APPLICATIONS OF PCR-BASED DNA-ANALYSIS TO GENETICS OF MALUS X DOMESTICA

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SUMMARY

The aim of the work presented here was to evaluate possible applications of the polymerase chain reaction (PCR) technology in the field of Malus x domestica breeding. A derived method, random amplified polymorphic DNA (RAPD), was to be used for this purpose.

In the first part, it was shown that commercially available apple cultivars can be identified by means of RAPD markers. Eleven cultivars could be distinguished by running a PCR with one decamer primer. It can be seen from this part that the variability in the apple genome pool is relatively small, but, on the other hand, that the RAPD method is sensitive enough to detect a reasonable amount of polymorphisms in this pool.

The bulked segregant analysis technique was used in the second part to search for molecular DNA markers linked to the resistance of apple plants against scab, caused by the ascomycete Venturia inaequalis (Cke.) Winter. This resistance, also called Vf, was originally introgressed from Malus floribunda 821. The PCR screening of bulked progeny DNAs of chosen apple crosses produced two markers segregating with the resistance. The genetic distance of these markers was greater than the commonly desired distance of 5 cM, but this low correlation is most certainly due to the small number of the progeny. Nevertheless the markers were also present in all of the Vf-resistant apple cultivars tested, and therefore the markers seem to be suited for the practical screening of future apple crosses.

In the third part, an attempt was made to assay genetic relationships of the individuals of three apple pedigrees. The individuals were screened with 100 decamer primers and the reaction products scored subsequently for presence or absence of polymorphic DNA fragments. The resulting banding pattern matrix was used to perform cluster and parsimony analysis. The correlation between the actual relationships among pedigree individuals and the results from similarity analysis was valid to only a limited extent, although there was a strong tendency for a progeny to be grouped together with the more outbred parent. In general, the individuals of apple pedigrees seem to be too closely related to each other to perform a similarity analysis based on RAPD-markers. However, the method would certainly be useful in characterisation of the many chance seedlings available. This would at least allow determination of the geographical background of those plants, thus allowing the breeder to make more informed decisions about future apple crosses.
ZUSAMMENFASSUNG

In der hier vorgelegten Arbeit sollten Anwendungen der Polymerase Kettenreaktion (polymerase chain reaction, PCR) für Forschung und Züchtung von Malus x domestica entwickelt werden. Dazu wurde die auf PCR basierende Methode der random amplified polymorphic DNA (RAPD) verwendet.


Die sogenannte "bulked segregant analysis" wurde angewendet, um molekulare DNA-Marker für die Resistenz von Apfelbäumen gegen den Ascomycet Venturia inaequalis (Cke.) Winter zu finden. Die auch "Vf" genannte Resistenz wurde 1914 aus Malus floribunda 821 in die Apfelzüchtung eingeführt. Das PCR-Screening der DNA aus resistenten und anfälligen Nachkommen einer Apfel-Kreuzung ergab zwei Marker, die mit der Vf-Resistenz vererbt werden. Die genetische Distanz der Marker ist zwar grösser als die erwünschten 5 cM, dies beruht aber höchstwahrscheinlich auf der geringen Anzahl der Individuen, die verwendet werden konnten. Trotzdem scheinen die Marker für eine Anwendung in der Züchtungs-Praxis geeignet zu sein, da sie in allen getesteten Vf-resistenten Apfelsorten vorhanden waren.


INTRODUCTION

The productivity of domestic crops and their improvement probably represents one of mankind's greatest achievements. For plants propagated vegetatively (e.g. apple), this improvement started thousands of years ago. Probably the best example for this process is grapevine, which has been grown for thousands of years. Different genotypes were cultivated in different geographical regions. The early growers planted and propagated vegetatively their own “cultivar” until they received better material from other growers. This way, active selection could take place to improve the quality of planting materials.

For crops that have to be propagated via seed, the situation is somewhat different. Until recently, improving quality and yield of these crops was a matter of chance in terms of selecting the right seed for the following cultivation cycle. In the beginning, this selection was certainly unconscious, because only the fittest plants survived to be used for a further cycle. Later on, conscious selection of plants with superior properties was added. But it is only since the second half of the 19th Century that breeding of domestic crops could be based upon a knowledge of heredity and genetics; it was only then that profound decisions could be made concerning the establishment of a breeding programme. From a historical point of view, improvement of genetic background influenced crop yield probably more than any other single factor [1].

However, breeders are challenged to achieve additional gains by the demands of human population growth and by a changing agricultural environment involving new agricultural practices and not least consumer preferences.

Until recently, the development of new tools for directed genetic manipulation of crop plants remained quite far behind other technological advances in agriculture. Almost all progress in breeding has been - and still is in the case of apple - based on phenotypic assessment of the genotype. As an example, Sax [7] associated size differences with seed-coat pattern and pigmentation in Phaseolus vulgaris. The limited availability of suitable markers hindered the application of genetic markers as an instrument in plant breeding. Furthermore, the phenotypic markers used until the 1970s were mostly morphological markers, which have important restrictions: a) only a limited number of morphological markers is available, b) most markers were recessive morphological mutations, and c) phenotypic markers are influenced by environmental conditions. The
introduction of isozymes overcame some of these disadvantages, but the numbers of loci examined and the numbers of polymorphisms detected were still limited.

The development of the restriction fragment length polymorphism (RFLP) technique enabled a direct identification of genotypes, because it is DNA-based rather than relying on phenotypes. RFLPs are produced by digestion of genomic DNA with restriction enzymes. Differences in DNA sequences, caused by mutations such as base pair changes, alter the length of restriction fragments, if the mutation is located at a recognition site of the restriction enzyme. Subsequently, agarose gel electrophoresis separates the restriction fragments according to their size, and a Southern analysis is performed: the DNA is blotted to a nylon membrane or a nitrocellulose filter. Fragments in this way immobilized are hybridized to a labelled DNA probe and visualized by autoradiography or colour reaction [8]. The method produces a virtually unlimited number of polymorphisms, but it is also rather time-consuming, requires expensive laboratory supplies and relatively large amounts of DNA.

In the second half of the 1980s, polymerase chain reaction (PCR) technology became the basis for several new genetic assays. Based on this technology, Williams et al. [11] and Welsh and McClelland [9] established a technique later referred to as random amplified polymorphic DNA (RAPD). The principle of RAPD is the binding or annealing of a single, usually 10 nucleotides long primer to the genomic DNA at two different sites on opposite strands of the DNA template. A subsequent thermocyclic amplification produces discrete DNA products, if the priming sites are within a suitable distance of each other. The randomness of the procedure is given by the fact that it is generally not known, if and where such palindromic binding sites exist in the genome. Separation of the amplification products according to their size, usually by agarose gel electrophoresis, allows the scoring of presence or absence of specific DNA fragments in the test samples. Amplification products that are present in different samples are homologous at each of their ends. The absence of a particular fragment therefore identifies a nucleotide sequence polymorphism at one of the priming sites. Each decamer primer will bind with a statistical probability to many loci in the genome, resulting in several amplified sequences of different length. Thus, the assay is an efficient way to screen for such polymorphisms.

The RAPD assay has several major advantages over the RFLP technique. Only very small quantities of genomic template DNA are required because of the amplifying nature of the PCR technique. Furthermore, DNA does not have to be as highly purified and of high quality as for RFLP analysis. The input of time and material is relatively low, and
Introduction

no radioactivity is needed. Because of the simplicity of the protocol, automation is feasible. One disadvantage of the method is the need to maintain very consistent reaction conditions in order to obtain reproducible results. Varying concentrations of template DNA, primers, magnesium and the polymerase enzyme affect the reaction as well as changing the annealing temperature and the thermocycling protocol. A drawback of RAPDs compared to RFLPs is their dominant rather than co-dominant character, therefore not allowing distinction of hetero- and homozygotes. Finally, the process of scoring the fragments may itself lead to errors. It is generally assumed that PCR fragments of the same size are identical, although co-migration of fragments of similar or equal size may occur. Such co-migration can be detected by eluting fragments from the gel and re-probing them via Southern analysis. As an alternative, polyacrylamide gel electrophoresis increases resolution of fragment separation.

In the last few years, RAPD technology has been shown to be a useful tool in genetic analysis in many biological systems. The three main areas of research where the method was applied are population genetics [12], the development of genetic maps and the targeting of genetic markers. The aim of the work presented here was to investigate the usefulness of the RAPD-PCR technology in some of these areas.

The first part of this thesis showed the potential of RAPD-PCR technology to discriminate between apple (Malus x domestica) cultivars. While RAPD markers had been used before for DNA fingerprinting of annual plants and animals [3, 4, 5, 10], this study seems to be one of the first that was performed on perennial, strongly outbreeding plants. Discrimination of apple cultivars is often difficult because of the many phenotypical descriptors that have to be assessed.

The goal of the second part was to find molecular DNA-markers for resistance against scab caused by the fungal pathogen Venturia inaequalis (Cke.) Wint. This resistance, also called Vf, was introgressed from Malus floribunda 821 and was regarded as durable until it was overcome by the fungus in the early 1990s [6]. Based upon segregation data, this resistance is assumed to be directed monogenically, although there are probably so-called minor genes that influence the phenotypical character of the resistance [2]. Apple breeders are particularly interested in having DNA markers since breeding of apple is extremely time-consuming due to the long generation cycle of the plant (at least 4 to 6 years). It would be therefore of much use to be able to identify progeny that fulfill one or (ideally) more selection criteria already at an early stage in the breeding programme without the need for cumbersome tests.
Finally an attempt was made to analyse individuals of three apple pedigrees regarding genetical relationships among the single plants. This was done by screening the individuals with a relatively large set of decamer primers, scoring the amplification products for presence or absence of polymorphic fragments and then computing genetic distances among the plants. The question was, if the RAPD-PCR technology is sensitive enough to correctly depict the genetic relationships of closely related organisms.

References

Identification of apple cultivars using RAPD markers

Abstract

Eleven apple cultivars were differentiated using RAPD markers obtained by PCR. Variability of the technique and of the origin of the DNA extract was analyzed. A set of bands consistent in presence or absence was chosen to create a differentiating band pattern. A key to differentiate apple cultivars using a commercially available primer is proposed.

Introduction

Discrimination of apple cultivars as they are being multiplied and grown is extremely important, as correct identification is usually not possible conventionally until fruit are produced [7]. The characterization of cultivars requires a large set of phenotypic data which is often difficult to assess and sometimes variable due to environmental influences. The term cultivar means today that all trees with the particular cultivar name are phenotypically equal and originate from the same ancestor by vegetative reproduction. This implies basically the same genome for all trees of a certain cultivar.

Although isoenzyme systems have been useful in cultivar identification [7], they are limited by the number of informative markers and give no direct assessment of the potential variation present in the genome. In addition, certain systems are prone to environmental or developmental variation. Direct assessment of genetic variation at the DNA level avoids such difficulties. Restriction fragment length polymorphisms (RFLPs) have been used to identify apple clones and seedlings [5, 6], but the technique is laborious and not suited for studies of a large number of samples [10]. Randomly amplified polymorphic DNA (RAPD) markers generated by Polymerase Chain Reaction (PCR) can be used to differentiate between morphologically indistinguishable strains and varieties [8, 9, 3]. DNA profiles based on arbitrarily primed PCR is both time and cost effective [2]. Furthermore, the availability of markers will aid in mapping genes coding for agronomically important characters. Such a molecular aid will increase efficiency and reduce the

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time-scale of plant breeding [4, 11]. In this work we tested the reliability of the RAPD-PCR as a tool for the identification of apple cultivars.

Material and Methods

The following apple cultivars were used in this study: Arlet, Cox’s Orange Pippin, Florina, Gala, Glockenapfel, Golden Delicious, Idared, Ingold, Ontario, Red Delicious and Spartan.

DNA isolation: Apple leaves of one tree of each cultivar were frozen immediately in liquid nitrogen and stored at -80 °C. Leave samples were taken from trees grown at Federal Research Station Wädenswil and Ingenieurschule Wädenswil, Switzerland. From cultivar Golden Delicious, five samples of leaves were taken from five scions (one sample/scion). The Golden Delicious trees were grown on M26 rootstocks. DNA was extracted as described by Dellaporta et al. [1] with the following modifications: after RNAse treatment 1 Volume of Chloroform-Isomylalcohol (24:1) was added, mixed and centrifuged at 6500 x g for 10 min. The upper aqueous phase was transferred to a new tube and 5 M NaCl was added to a final concentration of 0.2 M. Two volumes of cold ethanol (-20 °C ) was added, mixed and allowed to stand for 30 min at 4 °C. After centrifugation at 6500 x g, the supernatant was discarded and the DNA pellet was rinsed in 500 μl of 70 % ethanol. After centrifuging, the ethanol was discarded and the pellet air-dried. The DNA was dissolved in 100 μl of TE buffer pH 7.4.

Amplification conditions: Amplification reaction volumes were 25 μl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 100 M each of dATP, dCTP, dGTP and TTP (Boehringer), 0.28 μM Primer, 5 ng of genomic DNA and 1 U Taq DNA Polymerase (Boehringer). Amplification was performed in a Perkin Elmer Cetus Gene Amp PCR System 9600 programmed as follows: 2 cycles of 30 sec at 94 °C, 30 sec at 36 °C, 120 sec at 72 °C; 20 cycles of 20 sec at 94 °C, 15 sec at 36 °C, 15 sec at 45 °C, 90 sec at 72 °C; 19 cycles of 20 sec at 94 °C (increased 1 sec/cycle), 15 sec at 36 °C, 15 sec at 45 °C, 120 sec at 72 °C (increased 3 sec/cycle), followed by 10 min at 72 °C.

Amplification products were electrophoresed in 1.5 % agarose (Biorad) gels with 1 x TPE (0.09 M Tris-phosphate, 0.002 M EDTA) and stained with ethidium bromide (0.5 μg/ml).

The following primers were used: Primer P2 5’ACGAGGGGACT; E6 5’AA-GACCCCTC.
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Results

The arbitrarily primed DNA profiles of five separate DNA samples with two primers is illustrated in Fig. 1. This analysis was performed on DNA extracted from five Golden Delicious trees to show within-cultivar variation of amplification results. For each primer the DNA profiles are uniform over the five trees.

Fig. 1. Amplified DNA polymorphisms of five Golden Delicious scions. A and B were made with two different primers (P2; E6) in the same PCR run. PCR patterns using a particular primer do not differ between the inoculants. Electrophoresis was performed on Agarose gel (1.5%).

Fig. 2. Effect of DNA concentration in PCR reaction mixture. From left to right: Each lane is the 3 fold dilution of the previous lane. DNA was extracted from cultivar Glockenapfel. In A, primer P2, in B, primer E6 was used. Highest DNA concentration (first left lanes) was 55 ng DNA in 25 μl reaction mixture.
The results of PCR amplifications were robust over a wide range of DNA concentrations. With more than a 2000 fold change in DNA concentration the variation in the PCR amplifications were primarily quantitative (Fig. 2). This consistency is especially true for bands that are strongly amplified.

To show repeatability, PCR amplifications were performed four separate times with two separate, arbitrary primers on extracts from a set of two cultivars. Variation in the DNA profiles can be observed among the sequential PCR runs. Some bands in the DNA profiles (Fig. 3) were consistently amplified in each run, while others varied considerably.

A set of 11 arbitrarily chosen apple cultivars was subjected to several arbitrarily primed PCR runs. The banding patterns of those 11 cultivars showed consistently appearing bands throughout all cultivars as well as bands appearing only in some cultivars (Fig. 4). In order to determine consistently appearing bands, amplifications were repeated at five separate times (Fig. 5). For each apple cultivar a set of bands, appearing consistently in all five repetitions, was identified disregarding bands that appear throughout all cultivars. This set provided 14 RAPD markers which can be used to clearly distinguish among the 11 cultivars.

Scoring for the presence or absence of these markers results in a unique binary code for each cultivar (Table 1).

Fig. 3. Amplified DNA polymorphisms of extracts from cultivar Arlet (1) and Florina (2) using two primers (A: primer P2, B: primer E6). Four different PCR (I,II,III,IV) were performed at separate times. DNA concentration was 5 ng DNA in 25 μl reaction mixture.
**Fig. 4.** Banding patterns of 11 different apple cultivars in a simultaneous PCR using primer P2. DNA concentration was 5 ng DNA in 25 μl reaction mixture.

**Fig. 5.** Amplified DNA polymorphisms of extracts from 11 different apple cultivars. PCR was repeated at five separate times using primer P2. DNA concentration was 5 ng DNA in 25 μl reaction mixture.
Identification of apple cultivars

Discussion

In this study we demonstrated that arbitrarily chosen commercial decamer primers can be used to generate amplified segments of genomic DNA which differentiate apple cultivars. This method is rapid and simple and produces repeatable results. By pre-screening 24 10-mer primers for their informative content we found one primer that detects enough genetic variation among the 11 apple cultivars to allow for complete differentiation. By selecting only strongly (and therefore consistently) amplified DNA segments as informational bands, variation of minor bands resulting from different amplifications can be excluded.

The 14 bands allow a theoretical differentiation of 16384 band combinations, more than sufficient for all known apple varieties. However, the narrow gene pool of apple and the close relationship among many cultivars will require additional markers, generated by more primers, to fully characterize and distinguish a larger set of cultivars.

Given the results so far it should be possible to establish a standard set of primers which can be used to distinguish and characterize most common apple cultivars. If this system were to be generally used it would be useful to generate a set of amplified DNA fragments corresponding to the informative markers to serve as size standards for evaluating the presence or absence of particular bands. This would facilitate the comparison of results from different research groups.
Table 1. System for differentiation of 11 apple cultivars based on the presence (1) or absence (0) of chosen RAPD bands (Fig. 6).

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<th>Band No.(^a)</th>
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\(^a\) Bands number 2 and 10 were omitted since they are present in all cultivars and therefore not informative for differentiation.

The use of RAPD analysis in the identification and characterization of apple cultivars and breeding lines would be of considerable help to breeding institutes and nurseries. These markers will also be of use in the European apple genome mapping project [4].

References

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DNA-markers linked to the *Malus floribunda 821* scab resistance

Abstract

Breeding resistant apple plants is an alternative way to control fungal pathogens reducing the environmental impact due to the use of pesticides. The breeding of apple cultivars resistant to *Venturia inaequalis*, could be much improved by marker assisted selection. A molecular marker closely linked to the resistance locus called Vf could replace selection based on infection studies. To find such molecular markers, DNA of progenies from crossings of a resistant and a susceptible apple tree was subject to bulked segregant analysis. Two markers were found with a genetic distance of 10.6 % and 19.7 % recombination frequency to the Vf-locus.

Introduction

Apple production is a high quantity and quality output system that requires high technological and industrial input. This includes crop protection by intensive use of pesticides, which is the most questionable aspect in terms of environmental and consumer acceptability. Although alternative strategies have made some progress in disease control, fungal disease management still relies mainly on fungicides. Breeding of resistant plants is an alternative fungal pathogen control strategy, which is safer for the environment and yet effective against the fungus. Apple breeders started working with resistance against the major diseases of apple in 1926 [3]. Resistance against apple scab, caused by *Venturia inaequalis* (Cke.) Wint. is mostly introduced from wild *Malus* species, where *Malus floribunda* 821 is the most frequently used source of scab resistance. The resistance is assumed to be coded by one gene, called Vf.

This type of resistance was believed to be durable until it was overcome by *V. inaequalis* in Germany [19].

Apple growers will only make use of scab resistant cultivars if firstly, the cultivars match market requirements, and secondly, the resistance is durable. Durable resistance may be realized by breeding cultivars containing a combination of genes for specific re-

resistances [17]. Appropriate orchard planning and management should make resistance even more stable or longer lived [1]. A major requirement for resistance breeding and especially for pyramiding resistance genes is the ability to recognise functionally different resistances in order to select progeny accordingly [7, 14, 25].

To fulfil this, selection can be based on testing progenies with various pathogen races that allow the identification of functionally different resistances. This method requires inoculum of defined virulences, and the testing of progenies is very laborious and time-consuming. Testing such resistances may even be impractical, if by some reason the pathogen to be tested cannot be used in the test location. Breeding programmes also vary in classification of scab-resistant plants, depending on what classes are considered as resistant [15].

Based on the assumption that functionally different resistances are coded by different genes in the genome, selection of progenies by marking the corresponding genome segment offers a more elegant approach.

In recent years, DNA-markers have been widely used to construct linkage maps, and RFLP maps became a standard in genome mapping. Some RFLP markers also showed tight linkage to some human and plant genes [18, 28].

If only one phenotype is to be marked, i.e. if the phenotype is monogenically inherited, near isogenic lines (NILs) allow one to produce markers for a region of interest [13, 28]. Unfortunately, apple is a heterogamous, highly heterozygous plant with a long generation time, and therefore NILs are not available.

A new and powerful approach is given with the bulked segregant analysis [9, 16]. Bulked segregant analysis is based on utilization of a population segregating for a gene of interest. The population is screened for presence or absence of the phenotypical character to be targeted, i.e. scab resistance in the case of apple. DNA samples from resistant and susceptible individuals are then pooled to obtain a "resistant" and a "susceptible" bulk. The bulks will be enriched for alleles linked to the region of interest [9], as the rest of the genotype will be randomly distributed over the individuals of the bulks. The bulks are then subject to RAPD-PCR [26]. Polymorphic bands are supposed to result from the priming within or close to the target gene, which is present in the resistant bulk but absent in the susceptible. Segregation analysis for the polymorphic bands of the progeny allows for the calculation of genetic distances.
Material and Methods

Resistance screening

The progenies of crosses from the following apple cultivars were analyzed: Idared x M. floribunda 821 (IxM), P22R24A8 x K1R11A26 (PxK), where M. floribunda and P22R24A8 are the carriers of the Vf resistance. Progeny size was 248 (IxM) and 347 (PxK) plants. 29 resistant and 30 susceptible plants were used from the IxM progeny, and 13 and 17 from the PxK cross, respectively.

The inheritance of the Vf-gene was identified in two steps. Firstly, plants were infected in the greenhouse, where plants were sprayed with a suspension of spores from V. inaequalis. Disease was rated in classes 0 to 4 [2], according to the reaction type, where class 4 represents the highest susceptibility. Plants rated as 0 and 1 were subject to a second infection with controlled environmental conditions. Drops (7 μl) of a spore suspension (10⁵ conidia/ml) were deposited on the two youngest leaves of each seedling to be tested. The inoculum for this test was taken from sporulating lesions found on open-pollinated Golden Delicious seedlings. Plants were kept at 18 °C and 100 % rel. humidity for 48 hours and then put in a greenhouse. Assessment of susceptibility and resistance was performed after 10-12 days by macro- and microscopical studies after Gessler [6].

DNA extraction and RAPD amplification

DNA was extracted following a protocol by Dellaporta et al. [4] with the modifications of Koller et al. [11] and then diluted to a concentration of 1 ng/μl. For both crosses, two "resistant" and "susceptible" bulks consisting of 10 individuals were created by choosing individuals from the progeny previously scored as resistant or susceptible. Due to the low number of progeny available in the PxK cross, some individuals had to be used twice for composition of the bulks.

For screening, 400 random 10 base primers (Operon Technologies Inc.) were once used in a PCR under the following conditions:

Amplification reaction volume was 15 μl containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.9 mM MgCl₂, 100 M each of dATP, dCTP, dGTP and dTTP (Boehringer), 0.3 M Primer, 5 ng of genomic DNA and 1 U Taq DNA Polymerase (SuperTaq, Stehelin AG, Basel). Amplification was performed in a Perkin Elmer Cetus Gene Amp PCR System 9600 programmed as follows: 2 cycles of 30 sec at 94 °C, 30 sec at 36 °C, 120 sec at 72 °C; 20 cycles of 20 sec at 94 °C, 15 sec at 36 °C, 15 sec at 45 °C, 90 sec at 72 °C;
19 cycles of 20 sec at 94 °C (increased 1 sec/cycle), 15 sec at 36 °C, 15 sec at 45 °C, 120 sec at 72 °C (increased 3 sec/cycle), followed by 10 min at 72 °C.

Amplification products were electrophoresed in 1% agarose gels with 0.5x TBE (0.045 M Tris-borate, 0.001 M EDTA) and stained with ethidium bromide. Recombination frequencies were calculated using MAPMAKER [12], regarding the IxM progeny as a backcross generation and performing multi-point linkage analysis when the two molecular markers and Vf resistance was included in calculation.

**Results**

400 random decamer primers were screened, where about 5 to 10 fragments were scored per primer. Bulks of the two crosses were screened separately. Out of the 400 primers, only two showed polymorphic PCR products between the resistant and the susceptible bulks: M18 and U1. The size of the polymorphic fragments is 900 and 400 bp, respectively. These products appeared in the resistant bulks, but not in the bulks made of DNA extracts from susceptible plants. Accordingly, the fragments were present in the resistant parent (*Malus floribunda* 821) but not in the susceptible (Idared) (Figs. 1, 2). Screening the IxM progeny (i.e. the resistant and susceptible plants), 3 of 29 resistant plants did not show the M18900 fragment, whereas 2 of 30 susceptible plants had the fragment present (Table 1). The U1400 fragment was present in 8 susceptible plants and absent in 2 resistant plants.

![Fig. 1. RAPD-patterns showing the presence or absence of the M18900-fragment (arrow) in progeny and parents of the cross Idared x Malus floribunda 821. R1, R2: bulks made of 10 resistant progenies. S1, S2: bulks made of 10 susceptible progenies. RP, SP: resistant and susceptible individuals of the cross, respectively. M: Malus floribunda 821, I: Idared. SM: Size marker: 100 bp ladder (Gibco).](image-url)
Molecular markers

Fig. 2. RAPD-patterns showing the presence or absence of the U1400-fragment (arrow) in progeny and parents of the cross Idared x Malus floribunda 821. S1, S2: bulks made of 10 susceptible progenies. R1, R2: bulks made of 10 resistant progenies. M: Malus floribunda 821, I: Idared. RP, SP: resistant and susceptible individuals of the cross, respectively. SM: Size marker: 100 bp ladder (Gibco).

Fig. 3. Survey of the presence or absence of the M18000- and the U1400-fragment in several commercial apple cultivars. SM: Size marker: 100 bp ladder (Gibco). The fragment is present in the Vf-resistant Florina only (arrows), while it is absent in the other, susceptible cultivars.
From these results, genetic distances could be calculated using MAPMAKER. The distance Vf-M18900 was determined as 10.6 % recombination frequency, while the distance M18900-U1400 is 8.9 % recombination frequency. Both fragments were located on the same side on the linkage map with respect to the Vf-locus.

Appearance of the polymorphic bands from IXM was also tested on the PxK progeny. While band M18900 can be found in PCR products of those plants, primer U1 does not produce the polymorphic fragment. Because of the small number of individuals from this cross, genetic distances were not calculated.

Several scab resistant (+) and susceptible (-) apple cultivars were screened for marker M18900: Boskoop (-), Coop-13 (+), Florina (+), Glockenapfel (-), Golden Delicious (-), Idared (-), Jonafree (+), Jonagold (-), Maigold (-), Malus floribunda (+), Pinova (-), Spartan (-). Marker M18900 was present in the Vf-carriers Coop-13, Florina, Jonafree, M. floribunda, while it was absent in all the others. The presence and absence of markers M18900 and U1400 for some of these cultivars is shown in Fig. 3.

Table 1. Linkage data for two markers linked to the Vf-resistance locus based on the presence (+) or absence (-) of polymorphic DNA fragments. Data were obtained from selected resistant and susceptible plants of the cross Idared x Malus floribunda 821.

<table>
<thead>
<tr>
<th>Resistant (Vf)</th>
<th>U1400</th>
<th>M18900</th>
<th>nr. of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>23</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>n&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
<td>n</td>
<td>+</td>
<td>1</td>
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<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>19</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>2</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>4</td>
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<td>-</td>
<td>-</td>
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<td>2</td>
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<td>-</td>
<td>n</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>n</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Appearance of a DNA-fragment produced by PCR with decamer primers M18 and U1 (Operon Technologies).

<sup>b</sup>Scoring of the polymorphic fragment was not possible.
Discussion

The RFLP technique has been widely used in analysing plant populations, and some RFLP markers were shown to be tightly linked to genes coding for disease resistances [21, 28]. However, targeting particular genes by means of RFLP analysis is very time-consuming and requires the screening of a large number of clones. The bulked segregant analysis presented by Giovannoni et al. [9] and Michelmore et al. [16] overcomes this disadvantage. The technique has been shown to be useful in work with lettuce [16], tomato [13], bean [10] and oat [20]. All of these examples were performed on material from autogamous plants, where the parents of the progeny used were homozygous for a phenotypic marker. In this work, the supposed resistance gene, Vf, is heterozygous and present only in the resistant parent. As Melchinger [14] pointed out, the use of heterozygous bulks reduces the probability of a polymorphism to be detected by 50 %, since they are informative for the recombination events in one gamete only. This means that in our case, only markers in coupling with the resistance gene can be found, perhaps explaining the relatively low number of polymorphisms detected (2 polymorphic bands with 400 primers tested).

The usability of the bulked segregant analysis relies strongly on a precise resistance scoring. This is especially true for the rating of susceptible plants, where an unsuccessful infection can be due to resistance as well as to inappropriate infection conditions. Moreover, the expression of the Vf resistance is conditioned by modifiers [5, 22]. For this reason, the plants used in this study were tested for scab resistance at least twice, and only the extremely resistant and susceptible plants were used. Therefore, the plants employed here are a selected subset of the whole population, and the distance calculations may be slightly biased, although the $x^2$-test does not show a significant difference from the expected 1:1-segregation. This segregation is to expect when analysis is performed on a cross from a heterozygous resistant parent with a homozygous susceptible parent.

Due to the small sample size used for molecular screening and because of the fact that only a subset of a progeny could be investigated, only recombination frequencies are given instead of distances in centi Morgan. However, the LOD scores for all linkage analysis were always greater than 4, and the standard errors for linkages were as small as 0.0416 % (Vf-M18900) and 0.053 % (Vf-U1400). The backcross algorithm in MAPMAKER was applied because of the fact that the progeny can be considered to represent a backcross of the genomic region around the resistance gene. Only one map was produced using MAPMAKER, because, due to the heterozygous state of the resistant parent,
only markers in coupling with the resistance factor can be found. The statement that markers M18900 and U1400 are on the same side of the chromosome in respect to Vf is of course preliminary and not reliable, again because of the small sample size. Further investigations on large progenies should provide more precise information.

The size of the DNA bulks influences the results of the PCR analysis [9]. The pool size of 10 individuals per bulk seems to be a good compromise: smaller bulks do increase the risk of detecting homozygous regions other than the region of interest, larger bulks increase the probability of occurrence of individuals with double crossovers.

RAPD markers are not regarded to be reliably repeatable among different laboratories. However, fragment M18900 could be reproduced by the HRI in Wellesbourne (UK) (personal communication Dr. G. King).

As stated before, apple is generally self-sterile, i.e. it is a heterogamous plant. Apple breeders have therefore to change the recurrent parent from generation to generation [27]. Hence, true back-crossing and the creation of NILs or F2 populations is not possible.

A set of DNA markers closely linked to functionally different resistances would considerably facilitate pyramiding resistances in apple breeding. Breeders now have a tool to recognise functionally different resistances. By using DNA markers, it would be possible to screen progenies of appropriate parents for resistance markers. This way, laborious progeny testing for phenotypic, disease resistant behaviour against various pathogen races (sensu Gessler et al. [8]) could be avoided [15]. The use of two molecular markers bracketing the resistance gene should further improve the reliability of such a selection method [23] and allow to minimize the genetic drag of undesirable characters. Thus, the number of progeny individuals to work with can be reduced considerably already in an early stage of a breeding programme. Such a marker-facilitated selection could be much simpler and more reliable [14] than tests for resistance against scab, especially when breeding progenies have to be screened at a large scale.

Moreover, pyramiding can now also be made with resistances to which virulent races are not yet detected or not available.

References


The usefulness of pedigree assessment of apple cultivars determined by randomly amplified polymorphic DNA

Abstract

Knowing the genetic relationships among apple plants is of particular interest for breeders, because many of today’s commercially available cultivars are chance seedlings. Furthermore apple has a strong outbreeding character. In order to estimate the usefulness of a DNA-marker based similarity analysis, three existing apple pedigrees were used as experimental objects. The 18 individuals of the three pedigrees were analysed by randomly amplified polymorphic DNA markers. The resulting banding pattern matrix was used to perform cluster and parsimony analysis. The correlation between actual relationships among pedigree individuals and the results from similarity analysis was valid to only a limited extent, although there was a strong tendency for a progeny to be grouped together with the more outbred parent.

Introduction

Selection of appropriate parents in a plant breeding programme can be facilitated by knowledge of genetic similarities of the plants in question, allowing an efficient sampling and utilization of germplasm resources. Before the advent of DNA markers, the estimation of such genetic similarities has been based on biochemical or morphological markers [1,14]. Using phenotypic estimates for determining genetic relationships between plants or populations assumes that similarity in phenotype accurately represents similarity in genotype. But, as Cox et al. [1] mention, correspondence among biochemical or morphological markers and genetic relationships is never perfect, mainly due to the failure of phenotypes to differentiate genotypes correctly. Nienhuis et al. [11] state also, that identical phenotypic performance does not preclude base pair differences in relevant DNA sequences.

The advances in DNA analysis techniques provide new tools for direct assessment of genetic differences among individuals at the molecular level.

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Restriction fragment length polymorphisms (RFLPs) have been used to estimate genetic similarities in various crops such as maize [9] and Brassica [11]. However, the low level of DNA polymorphisms in some important crop species reduces the usefulness of RFLPs.

The development of random amplified polymorphic DNA (RAPD) markers allows faster and easier detection of polymorphisms than is possible with RFLPs.

This technique has several advantages compared to RFLPs, such as the low amount of DNA used and the technical simplicity of the methodology [18]. RAPD markers are now used in a wide range of analyses such as genotype identification, genetic mapping, phylogenetic studies, population and pedigree analyses [12, 18, 19].

As stated above, knowledge of genetic similarities among genotypes is useful in breeding programmes, where the breeder can use similarity information in order to choose appropriate parents for crosses. This is especially true in the case of apple, where no true backcrossing is possible due to a high level of self-incompatibility. Therefore, the breeder needs to know the genetical background of the non-recurrent parent. Many apple cultivars are of unknown provenance, and no information about ancestors is available. In addition, the generation cycle of apple is long (at least 4 to 6 years), and breeding of this crop is therefore time-consuming. Analysis of genetic relationships of apple

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Fig. 1. Pedigrees of the three apple varieties "Florina", "FAW7207" and "FAW7372". The underscored individuals in the "Florina" pedigree no longer exist and could not be included in this work.
Pedigree assessment
cultivars could help in making more informed decisions in choosing which genotypes to
cross.

In this study we wanted to investigate the usefulness of genetic similarity analysis in
apple breeding. It should be shown whether the given genetic relationship of three diffe-
rent apple pedigrees could be represented by the degree of similarity. The material used
here consisted of individuals of the pedigrees of the apple cultivar “Florina” and the two
selections “FAW7207” and “FAW7372”.

Material and Methods

Eighteen apple cultivars and experimental selections were used in this study. The
plants were selected according to the pedigrees of three scab resistant apple varieties,
“FAW7207”, “FAW7372” and “Florina” (Fig. 1). The cross Rome Beauty x Malus flor-
bunda 821 was made in 1914 [2]. The F1-progenies 9433-2-2 and 9433-2-8 no longer
exist. Most leaf material was taken from a collection orchard in Wädenswil (Switzer-
land); some material not available in Switzerland was kindly provided by the National
Germplasm Repository, Geneva, NY, USA.

DNA was extracted following a protocol by Dellaporta et al. [3] with modifications
by Koller et al. [6] and then diluted to a concentration of 1 ng/μl.

The 18 DNA samples were screened with 100 random 10 base primers (Kit
A,B,C,G,H from Operon Technologies Inc.), running PCR under the following condi-
tions: Amplification reaction volume was 15 μl containing 10 mM Tris-HCl pH 9.0, 50
mM KCl, 1.9 mM MgCl2, 100 M each of dATP, dCTP, dGTP and dTTP (Boehringer),
0.3 M Primer, 5 ng of genomic DNA and 1 U Taq DNA Polymerase (SuperTaq, Stehelin
AG, Basel). Amplification was performed in a Perkin Elmer Cetus Gene Amp PCR Sy-
ystem 9600 programmed as published in Koller et al. [6].

Amplification products were electrophoresed in 1.5 % agarose gels with 0.5x TBE
(0.045 M Tris-borate, 0.001 M EDTA) and stained with ethidium bromide.

Presence (1) or absence (0) of bands was scored for each of the 18 apple varieties.
The data were then put in separate matrices according to the three pedigrees. Calcula-
tions of coefficients of similarity (simple matching, Jaccard’s and Nei’s) [5,10] and clu-
ter analysis (using the UPGMA method) was performed with the Numerical taxonomy
and multivariate analysis system (NTSYS-pc) [13]. The PAUP software for phylogenetic
analysis [16] was used to generate dendrograms showing relationships among the varie-
ties. Heuristic search was performed with each of the three datasets, using bootstrapping with 1000 replications and the branch swapping / tree bisection option.

**Results**

One hundred 10-base primers were tested with the DNA of the 18 individuals of three apple pedigrees. Primers that produced a distinct banding pattern with polymorphic fragments were retested twice. Each of the selected primers produced about 5 to 15 bands. 52 polymorphic fragments were clearly scorable and reproducible throughout three repetitions. Presence or absence of those fragments was scored for all 18 varieties. Scoring data were then separated into three groups, corresponding to the three original pedigrees. Similarities among the individuals of each pedigree were estimated by calculating coefficients of similarity followed by cluster analysis.

In the case of pedigree "Florina", the use of the three above-mentioned coefficients of similarity produced three identical clustering trees. The relative similarities among the varieties were quite consistent for each coefficient. Analysis of the other two pedigrees

![Fig 2. Cluster dendrograms of the pedigree individuals of three apple varieties as determined from RAPD-DNA probes. The pairwise coefficients of similarity (Jaccard) were clustered using the UPGMA method of NTSYS-pc.](image-url)
"FAW7207" and "FAW7372" revealed trees where some subgroups were ordered differently. As a result, variety Jonathan (ancestor in pedigree "FAW7372") was correctly included in a node with its progeny Idared when Jaccard's coefficient was used. Applying simple matching or Nei's coefficients, Jonathan was put in an outer node and therefore regarded as less closely related to Idared.

Generally, subclusters consisted of a variety and one of its parents. This observation is valid for all three pedigrees with the exception of Malus floribunda, which, as a different species, is notably different from all Malus x domestica varieties.

Comparing the dendrograms resulting from the three types of similarity coefficients, Jaccard's coefficient gave the most reasonable result regarding representation of actual relationships for pedigrees "FAW7207" and "FAW7372", whereas the Nei's coefficient tree fitted best for "Florina". Cluster dendrograms (Jaccard's coefficient) for all three pedigrees are given in Fig. 2.

Parsimony analysis may be applied as an alternative approach to estimate genetic relationships. Using the Wagner parsimony analysis, a 50% majority-rule consensus tree was generated by heuristic search bootstrapping (1000 replications) with the branch swapping / tree bisection option. For each pedigree, scoring data of an additional, unrelated variety was added. This variety was then defined as the outgroup. The same gene-
eral observation could be made as with the cluster analysis, that a variety is put into a node with only one of its parents. This was valid for all three pedigrees. The majority-rule consensus trees are shown in Fig. 3.

Clear statements regarding relationships among the varieties of a pedigree can only be made for the most inner nodes. From the different dendrograms, it seems to be difficult to draw conclusions about relations among subclusters or groups. The fact that the dendrograms always shows a variety together with only one of its parents may be due to the graphical impossibility of representing the data in a format similar to the actual pedigree. The composition of the groups mentioned was consistent for almost any applied analyses with two exceptions. As stated above, parsimonial analysis revealed in pedigree "FAW7372" the same groups as in cluster analysis using the simple matching and the Nei's coefficient, and variety Wagener was put together with its progeny Idared. Application of Jaccard's coefficient grouped Idared together with its other parent, Jonathan. The other exception is located in pedigree "FAW7207", where Nei's coefficient showed a correlation between Kidd's Orange and Golden Delicious, although they are not related to each other regarding the actual pedigree.

Table 1. Presence (1) or absence (0) of polymorphic RAPD-fragments in 18 apple varieties. The names of the fragments are composed of the primer (Operon Technologies Inc.) and the arbitrary size of the band.

<table>
<thead>
<tr>
<th>apple variety</th>
<th>RAPD-fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malus floribunda</td>
<td>1 1 0 0 0 1 0 1 1 0 0 1 0 1 1 0 0 0 0 0 0 0 0 1 0 0 1 0 1 1 1 0 1 0 1 1 0 1 1 0 0 0 0 1 0 0 0 0 1 0 1 0 1 0 0 0 0 1 0 1 1 0 0 1 0</td>
</tr>
</tbody>
</table>
Discussion

The aim of this research was to perform a phylogenetic analysis of chosen apple pedigrees using RAPD markers and to investigate whether it could correctly represent the actual genetic similarities between the pedigree individuals. A good correlation between actual pedigree data and the molecular marker data would be of advantage for apple breeders, who are interested in the degree of genetic relationship among the plant genomes they choose for their breeding programmes. Many of today's commercially cultivated apple varieties are chance seedlings, and the geographical origin is often all that is known about them. Analysis of the similarities and relationships between such cultivars would widen remarkably the knowledge about their genetical background. In order to estimate the usefulness of a DNA-marker based similarity analysis, three existing apple pedigrees were used as experimental objects. In recent years, much work has been done in plant research to determine similarities among different plants, breeding lines or species sampled in different geographical regions [4, 9, 15, 17]. However, no published report could be found where a detailed plant pedigree was used to study genetic relationships.

In this study, the results correlated only partially with the given real situation. It was not possible to re-establish a complete pedigree by means of DNA-markers. Nevertheless, an notable consistency of clustering a variety together with one of its parents was observed. An explanation for this behaviour could be that many individuals of a pedigree are related to their ancestors as well as to their progeny. Minor differences in RAPD banding patterns may therefore result in an overproportionally distant clustering. Another reason may be that the models for calculating the similarities assume equal parental contributions and no selection [8]. But of course selection was involved during the breeding process of the three pedigrees, e.g. the selection for scab resistance originating from *Malus floribunda* 821. Finally, RAPD markers are dominant rather than co-dominant markers. Heterozygous parents may therefore appear more closely related to their progeny than to the other, more inbred parent [11]. In fact, almost all of the paired subclusters group a variety together with their more outbred parent.

Assuming that the RAPD-DNA fragments used in this study are not linked to specific genes, selection may be mirrored by the similarity coefficients. Although the number of markers is much too low to saturate a genome at a statistically representative level, this would explain the grouping of "FAW7207" and "FAW7372" together with Florina,
since the breeder was most certainly selecting for progeny with properties similar to Florina, e.g. scab resistance.

An important factor is to be conscious of incorrect or misleading data assessment. Given the example of a specific RAPD marker present in both parents of a cross, it may be possible that the marker is absent in their progeny because apple is highly heterozygous and RAPD fragments are dominant markers. Nevertheless, the genetical distance between the parents and the progeny that is missing this marker will probably be overestimated due to the low total number of markers. For this reason, markers showing this sharing pattern were omitted from similarity analysis.

The application of three different coefficients for clustering analysis produced quite consistent results. According to Lamboy [7], the Nei’s coefficient should generally be used for measuring similarities of closely-related organisms by means of RAPD data, as most of the similarities between RAPD samples are based on shared positive bands. Simple matching coefficients and Jaccard’s coefficients are less suited, since they display more percent bias when false positives or negatives are present. The general consistency of the clustering trees could therefore indicate an accurate scoring of the RAPD data.

Failure in correctly depicting the actual pedigree may also be due to the relatively low number of polymorphisms involved. Since the varieties of a pedigree are all closely related to each other, it might be necessary to screen many more primers in order to obtain more raw data. It cannot be ruled out that a possible source of discrepancy lies in the reported pedigrees of the cultivars; the DNA assessments may in some cases provide a more accurate representation of the relationships between apple plants than do the breeder’s records.

As mentioned above, the correlation between actual relationships among pedigree individuals and the results from similarity analysis was valid to only a limited extent only. For a future project, DNA from a wide set of apple chance seedlings should be extracted and subject to similarity analysis based on RAPD markers. This would probably allow clustering of these varieties in groups of common geographic origin.
References


Discussion

In 1868, when Marie Anne Smith realized with surprise that there was an apple tree growing in her back garden near Sydney [1], she was probably quite delighted about the tree itself and did not care too much about the properties of the apples to be expected. In fact all that she knew about the apple tree, which became well known as Grannie Smith, was the origin: her garden. This was the amount of knowledge available for virtually all apple cultivars until the beginning of this century. Only with the understanding of the behaviour of genetical factors was it possible to intentionally cross plants in order to combine selected properties of the parental plants.

Since then, classical breeding improved many of today’s crops in characters such as quality, yield and disease resistance. Apple breeders, however, have to contend with remarkable constraints. Their working subject, the apple tree, has a long juvenile phase and generation cycle of at least 4 to 6 years. This makes the development of a new apple variety an extremely long-term project. A second drawback is the high level of self-incompatibility. It is therefore not possible to establish a true backcross breeding programme, since the “recurrent” parent cannot be one of the ancestors. Even worse, some varieties cannot be crossed with anything at all due to polyploidy, e.g. the triploid Boskoop. Because of the outbreeding character, apple trees are generally very heterozygous. A third challenge for the breeder is the large number of demands that a new apple cultivar should fulfill, such as disease resistance, fruit colour, taste, storage capability and growth type. Disease resistance is of particular interest regarding the growing consciousness of consumers for environmental protection, and it is certainly a very powerful argument in a marketing strategy to popularise a new variety with the producer and consumer.

The ongoing process of resistances being overcome by the pathogens forced new strategies for breeders as well as for farmers. The idea of pyramiding functionally different resistance genes into one plant is not new [7]. For the interaction between Malus and Venturia inaequalis, the causal agent of apple scab, several resistance genes have been identified in various Malus sources, such as Vr (origin: Russian seedling), Vb (Hansen’s baccata #2) or Vbj (M. baccata jackii) by Dayton and Williams [11]. They analysed crosses between either susceptible and resistant apple plants as well as crosses
between two resistant apple plants in respect of segregation of the resistance. Segregation data showed that there must exist distinct resistance loci (as well as different alleles of some loci) since they segregated independently. It is not known until now, whether all those (functionally different) resistance loci are truly different, or if they became transferred to non-homologous chromosomes during the evolutionary process.

Nevertheless, the realisation of pyramiding resistances becomes possible only with a capability of re-identifying these different types of resistances in the progeny of a chosen cross. Until now this was done - if ever - by sequential infection tests. This procedure is not only very laborious and time-consuming, but it is sometimes impossible due to the lack of suitable inoculation material. Molecular DNA markers doubtlessly overcome such limitations.

The question remains, however, if the new technologies will find their way into practice. Apple breeders are certainly interested in the application of DNA markers to their breeding programmes. Using such markers, the breeder can screen a large number of progenies for the presence of one or several desired characters, e.g. one or more distinct loci for disease resistance (see publication 2 in this thesis). This way, the plants do not have to be exposed to plant pathogens such as *Venturia inaequalis*.

It will also be possible to recognize several genetic factors in a much shorter time than it would take to screen for e.g. scab resistance in the field. However, the costs of testing large progenies for presence of molecular markers for e.g. disease resistance genes are still too great to replace classical screening. In this context, RAPD markers are not necessarily a solution to that problem. According to Ragot and Hoisington [10], RAPD markers are the most economical markers when a relatively small sample set has to be tested, whereas RFLPs would be the markers of choice for larger sample sizes. But it has to be stated again that different markers reveal different information in terms of quality (dominance/co-dominance) and amount (one locus/many loci)[10]. Nevertheless, once a set of markers is available that scores for several important qualitative and quantitative characters, the size of the selection progeny can be reduced by a huge factor. This again would reduce the amount of plantation space, thus allowing the breeder to remarkably increase the number of offspring planted and/or the number of crosses performed.

Speaking of the Vf scab resistance, it is important to mention the so-called modifying genes [2]. These modifiers influence the phenotypic expression of the scab resistance. It is assumed that Vf carrying plants without modifiers are susceptible to apple scab. On the other hand, a plant carrying the modifiers cannot be resistant without the presen-
Discussion

ce of the major Vf gene. For example, in recent infection trials, *Malus floribunda* 821 showed to be susceptible against a certain isolate of *Venturia inaequalis*, while Florina, carrying Vf resistance originating from *M. floribunda* 821, was resistant [11]. This is a clear indication for the presence of modifiers. The existence of modifiers also explains why the progeny of a cross where Vf is present in one parent is so difficult to classify for resistance: The distribution of one or more modifiers leads to confusing situations regarding resistance expression. Moreover, the example mentioned before in this paragraph clearly shows that these modifying factors can originate from the susceptible parent, and are therefore inherited independently from the Vf locus. Considering all this information, one can say that DNA markers for the Vf locus are valuable and important, but the presence of a Vf marker (and therefore probably also the Vf gene) does not necessarily predict that the plant(s) will be totally scab resistant. It would therefore be of importance to have DNA markers for these modifying genes as well. But to find such markers will require big efforts, if it ever will be possible. The first problem here will be the difficulty of scoring and/or identifying the different modifying factors. Once that is possible, mapping for the modifiers would be possible by QTL analysis. This in turn will only be possible when a detailed genetic map for apple will be available. Such a detailed map has been worked out by Hemmat et al. [4]. and is also the objective of the European Apple Genome Mapping Project (EAGMAP) [6]. This map will probably contain more markers and therefore be more precise. Secondly, the apple populations have been distributed throughout different places in Europe. This enables to compare the many agronomically important factors that are assessed, therefore reducing the impact of environmental influences.

An alternative way to find molecular markers could be the application of doubled haploids by creating a set of such plants originating from an appropriate cross.

It has already been mentioned here that *Malus x domestica* represents a series of relatively closely related cultivars, many of which are chance seedlings of unknown provenance. Traits from wild *Malus* species are most certainly a suitable resource for improving apple. According to several authors [3, 9], much of existing genetic variation in plants is held by wild ancestors of crop species. The availability of such diverse germplasm and the characterization of their attributes is essential for the introgression of desirable traits. DNA markers are believed to be an especially valuable tool to accomplish this [8].
Until now, most publications about estimation of genetic relationships by means of molecular markers do not compare their results with real situations. Their values for relationships are therefore difficult to interpret, and it seems not to be clear to what extent these "artificial" data can reflect the situation in nature. In the third part of this work it was therefore attempted to re-establish given pedigrees of apples by RAPD marker data. The results were somewhat ambiguous. While it is obviously possible to confirm paternities, the individuals of a pedigree seem to be too closely related to each other to allow more detailed information to be drawn out. This may be especially true for a highly domesticated plant such as apple, where a cultivar is closely related to many other cultivars by the breeding and domestication process. It might well be that a RAPD marker based study is much more appropriate for a "natural" population. The idea here is that naturally evolved plants are, as a result from the longer evolution process, more different in genome among each other as are apple plants. But it is not necessarily known if this variability is great enough to let a RAPD marker based similarity analysis be more representative. It was, however, the aim of the third part of this thesis to evaluate the usefulness of such an analysis for *Malus x domestica*, and this usefulness can only be tested with material where the actual relationships between the plants are known.

The literature on the analysis of genetic relationships provides no firm consensus on the mathematical functions to be used. Depending on which function is applied, results can vary, although not considerably. Most authors rely on software packages that do the analysis for them. However, it is rarely clear whether the authors fully understand the mathematical basis and the biological meaning of the algorithms. Unless one has an extensive knowledge of these subjects, overinterpretation of results may be common.

Wild *Malus* species, for example, may be characterised therefore not by such relationship analysis, but rather by screening for molecular markers for specific characters, once they are sufficiently available cover the traits of interest.

Within a few years, RAPD markers have become widely used in virtually all biological research areas. Their usefulness for breeding purposes has been proved, and their general producibility facilitates mapping of genomes and the identification of qualitative and especially quantitative traits.

Although the first research projects working with RAPD markers were relatively enthusiastic about the new possibilities, there are also some points about the technique that have to be carefully discussed. One point is repeatability. With the increasing popularity of RAPD markers, it was also found that the amplifying procedure has its limitations in
Discussion

repeatability, not only among different experiments but also among different laboratories. The occurrence of bands that are not reliably amplified throughout many experiments is known. Such bands can be seen in the first publication of this thesis, where a fragment in apple cultivar Spartan was amplified only in three of five repetitions. This fragment was then scored as absent, since it was not consistently amplified in all repetitions, but such fragment should have been scored as "not known". This shows that it is not possible to give a general rule for numbers of repetitions necessary for reliable results. A second point is the scoring of reaction products, that is a problem that has not been solved, and perhaps never will be. It can only be said that poorly amplified fragments should not be scored, since such fragments may "disappear" even by artifacts such as poor gel staining or bad photographic documentation. Due to these general, method-inherent uncertainties it is also at least questionable if RAPD markers can be used for identification of individuals, cultivars or species, as it has been done in the first part of thesis and in other publications [5]. RAPD markers rather provide a way of distinguishing among samples in question. However, with the increasing number of coincident RAPD polymorphisms between the original and the sample also increases the certainty of the questioned sample.

The impact of molecular markers on crop improvement will be influenced by several factors, not least by their cost. RAPD-PCR technology requires much less financial input and infrastructure as RFLPs, but it is still too expensive for a routine screening of a large number of individuals, mainly because of the high price of the Taq-polymerase. Automatisation, further simplification of the technology and the possibility of screening for many desirable characters is necessary. The constant development of modified PCR technologies will certainly overcome the drawbacks of RAPDs. The conversion of a RAPD marker into sequence characterised amplified regions (SCARS), for example, reveals a way to greatly improve reliability and repeatability of a marker. This is achieved by partial sequencing of the chosen marker followed by designing two specific primers for it, each about 20 bases long. These primers will anneal only at the desired site because of their statistically unique sequence, therefore producing only one fragment. Other methods such as CAPS (cleaved amplified polymorphic sequences) transforms the usually dominant RAPDs into co-dominant markers: restriction digests of chosen PCR fragments can then provide information about the homo- or heterozygosity of a marker.

These and many further developments of PCR-based methods may well provide the need for analyses that are repeatable among different laboratories.
References


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