chosen detection limit. In this way a subset of 130 positions was made (Fig. 2). Similarities between patterns were calculated using the Simple Matching Coefficient; dendrograms were build using the UPGMA algorithm. Comparison of both figures clearly shows that the choice of data set is strongly determining the obtained dendrogram. E.g. when the limited subset of 130 positions was set (Fig. 2) the four winter persistent varieties "Gilbert Mullie", "Stewartstownian", "Palestrina" and "Blue Danube" are clustered together. In the full data set they are scattered over the dendrogram. Such phenomena are part of the dual problem that can occur when identification is based on markers that are not characterised: i) how many markers must be evaluated to get a good representation of the genetic distance between two individuals, and ii) do all detected markers have the same information content. As another strategy for identification, molecular markers will searched for that map at regular intervals over the chromosomes. This may enable a more objective and accurate identification of varieties since chromosomes are more homogeneously covered. We think this must allow to improve the reliability of these methods.

Conclusions

Major bottlenecks for molecular markers to be implemented in variety registration are i) the reproducibility of the marker technique, ii) the number of markers to be included, and iii) the information content of the used markers. Advantages of AFLP are that there is a high data output per reaction, the technique is well described, accessible (kits available) and robust. One of the major disadvantages of AFLP is that codominant scoring of AFLP is based on the band intensity since most of the detected markers are not allelic. In variety testing codominant scoring of AFLP markers is until now not applicable. However, due to the high data output of AFLP, in a short period a dense genetic map can be constructed. In this way, the structure of a chromosome can be defined with a high resolution. Such information will be at least an important supplement to the currently used morphological description of plant varieties.

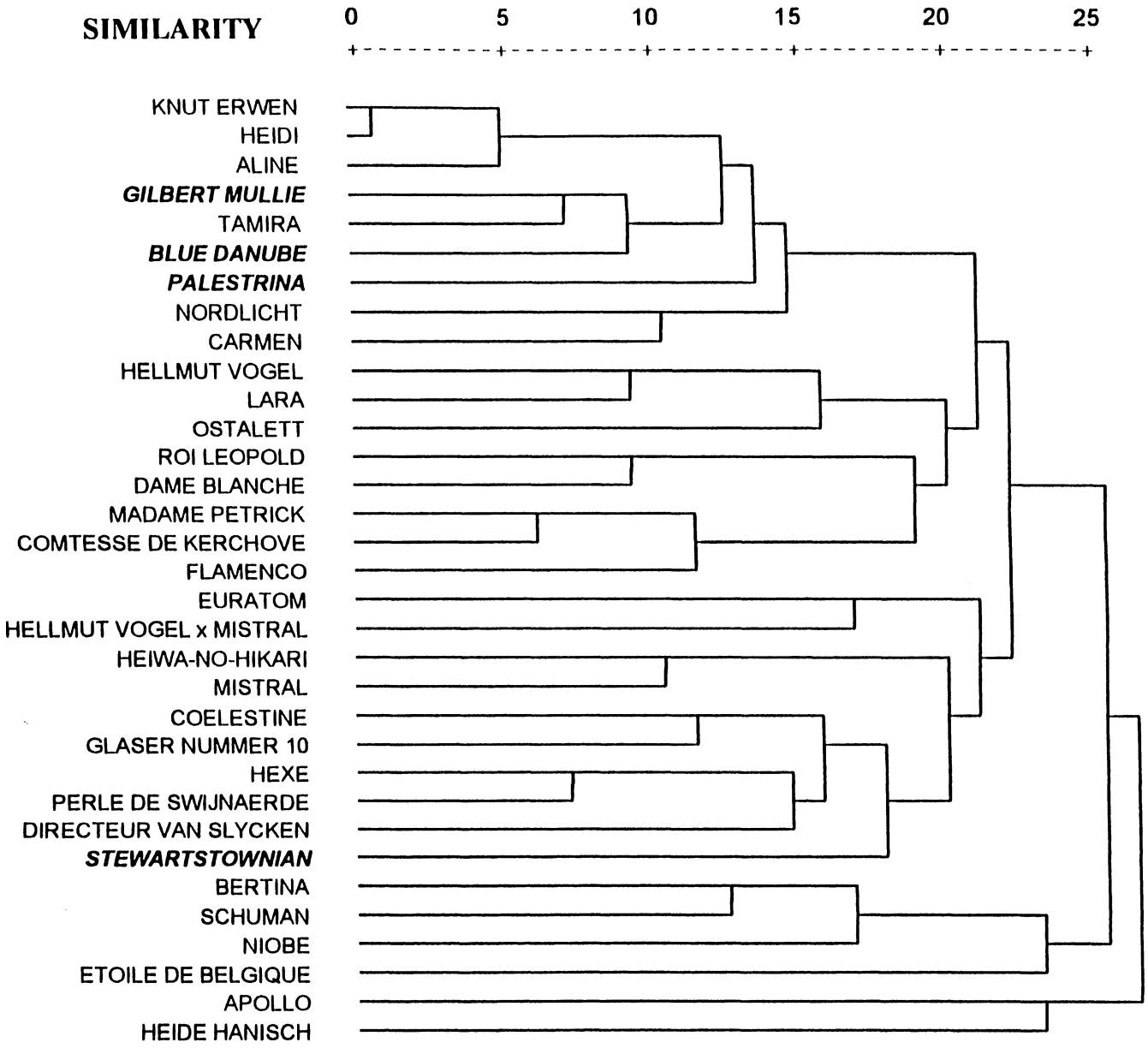

Figure 1: Dendrogram of 32 azalea varieties, based on the full data set (255 band catogeries; AFLP on ABI Prism 377; PC *Eco*RI ACT-*Mse*I CTA; Simple Matching Coefficient; UPGMA algorithm)

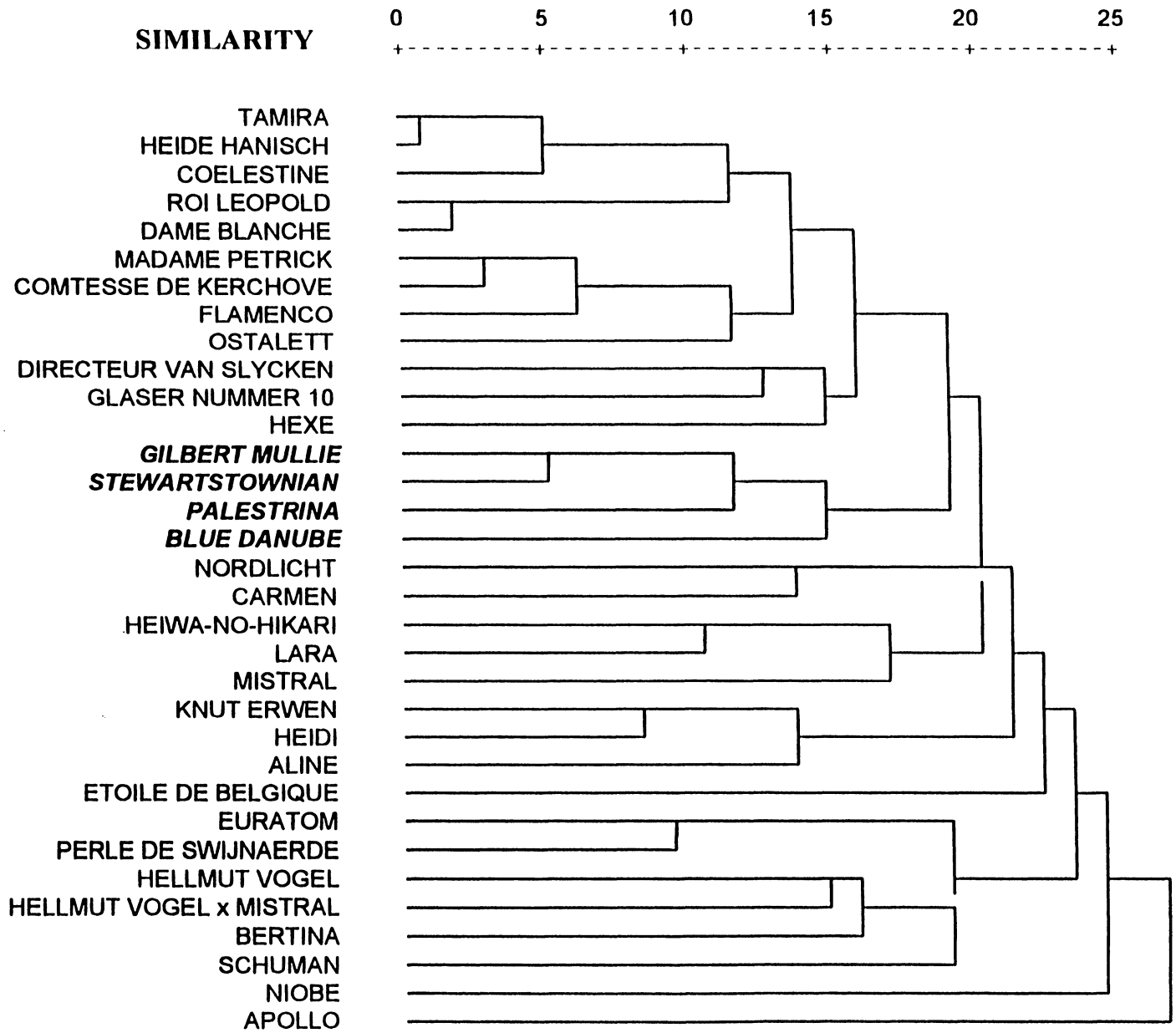

Figure 2: Dendrogram of 32 azalea varieties, based on a subset of band catogeries (130 band catogeries; AFLP on ABI Prism 377; PC *Eco*RI ACT-*Mse*I CTA; Simple Matching Coefficient; UPGMA algorithm)

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