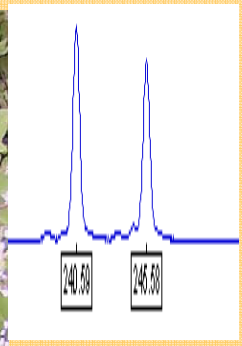
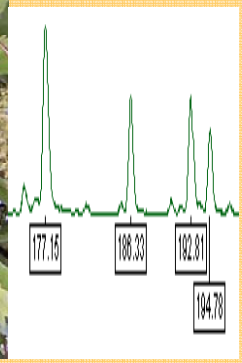
Madhav Pandey

Development of microsatellites in sycamore maple (*Acer pseudoplatanus* L.) and their application in population genetics





Institute of Forest Genetics and Forest Tree Breeding
Faculty of Forest Sciences and Forest Ecology
Georg-August University of Göttingen
Germany

Development of microsatellites in sycamore maple (*Acer pseudoplatanus* L.) and their application in population genetics

DISSERTATION

Submitted in partial fulfilment of the requirements for the degree of Doctor of Forestry Science at the Faculty of Forest Sciences and Forest Ecology, Georg-August University of Göttingen, Germany

by

Madhav Pandey

Born in Gulmi, Nepal

Göttingen, 2005

D7

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To my beloved family……

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1. Introduction

1.1. Introduction into the species

1.1.1. The genus Acer

The genus *Acer* belongs to the order of the Sapindales and the family Aceraceae that was created by TOURNEFORT in 1700. It contains approximately 150 species (DE JONG, 1976). The number of species might be higher than those presently identified, since several taxa from China, Indonesia and the Mediterranean regions are not yet sufficiently investigated. The genus *Acer* is subdivided into sixteen sections that are further subdivided into nineteen series (DE JONG, 1994).

Maple *(Acer* spp.*)* is found in the major part of temperate regions of the northern hemisphere and also in the southern Himalayas and in the mountains of Malaya, Indonesia and the Philippines. Only in Indonesia the genus crosses the equator to about 10° s. l. Maples are generally found in mountainous regions. In the south-east Himalayan region they can be found up to an altitude of 3,300 m.

1.1.2. Acer pseudoplatanus

Acer pseudoplatanus belongs to section *Acer* and series *Acer* of genus *Acer*. It is the tallest member of the genus *Acer*. This species is one of the 9 earliest-identified species of the genus which was recorded by LINNAEUS (1753). In English it is called ‘sycamore maple’ and in German ‘Bergahorn’.

1.1.2.1. Morphology

Acer pseudoplatanus is a tall tree with a round and dense crown that reaches a height of about 40 meters at the age of 150 years and can reach a diameter of 60 to 70 cm (SPETHMANN and NAMVAR, 1985). Sycamore can live up to 500 years (RUSANEN and MYKING, 2003). The leaves of sycamore maple are 5-lobed with toothed margins (see Figure 1).

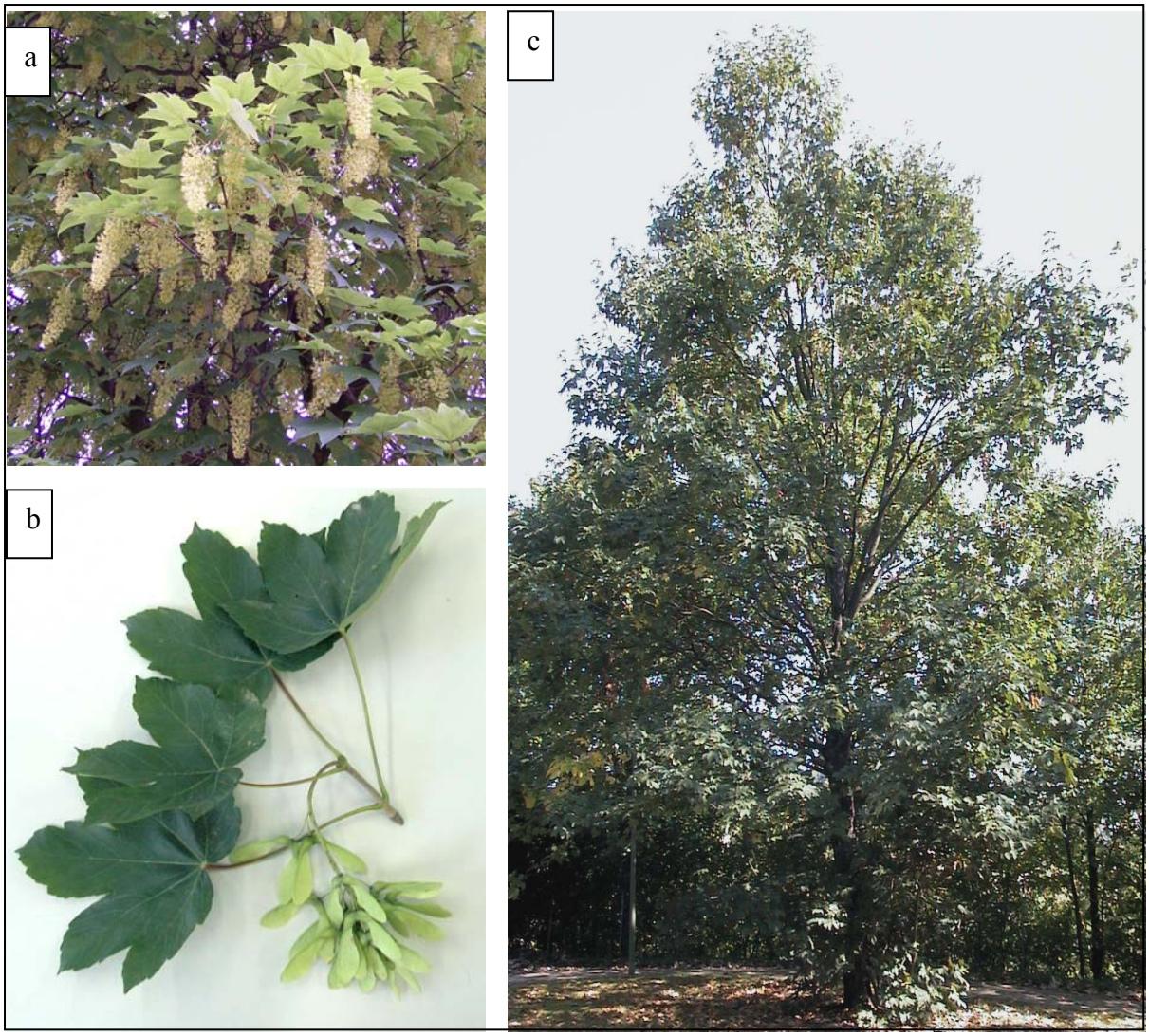

Figure 1. Photographs of *A pseudoplatanus*. a: flowers hanging on a tree; b: leaves and fruits; c: an adult tree.

They are coppery coloured when young and become dark green on top, and either purple, white or grey-green on the bottom at maturity. Buds are 8-10mm long, ovoid with few green scales whose margin is reddish, open with basal scales. Buds are bigger in size as compared to the buds of other maple species. The bark is gray-brown to red-brown, breaks up into large scales that often exfoliate.

1.1.2.2. Distribution

The natural distribution range of sycamore maple extends from Belgium to the Caucasian mountains and from northern Germany to southern Italy. The species does not occur in the northern and eastern parts of Europe (Figure 2). It is common in mountainous regions. This

mountain tree species is also found at low altitudes in northern Germany but its altitudinal distribution shifts upwards at lower latitudes. Sycamore maple can grow from 300 m up to 2,000 m altitude (SPAETHMANN and NAMVAR, 1985). In general, it is found in the altitudinal range from 700 m to 1,300 m and up to the tree line in many parts of Europe (BINGGELI, 1994).

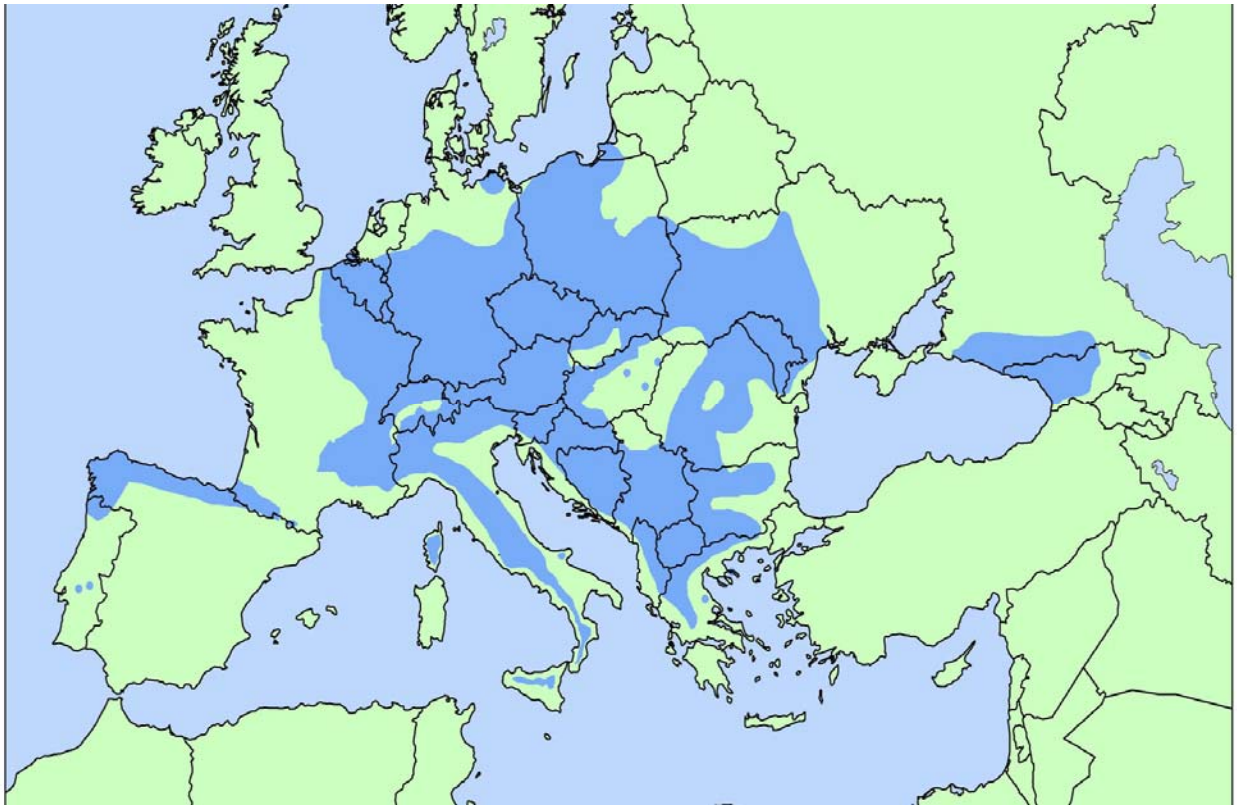

Figure 2. Distribution map of *A. pseudoplatanus* (RUSANEN and MYKING, 2003). The dark (blue) area represents the distribution of natural populations of *A. pseudoplatanus*.

1.1.2.3. Habitat and ecology

Sycamore maple prefers rich, deep, well-drained soils though it can also grow under less favourable conditions. It is the only maple tolerating salt-spray and can be frequently found near the ocean. It prefers shady and moist sites, usually strewn with rock fragments and boulders, chiefly on substrates rich in calcium, near places where water seeps out of the ground. In general it grows in mixed stands together with other forest tree species such as *Fagus* spp., *Fraxinus* spp., *Quercus* spp. Sycamore maple becomes the dominant canopy tree only in habitats which are not suitable to beech. In forests dominated by beech (*Fagus* sp.) sycamore maple's representation is very low due to its incapacity to compete with beech.

Sycamore-dominated forests are small and mainly found along moist ravines or on slopes where winter snowfalls are heavy (BINGGELI, 1994).

1.1.2.4. Flower and sexual system

Flowers are monoecious, yellow and hanging in 3-5 clusters, with 20-50 flowers on each stalk. They are opened from April to May, depending on the weather conditions (DE JONG, 1976). Most of the flowers are morphologically hermaphroditic but functionally unisexual. In most of the cases the male flowers also have ovaries and the female flowers have stamina but those are less developed and rudimentary (WEISER, 1973; DE JONG, 1976) (Figure 3). In each inflorescence both male and female flowers are present. The number of the male flowers are always higher and the duration of male flowering is always longer than the female flowering (RUSANEN and MYKING, 2003). The seeds of 5-10 mm diameter are paired in samaras, each seed with a 20-40 mm long wing to catch the wind and to rotate while they fall; this helps them to spread further from the parent tree. The seeds are mature in autumn, about 6 months after pollination.

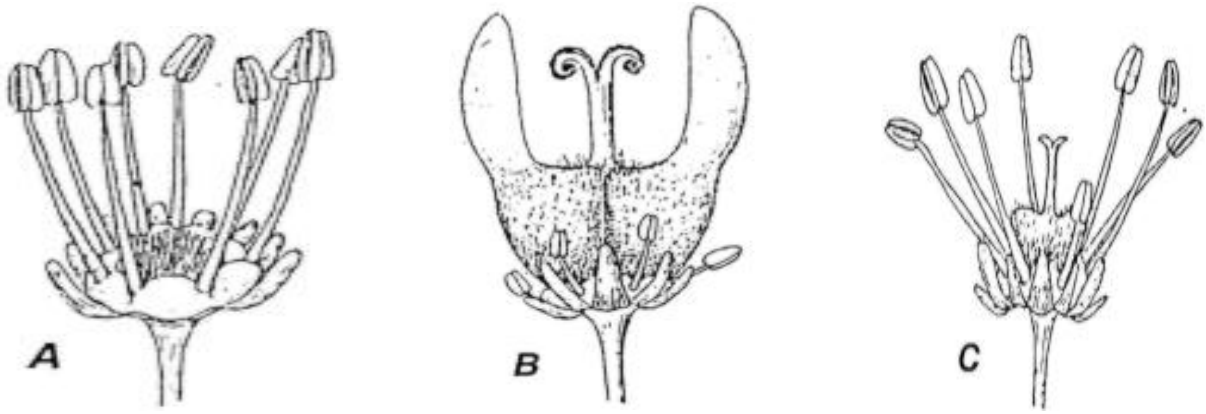

Figure 3. Flower types in sycamore maple (DE JONG, 1976) A. Morphologically and functionally male; B. Morphologically hermaphrodite but functionally female; C. Morphologically hermaphrodite but functionally male.

The species has been reported to be tetraploid with chromosomes 2n=4x=52 (DARLINGTON and WYLIE, 1955).

1.1.2.5. Pollination biology

Sycamore maple is commonly described as insect-pollinated. The flowers occur in early spring. They are the important for bee food (HOFFMAN, 1960). Because of the production of nectar and pollen, different insects belonging to the *Hymenoptera* and *Diptera* visit the flowers of *A. pseudoplatanus.* On the basis of its morphological characteristics and flowering ecology, especially the structure and distribution of pollen kits on the crown of pollen, HESSE (1979) categorised sycamore maple as an insect and wind-pollinated species. So, sycamore maple is considered as predominantly insect-pollinated with some degree of wind pollination.

1.1.2.6. Uses

The wood of sycamore maple is widely used for furniture making because of its creamy white colour, and because of the absence of unpleasant smell or taste. Its wood is rarely used for construction purposes due to its low durability but it can be used for outdoor construction after preservation treatment (RUSANEN and MYKING, 2003). It is also used for manufacturing music instruments, floor-parquet and for valuable handicrafts (WEDEL, 1964; KNIGGE and SCHULZ, 1966; KOLTZENBURG, 1974).

A. pseudoplatanus is popular for honey production. CRANE (1975) estimated that 200 to 500 pounds of honey per hectare can be produced by *A. pseudoplatanus* stands. Since there are many cultivars with different colours and patterns of leaves, it is widely planted in public gardens and for amenity purposes. Because of its strong resistance to wind it is also planted to serve as a wind breaker (OTERDOOM, 1994). *A. pseudoplatanus* plantations can also be used for the improvement of soil properties in bad site conditions. For example, it has been used in the colonization of power station ash heaps in Britain (OTERDOOM, 1994).

1.1.2.7. Propagation

Propagation of *A. pseudoplatanus* can be done by both sexual and vegetative means. The seeds are harvested during September to November. Seeds are used to produce seedlings in the nursery and after 1 year they can be planted in the planting site. In natural populations plenty of natural regeneration can be found in slightly open areas. Rootstocks are used for budding and for side-grafting. For some cultivars of *A. pseudoplatanus* hardwood grafting is used (VAN GELDEREN, 1994).

1.2. Polyploidy

Polyploidy is defined as the presence of more than two sets of chromosomes per nucleus. It has been recognized as a major source of evolution in angiosperms. Almost 50% of all angiosperm taxa are assumed to be of polyploid origin (GRANT, 1981). Polyploidy occurs only sporadically among animals (LEWIS, 1980). Although the phenomenon is widespread across the plant kingdom, some plant groups do not contain polyploid species. In gymnosperms, for example, no polyploids have yet been found in ginkgo or in cycads, and only few in conifers (GRANT, 1971). Polyploidy represents a special class of mutation and can occur via several routes: genomic doubling, gametic non-reduction and polyspermy (OTTO and WHITTON, 2000). The most widespread natural polyploidy is tetraploidy. In most cases, tetraploidy combines the genomes of two differently adapted, but cytogenetically closely allied taxa (DE WET, 1979). Polyploids are classified into two groups on the basis of their origin, autopolyploids and allopolyploids (KIHARA and ONO, 1926).

1.2.1 Autopolyploids

This type of polyploidy is described as a doubling of the diploid genome within a species. It is often assumed that polyploids forming multivalent during meiosis are autopolyploids. Autopolyploids are also called polysomic polyploids and can occur at the level of triploidy (3n) or higher ploidy levels (GRANT, 1981). Autopolyploids with small chromosomes or low chiasma frequencies may exhibit disomic inheritance immediately after their formation (STEBBINS, 1938).

1.2.2 Allopolyploids

Polyploids which form bivalents during meiosis are called allopolyploids. They exhibit disomic segregation. Generally, allopolyploids are considered to be much more common than autopolyploids. However, the occurrence of autopolyploidy may be greatly underestimated (SOLTIS and SOLTIS, 2000). Allopolyploids can be further divided into two groups:

a. Segmental allopolyploids: They are the result of hybridization between partially cross-fertile progenitors, i.e. those that have a certain degree of homology in chromosome make-up but are sufficiently heterologous for the progenitors to be almost cross-sterile at the diploid level (THOMPSON and LUMARET, 1992).

Segmental allopolyploids are essentially intermediate forms between auto- and allopolyploids (STEBBINS, 1950).

b. Genomic allopolyploids: They have almost completely cross-sterile ancestors and contain two complete genomes, each of which shows disomic inheritance. Unlike segmental allopolyploids, these types of polyploids do not segregate for characters by which the progenitor species differ.

1.2.3. Genetic consequences of polyploidy

Polyploidy has been considered a major cause of evolution and speciation (SCHULTZ, 1980; SOLTIS and SOLTIS, 1995). One of the major consequences of natural polyploidization is the creation of new species such as new species of wheat (*Triticum aestivum*), tobacco (*Nicotiana tabaccum*), and upland cotton (*Gossypium hirsutum*) formed due to the increase in ploidy level through different routes (SWANSON et al. 1981). Natural polyploidization is also common in the genus *Acer* such as *A. carpinifolium, A. caesium, A. pseudoplatanus.*

The combination of genomes from different individuals with three or more adapted genomes is a reason for the success of polyploids (LEWIS, 1979). Appropriate combinations of multiple genomes could be a reason for forming highly competitive and adaptive polyploid biotypes, producing new, vigorous and well-adapted cytotypes (BINGHAM, 1979). The adaptation benefit of polyploids is due to the presence of more alleles as compared to diploids. Thus each individual has a greater chance of carrying a new beneficial mutation and can have a lower deleterious load than the individuals of lower ploidy level (PAQUIN and ADAMS, 1983).

Though the genetic changes due to polyploidy depend on the type of polyploidy, there is potentially more internal genetic variation in both types of polyploidy (THOMPSON and LUMARET, 1992). In autopolyploids, inheritance is polysomic due to the occurrence of multivalent formation during meiosis. In general, polysomic inheritance produces more heterozygous progeny due to an increase in the proportion of loci in heterozygous state as compared to disomic inheritance.

1.3. Molecular markers

The invention of DNA gene markers has played an important role in the field of genetics. The use of DNA gene markers was initiated by BOTSTEIN *et al*. (1980). In the beginning, DNA gene markers were used in human genetics, but soon after their development they were used also in plant genetics. Along with the increase in knowledge on the genetic properties of DNA, numerous new techniques for detecting DNA polymorphisms are evolving. The isolation of restriction enzymes and the polymerase chain reaction (PCR) have allowed us to assess polymorphism directly at the DNA level (GLAUBITZ and MORAN, 2000). There are several DNA gene markers in use in forest genetics. Commonly used markers are: Restriction Fragment Length Polymorphisms (RFLPs), Amplified Fragment Length Polymorphisms (AFLPs), Random Amplified DNA Polymorphisms (RAPDs), Inter Simple Sequence Repeats (ISSRs), Single Nucleotide Polymorphisms (SNPs) and Simple Sequence Repeats or microsatellites (SSRs). Since microsatellite gene markers were used for this study, only this marker type will be discussed in more detail here.

1.3.1. Microsatellites or Simple Sequence Repeats (SSRs)

Microsatellites are sequences composed of tandem repeats from one to six bases in length which are arranged head-to-tail generally without interruption (HANCOCK, 1999). They are also known as Short Tandem Repeats (STR) or Simple Sequence Repeats (SSRs). Depending on the number of nucleotides per motif, microsatellites are divided into mononucleotide (motif with a single nucleotide), dinucleotide (motif with two nucleotides), trinucleotide (motif with three nucleotides), and so on. On the basis of purity, microsatellites are divided into two types; one is uninterrupted (pure microsatellite motif) and another is interrupted (not pure but mixed with other nucleotides within the repeat motif). Microsatellites are mostly found in non-coding regions (such as introns) of the genome and are very rarely found in coding regions (exons) of the genome (HANCOCK, 1995). The most polymorphic and therefore the most useful microsatellites are uninterrupted microsatellites (WEBER, 1990). In genomes of almost every organism so far studied microsatellites have been detected in higher frequencies than they were expected (HANCOCK, 1999). Microsatellites seem to be distributed evenly throughout the genome. EDWARDS *et al.* (1991) investigated microsatellite loci in the human genome. They observed that at least one SSR repeat was present at 300 to 500 kbs.

The reason for the high variability of microsatellites is their high mutation rate. The mutation rates in microsatellites are higher as compared to rates of point mutation, which are of the order of 10^{-9} to 10^{-10} (HANCOCK, 1999). LEVINSON and GUTMAN (1987) estimated about 10^{-2} events per replication in *E. coli* and WEBER and WONG (1993) suggested 10^{-3} events per locus and generation in humans. In *Drosophila* relatively lower rates of mutation of about $6x10^{-6}$ were observed (SCHUG *et al.* 1997). In general, the mutation rate of microsatellite sequences is 10^{-3} to 10^{-5} per locus and per generation (EDWARDS *et al.* 1992; SCHLÖTTERER and TAUTZ, 1992; BOWCOCK *et al.* 1994; FORBES *et al.* 1995).
Microsatellites seem to be less abundant in plants as compared to vertebrates (LAGERCRANTZ *et al.* 1993). In terms of the type of repeat motifs, the most common dinucleotide repeat $(GT)_n$ in human genomes seems to be the most-scarce in plant genomes, while $(AT)_n$ is most common in plants (LAGERCRANTZ *et al.* 1993).

1.3.2. Evolution of microsatellites (SSRs)

Microsatellites are useful tools for evolutionary and genetic studies due to their inherent instability. To understand the mechanism of instability of microsatellite repeats we also have to understand why the instability varies within and between species (EISEN, 1999). There are two different models to describe the evolution of microsatellites:

A. Unequal Crossing Over (UCO)

One of the reasons of microsatellite evolution is an elevated rate of unequal crossing over (EISEN, 1999). It is due to the recombination of two homologous chromosomes which are aligned imperfectly during the crossing over (SMITH, 1973). The hypothesis behind this model is that the presence of repeats increases the likelihood of misalignment between the homologous chromosomes (EISEN, 1999).

B. Slip-strand mispairing (SSM)

Another model of microsatellite mutation is the slip-strand mispairing (SSM) model, which was first proposed by FRESCO and ALBERTS (1960). In this model slippage of DNA polymerase occurs during the replication of DNA causing the template strand and the newly replicated strands to be temporarily unaligned. In order to continue the replication process, the strand must realign and a mutation will be created if this realignment is not perfect. The

hypothesis behind this model is that the presence of repeats in template DNA increases the chance of DNA polymerase slippage, because repeats can easily be looped out of the DNA double helix (STREISINGER *et al.* 1966). This model appears to be a predominant mode of microsatellite evolution (WOLFF *et al.* 1989). Although microsatellite instability is an integral part of the SSM model, not all the errors generated due to SSM lead to mutations. Some of them are repaired by error correction mechanisms (EISEN, 1999). There are two pathways of error correction; one is exonucleolytic proofreading and the other is post–replication mismatch repair. So, in order to understand the complete mechanism, these two factors should be included in the SSM mutation model (EISEN, 1999) (Figure 4).

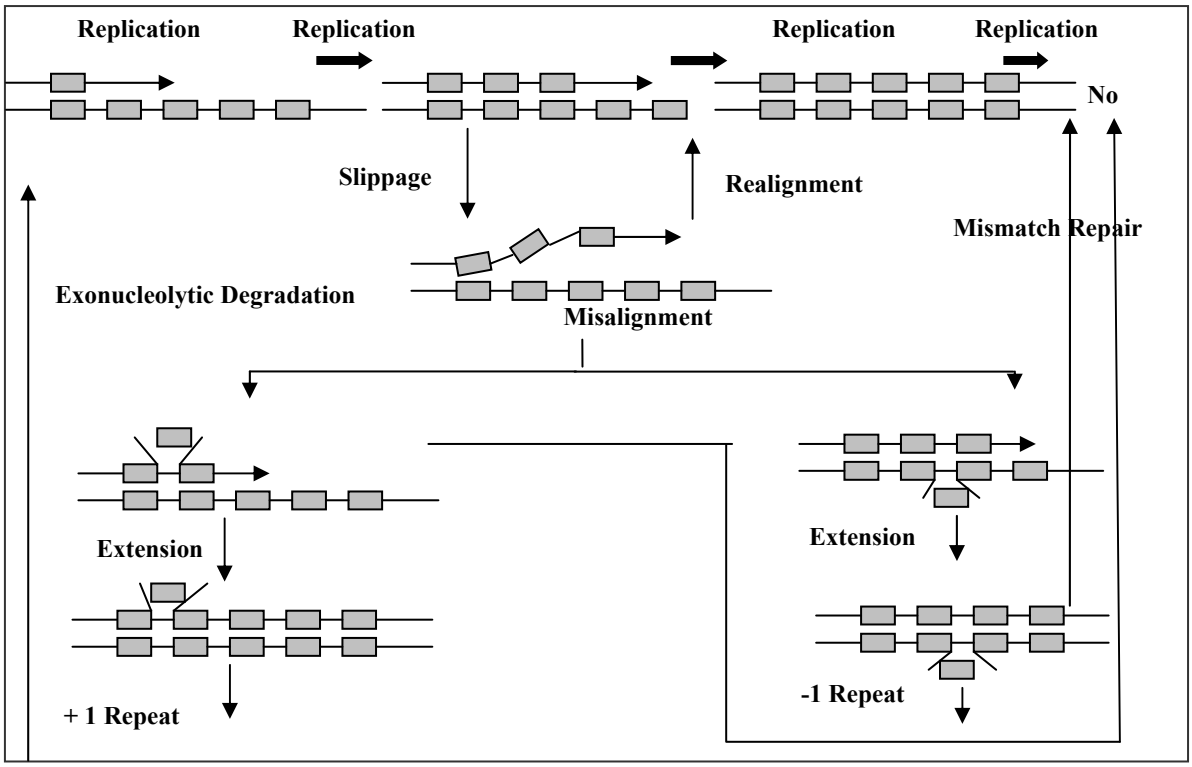

Figure 4. Diagrammatic presentation of the slip-strand mispairing model of microsatellite mutation (EISEN, 1999).

1.3.3. Theoretical models of microsatellite mutation

Microsatellite gene markers became popular in the field of evolutionary genetics and replaced or complemented other markers for different applications. A detailed understanding of the mutational process guiding the evolution of microsatellites is necessary to optimize the information obtained from these markers (ESTOUP and CORNUET, 1999). In order to

understand the evolution of microsatellite loci, different theoretical mutation models have been developed. Three important models are briefly discussed below:

A. Infinite allele model (IAM) (KIMURA and CROW, 1964)

In this mutation model any number of tandem repeats can evolve and always results in a new allele status not previously existing in the population.
This mechanism mostly causes small changes in the repeat numbers such as alleles of similar lengths. They are expected to be more closely related to each other than alleles of completely different sizes.

B. Stepwise mutation model (SMM) (KIMURA and OHTA, 1978)

The SMM better describes this kind of evolutionary process more accurately. Under the SMM; each mutation creates a novel allele either by adding or deleting a single repeat with the same probability in both directions.

Consequently, more different alleles in terms of sizes are also evolutionarily more distant than alleles having similar sizes: therefore, SMM has a "memory". In this model new alleles are not necessarily always generated. Alleles possibly mutate towards allele states already present in the population.

C. K-allele model (KAM) (CROW and KIMURA, 1970)

Under this model, there are K possible allelic states, and any allele has a constant probability of mutating towards any of the K-1 allelic states existing before. Due to size constraints acting on microsatellite loci, the KAM seems to be more realistic than the IAM.

1.3.4. Application of microsatellite markers

Because of their high level of polymorphism and their co-dominant nature, microsatellites are useful for many purposes in genetic studies. Main applications of microsatellite gene makers are listed as below:

- Genetic mapping and linkage analysis (e.g. WEISSENBACH *et al.* 1992; ROBINSON *et al.* 2001).

- Paternity and kinship analysis (e.g. QUELLER *et al.* 1993).
- Genetic variation within and among species (e.g. GOTTELLI *et al.* 1994; ROY *et al.* 1994).
- Genetic variation within populations and differentiation among populations (e.g. BOWCOCK *et al.* 1994; FORBES *et al.* 1995; ESTOUP *et al.* 1996; LADE *et al.* 1996; GARCIA *et al.* 2004).
- Estimation of effective population size (e.g. ALLEN *et al.*, 1995).
- Gene flow and mating system (e.g. KAMEYAMA *et al.* 2000; WHITE *et al.* 2002; DECARLI, 2003; GAIOTTO, 2003; IMBERT and LEFEVRE, 2003).
- Study of phylogeny (e.g. TAKEZAKI and NEI, 1996; VANDER-ZWAN *et al.* 2000; THOMAS *et al*. 2003; STENSON *et al.* 2004).

1.3.5. Advantages of microsatellite gene markers

Advantages of microsatellite gene markers are:

- Low quantities of template DNA required (10-100 ng per reaction).
- High genomic abundance and possible random distribution throughout the genome.
- High level of polymorphism.
- Band profiles can be interpreted in terms of loci and alleles.
- Co-dominant inheritance of alleles. However, recessive "null-alleles" have been reported.
- Allele sizes can be determined with an accuracy of 1 bp, allowing accurate comparison across different gels.
- High reproducibility. However, there are exceptions to this general condition as will be mentioned below.
- Different microsatellites may be multiplexed in PCR or on gels in capillaries.
- Wide range of applications.
- Amenable to automation.

1.3.6. Disadvantages

Despite many advantages, the work with microsatellite gene markers poses the following problems:

- SSR development is practically complex and expensive and may yield only a small number of useful microsatellite loci. For example, KELLEY and WILLIS (1998) screened 150,000 plaques with SSR probes, and only 179 positive plaques could be sequenced.
- Heterozygotes may be misclassified as homozygotes when null-alleles occur due to mutations in the primer-binding sites.
- Stutter bands on gels may complicate accurate scoring of polymorphisms.
- The underlying mutation model (infinite allele model or stepwise mutation model) is usually unknown.
- Homoplasy due to different forward and backward mutations may underestimate genetic divergence. Mutations (insertion/deletion) may also occur in SSR flanking regions.

1.3.7. Development of microsatellite gene markers

The first microsatellites were developed in humans. Soon after their application in human genetics due to their wide range of applicability they became also popular among plant geneticists. Meanwhile there are numerous reports on microsatellite development in plant species. One of the first tree species for which microsatellites were developed was *Pinus radiata* D. Don (radiata pine) (SMITH and DEVEY, 1994). A search in the Journal "Molecular Ecology Notes" resulted in 112 reports of microsatellite development in forest tree species published since then. This indicates how fast their application in forestry is growing. Some examples of tree species for which microsatellites have been developed at the early stages are: *Quercus* spp. (oaks) (DOW *et al.* 1995; BARRET *et al.* 1997; STEINKELLNER *et al.* 1997; ISAGI and SUHANDONO, 1997), *Eucalyptus* (BYRNE *et al.* 1996), *Pinus strobus* (ECHT *et al.* 1996), *Picea abies* (PFEIFFER *et al.* 1997), and many tropical tree species (CHASE *et al.* 1996; WHITE and POWELL, 1997; DAWSON *et al.* 1997). Mononucleotide microsatellites have also been detected in the chloroplast genome of

pine trees (VENDRAMIN *et al.* 1996) and of angiosperms (WEISING and GARDNER, 1999).

One of the main reasons of widespread microsatellite development in many species of animals and plants is due to the technical advances made in recent years (ZANE, *et al.* 2002). The traditional method for microsatellite development has been to construct a partial genomic library of the targeted species and screening thousands of clones through colony hybridization with probes containing repeats (RASSMANN *et al.* 1991). Although this method is relatively simple, it may become tedious and inefficient for species with low microsatellite frequencies. The average percentage of getting positive clones estimated from 115 species of plant and animal species using this method ranged from 0.4 to 2.3 (ZANE *et al.* 1991). Some authors have modified the randomly amplified polymorphic DNA (RAPD) technique for the amplification of unknown microsatellites by either using repeat-anchored primers (WU *et al.* 1994) or using RAPD primers and Southern Hybridization of polymerase chain reaction bands with microsatellite probes (CIFARELLI *et al.* 1995; RICHARDSON *et al.* 1995). The drawback of this method is that these microsatellites are not useful for single locus analysis, since there is no information on microsatellite flanking regions.

Another approach of microsatellite development is the production of genomic libraries that are highly enriched for specific microsatellite repeats using a primer extension reaction (OSTRANDER *et al.* 1992; PAETKAU, 1999). Although the success rate of getting positive clones is higher, application of this method is not very efficient since the method involves numerous steps.

A further kind of microsatellite isolation methods is selective hybridization (KARAGYOZOV *et al.* 1993; ARMOUR *et al*. 1994; KIJAS *et al.* 1994). The main feature of this method is to hybridize selected artificial microsatellite probes with restricted-ligated genomic DNA fragments and to capture the hybridized fragments. There are some variations of this method, especially for artificial probe hybridization; one is hybridization to a nylon membrane (KARAGYOZOV *et al.* 1993; ARMOUR *et al.* 1994), another is hybridization of 5' biotinylated oligo-probes, binding to streptavidin-coated beads, and capturing with the magnet particles (KANDPAL *et al.* 1994; KIJAS *et al.* 1994; FISCHER and BACHMANN, 1998). This method has frequently been used and the enrichment efficiency is reported to be 20 to 90% (ZANE *et al.* 2002). The latest method of microsatellite isolation is the Fast

Isolation by AFLP Sequences Containing Repeats (FIASCO) (ZANE *et al.* 2002). In this method the AFLP fragments are hybridized to the biotinylated probes. The rest of the procedure is similar to the selective hybridization method. ZANE *et al.* (2002) reported 50 to 90% of clones containing dinucleotide microsatellite repeats. The cost and time required for the isolation of microsatellites and their success are the major draw-backs limiting their application to the study of the genetics of an even wider range of plant and animal species (ZANE *et al.* 2002)

1.4. Genetic variation and genetic structure

1.4.1. Genetic variation

Genetic variation is the fundamental requirement of living organisms to keep on their existence in heterogeneous and changing environmental conditions. The genetic variation of a biological collective can be defined as the occurrence of different genetic variants (HATTEMER, 1991). Genetic variation between individuals of any animal or plant species and between those species is the product of the evolutionary development process of the living world (DOBZHANSKY, 1967).
Compared to other ecologically or economically significant species, trees are extremely long-lived and grow in a much more heterogeneous environment. Thus, in order to adapt under such complex complex environmental conditions, trees need a high level of genetic variation (MÜLLER-STARCK and GREGORIUS, 1986).

Most of the recent experimental studies aimed at the measurement of genetic variation are usually based on genetic markers. However, the observation of phenotypic variation in quantitative traits has also been used to gain important insights into the patterns of genetic variations. The basic parameters for estimating the genetic variation within populations are allelic and genotypic structure.

Genetic variation can be measured at different hierarchical stages, e.g. between species and within species. The variation between species is easily recognizable as they are morphologically distinct. The variation within species is difficult to predict on the basis of morphology. Hence, the study of genetic characteristics is the only way to explore the genetic variation within species.

1.4.2. Spatial genetic structure

The study of spatial genetic structure deals with the distribution pattern of genetic variants of individuals or groups of individuals within a population. The study of genetic structure within the population is important in order to understand micro-geographical evolutionary processes such as family structures or micro-site selection effects (KNOWLES, 1991). The spatial distribution of genetic information connects a central problem of ecological genetics, i.e. the significance of evolutionary factors for the dynamics of genetic structures, and application-oriented problems regarding the conservation and utilization of this variation (FINKELDEY and MATYAS, 2003). The genetic variation over short distances may occur either due to spatially variable selection or due to localized genetic drift, if the transport of genetic information restricted (ENDLER, 1977).

In plant populations the phenomenon of spatial genetic structure is common, since plants are immobile and the dispersal of pollen and diaspores is spatially limited. Contrary to the theoretical expectation, the spatial structure in plant populations is rarely consistent across loci or sites and, when found, may be very weak (SMOUSE and PEAKALL, 1999). There are many studies which reported minor spatial structure on a micro-spatial scale (HEYWOOD, 1991; SCHNABEL *et al.* 1991; PERRY and KNOWLES, 1991; BERG and HAMRICK, 1995).

As has been mentioned, a significant departure from random spatial distribution of genotypes in plant populations can be due to spatially varying selection and/or to local dispersal of genes (TURNER *et al,* 1982; BRADSHAW, 1984). In most plant populations, significant variation of genetic structure over small distances results from restricted seed dispersal such as siblings or parents and offspring growing close to one another, sometimes despite the possibility of long-distance pollen flow (CAMPBELL and DOOLEY 1992; LOISELLE *et al.* 1995; VORNAM *et al.* 2004; CRUSE-SANDERS and HAMRICK, 2004). On the other hand, plant species with overlapping seed shadows, high out-crossing rates, rare seedling establishment, and/or recruitment away from the maternal plant are expected to display minor genetic structure within populations (HAMRICK and LOVELESS 1986; UENO *et al.* 2000).

Information on spatial population genetic structure is very important in order to understand gene transfer within the population, which may also contribute to understand how populations respond to selection (EPPERSON, 1989). The lack of knowledge about the

genetic structure of the population can lead to a biased assessment of other biological phenomena within plant populations. If we, for instance, assume that the population does not have population substructure although it is in fact present, we are liable to biased estimation of mating system parameters (ELLSTRAND *et al.* 1978; RITLAND, 1985). For example, disregarding an existent spatial genetic structure may result in biased estimates of mating system parameters.

1.5. Movement of genetic information

Genetic information can be moved within a population, between populations, and on the landscape or regional (meta-population) level. Gene flow or gene transfer between populations of living organisms is a very important phenomenon for maintaining their genetic diversity. The gene flow among populations genetically homogenizes spatially distinct populations (SLATKIN, 1985; ELLSTRAND, 1992). In most plants there are two basic mechanisms of gene transfer, i.e. by male gametes and seeds. Genetic information of some plants is also transported by vegetative propagules.

Gene flow between different species is much less common and is increasingly less likely between more distantly related species. The likelihood of successful cross-pollination and formation of a hybrid individual between two related species initially depends on their proximity, synchronisation of flowering, and many biological barriers that must be overcome. However, gene flow among species has played a key role in the evolution of closely related plant species through hybrid formation.

Extensive gene flow from natural populations may cause a problem in tree breeding and in gene conservation areas due to the introduction of “undesirable” or “inferior” genes. Influx of effective pollen from unimproved stands into seed orchards and selected seed collection areas reduces the expected genetic gain. These should therefore be kept isolated (EL-KASSABY and RITLAND, 1986; ADAMS and BIRKES, 1989; HATTEMER *et al.* 1993).

There are direct and indirect methods to measure gene flow. Direct methods are based on the observation of the dispersal of pollen and seeds, which gives an estimate of potential gene flow. To track the gene movement directly, parentage analysis of seeds is required (ROEDER *et al*. 1989; ADAMS and BIRKES, 1991; DEVLIN and ELLSTRAND, 1990;

SMOUSE and MEAGHER, 1994). Paternity analysis is usually based on progenies from known maternal parents to assign paternity to a set of potential pollen donors.

Indirect methods use the spatial distribution of genetic variation to infer average amounts of gene dispersal over time, including rare long-distance dispersal events. For example, the genetic differentiation F_{ST} (WRIGHT, 1969) is a commonly used parameter that under certain assumptions is inversely related to gene flow among populations.

Since the mid-1960s, enzyme gene loci have been used to estimate gene flow. Recently, microsatellites have become the markers of choice for gene flow studies, because the number of polymorphic loci and the number of alleles per locus tend to be higher than those of enzyme gene loci.

1.6. Genetic variation of Acer pseudoplatanus

The first insight into the genetics of this species was an observation of the chromosome numbers (DARLINGTON and WYLIE, 1955). *A. pseudoplatanus* was identified as a tetraploid species with a chromosome number of 2n=4x =52. The basic chromosome number for the genus *Acer* is 13. It is classified as an autotetraploid species because of its multisomic inheritance pattern. Due to its autotetraploid characteristics and complex reproduction system, there have been much fewer studies on the population genetics of this species conducted as compared to diploid tree species of the genus *Acer* (e.g. PERRY and KNOWLES, 1989; PERRY and KNOWLES, 1991; YOUNG and MERRIAM, 1994; BENDIXEN, 2001; FORE *et al.* 1991; BALLAL *et al.* 1994; RUSANEN *et al*. 2000).

Marker-based genetic studies in this species were initiated by KONNERT (1992) who identified individuals of a clone with three highly variable enzyme systems. Furthermore, KONNERT *et al.* (2001) investigated the inheritance pattern at 25 enzyme gene loci in single tree progenies. The mode of inheritance was confirmed for 17 gene loci coding for 11 enzyme systems. These authors reported the complexities in interpreting the zymograms of some of the enzyme systems due to the tetrasomic inheritance pattern of the species.

Investigation of chloroplast DNA markers in 19 populations of *A. pseudoplatanus* from different parts of Europe have led to the identification of 22 different haplotypes (PETIT *et*

al. 2003). BITTKAU and MÜLLER-STARCK (subm.) found that the polymorphism in chloroplast DNA of *A. pseudoplatanus* varies predominantly among populations. However, several variants were detected in some of the few central European populations examined so far.

NGERNPRASIRTSIRI and KOBAYASHI (1990) have constructed a complete physical map of the amyloplast (leucoplast) DNA. They identified that the sycamore amyloplast genome was composed of 141.7 kbp nucleotides with the same gene arrangement as that of tobacco chloroplasts. Chemical and physiological studies have also been carried out in *A. pseudoplatanus* (e.g. NGERNPRASIRTSIRI *et al.* 1988; NGERNPRASIRTSIRI *et al.* 1989; VIALE *et al*. 1991; LAFAYETTE *et al.* 1995).

CUNDALL *et al.* (1998) have investigated the performance of provenances from Britain, Denmark and Germany. They reported that in the nursery stage the performance (height, growth and root-collar diameter) of British provenances was better than that of the Danish and German provenances. However, after transplanting to the field test the provenances of Denmark and Germany performed better than the British provenances.

1.7. Objectives and research hypotheses

The present study has the following objectives:

- To develop variable microsatellite gene markers for *A. pseudoplatanus*,
- To study the genetic structure within two natural populations of *A. pseudoplatanus* and their differentiation,
- To study the spatial genetic structure of the adult trees and the natural regeneration of *A. pseudoplatanus* populations.
- To infer the dispersal of genes within the populations.

To achieve the above-mentioned objectives, the following research hypotheses were tested:

- Microsatellites are highly variable in *A. pseudoplatanus*. The tetraploidy of *A. pseudoplatanus* is reflected at microsatellite gene loci.
- The genetic structures within the two populations of *A. pseudoplatanus* differ.
- Gene transfer within the populations is random.
- The spatial genetic analysis within populations reveals family structures.

2. Materials and Methods

2.1. Plant material

2.1.1. Introduction to research areas

2.1.1.1. Södderich population

The population Södderich is part of the Reinhausen forest district and is located about 10 km east of Göttingen (longitude: 010° 01’ 52’’E; latitude: 51° 33’ 43’’ N; altitude: 317 m above sea level). The area is about 3 ha in size. Depending on their origin, the Södderich population is divided into the following two sub-compartments (Figure 5):

Sub-compartment 2b1

It is a mixed stand of beech (*Fagus sylvatica*), ash (*Fraxinus execlsior*), pedunculate oak (*Quercus robur*) and sycamore maple (*Acer pseudoplatanus*). According to records of the Reinhausen district forest office, the stand originated from natural regeneration and is 142 years old. It is dominated by beech with 72 % of standing gross volume, 47 cm average breast height diameter (DBH) and 32 m top height. Ash comprised 16 % of the standing gross volume, possessed 46 cm average DBH and 32 m top height. Sycamore maple is the third-most frequent species with 7 % of standing gross volume, 47 cm average diameters at breast-height and 34 m top height. The least represented species is pedunculate oak with 5 % of standing gross volume, 49 cm average DBH and 30 m top height. The stand has been approved for the collection of selected forest reproductive material for sycamore maple that can be marketed (according to EU Directives 55/404 and 1999/105). A map of all sampled sycamore maple trees is given in Figures 5 and 6 and a photograph of the population is shown in Figure 8.

Sub-compartment 2b2

The sub-compartment 2b2 is located in the north-eastern part of the stand where most of the trees are sycamore maples. In this part of the stand maples trees are 52 years old, were mostly planted and have only partly originated from natural regeneration.

2.1.1.2 Weißwassertal population

The Weißwassertal population is located about 13 km north-east of Göttingen (longitude: 010° 04’ 51’’E; latitude: 51° 34’ 28’’ N; altitude: 224 m above sea level) and also belongs to the Reinhausen forest district. It is about 3 kilometers away from the Södderich population. A photograph of a part of the population is given in Figure 8.

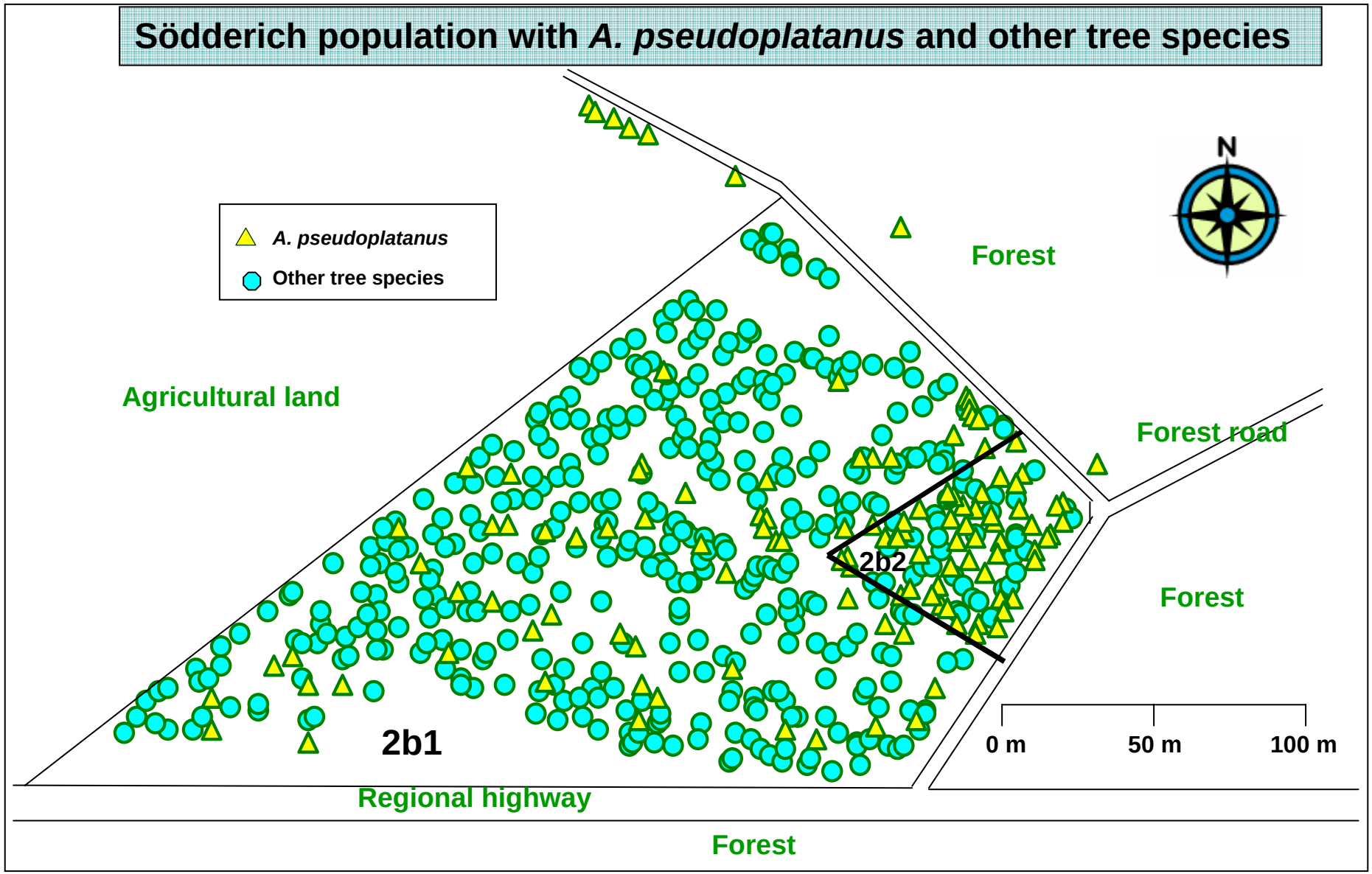

Figure 5. Map of *A. pseudoplatanus* and other tree species in the Södderich population.

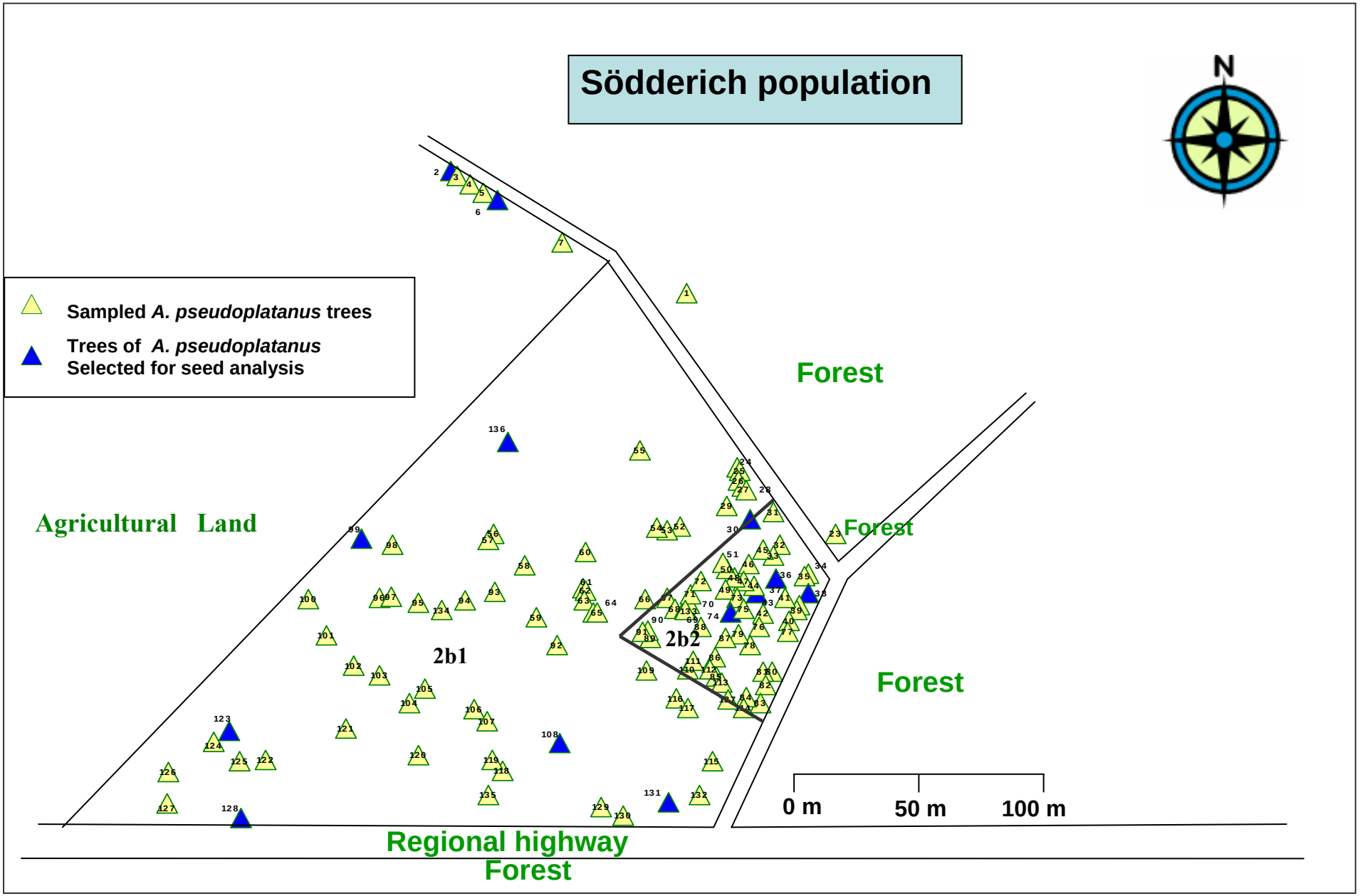

Figure 6. Map of all sampled *A. pseudoplatanus* trees in the Södderich population with their identification numbers.

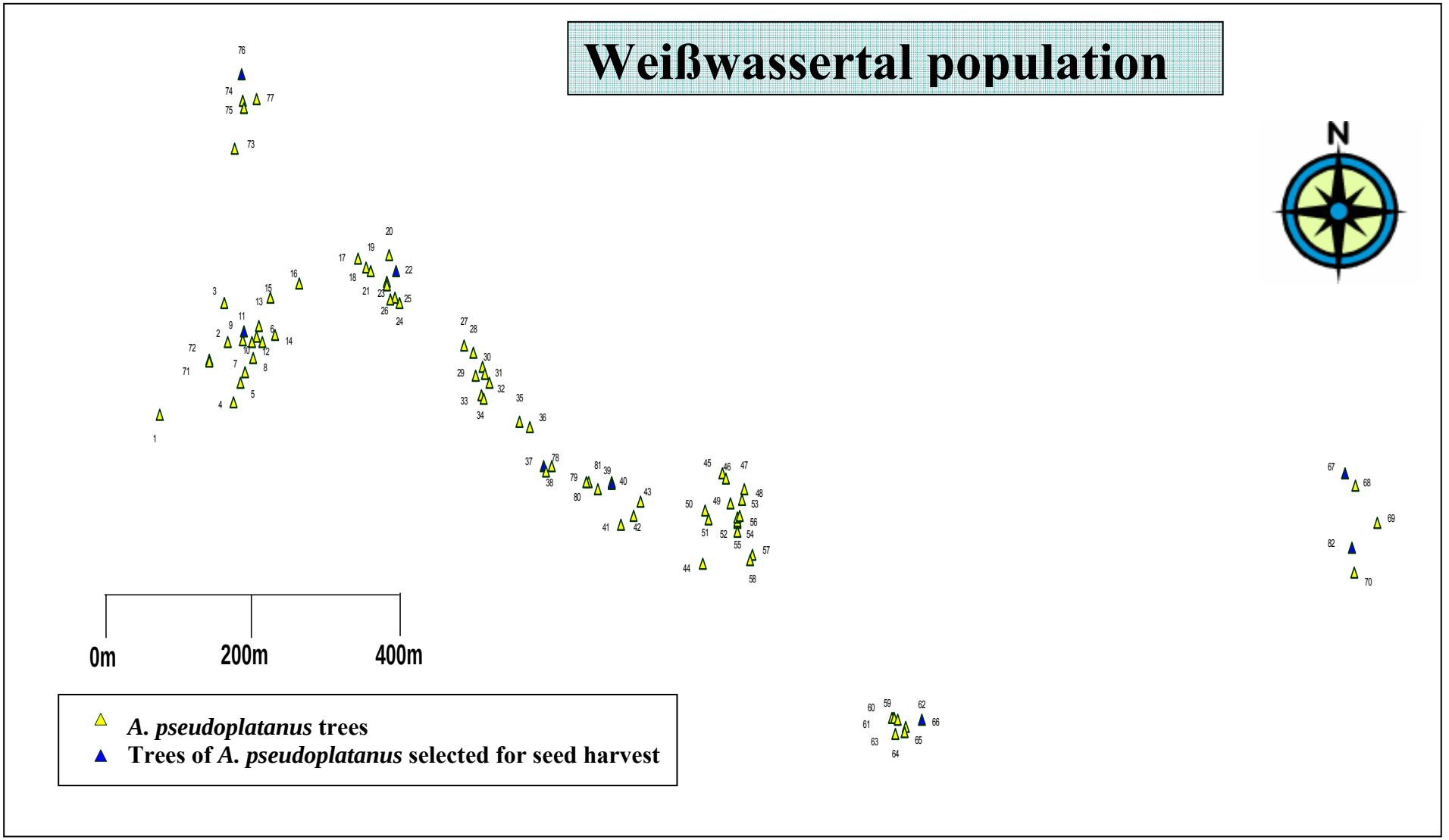

Figure 7. Map of all sampled *A. pseudoplatanus* trees of the Weißwassertal population with their identification number.

According to the records of the Reinhausen district forest office it is a mixed stand of beech (*Fagus sylvatica*), ash (*Fraxinus excelsior*), sycamore maple (*Acer pseudoplatanus*) and cherry (*Prunus avium*). Beech has 61 % of the standing gross volume and 28 cm average (DBH), followed by ash with 31 % and 34 cm, respectively. Sycamore maple comprises 5 % of the standing gross volume with 28 cm average DBH. Wild cherry represents only 2% of the gross standing volume with 28 cm average DBH. The top height of all tree species is 28 m. This population is stretched over 2.5 km along the narrow Weißwassertal valley. The western part of the stand is relatively open as compared to other parts. In the eastern part the ridges on both sides of the valley are covered with coniferous species. The location of all sampled sycamore maple trees are given in Figure 7.

2.1.2. Material collection

2.1.2.1. Buds

Buds of 137 (122 from the Södderich population and 15 (not shown in map) from nearby) adult trees in the Södderich population and 82 in the Weißwassertal were collected during February, 2003. In both populations the location of each individual was determined (Figures 5, 6 and 7). Buds were collected for DNA extraction. The genotypes at six microsatellite loci (table 2) were used for the analysis of genetic variation within and between populations and for the analysis of spatial genetic structures (in the Södderich 122 trees) in both populations. The genotypes of the adult trees were also used for the study of some aspects of pollen dispersal by paternity analysis. We used a shotgun (Figure 8) and a branch-pruner to collect the buds. Small branches of the tall trees that were not within reach of the branch-pruner were shot down. After collection, the buds were immediately stored at -20°C.

2.1.2.2. Leaves

Leaves were also collected from natural regeneration of the Södderich population. In total, leaves from 115 natural saplings were collected during July, 2004. Each individual was genotyped at 6 microsatellite loci (table 2). The genotype data were used to assess genetic variation within the natural regeneration and to compare it to the adult trees. They were also used for the analysis of spatial genetic structure. If there existed natural regeneration in the respective part of the stand, the sapling nearest to an adult tree was sampled. Mapping of all saplings was carried out with reference to their nearest adult tree (Figure 9). Because of the pronounced heterogeneity of the density of natural regeneration, the nearest adult tree may

either grow very close or at some distance. In total, leaves from 115 natural saplings were collected. The leaves were stored at -20°C immediately after collection.

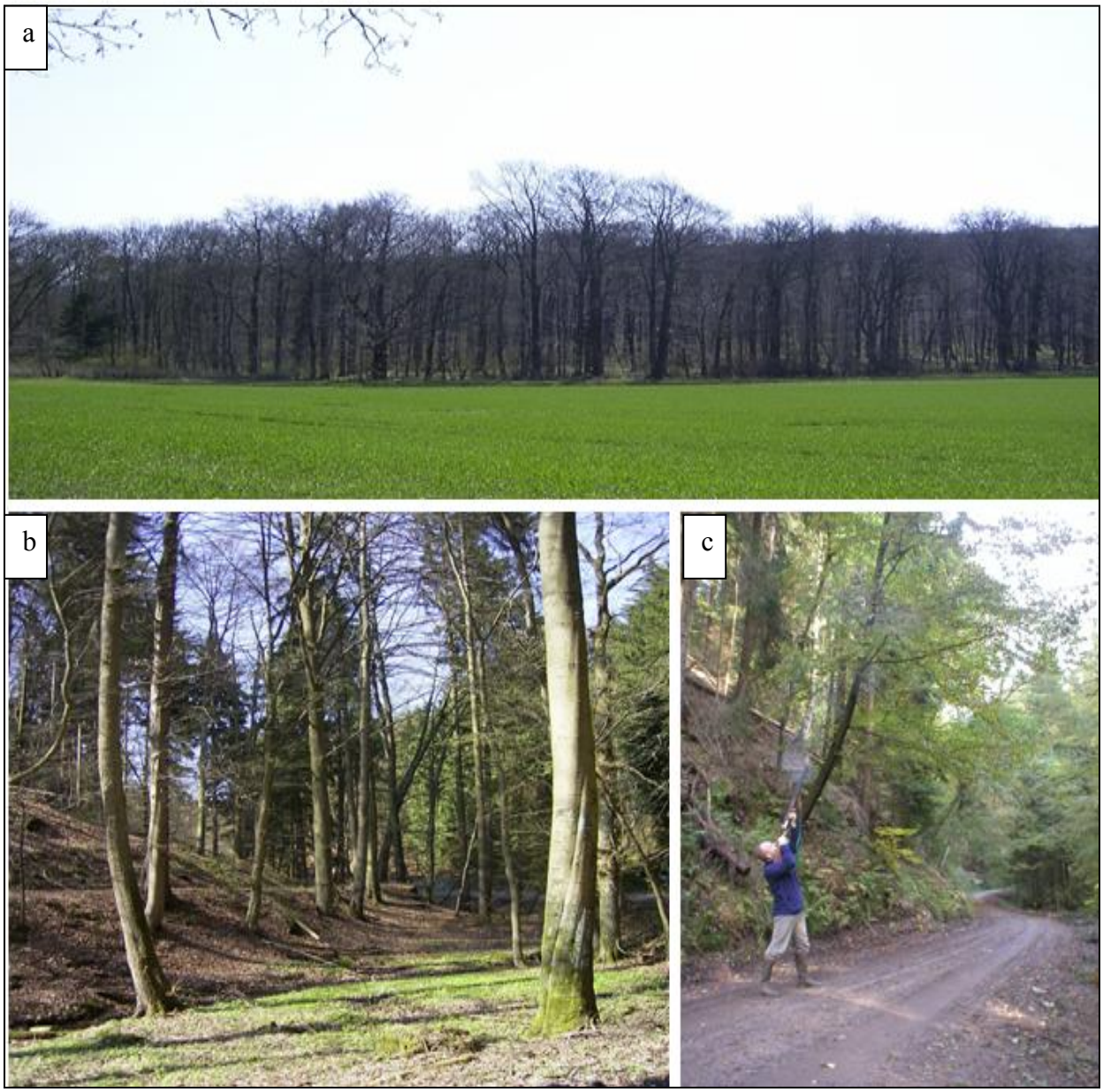

Figure 8. Photos of two *A. pseudoplatanus* and method of seed and bud collection. a: Södderich population; b: a part of the Weißwassertal population; c: collection of seed by shotgun.

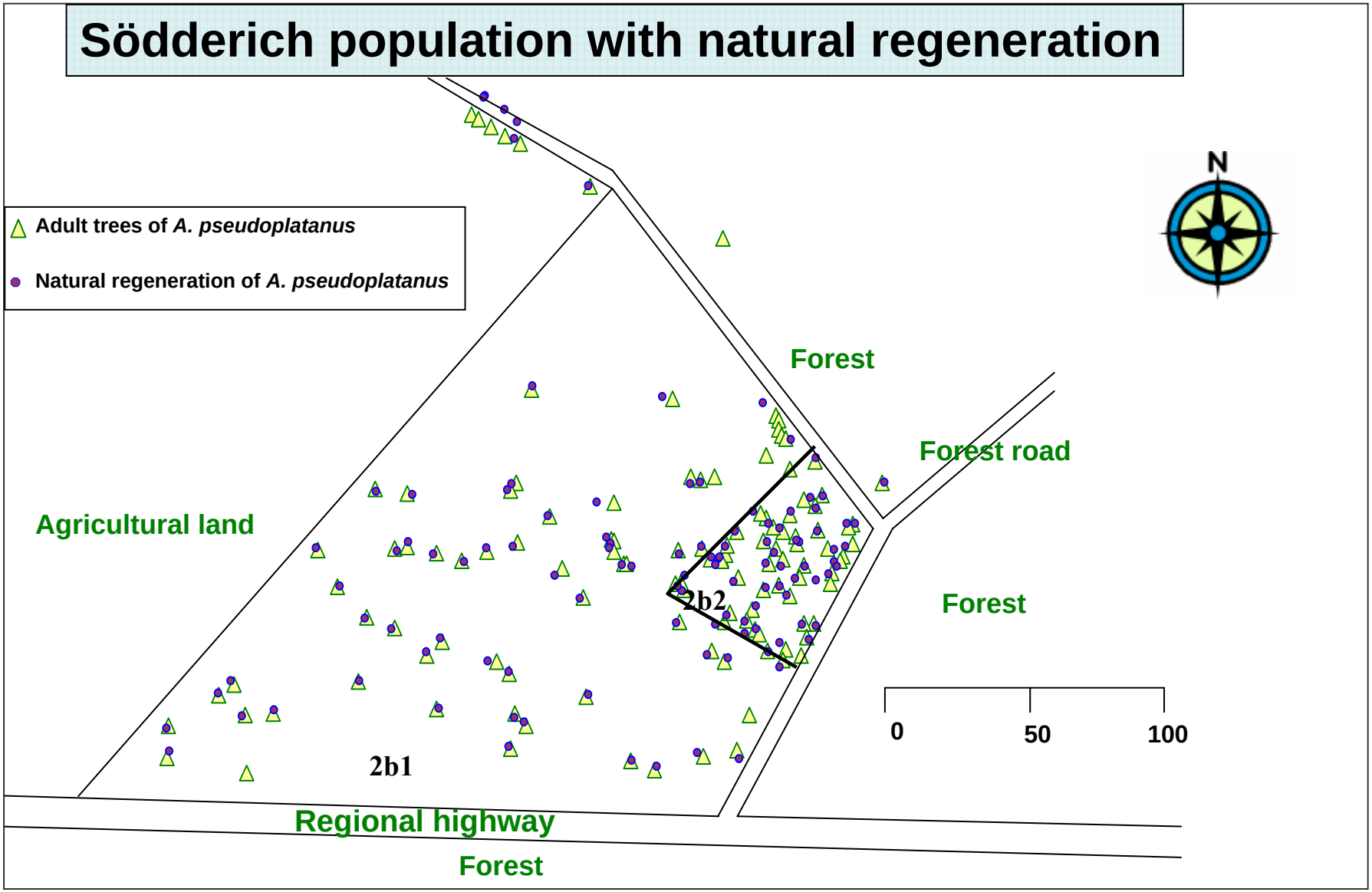

Figure 9. Ground locations of all sampled adult *A. pseudoplatanus* trees and natural regeneration.

2.1.2.3. Seeds

Thirteen trees in the Södderich and eight in the Weißwassertal population were selected for seed collection. The trees were selected in such a way that they represent the population in size, age and spatial distribution. The selected trees are shown in Figures 6 and 7. At least 50 seeds were collected from each tree during September 2004. Thirty-two seeds from each tree were genotyped at five microsatellite gene loci, *i.e. MAP-2, MAP-9, MAP-33, MAP-40* and *MAP-46.* The microsatellites were selected on the basis of their higher variability and easily interpretable bands (table 2). The genotype data were used to survey the genetic variation in the progenies and to compare it to the genetic variation of the adult population. They were further used to analyse certain aspects of the reproduction system. Material was collected as explained above for buds. Seeds were stored at -20°C until analysis.

2.2. Laboratory methods

2.2.1. Development of microsatellite (SSRs) gene markers

In order to develop microsatellite gene markers in sycamore maple, the protocol developed by FISCHER and BACHMANN (1998) was used.

2.2.1.1. Isolation of genomic DNA

DNA from young buds of *A. pseudoplatanus* was isolated using the DNeasy® Plant mini kit (QIAGEN, Hilden). Very good quality of DNA is required. DNA should be free from any contaminants, e. g**.** residual polysaccharides, proteins, RNA, remnants of phenol, chloroform, etc. The DNA was precipitated with ethanol to get it in concentrated form and free from other contaminants. Six micrograms DNA were used for SSR development.

2.2.1.2. Digestion of genomic DNA

Total genomic DNA is digested by blunt-end-generating restriction endonuclease *RsaI* that recognizes the restriction site (GT/AC) generating blunt-end double-stranded DNA fragments (Figure 10). The reaction mix and the conditions required for the digestion of genomic DNA are described in Appendix 1.

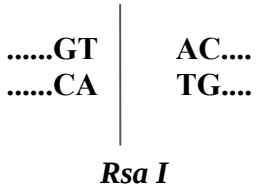

Figure 10. The *Rsa I* restriction site in the DNA sequence.

2.2.1.3. Ligation of the adapters

5'- phosphorylated 21-mer and 25-mer adapters with overlapping complementary sequences were ligated to the blunt-end termini of the restricted DNA fragments. The recipe of reaction volume and reaction conditions is given in Appendix 1.2. Sequences of adapters can be recognized by the restriction enzyme *Mlu I* which cuts the DNA creating bold type or sticky-ended fragments. After ligation the product was purified using GeneClean™ II "*glassmilk*" (Silicate DNA purification, Fa. Bio101). After purification the restriction–ligation DNA fragments were eluted in 10 µl distilled water. In order to check the success of the restriction-ligation process a PCR test was done using 1 µl DNA as template and the 21-mer oligonucleotide as a primer. The reaction mix and the conditions required for the digestion of genomic DNA are described in Appendix 2. Figure 11 shows the process of ligation.

a.

21-mer	5′-CTCTTGCTTACGCGTGGACTA-3′	+	ACNNNNNN
25-mer	3′-ACACGAGAACGAATGCGCACCTGAT-5P	+	TGNNNNNN
	Adapters		**DNA fragments**

b. 21-mer 5′-CTCTTGCTT**ACGCGT**GGACTA-3′

25-mer 3′-ACACGAGAACGAA**TGCGCA**CCTGAT-Pho-5′

Mlu I restriction

c. CTCTTGCTTACGCG TGGACTANNNNN

ACACGAGAACGAAT GCGCACCTGATNNNNNN

Figure 11. a. Sequences of 21-mer and 25-mer adapters and their binding site in DNA fragments; b. *Mlu I* recognition sites in the adapter sequence are underlined; c. Sticky-end created after *Mlu I* restriction of adapter sequences.

2.2.1.4. Hybridization of oligonucleotide probes

In this step the *Rsa I* restricted and adapter-ligated genomic DNA is hybridized to biotinylated microsatellite oligonucleotide probes. These oligonucleotide probes are artificial oligonucleotides constructed with an oligomer sequence complementary to the microsatellite sequence that were to be isolated. The oligonucleotide-probes $(CA)_{10}$, $(GAA)_8$ and $(AAC)_8$ were selected on the basis of previous experience about their abundance in plants with large genome sizes (FISCHER and BACHMANN, 1998). The recipe of different hybridization steps and the required conditions are described in Appendix 3. Figure 12 shows different steps of the hybridization process.

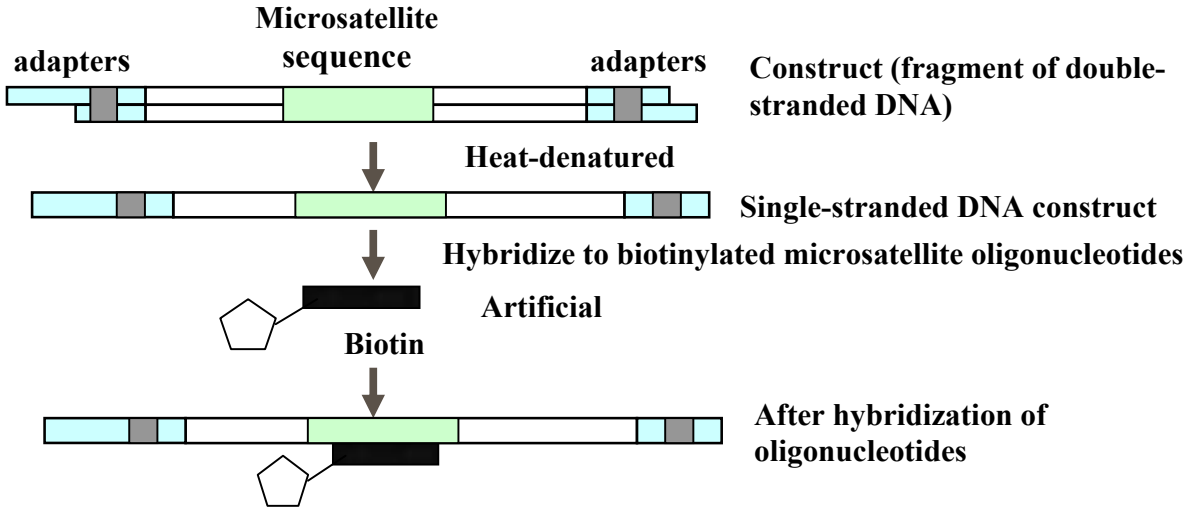

Figure 12. Different steps of the hybridization process (Adapted from FISCHER and BACHMANN (1998)).

2.2.1.5. Magnetic separation

The hybrids (constructs + biotinylated microsatellite oligonucleotides) were bound to streptavidin-coated magnetic beads (Dynabeads, Dynal GmbH) and the fragments with microsatellite sequences were captured by magnetic separation. DNA fragments without hybrids were washed away. The procedure is described in detail in Appendix 4. Figure 13 shows the process of magnetic separation.

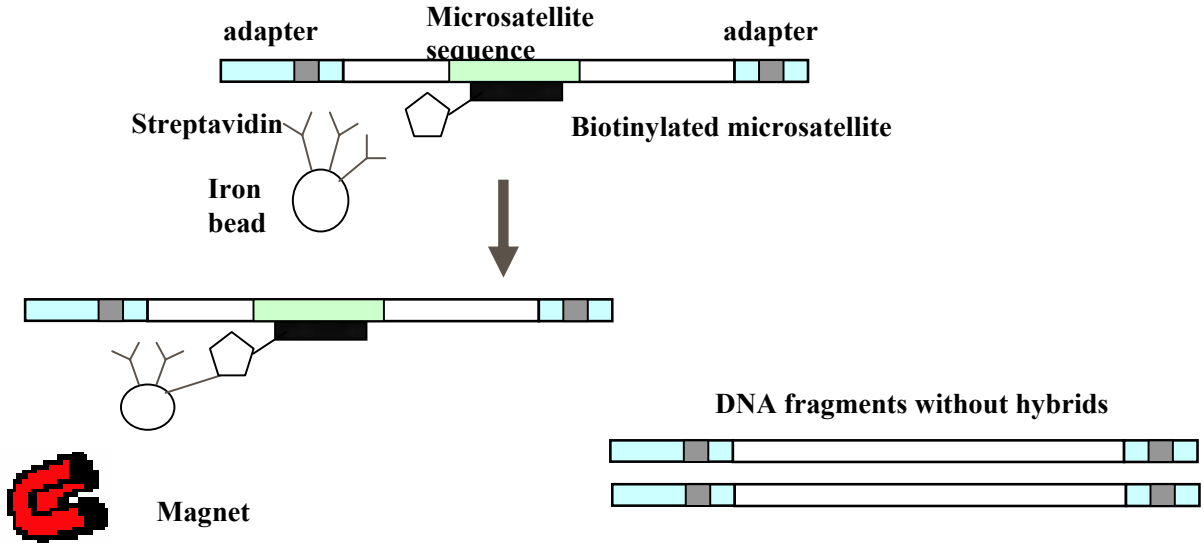

Figure 13. Processes and steps of magnetic separation (Adapted from FISCHER and BACHMANN (1998)).

2.2.1.6. Elution

In this step the DNA fragments which were hybridized to biotinylated microsatellite oligonucleotides were eluted from the iron beads. With the elution the whole hybridization process was repeated again to increase the efficiency of hybridization, which is known as double enrichment. This was performed by means of successive washings under different stringency conditions and different buffer concentrations. At the end of this step 10 µl of enriched DNA sample were recovered. The recipe and conditions of elution are given in Appendix 5.

2.2.1.7. PCR amplification

PCR amplification was performed using the elution that was obtained after hybridization as template. The 21-mer oligonucleotide was used as primer. This step is done to get sufficient amounts of DNA fragments for further processing. The recipe of PCR reaction volume and

thermocycler profiles are provided in Appendix 6. The PCR product was purified using GeneClean™ II "*glassmilk*" (Silicate DNA purification, Fa. Bio101) and was eluted with 10 µl distilled water.

2.2.1.8. Restriction of elution with Mlu I and vector with Bssh II

The elution received in step 2.2.1.7 was restricted with *Mlu I* to get vector-compatible sticky-ended fragments in order to ligate these fragments into a plasmid vector. After restriction the reaction was purified using GeneClean™ II "*glassmilk*" (Silicate DNA purification, Fa. Bio101).

The vector was digested with *BssH II* enzyme, then double-dephosphorylated using shrimp alkaline phosphotase (SAP, USB) and purified using GeneClean™ II "*glassmilk*" (Silicate DNA purification, Fa. Bio101). We used pCR-Script Amp SK (+) (STRATAGENE) as a plasmid vector. The restriction reaction was purified using GeneClean™ II "*glassmilk*" (Silicate DNA purification, Fa. Bio101) and was eluted with 10 µl distilled water. The recipe of the reaction volume and conditions for both restrictions are described in Appendix 7. The process of digestion of DNA and of the vector is shown in Figure 14.

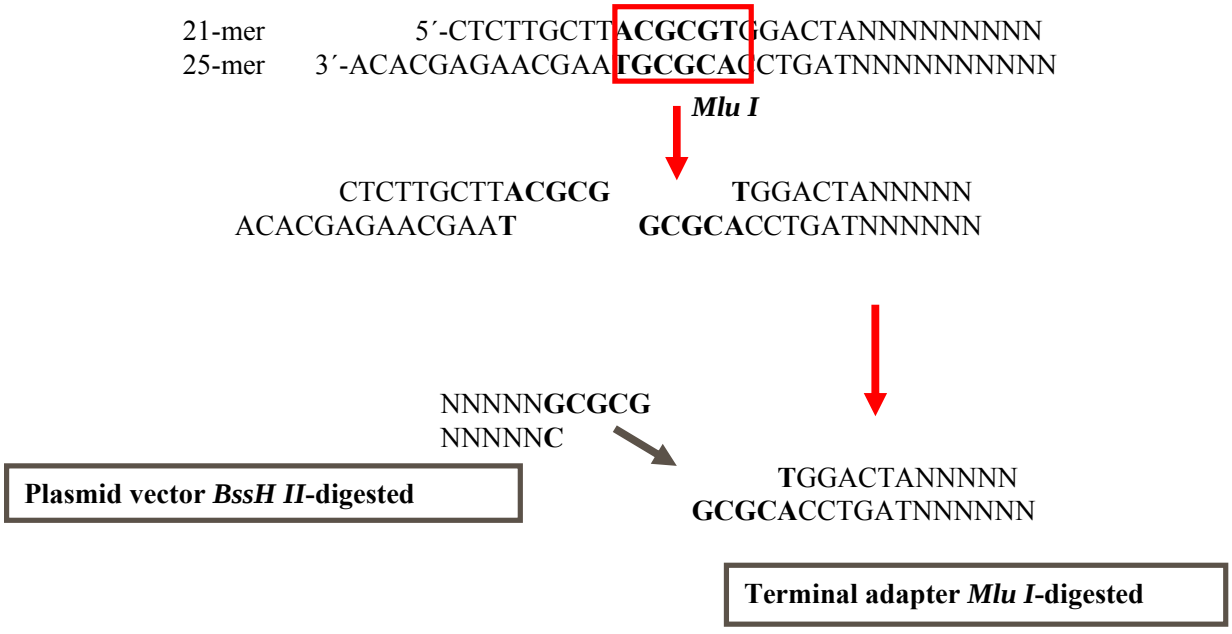

Figure 14. The process of DNA and vector digestion (Adapted from FISCHER (2000))

2.2.1.9. Cloning into a plasmid vector

In this step the *Mlu I* digested DNA fragments were cloned into a plasmid vector. The detailed procedure of this step is given in Appendix 8. The cloning site in the vector is shown in Figure 15.

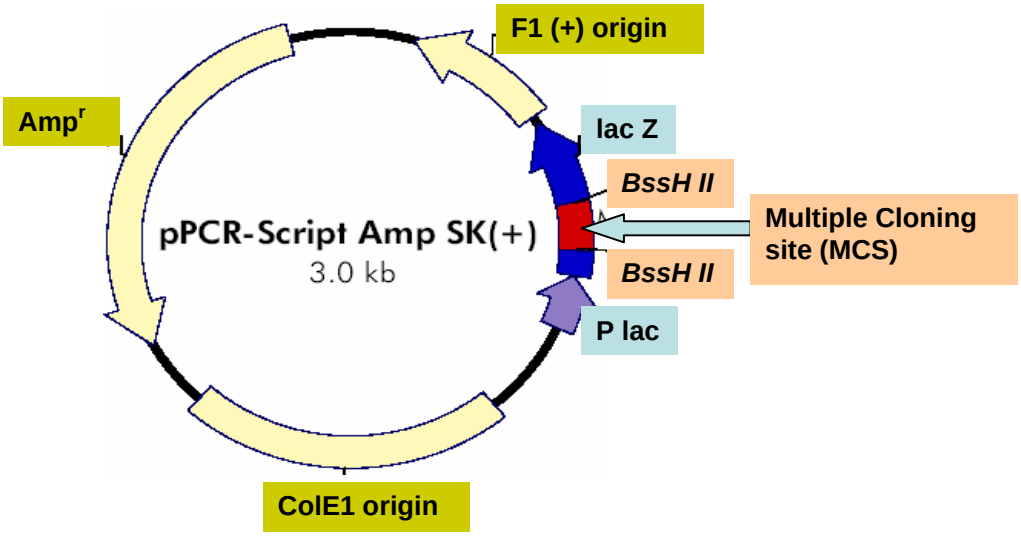

Figure 15. Map of a circular Phagemid-Vectors pCR-Script with *Ampicillin* resistance (Adapted from STRATAGENE)

2.2.1.10 Bacterial transformation

The ligated vector fragments were transformed into *Escherichia coli* XL1-BlueStrain bacterial cells (STRATAGENE) and plated onto LB agar medium. A heat-shock method was used for transformation. The detailed procedures of bacterial transformation are given in Appendix 9.

2.2.1.11 Colony PCR

After bacterial transformation the colonies were picked up and transferred onto grid plates with LB agar medium, so that it was possible to assign a number to each colony. PCR was performed with the colonies using vector primers. The recipe of PCR reaction volume and thermocycler profiles are given in Appendix 10.

Sequences of vector primers:

Vec-F-5’ AAGGCGATTAAGTTGGG- 3’

Vec-R-5’GGCTCGTATCTTGTGTGG- 3’

2.2.1.12. Sequencing of colony DNA

The PCR product received from colony PCR was separated in agarose gel (2%) electrophoresis and purified using QIAEX^{TM}II gel extraction kit (QIAGEN). The elution received after gel purification was used for the sequence reaction. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and carried out with the ABI PRISM® 3100 Genetic Analyser (Applied Biosystems / HITACHI).

2.2.1.13. Primer design

After sequencing of colony DNA good sequences with a microsatellite repeat motif were selected to design the primers. Sequences with sufficient nucleotides on both sides of the microsatellite were selected, so that there was a better choice for the priming site. PRIMER 3 computer software (ROZEN and SKALETSKY, 2000) was used for designing the primers. In order to obtain good primers, parameters presented in table 1 were selected.

Table 1. Parameters including their values and meanings which were considered to design microsatellite primers for *A. pseudoplatanus*.

Parameters	Values			Meaning
	Min.	Standard	Max.	
Primer length (bp)	18	20	27	Primer length depends on melting temperature (T_m)
Primer T_m (°C)	57	60	63	Melting temperature (T_m) depends on primer length and % of GC content
Primer T_m variance	1	-	2	Maximum differences between the T_m of both primers
GC content (%)	20	50-60	80	Minimum GC content depends on T_m together
Salt con. (mMol)	-	50	-	Total salt concentration, activation according to T_m
Product size (bp)	90	-	400	Length of PCR products, relevant for the analysis
3'-end Dimers	-	2	-	Complementary part between both primers from the 3'-end of the primers
G/C clamping	-	-	-	G or C nucleotide at the 3'-end of the primer sequence

2.2.1.14. Primer optimization

At first the primers were tested on a few samples of *A. pseudoplatanus.* Different PCR conditions were tried wherever necessary. Gradient PCR programmes with different annealing temperatures ranging from 50 to 66 °C were used to determine the optimum annealing temperature using a PTC-200 Gradient cycler (MJ RESEARCH).

The maximum allowed melting temperature (T_m) was calculated using the Primer 3 software (ROZEN and SKALETSKY, 2000) according to the formula described by BOLTON and MCCARTHY (1962) as presented in SAMBROOK *et al.* (1989):

$T_m = 81.5 + 16.6(\log_{10}([Na+])) + 0.41(\%GC) - 600/length$,

where

[Na+] is the molar sodium concentration, (%GC) is the percent of Gs and Cs in the sequence, and length is the length of the sequence.

The annealing temperature (T_a) was calculated by subtracting 5°C from the estimated melting temperature.

The number of PCR cycles was taken into consideration. Also different concentrations of template DNA and primer were tried.

2.2.1.15. Sequencing

Sequencing was carried out for two purposes, *i. e*. colony sequencing to develop microsatellite primers and to proof the homology of amplification products of eight microsatellite loci. For sequencing the method described by SANGER *et al.* (1977) was applied. In this method 2', 3'-dideoxynucleotide triphosphates (ddNTPs) play a vital role that differ from deoxynucleotides by the possession of a hydrogen atom attached to the 3' carbon rather than an OH group (Figure 16). These molecules terminate the DNA chain elongation because they cannot form a phosphodiester bond with the next deoxynucleotide.

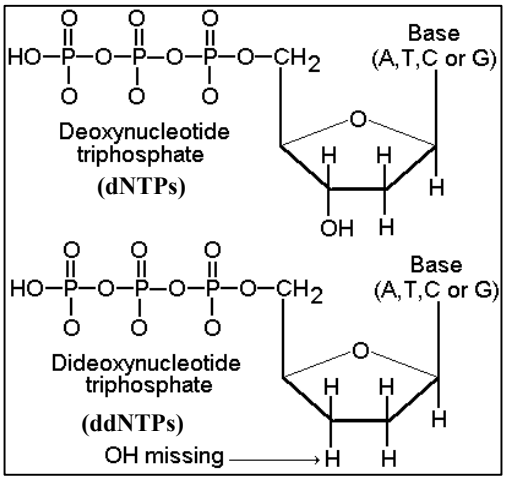

Figure 16. The different chemical constitutions of dNTPs and ddNTPs terminators (Adapted form:http://www.food.rdg.ac.uk/online/fs761/Topic5/Topic5.htm).

2.2.1.16. The procedure for sequencing

After PCR amplification of targeted DNA the PCR product was run on an agarose gel and the targeted band was excised from the gel. The DNA fragments with agarose gel were purified using the QIAEX^{TM}II gel extraction kit (QIAGEN). Then the purified DNA was sequenced using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems). The recipe of sequence reaction and thermocycler profile is given in Appendix 11.

2.2.1.17. Comparison of sequences

The sequences of the amplification products of different microsatellite loci were compared to the original clone sequences from which the primers were designed. This comparison is important in order to check the homology of the products. The DNA sequences were analyzed and edited using the computer programme "DNA Sequencing Analysis Software V 3.7" (Applied Biosystems). Then the sequences were compared using the computer programme "Clustal W multiple alignment" (THOMPSON *et al.* 1994).

2.2.2. Application of microsatellite markers in population genetics

2.2.2.1 DNA Isolation

DNA isolation from buds, leaves and seeds was carried out using DNeasy 96 Plant Kit (Cat. No. 69181; QIAGEN, Hilden). The DNA was eluted in 100 µl elution buffer. High quality DNA from the above-mentioned material of *A. pseudoplatanus* was obtained with this method.

In order to check the success of DNA isolation, 4 µl of DNA from each sample was tested on 1% agarose gel. Depending on their concentration, the DNA samples were diluted for PCR analysis.

2.2.2.2. DNA amplification with microsatellite primers

For the amplification of DNA samples from buds, leaves and seeds, microsatellite primers developed by PANDEY *et al.* (2004) were used. The PCR amplification was carried out using a PTC-200 Gradient cycler (MJ RESEARCH). The sequences of primers and their annealing temperature are given in table 2. The combination of PCR reaction volume and thermocycler profile was as following:

PCR-reaction mix:

1.2 µl Template DNA (5-10 ng)

1.2 µl F Primer (0.2 µM)

1.2 µl R Primer (0.2 µM)

7.5 µl Hot StarTaq Master Mix (Qiagen, Hilden)

3.9 µl H_2O

15.0 µl final volume

Thermocycler profile:

95° C	15 min.	
94° C	45 sec.	X 35 cycles
56° C - 64°C	1 min.	
72° C	45 sec.	
72° C	8 min.	
8° C	forever	

The PCR products were tested in 2% agarose gel to check the success of PCR amplification and at the same time to estimate the intensity of bands. The intensity of the PCR product of each sample was used in order to dilute the samples for capillary electrophoresis.

Table 2. Six microsatellite loci with their primer sequences (PANDEY *et al.* 2004). T_a: annealing temperature.

Locus name	Primer Sequences (5'-3') and type of labelling dye	T_a (°C)
MAP-2	F: (HEX) CATTAAACACATTTAAGCAAAACAAG	56
	R: ATCGGTTTGACATTGAGTGG	
MAP-9	F: (FAM) ACAATAAAAGAGCCCACATAGATAG	56
	R: TCTCTTCAATTGCAAGGCTTC	
MAP-12	F: (HEX) CAAAGACCCCAAAACTGTAAAGAC	64
	R:AAATATAAAGACATCGGAAAGTTGAG	
MAP-33	F: (FAM) GCAATGAACACATATACAAACAAGAG	64
	R: GCAACAAATGCCCTCTCAAG	
MAP-40	F: (FAM) TGCAGGGACACAAATGAATG	64
	R: GTGCATGTCTGTTAGGATTTTGG	
MAP-46	F: (FAM) CATAATGTAGGGACACATATGAATG	64
	R: GAGCGTCAAAGATTGACTTGG	

2.2.2.3. Electrophoresis

2.2.3.1. Agarose gel electrophoresis

Agarose gel electrophoresis was used to check the success of DNA isolation and also to test the intensity of PCR products. For DNA checks 1% agarose gel and for PCR check 2% agarose gels were used. Gels were stained with ethidium bromide and the bands were visualized under ultra violate light.

2.2.3.2. Capillary electrophoresis

Capillary electrophoresis was used for microsatellite fragment analysis and DNA sequencing. An ABI PRISM® 3100 Genetic Analyser (Applied Biosystem/ HITACHI) was used for the capillary

electrophoresis. After checking the PCR product on a 2% gel it is diluted depending on the intensity of bands in agarose gel to get an optimum intensity of peaks (500 to 1000 flourescence intensity) for capillary electrophoresis. Then 2 µl of the diluted PCR product were mixed with 12 µl HiDye Formamide (Applied Biosystems) and 0.01µl GS 500 Rox ^{TM} (Applied Biosystems) internal size standard. Thereafter, the mixture was denatured at 90° C for 2 minutes. After denaturation the samples were put on ice immediately in order to cool them down. Then the samples were loaded to the ABI PRISM® 3100 (Applied Biosystems/HITACHI) for capillary electrophoresis (Figure 17).

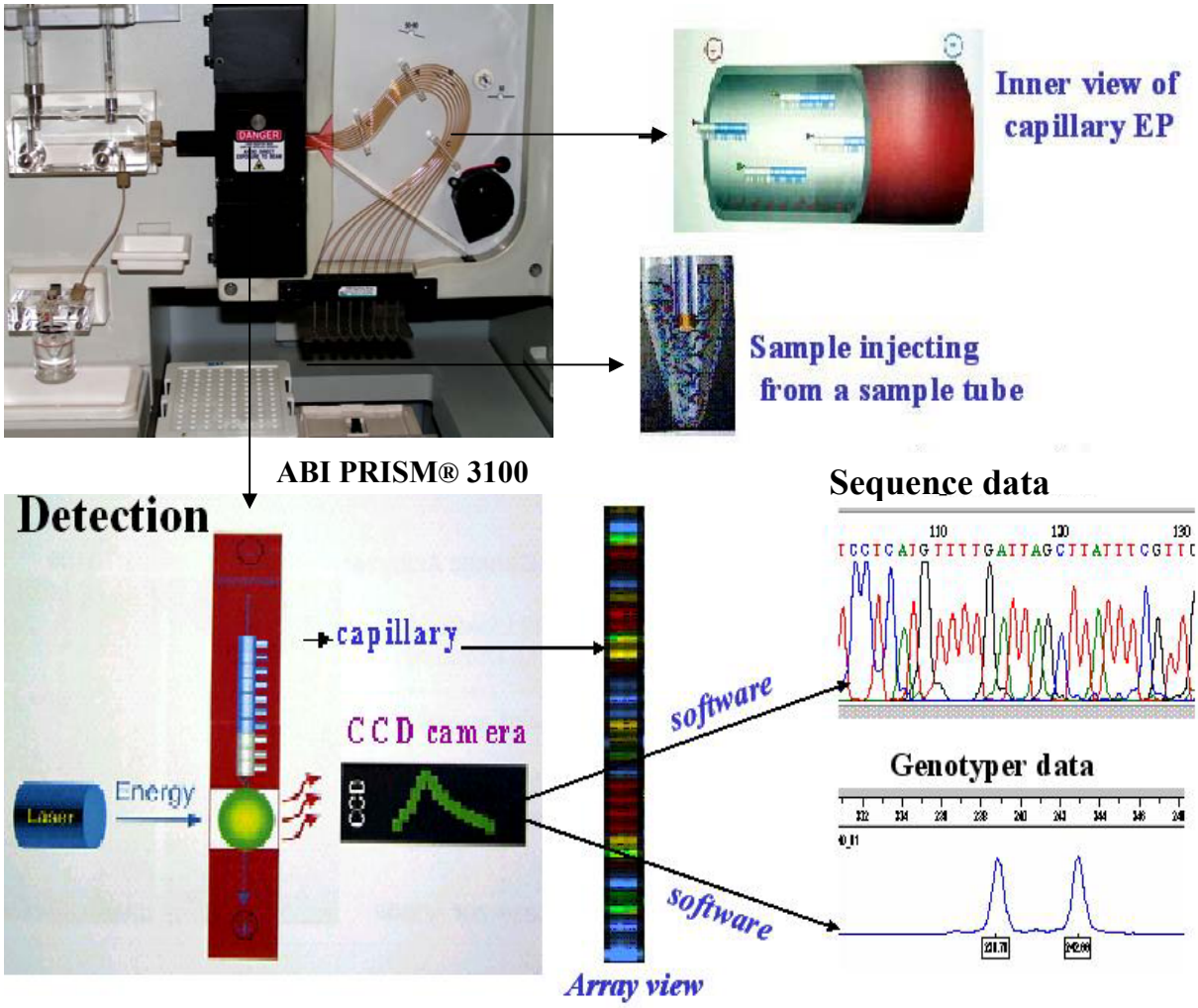

Figure 17. Steps and processes in capillary electrophoresis for DNA sequencing and DNA fragment length analysis (Adapted from Applied Biosystems)

2.2.3. Data interpretation

The data achieved from capillary electrophoresis were interpreted using GENESCAN 3.7 and the GENOTYPER 3.7 computer programmes (both from Applied Biosystems).

2.3. Data analysis

2.3.1. Genetic variation within and between populations

Since *A. pseudoplatanus* is an autotetraploid species, individual trees possess between one to four alleles at a locus. Therefore it is difficult to determine the exact number of copies of an allele present in a particular heterozygote. This makes interpretation of genotypes complex so that we were not able to calculate the genetic variation parameters that are conventionally used for diploids, such as observed (H_o) and expected heterozygosity (H_e).

2.3.1.1. Genetic variation within population

2.3.1.1.1. Average number of alleles per locus (A/L)

The simplest genetic variation parameter used to measure the genetic diversity of a population is the total number of alleles observed at all gene loci divided by the total number of gene loci. It was calculated as following:

$$\text{Average no. of alleles per locus (A/L)} = \sum_a n_a \Big/ L$$

where

n_a: Total number of alleles observed at all loci within the population

L: Total number of loci considered.

2.3.1.1.2. Frequencies of allelic types

The absolute frequencies of all fragments present in the populations were counted. In order to compare the frequencies of each fragment detected at six microsatellite loci, the relative frequencies of each fragment observed in the populations were calculated. These frequencies are not allelic frequencies in a strict sense but relative frequencies of particular genetic traits (on a fragments of a particular size) which were interpreted as allelic types or, for sake of brevity, alleles in the discussion.

2.3.1.2. Genetic differentiation between populations

Genetic distances based on the relative frequencies of each fragment as described in 2.3.1.1.2 were calculated using following the genetic distance parameters:

2.3.1.2.1. Genetic distance (d_0)

The genetic distance d_o represents the proportion of genetic types (alleles, gametes, genotypes) that are not shared between two populations (GREGORIUS, 1974). The value of the genetic distance d_o will be 1.0 only in the case if two populations do not have common genetic types. It can be calculated with the following expression:

$$0 \leq \qquad d_0(x, y) = \frac{1}{2} \sum_i \left| \; x_i - y_i \; \right| \quad \leq 1$$

where

x_i, y_i = frequency of allele i in populations x and y

$d_0(x, y)$ = genetic distance d_0 between two populations x and y

The gene pool value of the genetic distance can be calculated by taking the arithmetic mean of all L gene loci as follows:

$$\overline{d}_0 = \frac{1}{L} \sum_a d_{0(a)}$$

2.3.1.2.2 Nei's Minimum genetic distance (d_m)

The minimum Nei's genetic distance d_m (NEI, 1973) was calculated as following:

$$d_m(x, y) = \frac{1}{2} \sum_i \left| x_i - y_i \right|^2$$

where

x_i = frequency of allele i in population x,

y_i = frequency of allele i in populations y,

$d_m(x, y)$ = minimum genetic distance between two populations x and y.

2.3.2. Spatial genetic structure of populations

Spatial genetic structures of the Södderich and Weißwassertal populations were analysed using the following two methods:

2.3.2.1. Spatial distribution of alleles

To visualize the spatial distribution of selected alleles, two-dimensional and three-dimensional interpolated maps of the allele frequencies were constructed. For this purpose, the presence and absence of the selected allele in a given geographical position was coded with '1' and '0', respectively. The data were further evaluated by 'semi-variance' analyses. After performing a semi-variance analysis 'Kriging analysis' was carried out with the following parameters:

Type of Kriging analysis: Punctual Kriging analysis
Search radius: The maximum distance of the population was taken in to consideration, *i.e.* 286 m in the Södderich and 2,009 m in the Weißwassertal population.
Number of neighbours: 16
Grid interval: 4 m in the Södderich and 21 m in the Weißwassertal population.
After performing the 'Kriging analysis', two-dimensional and three-dimensional interpolated spatial distribution maps of selected alleles were constructed. All of the above-mentioned analyses and construction of interpolated spatial distribution maps the GS+ (Geostatistics for the

environmental science, version 3.1.7, Gamma Design Software, Michigan, USA) computer programme was used.

2.3.2.2. Spatial autocorrelation

Spatial genetic structures of the adult trees in the two populations, *i.e.* Södderich and Weißwassertal and the natural regeneration of the Södderich population were calculated following the method of SMOUSE and PEAKALL (1999). In order to calculate spatial genetic structure, fragments of six microsatellite loci (*MAP-2, MAP-9,* MAP-12, *MAP-33, MAP-40* and *MAP-46*) were scored as presence or absence (0, 1). At first matrices C and $X^{(h)}$ of genetic and geographic distances were calculated from the genetic data and the geographical coordinates of the trees, respectively. Spatial autocorrelation coefficients were calculated by the following formula:

$$r^{(h)} = \left(\sum_{i \neq j}^{N} x_{ij}^{(h)} c_{ij} \right) \Big/ \left(\sum_{i=1}^{N} x_{ii}^{(h)} c_{ii} \right)$$

where

$r^{(h)}$ = spatial autocorrelation coefficient, which is bounded by (-1, +1). The "0" means that there is no autocorrelation.

Index *h* denotes distance classes, *h* = 1, 2, 3, …, and so on.

C = genetic covariance matrix consisting of elements c_{ij} (see SMOUSE and PEAKALL, 1999).

$X^{(h)}$ = geospatial matrix among individuals consisting elements x_{ij} (see SMOUSE and PEAKALL, 1999).

The numerator is the sum of all N (N-1) off-diagonal "element by element" products of c_{ij} and $x_{ij}^{(h)}$. The denominator is the sum of all N main-diagonal "element by element" products of those same two matrices.

For the calculation of distance matrices and the spatial autocorrelation coefficients, the computer programme GenALEx V6.0 (PEAKALL and SMOUSE, 2001) was used. Different types of

distance classes were used for the calculation of spatial autocorrelation, *i.e.* even distance classes and distance classes with width proportional to sample size.

2.3.3. Segregation analyses

The utilization of microsatellites for inference on elements of the mating system requires knowledge of their inheritance. Since offspring from artificial crosses were not available, the segregation among the seeds of single trees collected after open pollination was used in order to check whether the empirical data comply with our expectations. The respective criteria are not as stringent as the compliance with Mendelian expectations in pairs of mates and their offspring in a diploid species. Some minimum criteria are, nevertheless, listed below.

The segregation at six microsatellite loci in the progenies of single seed trees was tentatively analysed (table 3). The trees possess between one and four alleles. If a tree possesses four alleles, it is expected that a minimum of two are present in each offspring and if a tree possesses only one allele, this allele should always be present in any progeny of that tree. However, seed trees possessing two or three bands make it difficult to infer the number of copies of their alleles.

A further look at the examples of genotypes in table 3 helped to formulate criteria for the validity of the genetic interpretation of chromatograms as shown in Figures 24 through 31. Let p denote the number of alleles borne by the seed parent and f denote the number of those borne by the seed. c is the number of alleles shared by the seed parent and the seed.

The hypothesis of maternity was rejected in the following situations:

(1) $c = 0$. Any seed must bear at least one allele of the maternal parent or $c > 0$. Otherwise, the genetic interpretation of the laboratory findings could not be entrusted in this case. Mistakes in assigning a seed to the wrong seed tree in the field could be safely excluded.

(2) $p = 4$; $c < 2$. Another criterion of the validity of parent-offspring combinations was consistency of the number of allelic types present in the seed parent and its offspring. If the seed parent bears four allelic types, then every seed must have received *any two* of them.

Seed trees bearing three different allelic types may produce seeds bearing only one of them. In this case it was assumed that the seed parent possesses this very type in twofold. Consequently, a seed parent possessing allelic types 158, 170, and 182 at ***MAP-2*** as is shown for example, in the first column of table 3 is able to produce only one type of homozygotes, *i.e. either* (158) *or* (182), or *either* (158 **O**) *or* (182 **O**), respectively. The symbol **O** in the genotype of a seed is used for any allelic type that is not present in the seed parent and must have been contributed by the pollen parent. A seed parent bearing two allelic types may transmit one or both of them depending on their gene doses.

(3) $p = 1$; $f > 3$. Since the pollen parent contributed only one or two different alleles to the seed, a seed tree possessing only one allele cannot possibly produce seeds with four alleles.

Analyses of this kind do not permit to assign complete genotypes to parents or progeny. However, they were used for testing the plausibility of the laboratory findings. Detected inconsistencies are most likely due to incorrect scoring of the peaks and to the presence of null alleles. If those tests failed to establish the consistency, the respective seed was not included in further analyses such as the inference on self-fertilization events. For instance, all entries of table 3 except the one just mentioned proved to be consistent with the above criteria. However, *MAP-2* of all seeds with genotypes (158) or (158 **O**) had to be excluded from subsequent analyses. The decision to omit just these genotypes rather than (182) and (182 **O**) was more or less arbitrarily based on their smaller numbers, i.e. seven and eight. The eventuality that allele 170 was erroneously scored in the seed tree was refuted on the basis of repeated laboratory analyses.

Table 3. Examples of segregation at six microsatellite loci among offspring of seed trees. An allelic type that is not present in the seed tree is denoted by 'O'. The examples are taken from the data of different trees.

MAP-2		***MAP-9***		***MAP-12***		***MAP-33***		***MAP-40***		***MAP-46***	
Genotype of seed tree: 158 170 182		Genotype of seed tree: 102 110		Genotype of seed tree: 152 160		Genotype of seed tree: 160 180		Genotype of seed tree: 244		Genotype of seed tree: 164 178 188 190	
Genotypes of seeds	No.	Genotypes of seeds	No.	Genotypes of seeds	No.	Genotypes of seeds	No.	Genotypes of seeds	No.	Genotypes of seeds	No.
158	4	102	11	152	15	160	7	244	13	164 188	1
182	3	102 110	21	160	4	180	12	244 **O**	19	178 188	1
158 182	5	102 **O**	5	152 160	16	160 180	5			164 178 188	1
170 182	4	102 110 **O**	7	152 **O**	8	160 **O**	6			164 178 190	1
158 170 182	4					180 **O**	13			164 178 188 190	1
158 **O**	3									164 188 190	1
182 **O**	5									164 178 **O**	4
158 170 **O**	2									164 188 **O**	9
158 182 **O**	1									178 188 **O**	5
170 182 **O**	1									178 190 **O**	2
										164 178 190 **O**	2
										164 188 190 **O**	1
										178 188 190 **O**	3
Total	**32**		**44**		**43**		**43**		**32**		**32**

2.3.4 . Estimation of the proportion of self-fertilization

One important element of the reproduction system of a co-sexual plant species is the proportion of self-fertilization. A seed was considered to be due to self-fertilization if only maternal variants were encountered at all microsatellite loci. This approach based on the principle of simple exclusion leads to an estimate of the upper bound of self-fertilization, since cross-pollen and "foreign" pollen may have contributed alleles possessed also by the seed parent.

If a seed possessed no maternal allele at some microsatellite locus, the respective seed was excluded and the estimation was based on the other seeds only (see section 2.3.3). Additional criteria for discarding the information at a given locus were developed by inspection of the data.

The hypothesis of self-fertilization was rejected, if in addition to the criteria for maternity listed in subsection 2.3.3 one of the five following criteria is not fulfilled.

(4) $c < f$. This criterion is more stringent than no. (1) introduced for the decision on descent from the given seed tree. Any effective allele contributed by cross-pollen would lead to this condition.
(5) $p = 4$; $c \neq 2$. For obvious reasons, a tree bearing $p = 4$ alleles is expected to transmit exactly two of them to its offspring arising from self-fertilization
(6) $p = 3$; $f > 2$. If the seed tree bears three alleles, seeds can possess either one or two of them, but not more, so that f can at most be equal to 2.
(7) $p = 2$; $f > 2$. If the seed parent bears two alleles, seeds possess either one or both of these but never three.
(8) $p = 1$; $f > 1$. It is almost needless to say that if the seed parent is homozygous, the seeds must carry only this very allele under the given conditions.

MAP-12 was omitted from the analysis because moderately many seeds had genotypes that were not consistent with that of the respective seed trees.

3. Results

3.1. Microsatellite development

3.1.1. Microsatellite enrichment

DNA isolation with the DNeasy® Plant mini kit (QIAGEN, Hilden) was successful. A sufficient amount of DNA was available for the restriction-ligation reaction. Since the quality of DNA is very important. Unnecessary products, *i.e.* residual polysaccharides, proteins, RNA, and residual phenol were removed by cold ethanol precipitation.

Restriction of 6 µg genomic DNA with *Rsa I* enzyme and ligation of the sticky end 21-mer and 25-mer adapters to the restricted genomic DNA fragments produced fragments of different sizes (200 to 1000bp). Figure 18 shows the restriction-ligation fragments after purification using GeneClean™ II "*glassmilk*" (Silicate DNA purification, Fa. Bio101).

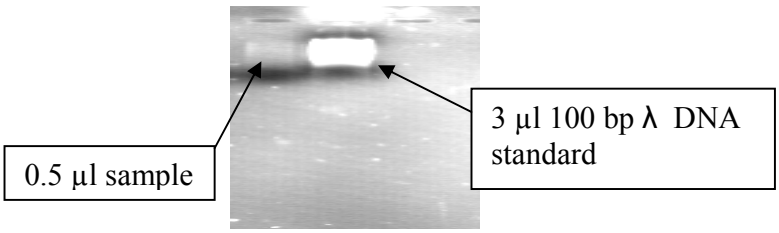

Figure 18. Agarose gel (1%) photograph of the restriction-ligation of genomic DNA of *A. pseudoplatanus*.

The hybridization of biotinylated artificial microsatellite oligonucleotides with the prepared genomic DNA fragments was successful and was sufficient to obtain microsatellite-enriched genomic DNA. The success of the first enrichment was tested by using the 21-mer oligonucleotide as primer. The PCR amplification test of the first enrichment on 1% agarose gel is shown in Figure 19 a. The presence of strong fragments can be seen within a series of continuous fragments. After the second enrichment it was possible to get a continuous series of fragments with different sizes (200 bp to 1000 bp) (Figure 19 b).

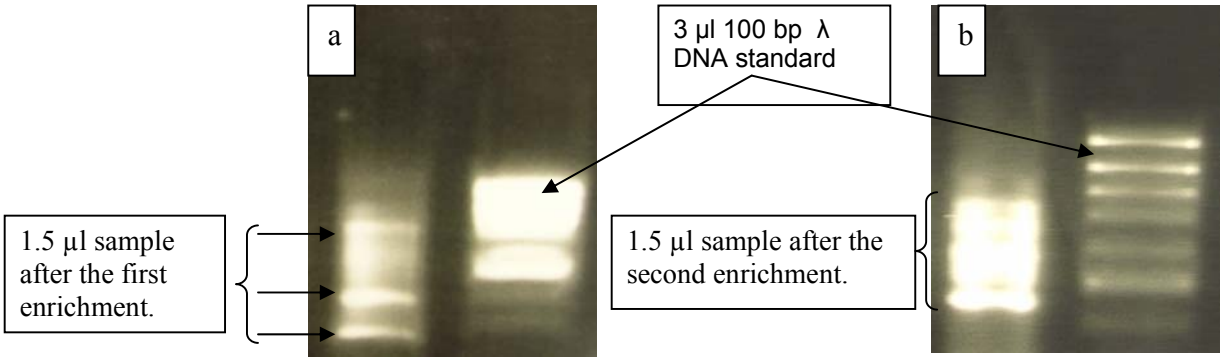

Figure 19. Agarose gel (1%) photograph after enrichment of biotinylated microsatellite oligonucleotides with the prepared genomic DNA of *A. pseudoplatanus*; a: after the first enrichment; b: after the second enrichment.

The enriched genomic DNA fragments were restricted with the enzyme *Mlu I* and were purified using GeneClean™ II "*glassmilk*" (Silicate DNA purification, Fa. Bio101). The test of the restriction reaction on 1% agarose gel is shown in Figure 20. Many fragments of different sizes distributed continuously were observed.

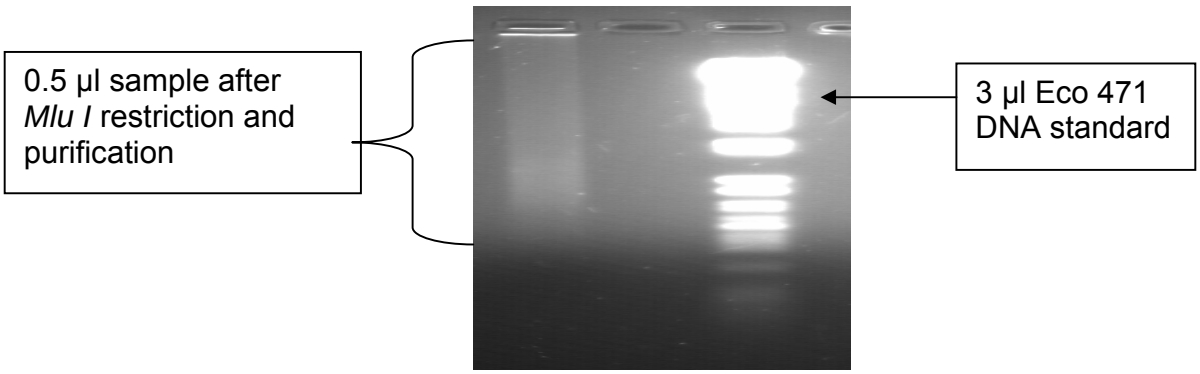

Figure 20. Agarose gel (1%) photograph after *Mlu I* restriction of the enriched genomic DNA of *A. pseudoplatanus.*

The pCR-Script Amp SK (+) (STRATAGENE) vector was restricted with *BssH II* enzyme and was dephosphorylated with shrimp alkaline phosphatase (SAP, USB). The restriction of the vector was done to linearise and to create a gap in the vector, so that the enriched genomic DNA can be cloned into the vector. After restriction and dephosphorylation, the reaction mix was run on a 1 % agarose gel. After staining we could see two vector bands. The upper band was stronger than the lower band. The lower band of 173 bp was the part of the vector DNA which was removed to create a gap for cloning. The stronger band was the vector DNA with a gap. This band was isolated from the gel and purified using GeneClean™ II "*glassmilk*" (Silicate DNA

purification, Fa. Bio101). Figure 21 shows the gel photo of the vector after gel purification. Then the enriched genomic DNA fragments were ligated into the vector.

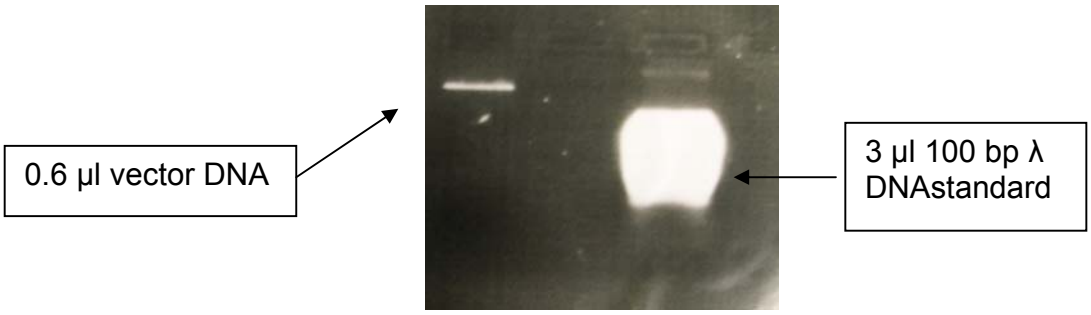

Figure 21. Agarose gel (1%) photograph of the restricted, SAP dephosphorylated, and gel purified pCR-Script Amp SK (+) (STRATAGENE) vector.

After ligating the genomic DNA into the vector, it was transformed into super-competent cells (*E. coli* XL1-Blue, STRATAGENE). Then the cells were plated onto LB-medium and the plates were incubated at 37 °C for 12 hours. A total of 90 colonies were obtained. Since the 'LacZ – gene' of the vector DNA was damaged after the restriction with *BssH II* enzymes, no "blue-white" selection was made and all colonies were selected for sequencing.

The colonies were amplified by colony PCR with vector primers. PCR products visible as strong bands were received (Figure 22 a). These bands were extracted from the gel and purified using GeneClean™ II "*glassmilk*" (Silicate DNA purification, Fa. Bio101) and used for sequencing. Figure 22 b shows the PCR products after gel purification.

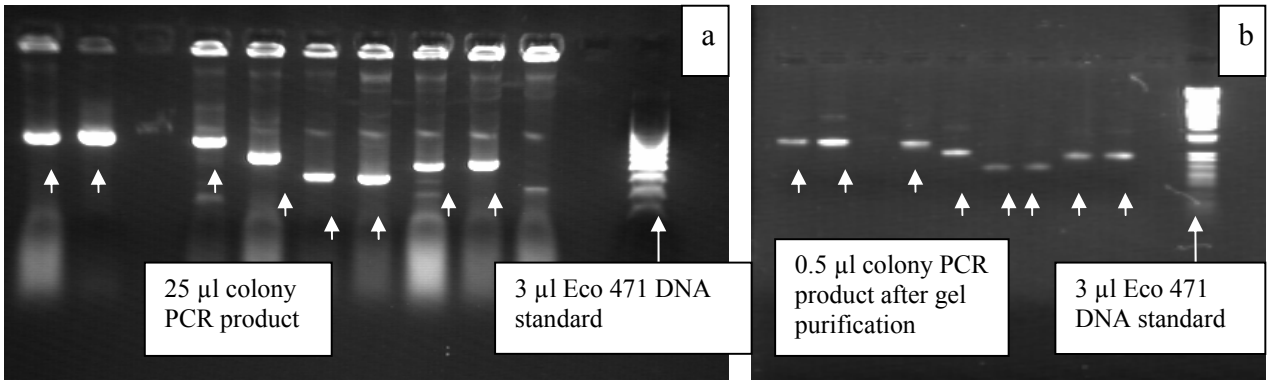

Figure 22. Agarose gel (1.5%) photograph of the test of colony PCR, short arrows show the colony DNA.

Sequencing of all 90 colonies produced 54 (60%) sequences containing at least one microsatellite. Out of 54 sequences 48 (89 %) contained dinucleotide microsatellite motifs

(41 GT/CA, (76 %), 4 GA/CT (5 %), 2 TA/AT (4 %)), two contained mononucleotide motifs A/T (4 %) and five contained trinucleotide motifs CTT/GAA (9 %). Twenty-four sequences containing 19 dinucleotide repeats (nine $(GT)_n$, eight $(CA)_n$, one $(GA)_9$, and one $(CT)_{15}$) and five trinucleotide repeats (three $(CTT)_n$ and two $(GAA)_n$) with simple and non-interrupted microsatellites having sufficient numbers of nucleotides on both sides of the repeats were selected for primer design. An example of a sequence containing the microsatellite motif $(GT)_7$ is shown in Figure 23. Sixteen (67 %) out of 24 primer pairs were able to amplify fragments of expected sizes. Finally eight primer pairs producing reproducible and easily scorable bands were optimized.

Figure 23. Example of a sequence containing microsatellite repeat motif $(GT)_7$.

The eight primer pairs were tested in 96 individual trees from the Södderich population. The results on the number of alleles and allele size ranges are presented in table 4. All eight primer pairs produced a moderate to high level of polymorphism with allele numbers ranging from 3 to 16. *MAP-2, MAP-12, MAP-33* and *MAP-46* were highly variable with allele numbers of 16, 10, 14 and 16, respectively, while *MAP-10* was least variable with only three alleles. *MAP-9, MAP-34* and *MAP-40* were moderately variable with allele numbers of 7, 9 and 5, respectively.

Chromatograms produced with Genescan 3.7 and Genotypes 3.7 software (Applied Biosystems) of the eight microsatellite loci are presented in Figures 24 to 31.

MAP-2

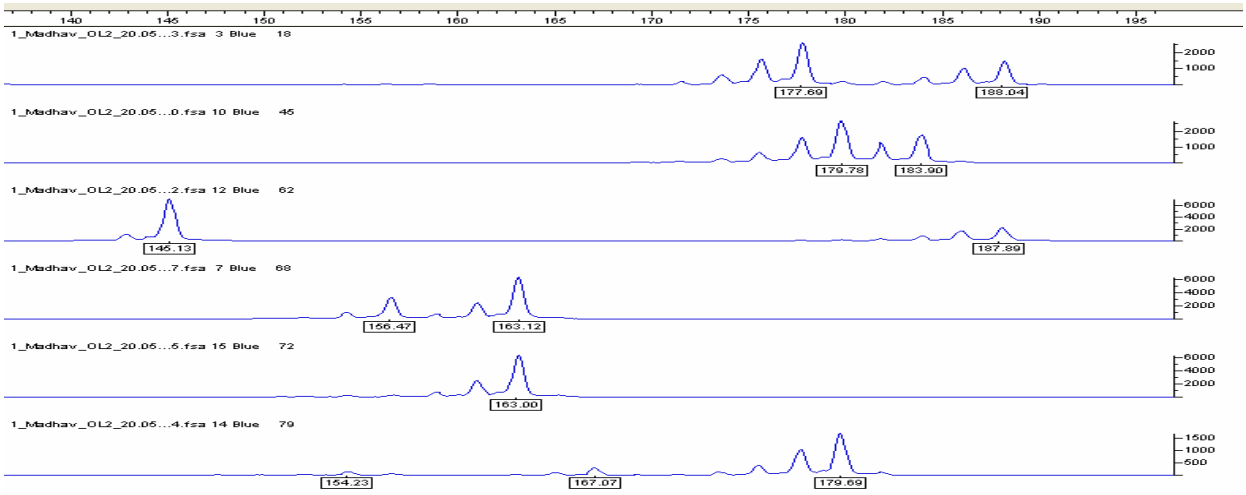

Figure 24. Chromatogram showing amplification products of *MAP-2* in six selected trees

MAP-9

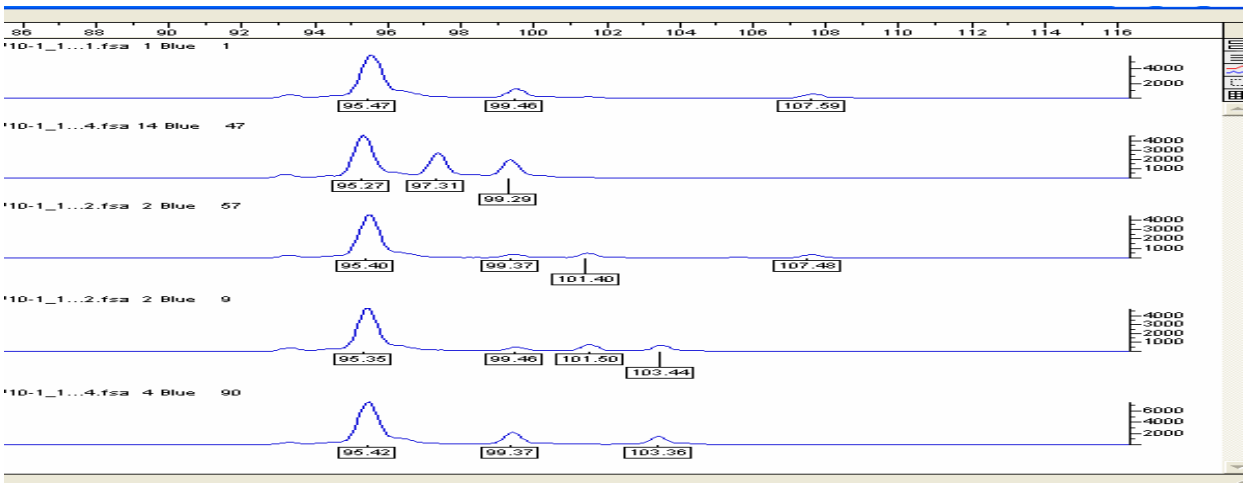

Figure 25. Chromatogram showing amplification products of *MAP-9* in five selected trees.

Table 4. Characterization of eight microsatellite gene markers for *A. pseudoplatanus* (PANDEY *et al.* 2004). T_a : Annealing temperature

Locus Name	Repeat Sequence	Primer sequences (5'-3') and type of labelling dye	Cloned allele size	T_a (°C)	Alleles size range (bp)	Alleles per individual	Total number of alleles	Sequenced allele sizes	GenBank accession number
MAP2	$(GT)_{23}$	F: (FAM) CATTAAACACATTTAAGCAAAACAAG R: ATCGGTTTGACATTGAGTGG	178	56	144 - 198	1-4	16	144,156,188	AJ620722
MAP9	$(GA)_8$	F: (FAM) ACAATAAAAGAGCCCACATAGATAG R: TCTCTTCAATTGCAAGGCTTC	100	56	96 -108	2-4	7	100, 108	AJ620723
MAP10	$(GT)_2$ TT $(GT)_4$	F: (HEX) CCACGATCTGGGGTACTGAG R: CTCTTCTTCGCACCTTTTGG	106	56	92 - 110	2-3	3	92, 106	AJ620724
MAP12	$(GT)_7$	F: (HEX) CAAAGACCCCAAAACTGTAAAGAC R: AAATATAAAGACATCGGAAAGTTGAG	150	64	142 - 178	1-4	10	150,156,172	AJ620725
MAP33	$(GT)_{18}$	F: (FAM) GCAATGAACACATATACAAACAAGAG R: GCAACAAATGCCCTCTCAAG	156	64	146 - 182	1-4	14	150,164,172	AJ620726
MAP34	$(CA)_{21}$	F: (FAM) ACCATTCTCACCCCTCCATC R: TAAGTGGGAACATGGCAAGG	150	66	120 - 162	2-4	9	120,130,162	AJ620727
MAP40	$(GT)_6$	F: (FAM) TGCAGGGACACAAATGAATG R: GTGCATGTCTGTTAGGATTTTGG	246	64	238 - 246	1-3	5	242, 246	AJ620728
MAP46	$(GT)_8$GAT $(GT)_8$	F:(FAM)CATAATGTAGGGACACATATGAATG R: GAGCGTCAAAGATTGACTTGG	176	64	152 - 194	1-4	16	152,166,186	AJ620729

MAP-10

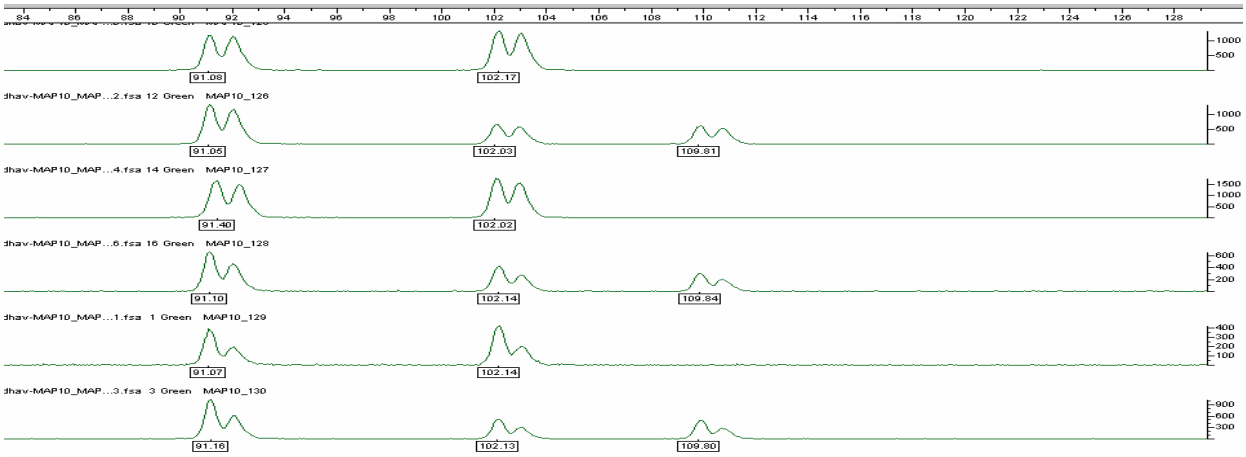

Figure 26. Chromatogram showing amplification products of *MAP-10* in six selected trees.

MAP-12

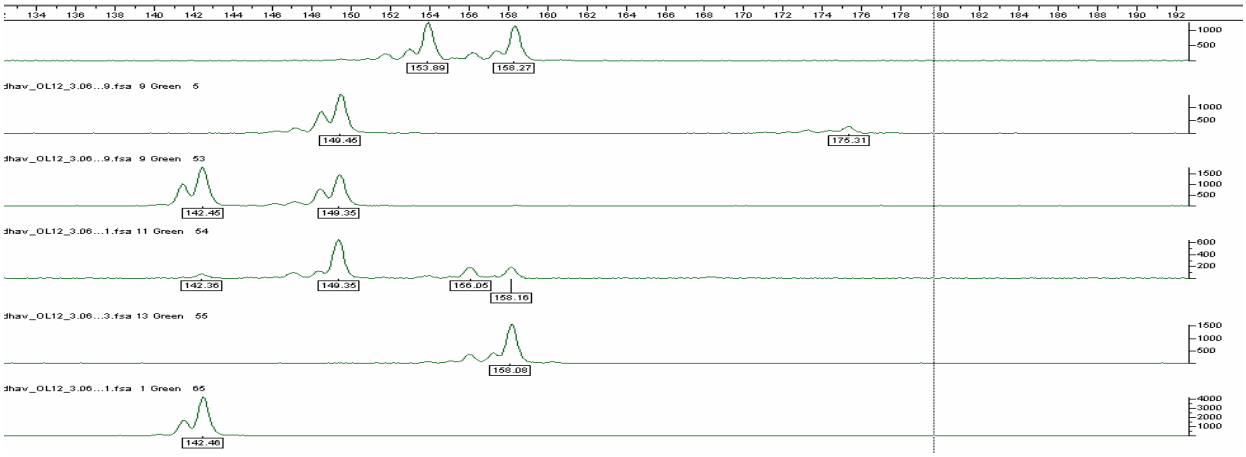

Figure 27. Chromatogram showing amplification products of *MAP-12* in six selected trees.

MAP-33

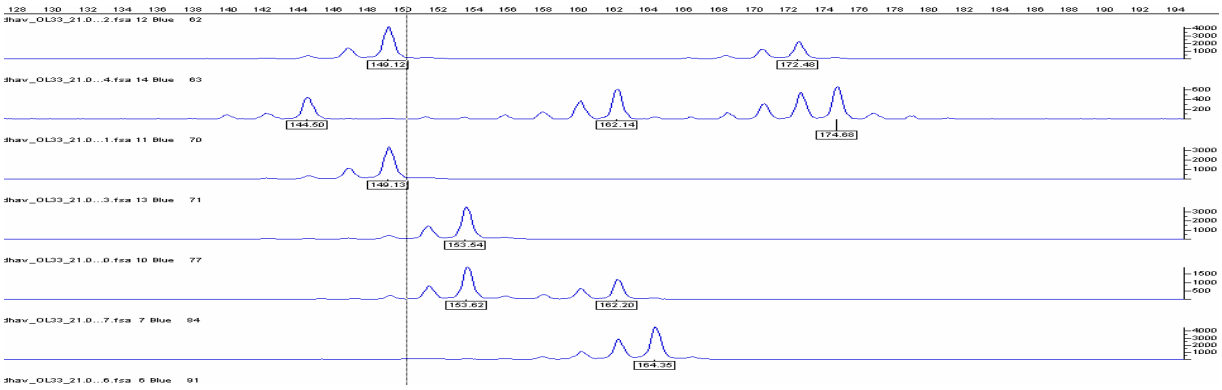

Figure 28. Chromatogram showing amplification products of *MAP-33* in six selected trees.

MAP-34

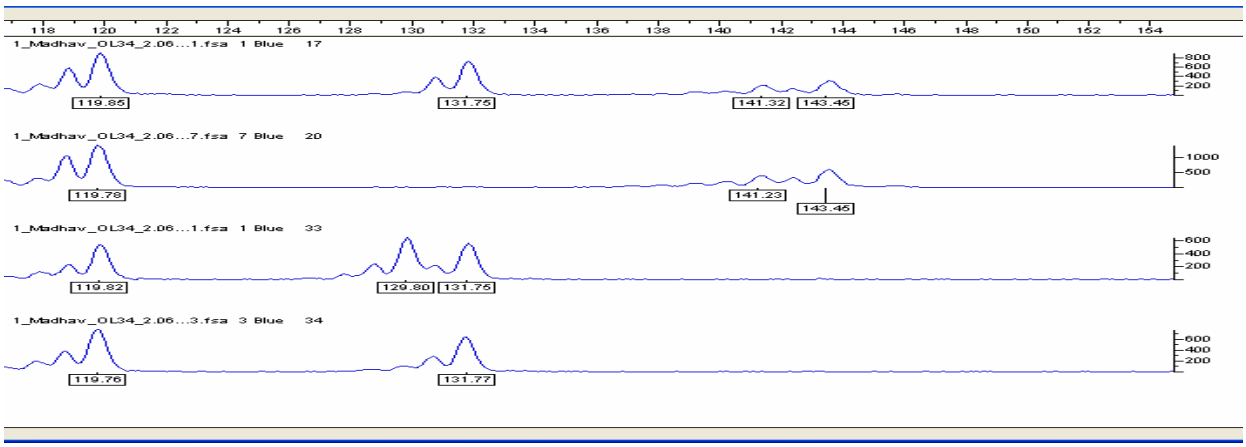

Figure 29.Chromatogram showing amplification products of *MAP-34* in four selected trees.

MAP-40

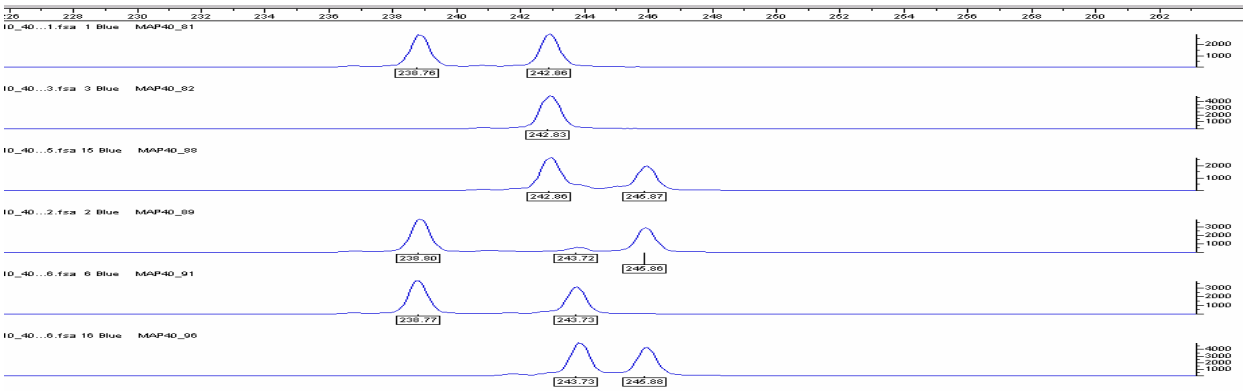

Figure 30. Chromatogram showing amplification products of *MAP-40* in six selected trees.

MAP-46

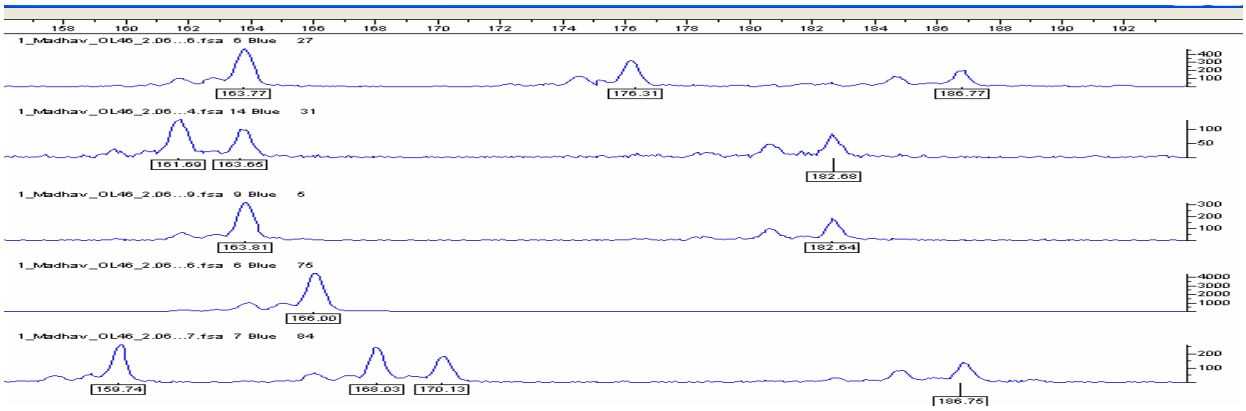

Figure 31. Chromatogram showing amplification products of *MAP-46* in six selected trees.

3.1.2. Sequencing of selected amplified products

Sequencing of selected alleles generated by each of the eight primer pairs showed that the variation in fragment length of different alleles was mainly due to the variation in the microsatellite region. Microsatellite flanking regions were conserved. This suggested that amplification products were different alleles of one gene locus. The sequence alignments of the sequenced alleles of eight microsatellites loci are presented in Figure 32. The sequence alignment of microsatellite locus *MAP-33* showed that there was an indel of two bases in the flanking region in allele 164 and 174. The gene loci *MAP-9* and *MAP-10* were tightly liked because an additional microsatellite motif ($(GT)_2$ TT $(GT)_4$) in the same fragment was detected after sequencing of the amplified products. New primers were designed to amplify both of microsatellites separately (Figure 25 and 26).

3.1.3. Cross-species amplification

The results of testing microsatellite primers developed for *A. pseudoplatanus* in 21 other species of genus *Acer* revealed that most of the primer pairs produced amplification products of the expected size in the majority of the species tested (table 5). In 15 out of the 21 species all eight primer pairs produced amplification products of the expected size. In case of *A. neglectum* and *A. tegmentosum,* four and three primer pairs could not produce any amplification product, respectively. Primer pairs for *MAP-9* and *MAP-40* produced amplification products of the expected size in all 21 species. Since only one sample per species was tested, there is no information about the level of polymorphism. Their ability to produce amplification products in most of the tested species (92% cases) indicates the possibility of applying the microsatellite gene markers for the study of the genetic structure and reproduction systems of other maple species.

Table 5. Cross-species amplification in 21 species (one sample per species) of the genus *Acer* using eight microsatellite primer pairs developed for *Acer pseudoplatanus* (PANDEY *et al.* 2004). 'X' indicates presence of fragments in the expected size range, '0'indicates no amplification.

Acer species	Primer pairs							
	MAP- 2	***MAP-9***	***MAP-10***	***MAP-12***	***MAP-33***	***MAP-34***	***MAP-40***	***MAP-46***
A. barbinerve Maxim.	X	X	0	X	X	X	X	0
A. campestre L.	X	X	X	X	X	X	X	X
A. capillipes Maxim.	X	X	X	X	X	X	X	X
A. caudatum Wall.	X	X	X	X	X	X	X	X
A. cissifolium K. Koch	X	X	X	X	X	X	X	X
A. davidii Franch.	X	X	X	X	X	X	X	X
A. grosseri Pax ex Engl.	X	X	X	X	0	X	X	X
A. komarovii Pojark.	X	X	X	X	X	X	X	X
A. lobelii Tenore	X	X	X	X	X	X	X	X
A. mono Maxim.	X	X	X	X	0	0	X	X
A. monspessulanum L.	X	X	X	X	X	X	X	X
A. neglectum Annae	0	X	X	0	0	0	X	X
A. opalus Mill.	X	X	0	X	X	X	X	X
A. obtusatum Waldst.	X	X	X	X	X	X	X	X
A. platanoides L.	X	X	X	X	X	X	X	X
A. rubrum L.	X	X	X	X	X	X	X	X
A. rufinerve Sieb. et Zucc.	X	X	X	X	X	X	X	X
A. saccharum Marshall	X	X	X	X	X	X	X	X
A. saccharinum L.	X	X	X	X	X	X	X	X
A. skutchii Rehder	X	X	X	X	X	X	X	X
A. tegmentosum Maxim.	0	X	X	0	X	0	X	X

MAP-2

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55         65         75         85         95        105
MAP2-146        CATTAAACAC ATTTAAGCAA AACAAGTGTG TGTGTGTGT- ---------- ---------- ---------- ---------- -----GAATT TTGCTTCACG CATAAGTAAA
MAP2-156        CATTAAACAC ATTTAAGCAA AACAAGTGTG TGTGTGTGTG TGTGTGTGT- ---------- ---------- ---------- -----GAATT TTGCTTCAAG CATAAGTAAA
MAP2-178        CATTAAACAC ATTTAAGCAA AACAAGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGT---- ------GAATT TTGCTTCCGC AATAAGTAAA
MAP2-188        CATTAAACAC ATTTAAGCAA AACAAGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGAATT TTGCTTCACG CATAAGTAAA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|...
                   115        125        135        145        155        165        175        185
MAP2-146        GAAGTAGATG AATCATCAAC ATGCAAACCA TATCCAATAT CTATTCATCT TAAGCTTTCC ACTCAATGTC AAACCGAT
MAP2-156        GAAGTAGATG AATCATCAAC ATGCAAACCN NNNNNNNNNN NNNNNNNNNN NNNNNNNNCC ACTCAATGTC AAACCGAT
MAP2-178        GAAGTAGATG AATCATCAAC ATGCAAACCA TATCCAATAT CTATTCATCT TAAGCTTTCC ACTCAATGTC AAACCGAT
MAP2-188        GAAGTAGATG AATCATCAAC ATGCAAACCA TATCCAATAT CTATCNNNNN NNNNNNNNCC ACTCAATGTC AAACCGAT
```

MAP-9

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|...
                    5         15         25         35         45         55         65         75         85         95        105
MAP9-100        ACAATAAAAG AGCCCACATA GATAGATCAA GAGAGAGAGA GAGAGAG--- -----TTTCA AACGACAGCC CACGATCTGG GGTACTGAGA ACCTTGCAAT TGAAGAGA
MAP9-108        ACAATAAAAG AGCCCACATA GATAGATCAA GAGAGAGAGA GAGAGAGTAG AGAGATTTCA AACGACAGCC CACGATGTAG GGTACTGAGA ACCTTGCAAT TGAAGAGA
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MAP-10

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
                    5         15         25         35         45         55         65         75         85         95        105
MAP10-106       CCACGATCTG GGGTACTGAG AAGCCTTGCA ATTGAAGAGA GTGTTTGTGT GTGTTCCCCA CAAGAGAGAA TCATCAATCC ATCATGCCAA AAGGTGCGAA GAAGAG
MAP10-96        CCACGATCTG GGGTACTGAG AAACCTTGCA ATTGAAGAGA GTGTT----- -----CCCCA CAAGAGAGAA TCATCAATCC ATCATGCCAA AAGGTGCGAA GAAGAG
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MAP-12

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55         65         75         85         95        105
MAP12-150       CAAAGACCCC AAAACTGTAA AGACATGGTG TGTGTGTGTG T--------- ---------- ---TCCAGCT ACAATCATTC TCAAGATAAT GAATTTGAGA TTTTTTCAAG
MAP12-156       CAAAGACCCC AAAACTGTAA AGACATGGTG TGTGTGTGTG TGTGTGT--- ---------- ---TCCAGCT ACAATCATTC TCAAGATAAT GAATTTGAGA TTTTTTCAAG
MAP12-172       CAAAGACCCC AAAACTGTAA AGACATGGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTTCCAGCT ACAATCATTC TCAAGATAAT GAATTTGAGA TTTTTTCAAG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
                   115        125        135        145        155        165     172
MAP12-150       TACTAGAACT ATTCTTCTAA ATTATCCTAT ATTTCTCTCA ACTTTCCGAT GTCTTTATAT TT
MAP12-156       TACTAGAACT ATTCTTCTAA ATTATCCTAT ATTTCTCTCA ACTTTCCGAT GTCTTTATAT TT
MAP12-172       TACTAGAACT ATTCTTCTAA ATTATCCTAT ATTTCTCTCA ACTTTCCGAT GTCTTTATAT TT
```

MAP-33

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55         65         75         85         95        105
MAP33-150       GCAATGAACA CATATACAAA CAAGAGAGGT GTGTGTGTGT GTGTGTGTGT GTGT------ ---------- ------CAA- -CTATTTTTT CAATGTAATT AAGCACAAGT
MAP33-156       GCAATGAACA CATATACAAA CAAGAGAGGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGT------ ------CAA- -CTATTTTTT CAATGTAATT AAGCACAAGT
MAP33-164       GCAATGAACA CATATACAAA CAAGAGAGGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGT-- ------CAAC ACTATTTTTT CAATGTAATT AAGCACAAGT
MAP33-174       GCAATGAACA CATATACAAA CAAGAGAGGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTCAAC ACTATTTTTT CAATGTAATT AAGCACAAGT
```

continued

Figure 32. Alignments of the selected allele sequences of the eight microsatellite loci.

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....
                   115        125        135        145        155        165        174
MAP33-148       GTTTATACCT TTATTCTTCT AAATTATCCT ATATTTCTCT CAACTTTCCG ATGTCTTTAT ATTT
MAP33-156       GTTTATACCT TTATTCTTCT AAATTATCCT ATATTTCTCT CAACTTTCCG ATGTCTTTAT ATTT
MAP33-164       GTTTATACCT TTATTCTTCT AAATTATCCT ATATTTCTCT CAACTTTCCG ATGTCTTTAT ATTT
MAP33-174       GTTTATACCT TTATTCTTCT AAATTATCCT ATATTTCTCT CAACTTTCCG ATGTCTTTAT ATTT


MAP-34

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5          15         25         35         45         55         65         75         85         95         105
MAP34-120       ACCATTCTCA CCCCTCCATC NNNNNTTATC TTTCTATCTC ATCCCCATGC TTACACACAC ACACA----- ---------- ---------- ----CGAACA TGCTTACACC
MAP34-150       ACCATTCTCA CCCCTCCATC TTCTCTTATC TTTCTATCTC ATCCCCATGC TTACACACAC ACACACACAC ACACACACAC ACACACACAC ACACGAACA TGCTTACACC
MAP33-162       ACCATTCTCA CCCCTCCATC NNNNNTTATC TTTCTATCTC ATCCCCATGC TTACACACAC ACACACACAC ACACACACAC ACACACACAC ACACCNNNNN NNNNNNNNNN

                ....|....| ....|....| ....|....| ....|....| ....|
                   115        125        135        145        155
MAP34-120       AACTTCGATG TCTTTATATT TCCTTGCCAT GTTCCCACTT A....
MAP34-150       AACTTCGATG TCTTTATATT TCCTTGCCAT GTTCCCACTT A....
MAP33-162       NNNNNNNNNN NNNNNNNNNN NNNNNCCTTG CCATGTTCCC ACTTA


MAP-40

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5          15         25         35         45         55         65         75         85         95         105
MAP40-242       TGCAGGGACA CAAATGAATG TCATGTGTGT G----CCTAT GCCTTGTAAG AGGAATGAGG TCAATTATAC CCTTGTTGAG CAATGTAGCT TATTCCCCTT TTTAGACACC
MAP40-246       TGCAGGGACA CAAATGAATG TCATGTGTGT GTGTGCCTAT GCCTTGTAAG AGGAATGAGG TCAATTATAC CCTTGTTGAG CAATGTAGCT TATTCCCCTT TTTAGACACC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   115        125        135        145        155        165        175        185        195        205        215
MAP40-242       ATCTTGCATG AAACGTAGCC GAGTATTTCT TTGAAGCTTT GAATTATCAT TTCTAAGACC ACTAATCAAT ACAAAAAAAT CATCATTTTG ATATGGATAT ACACTAACAC
MAP40-246       ATCTTGCATG AAACGTAGCC GAGTATTTCT TTGAAGCTTT GAATTATCAT TTCTAAGACC ACTAATCAAT ACAAAAAAAT CATCATTTTG ATATGGATAT ACACTAACAC

                ....|....| ....|....| ....|.
                   225        235        245
MAP40-242       AAACCAAAAT CCTAACAGAC ATGCAC
MAP40-246       AAACCAAAAT CCTAACAGAC ATGCAC


MAP-46

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5          15         25         35         45         55         65         75         85         95         105
MAP46-176       CATAATGTAG GGAACATATG AATGAATTGT GTGTGTGTGT GTGTGATGTG TGTGTGTGTG TGTG------ ----CCTAAC CCTTGTAGAG GAATGAGGTC AATATACCTT
MAP46-186       CATAATGTAG GGAACATATG AATGAATTAT GTGTGTGTGT GTGTGATGTG TGTGTGTGTG TGTGTGTGTG TGTGCCTAAC NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
                    115        125        135        145        155        165        175        185
MAP46-176       TGTAGAGCAA TGTAGCTTAA TCATCTTCGA AGCCTTCATC TTTCATGAAC CGTGACCAAG TCAATCTTTG ACGCTC
MAP46-186       NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNCCAAG TCAATCTTTG ACGCTC
```

Figure 32. (Continued) Alignments of the selected allele sequences of the eight microsatellite loci.

3.2. Genetic variation within and between adult trees in the Södderich and the Weißwassertal populations.

3.2.1. Genetic variation

The segregation of SSR variants within progenies is in most cases compatible with the conditions expected for tetrasomic inheritance (section 2.3). Therefore, it is justified to consider these variants as alleles.

Unfortunately, the observations of genotypes are still incomplete in all partial heterozygotes, since individuals showing the presence of two or three alleles do not reveal the number of copies present in their genotype. We assumed that in homozygotes one allele is present in four copies. In the case of quadruplex heterozygotes we neither have a problem. However, not knowing the allele doses in the duplex and triplex heterozygotes, it was possible to score the presence of allelic types but not to precisely assign complete genotypes to individuals. Nevertheless, the term genotype was used rather than phenotype. After all, the presence or absence of allelic variants of microsatellites is independent of the environment of the respective individuals.

As a consequence of the problems outlined above it was not possible to derive unbiased estimates of the allele frequencies in the two populations. What is possible is to present just the frequencies of individuals bearing at least one copy of the scored allelic types. These estimates allow only for approximation of the true allele frequencies. As ZHANG *et al.* (submitted) have demonstrated by their model-based analysis of the two populations their more exact allele frequencies differ only moderately from our approximations (see Figure 33). Some obvious differences between the two frequency structures in Figure 33 can be explained by the provisional data basis of the upper graphs. In particular, all alleles at *MAP-2* had formerly been scored with two base pairs less. In all, it seems still appropriate to infer on population differentiation on the basis of the approximate allele frequencies.

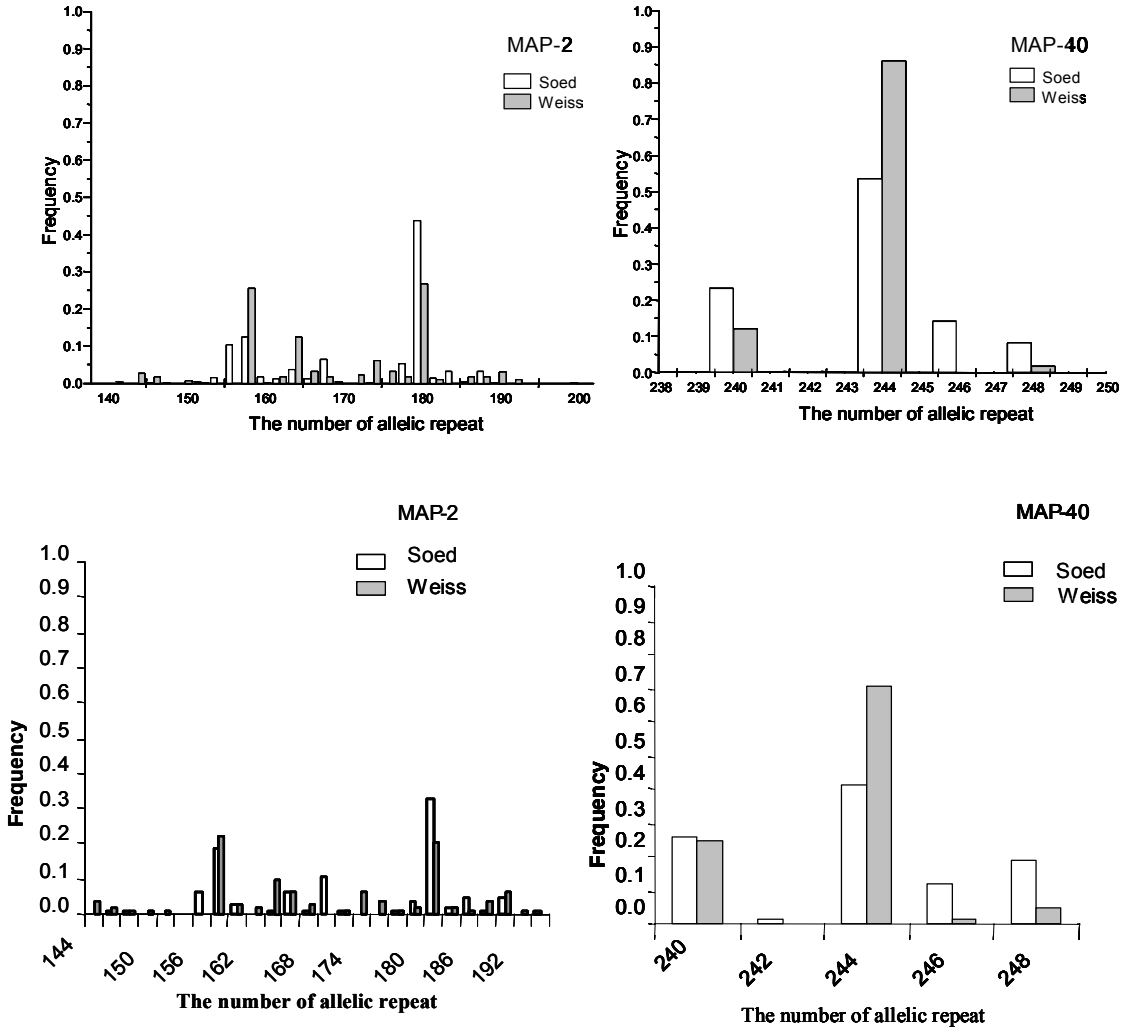

Figure 33. Above: sample of allelic frequencies of *MAP-2* and *MAP-40* in the Södderich and Weißwassertal populations according to ZHANG *et al.* (submitted). Below: approximate frequencies as described in the text.

3.2.1.1. Allelic multiplicities

Alleles observed at the six microsatellite loci in the Södderich and the Weißwassertal populations are presented in table 6. Six out of eight microsatellite loci were selected depending on their variability and the simplicity of scoring the bands. The total number of alleles found in the Weißwassertal population (85) was slightly higher than in the Södderich population (80). Accordingly, the average number of alleles observed in the Weißwassertal population was 14.2 and in the Södderich population 13.3. This is remarkable in view of the smaller size of the

Weißwassertal population. The numbers of alleles observed at microsatellite loci *MAP-2*, *MAP-12* and *MAP-33* were larger in the Weißwassertal as compared to the Södderich population (table 6). On the other hand, the microsatellite loci *MAP-9*, *MAP-12* and *MAP-46* have more alleles in the Södderich population than in the Weißwassertal.

The results of the counts of alleles present at each locus (table 6) shows that some alleles are only found in one of the two populations. For instance, at locus *MAP-2*, the alleles 144 (n=6), 150 (n=1), 152 (n=2), 162 (n=3), 174 (n=10), 176 (n=6) and 192 (n=2) were found only in the Weißwassertal and the alleles 154 (n=1), 156 (n=20), 170 (n=33), and 200 (n=3) were found only in the Södderich population. Allele 170 with its moderate frequency indicates that not only rare alleles are specific for one of the two populations.

At locus *MAP-9*, the alleles 100 (n=2) and 110 (n=5) were only found in the Södderich population. At *MAP-12*, the Södderich population has only allele 180 (n=4) as a specific allele, while the Weißwassertal population has 6 specific alleles, i.e. 150 (n=2), 162 (n=5), 164(n=6), 166 (n=1), 168 (n=1) and 190 (n=1). Likewise, at *MAP-33*, the alleles 170 (n=1) and 180 (n=1) were found only in the Södderich population, while the alleles 138 (n=1), 140 (n=2), 156 (n=2) and 178 (n=2) occurred only in the Weißwassertal population. In case of *MAP-40* allele 242 (n=4) was only detected in the Södderich population. In contrast to *MAP-2*, *MAP-12* and *MAP-33*, *MAP-46* has three alleles i.e. 182 (n=3), 186 (n=1) and 198 (n=1), which were specific to the Södderich population as compared to the Weißwassertal population with only allele 160 (n=2) as a specific type.

Table 6. Absolute number of trees (n) with alleles of six microsatellite loci were encountered in Södderich and Weißwassertal population.

Locus		*MAP-2*			*MAP-9*			*MAP-12*			*MAP-33*			*MAP-40*			*MAP-46*	
		Frequency (n)			Frequency (n)			Frequency (n)			Frequency (n)			Frequency (n)			Frequency (n)	
	Alleles (bp)	Södderich	Weißwassertal	Alleles	Södderich	Weißwassertal	Alleles	Södderich	Weißwassertal	Alleles	Södderich	Weißwassertal	Alleles	Södderich	Weißwassertal	Alleles	Södderich	Weißwassertal
1	144	0	6	100	2	0	144	7	2	136	2	3	240	61	28	160	0	2
2	146	2	3	102	135	82	150	0	2	138	0	1	242	4	0	162	17	6
3	148	2	1	104	32	19	152	106	50	140	0	2	244	100	82	164	45	33
4	150	0	1	106	10	15	154	14	27	142	1	1	246	29	1	166	38	24
5	152	0	2	108	9	8	156	15	16	144	2	1	248	46	5	168	85	58
6	154	1	0	110	5	0	158	17	3	146	40	2				170	11	14
7	156	20	0				160	22	28	148	2	2				172	5	6
8	158	56	37				162	0	5	150	24	19				174	4	6
9	160	8	4				164	0	6	152	9	8				176	7	2
10	162	0	3				166	0	1	154	25	15				178	32	8
11	164	2	16				168	0	1	156	0	2				180	1	1
12	166	19	11				172	2	9	158	14	6				182	3	0
13	168	3	4				174	3	4	160	17	8				184	17	19
14	170	33	0				176	2	2	162	11	8				186	1	0
15	172	3	2				178	6	3	164	27	22				188	67	24
16	174	0	10				180	4	0	166	38	11				190	20	20
17	176	0	6				190	0	1	168	14	10				192	8	4
18	178	3	2							170	1	0				194	3	9
19	180	12	3							172	4	3				196	4	7
20	182	101	35							174	51	43				198	1	0
21	184	6	3							176	6	6						
22	186	14	2							178	0	2						
23	188	3	6							180	1	0						
24	190	14	10							182	1	1						
25	192	0	2															
26	200	3	0															
No. of variants		**19**	**22**		**6**	**4**		**11**	**16**		**20**	**22**		**5**	**4**		**19**	**17**
Average number of alleles in the Södderich population = 13.30										**Total number of alleles= 80**								
Average number of alleles in the Weißwassertal population= 14.20										**Total number of alleles= 85**								

3. 2.1.2.Observed heterozygosity (H_o) and expected heterozygosity (H_e)

ZHANG *et al.* (submitted) have developed a method for the estimation of genetic diversity from the genetic data of autotetraploid species. Pertinent results of their analysis of genetic variation within the Södderich and the Weißwassertal populations of *A. pseudoplatanus* at six microsatellite loci are shown in table 7.

The average observed heterozygosity in the Weißwassertal (H_o=0.55) population is slightly higher than that in the Södderich population (H_o= 0.54). However, both of the populations have quite similar values of expected heterozygosity (H_e), *i.e.* Södderich: 0.5730 and Weißwassertal: 0.5736.

In both of the populations *MAP-46* was the most variable gene locus (Södderich: H_o= 0.71; H_e=0.72 and Weißwassertal: H_o and H_e =0.80). *MAP-12* with H_o=0.32 and H_e= 0.41 was the least variable locus in the Södderich population, while in the Weißwassertal it was *MAP-40* with H_o=0.23 and H_e= 0.23.

Significant deviation of the observed from the expected heterozygosity was found in four (*MAP-2*, *MAP-33*, *MAP-40* and *MAP-46*) of the six microsatellite loci in the Södderich population, while in case of the Weißwassertal population, no significant deviation at any of the six loci was found.

Table 7. Analyses of six microsatellite marker loci from the two *A. pseudoplatanus* populations Weißwassertal and Södderich according to ZHANG *et al.* (submitted). *n* is the number of individuals scored; H_o and H_e denote the observed and the expected heterozygosity under HW structure, respectively. χ^2 (df) is the chi-square value (degrees of freedom).

Population	Marker	*n*	H_o	H_e	χ^2	(df)
Södderich	*MAP-2*	132	0.6121	0.6026	27.85*	(15)
	MAP-9	133	0.6901	0.6144	9.86	(6)
	MAP-12	127	0.3234	0.4063	6.94	(11)
	MAP-33	127	0.5095	0.6363	49.65**	(20)
	MAP-40	133	0.4119	0.4584	34.63**	(10)
	MAP-46	133	0.7103	0.7236	52.15**	(20)
	Mean		**0.5429**	**0.5730**		
Weißwassertal	*MAP-2*	77	0.5455	0.6139	7.18	(6)
	MAP-9	80	0.6833	0.6046	3.56	(3)
	MAP-12	78	0.4960	0.5809	16.54	(12)
	MAP-33	78	0.5596	0.6103	12.04	(8)
	MAP-40	74	0.2348	0.2299	0.43	(3)
	MAP-46	80	0.7986	0.7984	6.93	(8)
	Mean		**0.5530**	**0.5736**		

3.2.2. Genetic differentiation between the Södderich and the Weißwassertal populations

The results on genetic differentiation between the Södderich and the Weißwassertal populations are presented in table 8. The average genetic distance estimated as d_o was remarkable; it amounted to 0.25. Estimates for the individual gene loci ranged from 0.09 to 0.37. The largest genetic distance was observed at locus *MAP-2* (d_0 = 0.37) and the lowest was at locus *MAP-9* (d_0 = 0.09). The same is, of course, true for the minimum distances (d_m). These estimates are just smaller. The average genetic distance calculated as d_m is 0.025. In case of d_m, locus *MAP-40*

showed the highest differentiation with a value 0.060, while *MAP-9* showed the least with the value 0.004.

Table 8. Genetic distances between the Södderich and the Weißwassertal populations at six microsatellite loci. d_0: genetic distance (GREGORIUS, 1974); d_m: Nei's minimum genetic distance (NEI, 1973)

Locus	*MAP-2*	*MAP-9*	*MAP-12*	*MAP-33*	*MAP-40*	*MAP-46*	Average
d_0	0.37	0.09	0.35	0.23	0.29	0.19	**0.25**
d_m	0.025	0.004	0.040	0.015	0.060	0.005	**0.025**

3.2.3. Genetic variation of the natural regeneration in the Södderich population

The relative allelic frequencies of the adult trees and their natural regeneration in the Södderich population are compared in table 9. The results show that the average number of alleles in the natural regeneration was 11.50 with a total number of alleles 69. Locus *MAP-2* possessed the highest number of alleles with a value of 18 and the lowest number of alleles was observed at *MAP-40* with only 4 alleles.

3.2.4. Genetic differentiation between the adult trees and the natural regeneration in the Södderich population

According to table 9, the average number of observed alleles is larger (13.3) in the adult trees as compared to the natural regeneration (11.5). Four microsatellite loci possess more alleles in the adult trees than in the natural regeneration. *MAP-12* is more variable in the natural regeneration with one allele more than in the adult trees. In the natural regeneration, some new alleles were detected at *MAP-2* (allele 202), *MAP-12* (alleles: 162, 168 and 190) and at *MAP-33* (allele 178) which were not present in adult trees.

The genetic differentiation between adult trees and the natural regeneration is given in table 10. The genetic distances (d_0) ranged from 0.03 (*MAP-9*) to 0.28 (*MAP-33*) and the average value at six microsatellite loci was 0.17. The average minimum genetic distance d_m is 0.010.

Table 9. Relative number of adult trees and natural regeneration with certain alleles at six microsatellite loci in the Södderich population

Locus		*MAP-2*			*MAP-9*			*MAP-12*			*MAP-33*			*MAP-40*			*MAP-46*		
			Frequency			Frequency			Frequency			Frequency			Frequency			Frequency	
	Alleles (bp)		Adult trees	Natural Regeneration	Alleles	Adult trees	Natural Regeneration	Alleles	Adult trees	Natural Regeneration	Alleles	Adult trees	Natural Regeneration	Alleles	Adult trees	Natural Regeneration	Alleles	Adult trees	Natural Regeneration
1	146	0.01	0.02	100	0.01	0.01	144	0.04	0.06	136	0.01	0.00	240	0.25	0.36	162	0.05	0.03
2	148	0.01	0.01	102	0.70	0.70	152	0.54	0.46	142	0.01	0.00	242	0.02	0.00	164	0.12	0.09
3	154	0.01	0.00	104	0.17	0.18	154	0.07	0.11	144	0.01	0.00	244	0.42	0.36	166	0.10	0.07
4	156	0.07	0.00	106	0.05	0.03	156	0.08	0.11	146	0.14	0.00	246	0.12	0.14	168	0.23	0.30
5	158	0.18	0.20	108	0.05	0.03	158	0.09	0.06	148	0.01	0.00	248	0.19	0.14	170	0.03	0.05
6	160	0.03	0.03	110	0.03	0.04	160	0.11	0.10	150	0.08	0.10				172	0.01	0.01
7	164	0.01	0.10				162	0.00	0.01	152	0.03	0.03				174	0.01	0.01
8	166	0.06	0.01				168	0.00	0.01	154	0.09	0.10				176	0.02	0.00
9	168	0.01	0.01				172	0.01	0.00	158	0.05	0.17				178	0.09	0.06
10	170	0.11	0.07				174	0.02	0.01	160	0.06	0.03				180	0.01	0.00
11	172	0.01	0.01				176	0.01	0.00	162	0.04	0.00				182	0.01	0.00
12	178	0.01	0.01				178	0.03	0.02	164	0.09	0.12				184	0.05	0.08
13	180	0.04	0.02				180	0.02	0.04	166	0.13	0.15				188	0.18	0.21
14	182	0.33	0.33				190	0.00	0.01	168	0.05	0.06				190	0.05	0.03
15	184	0.02	0.02							170	0.01	0.00				192	0.02	0.01
16	186	0.05	0.03							172	0.01	0.01				194	0.01	0.00
17	188	0.01	0.09							174	0.18	0.20				196	0.01	0.02
18	190	0.05	0.06							176	0.02	0.01				198	0.01	0.00
19	200	0.01	0.01							178	0.00	0.01						
20	202	0.00	0.01							180	0.01	0.00						
21										182	0.01	0.00						
No. of variants		**19**	**18**		**6**	**6**		**11**	**12**		**20**	**12**		**5**	**4**		**19**	**17**
Average number of alleles in adult trees = 13.30								**Total number of alleles=80**										
Average number of alleles in natural regeneration = 11.50								**Total number of alleles=69**										

Table 10. Genetic distances between the adult trees and natural regeneration in Södderich population at six microsatellite loci. d_0: genetic distance (GREGORIUS, 1974); d_m: Nei's minimum genetic distance (NEI, 1973).

Locus	*MAP-2*	*MAP-9*	*MAP-12*	*MAP-33*	*MAP-40*	*MAP-46*	Average
d_0	0.23	0.03	0.15	0.28	0.13	0.17	**0.17**
d_m	0.010	0.001	0.006	0.020	0.010	0.010	**0.010**

3.2.5. Comparison between two sub-compartments (2b1 and 2b2) in the Södderich population

The two sub-compartments, i.e. 2b1 and 2b2, in the Södderich population have a different origin; 2b1 originated from natural regeneration and 2b2 is from a combination of natural regeneration and planting. The results of genetic variation within the sub-compartments and genetic differentiation between the sub-compartments are given in table 11.

The average number of alleles observed in sub-compartment 2b2 was higher (12.5) than in 2b1 (11.5). The sub-compartment 2b2 has a larger number of alleles in case of *MAP-2* (2b1: 14 and 2b2: 19), *MAP-9* (2b1: 5 and 2b2: 6) and *MAP-33* (2b1: 18 and 2b2: 19) as compared to sub-compartment 2b1. At locus *MAP-2*, 2b2 has five alleles more. *MAP-40* and *MAP-46* have exactly the same number of alleles in the both sub-compartments.

According to the table 11 the genetic distances (d_0) between the sub-compartments were very low at locus *MAP-9* and *MAP-40* with the values of 0.04 and 0.06, respectively. The other four loci (*MAP-2*, *MAP-12, MAP-33* and *MAP-46*) showed higher genetic distances ranging the values of d_0 from 0.12 to 0.19. The average value of genetic distance d_0 between two populations was 0.13. The genetic differentiations between the two sub compartments measured as d_m ranged from 0.001 to 0.009 and the average distance was less than 1% with the value 0.006.

Table 11. Genetic variation within the sub-compartment 2b1 and 2b2 and genetic differentiation between the compartments of the Södderich population at six microsatellite loci. d_0: genetic distance (GREGORIUS, 1974); d_m: Nei's minimum genetic distance (NEI, 1973)

Locus	Number of alleles		d_0	d_m
	Sub-compartment 2b1	Sub-compartment 2b2		
MAP-2	14	19	0.12	0.009
MAP-9	5	6	0.04	0.001
MAP-12	11	9	0.17	0.008
MAP-33	18	19	0.19	0.008
MAP-40	5	5	0.06	0.006
MAP-46	17	17	0.17	0.003
Total	**69**	**75**		
Average	**11.5**	**12.5**	**0.13**	**0.006**

3.3. Spatial genetic structure of adult trees in the Södderich and the Weißwassertal populations

3.3.1. Distribution pattern of pairs of data

The distribution of the number of pairs of trees in the Södderich and the Weißwassertal populations is shown in Figure 34. The frequency distribution curve in the Södderich population shows that the highest numbers of trees pairs are present in the distance class of 40 to 60 m. After 60 m the numbers of trees decrease gradually up to 280 m. In the less dense and more or less linear Weißwassertal population most of the trees are present in a distance class between 100 and 300 m. Then the numbers fall abruptly at the distance class 400 m and go down up to 1,100m. After 1,100 m the distribution curve is almost flat up to 1,700 m and then there are few more individuals at 1,800 m distance. This almost disjunct distribution of distances between tree individuals is due to the wide gap between small clumps of trees (Figure 7).

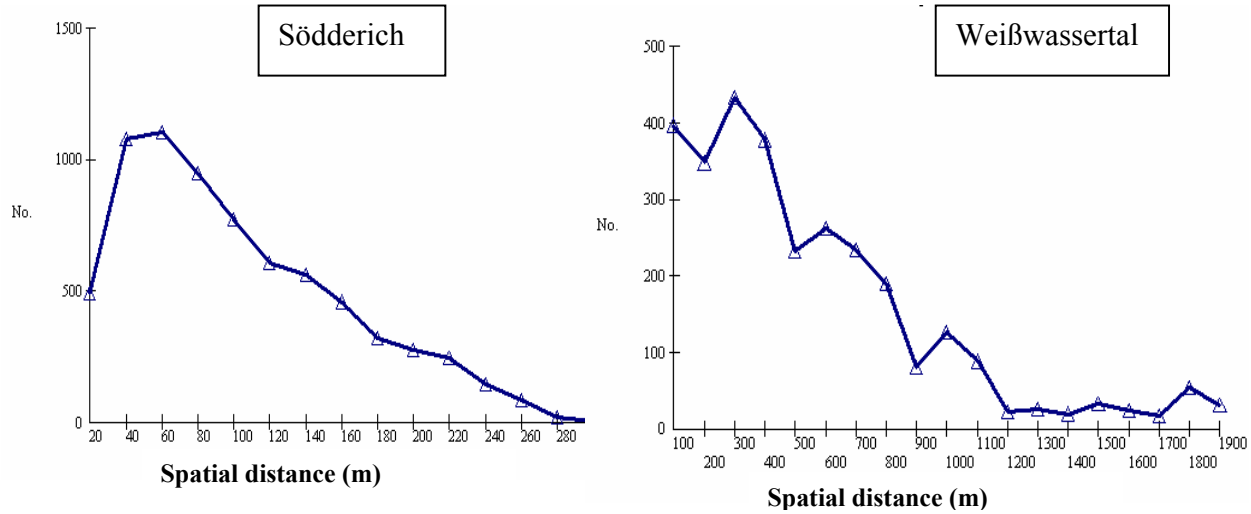

Figure 34. Frequency distribution of the numbers of tree pairs over distance classes in the Södderich and the Weißwassertal populations.

The extreme imbalance existing in the numbers of tree pairs falling into the various distance classes made it advisable to compute spatial autocorrelograms also by defining the width of distance classes according to numbers of tree pairs.

3.3.2. Spatial genetic structure of the adult trees in the Södderich population

Two-dimensional interpolated spatial distribution maps of some alleles at selected microsatellite loci are shown in Figure 35. The interpolation was carried out with the 'semivariance analysis' and 'punctual kriging analysis' using GS+ (Geostatistics for the Environmental Science, version 3.1.7) computer programme. The distribution of allele 182 of *MAP-2* was almost random all over the population. Colours representing higher interpolated frequencies dominated the whole area of the population. The allele 186 at the same locus was mostly concentrated in the south-eastern part (Figure 6) of the population with two clumps in the north and the west. Allele 152 of *MAP-12* was distributed mostly in the central and the in north-eastern part (sub compartment 2b2). It was less frequent in the western part of the population. Also allele 158 of *MAP-12* was mainly concentrated in the north-eastern part of the population. Allele 240 of *MAP-40* was distributed over the population in a different way. It has a concentration in north-south centre strip and some smaller clumps on either side. Allele 244 of that same locus is mainly concentrated on a strip running from east to west through the centre of the population. There was a pronounced deficiency of the allele in the southern and northern part of the population.

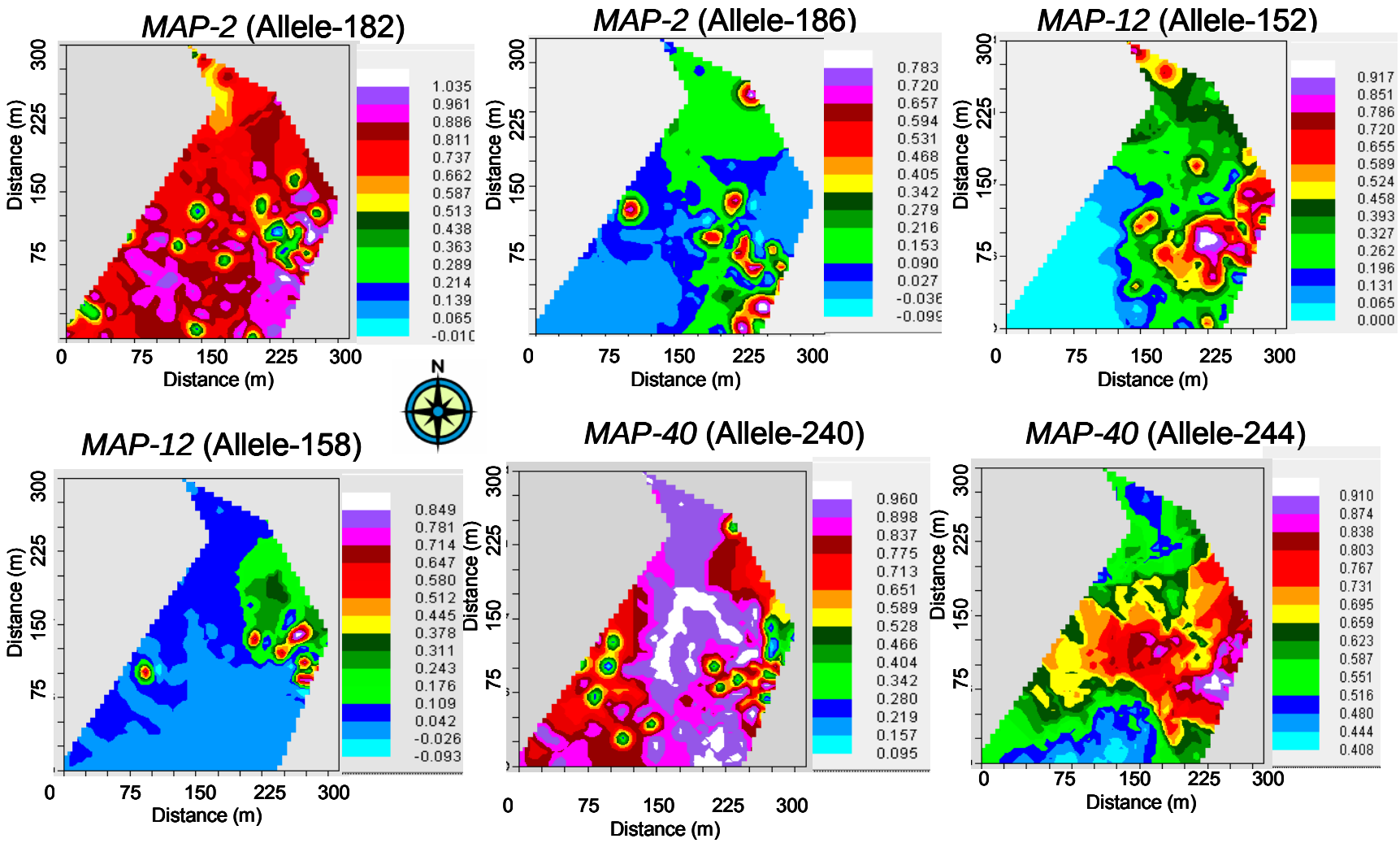

Figure 35. Two-dimensional map of interpolated spatial distribution of selected alleles of three microsatellite loci in the Södderich population. The right box of each map shows the scale of interpolated frequency of that allele represented by different colour.

The spatial correlograms of multi-locus genotypes of the Södderich adult trees are presented in Figure 36. The spatial autocorrelation analysis was carried out on the basis of two types of spatial distance classes, one on the basis of even distance classes and another based on even sample sizes. The solid line shows the spatial autocorrelation in each distance class. The dotted lines below and above the zero line show the lower and upper 95% confidence limits.

Both of the correlograms (solid line) (Figure 36) show that there was a significant spatial genetic structure up to the distance of 40 m. Positive values for the autocorrelation coefficient were found and up to about 50 m. After 50 m the spatial autocorrelation line fell below the zero line and was slightly negatively significant at 100 m and 220 m in the case of even distance classes, and at 70 m and 96 m in the case of even sample size. Thereafter the curve was almost flat and no significant spatial genetic structure was observed.

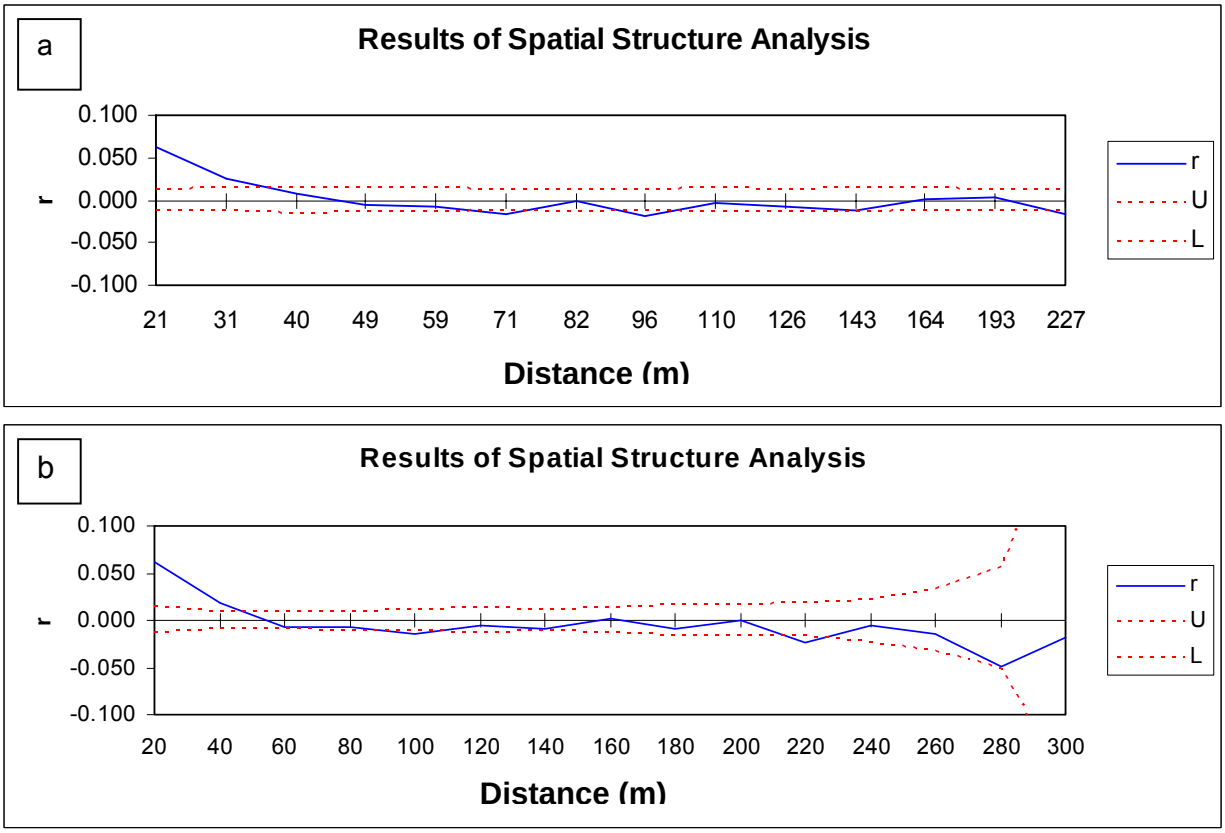

Figure 36. Correlograms (solid line) showing spatial genetic structures at six microsatellite loci of the adult trees in the Södderich population, with 95% confidence regions indicated with dotted lines, which were obtained after 999 permutations. r – coefficient spatial autocorrelation (SMOUSE and PEAKALL, 1999); u- upper confidence limit (95%); l- lower confidence limit (95%). a: based on equal sample size in each distance class, b: based on equal distance classes.

3.3.3. Spatial genetic structure of the Weißwassertal population

Since the Weißwassertal population possessed strongly elongated shape, the results of allelic distribution were better visible in three-dimensional than in to two-dimensional maps. Three-dimensional maps of the interpolated spatial distribution of six alleles at five microsatellite loci in this population are shown in Figure 37. Allele 182 of *MAP-2* was distributed almost equally in the population, while allele 166 was mainly found in the centre of the population. Allele 104 of *MAP-9* was relatively frequent in the eastern and western part of the population but was absent in the centre. Allele 150 of *MAP-33* was mostly concentrated in the western and central part of the population. Allele 240 of *MAP-40* was most frequent in all parts of the population, while allele 248 was concentrated around the middle.

The results of the spatial autocorrelation analysis in the Weißwassertal population are presented in Figure 38. There are three types of correlograms, *i.e.* a. based on equal sample sizes, b. based on equal distance classes and c. based on selected distance classes of up to 1,100 m. Correlograms ‘b’ and ‘c’ showed positive auto-correlation up to a distance class of about 300 m. After 300 m the curve fell below the zero line indicating negative autocorrelation. Significant negative autocorrelation was observed within the distance class of 600 to 800 m in correlogram ‘c’. Thereafter the curve did not show any significant clustering. In case of correlogram ‘b’ the confidence interval became wider in the higher distance classes due to the presence of very few pairs beyond 1,000 meters (Figure 7 and 34).

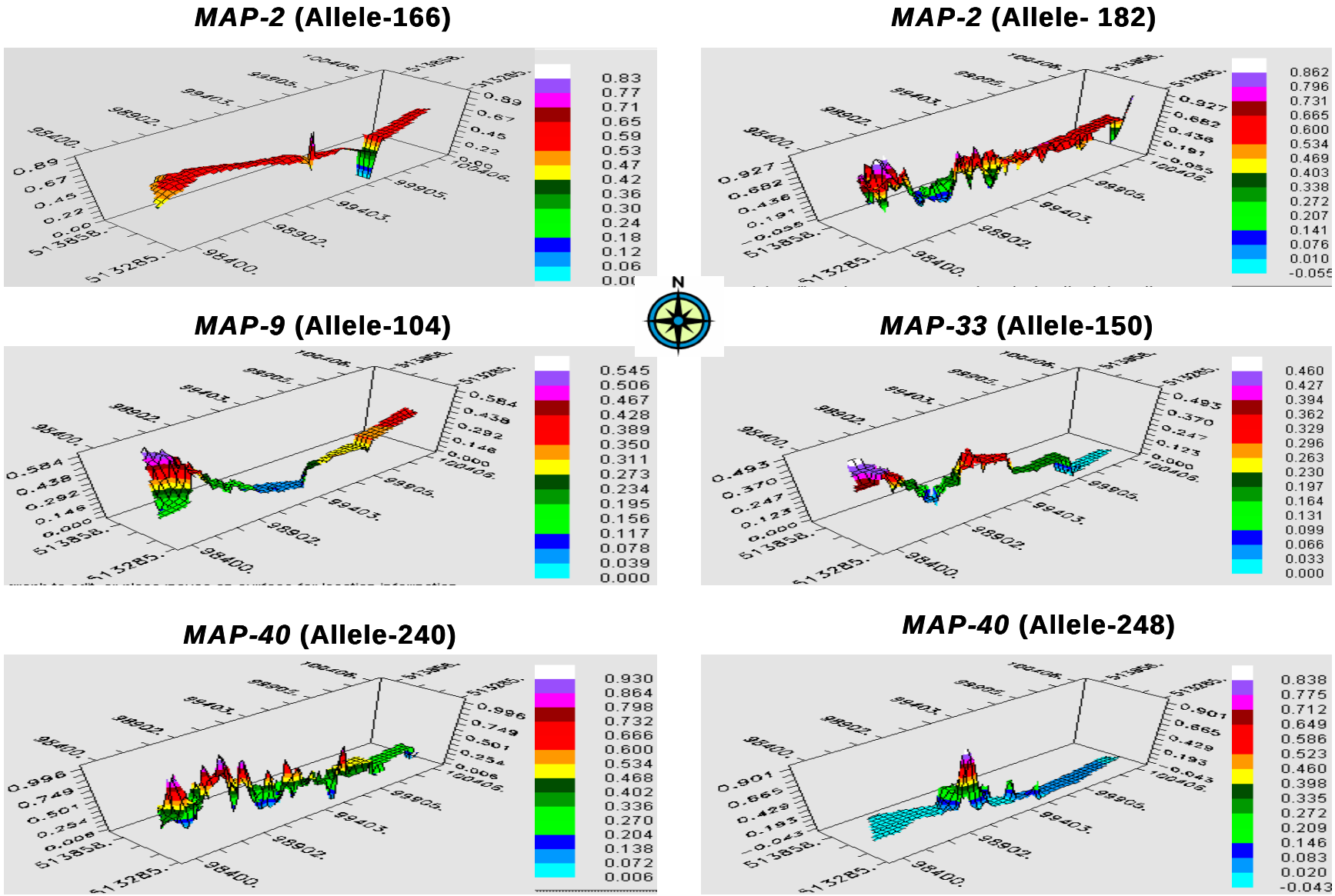

Figure 37. Three-dimensional map of interpolated spatial distribution of selected alleles of five microsatellite loci in the Weißwassertal population. The right box of each map shows the scale of interpolated frequency of that allele represented with different colour. In the map the horizontal scale represents the distance class X-axis (998400=0 m, 998902= 502m, 999403= 1003 m, 999905= 1505 m and 1000406= 2006 m) Y-axis (513285=0 m and 513858= 573 m). Z- values (interpolated frequencies) are different for different maps.

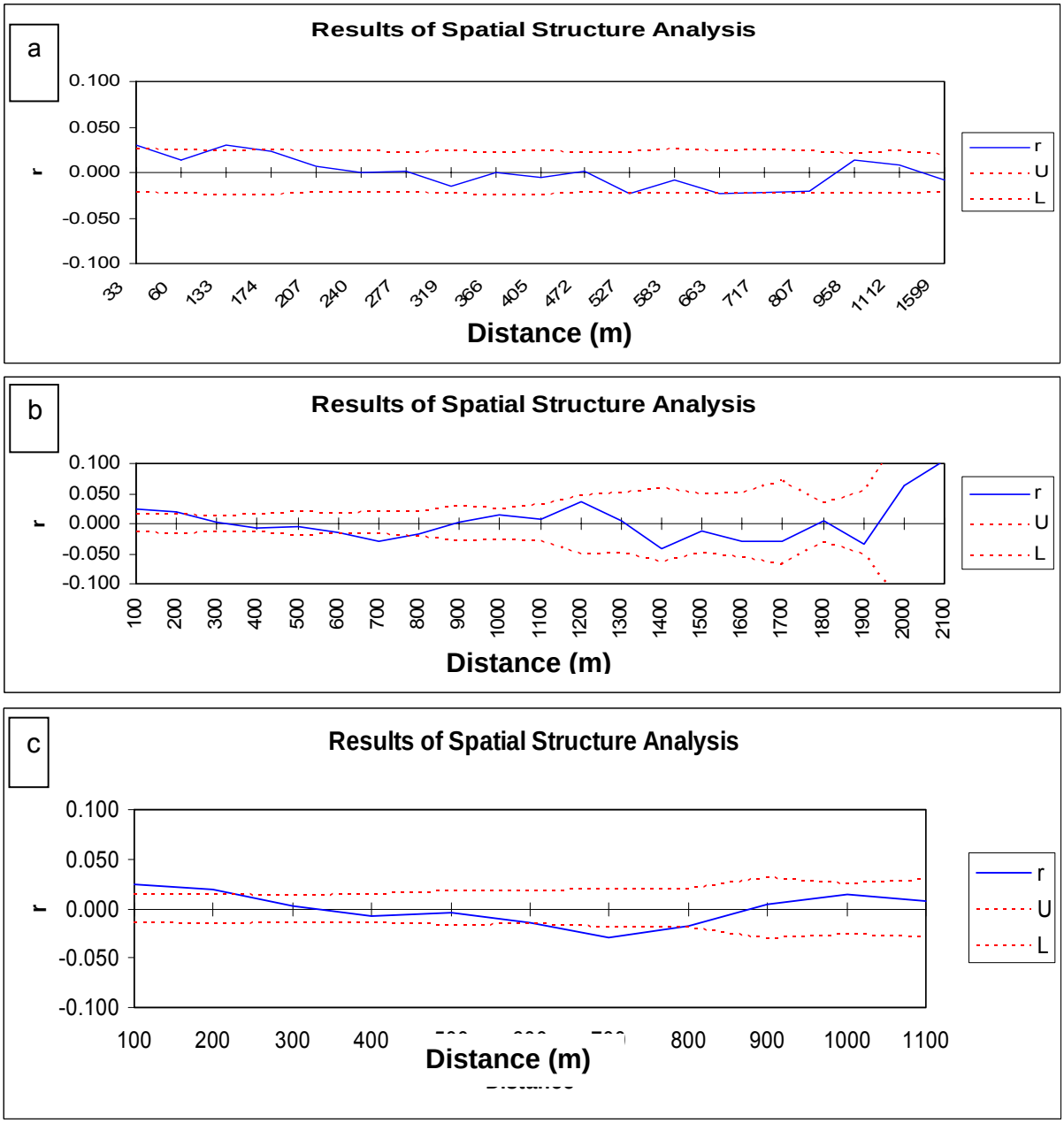

Figure 38. Correlograms (solid lines) showing spatial genetic structures at six microsatellite loci in the Weißwassertal population, with 95% confidence regions indicated with dotted lines, which were obtained after 999 permutations. r – coefficient of spatial autocorrelation (SMOUSE and PEAKALL, 1999); u- upper confidence limit (95%); l- lower confidence limit (95%). a: based on equal distance classes, b: based on equal sample size in each distance class, c: based on selected distances up to 1,100 m.

3.3.4. Natural regeneration in the Söderich population

The results of the spatial autocorrelation analysis of natural regeneration of the Söderich population are presented in Figure 39. Both of the correlograms show positively significant spatial autocorrelation up to a distance class of around 40 m. The autocorrelation in this domain is less close in the natural regeneration than in the adult trees. Then the curve is almost flat up to 130 m. Thereafter, it showed a slight but significant negative autocorrelation up to 200 m in correlogram 'a'.

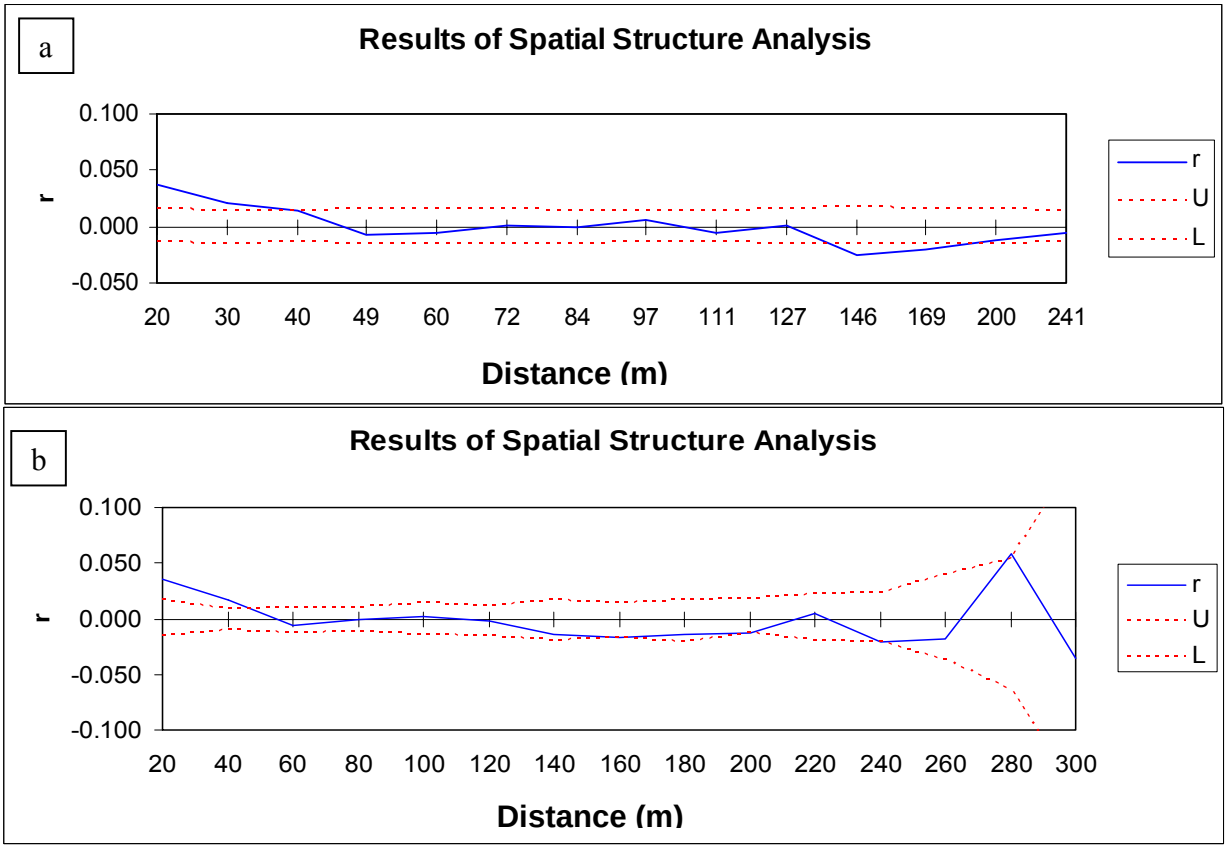

Figure 39. Correlograms (solid lines) showing the spatial genetic structures at six microsatellite loci in the natural regeneration in the Söderich population, with 95% confidence regions indicated with dotted lines, which were obtained after 999 permutations. r – coefficient of spatial autocorrelation (SMOUSE and PEAKALL, 1999); u- upper confidence limit (95%); l- lower confidence limit (95%). a: based on equal sample size in each distance class, b: based on equal distance classes.

3.3.5. Natural regeneration and adult trees in the Söderich population combined

The data of the adult trees and the natural regeneration were also subjected to a combined analysis to get an impression about the overall spatial genetic structure in the Söderich population. The results of the spatial autocorrelation analysis are presented in Figure 40. Both of the correlograms show a significant positive autocorrelation up to 40 m. In this joint analysis the initial correlation is less than that estimated in the adult trees (cf. Figure 36). In correlogram 'a', there is a significant negative auto-correlation within the distance classes of 130 to 200 m, while in correlogram 'b' the negative autocorrelation is not significant.

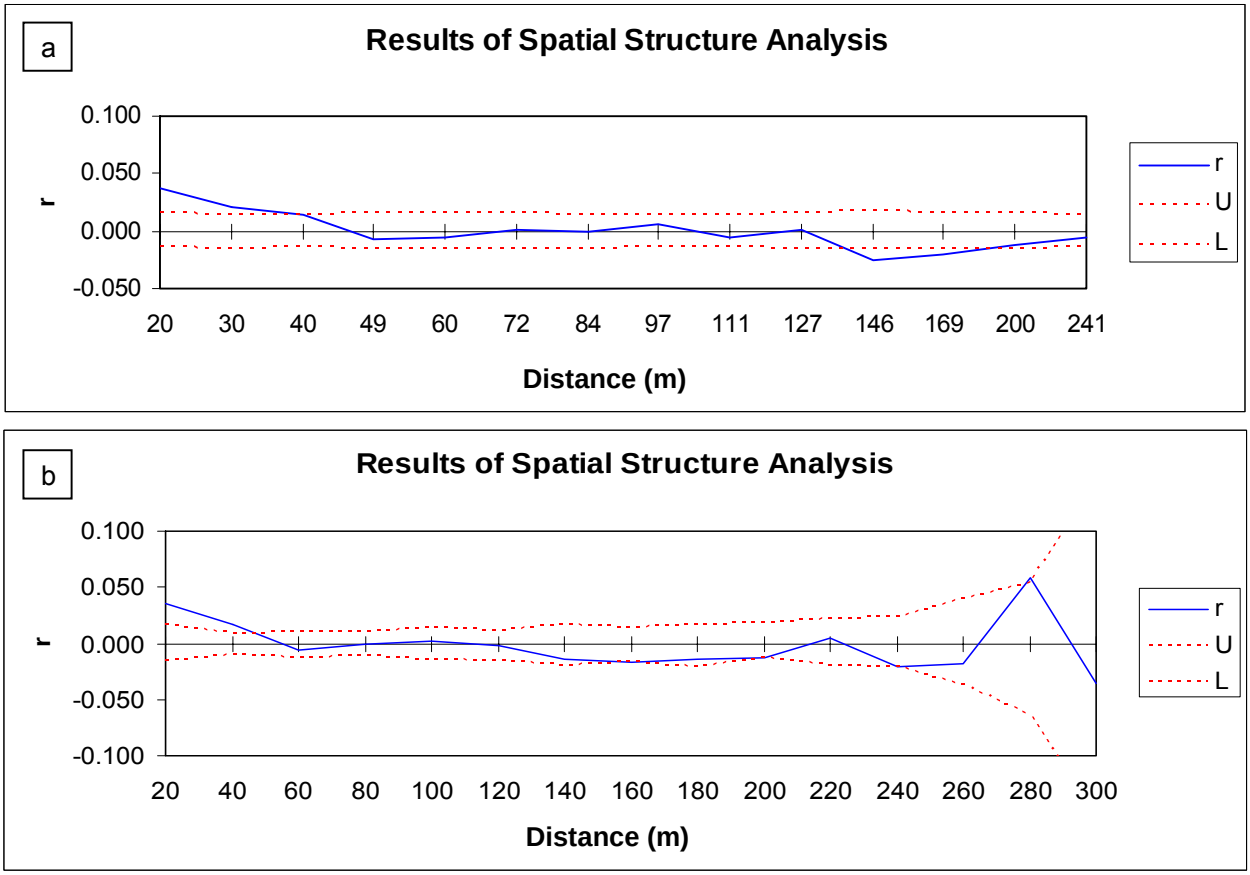

Figure 40. Correlograms (solid lines) showing the combined results of spatial genetic structures multilocus genotypes in adult trees and natural regeneration in the Söderich population, with 95% confidence regions indicated with dotted lines, which were obtained after 999 permutations. r –coefficient of spatial autocorrelation (SMOUSE and PEAKALL, 1999); u- upper confidence limit (95%); l- lower confidence limit (95%). a : based on equal sample size in each distance class, b: based on equal distance classes.

3.4. Proportion of self-fertilization

Proportions of maximum self-fertilization or minimum out-crossing in the two populations were estimated according to the exclusion principle on the basis of the criteria described above. The maximum rate of self-fertilization in the Södderich population ranged from 0 to 22 % (table 12). The average self-fertilization rate for these 13 trees was then 8 %. Eleven percent of the seeds had to be discarded due to their failure to meet the criteria (1) to (3) as described in sub-section 2.3.3. The seeds that did not meet the criteria were mainly from the trees 30, 123 and 128. The average value of external pollen that became effective in the Södderich population was 2 %.

In the Weißwassertal population the average maximum self-fertilization rates for 8 seed trees was 13 % and it ranged among individual trees from 3 to 40 %. The percentage of seed that did not meet the criteria (14 %) is slightly higher and was mainly confined to trees 66 and 82. Average external pollen observed in the Weißwassertal was quite high (13 %) as compared to the Södderich population. Remarkably, trees 11 and 82 received considerably high percentages of external effective pollen with the values 41 and 20 %.

The overall maximum self-fertilization rate of the two populations was 11 %. In total, 12 % seeds did not meet the minimum criteria to be considered for the validity of this analysis. All external effective pollen could have come from the other population and vice versa. The distance between two populations is about 3 km. Supposedly the survey of the 137 + 82 trees with the two populations detected most of the alleles that exist in the species.

Table 12. Multilocus (5 microsatellite loci) estimation of the maximum proportion of self-fertilization in the Södderich and Weißwassertal populations (seeds from 13 and 8 trees, respectively). The percentages of self-and cross-fertilization were computed on the basis of the used seeds. Data on fertilization by external pollen in the right-most column refer to percentages of cross-fertilized seeds.

Södderich population							
	No. of seeds				%	%	%
Tree No.	Analysed	Omitted	Used		Self-fertilization	Out-crossing	External pollen
2	32	0	32		0	100	3
6	32	0	32		9	91	3
30	32	10	22		0	100	5
36	32	0	32		3	97	0
38	32	0	32		19	81	0
43	32	0	32		22	78	0
74	32	4	28		0	100	0
99	32	0	32		3	97	3
108	31	1	30		6	94	4
123	32	9	23		17	83	0
128	32	18	14		14	86	0
131	32	0	32		9	91	3
136	31	2	29		7	93	0
Total	**414**	**44**	**370**	**Average**	**8**	**92**	**2**
Weißwassertal population							
	No. of seeds				%	%	%
Tree No.	Analysed	Omitted	Used		Self-fertilization	Out-crossing	External pollen
11	32	3	29		7	93	41
22	31	0	31		13	87	0
37	29	1	28		11	89	8
39	32	3	29		17	83	0
66	32	22	10		10	90	11
67	32	0	32		6	94	13
76	31	0	31		3	97	7
82	32	7	25		40	60	20
Total	**251**	**36**	**215**	**Average**	**13**	**87**	**13**
				Species average	**11**	**89**	**8**

4. Discussion

4.1. Microsatellite marker development in A. pseudoplatanus

4.1.1. Efficiency of enrichment protocol

Microsatellites became frequently used marker types because of their wide applicability and their high variability. Its application is widening along with new technological advances for isolating microsatellite markers (ZANE, *et al.* 2002).

The efficiency of microsatellite hybridization in the present study using the protocol of FISCHER and BACHMANN (1998) was 60% (out of 90 colonies sequenced, 54 contained the microsatellite repeats of more than 5 repeat units and at least 10bps length). Authors using similar protocols have reported levels of enrichment between 11% and 99% (EDWARDS *et al.* 1996; PAETKAU, 1999; RODRIGUES *et al.* 2002; SANEYOSHI *et al.* 2003, MOTTURA *et al.* 2004). If we compare the efficiency in the present study to the above- mentioned studies, it can be considered as medium level. SQUIRREL *et al.* (2003) compared the efficiency level of microsatellite enrichment in 71 plant species in which different protocols of microsatellite isolation were used. They found that an enriched library, *i.e.* a library achieved after hybridizing selected artificial microsatellite motifs to genomic DNA, was more efficient (64%) as compared to the non-enriched libraries constructed without hybridization of selected artificial microsatellite motifs to genomic DNA (51.3%). There not many data available on other methods of microsatellite isolation, *i.e.* from AFLPs (YAMANTO *et al.* 2002), RAPDs (UENO *et al.* 1999), random amplified microsatellite PCR (RAMP) (VANDER STAPPEN *et al.* 1999), 5'- anchor (KUMAR *et al.* 2002) and expressed sequences tags (ESTs) (WOODHEAD *et al.* 2003). In the above cited five studies the average efficiency of microsatellite enrichment was 65 %.

FISCHER (2000) sequenced 243 colonies using a similar protocol as in the present study. Among 243 colonies sequenced, 182 (75%) contained microsatellite repeat motifs. Thus the efficiency of microsatellite isolation in *A. pseudoplatanus* was slightly lower (60%) in this study as compared to the isolation of microsatellites in *Allium cepa* by FISCHER (2000) using a similar protocol. The higher efficiency in *Allium cepa* could be due to the use of six different

oligo-probes ($(CT)_{10}$, $(CA)_{10}$, $(TAA)_8$, $(GAA)_8$, $(AAC)_8$ and $(GGT)_8$) for hybridization which may have increased the chance of getting more fragments with microsatellite repeats, while in this study only three oligo-probes ($(CA)_{10}$, $(GAA)_8$, $(AAC)_8$) were used.

MOTTURA *et al.* (2004) also used the same protocol to develop microsatellite gene markers in *Prosopis chilensis* with some modifications in hybridization, since only a single enrichment was performed and in cloning, since a different vector was used and "blue/white screening" was performed. They reported that only 16% of all detected SSR sequences contained the complementary sequences to the oligo-probes ($(CA)_{10}$, $(GAA)_8$, $(AAC)_8$) used for the hybridization. Since they used the same oligo-probes as in the present study, the results are comparable. In contrast to their result, in this study the presence of microsatellite sequences complementary to the oligo-probes was considerably higher with the value of 82.60 %. Furthermore, they reported that 30.8 % of sequences contained a microsatellite repeat motif of at least 5 repeat units and a minimum length of 10 bp. In contrast to that, in this study 60% of sequences contained a microsatellite repeat motif according to the above-mentioned criteria. The reason of the lower complementing to the oligo-probes and for the lower enrichment efficiency could be the performance of only a single hybridization, while in the present study two successive hybridizations were performed. This double hybridization may have played a vital role in increasing the efficiency of hybridization of the oligo-probes with the DNA fragments.

The efficiencies of microsatellite enrichment in 12 tree species including this study using different enrichment protocols are given in table 13. The efficiency of obtaining sequences with microsatellites ranged from 28 to 72 %. The average efficiency in *A. pseudoplatanus* (60%) is still higher than the average efficiency of the other eleven tree species (53%) although in the case of *A. pseudoplatanus* no screening was carried out. The efficiency might have been higher if a screening was carried out.

Table 13. Efficiency of microsatellite primer development in 12 tree species. Φ: percentage values representing the percentage of sequences retained from the previous stage. †: sequences with at least 5 microsatellite repeats and at least 10bp long.

Species	Screening	No. of clones sequenced	% of sequenced inserts with a unique microsatellite† ϕ	% of SSR for which primers could be designed ϕ	% of primers that amplified the expected product size ϕ	% of primers with polymorphic loci ϕ	References
Sorbus torminalis	Yes	100	28	32	28	88	ODDOU-MURATORIO *et al.* (2001)
Castanopsis cuspidata	Yes	135	51	39	39	100	UENO *et al*. (2000)
Cajanus cajan	Yes	279	15	100	48	50	BURNS *et al*. (2001)
Cocos nucifera	Yes	120	75	28	93	100	PERERA *et al*. (1999)
Eucalyptus globulus	Yes	105	_	12	100	92	STEANE *et al*. (2001)
Morus indica	No	72	79	26	-	40	AGGARWAL *et al.* (2004)
Neobalanocarpus heimii	Yes	16	94	87	62	100	IWATA *et al*. (2000)
Pinus sylvestris	Yes	90	72	57	-	19	SORANZO *et al.* (1998)
Ulmus laevis	No	120	16	100	44	88	WHITELEY *et al.* (2003)
Swietenia humilis	Yes	60	68	40	-	33	WHITE *et al*. (1997)
Prosopis chilensis	Yes	120	30	49	50	67	MOTTURA *et al.* (in press)
Mean		**111**	**53**	**52**	**58**	**71**	
Acer pseudoplatanus	No	90	60	45	67	50	PANDEY *et al.* (2004)

4.1.2. Dinucleotide vs trinucleotide microsatellite repeats

In this study dinucleotide repeats were the most abundant microsatellite repeats representing 87.05% (CA/GT=75.92%, GA/CT= 5.4%, TA/AT=3.70%) of the sequences with microsatellites, while trinucleotide repeats represented 9.25% (only CTT/GAA) followed by mononucleotides with 3.7% (with only one repeat type A/T). No microsatellite repeat containing AAC/TTG was detected even though it was used for hybridization. SCOTTI *et al.* (1999) reported quite similar results in their study in *Araucaria cunninghamii* and *Pinus elliotti.* They found that the percentage of dinucleotide microsatellite repeats was higher (*A. cunninghamii* =87.5% and *P. elliottii* = 90.70%) as compared to trinucleotide repeats (*A. cunninghamii* =12.5% and *P. elliottii* = 9.30%) although they used eleven different types of trinucleotide and only two dinucleotide oligo-probes for hybridization. MOTTURA *et al.* (2004) have also reported a higher percentage of dinucleotide repeats (69%) as compared to trinucleotide repeats (31%), even though trinucleotides were used for hybridization.

In contrast to the above findings, MORGANTE *et al.* (2002) found that trinucleotide microsatellite repeats are almost two times more frequent than the dinucleotide repeats per mega base pair in plant genomes. They performed a sequence database search in *Arabidopsis* (dinucleotide = 87.4 and trinucleotide = 159.3), rice (dinucleotide = 62.8 and trinucleotide=113), soybean (dinucleotide = 47.6 and trinucleotide = 76.8), maize (dinucleotide = 30.7 and trinucleotide = 92.6), and wheat (dinucleotide = 44.7 and trinucleotide = 89.2).

The lower level of trinucleotide microsatellite repeats in the above-mentioned forest tree species as compared to agricultural crops could be due to the different composition in sequences of genomic DNA of the plant species. On the other hand, it may also due to the lack of optimum enrichment conditions (e.g. annealing temperature, duration of annealing, concentration of buffers, etc.) required for specific trinucleotide repeats.

In this study the efficiency of hybridization of CA/GT microsatellite repeats is high (75.92%). YUE *et al.* (2000) reported similar levels of efficiency of the microsatellite CA/GT repeat. They reported that out of 45 sequences, 35 (80%) were with CA/GT microsatellite motifs.

However, it is reported that the CA/GT repeat is common in animals and the AT/TA motif is common in plants (LAGERCRANTZ *et al.* 1993). Since no AT/TA oligo-probe was used for hybridization in this study, a comparison regarding the enrichment of this motif was not possible.

4.1.3. Primer design and optimization

4.1.3.1. Efficiency of primers

In this study, out of 54 sequences that contained microsatellite repeat motifs, 24 sequences (44.44 %) were suitable for primer design. The rest of the sequences contained either disturbed (imperfect) microsatellites or they were too close to the vector sequence to design primers. Table 13 shows the percentage of sequences suitable for primer design ranging from 12 to 100%. The average for the 11 tree species mentioned in table 13 is 52 % which is slightly higher than the result in the present study. The efficiency to amplify a product of the expected size is higher in this study as compared to the average efficiency reported for 11 other tree species (table 13). The percentage of amplifying fragments of expected size is also larger than in other polyploid species; *i.e. Medicago sativa* (5%, DIWAN *et al*. 1997*), Ipomoea batatas* (14.2%, BUTELER *et al.* 1999) and hexaploid wheat (32%, BRYAN *et al.* 1997). Finally, the percentage of primers that were polymorphic for *A. pseudoplatanus* is relatively low (50%) in comparison to the average value for 11 other tree species (71%).

SQUIRRELL *et al.* (2003) presented the level of attrition in different stages of microsatellite development in a diagramme (Figure 41). The level of final attrition in this study is slightly higher (91%) than SQUIRRELL *et al.* (2003) have estimated (83 %).

4.1.3.2. Optimization of primers

Although amplification products of expected size were observe, it was difficult to obtain clearly interpretable bands. Amplification was improved after optimizing PCR conditions, *i.e.* amount of DNA, amount of primers, and annealing temperature. Annealing temperatures estimated on the basis of BOLTON and McCARTHY (1962) described by SAMBROOK *et al.* (1989) did not always correspond to the optimum T_a observed, on the basis of signal strength of the amplicon. Nevertheless, the formula provided a starting point to develop optimized amplification programs for numerous microsatellite loci. The use of the gradient PCR cycle with different annealing temperatures at a time proved to be very useful to find the best annealing temperature for each primer pair.

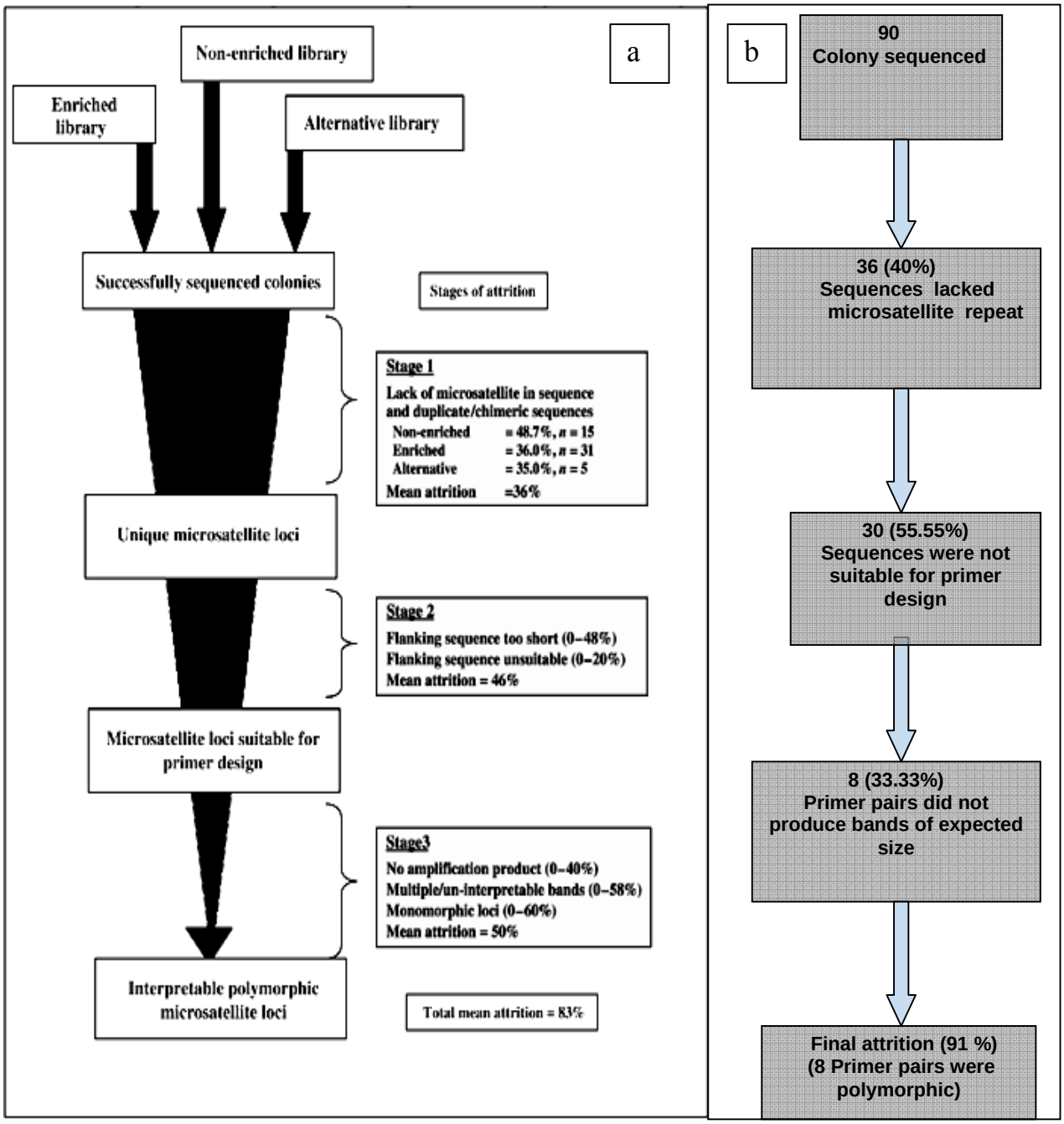

Figure 41. Diagrammatic representation of microsatellite attrition. Diagramme (a) is from SQUIRRELL *et al.* (2003). The figure illustrates average attrition at several stages in the isolation of microsatellites primers. Starting with successfully sequenced clones, attrition occurs due to (1) lack of microsatellites and duplicate/chimeric sequences, (2) unsuitability for primer design and (3) nonmendelian, nonpolymorphic products. The mean percentage attrition given for each stage is the percentage loss from the previous stage. The right diagramme (b) is based on the present study.

4.1.3.3. Problem of stutter bands

Even after optimizing the PCR protocol, the problem of stutter bands was not completely solved. In some cases, the higher annealing temperature reduced the number of stutter bands. Stutter bands result from slippage of DNA polymerase over the template DNA and they are common when the amplicon is comprised of dinucleotide repeats (BROWN *et al*, 1996). The primary cause of stutter bands under normal PCR conditions is a change in the number of repeat units due to slipped strand extension by the *Taq* DNA polymerase. In general, the "mutation" rate in the template DNA increases with the increase in the number of repeat units, which can also have happened during the PCR (SHINDE *et al.* 2003). The finding of this study also confirms the above-mentioned correlation between number of repeat unit and the occurrence of stutter bands since we observed that alleles with longer microsatellite repeats (*MAP-2, MAP-33, MAP-34, MAP-46*) possess more stutter bands as compared to alleles with shorter microsatellite repeats (*MAP-9*, *MAP-10*, *MAP-12*, *MAP-40*) (see Figure 24-31).

Since stutter bands were weaker in intensity as compared to the "real" bands and since they were usually smaller than the "real" band interpretation of genotypes could be done without problems even stutter bands occurred.

4.1.4. Validation of amplification products

In principal, mechanisms of generating allelic variation in microsatellites are due to changes in the microsatellite repeat motif (STRAND *et al.* 1993; JIN *et al.* 1996; KIMMEL *et al.* 1996). However, sequence studies of some animal and plant microsatellite alleles indicated that the variation in alleles may also arise due to different complex phenomena such as nucleotide substitution, insertions/deletions (indels) in the flanking region of the microsatellite repeat, and changes in composition of the repeat unit (GRIMALDI and CROAU-ROY, 1997; MACAUBAS *et al.* 1997; BUTELER *et al.* 1999, CURTU *et al.* 2004). Sequencing of alleles revealed that length variation in fragments was usually caused by different numbers of repeats.

In *MAP-33* an indel of two base pairs was detected in the microsatellite flanking region. An additional microsatellite was detected by sequencing the amplified products of one primer pair and new primers (*MAP-9* and *MAP-10*) were designed to amplify these microsatellites separately.

4.1.5. Problem of interpreting microsatellite genotypes in autotetraploid species

Because of the autotetraploidy of *A. pseudoplatanus,* a single individual can possess one to four bands or fragments at any SSR gene locus. In case of one and four bands it was straight forward to assign a complete genotype. However, it was impossible to assign a complete genotype for the individuals possessing two or three bands. The complication in case of two or three bands was due to the inability to determine the exact "dose" of alleles. Due to this reason, strictly speaking only the allelic phenotypes of each individual observed at each locus were scored. This incomplete information on the genotype creates many limitations to calculate frequently used population genetic parameters such as H_o, H_e, and F_{ST} and to compare the findings to other studies.

Similar problems were reported by many authors (e.g. SAMADI *et al.* 1999; MENGONI *et al.* 2000; KORBECKA *et al.* 2003; LIAN *et al.* 2003; SALTONSTAL, 2003; KOSMAN and LEONARD, 2005) who developed or used microsatellite markers in polyploid species. Many of them scored the bands according to their presence or absence i.e. as dominant markers. KORBECKA *et al.* (2003) scored the genotypes of two or three bands as incomplete and treated individuals with two bands as having two missing data, and individuals with three bands as having one missing data. The authors accepted that their interpretation underestimates the allelic frequencies and the population genetic parameters estimated from such interpretation are not much reliable. Difficulties to interpret banding patterns of polyploids are not limited to microsatellites, but the problem also exists in isozyme analysis. For example, KONNERT *et al.* (2000) reported the complexities of scoring bands at particular isozyme gene loci in *A. pseudoplatanus.* However, LEINEMANN (2000) was able to use the staining intensity of zymogram bands for assigning complete genotypes to individuals in autotetraploid *Prunus spinosa* L.

4.1.6. Cross-species amplification of microsatellites

The probability of successful cross-species amplification of microsatellites depends on the relatedness between species. The eight microsatellite loci developed for *A. pseudoplatanus* were tested in 21 other species of genus *Acer*. The amplification of fragments with expected size was very high (92 %) as revealed by a comparison to 10 other studies (table 14). The flanking regions of the sequences used for microsatellite development may be relatively conserved in the genus *Acer* due to close relatedness of the species to *A. pseudoplatanus.* There are, however, some examples where microsatellite loci have been conserved over very long evolutionary distances, such as in different genera of turtles for over 300 million of years (FIZSIMMONS *et al.* 1995) or in fish species for over 450 million years (RICO *et al.* 1996). The transferability is usually considerably reduced beyond the genus level. GUPTA and VARSHNEY (2000) tried 35 microsatellite loci developed from members as divergent as those from dicots (Myrtaceae), monocots (Poaceae) and gymnosperms (*Pinus*) for a study of transferability. Success was achieved in 80% when transferability was tested within a genus and was considerably reduced to 20%, when tested with species of another genus. ECHT *et al.* (1999) also reported a similar result to those of GUPTA and VARSHNEY (2000). Their studies showed that it is possible to share primers among members of the subgenera of *Pinus* with an amplification success rate of 29 % when primer pairs were tested among subgenus *Strobus* and subgenus *Pinus*.

The polymorphism of the markers developed for *A. pseudoplatanus* was not determined for other *Acer* species since they were tested in only one sample per species.

Table 14. Efficiency of cross-species amplification of microsatellite markers in different plant species within the same genus. -: not investigated or not reported.

The species for which the primers were developed originally	Number of species tested	Total primer tested	% of Amplification	% of Polymorph -ism	References
Amsonia kearneyana	5	12	78	-	TOPINKA *et al.* (2004)
Bambusa arundinacea	17	6	59	-	NAYAK and ROUTI (2005)
Coffea arabica L.	17	9	90	48	BARUAH *et al.* (2003)
Ficus carica	17	8	86	-	KHADARI *et al*. 2001
Manihot esculenta	6	10	100	100	ROA *et al.* (2000)
Melaleuca alternifolia	7	35	80	-	ROSSETTO *et al.* (1999)
Morus indica	14	6	100	77	AGGARWAL *et al.* (2004)
Prosopis chilensis	7	6	83	74	MOTTURA *et al.* (in press)
Solidago sempervirens	11	9	50	-	WIECZOREK and GEBER(2002)
Ulmus laevis Pall.	4	7	69	59	WHITELEY *et al.* (2003)
Mean	**11**	**11**	**80**	**72**	
Acer pseudoplatanus	21	8	92	-	PANDEY *et al.* (2004)

4.2. Genetic variation within and between the Södderich and Weißwassertal populations

4.2.1. Genetic variation within populations

4.2.1.1. Genetic variation in the Södderich and Weißwassertal populations

The average numbers of alleles per gene locus observed in the Södderich and Weißwassertal populations of *A. pseudoplatanus* were 13.3 (total number of alleles= 80) and 14.2 (total number of alleles=85), respectively. Although the genetic diversity at the level of alleles was slightly higher in the Weißwassertal population, the observed (Södderich: 0.54 and Weißwassertal: 0.55) and expected heterozygosities (Södderich: 0.57 and Weißwassertal: 0.57) were similar (table 7). The Weißwassertal population is considerably smaller (N=82) as compared to the Södderich population (N=137). Still more alleles were observed in the smaller Weißwassertal population. Considering the shape of the populations, the Weißwassertal population is stretched up to about 2 km along the narrow valley of Weißwassertal and is composed of several small clusters of individuals, while the Södderich population is more compact and almost square (300m x 300m). At first sight, the higher genetic diversity in the Weißwassertal population could be due to the stretched population shape. The population experiences heterogeneous micro-sites and thus more genetic diversity might be the required to adapt to such sites. This hypothesis is supported by the detection of 5 specific alleles in the Weißwassertal population as compared to the Södderich population (table 6). However, it is not clear, whether microsatellites are eventually relevant for adaptation. This will be discussed in more detail in subsection 4.3.2.

The total average number of alleles per population (82.5) observed in the two populations of *A. pseudoplatanus* in this study is similar to the average value (83.2 ± 63.0) observed in 106 populations of 104 studies in plant species using the microsatellite gene markers as reviewed by NYBOM (2004) (table 15). Observed and expected heterozygosities in this review were 0.58 ± 0.22 and 0.61 ± 0.21, respectively, which are slightly higher than in this review. The number of microsatellite markers used in the present study was slightly lower (6) than the average number of loci (8.4 ± 6.7) used for the 104 studies in other plant species. The number of populations (2) was also lower in this study than the average relevant in the review (4.1 ± 6.1) of NYBOM (2004).

Table 15. Database of the review on life history traits and STMS marker diversity. Number of studies, mean and standard deviation are given for each of five sampling strategy parameters: number of populations, number of plants per population, maximum geographical distance between sampled populations, number of polymorphic loci and number of polymorphic alleles, and for four genetic parameters: population differentiation measured with F_{ST} and R_{ST}, and mean within-population diversity measured as H_e and H_o (NYBOM, 2004).

Parameter	*N*	Mean ± SD
Populations	106	4.1 ± 6.1
Plants	104	51.5 ± 55.4
Distance (km)	37	1103 ± 2694
Loci	105	8.4 ± 6.7
Alleles	90	83.2 ± 63.0
F_{ST}	33	0.26 ± 0.17
R_{ST}	18	0.24 ± 0.21
H_e	104	0.61 ± 0.21
H_o	80	0.58 ± 0.22

A compilation of findings of different studies using microsatellite gene markers is given in table 16 comparing the findings of the present study with the relevant groups of plants, *i.e.* tree species, diploids, polyploids. Since the studies in polyploid tree species are much lower in numbers than those in diploid tree species, other plant groups such as herbs and shrubs are also listed in the table. The average allelic multiplicity (13.80) in this study is relatively higher than the average number of alleles (11.90) of eight polyploid plant species and is almost double in comparison to eight diploid tree species. The average observed heterozygosity in this study is relatively higher than in the eight diploid tree species (H_o= 0.50) and slightly lower than average in eight polyploid plant species (see table 16). On the other hand, the expected heterozygosity is lower than the average values of both diploid (H_e= 0.61) and polyploid (H_e =0.69) groups. The genetic diversity observed in 5 microsatellite loci in *Acer skutchii* was considerably lower (A/L=2; H_e= 0.20) (LARA-GÓMEZ *et al.* submitted) than in *A. pseudoplatanus*, although they used four same microsatellite as in this study. It is obvious that the variability of microsatellite can be reduced when they are applied in other taxa even of the same genus. The genetic diversity

of this *A. pseudoplatanus* is lower at microsatellite gene loci in comparison to its co-growing species, *Fagus sylvatica*, *Quercus robur* and *Q. petraea* (see table 16). Although *A. pseudoplatanus* has a slightly higher average number of alleles per locus as compared to *F. sylvatica*.

For only two out of eight studies on polyploid plant species, observed and expected heterozygosities were calculated (e.g. GONZÁLEZ-PÉREZ *et al.* 2004; TRUONG *et. al* 2005) and for two plant species only a minimum observed heterozygosity was estimated (e.g. BUTELER, 1999). All mentioned authors in table 16 who developed microsatellites for polyploid species reported problems of interpreting polyploid genotypes. There is an urgent need to develop appropriate interpretation tools for polyploids (e.g. ZHANG *et al.* submitted).

4.2.1.2. Genetic variation in the natural regeneration

Genetic variation measured as average number of alleles in the natural regeneration was slightly lower than in the adult trees of the Södderich population. This lower variation could be due to the smaller sample size (N=115) in comparison to adult trees (N=137). At locus MAP-33, out of 20 alleles detected in adult trees, only, 12 were present in the natural regeneration. It has to be added that seven out of eight missing alleles were detected only once in the adult trees. The absence of these rare alleles in the natural regeneration could hardly be either due to selection or due to drift effects.

Five (7.2%) new alleles which were not present in the adult trees were detected in the natural regeneration. This indicates that an influx of external pollen or/and migration of seeds occurred in the population from neighbouring populations of A. pseudoplatanus. Wang (2001) reported that in Fagus sylvatica 0.7 to 1.4 % of external effective pollen was detected in the progeny of individual seed trees on the basis of new alleles at isozyme gene loci. The higher proportion of external alleles detected in the present study could be due to the use of highly variable microsatellite markers.

Table 16. Genetic variation in different plant species using microsatellite markers.

Species	Plant group	No.	No. of Population	Ploidy level	No. of loci	Average numbers of alleles per locus	H_o	H_e	References
Acer skutchii	Tree	80	3	diploid	5	2	-	0.20	LARA-GÓMEZ *et al.* (submitted)
Fagus sylvatica	Tree	99	1	diploid	4	10.80	0.57	0.77	VORNAMM *et al.* (2004)
Pinus sylvastris	Tree	14	1	diploid	7	7.70	-	0.85	SORANZO *et al.* (1998)
Pinus flexuosa	Tree	20	1	diploid	6	8.70	0.50	0.72	MOTTURA *et al* (in press)
Pinus resinosa	Tree	518	17	diploid	5	9.00	0.19	0.50	BOYS *et al*. (2005)
Populus tremuloides	Tree	189	11	diploid	16	8.20	0.40	0.48	COLE (2005)
Quercus robur and Q. petraea	Tree	355	1	diploid	6	21.70	0.81	0.87	STREIFF *et al.* (1998)
Ulmus laevis	Tree	43	5	diploid	6	4.70	0.52	0.52	WHITELEY *et al.* (2003)
Mean		**165**	**5**		**6.87**	**8.63**	**0.50**	**0.61**	
Bencomia exstipulata and *B. caudata*	Shrub	75	2	autotetraploid	5	6.20	0.43	0.64	GONZÁLEZ-PÉREZ *et al.* (2004)
Betula pubescens	Tree	452	3	tetraploid	9	26.30	0.67	0.73	TRUONG *et. al* (2005)
Ipomoea. batatas	Herb	18	1	hexaploid	5	5.60	0.59	-	BUTELER *et al.* (1999)
I. trifidia	Herb	42	1	tetraploid	3	6.70	0.58	-	BUTELER *et al.* (1999)
Limonium narbonense	Grass	30	1	tetraploid	16	9.10			PALOP *et. al* (2000)
Phragmites australis	Grass	654	4	polyploid	10	6	-	-	SALTONSTALL (2003)
Rubus alceifolius	Shrub	63	3	polyploid	8	19	-	-	AMSELLEM *et al*. (2001)
Salix reinii	Tree	18	1	polyploid	7	16.40	-	-	LIAN *et al*. (2001)
Mean		**169**	**2**		**7.90**	**11.90**	**0.57**	**0.69**	
Acer pseudoplatanus	Tree	219	2	autotetraploid	6	13.80	0.55	0.57	Present study

4.2.1.3. Genetic variation between two sub-compartments (2b1 and 2b2) in the Södderich population

Genetic variation in sub-compartment 2b2 was slightly higher than in sub-compartment 2b1 with an average number of alleles 12.5 and 11.5, respectively. The possible explanation of higher genetic variation in sub-compartment 2b2 is eventually the introduction of planting material with new genetic information. The planting stock was presumably of local origin. However, there is no record on this at the forest office.

4.2.2. Genetic differentiation between populations

The average genetic distance between two populations with d_0 = 0.25 was remarkable. Genetic distances among four populations of *Fraxinus excelsior* L. using four microsatellite markers was still higher (average d_0 = 0.50) (HÖLTKEN *et al.* 2003) than the genetic distance estimated in the present study. The authors argued that the different growing conditions could be the reason for this high genetic differentiation among populations.

The genetic distance estimated in this study was much higher than the average genetic distance of *Quercus robur* (0.057), *Q. petraea* (0.056) *and Q. pubescens* (0.069) as reported by FINKELDEY and MÁTYÁS (2003) using isozyme gene markers. However, these authors reported strong differentiation among Quercus populations at maternally inherited cpDNA markers.

Very low genetic differentiation (F_{st} = 0.075) was observed among populations of *Acer skutchii* using four of the microsatellites as in *A. pseudoplatanus* (LARA-GÓMEZ *et al.* submitted).

The average genetic distance between the adult trees and the natural regeneration of the Södderich population was d_0 = 0.17 and between the two sub-compartments of the same population it was d_0 = 0.13. These distances are lower than the distances between the Weißwassertal and the Södderich populations. Private alleles (see table 6 and 9) in adults, processes involved in natural regeneration and minor heterogeneity of sub-compartments eventually contribute to those estimates of genetic differentiation.

In his review NYBOM (2004) reported the mean genetic differentiation from 33 studies (F_{st} = 0.26 ± 0.17) (table 15) using microsatellite markers being almost equal to the average genetic distance (d_0 = 0.25) observed in the present study. However, different differentiation parameters were used.

4.3. Spatial genetic structure

4.3.1. Spatial distribution of alleles

In both populations, some alleles showed random distribution within the populations, while others showed clumping in certain parts of the populations. Most of the frequent alleles were distributed more or less randomly within the populations. The alleles which showed a non-random distribution were mostly rare alleles. These rare alleles may have contributed the significant spatial genetic structure in two populations.

DECARLI (2003) found that within the population of *Fagus sylvatica* some alleles of some of the isozyme gene loci showed clumping. VORNAM *et al.* (2004) also detected some genotypes at a microsatellite locus showing a clustered distribution within the same population. They argued that this clustering could be due to limited gene flow by seed dispersal and preferential mating among possibly related neighbours.

WILLIS and COFFMAN (1975) reported that a spatial distribution pattern in an even-aged group of sugar maple (*Acer saccharum*) was due to the replacement of old trees with new regeneration in a short period of time. This argument is eventually relevant to the Södderich population where a sub-compartment 2b1 (see Figure 6) is relatively young, more or less even-aged, and established by a combination of natural regeneration and planting. The spatial distribution of alleles 152 and 158 of *MAP-12* (see Figure 35) has revealed groups of similar genotypes in this sub-compartment, while the same alleles were either completely absent or found in very low frequency in compartment 2b2, where trees of different ages are found due to its origin by natural regeneration.

4.3.2. Spatial autocorrelation analysis

Both of the populations showed a small-scale spatial genetic clustering. In the Södderich population clumping of individuals with similar genetic information up to 40 m distance was

observed, while in the Weißwassertal it was up to 300 m. The results showed that most genotypes of the adult trees are randomly distributed within the populations of *A. pseudoplatanus*. The distance between tree-clumps in the Weißwassertal is due to the elongated form of this population. The Södderich population possesses higher density and has more or less square form. Because of this structure in the Södderich population, there is a good possibility of unrestricted gene flow among trees which may be the main cause of the weaker spatial genetic structure as compared to the Weißwassertal population. In the Weißwassertal the strongly stretched form and the disjunct distribution of individuals may have resulted in only localized gene flow through both pollen and seed dispersal.

The spatial structure of *A. pseudoplatanus* observed in the Södderich population is slightly more pronounced in comparison to the findings of PERRY and KNOWLES (1991) who have detected a significant genetic structure (20-32 m) in three natural populations of sugar maple (*Acer saccharum*) in north-western Ontario, Canada. Since the two species have more or less similar morphology, pollination biology and seed dispersal mechanisms, the results are comparable. However, these authors used other markers. The spatial genetic clustering up to a distance of 300 m observed in the Weißwassertal population is much wider than the result reported by PERRY and KNOWLES (1991). However, these differences are easily explained by the stretched distribution of the trees along the Weißwassertal.

Factors that may contribute to genetic structure include restricted pollen and seed dispersal and spatially varying selection. The author is not aware of any information concerning seed dispersal in natural populations of *A. pseudoplatanus*. However, due to the wings attached to the fruit the potential dispersal distance of *A. pseudoplatanus* seeds is large. It can fly hundreds of meters in strong winds in open areas. In dense forest stands the samaras can strike other trees and shrubs in the course of flight. Due to this effect, seed dispersal is expected to be much more restricted.

Restricted gene flow due to limited pollen dispersal contributes to the local clumping of individuals with similar genetic information (PERRY and KNOWLES, 1991). This factor is less likely to have an effect in the Södderich population which is relatively small and dense. In contrast, the trees of the Weißwassertal population occur in clumps and pollination may be largely restricted to trees of the same clump.

Selection due to microhabitat heterogeneity has been considered as another factor responsible for spatial genetic structure in plant species by some authors such as HAMRICK and HOLDEN (1979), TURKINGTON and HARPER (1979), LINHART *et al.* (1981) and EPPERSON and ALLARD (1989). Since the Södderich population is only about 3 ha, more or less square and obviously similar site conditions, selection is unlikely to cause the small-scale spatial genetic structure. However, the effect of selection in the Weißwassertal population cannot be ruled out, because the stand is stretched for about 2 km in length. Thus the microhabitats are possibly heterogeneous. Some trees are growing at the slope of the Weißwassertal valley, while others were on flat ground. These factors may have created a microhabitat controlling the levels of availability of sun light, moisture, nutrients, and other factors that are essential for the establishment of natural regeneration and the growth of trees.

Different levels of spatial genetic structure in *Fagus sylvatica* have been observed by different authors. DOUNAVI (2000) found that a significant spatial autocorrelation occurred in beech exists between distances of about 20 to 30 m, while WANG (2001) reported a slightly higher level of significant autocorrelation up to 40 m distance in the same species. Furthermore, VORNAM *et al.* (2004) found a significant autocorrelation up to 30 m. Most of these authors argued that micro-environmental selection and restricted gene flow should be considered for the interpretation of the spatial distribution of genetic information. It is remarkable that the spatial genetic structure observed for the same species using different gene markers showed similar results. VORNAM *et al.* (2004) used microsatellite markers, while DOUNAVI (2000) and WANG (2001) had used allozymes. In addition to this, STREIFF *et al.* (1998) found similar results in *Quercus robur* and *Q. petraea* by using microsatellite and allozyme gene markers. So, the results of the present study using microsatellite as gene markers can be compared to the results of other studies that used different gene markers. It must not be forgotten that microsatellites are not expressed phenotypically and therefore most probably have no relevance for natural selection. A primary spatial structure brought about by restricted gene flow is then hardly modified by natural selection. The authors cited above studied spatial genetic structures during the seventies and eighties of the former century and could eventually not discriminate between the effects of the reproduction system and selection. However, the similarity of the results of WANG (2001) and VORNAM *et al.* (2004) derived from the very same stand of beech by the use of allozymes and microsatellites supports the conclusion that restricted gene flow is

the prime causal factor of spatial genetic structure and selection did not subsequently modify the family structures.

SCHNABEL *et al.* (1991) studied the genetic structure of diploid (*Maclura pomifera*) and autotetraploid (*Gleditsia triacanthos*) co-occurring tree species and found that the spatial genetic structure was slightly lower in the autotetraploid as compared to the diploid species. They argued that the rate of heterozygosity losses in autotetraploid populations due to selfing and mating among relatives is much slower than for the diploids, which would inhibit the development of spatial genetic structure as a result of limited gene flow in polyploids. The argument is relevant to the lower level of significant genetic structure observed in *A. pseudoplatanus,* which is also an autotetraploid tree species. However, the effects of polyploidization may differ depending on the pre-existing systems of incompatibility or self-sterility.

If selection were the mechanism generating within-population spatial genetic structure, then spatial associations may take time to develop and become more obvious only in older cohorts. This hypothesis can be rejected in the Södderich population, since the results of natural regeneration and adult trees of the same population showed a similar level of spatial genetic structure.

Spatial genetic structure of tree species can also be affected by the impact of flowering phenology involved in the mating system (YOUNG and MERRIAM, 1994). Individual trees of *A. pseudoplatanus* are heterodichogamous, being either protogynous or protandrous (DE JONG, 1976). Due to this characteristic there exists a higher probability of individuals with asynchronous flowering within the population. In this case, the spatial distribution of potential mates depends on the temporal distribution of mature male and female flowers which may result in genetic correlation among half-sib progenies at some spatial scale. Consequently, the origin of part of such progeny could lead to a local clustering of individuals bearing similar genetic information. YOUNG *et al.* (1993) and YOUNG and MERRIAM (1994) have considered the above-mentioned mechanisms to be one of the possible causes of the spatial genetic structure observed in *A. saccharum* which is also a heterodichogamous tree species (GABRIEL, 1968). BENDIXEN (2001) reported that *A. campestre,* another member the same genus, is also heterodichogamous.

4.4. Proportion of self-fertilization in A. pseudoplatanus

The amount of self-fertilization is an important component of the mating in a population because of its direct effect on inbreeding and the distribution of genetic diversity within and among populations (CHARLESWORTH and CHARLESWORTH, 1987; HAMRICK and GODT, 1989). The rate of self-fertilization in plants is usually influenced by genetic and environmental factors. Self-incompatibility, dioecious sexual system and postzygotic self-sterility are some of the main factors that determine a low frequency of offspring arising from self-fertilization or exclude self-fertilization completely (FINKELDEY, 2001). The spatial distribution and density of the trees in the population might influence this aspect of the mating pattern of insect-pollinated plant species (BARRET et al. 2004). Small population size acts to reduce the frequency of pollinator visits and thus increases the self-pollination and eventually local inbreeding (ÅGREN, 1996).

The maximum self-fertilization rate observed in individual trees of *A. pseudoplatanus* in this study ranged from 0 to 40 % and the average rate of the two populations was 11 %. Since *A. pseudoplatanus* has been reported to be a self-compatible species, the occurrence of self-fertilization was expected because of the possible overlapping of female and male flowering period of the same individual (DE JONG, 1976). However, out-crossing is eventually promoted by the special structure of flowers (morphologically hermaphroditic but functionally unisexual) (HALL, 1967).

The self-fertilization rate observed in this study is almost double than that in *Echium vulgare* (5.43%) (KORBECKA, 2004) which is also an autotetraploid. SOLTIS and SOLTIS (1990) reported relatively higher rate of selfing (23.60 %) in tetraploid fern *(Polystichum californicum)*. Furthermore, the selfing rate observed in tetraploid *Pteris dispar* was remarkably high with 84 % (MASUYAMA and WATANO, 1990) as compared to the above studies.

There are very few reported estimates of self-fertilization rates in polyploid tree species, largely because the polyploid plants lack the sufficient attention from researchers due to their complex reproduction systems. RIBEIRO and LOVATO (2004) estimated out-crossing rates

of 31 to 84 % in a neo-tropical polyploid tree species, *Senna multijuga.* They indicated a high rate of self-fertilization (16 to 69%) in that species.

Here, it may be relevant to review the self-fertilization rates of some of the co-growing tree species of *A. pseudoplataus.* WANG (2001) reported maximum self-fertilization rates in beech from 5.6 to 24.1% for individual trees and an average 10.66 % for four populations. The average self-fertilization rates observed in beech and in the present study are almost similar. Estimates of self-fertilization rates in two species of oak, *Quercus petraea* and *Q. robur* that are also co-growing tree species of *A. pseudoplatanus* were 0 and 5 %, respectively. BENDIXEN (2001) reported from 0 to 100 % self-fertilization among individual seed trees of diploid *A. campestre* using the method of simple exclusion as was used in the present study. FROMM (2001) reported an average self-fertilization rate 38.70 % in *Tilia cordata* also using the simple exclusion method. He observed quite heterogeneous values (0 to 89 %) of self-fertilization among individual seed trees. The self-fertilization rate within a stand with many trees was considerably lower (25.1 %) than in trees growing in isolation (64.8 %). He argued that the self-fertilization rate was related with the distance of the nearest flowering trees.

Since the spatial distribution of the trees might influence the feeding behaviour of pollinators and, hence, the mating pattern of insect-pollinated plant species (BARRET *et al.* 2004), the proportion of self-fertilization in the Weißwassertal where small clumps of trees are isolated by moderately wide distances with 13 % may have exceeded that of the Södderich population (8 %). LIENGSIRI *et al.* (1998) suggested that the differences in out-crossing rates seen among 11 populations of *Pterocarpus macrocarpus* were attributable to the degree of habitat disturbance and the density and distribution of flowering trees. In the Weißwassertal population there are isolated clumps of only few individuals (see Figure 7). Although habitat disturbance was not observed, the low density of flowering trees could have limited the availability of sufficient pollen from other trees. This argument is supported by the tree number 82 in the Weißwassertal that possessed a considerably higher rate of self-fertilization (40 %) and is in isolation with very few other trees. On the other hand, if we consider the self-fertilization rate of tree 38 (19%) and tree 43 (22%) (see Figure 6) in the Södderich population, the argument of higher self-fertilization due to isolation could not be the only explanation. In order to explain the mating pattern of plants, numerous other factors also need to be considered, *i.e.* the type of pollen vectors, their behavior, weather conditions at the time of flowering, flowering phenology and others. FRANCESCHINELLI and BAWA

(2000) have shown that variation in pollinator behavior, plant density, and number of flowers per plant can alter mating system parameters.
The estimate of the proportion of external effective pollen in the Weißwassertal (13 %) was remarkably higher than in the Södderich population (2 %). In the Weißwassertal, occurrence of effective external pollen in individual trees is very heterogeneous ranging from 0 to 41 %, while in the Södderich population it is almost similar with the values 0 to 5 %. In the Weißwassertal population trees 11 and 82 possess the highest rate of external pollen with 41 % and 20 %, respectively. This showed that pollen is transported over at least moderate distance by insects from nearby populations. FINKELDEY *et al.* (1999) reported 62 % out-crossing rate in an isolated seed tree of *Pterocarpus indicus* and argued that the long-distance movement of pollen can occur in insect-pollinated tree species with unknown pollinators.

The total percentages of seeds that did not meet the minimum criteria of descent to be considered for the estimation of self-fertilization rates were 11 and 14% in the Södderich and the Weißwassertal population, respectively. In most of the cases the inconsistencies were observed at the microsatellite locus *MAP-33*. This could be due to the presence of null alleles at that locus. In fact, inspection of the data showed that the trees producing seeds that did not meet the criteria formulated in section 2.3.3 were homozygous at some gene loci, particularly at *MAP-33*. The existence of a non-detected null-allele present in two-fold or even more so in three-fold is, of course, to be expected to lead to a certain proportion of offspring possessing a different visible band, *i.e*. sharing no expressed allele with the seed parent at the respective gene locus. Unfortunately, appropriate tests of the hypothesis that moderate frequencies of null-alleles are the source of inconsistencies between seed trees and seed would have required much time and had been beyond the scope of the present study.

Self-fertilization is one of the thinkable sources of spatial genetic structure in populations. However, in view of the moderate to low estimated percentages of self-fertilization, the existing spatial autocorrelations indicate that in spite of some external pollen considerable part of the effective pollen is transferred only over somewhat short distances within stands. It must also be considered that the presented estimates refer to maximum proportions, i.e. the true proportions of self-fertilization may be much smaller. Last but not least, nothing is known about the chance of seeds arising from self-fertilization to seedlings or even adult trees. Taking these types of evidence together, short distances of effective pollen transfer involved in cross-fertilization may be inferred.

4.5. Practical application of the results of this study

In order to understand the genetic system of populations, an inventory of their genetic structure is essential. Those genetic structures of populations can serve as an indicator of their adaptation and their adaptative capacity (HATTEMER and ZIEHE, 1997; ZIEHE *et al.* 1999). Allelic diversity and the heterozygosity have often been used to quantify the genetic variation of a population which can serve as basis for the conservation of genetic resources of forest tree species (however see FINKELDEY, 1993). The present study revealed an average number of alleles of 13.30 and 14.20 and an estimated heterozygosity according to ZHANG *et al.* (submitted) of 0.54 to 0.57 at six microsatellite loci. Since the overall genetic diversity in both populations is high and similar, both of the stands could be of value for the conservation of genetic resources. Genetic variation of other *A. pseudoplatanus* populations is unknown. Thus, it is premature to overemphasize the importance of the result for genetic resource conservation. Nevertheless, the detection of private alleles (13 in the Södderich and 18 in the Weißwassertal populations) and the large genetic distance between the populations ($d_0 = 0.25$) indicates considerable differences of the genetic constitution of both populations.

The analysis of spatial genetic structure in the two populations of *A. pseudoplatanus* confirmed that there exists a small-scale family structure (Södderich: 40 m and Weißwassertal: 300 m). The result is relevant for selecting individual trees for the purpose of seed collection in stands of the respective dimensions. In order to avoid the collection of seeds from individuals with similar genetic characteristics, the distance between seed trees should be kept above 40 m in the Södderich and above 300 m in the Weißwassertal population. This will be useful to maintain genetic diversity in seed material and reduce the possibility later inbreeding in plantations. The result is particularly important for the Södderich population, since the stand has been approved for the collection of reproductive material according to EU rules.

The spatial autocorrelation analyses of natural regeneration and adult trees in the Södderich population showed similar spatial genetic structure (see Figure 36 and 39) which provides evidence for restricted gene flow either by limited seed migration or/and limited pollen dispersal (WRIGHT, 1931; BRADWHAW, 1972; TURNER *et al.* 1982). The likelihood of limited seed migration is higher, since the population is mixed with other species in high

density (see Figure 5) that may have acted as a barrier for effective seed dispersal. Thinning of unwanted or less valuable species could improve the conditions for seed dispersal.

In the Weißwassertal population, formation of a family structure was mainly due to the long distance between small clumps of trees. In order to get a random genetic structure in stands of tree species that are conventionally regenerated naturally, the connectivity between clumps should be improved by planting in the gaps. This will eventually also be helpful to reduce the mating of relatives and thus decrease inbreeding depression. For planting, priority should be given to the reproductive material collected from the same population due to its degree of adaptation acquired during evolution on that site.

External effective pollen was detected among the offspring of the Södderich (2 %) and the Weißwassertal (13 %) populations. Furthermore, five external alleles were found in the natural regeneration which were not present in the adult trees of the same population. This indicates that gene transfer between populations of *A. pseudoplatanus* presumably by pollen dispersal does take place. This is important for maintaining high genetic diversity and for reducing the genetic differentiation between populations. However, this result is also a matter of concern for tree breeding of the species, if the populations should be kept in isolation to avoid "contamination" through influx of external pollen (ADAMS and BIRKES, 1989; WHEELER *et al.* 1992). More studies are needed to gain better knowledge regarding this matter. The mechanisms, effectiveness and the maximum distance of seed migration and pollen dispersal in *A. psueudoplatanus* need be studied in more detail.

4.6. Perspectives

Although 30-50% of all angiospermous plant species are polyploid, the development of microsatellite markers for polyploid plant species has lagged behind that of diploid species (ASHLEY *et al.* 2003). One of the problems is due to the poorly understood and potentially complex polysomic inheritance patterns in autotetraploids (LIAN *et al.* 2003). Since *A. pseudoplatanus* is an autotetraploid, the microsatellites developed for the species show a complex inheritance pattern which posed many limitations in estimating genetic variation parameters (e.g. H_o, H_e, F_{ST},) and limited their use for analyses of the reproduction system as, for instance, the estimation of pollen transfer distances. The evaluation of inheritance patterns of the markers in *A. pseudoplatanus* by examining parental and progeny genotypes

from controlled crosses is essential to address these problems (WU *et al.* 2001; ASHLEY *et al.* 2003).

Important genetic traits in plants are quantitative trait loci (QTLs). Identification of individual loci controlling the variation of different economically important quantitative traits, *e.g.* stem growth, form, leaf phenology, wood quality, and disease resistance can serve as basis for modern tree breeding programmes (BRADSHAW and GRATTAPAGLIA, 1994; PLOMION and DUREL, 1996; VERHAGEN *et al.* 1997; VERHAGEN *et al.* 1998). The use of microsatellite markers in QTL mapping is becoming ever more popular because of their high variability. Since *A. pseudoplatanus* is also an economically important tree species, breeding strategies on the basis of molecular markers could become important in future. Although the microsatellite loci developed for this study are too few for efficient QTL mapping, the success of isolating microsatellites in this study for the species could nevertheless serve as a basis for the development of a large number of microsatellite loci (>100) necessary for the purpose of QTL mapping.

The successful amplification of DNA of expected fragment sizes suggests the usefulness of the developed markers also for other *Acer* species.

Microsatellites have been applied in phylogenetics because of their high conservation of flanking regions. There exists evidence that some species have consistently larger microsatellites than others, implying that there is a common factor operating across loci (AMOS and RUBINSZTEIN, 1996; CRAWFORD *et al.* 1998). So far, information on the dynamics of microsatellite evolution has come mainly from studies of one or a few closely related species. A larger perspective on the evolution of microsatellites could be provided by the study of a larger-scale sequence variation in a phylogenetic context (ZHU *et al.* 2000). Some studies have already proved their applicability in this context (MUIR *et al.* 2000; ZHU *et al.* 2000; RALLO *et al.* 2003). The microsatellites developed in this study may also be used for phylogenetic studies within the genus *Acer*.

The use of microsatellite markers in forensic applications was introduced by JEFFREYS *et al.* (1985). A main benefit of microsatellite markers for forensic application is that they require very small amounts of DNA (BALDING, 1999). Since *A. pseudoplatanus* is widely planted along roadsides and in parks, some criminal cases may be solved on the basis of

evidence derived from trees of this species. The microsatellite markers developed in this study can be used to identify the particular tree which might be related to criminal offences.

The microsatellites developed for *A. pseudoplatanus* may also be used to identify clones and to check clonal identity *e.g.* in clonal seed orchards.

Finally, the markers have a potential also for the icharacterization of provenances of *A. pseudoplatanus.* Since the hyper-variability of microsatellites may become a problem for the discrimination of geographical races, the use of nuclear microsatellites in combination with chloroplast markers can provide better understanding of geographic variation of the species (SCALA *et al.* 1999).

5. Summary

Sycamore maple (*Acer pseudoplatanus* L.) is an indigenous hardwood in Germany and in other central-European countries. It belongs to section *Acer* of series *Acer* of genus *Acer*. It forms the tallest trees in the genus *Acer*. Although it is common in mountainous regions, it is also found at low altitudes in northern Germany. The species has been reported to be tetraploid (2n=4x=52) and is considered as predominantly insect-pollinated with some degree of wind-pollination. The species is not only important for garden and landscape management but also for forestry because of its economic value. The wood of the species is of high quality with creamy white colour, has no unpleasant smells and is therefore used for a variety of purposes, *i.e.* furniture, music instruments, floor-parqueting, and for valuable handicrafts.

Despite its economic and ecological importance, there exists very little information about the genetic structure and reproduction system of the species. Until now only limited studies have been carried out on the basis of isozymes. However, highly polymorphic genetic markers are indispensable for the study of genetic structure and system of reproduction (mating system, gene flow). Many studies have already proven that nuclear microsatellite markers are highly suitable for the study of these aspects in many different organisms due to their high variability and co-dominant characteristics.

The aim of this dissertation was first to develop highly polymorphic microsatellite markers and to use them for the study of genetic structure and transfer of genetic information within two natural populations of *A. pseudoplatanus*.

In order to develop microsatellite markers the protocol developed by FISCHER and BACHMANN (1998) was used. One of the main advantages of this protocol is that it is not based on radioactive substances. This protocol is rather based on selective hybridization of biotinylated oligo-probes to the genomic DNA and on capturing the fragments containing microsatellites by magnetic particles. Two trinucleotide ($(AAC)_8$ and $(GAA)_8$) and one dinucleotide ($(CA)_{10}$) artificial oligo-probes were used for hybridization to the genomic DNA of *A. pseudoplatanus*. Double hybridization was performed to increase the efficiency of microsatellite enrichment. The enriched fragments were ligated into the *BssH II,* digested, double-dephosphorylated and the gel-purified pCRscript vector. Thereafter, the fragments were transformed into XL1-Blue *E. coli* bacteria and a total 90 colonies were obtained.

Sequencing of the colonies produced 54 (60%) sequences containing at least one microsatellite more than 5 repeats and at least 10 bp long. As far as the type of microsatellite is concerned, dinucleotide repeats were the most abundant with 87 % as compared to trinucleotide repeat motifs with 13 %. Out of 54 sequences that contained microsatellite repeat motifs, 24 (44 %) sequences were suitable for the primer design. Sixteen out of 24 primer pairs (67 %) produced the expected fragment size. Finally, eight primers produced polymorphic and well-scorable bands. The final efficiency from the enriched colony to getting the polymorphic primers was 9 %. At first these eight primers were characterized in 96 adult trees of the Södderich population near Göttingen, Germany. All eight primers show moderate to high levels of polymorphisms producing 3 to 16 alleles. The validity of variants as alleles of a single gene locus was proved by sequencing several fragments for each primer pairs. As expected, the numbers of different alleles varied from one to four per individual since the *A. pseudoplatanus* is an autotetraploid species. The complex inheritance pattern posed limitations in estimating genetic variation parameters (***e.g.*** H_o, H_e, F_{ST}) and their use in an analysis of the reproduction system, i.e. gene flow and mating system.

These eight primers were tested in 21 species of the genus *Acer* in order to know their transferability to other species of the same genus. The amplification of fragments with expected size was obtained in 92% of all cases. This indicates their applicability for the study of genetic variation and reproduction systems of those species.

The developed microsatellite markers were applied to study the genetic variation, genetic structure and aspects of pollen transfer in two natural populations of *A. pseudoplatanus*, ***i.e.,*** Södderich and Weißwassertal. The populations are part of the Reinhausen forest district and situated near Göttingen, Germany. The DNA extracted from buds of 137 trees in the Södderich population and 82 adult trees in the Weißwassertal were used to genotype at six microsatellite loci, *i.e., MAP-2, MAP-9, MAP-12, MAP-33, MAP-40, and MAP-46*. In addition to that, leaves of 115 saplings in the natural regeneration were sampled in the Södderich population to investigate their genetic variation and genetic structure and to compare them with the results of the adult trees. They were genotyped at the above-mentioned six microsatellite loci. Furthermore, a comparison of genetic variation between two sub-compartments (2b1 and 2b2) was performed. The ground locations of all sampled trees and the natural regeneration were mapped.

A total of 80 alleles (average number of alleles per locus = 13.30) in the Södderich population and 85 alleles (average number of alleles per locus = 14.20) in the Weißwassertal were encountered at the six microsatellite loci. The average observed and expected heterozygosities (H_o and H_e) were 0.54 and 0.57 in the Södderich population and 0.55 and 0.57 in the Weißwassertal, respectively. The results indicated similar genetic variation in both of the populations. In spite of their similar genetic variation, the genetic distance between the population was considerable (d_0 = 0.25). The presence of private alleles in both populations and unbalanced frequency distributions of the alleles in both populations are mainly responsible for this pronounced genetic differentiation of the populations.

Genetic multiplicity in the natural regeneration of the Södderich population with a total number of 69 detected alleles (average per locus =11.50) was slightly lower than that in the adult trees with 80 and 13.30, respectively. Five external alleles were found in the natural regeneration that were not present in the adult trees, which is the evidence of the influx of external effective pollen from neighbouring populations of *A. pseudoplatanus*, possibly also of seed migration. Genetic multiplicity estimated by the average number of alleles in two sub-compartments (2b1 and 2b2) of the Södderich population showed that 2b2 (average number of alleles = 12.50) possessed more genetic variation than 2b1 (average number of alleles = 11.50), although 2b1 comprised more trees.

Spatial genetic structure in the two populations of *A. pseudoplatanus* was studied by mapping the spatial distribution of alleles in the population and analysed by estimating spatial autocorrelations. Significant spatial genetic clustering up to 40 m distance was observed in the Södderich population and up to 300 m in the Weißwassertal. The occurrence of the moderate levels of spatial genetic structures could be due to restricted gene flow within the dense population embedded in a mixed forest at the Södderich and long distances between clumps of trees in the Weißwassertal population. Although the most frequent alleles were distributed more or less randomly over the populations, some of the less frequent alleles were clustered in certain parts of the populations. Similar results of spatial genetic structure were observed in the natural regeneration of the Södderich population, which also indicated that the gene flow within the population was not completely random.

In order to estimate the self-fertilization rate of *A. pseudoplatanus*, 13 seed-trees in the Södderich population and 8 in the Weißwassertal were selected and mostly 32 offspring per

seed tree were genotyped at five microsatellite loci, *i.e., MAP-2, MAP-9, MAP-33, MAP-40,* and *MAP-46*. The overall maximum self-fertilization rate for *A. pseudoplatanus* was estimated to be 11 % by the exclusion principle. The Weißwassertal (13 %) showed little more self-fertilization than the Södderich population (8 %). The proportions of self-fertilization were more heterogeneous among individual seed trees in the Weißwassertal (3 to 40 %) than in the Södderich (0 to 22 %) population. Influx of external effective pollen was detected in both of the populations. The percentage of external effective pollen in seeds produced by the Weißwassertal population (13 %) exceeded that in the Södderich population (2 %). The moderate long-distance transport of effective pollen and the low proportion of self-fertilization find their joint expression in moderate spatial genetic structures and in remarkable genetic differentiation of the two populations.

Practical applications of the above-mentioned results were discussed. Due to the genetic distance between the two populations and the occurrence of several private alleles, populations under the given conditions could become eligible for *in-situ* conservation of genetic resources of *A. pseudoplatanus* if other criteria were fulfillied. The influx of external pollen into the populations indicated that especial attention should be given to sufficient isolation of seed orchards from pollen contamination. The results of spatial genetic structures are relevant for locating the trees for seed collection. The results showed that minimum distances among seed trees should be 40 m in the Södderich population and 300 m in the Weißwassertal.

The microsatellite markers developed in the present study are the first not only in *A. pseudoplatanus* but also for the genus *Acer*. Owing to their variability, they may be used for various purposes, *i.e.* to study genetic variation and aspects of the reproduction system, the identification of orchard clones, forensic application, phylogenetic studies, mapping quantitative trait loci (QTLs), and provenance charecterization. However, the existence of null-alleles has to be clarified before large-scale application.

Die Entwicklung von Mikrosatelliten bei Bergahorn (*Acer pseudoplatanus* L.) und deren Anwendung in der Populationsgenetik

6. Zusammenfassung

Der Bergahorn (*Acer pseudoplatanus* L.) ist eine in Deutschland und anderen mitteleuropäischen Ländern heimische Edellaubbaumart. Er gehört zur Sektion *Acer* der Gattung *Acer* und weist unter allen Arten dieser Gattung das größte Höhenwachstum auf. Obwohl der Bergahorn in allen Bergregionen verbreitet ist, tritt er auch in tieferen Lagen Norddeutschlands auf. Die Art ist autotetraploid und gilt als vorwiegend durch Insekten bestäubt, wenn auch mit einem geringen Anteil von Windbestäubung. Der Bergahorn hat Bedeutung nicht nur für den Garten- und Landschaftsbau, sondern wegen seines wirtschaftlichen Werts auch für die Forstwirtschaft. Das qualitativ hochwertige Holz ist von cremeweißer Farbe; es wird zu vielen verschiedenen Zwecken benutzt, darunter für die Möbelherstellung, für den Bau von Musikinstrumenten, für Fußböden und im Kunstgewerbe.

Trotz der wirtschaftlichen und ökologischen Bedeutung des Bergahorns herrscht über seine genetische Strukturen und sein Reproduktionssystem weitgehend Unkenntnis. Begrenzte Untersuchungen wurden bisher mittels Enzymgenloci angestellt. Indessen sind für die Analyse genetischer Strukturen und des Reproduktionssystems (Paarungs- und Genflusssystem) hochvariable genetische Marker unentbehrlich. In zahlreichen Studien hat sich gezeigt, dass Kernmikrosatelliten wegen ihrer hohen Variabilität und ihrer kodominanten Ausprägung für die Untersuchung dieser Aspekte bei vielen Organismen geeignet sind.

Ziel der vorliegenden Dissertation war es, hochgradig polymorphe Mikrosatellitenmarker zur entwickeln und bei der Untersuchung genetischer Strukturen und des Transports genetischer Information in zwei Populationen des Bergahorns einzusetzen.

Die Mikrosatellitenmarker wurden mit dem Verfahren von FISCHER und BACHMANN (1998) entwickelt. Einer der Hauptvorteile dieses Verfahrens ist der Verzicht auf die Verwendung radioaktiver Substanzen. Das Verfahren beruht vielmehr auf der selektiven Hybridisierung biotinylierter Oligo-Proben mit Kern-DNA und der Anreicherung der Mikrosatelliten mittels

magnetischer Partikel. Für die Hybridisierung wurden trinukleotide ($(AAC)_8$ und $(GAA)_8$) sowie eine dinukleotide ($(CA)_{10}$) Oligo-Proben verwendet. Eine Wiederholung des Anreicherungsschrittes (doppelte Anreicherung) sollte die Effizienz der Mikrosatellitenisolierung erhöhen. Die angereicherten Fragmente wurden in den mit *BssH II* geschnittenen, doppelt dephosphorilierten pCRscript-Vektor eingebaut und in XL1-Bluebakterien ("superkompetente" Zellen, *E. coli*) transformiert. Es wurden 90 Kolonien erhalten.

Die Sequenzierung dieser Kolonien ergab 54 (oder 60%) Sequenzen, welche mindestens einen Mikrosatelliten mit mehr als fünf Wiederholungsmotiven (Repeats) von wenigstens 10bp Länge enthielten. Dinukleotide Repeats waren mit 87% wesentlich häufiger vertreten als trinukleotide. Unter den 54 Sequenzen mit Repeat-Motiven waren 24 (44%) für die Konstruktion von Primern brauchbar. 16 der 24 Primer-Paare (d.i. 67%) lieferten Fragmente der erwarteten Größe. Schließlich ergaben acht Primer polymorphe und gut anzusprechende Banden. Die endgültige Effizienz von der angereicherten Kolonie bis zum Erhalt der polymorphen Primer betrug 9%. Zunächst wurden 96 Bäume der Population Södderich bei Göttingen mithilfe von 8 Mikrosatelliten charakterisiert. Alle acht Primer wiesen mit 3 bis 16 Allelen einen mittleren bis hohen Grad von Polymorphismus auf. Die Interpretation der jeweiligen Varianten als Produkte *eines* Genlocus wurde durch Sequenzierung verschiedener Fragmente für jedes Primerpaar nachgewiesen. Wie nach der Autotetraploidie des Bergahorns zu erwarten war, variierte die Anzahl verschiedener Allele eines Individuums zwischen 1 und 4. Der komplexe Vererbungsmodus warf Probleme bei der Schätzung von Variationsparametern (z.B. H_o, H_e und F_{ST}) und der Analyse des Reproduktionssystems (Genfluss- sowie Paarungssystem) auf.

Diese acht Primer wurden in 21 *Acer*-Arten getestet, um ihre Übertragbarkeit auf diese anderen Arten in Erfahrung zu bringen. In 92% aller Fälle gelang die Amplifizierung von Fragmenten der erwarteten Länge. Daraus folgt grund-sätzlich die Verwendbarkeit der Mikrosatelliten zur Analyse von genetischer Variation und Reproduktionssystem auch dieser Arten.

Die entwickelten Mikrosatelliten wurden zur Untersuchung genetischer Variation, genetischer Strukturen und gewisser Aspekte des Transports effektiven Pollens in zwei Populationen des Bergahorns, Södderich und Weißwassertal, eingesetzt. Die beiden betreffenden Bestände liegen im Bereich des Forstamtes Reinhausen bei Göttingen. Die aus

den Knospen von 137 kartierten Bäumen der Population Södderich und 82 aus der Population Weißwassertal extrahierte DNA wurde zur Zuordnung von Genotypen an sechs Mikrosatelliten-Genloci, d.i. *MAP-2, MAP-9, MAP-12, MAP-33, MAP-40* und *MAP-46*, benutzt. Zusätzlich wurden von 115 jeweils einem Altbaum am nächsten stehenden Jungwüchsen aus der Naturverjüngung am Södderich zum Zwecke des Vergleichs mit dem Altbestand Knospen geworben und an den sechs Genloci charakterisiert. Ferner wurden zwei etwas unterschiedlich entstandene Unterabteilungen des Bestandes am Södderich verglichen.

Insgesamt traten an den sechs Mikrosatelliten-Genorten in der Population Södderich 80 (durchschnittlich 13,3 je Genort) und in der Population Weißwassertal 85 (durchschnittlich 14,2) Allele auf. Die Maße der beobachteten und erwarteten Heterozygotie (H_o bzw. H_e) betrugen am Södderich 0,54 bzw. 0,57 und im Weißwassertal 0,55 bzw. 0,57. Die beiden Populationen wiesen also ähnliche genetische Variation auf, unterschieden sich aber dennoch sehr deutlich voneinander (d_0=0,25). Für diesen Grad der Differenzierung waren die allgemein etwas unausgewogenen (näherungsweisen) Häufigkeitsverteilungen der Allele und die Anwesenheit privater Allele in den beiden Populationen maßgeblich.

Die genetische Vielfalt in der Naturverjüngung der Population Södderich reichte mit 69 (durchschnittlich 11,5) gefundenen Allelen nicht ganz an die der Altbäume heran. Trotzdem kamen fünf Allele unter den Altbäumen nicht vor, waren also aus anderen Populationen des Bergahorns durch Externpollen, möglicherweise auch durch Samenmigration, eingetragen. Die genetische Vielfalt war in der aus Naturverjüngung hervorgegangenen Unterabteilung 2b1 mit durchschnittlich 11,5 Allelen je Genort etwas geringer als in der teilweise gepflanzten 2b2 mit 12,5 Allelen, obwohl 2b1 etwas mehr Bäume umfasste.

Räumliche genetische Strukturen in den beiden Populationen wurden durch die Verteilung allelischer Varianten graphisch dargestellt und durch die Schätzung von Autokorrelationen analysiert. Signifikante genetische Strukturierung wurde am Södderich bis zu einem Abstand von 40m und im Weißwassertal bis zu einem Abstand von 300m festgestellt. Die mäßig intensiv ausgeprägten Strukturen dürften am Södderich auf beschränkten Genfluß in der in einen Mischbestand eingebetteten Population, im Weißwassertal zusätzlich auf die etwas größeren Abstände zwischen einzelnen Baumgruppen zurückzuführen sein. Während die häufigsten Allele mehr oder weniger zufallsmäßig über die Populationen verteilt waren,

traten die selteneren geklumpt auf. Ähnliche Verhältnisse wurden in der Naturverjüngung am Södderich angetroffen – ein Hinweis auf den nicht zufallsmäßigen Genfluß in dieser Population.

Zur Schätzung des Selbstbefruchtungsanteils des Bergahorns wurden am Södderich 13, im Weißwassertal zusätzlich 8 Bäume beerntet und je bis 32 ihrer Samen an fünf Mikrosatelliten-Genorten (wegen aufgetretener Unsicherheiten ohne *MAP-12*) charakterisiert. Der nach dem Ausschlußprinzip geschätzte maximale Anteil der Selbstbefruchtung war im Weißwassertal mit 13 % etwas größer als am Södderich (8%) und betrug im Durchschnitt 11 %. Im Weißwassertal waren die für einzelne Samenbäume geschätzen Anteile mit Werten zwischen 3 und 40 % etwas heterogener als am Södderich, wo sie zwischen 0 und 22% schwankten. Unter den im Weißwasertal erzeugten Samen betrug der Anteil externer effektiver Pollen mit 13 % mehr als am Södderich mit nur 2 %. Der moderate Ferntransport effektiven Pollens und der geringe Anteil der Selbstbefruchtung fanden ihren gemeinsamen Ausdruck in mäßig stark ausgeprägten räumlichen genetischen Strukturen und in einer bemerkenswerten Differenzierung der beiden Populationen.

Praktische Anwendungen der erzielten Ergebnisse werden diskutiert. Der genetische Abstand zwischen den beiden Populationen und das Auftreten privater Allele läßt in derartigen Populationen unter den gegebenen Umständen die Erhaltung genetischer Ressourcen des Bergahorns *in situ* als denkbar erscheinen. Angesichts des Eintrags von Externpollen ist auf die ausreichende Isolation von Samenplantagen vor Pollenkontamination zu achten. Die Befunde zu räumlichen genetischen Strukturen sind von Bedeutung für die Plazierung von Erntebäumen bei der Saatgutgewinnung: In einem Bestand wie dem am Södderich sollten Abstände von 40m, in einem langestreckten Bestand wie dem im Weißwassertal gar von 300m eingehalten werden.

Die in der vorliegenden Arbeit entwickelten Mikrosateliten sind die ersten für die Gattung *Acer*. Dank ihrer hohen Variation sind sie für verschiedene Zwecke verwendbar, etwa für die Einschätzung genetischer Variation und die Untersuchung gewisser Aspekte des Reproduktionssystems, die Identifikation von Samenplantagenklonen, forensische Untersuchungen, phylogenetische Untersuchungen, die Kartierung der an der Kontrolle quantitativer Merkmale beteiligten Genorte und die charakterisierung von Provenienzen. Vor großmaßstablicher Anwendung gilt es allerdings die Anwesenheit von Nullallelen zu klären.

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8. Appendices

Appendix 1. Digestion of genomic DNA with Rsa I

Reaction mix:

12 µl total genomic (~ 6 mg) DNA
2.5 µl 10x restriction buffer
3.0 µl *Rsa I* (10 U /µl)
7.5 µl distilled water (H_2O)
25.0 µl total volume

- After preparation the reaction mixture was incubated at 37°C for 1.5 hours.

Appendix 2. ligation of the adaptors

- Prepare a mixture of adapter 1(100pmol/µl) and adapter 2 (100pmol/µl)
- Add to the digested DNA:

  2.3 µl T-4 Ligase (4U/µl) (Stratagene)
  3.5 µl ATP (10 mM)
  5.1 µl Adapter-mix (10 pm/µl)

- Incubate the reaction at 37° C for 2 hours.
- Then incubate at 95° C for 10 minutes to inactivate enzymes.
- Then leave the reaction briefly at room temperature.
- Clean the reaction with GeneClean™ II "*glassmilk*" (Silicate DNA purification, Fa. Bio101) or using any other purification kit. The detailed procedure of cleaning is given in the manufacturer's protocol.
- To test the success of restriction-ligation, perform PCR with elution achieved after purification.

PCR mix:

9.7 µl H_2O

1.5 µl PCR Buffer (10x)

1.0 µl $MgCl_2$

0.7 µl dNTPs (10 mM)

2.0 µl 21-mer Primer (10 pm/µl)

0.1 µl Taq DNA Polymerase (5 U/µl)

1.0 µl DNA (purified after restriction-ligation)

15.0 µl final volume

PCR Programme:

94° C 5 mins

94° C 30 sec.

56° C 30 sec. } 30 cycles

72° C 1 min.

72° C 7 mins.

8°C forever

- Test the PCR product in 1 % agarose gel. If the expected band is present, then start with next step.

Appendix 3. Hybridization of biotinylated microsatellite oligonucleotides to genomic DNA fragments

Preparation of oligonucleotide pool:

Oligonucleotides	Melting Temp. °C	Annealing Temp. °C	Mean Annealing Temp. °C
$(CA)_{10}$	80.4	75.4	75.0
$(GAA)_8$	78.0	73.0	75.0
$(AAC)_8$	82.2	77.0	75.0

- Mix 1.0 µl of 10 µM solutions from each biotinylated microsatellite oligonucleotide.
- Add 6.5 µl H_2O to the above mixture, so the final volume is 9.5 µl.

Hybridization mixture:

26.0 µl H_2O

19.5 µl 20 x SSC buffer

10.0 µl DNA (DNA fragments with adapters)

9.5 µl oligonucleotide pool

65.0 µl final volume

Preparation of streptavidin coated Dynabeads (10mg/ml):

- Suspend the beads by shaking gently.
- Take 30 µl of suspended Dynabeads and pipette into a tube.
- Wash the beads twice with 1x TE buffer.
- After each washing capture the beads by putting the tube in the MPC (Magnetic Particle Collecting Unit), wait 2-3 minutes until the beads are concentrated, remove the supernatant buffer carefully with a pipette while the tube remains in the MPC.
- Wash the beads twice with 1 ml. 6x SSC buffer in the same way as in the previous step.
- Collect the beads in the tube.

Appendix 4. Indirect capture hybridization

- Incubate the hybridization mix (65.0 µl) at 98° C for 5 minutes and allow slow cooling (0.1° C/sec.) to T_A (75° C) in the thermocycler and incubate for 20 minutes.
- Quickly pipet the hybridization mix (65.0µl) to 6x SSC pre-washed dynabeads (35.0 µl), now the total volume is 100 µl.
- Re-suspend the dynabeads and allow 20 minutes moderately agitated incubation.
- Wash twice with 1ml 2x SSC + 0.1% SDS (5 minutes each) at room temperature and apply moderate agitation. After each washing collect the dynabeads placing the tube in the MPC, wait 2-3 minutes and remove the supernatant with a pipet.
- Wash twice in 1x SSC at room temperature (5 minutes each), continue moderate agitation and collect the dynabeads as in the previous step.
- Wash with 1x SSC buffer at T_A (75°C) once for 2 minutes and once for 5 minutes. Collect the dynabeads as in the previous step and remove the washing buffer immediately, but do not leave open to air-dry.

Appendix 5. Elution

- Add following solutions to the beads:
  -20 µl 0.1 M NaOH + 0.1 M NaCl and mix well (prepare the mix with 17.5 µl H_2O + 2 µl 1 N NaOH+ 0.5µl 4M NaCl).
  -10 µl 2 M HCl (8 µl H_2O + 2 µl 1N HCl).
  -2.2 µl 0.1 M Tris-HCl pH 7.5.
- Mix and centrifuge at 13000 rpm for 5 minutes.
- Take off 2.2 µl elute.
- Concentrate the DNA and remove salt and low molecular weight components by passage through a Microcon spin filter (Microcon YM-100, color code "Blue", Membrane NMWL 100,000, Cat N°-42412).
- Dilute the sample (2.3 µl elute) to 500 µl with H_2O in the filter unit and place the filter in the tube.
- Centrifuge at 13000 rpm for about 7 minutes until about 10 µl remains in the filter.
- Invert the filter to a new tube and spin briefly at 3700 rpm to collect the sample, which yield about 10 µl enriched DNA sample.

Appendix 6. PCR Amplification

- The receipt for the PCR of enriched DNA samples with 21-mer primer is as follows:

PCR mixture:	**PCR programme :**		
30.5 µl H_20	94° C	5 min.	
7.0 µl 10x PCR buffer	94° C	30 sec.	
1.0 µl dNTPs (10mM)	54° C	30 sec.	X 30 cycles
10.0 µl primer (21-mer, 10pm/µl)	72° C	1 min.	
0.50 µl Taq DNA polymerase (5 U/µl)	72° C	7 min.	
1.0 µl enriched DNA	8° C	forever	
50 µl final volume			

Appendix 7. Restriction of enriched DNA and vector

Elution restriction mix:

7.0 µl enriched DNA

4.0 µl *Mlu I* (1u /µl)

2.0 µl 10x buffer

7.0 µl H_2O

20.0 µl final volume (incubate reaction at 37°C for 2 hours)

- After 2 hours spin the reaction briefly and incubate at 98°C for 10 minutes to neutralize the enzyme.
- Then put the tube on ice.
- Purify the DNA with GeneClean™ II "*glassmilk*" (Silicate DNA purification, Fa. Bio101) and elute with 10.0 µl distilled water.

Vector restriction mix:

10.0 µl Vector (pCR-Script Amp SK (+) (Stratagene))

1.0 µl *BssH II* (10 U /µl)

2.0 µl 10x Buffer

7.0 µl H_2O

20.0 µl final volume (Incubate reaction at 50°C for 2 hours)

SAP restriction after vector restriction:

2.0 µl *Bssh II* restricted and purified vector (pCR-Script Amp SK (+) (Stratagene))

4.0 µl SAP (shrimp alkaline phosphotase, USB)

2.0 µl Buffer

2.0 µl H_2O

10.0 µl final volume (Incubate reaction at 37°C for 2 hours)

Appendix 8. Cloning to vector

Mixture for cloning:

Ingredients	**Reaction I**	**Reaction II**	**Control**
H2O	2.4	2.0	2.8
Vector (100ng/µl)	1.0	1.0	1.0
Insert (DNA) (20ng/µl)	0.4	0.8	0.0
1 x KGB Buffer (Mc CLELLAND *et al.*1988)	6.0	6.0	6.0
T4-Ligase, diluted (0.1u/µl)	1.0	1.0	1.0
ATP 10 mM	1.2	1.2	1.2
Final volume	**12 µl**	**12 µl**	**12 µl**

- Incubate these reactions at 37° C for 2 hours.
- Centrifuge briefly after one hour of incubation to concentrate the volume at the bottom of the tube.

Appendix 9. Bacterial transformation

Take the competent cells out from -80°C and put in ice for about 30 minutes before transformation.

- Prepare the following mixture:
  2.0 µl cloning reaction
  50 µl competent Bacteria cell
  52 µl final volume
- Mix the gently and incubate on ice for 20 minutes.
- After incubating on ice for 20 minutes, incubate at 42 °C for 50 seconds.
- Incubate again on ice for 2-3 minutes.
- Then add 600 µl SOC medium to the reaction and incubate at 37°C for 45 minutes.
- Pipette 100 µl in one plate and 50 µl in another plate.
- Add some medium (SOC) to cover the bottom of plate.

- Allow to dry the medium on the plate for 15 minutes at room temperature.
- Incubate the plates at 37°C for about 12 hours.

Appendix 10. Colony PCR

PCR reaction mix:

11.9 µl H_20

3.0 µl 10x PCR buffer

2.6 µl dNTPs (10 mM)

6.0 µl Vec. F primer (10 pm/µl)

6.0 µl Vec. R primer (10 pm/µl)

0.075 µl Taq DNA polymerase (1 U /µl)

pick the cells with 200 µl pipeting tip and dip the tip in to the reaction

≈ **30 µl** final volume

Thermocycler profile:

95° C	3 min.	
95° C	30 sec.	
45° C	30 sec.	X 35 cycles
72° C	1.5 min	
72° C	5 min.	
8° C	forever	

Appendix 11. Sequencing

Sequence reaction mix (10 µl)

1.0 µl Ready Reaction Premix (includes: dNTPs, ddNTPs terminators, DNA polymerase) (Applied Biosystems)

1.5 µl BigDye Sequencing Buffer v 3.1(5X)

10-100 ng Template DNA

4.0 µl HPLC-H_2O

Thermocycler profile (25 cycles):

96° C 10 sec.

45-50 ° C 10 sec.

60 ° C 4 min.

After performing the sequence reaction, the product was purified by the ethanol precipitation.

- The following solutions were added in 10 µl PCR product:

  40.0 µl distilled H2O

  5.0 µl 3M NaAc (pH 6.0)

  150. 0 µl 100% ethanol
- After mixing the solutions the mixture was vertexed for few seconds. Then it was centrifuged for 25 minutes at 13,000 rpm.
- After centrifugation the solution was carefully discarded and the tube was incubated at 50°C for 15 minutes to dry the pellet.
- Then 250 µl 70% ethanol was carefully (not to disturb the pellet) added to the tube and centrifuged for 10 minutes at 13,000 rpm.
- Then the solution was discarded carefully and the pellet was dried at 50°C for 55 minutes.
- To elute the DNA 15 µl HPLC-H_2O was added to the pellet and intensive (ca. 5 minutes) vertexed. After vortex the tube with the elution was incubated for 5 minutes at room temperature.

14 µl of eluted DNA was used for capillary electrophoresis. This was carried out using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems / HITACHI)

Curriculum Vitae

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