AFLP-Based Identification Procedures for Woody Ornamentals

J.M. Van Huylenbroeck, E. Calsyn, F. Jeanneteau, J. De Riek and E. Van Bockstaele
Department of Plant Genetics and Breeding, CLO-Gent
Caritasstraat 21
9090 Melle
Belgium

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Abstract
Within the European Community traded ornamental plants have to fulfil standard quality requirements. Among these correct nomenclature and identification of the cultivar are important criteria. Until now control of authenticity of the produced plant material is based only on morphological descriptions. This causes difficulties especially when identification has to be done in wintertime or on juvenile plant material. Recently new DNA-based techniques have offered possibilities for fast characterisation and control of authenticity of a plant. A highly reproducible standard procedure for AFLP-based identification and control of authenticity of vegetatively multiplied woody ornamentals was developed using *Acer*, *Tilia*, *Syringa* and ornamental *Prunus*, and *Pyrus* as test genera. Starting from true to type plant material originating from nuclear stock plants a database of fingerprints patterns were compiled. Statistical analysis of the data revealed that between most cultivars of all species the genetic distance was high enough for correct identification. Further more the protocol for DNA extraction and analysis starting from winter buds was optimised.

INTRODUCTION
Within the European Community traded fruit trees and ornamental plants have to fulfil standard quality requirements. For fruit trees this is called CAC-material (Conformitas Agraria Communitatis), while for ornamentals the indication EU-quality is used. The minimum requirements are situated on phytosanitary (free from harmful organisms), physiological, morphological and genetic level. For this last criterion a correct nomenclature (genus, species and cultivar) and identification are very important. Each producer is obliged to check that during the whole trade chain the plant material is conform the legislation. To be able to guarantee authenticity of the produced plants, it is important to have objective control mechanisms.

Until now control of authenticity of the produced plant material is based only on morphological descriptions. This causes difficulties especially when identification has to be done in wintertime (when most plants are sold) or on juvenile plant material, since in these stages important morphological characteristics necessary for correct identification are not present. Recently new DNA-based techniques have offered possibilities for fast characterisation and cultivar identification also in ornamental plants (Barcaccia et al., 1999; Han et al., 1999; Torres et al., 1993). Among these the AFLP (Amplified Fragment Length Polymorphism) technique (Vos et al., 1995) has proved to produce high numbers of markers in a single PCR-amplification with a high reproducibility. The analysis of cultivars at the DNA level also provides a potentially powerful method for cultivar identification, control and protection of intellectual property rights through Plant Breeders’ Rights (Preston et al., 1999; De Riek, 2001).

In woody plants most research on cultivar identification and genetic fingerprinting using DNA marker technology is focused on fruit tree species as *Prunus* and *Malus* (Wunsch and Hormaza, 2002). Identification methods for woody ornamentals using isozyme analysis (i.e. genus *Acer* by Tobolski and Kemery, 1992; genus *Tilia* by Maurer and Tabel, 1995), RAPD markers (i.e. genus *Syringa* by Marsolais et al., 1993) and AFLP-markers (i.e. roses by Zhang et al., 2001; *Syringa* by Pfosser et al., 2000; *Hibiscus*...
by Van Huylenbroeck et al., 2000) are published.

The aim of present study was to develop and optimise a highly reproducible standard procedure for AFLP-based identification and control of authenticity of vegetatively multiplied woody ornamentals using Acer, Tilia, Syringa and ornamental Prunus and Pyrus as test species.

MATERIALS AND METHODS

Plant Material

To generate reference fingerprint patterns, leaf material of true-to-type Acer, Tilia, Syringa and ornamental Prunus and Pyrus cultivars, originating from well known and described nuclear stock plants, were collected at the Costentpark, Destelbergen (Belgium). Directly after sampling, leaf material was stored in liquid nitrogen and kept at -80°C before freeze-drying for 48 hours. Dried material was stored at room temperature under vacuum conditions until DNA-extraction.

DNA Isolation, AFLP Reactions and PAGE

DNA isolation was performed as in De Riek et al. (1999). AFLP reactions were run on an ABI Prism 377 DNA Sequencer using the commercially available kit for fluorescent fragment detection (Perkin-Elmer, 1995). EcoRI and MseI were used for DNA digestion. Selective amplification was done using five fluorescent-labelled EcoRI-MseI primer combinations with six selective bases: EcoRI-ACT/MseI-CTG, EcoRI-ACA/MseI-CTG, EcoRI-ACG/MseI-CAA, EcoRI-AGC/MseI-CAT and EcoRI-ACC/MseI-CAT (De Riek et al., 1999).

Data Analysis

For the scoring of the AFLP fingerprints, the settings for semi-automated scoring of fluorescent reactions defined by De Riek et al. (1999) were applied. Statistical analysis was based on a differing number of scored AFLP markers, depending on the species. Calculation of similarity coefficients, construction of dendrograms (UPGMA) and principal co-ordinates analysis were performed by the modules SIMIL, CLUSTER and PCOORD of the “R package” (Legendre and Vaudor, 1991).

RESULTS AND DISCUSSION

Effectiveness of the Used Primer Combinations

Randomly selected AFLP primer combinations with six selective bases were used. They generate approximately 70 to 90 AFLP fragments per reaction. Intense bands, indicative for repetitive fragments, were not observed in the used sets. Depending on the variable number of cultivars per species analysed a differing number of AFLP markers were scored per species (a total number for the five PC analysed): Acer 1497 markers, Prunus 1372, Pyrus 617, Syringa 1016 and Tilia 1117.

Evaluation of Data by Multivariate Analysis

As a first entry to the data analysis, the data from the scored AFLP reactions were taken into a multivariate analysis. Dendrograms were constructed using the UPGMA algorithm based on the pair wise distance matrices (Jaccard similarity coefficients). For the species studied (except for Pyrus were only three cultivars were tested), these dendrograms are shown in Fig. 1 to 4. In general, cultivars belonging to the same species are clustered together; also duplicate samples from the same cultivar are always grouped at the highest levels of similarities.

Step-by-step Determination Model

Although computationally feasible and fast, multivariate analysis is not an easy interpretable way for general usage to identify an unknown sample. For that purpose a
step-by-step determination model was elaborated based on the presence or absence of unique markers in certain accessions or groups of accessions. This is exemplified in Fig. 5 for the cultivars analysed within the Acer species. First, from the database with the scored AFLP data, those markers were selected that uniquely can identify an accession (or groups of accessions) at a certain level. Due to the high degree of polymorphism in the material (and the fact that the inside variation within a cultivar is non existing as it all concerns vegetatively propagated material) quite a number of such markers could be identified. The system does not imply that all the cultivars belonging to a certain branch of the decision tree all show the full set of these unique markers: some of them might be present; others will be absent. However, when a subset of these unique markers is present, it is conclusive for the assignment to a certain branch. The number of markers indicated per PC in Fig. 5 only are valid within a certain level of the decision tree: e.g. a marker that is conclusive to identify cultivar Z within species X might very well be not unique when species X is compared to species Y. The system is designed with an automated classification system in mind that easily can be transformed into a database search algorithm.

For the ease of determination, the Acer species were grouped into two classes: 1.) A. campestre, A. pseudoplatanus, A. negundo and A. platanoides, and 2.) A. rubrum, A. davidii, A. saccharinum and A. lobelii. Although this subdivision was arbitrarily chosen (a group of species for which multiple cultivars were analysed versus those that were single accessions), the AFLP data generated enough uniquely present markers that could support this subdivision (Fig. 5). As an example the decision tree for the A. platanoides cultivars is shown. It can be seen from the example that the number of unique markers that have the capacity to identify a certain accession is decreasing when the level increases from the genus up to the cultivar. It might be very well possible that, especially when one is willing to lower the analysis costs by reducing the number of AFLP primer combinations tested on each sample, one runs out of unique markers at the cultivar level. However, this step-by-step determination model is the first (but fast) entrance to a finally correct classification: in a pair-wise comparison between the final selection of assignment candidates, all the available markers have to be taken into account.

A Robust Testing System

The ultimate goal of the testing system is to overcome misidentification of plant material at the moment of trade i.e., in wintertime when most plants are sold to the customers and when cuttings are also marketed. To be able to react quickly under market conditions, a fast and reliable DNA isolation protocol that can work on buds was needed. Due to the fact that for AFLP reactions only a limited amount of (good quality) DNA is needed, some simple modifications to the standard DNA isolation protocol were sufficient to function on buds. Scales, which appeared to be the most recalcitrant, were peeled of manually before grinding the material in liquid nitrogen. To prevent browning an extra amount of insoluble polyvinylpyrrolidone (PVPP) was directly added in the mortar at grinding. An augmentation of the concentration of β-mercaptoethanol or dithiothreitol in the extraction buffer was not necessary. AFLP patterns generated from buds were compared to profiles on leaves in the database. No significant differences between them were observed.

Although replicate samples taken at the moment the initial database starting from leaf material was constructed always appeared to cluster together (see also Fig. 1 to 4), a final proof of robustness can only be achieved when samples unknown to the examiner are correctly classified. For that purpose, next winter shoots will be collected from several species. Starting from buds, we will try to make a classification based on the AFLP patterns of the unknown samples and verify it when names are disclosed. In this way, it must be possible to fine-tune small shortcomings in the determination models that can not be checked at this moment.

Application of above described procedures will allow efficient control on authenticity of vegetatively multiplied woody ornamentals. A database containing fingerprint patterns of nuclear stock plants of other species is currently under construction.
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Literature Cited
Fig. 1. Ordination of the *Syringa* gene pool based on AFLP data (Jaccard similarity coefficients, UPGMA clustering).
Fig. 2. Ordination of the *Acer* gene pool based on AFLP data (Jaccard similarity coefficients, UPGMA clustering).

Fig. 3. Ordination of the *Tilia* gene pool based on AFLP data (Jaccard similarity coefficients, UPGMA clustering).
Fig. 4. Ordination of the ornamental *Prunus* gene pool based on AFLP data (Jaccard similarity coefficients, UPGMA clustering).
Fig. 5. Step-by-step determination model for ornamental Acer cultivars based on AFLP data. For each level the numbers of unique markers discriminating between groups of species, species and cultivars are given.