# Genetic processes in Scots pine (*Pinus sylvestris* L.) in the Chernobyl exclusion zone

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*To my family*

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# 1. General introduction

Environmental changes require fast adaptive responses by plant populations to ensure survival and reproduction. Plants with a short life span are able to evolve rapidly in response to environmental stress if strong selection acts over several generations. However, long-lived woody plants that dominate in most terrestrial ecosystems have long generation cycles. This makes woody plants less prone to selective changes of genetic structures. Under stress conditions trees have to involve mechanisms of fast adaptive responses such as phenotypic plasticity (Schlichting and Smith 2002), epigenetic effects or genome methylation (Kalisz and Kramer 2008). The development of germ cells from somatic cells in plants assures that all genome changes or somatic mutations may be inherited and transferred to the next generation (Ledig 1986).

Ionizing radiation is well known as a strong stress factor which causes organism's reaction even at low levels of irradiation (Real et al. 2004). A wide range of intragenic and intergenic mutative changes is induced by the influence of radiation (Evans and DeMarini 1999). The types of mutation events caused by radiation are the same for all living organisms and vary from simple base substitutions to single and double-strand breaks of DNA (Grosovsky et al. 1988). Significant changes in the structure or the function of the genome due to natural mutation processes are rare, but a severe damage factor such as high radiation may strongly affect the genome organization. An increase in mutation rates is only a part of the complex reaction of an organism to the exposure to ionizing radiation. Under the pressure of radiation exposure, selection processes should play an important role to ensure surviving and successful reproduction of pre-adapted organisms.

The response mechanisms of plants to radiation exposure are similar to those caused by other stress factors (Holst and Nagel 1997). Reactions to sudden stress exposure are uniform for different kinds of stress due to the same pathways involved in stress response. Thus, the investigation of radiation effects on plant populations may also help to better understand adaptation to less severe environmental changes and their impact on selection.

## 1.1. The Chernobyl accident and its influence of radiation exposure on biota

The accident on 26 April 1986 at the 4th unit of the Chernobyl Nuclear Power Plant (ChNPP) resulted in the largest accidental release of radioactive materials reported. Nearly 200 radioactive isotopes in different phases and chemical forms were released to the

atmosphere and subsequently contaminated the environment through radioactive fallouts. The level of background gamma-irradiation in many parts of the contaminated zone was measured as 1-20 mR/h. The accident contaminated over 145,000 $km^2$ of the territory of the Ukraine, the Republic of Belarus and the Russian Federation with the density of radioactive pollution exceeding 37 $kBq/m^2$ ($10^3$ Ci/ $km^2$). The radioactive cloud following the explosion at the ChNPP also affected Sweden, Norway, Poland, the United Kingdom, Austria, Germany, Finland and Switzerland (National Report of Ukraine 2006). An area of more than 200 000 $km^2$ in Europe was contaminated with radiocaesium (Environmental consequences of the Chernobyl accident 2006).

Most organisms on the territory of the 30-km ChNPP exclusion zone were exposed to high doses of acute radiation during the first days after the accident and continued to be exposed to chronic radiation to the present days. Both acute radiation effects (death by radiation of plants and animals, loss of reproduction, etc.) and long term effects (change of biodiversity, cytogenetic anomalies, etc.) have been observed in the affected areas. The doses of radiation received by biota and their effects varied widely depending on the sensitivity of exposed species to the influence of radiation – from exposures that are lethal at least for the most radiosensitive species to exposures similar to background levels of natural radioactivity (National Report of Ukraine 2006). Radiation induced loss of different plants and animal species. In areas with high surface contamination radiosensitive species were lost, such as pine and soil invertebrates, and only highly radioresistant species survived, including lichen and certain species of moss. This resulted in the change of biota composition due to disturbance of the trophic links, initiation of succession process and formation of new trophic chains, which significantly changed the biocenosis structure (Environmental consequences of the Chernobyl accident 2006). Generally, seed plants can be pointed out as the most affected group of organisms due to the influence of radiation while they are unable to leave contaminated areas. But even though radiation obviously strongly affects their existence, plants have continued growing even in the most radioactively contaminated areas. The surviving vegetation in the Chernobyl zone has attracted attention to the question how organisms adapt to ionizing radiation and which mechanisms of response are involved in the process of adaptation.

### 1.2. Radiation effects on plants in the Chernobyl exclusion zone

Investigation of radiation effects on plants has a very long history and much of the information about the effects on humans was obtained from studies on plants. The broad range of radiosensitivity within the plant kingdom makes it possible to observe all known effects of radiation exposure investigating different plant species. Effects of radiation on plants frequently depend on molecular and cellular characteristics which determine reactions to radiation stress. Such characteristics as DNA content, amount of heterochromatin, number, size and structure of chromosomes, number of nuclei, ploidy, type of reproduction, duration of mitosis and meiosis, length of dormant period and the stage of the life cycle are responsible for the sensitivity or the tolerance to radiation (Sparrow and Woodwell 1962).

Concurrency of the Chernobyl accident with the period of accelerated growth and reproduction in plants resulted in much stronger damaging effects of radiation that affected also the next generations. Due to the high dose of radiation short term sterility and reduction in productivity were observed for some species. Plants growing in fields demonstrated growth and developmental problems. Spot necroses on leaves, withered tips of leaves and inhibition of photosynthesis, transpiration and metabolite synthesis were detected, as well as an increased incidence of chromosome aberrations in meristem cells (Shevchenko et al. 1996). For pines growing around the nuclear station high mortality rate, reproduction anomalies, change in stand viability and different radiomorphoses depending on the absorbed dose were observed (Tikhomirov and Shcheglov 1994; Arkhipov et al. 1994). Cytogenetic analysis of cells from the root meristem of *Secale cereale* and *Triticum aestivum* revealed a high frequency of chromosome aberrations (Geraskin et al. 2003); the same results were described for *Pinus sylvestris* (Kal'chenko and Fedotov 2001). An increased frequency of embryonic lethal mutants was observed for several generations of *Arabidopsis thaliana Heynh.* (L.) (Abramov 1992). DNA damage with radiation resulted in increased incidences of single-stand breaks (Syomov et al. 1992). For some plants a higher frequency of homologous recombinations was observed (Kovalchuk et al. 1998, 2000). Increase in mutation rates was demonstrated for *Triticum sativum* with microsatellite markers (Kovalchuk et al. 2000) and for *Pinus sylvestris* in isozyme analysis (Kal'chenko et al. 1993).

The years following the accident, did not only reveal damaging effects of radiation, but also clear evidence of an on-going adaptation process in the Chernobyl zone.

Hypermethylation of genomic DNA that was detected in *Pinus sylvestris* (Kovalchuk et al. 2003) and *Arabidopsis thaliana* (Kovalchuk et al. 2004) after exposure to radiation is also regarded as one of the protective mechanisms of the genome. Moreover, changes in the expression of radical scavenging and DNA-repair genes were observed in *Pinus sylvestris* (Zelena et al. 2005) and *Arabidopsis thaliana* (Kovalchuk et al. 2004).

Many years have passed from the Chernobyl accident, but there are only few studies about mutation processes affecting the genome and its consequences, though radiation is one of the strongest existing mutagenic factors. No information is available about selection processes influencing plant populations at the Chernobyl zone, though they play a very important role in adaptation of populations in stress conditions. These two aspects of response to radiation deserve more attention since both of them can have a strong influence on the adaptation and survival of populations in conditions of radiation exposure.

### 1.3. Mutation detection with microsatellite and AFLP markers

Genomic microsatellites (Simple Sequence Repeats; SSRs) were discovered in the early 1980s. Tandem repeats are found in the DNA of all investigated higher organisms. They belong to the most variable types of DNA sequences in the genome. In contrast to unique DNA, microsatellite polymorphism derives mainly from variability in length of alleles caused by a difference in the number of repeat units (Weising et al. 2005). Due to the high level of polymorphism and the mostly neutral nature of SSRs, mutations occur more frequently in microsatellites. Estimates of mutation rates for SSRs are very high ($10^{-2}$-$10^{-6}$ events per locus per generation), as compared to the rates of point mutations at coding gene loci (Weising et al. 2005). The high rate of mutations at microsatellite loci makes it possible to observe mutation events directly. Observations of spontaneous mutations in SSRs showed that the mutation process is affected by various factors including allele size, repeated motif, GC content in DNA, cell division (mitotic or meiotic process), chromosome position, genotype and sex (Ellegren 2004). Though most simple repeats are part of non-coding DNA, either intergenic sequences or introns, there is increasing evidence that microsatellite structure may have a functional importance for gene translation, transcription, recombination, DNA replication, chromatin organization, and cell cycle (Li et al. 2002). An over-representation of SSR repeats was demonstrated in stress response genes (Rocha et al. 2002) suggesting the importance of SSR motifs for adaptation. Due to the functional importance of some microsatellites, selection is expected

to act against change of SSR size at those loci. On the other hand, most of the microsatellites that are used as genetic markers are assumed to evolve neutrally and therefore, their frequency and distribution should reflect the natural mutation process. All these characteristics make microsatellites useful tools to study how the overall genomic mutation rate is affected by stress factors, for example radiation. In studies of humans exposed to high level of irradiation after the Chernobyl accident, application of microsatellites did not show any increase in the mutation rates (Nikiforov et al. 1998; Furitsu et al. 2005), but significant increases in microsatellite mutations have already been observed in wheat (Kovalchuk et al. 2000) and barn swallows (Ellegren et al. 1997) from the highly contaminated areas of the Chernobyl exclusion zone.

The amplified fragment length polymorphism (AFLP; Vos *et al*. 1995) method is a technique with which individuals can be scored simultaneously for many different DNA regions distributed randomly throughout the genome, resulting in a unique, reproducible fingerprint and allowing an assessment of genome-wide variation. Mutation rates for AFLPs were empirically estimated as $10^{-5}$-$10^{-6}$ per locus per generation (Mariette et al. 2001; Kropf et al. 2009), but no experimental data are available. In AFLPs, mutations can occur at restriction sites, primer elongation binding sites or due to insertion/deletions (indels) in the amplified region, resulting in the loss and/or the appearance of a 'new' AFLP band (Li 2007; Kropf et al. 2009). These anonymous markers consist largely of non-coding DNA (Shirasawa et al. 2004). In plants non-coding DNA is largely represented by repeated sequences (Kubis et al. 1998), which have high mutation rates. The influence of a strong DNA damaging factor, such as radiation, may create an additional mutation load to the genome. Thus, it could be possible to observe higher mutation rates for AFLP markers as predicted by theoretical estimations.

The AFLP technique allows the amplification of genomic fragments without any knowledge of sequence information and is expected to cover the genome better than SSRs, since many loci are simultaneously scored. Microsatellites, on the other hand, have high natural mutation rates. Mutation mechanisms differ between both markers types that can eventually result in different mutation rates. A combination of these contrasting properties for investigations of the same material can be useful for an evaluation of the influence of radiation on different parts of the genome.

## 1.4. Evidence for selection assessed at AFLP markers

Evolutional adaptation of a population to environmental changes strongly depends on genetic variation available in a population. High level of genetic variation provides sufficient numbers of allelic variants which will be the basis for selection promoting the survival of the population. Under the influence of different environmental conditions, populations become genetically differentiated due to differences in the allele combinations involved in adaptation processes (Hansson and Westerberg 2002). Changes of genetic structures have been observed in response to strong environmental stress for a number of plant species including forest trees: industrial pollution for *Pinus sylvestris* (Dukharev et al. 1992; Prus-Glowacki et al. 1999) and *Picea abies* (Longauer et al. 2004); water and nutrient stress for *Pinus edulis* (Cobb et al. 1994; Mitton and Duran 2004); temperature for *Betula pendula* (Kelly et al. 2003).

Though only few genes may be involved by selection in adaptation process, they have main functional roles (Black et al. 2001; Luikart et al. 2003). Various methods have been developed for identification of these genes (Vasemägi and Primmer 2005). QTL (Quantitative trait loci) approaches can identify gene regions associated with phenotypes under selection (Neale and Savolainen 2004; Gailing et al. 2009), and candidate gene approaches can characterize selection at the level of sequences variation (Nielsen 2005). Unfortunately, application of these methods is limited in case of non-model species because of the lack of sequence information and not enough knowledge about gene functions (Ford 2002; Erickson et al. 2004).

In case of non-model species, alternative method have been proposed, which enable genome selection studies in the absence of prior knowledge about the selectively advantageous genes or phenotypes (Storz 2005). The development of the amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995) allows investigation of hundreds random markers covering large parts of the genome. This method may be applied to any species without any prior sequence knowledge, and allows obtaining highly reproducible and informative data. The comparison of genetic variation at many loci across the genome might identify loci showing atypical differentiation, which are likely to be linked to the genomic regions affected by selection (Black et al. 2001). Large differences at the allele frequencies between populations can be interpreted as evidence for adaptive genetic differentiation (Linhart and Grant 1996; Nevo 2001). AFLP variation makes it possible to reveal the effects of natural selection using the $F_{ST}$ outlier method, which

assumes that loci directly affected by selection are expected to show a higher $F_{ST}$ value in comparison to 'neutral' loci. The $F_{ST}$ outlier method has been used to detect candidate loci under natural selection in numerous species (for review see Galindo et al. 2009). AFLP has become the method of choice for many studies on plants and animals, and especially useful in the investigation of adaptation processes and selection (Meudt and Clarke 2007).

Evidences of selection are difficult to observe in long-living organisms, especially if a severe environmental change has been recently imposed on a population. In this case, the first signs of differences in adaptation potential will be revealed through strong phenotype differences between organisms. Observing of genetic differentiation in pairs of neighboring individuals which experienced similar stress conditions but exhibit contrasting phenotypes can be considered as a sign of adaptation and, thus, selection process (Müller-Starck 1985; Ziehe et al. 1999). Application of AFLP markers and the $F_{ST}$ outlier method to such material will provide information about number of loci and part of the genome involved in adaptation and influenced by selection.

### 1.5. *Pinus sylvestris* as a model species for investigation of radiation effects

*Pinus sylvestris* (Scots pine) belongs to the family *Pinaceae*, genus *Pinus*, subgenus *Pinus* (Fajon 1984). It is one of the main forest-forming species, native to Europe and Asia, from Norway to Spain, and to eastern Siberia. *P. sylvestris* is an evergreen coniferous tree growing up to 40 m in height and 1.7 m trunk diameter. The lifespan is normally 150–300 years. The stem is straight with thick bark, scaly dark grey-brown on the lower trunk, and thin, flaky and orange on the upper trunk and branches. The crown is variable, with a variety of shapes common in wild populations from level branches to near-fastigiate; open ovoid-conic when young, and usually eventually becoming dense, broadly domed or even flat-topped. The shoots are light brown, with a spirally arranged scale-like pattern. On mature trees the leaves ("needles") are a glaucous blue-green, often darker green to dark yellow-green in winter, 2.5–5 cm long and 1–2 mm broad, produced in fascicles of two with a persistent grey 5–10 mm basal sheath. *P. sylvestris* is wind-pollinated and monoecious. The seed (female) cones are red at pollination, then pale brown, globose and 4–8 mm diameter in their first year, expanding to full size in their second year, pointed ovoid-conic, green, then grey-green to yellow-brown at maturity, 3–7.5 cm in length. The cone scales have a flat to pyramidal apophysis, with a small prickle on the umbo. The pollen (male) cones are yellow, occasionally pink, 8–12 mm long; pollen release is in

middle to late spring. The seeds are blackish, 3–5 mm long with a pale brown 12–20 mm wing; they are released when the cones open in spring 22–24 months after pollination (Fajon 1984). The species is diploid, with 12 pair of chromosomes, which are morphologically similar (Saylor 1961); the genome size is 24,600 Mb (Ahuja et al. 2005).

High level of genetic diversity, strong stress response and a long history of ecological genetic research (Morgenstern 1996; Neale and Savolainen 2004; Guevara et al. 2005) made pines a good model to study genetic effects in alternating environment. With a large genome size as compared to most other plant species (Ahuja et al. 2005), *P. sylvestris* also posses one of the highest sensitivity to radiation ($LD_{50}$ is 5–20 Gy) among the so far investigated species (Sarapul'tsev et al. 1993). Experiments on *P. sylvestris* started in the early 1960s (Sparrow and Woodwell 1962) and demonstrated very low radiotolerance and similar radiosensitivity between pine and human cells. Due to the high capacity for accumulating numerous chemical pollutants, Scots pine became one of the main natural test objects for ecological genetic monitoring (Dukharev et al. 1992; Micieta et al. 1998; Prus-Glowacki et al. 1999) and after the Chernobyl accident one of the most useful species for investigation of radiation effects on organisms because of strong and easy detectable reaction even to low-dose exposure. Different types of morphological abnormalities (Arkhipov et al. 1994), mutagenesis of enzyme loci (Kal'chenko et al. 1995), replacement of radiosensitive cells by more radioresistant cells (Kal'chenko and Fedotov 2001), changes in the spectrum of functionally active genes (Zelena et al. 2005), increase in the cytogenetic aberration rate (Geras'kin et al. 2005), and hypermethylation of the DNA (Kovalchuk et al., 2003) have been reported for pines irradiated after the Chernobyl accident.

### 1.6. Rationale

The accident at the Chernobyl Nuclear Power Plant created unique conditions for the investigation of the responses of biological systems to the influence of a strong stress factor, from the genomic level to the population level. Large territories with variety of species fell under the influence of high-level radiation exposure well known for its damaging effects on all organisms (Real et al. 2004). Chronic irradiation during the years after the accident provided permanent stress influence on biota, especially on plants, which are unable to leave the contaminated areas (IAEA "Environmental consequences of the Chernobyl accident", 2006). Thus, to ensure surviving, plants had to react to such

unfavorable conditions by adaptations to harmful effects and possibly also by selection processes. Due to high sensitivity (Sarapul'tsev et al. 1993), Scots pine (*Pinus sylvestris*) growing on heavily contaminated territories around the Chernobyl station is an excellent object for investigation of radiation exposure. Various effects were observed on the organism and cell levels, but very little is known about mutation rates, genes involved in adaptation response and population reactions. Thus, investigation of pines of different age and irradiation conditions can provide information about changes in mutation rates and the nature of mutations. Comparison of genetic structures in pairs of pine trees with contrasting phenotypic characteristics can reveal signs of on-going selection process and show which parts of the genome are involved in the adaptation process.

This thesis contains not only a case study on adaptation of *P. sylvestris* to conditions of high radiation exposure, but also contributes to the general understanding of adaptation processes and the role of selection in plants response to changing environmental conditions.

### 1.7. Hypothesis and objectives of the study

The hypothesis that genetic structures of pine populations growing in the Chernobyl exclusion zone are influenced both by mutation and by selective processes in areas of high radioactivity was tested using microsatellite and AFLP markers. Microsatellites as neutral markers with high mutation rates can provide us with information about mutation processes in the DNA of irradiated trees, whereas AFLPs as markers spread through the whole genome are often used to detect changes introduced by selection in the genetic organisation of populations.

The main objectives to confirm our hypothesis were:

- to assess changes of genetic structure of pine populations under the influence of radiation
- to compare mutation rates in pines of different age and irradiation conditions
- to examine the effect of radiation on selection in pines with contrasting phenotypic characteristics in comparison to pines in areas with low radiation

### 1.8. Study area

The most affected ecosystem by radiation exposure was the pine forest around the Chernobyl Nuclear Power Plant. According to the pines response to radiation, four zones

were identified in the exclusion area: with lethal, sublethal, medium and moderate damage of trees (Kozubov et al. 1990). In the lethal damage zone, there was complete loss of pine trees of any age and this area was named “Red forest”. The total area of loss of all pines exceeded 600 ha. In certain areas of the “Red forest” even the loss of other, more radioresistant tree species as *Betula pendula* and *Alnus glutinosa* was observed. The absorbed doses for the trees in these forest areas had exceeded 200–300 Gy.

Due to the huge levels of radioactivity of the dead trees and contaminated soil, it was decided to bury them in trenches created on the territory of the “Red forest” (Fig. 1).

Figure 1: Place of radioactive waste storage in the “Red forest” (Photo Kuchma M.D.)

New pine plantations were established in 1989 above the trenches to prevent soil erosion and, as its consequence, secondary dust contamination of the surrounding territories with radioactive materials. Young pine trees growing on this territory showed various, but often strong abnormalities in tree morphology (Fig. 2).

In the second year after the accident, various morphological abnormalities occurred in the sublethal damage zone, the area “Prypiat” with old pine stands including gigantic sizes of leaves, fasciation (flattened tissues), off-plan branching, loss of organs etc. The average radiation doses in this zone until 1991 were about 50 Gy. The medium and moderate damage zones cover a territory more than hundreds of thousands of hectares. In this zone,

the trees suffer from growth inhibition, untimely needle shedding, radiomorphoses and intensive branch-out (Arkhipov et al. 1994; National Report of Ukraine 2006). Over the time the "Red forest" area became the sublethal damage zone and the "Prypiat" area got the status of the medium damage zone. Even 20 years after the accident, trees show various symptoms of strong radioactive damage.

Pine rosette forms at open surface

Young Scots pine shoots with metamorphized needles

Inhibition of apical dominance among Scots pines

Cluster of the large buds on Scots pine shoot tops

Figure 2: Examples of morphological abnormalities among coniferous trees (Photos Kuchma M.D.)

## 2. Material and methods

### 2.1. Plant material and sampling strategy

Fresh needles were collected from pine trees (*Pinus sylvestris*) of different age and irradiation conditions (See Table 1 of Manuscript II for details). The trees were divided in 5 different groups (demes) – 3 irradiated populations sampled in the Chernobyl exclusion zone (Fig. 3) and 2 control populations.

Deme P1 (Fig. 4) is represented by about 50 years old trees growing on heavily radiation-exposed sites close to the nuclear power plant ("Prypiat"). In the age of 30 years they have been exposed to acute high radiation during the accident in 1986, followed by chronic irradiation until now.

Deme P2 and deme P3 (Fig. 6) are composed of trees derived from pine plantations established after the accident on heavily contaminated soils close to the nuclear power plant. Material was collected from 20 years old trees which have been planted after the accident and were exposed to chronic irradiation during the whole time. Needles were samples from directly neighboring pairs of trees with contrasting phenotypic appearance: one tree with no or minor damage and one tree with strong damage symptoms (abnormalities in growth, morphogenesis, buds and shoots formation, etc.). Fifty pairs of trees were sampled in the so called "Red forest" area. Deme P2 consists of trees with strong damage (irradiated damaged) and deme P3 consists of trees with no or only minor damage symptoms (irradiated healthy).

Deme P4 (Fig. 7) and deme P5 (Fig. 5) are represented by trees which were randomly selected in the plantations established in the area with low radiation exposure. These pines were planted in the same years as the trees in the Chernobyl exclusion zone (see above) and have the same age and origin. Demes P4 and P5 served as controls for demes P1 and P2-P3, respectively.

Needles were harvested from four branches with different orientation (North, East, South, West) from each tree. In total, needles were sampled from 264 trees (See Table 1 of Manuscript II, Appendix 1). All samples were stored in silica gel until DNA extraction and genotyping.

## 2.2. Radiation measurements

The radiation exposure of the plant material was quantified by measuring the radiation dose on site and by calculation of the absorbed radiation doses from the main dose-forming radionuclides (See Table 1 of Manuscript II, Appendix 1). The present radiation dose measured for Chernobyl plots is 0.6 – 4.4 mR/h, about 60 – 400-times higher than the background levels measured for the control plots (0,005-0,008 mR/h). The accumulated dose was 9.8 Gy/year for the "Red forest", 5.7 Gy/year for the "Prypiat" area and $3x10^{-6}$ Gy/year for the control plots.

Figure 3: Sampling plots in the Chernobyl exclusion zone

Figure 4: P1 – trees, planted before the accident and exposed to acute and chronic irradiation (Photo Kuchma O.)

Figure 5: P5 – trees, which were used as control for P1, from a “clean” area (Photo Kuchma O.)

Figure 6: P2 and P3 – trees, planted after the accident with strong and minor damage symptoms, respectively; exposed to chronic irradiation (Photo Kuchma O.)

Figure 7: P4 – trees, which were used as control for P1, from a “clean” area (Photo Kuchma O.)

### 2.3. DNA isolation

Genomic DNA was extracted from dried needles using the DNeasy 96 Plant Kit protocol of QIAGEN (QIAGEN GmbH, Hilden, Germany).

### 2.4. Nuclear microsatellites

Nine polymorphic microsatellite primers (Soranzo et al. 1998, Liewlaksaneeyanawin et al.2004; González-Martínez et al.2004) were used to screen 843 samples from 5 demes. PCR reactions were performed as described in the original Manuscripts with minor modifications. Population genetic diversity parameters and differentiation parameters were calculated by using GenAlEx 6.1 (Peakall and Smouse 2001). Mutation rates were calculated as the number of mutations per locus per generation. Statistical significance was confirmed by the $\chi 2$-test. Details on the methods and data analyses are descrided in Manuscript II and Appendix 2.

### 2.5. DNA cloning and sequencing

All alleles with mutations in microsatellites were cloned and sequenced to confirm mutation events and to determine the molecular basis for size variation. Comparative sequencing of 8 samples of the three microsatellites loci was performed. The selected sequences were analyzed at least in five clones and compared to the non-mutated type from the same tree. Details on the method and data analyses are descrided in Manuscript II and Appendix 2.

### 2.6. AFLP analyses

A total of 533 samples from demes P2, P3, P4 were analyzed with AFLP markers using the selective primer combination *Eco*RI-ACA and *Mse*I-GAAC (nomenclature according to Keygene N. V.). A total of 222 fragments with 100% reproducibility in size range from 75 bp to 324 bp were manually scored. Calculation of genetic diversity between population was done with AFLP-SURV version 1.0 (Vekemans et al. 2002). Population differentiation and outlier loci detection were obtained by using DFDIST (modified from Beaumont and Balding 2004). Significance test was performed by using $\chi 2$-test with Bonferroni correction. Mutation rates were calculated in the same way as for microsatellites. Details on the methods and data analyses are descrided in Manuscript I and Appendix 2.

## 3. Summary of results

### 3.1. Genetic diversity and overall differentiation

Genetic variation at nine nuclear microsatellites loci (nSSRs) was examined in 264 trees from 5 demes (three irradiated demes from the Chernobyl exclusion zone and 2 controls from clean areas). A total of 191 microsatellite alleles were generated (Manuscript II). The number of alleles per locus ranged from 5 for LOP3 to 36 for SPAC7.14. High overall genetic variation among demes was observed at all investigated microsatellite loci. The mean number of alleles scored was 14.5 for P1-P5 and 15.2 for P2-P3-P4 comparisons. The mean values of observed heterozygosity were 0.600 for group P1-P5 and 0.605 for group P2-P3-P4, respectively. The mean $F_{ST}$ values showed low differentiation between populations with $F_{ST}$ 0.012 and 0.009 for groups of old (P1-P5) and young (P2-P3-P4) trees, respectively (Appendix 3).

A total of the 164 individuals from demes P2, P3 and P4 were analysed with the AFLP technique, and 222 bands were scored. All populations demonstrated high level of genetic diversity with a percentage of polymorphic loci (*PPL*) ranging from 74.3% to 77.9% and a heterozygosity ($H_e$) ranging from 0.224 to 0.239. The mean heterozygosity over all samples and percentage of polymorphic loci are 0.231 and 76.1%, respectively. The global $F_{ST}$ between demes generated from DFDIST was 0.0454 and indicated low differentiation between all populations. The pairwise $F_{ST}$ analysis demonstrated moderate level of differentiation between P2-P4 and P3-P4 groups (exposed to radiation *versus* control) with $F_{ST}$ 0.0562 and 0.0619, respectively, meanwhile group P2-P3, which consists of the demes exposed to high radiation with or without exhibiting strong symptoms of damage, showed low genetic differentiation with $F_{ST} = 0.0187$. More details are given in Manuscript I.

### 3.2. Mutation analysis

Mutation events were detected both at microsatellite and AFLP markers. Only four mutations were observed with microsatellites in demes P1, P2 and P3 and no mutations were found in the control plots (P4 and P5). Three mutations were revealed in the group of young trees and one in the group of old trees. Mutations are represented by different types of deletions or loss of the allele and were found in the repeat motifs of three different microsatellite loci (LOP5, PtTX3107 and PtTX2146) (Appendixes 4 and 5). In case of AFLP markers, a mutation analysis was performed only for the group of young trees (P2,

P3, P4). Mutations were detected in both irradiated demes – 34 and 32 mutation events in P2 and P3, respectively, and also in the control – 12 mutations in P4 (Table 6 in Manuscript II). The nature of mutation within AFLP loci was represented by both the loss of whole fragments and the appearance of new fragments; in some samples single nucleotide insertions/deletions were observed.

Analysis of mutation rates in microsatellites showed no significant statistical differences in the comparison of demes P1-P5 and demes P3-P4, but a highly significant statistical difference ($P<0.0001$) was found in the P2-P4 group. The mutation rates in irradiated groups are $2.8 \times 10^{-4}$ (1/3546) for deme P1, $7.1 \times 10^{-4}$ (2/2808) in deme P2 and $3.3 \times 10^{-4}$ (1/3006) for deme P3. For AFLP loci, the estimates of mutation rates are $1.06 \times 10^{-3}$ (12/23668) for control (deme P4), $3.99 \times 10^{-3}$ (34/17483) and $3.74 \times 10^{-3}$ (32/17699) for demes P2 and P3, respectively. After AFLP analysis, a highly significant statistical difference ($P<0.0001$) was found for the demes P2 and P3 compared to the control group P4 with difference in 3.8- and 3.5 times between irradiated demes (P2 and P3, respectively) and control. More details are described in Manuscript II.

### 3.3. Outlier loci detection

Applying of $\chi 2$-test with Bonferroni correction revealed 23 loci out of 222 as significant above 99% confidence level. No loci with significantly different allele frequencies were detected in pairwise comparisons between P2 and P3, while the number of loci for P2-P4 and P3-P4 was 7 and 17, respectively. Analysis of all three demes (P2-P3-P4) revealed 19 loci significantly deviated after Bonferroni correction.

Outlier loci analysis was performed with DFDIST software among 222 AFLP loci in three demes (P2, P3, P4) and revealed in total 15 loci potentially under the pressure of selection. These loci deserve special attention due to higher differentiation than expected at the 99% confidence level and big differences in allele frequencies between populations. Pairwise comparisons identified 3 outliers in the group P2-P3, 7 loci in the group P2-P4 and 8 outliers in the group P3-P4. The global analysis on all three demes revealed 8 loci lying above the 99% confidence line. Some loci showed significant values only in one of the pairwise comparisons, but were lying above the 95% confidence level in the global analysis, as well; so they still can be considered as "true" outliers. Most of the identified loci can be divided into two groups: overrepresention of the dominant allele in irradiated

populations (8 loci) or control population (7 loci) with differences in allele frequencies from 19% to 92% between demes in at least one comparison.

Mostly the same loci were detected with DFDIST and $\chi 2$-test with Bonferroni correction that confirms their outlier behavior. Five loci in the comparison P2-P4, seven loci in the P3-P4 pair and eight loci in the global analysis were detected as highly significant with both tests. Observed deviations can be explained by differences in procedure of data analysis. In total, 6 % of the observed loci were identified as candidate loci under the influence of selection. Detailed results are reported in Manuscript I.

## 4. General discussion and conclusions

Changes in environmental conditions, such as global climate change, chemical and radioactive pollution affect the genetics of natural populations in different ways. Many interacting biological mechanisms are involved in the response, which eventually contribute to the survival of an organism or a population. Genetic variability can be altered by the appearance of new mutations, changes on the level of genes expression and in allele frequencies. Thus, stressful environmental conditions can affect genetic systems at a variety of levels of biological organization (Bickham et al. 2000). At the molecular level, interaction of a stress factor with DNA will create somatic lesions that can cause cell or tissue damage and lead to strong health effects on individuals. This can affect the reproductive capacity or result in high mortality rates that, in turn, can cause a decrease in population size and result in a reduction of genetic variability in populations. Changes in allele frequencies might appear from selections at loci important for the survival in stress environments or by chance in fixation of deleterious alleles in small populations. All of these might, in the end, lead to a reduction in the average fitness of populations, or in the adaptation of the population to the new environment through surviving and reproduction of individuals with the most fitting characteristics. Therefore, the occurrence of effects at the molecular level may also initiate a cascade of responses at higher levels including tissue, organism health, reproduction, population demographics, population genetics, and finally, evolutionary processes including the appearance of adapted populations with some unique features (Bickham and Smolen 1994, Theodorakis et al. 1997). Knowledge of the mechanisms of response of the individual and populations to stress factors can allow us to predict their reactions to a changed environment.

Plant species demonstrate great morphological and functional variation, much of which is adaptive (Wright and Gaut 2005, Kalisz and Kramer 2008). Plants are immobile organisms and thus must respond to changes in environmental conditions through physiological and evolutionary adaptation processes at their growing sites. It is possible for plants with a relatively short life cycle to improve adaptedness to environmental stress very fast within several generations, due to the selection of the most favourable genotypes. However, this does not hold for species with a long life cycle, such as trees. Tree survival depends on the capacity to develop plastic reactions to the changing environmental conditions, involving ways of fast genetic response, such as changes in mechanisms of gene regulation and

expression, DNA methylation etc. These processes possibly lead to different viabilities or fertilities of different genotypes, ultimately resulting in changes of genetic structures within populations. The absence of a germline in plants implies that all changes (including mutations) in the genome can be inherited. Complex responses to stress make plants useful objects for studies on the influence of different environmental factors to a single organism or a population. Long-living plants growing under outdoor conditions give us the possibility to investigate reactions to the stress factors under semi-natural conditions. These reactions cannot be studied in laboratory experiments. The universality of the fundamental mechanisms underlying these responses allows to apply the obtained knowledge also to another species.

Ionizing radiation is known to affect plants in many ways, depending upon the species, plant physiology, and genome organization (Holst and Nagel, 1997). During the years after the Chernobyl accident various effects and responses of plants, including forest tree species, to acute and chronic irradiation were observed, such as death of radiosensitive plants and radiomorphoses (Arkhipov et al., 1994), an increased frequency of chlorophyll and embryonic lethal mutations (Abramov et al. 1992; Bubryak et al. 1992; Shevchencko et al. 1996; Kovalchuk et al. 2000), an increased frequency of chromosome aberrations (Kal'chenko and Fedotov 2001; Oudalova et al. 2005), changes in levels of DNA methylation (Kovalchuk et al. 2003) and gene expression (Zelena et al. 2005), etc. The main object for most of these studies was Scots pine (*Pinus sylvestris*), a species with high level of genetic diversity and a high sensitivity to ionizing radiation. Thus, *P. sylvestris* becam one of the model organisms used for the analysis of radiation effects on biological systems.

### 4.1. Changes in mutation rates

Mutations are one of the sources of genetic variation on which selection acts. Although most mutations are either selectively neutral or detrimental, a few favourable ones allow for adaptation to a changing environment and create the basis for the evolution of the species. Strongly deleterious mutations will be directly removed from the population. Effects of slightly deleterious mutations are only minor. They and neutral mutations have the potential to be spread within the population and persist for much longer periods of time, eventually affecting the viability and fertility of the population. Induced heritable mutations lower the reproductive capacity of a population since affected individuals may

have low viability and fertility. Thus, a high mutation rate has damaging effects and results in reduced average fitness of the population. The most commonly investigated and detected mutations occure in somatic cells. Genetic damage in somatic tissues can have a number of immediate consequences to the cells involved, including derangements in cell functions or cell death. In case of plants, somatic mutations quite easily can be transformed to heritable mutations that create opportunities for investigation of changes in mutation rates under the influence of stress factors, including radiation.

Most studies concerning changes in mutation rates after the Chernobyl accident were conducted on humans and animals using microsatellite or minisatellite markers. Investigations of germline mutations in several studies showed an increase in rates (Ellegren et al. 1997; Dubrova et al. 1998; Dubrova et al. 2002), but mostly no effects were observed, though both types of markers have very high mutation rates compared to other DNA markers (Weising et al. 2005). Only few studies were performed on plant species, such as *Pinus sylvestris* (Shevchenko et al. 1996), *Arabidopsis thaliana* (Abramov et al. 1992), and *Triticum sativum* (Kovalchuk et al. 2000). Rates of somatic mutations were examined and in all cases a significant increase was detected.

After performing microsatellite analysis, the irradiated populations showed mutation rates, which correspond with general estimations of mutation frequencies of SSRs – $10^{-4}$ mutations per locus per generation. No mutations were found in controls and only few in irradiated groups. A comparison of pines with different age showed no difference in the number of mutations between deme P1 and demes P2-P3. A highly significant difference in the mutation rate ($P<0.0001$) was detected only between the most radiation-damaged samples and the contol (P2-P4 comparison). According to the literature, there are a lot of factors that can influence the mutation rates in microsatellites (Li et al. 2002) and there is proof of an active functional role of some microsatellites in cell processes (Ellegren 2004). An improved DNA repair capacity in irradiated material was detected in the study of *Betula verrucosa* and *Oenothera biennis* from the Chernobyl exclusion zone (Boubriak et al. 2008). This can be an explanation for low mutation rates in microsatellites if we assume that some of the investigated SSR loci are linked to the actively expressed DNA regions where reparation process removes most of DNA damages.

Very little is known about mutation rates of AFLPs. According to some estimations, the mutation rates for AFLP are$10^{-5}$-$10^{-6}$ per locus per generation (Mariette et al. 2001; Kropf et al. 2009). Our study is one of the first attempts to apply AFLP markers for the

investigation of mutation rates. AFLPs screening covers not only repetitive DNA, but in principle all genomic regions including functionally active genes. Thus it can provide information about mutation processes in the whole genome. Mutation rates obtained for AFLPs in our study were higher than in microsatellites – $10^{-3}$ per locus per generation which may relate to the very high amount of repetitive DNA in the pine genome. Mutations were detected in both control and irradiated groups of pines. The difference was more than 3-fold and highly significant ($P<0.0001$) for the both comparisons – irradiated damaged *versus* control (P2-P4) and irradiated healthy *versus* control (P3-P4), indicating strong DNA damage in irradiated samples. All obtained results suggest that AFLPs may be applied as an additional marker system for the detection of mutation events under the influence of radiation.

Our results demonstrate that a mutation process is still taking place in the Chernobyl exclusion zone and that it is one of the reactions of organism to irradiation. Different types of markers, such as SSRs and AFLPs, can be successfully applied together and complement each other in the investigation of mutation rates.

### 4.2. Selection process under the influence of radiation

A change of genetic structures due to selection is one of the responses of populations to a stressful environment and results in evolutionary adaptations. The persistence of a population in a new environment depends on its vulnerability to these environmental changes, which is determined by its genetic variability and physiological tolerance. High level of genetic diversity promotes thew survival of a population under the influence of a stress factor. It is the basis for selection processes; a high number of allelic variants at adaptive loci potentially determines the future of the population.

Numerous experimental studies were performed to investigate connections between “new” adaptations and the selection process. Many investigations aimed to observe changes of genetic structures in plant populations, including the trees *Betula pendula* (Kelly et al. 2003), *Pinus edulis* (Cobb et al. 1994; Mitton et al. 1998; Mitton and Duran 2004), *Picea abies* (Bozhko et al. 2003; Longauer et al. 2004) and *Pinus sylvestris* (Dukharev et al. 1992; Prus-Glowacki et al. 1999). The selection process cannot be easily observed for long-livingtree species, but might be detected under severe change of environmental conditions, when sudden and long-lasting changes of the environment will induce an

adaptive response of the organism and selection process in the population. Such conditions are available in areas of industrial pollution or nuclear accidents.

The accident of the Chernobyl nuclear power plant in April 1986 created unique conditions for an investigation of the influence of radiation as a strong and long-lasting stress factor on plants. Under a radiation exposure of several orders of magnitude above natural levels, selection is expected to enhance the survival of a population. The most affected species was *P. sylvestris* planted on the territories surrounding the nuclear station. The naturally high sensitivity to stress factors due to the big genome size and strongly pronounced reactions through phenotypic variability made Scots pine one of the main species for the investigation of radiation effects on organisms. Different types of morphological abnormalities (Arkhipov et al. 1994), mutagenesis at enzyme loci (Kal'chenko et al. 1995) replacement of radiosensitive cells by more radioresistant cells (Kal'chenko and Fedotov 2001), epigenetic variability (Zelena et al. 2005), an increase in the cytogenetic aberration rate (Geras'kin et al. 2005), and hypermethylation of the DNA (Kovalchuk et al., 2003) have been reported for pines irradiated after the Chernobyl accident. Many pine trees show drastic phenotypic differences under similar irradiation conditions that indicate a variation in their adaptation ability and this can be the result of the ongoing selection process.

We used AFLP markers to reveal genome regions under the influence of selection and evaluated which part of the genome can be involved in an adaptation response. The application of the DFDIST software and tests for the significance of differences of genetic structures between irradiated and control demes allowed us to identify outlier loci possibly targeted by the selection process. Though most of the scored loci most probably belong to non-coding DNA that is common for pines, detected outliers could be linked to the loci responsible for different adaptive responses.

A comparison of genetic structures of trees which demonstrate strong phenotypic differences under comparable irradiation conditions (demes P2 and P3) and the control (deme P4) revealed loci which can be responsible for such contrasting characteristics. In total, 6% of scored loci (sum of outlier loci observed in any of the pairwise comparisons) showed strong outlier behavior and can be considered as candidates for the adaptation response between compared populations. The pairwise estimates detected 1.4-3.6% outlier loci.

Our results are comparable with results of other studies where the percentage of outlier loci involved in the selection process varied between 1% and 6%; only for interspecific

comparisons the percentage of detected loci was about 12-16% (See Manuscript I). A comparison of the different statistical methods revealed that both tests highlighted mostly the same outlier loci, and minor differences can be explained by specificities in the analysis of the data sets. Generally, the results of both tests complement each other and suggest that a relevant part of the genome is affected by the adaptation process.

We used stringent conditions for the detection of outliers. Our populations are strictly comparable. All trees were planted in the same year. Their genetic similarity was proven by the analysis of genetic differentiation with AFLP markers using additional functions of the DFDIST software (Manuscript I). Only strictly repeatable loci were analyzed, positive and negative controls were included in all analyses, and all fragments were manually scored. To avoid scoring of false positives in outlier detection, we followed the suggestions by Caballero (2008) for the detection of candidate loci for selection using the $F_{ST}$ approach. Low differentiation between all populations as expected for pines was indicated with mean trimmed $F_{ST} = 0.021$, which represents 'neutral' $F_{ST}$ values calculated after excluding of 30% of outlier loci with the highest and with the lowest $F_{ST}$. Meanwhile, untrimmed $F_{ST}$ values calculated with a full data set showed moderate levels of differentiation, if populations planted on irradiated sites (P2 and P3) were compared to the control (P4). This demonstrates the influence of detected outlier loci on the overall differentiation between these populations. Low differentiation was observed between two irradiated populations with contrasting phenotypic characteristics (P2 *versus* P3). Only 3 loci were identified as outlier in this comparison, which did not strongly elevate the overall differentiation. Thus, the investigated markers are most likely not the main reason for strong differences in phenotypes, but they may still be crucial for the survival of both 'damaged' and 'healthy' plants planted under high radiation.

Pairwise comparisons revealed that up to 8 out of 222 investigated loci were outliers showing an unexpectedly strong differentiation between different demes. In total, our genome scan pointed to 15 loci potentially involved in the adaptation to the influence of radiation. Even if the chance that at least some of the detected loci are the part of the genes involved in adaptation is quite small, they may still be linked to the regions of the genome targeted by the selection process. Our results demonstrate that complex selective responses to radiation involving numerous genomic regions is taking place in the Chernobyl exclusion zone and they prove the usefulness of the outlier detection methods based on

AFLP analyses to study the adaptation of long-living organisms like forest trees to rapid and extreme environmental change.

The results of our study are only a first step in understanding the complex reactions involved in the adaptation of *P.sylvestris* growing under the influence of radiation. The method of outlier loci detection revealed evidence of an ongoing selection process in pines from the Chernobyl exclusion zone, but an identification of the genes responsible for survival in such severe conditions requires applications of other valid approaches. An analysis of gene expression and sequencing of candidate genes can help us to understand which genes are crucial for a successful performance of pines under high radiation exposure. These trees may be considered as good models for further analysis of the adaptation and selection process of long-living plants to extreme environmental change and provide better insight into the mechanisms underlying responses of an organism to stressful conditions.

## 5. Summary

Due to their immobility, plants have to tolerate unfavorable environmental conditions and make use of different adaptive mechanisms to survive and reproduce successfully under stress. An adaptation of plants to new conditions can be achieved by selection processes based on the genetic diversity of populations, or by mechanisms of fast genetic responses such as changes in the level of DNA-methylation, gene expression or phenotypic plasticity.

It is often difficult to detect the organism’s or population’s response to stress factors due to slow changes of environmental conditions or delayed reactions of plants. From this point of view, the investigation of reactions under extreme environmental change offer unique opportunities to study adaptation mechanisms. Areas with a strong anthropogenic impact on the environment represent ideal places for research on adaptation or selection processes.

For many decades, ionizing radiation is well known as a strong damaging and stress factor. Radiation exposure causes heavy damages of the DNA. This leads to a decrease in fitness in the present generation and inheritable mutations which reveal their effects in later generations. On the other hand, radiation exposure activates adaptation processes to ensure survival. The investigation of the influence of radiation at different levels of life organization from the DNA level to the population level can help to elucidate response mechanisms to changing environments.

After the accident in 1986 at the Chernobyl Nuclear Power Plant, the surrounding territories were contaminated with high amounts of radioactive material and even now the doses of irradiation exceed the level of the background irradiation more than hundred times in many areas. The exclusion zone became a natural laboratory for the investigation of effects of radiation on the biocenosis. *Pinus sylvestris* is one of the most wildly used species in radiation research due to its very high sensitivity to radiation exposure and dominance in forest ecosystems of the exclusion zone.

Mutation rates and selection processes under the influence of radiation in the Scots pine (*Pinus sylvestris*) of different age and irradiation conditions collected in the Chernobyl exclusion zone and control areas were investigated. The main objectives of this study are: 1) an assessment of changes of the genetic structure of pine populations under the influence of radiation; 2) a comparison of mutation rates in pines of different age and

irradiation conditions; 3) an examination of the effect of radiation on selection processes in pines with contrasting phenotypic characteristics.

Within the Chernobyl area, different sets of pine trees were collected. Deme P1 consists of 50 year old trees, which were exposed to acute irradiation during the accident and to chronic irradiation after the accident. Deme P2 is represented by 20 year old trees planted after the accident with heavy irradiation symptoms and exposed only to chronic irradiation. Deme P3 is a group of trees corresponding to deme P2 but with only minor irradiation symptoms. Control trees were collected from areas with natural radiation levels of the same age and origin. Demes P4 and P5 are the controls for demes P2-P3 and deme P1, respectively.

A set of nine polymorphic nuclear microsatellites (SSRs) was used to analyse the genetic variation for 843 samples and to evaluate changes in mutation rates under the influence of radiation. In total, 191 microsatellite alleles were scored. A high level of genetic diversity was revealed for the groups of older trees (P1 and P5; average $H_e$=0.756) and younger plants (P2, P3, and P4; average $H_e$=0.749). The population differentiation between the demes of the same age group is very low ($F_{ST}$=0.012 and $F_{ST}$=0.009 for P1-P5 and P2-P3-P4, respectively); this is consistent with their common origin. Only 4 mutation events were detected in the irradiated groups and no mutations in the controls. Mutations were represented by deletions in repeat motifs and by one null allele. Three mutations were revealed in the group of young trees and one in the group of old trees. Calculated mutation rates vary from $2.8x10^{-4}$ to $7.1x10^{-4}$ confirming general estimates available for microsatellites. A highly significant difference ($P$<0.0001) was only observed by comparing irradiated damaged trees (P2) *versus* control (P4) trees. Generally, the number of mutations detected by microsatellite markers was not as high as expected due to the natural high mutation rates of SSRs. This phenomenon may be explained by an active repair process under radiation exposure.

AFLP markers were used to analyse the genetic differences, selection processes and mutation rates between pairs of pine trees showing contrasting phenotypic characteristics (demes P2 and P3) and control trees (deme P4). 222 AFLP loci were scored. A high level of genetic diversity was found in all populations with a mean heterozygosity of 0.231 over all samples. Outlier analysis with DFDIST and $\chi$2-test with Bonferroni correction were performed to detect loci under selection. Trimmed $F_{ST}$ values with a mean of $F_{ST}$=0.0208 indicate, similar to the microsatellite analysis, low differentiation between all three

populations. Untrimmed $F_{ST}$ values showed a moderate level of genetic differentiation between the groups P2-P4 ($F_{ST}$=0.0562) and P3-P4 ($F_{ST}$=0.0619); low differentiation was observed in the group P2-P3 ($F_{ST}$=0.0187). Out of 222 scored AFLP loci, fifteen were identified as outlier loci ($P$<0.01) by DFDIST; most of them were significant ($P$<0.01) in $\chi 2$-test with Bonferroni correction. A similar number of outliers was detected for the pairwise comparisons between both irradiated demes and the control: 7 loci in pair P2-P4 and 8 loci in pair P3-P4. Only 3 loci were identified in the comparison of irradiated demes to each other (P2-P3). The outlier loci can be divided in two groups: the dominant allele is either overrepresented in the irradiated population or in the control with difference in frequencies ranging from 19% to 92% which might depend on their functional role in the genome. The comparison between the different populations indicates that in total more than 6% of the detected loci are candidates for an adaptive response.

Mutation rates obtained with AFLP markers were estimated as $1.06 \times 10^{-3}$ for the control deme (P4) and $3.99 \times 10^{-3}$ and $3.74 \times 10^{-3}$ for demes P2 and P3, respectively. The number of mutation events detected by AFLP-analyses was much higher than by SSR-analysis. Mutations were represented by the appearance of new fragments, the loss of fragments, and one nucleotide insertion-deletion. The number of mutations was three times higher in both irradiated populations compared to the control population (34, 32 and 12 mutation events in the trees of deme P2, P3 and P4, respectively). The comparison of the demes P2-P4 and P3-P4 showed highly significant differences between them ($P$<0.0001). AFLP-markers cover the whole genome; their high potential for analysing mutation events was demonstrated.

The results of this study show that a high number of mutations occur across the whole genome of *P. sylvestris*; selection processes were also observed among pines of the Chernobyl exclusion zone. If most of the occurring mutations are deleterious or decrease the fitness of organisms, pines had to activate protective, repairing and adaptive mechanisms in order to survive in conditions of permanent radiation exposure. It is difficult to distinguish which part of the DNA is affected by mutation processes or influenced by selection. More detailed studies are needed in this regard. Sequencing of candidate genes and the transcriptome will provide information about changes in nucleotides composition and their influence on protein structure or functions. Changes in gene expression can demonstrate which genes are crucial for surviving in stress conditions and are targets for selection processes. Further investigation of *P. sylvestris* from the

Chernobyl exclusion zone could provide new information about rapid adaptation of long-living plants to extreme environmental change.

# 6. Zusammenfassung

Pflanzen müssen aufgrund ihrer Immobilität ungünstige Umweltbedingungen tolerieren und verschiedene Anpassungsmechanismen aktivieren, um unter Stressbedingungen zu überleben und erfolgreich zu reproduzieren. Die Anpassung von Pflanzen an neue Umweltbedingungen kann zum einen durch Selektionsprozesse, die auf der genetischen Diversität von Populationen basieren, oder zum anderen durch schnelle genetische Responsmechanismen, wie Veränderungen im Methylierungsgrad der DNA, der Genexpression oder der phenotypischen Plastizität, erreicht werden.

Oft ist es schwierig, die Stressantwort eines Organismus oder einer Population zu entdecken, da sich die Umweltbedingungen nur sehr langsam verändern können oder die Stressantwort der Pflanzen erst verzögert erfolgt. Somit bieten Untersuchungen unter extremen Umweltbedingungen, einmalige Gelegenheiten, Anpassungsmechanismen zu analysieren. Gebiete mit starkem anthropogenem Einfluss auf die Umwelt, stellen ideale Orte dar, um Anpassungs- und Selektionsprozesse zu studieren.

Seit vielen Jahrzehnten ist bekannt, dass ionisierende Strahlung ein extrem schädlicher Stressfaktor ist. Radioaktive Strahlung bewirkt eine starke Schädigung der DNA. Dies führt zu einem Verlust der Fitness in der gegenwärtigen Generation und zu vererbbaren Mutationen, die erst in den späteren Generationen sichtbar werden. Untersuchungen zum Einfluss von Radioaktivität auf verschiedenen Ebenen eines Organismus, wie etwa von der DNA-Ebene eines einzelnen Individuums bis hin zur Populations-Ebene, können helfen, den Mechanismus der Stressantwort auf sich ändernde Umweltbedingungen besser zu verstehen.

Nach dem Reaktorunfall 1986 im Kernkraftwerk Tschernobyl wurden große Gebiete mit radioaktivem Material kontaminiert; bis heute übersteigt die Strahlendosis in vielen Gebieten mehr als das hundertfache der natürlichen Strahlung. Die Sperrzone in Tschernobyl wurde somit ein natürliches Labor, um den Einfluss von Radioaktivität auf die Biozönose zu untersuchen. Aufgrund ihrer hohen Sensitivität gegenüber Strahlenbelastung und ihrer Dominanz in den Waldökosystemen der Sperrzone ist *Pinus sylvestris* eine der meist untersuchten Spezies bezüglich der Auswirkungen von Radioaktivität.

In Kiefern (*Pinus sylvestris*) unterschiedlichen Alters und mit unterschiedlicher Strahlenbelastung, die in der Sperrzone beerntet wurden, wurden Mutationsraten und

Selektionsprozesse im Vergleich zu Kontrollkollektiven analysiert. Die Hauptziele dieser Untersuchung sind:

1) die Einschätzung der Veränderung genetischer Strukturen in Kiefernpopulationen unter dem Einfluss radioaktiver Strahlung;

2) der Vergleich von Mutationsraten in Kiefern unterschiedlichen Alters und mit unterschiedlicher Strahlenbelastung;

3) die Untersuchungen zum Einfluss der Strahlung auf Selektionsprozesse in Kiefern mit unterschiedlichen phänotypischen Charakteristika.

Im Gebiet um Tschernobyl wurden verschiedene Kiefernkollektive (Deme) beerntet. Kollektiv P1 besteht aus 50 Jahre alten Kiefern, die während des Unfalls akuter und nach dem Unfall chronischer radioaktiver Strahlung ausgesetzt waren. Kollektiv P2 wird von 20 Jahre alten Kiefern gebildet, die nach dem Unfall gepflanzt wurden und somit nur chronischer Strahlung ausgesetzt waren, aber starke Symptome zeigen. Kollektiv P3 entspricht Kollektiv P2, allerdings mit nur geringen Symptomen radioaktiver Belastung. Aus Gebieten mit natürlicher Hintergrundstrahlung wurden Kontrollkollektive gleichen Alters und gleichen Ursprungs gesammelt. Die Kollektive P4 und P5 sind somit die Kontrollkollektive für die Kollektive P2-P3 beziehungsweise P1.

Ein Set von neun polymorphen, kern-kodierten Mikrosatelliten- (SSR) Markern wurde benutzt, um die genetische Variation und Änderungen in den Mutationsraten unter dem Einfluss von Strahlung in 843 Bäumen zu analysieren. Insgesamt wurden 191 Mikrosatelliten- Allele untersucht. Hohe genetische Diversität wurde sowohl in der Gruppe der älteren Bäume (P1 und P5; $H_e$=0.756 im Mittel) als auch in der der jüngeren Bäume gefunden (P2, P3 und P4; $H_e$=0.749 im Mittel). Die Differenzierung zwischen den Populationen gleichen Alters ist sehr gering ($F_{ST}$=0.012 und $F_{ST}$=0.009 für P1-P5, beziehungsweise P2-P3-P4). Dieses Ergebnis ist konsistent mit der Tatsache, dass die Kollektive einen gleichen Ursprung haben. In der Gruppe der stark belasteten Pflanzen konnten lediglich vier Mutationsereignisse gefunden werden und keine in den Kontrollgruppen. Die Mutationen stellen Deletionen im Wiederholungsmotiv und ein Null-Allel dar. Während im Kollektiv der jüngeren Bäume drei Mutationen gefunden wurden, zeigte das Kollektiv der älteren Bäume nur eine. Die berechneten Mutationsraten variieren von $2.8 \times 10^{-4}$ bis $7.1 \times 10^{-4}$, was generell den Schätzungen bei Mikrosatelliten-Markern entspricht. Eine signifikant höhere Differenz ($P<0.0001$) wurde nur im Vergleich der stark geschädigten Bäume (P2) mit dem Kontrollkollektiv (P4) gefunden. Generell war die

Anzahl der Mutationen, die mit Hilfe von Mikrosatelliten-Markern detektiert wurden, nicht so hoch wie erwartet, zumal Mikrosatellitenmotive natürlich-bedingte, hohe Mutationsraten zeigen. Dieses Phänomen kann mit einem aktiven Reparaturmechanismus erklärt werden, der unter Strahlenbelastung induziert wird.

Ferner wurden AFLP-Marker benutzt, um genetische Differenzierung, Selektionsprozesse und Mutationsraten in Paaren von Bäumen mit unterschiedlichem Phänotyp (P2 undP3) gegenüber dem Kontrollkollektiv (P4) zu analysieren. Es wurden 222 AFLP-Loci untersucht. In allen Populationen wurde eine hohe genetische Diversität mit einer mittleren Heterozygotie, gemittelt über alle Proben, von 0.231 gefunden. Das Programm DFDIST und ein $\chi 2$-Test mit Bonferroni Korrektur wurde benutzt, um Genorte zu finden, die möglicherweise unter Selektion stehen („Outlier“). Angepasste $F_{ST}$ Werte, im Mittel mit $F_{ST}=0.0208$, zeigen ebenso wie die Mikrosatelliten-Analyse eine niedrige Differenzierung zwischen den drei Populationen. Mittlere $F_{st}$ Werte auf Basis aller Marker zeigen eine moderate Differenzierung zwischen den Gruppen P2-P4 ($F_{ST}=0.0562$) und P3-P4 ($F_{ST}=0.0619$) und eine geringe Differenzierung in der Gruppe P2-P3 ($F_{ST}=0.0187$). Mit Hilfe von DFDIST konnten unter den 222 analysierten AFLP-Loci 15 Genorte als „Outlier“ ($P<0.01$) identifiziert werden, von denen die meisten auch im $\chi 2$-Test mit Bonferroni Korrektur ($P<0.01$) als signifikant eingestuft wurden. Eine ähnliche Anzahl von „Outlier“ wurde auch im paarweisen Vergleich zwischen den beiden belasteten Kollektiven und der Kontrolle gefunden: 7 Genorte für das Paar P2-P4 und 8 Genorte für das Paar P3-P4. Lediglich 3 „Outlier“ wurden im direkten Vergleich der beiden belasteten Kollektive P2-P3 detektiert. Die „Outlier“-Genorte können in zwei Gruppen eingeteilt werden, in denen das dominante Allel entweder in der Gruppe der belasteten Populationen überrepräsentiert ist oder in der Kontrolle, wobei die Frequenzunterschiede von 19% bis 92% reichen können. Diese Schwankung ist möglicherweise abhängig von der Funktion des Allels im Genom. Der Vergleich der verschiedenen Populationen zeigt, dass mehr als 6% der detektierten Loci potentielle Kandidatengene sind, die an der adaptiven Stressantwort beteiligt sind.

Die Mutationsraten, die mit Hilfe der AFLP-Marker bestimmt wurden, betragen für das Kontrollkollektiv (P4) $1.06x10^{-3}$ und $3.99x10^{-3}$ beziehungsweise $3.74x10^{-3}$ für die Kollektive P2 und P3. Die Anzahl der Mutationsereignisse war deutlich höher als bei der Mikrosatelliten-Analyse. Das Auftauchen neuer Fragmente, der Verlust von Fragmenten und Insertionen und Deletionen von einem Nukleotid wurden als Mutationen gewertet. Die

Anzahl der Mutationen in den beiden belasteten Populationen war um das dreifache höher als in der Kontrollpopulation (34, 32 und 12 Mutationsereignisse in P2, P3 und P4). Der Vergleich der Kollektive P2-P4 und P3-P4 zeigt eine deutliche Differenzierung der Kollektive ($P<0.0001$). Es konnte gezeigt werden, dass für die Analyse von Mutationsereignissen AFLP-Marker, die sich über das gesamte Genom erstrecken, ein hohes Potential darstellen.

Die Ergebnisse dieser Studie zeigen, dass über das gesamte Genom von *P. sylvestris* verteilt Mutationen stattfinden, ebenso konnten Selektionsprozesse unter den Kiefern aus der Sperrzone von Tschernobyl beobachtet werden. Sollten die meisten Mutationen schädlich sein, oder die Fitness der Organismen reduzieren, dann müssen Kiefern Mechanismen aktivieren, die schützend- reparierend- und anpassungsrelevant sind, um ein Überleben unter permanenter radioaktiver Strahlung zu ermöglichen. Bisher ist es schwierig zu unterscheiden, welcher Teil der DNA von Mutationen betroffen oder von Selektion beeinflusst wird; hierzu sind weitere detaillierte Studien notwendig. So kann die Sequenzierung von Kandidatengenen und des Transkriptoms weitere Informationen darüber liefern, welche Veränderungen in der Nukleotidfolge vorliegen und wie diese die Proteinstruktur oder Funktion beeinflussen. Veränderungen in der Genexpression können zeigen, welche Gene für Selektionsprozesse und das Überleben unter Stressbedingungen eine Rolle spielen. Weitere Untersuchungen von *P. sylvestris* aus der Sperrzone von Tschernobyl könnten neue Informationen über schnelle Anpassungsmechanismen von langlebigen Pflanzen an extreme Veränderungen der Umwelt liefern.

# 7. Manuscripts

## I. Evidence for selection in response to radiation exposure: *Pinus sylvestris* in the Chernobyl exclusion zone

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**Abstract**

Changes of genetic structures due to viability selection are likely to be observed in populations exposed to rapidly and extremely changing environmental conditions after catastrophic events. However, very little is known about the extent of selective responses and in particular the proportion of the genome involved in putatively adaptive reactions for non-model plants. We used amplified fragment length polymorphisms (AFLPs), an anonymous marker technique, in order to investigate genetic differences between pine (*Pinus sylvestris*) trees which were partially exposed to extreme environmental conditions. Genetic variation patterns of pines exposed to high radiation in the Chernobyl exclusion zone with or without phenotypic stress symptoms were compared to control trees with a similar origin. Six percent of the investigated loci (15 of 222 loci) were identified as candidate regions for selective responses due to high differentiation between the groups of irradiated vs. non-irradiated trees. Moderate differentiation was observed between groups of trees showing either weak or strong phenotypic responses to high radiation levels. Our results indicate that genetic responses to extreme environmental change such as the long-term exposure of trees to radiation in the Chernobyl exclusion zone may not be restricted to few loci under strong selection, but may involve adaptive changes at a comparatively large part of the genome.

Key words: Adaptation, radiation, *Pinus sylvestris*, selection, AFLP, outlier loci

## Introduction

Exposure of plant populations to extreme environmental change requires adaptive responses to ensure survival and reproduction. Most terrestrial ecosystems are dominated by long-lived woody plants. Rapid evolution in response to environmental stress seems possible in plants with a short life span if strong selection acts uniformly over several generations. However, woody plants with long generation cycles and late beginning of flowering appear less prone to selective changes of genetic structures. Other mechanisms of adaptation such as phenotypic plasticity (Schlichting and Smith 2002), epigenetic effects, or genome methylation (Kalisz and Kramer 2008) are possibly involved in adaptive responses of trees.

One from the most basic unsolved problems of evolutionary biology concerns the number and location of genes involved in adaptation and selection (Storz 2005). Genetic differentiation among populations is not uniform across the genome (Luikart et al. 2003). Populations exposed to different environments are expected to become genetically differentiated from each other at 'adaptive' genes and possibly closely associated genomic regions (Hansson and Westerberg 2002). Developing adaptations to local conditions, selection may only involve a few genes, but these genes will have main functional roles (Black et al. 2001; Luikart et al. 2003). The identification of these genes is a challenging task which has rarely been achieved for non-model plants. The comparison of genetic variation patterns at many molecular marker loci across the genome may be used to identify loci with atypical differentiation, which are likely to be linked to the genomic regions affected by selection (Black et al. 2001). Large between-population allele frequency differences at the loci can be interpreted as the evidence of adaptive genetic differentiation in this case (Linhart and Grant 1996; Nevo 2001).

Changes of genetic structures have been observed in response to strong environmental stress for a number of plant species including forest trees: industrial pollution for *Pinus sylvestris* (Dukharev et al. 1992; Prus-Glowacki et al. 1999) and *Picea abies* (Longauer et al. 2004); water and nutrient stress for *Pinus edulis* (Cobb et al. 1994; Mitton and Duran 2004); temperature for *Betula pendula* (Kelly et al. 2003).

Regions of the genome that are influenced by selection are likely to be functionally-active parts of DNA (Nielsen 2005). Various methods have been developed to reveal genomic regions that can be the target of natural selection (Vasemägi and Primmer 2005).

Associations between adaptive phenotypic traits and underlying genotypes are investigated by QTL (Quantitative trait loci) approaches in known pedigrees (Gailing et al. 2009) or in association populations of unrelated plants (Neale and Savolainen 2004). Since a genome-wide scanning of genetic variation is not yet feasible for most non-model plants, genetic diversity is frequently assessed at few *a priori* selected candidate genes. The 'candidate gene' approach has recently been questioned since most adaptive diversity may not be detected (Storz 2005). This is particularly risky in case of non-model plants with limited sequence information and even less knowledge about gene functions (Ford 2002; Erickson et al. 2004).

Alternative methods have been proposed, which enable genome selection studies in the absence of prior knowledge about the selectively advantageous gene or phenotype (Storz 2005). The development of the amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995) allows to investigate hundreds of random markers covering large parts of the genome, albeit in low resolution. This method may be applied to any species of interest without any prior sequence knowledge, and allows obtaining highly reproducible and informative data. Nowadays AFLP is one of the most used methods in the fields of conservation and evolutionary genetics and ecology (Mueller and Wolfenbarger 1999; Bonin et al. 2007; Meudt and Clarke 2007).

AFLP variation makes possible to reveal the effects of natural selection using the $F_{ST}$ outlier method, which assumes that loci directly affected by selection are expected to show a higher differentiation in comparison to 'neutral' loci. The $F_{ST}$ outlier method has been used to detect candidate loci under natural selection in numerous species, for example, *Coregonus clupeaformis* (Campbell and Bernatchez 2004), *Quercus robur* and *Q. petraea* (Scotti-Saintagne et al. 2004), *Picea abies* (Achere´ et al. 2005), *Rana temporaria* (Bonin et al. 2006), *Fagus sylvatica* (Jump et al. 2006), *Hesperostipa comata* and *Sporobolus airoides* (Mealor and Hild 2006), *Hylobius abietis* (Joost et al. 2007), *Littorina saxatilis* (Galindo et al. 2009).

Selection is particularly difficult to detect if the underlying environmental variation did not persist for many generations, but if a drastic environmental change has been recently imposed on a population of long-living organisms. In this case, viability selection may not yet have wiped out all non-adapted genotypes, and it is possible to compare pairs of neighboring individuals which experienced similar stress conditions but exhibit contrasting phenotypes. A significant difference of genetic structures between the 'sensitive' and the

‘tolerant’ group indicates that the respective loci may be involved in adaptation and, thus, in the selection process (Müller-Starck 1985; Ziehe et al. 1999).

Ionizing radiation is well known as a strong stress factor which causes organism’s reaction even at low levels of irradiation (Real et al. 2004). The response mechanisms of plants to radiation exposure are comparable to other stress factors since metabolic pathways in reaction to sudden stress exposure are rather uniform irrespective of the specific type of stress (Holst and Nagel 1997). Thus, the investigation of radiation effects on populations may also help to better understand adaptation to less severe environmental change and its impact on selection.

After the explosion of the Chernobyl nuclear power plant in April 1986, surrounding territories were contaminated with large amounts of radioactive materials. Plantations of Scots pine (*Pinus sylvestris* L.) which were the dominating vegetation in the vicinity of the exploded reactor block were severely affected by ionizing radiation. Pines growing in the direct vicinity of the nuclear power plant received radiation doses up to 100 Grays (Gy). Most of these trees died; the area with the dead trees became known as “Red forest”. It was turned to the places of nuclear wastes storage. In order to prevent secondary dust contamination with soil erosion, this area was again afforested with pine seedlings. During the years after the accident high mortality rates were observed in the plantations. Most surviving trees show decreased growth, low vitality, and an increase in the frequency of morphological abnormalities (Arkhipov et al. 1994; Kal'chenko et al. 2001).

In this study, we compared pine seedlings from radiation-exposed and non-radiated control areas to observe signs of selection in response to extreme environmental stress. Sampling in the “Red forest” was performed by the identification of neighboring plants with strong and weak damage followed by the collection of tissue samples from these pairs of trees with contrasting phenotypes. We used AFLP markers for genotyping all sampled seedlings, calculated frequency distributions of fragments and alleles, and estimated genetic differentiation between different demes with the objective to identify candidate regions involved in selection. We tested the ‘null’ hypothesis that genetic structures of seedlings which were exposed to radiation do not significantly differ from seedlings of the same origin in control areas, and that the genetic structures of seedlings with strong symptoms of damage do not differ from seedlings with no or minor visible damage.

## Materials and methods

### Study species, sites and sampling

*Pinus sylvestris* (Scots pine) is a monoecious, diploid, fast growing evergreen conifer, capable of survival infertile soils. It is native to Europe and Asia, from Norway to Spain, and to eastern Siberia. Scots pine is a good model to study the genetics of adaptation because of high level of genetic diversity, strong stress response and long history of ecological genetic research (Morgenstern 1996; Neale and Savolainen 2004; Guevara et al. 2005). A large genome size as compared to the most other plant species - 24,6 Mb (Ahuja et al. 2005) - and big interphase chromosomes – 61.1 µm3 (Conger et al. 1982) - possibly contribute to one of the highest sensitivities to radiation ($LD_{50}$ is 5–20 Gy) among species investigated by Sarapul'tsev et al. (1993).

We sampled material (fresh needles) from pine plantations established after the accident on heavily contaminated soils close to the nuclear power plant. Material was collected from 20 years old trees which have been planted after the accident and exposed to chronic irradiation throughout its persistence at the site. Needles were samples from directly neighboring pairs of trees with contrasting phenotypic appearance: one tree with no or minor damage and one tree with strong damage symptoms (abnormalities in growth, morphogenesis, buds and shoots formation, yellowish needles etc.). Fifty tree pairs were sampled in the "Red forest" area. Deme P2 consists of trees with strong damage (irradiated damaged), deme P3 consists of trees with no or only minor damage symptoms (irradiated healthy). We also sampled 66 pine trees randomly selected in the plantation established in the area with low radiation exposure, which will be used as control plot. These pines were planted in the same year as seedlings on the contaminated soil; they have the same age and origin. Thus, the genetic structures of this deme P4, which will serve as a control in our study, is assumed to reflect the structures of the trees planted without selection in response to radiation. The high survival rate of the trees from the control plantation allows us to preclude strong selection making them suitable material for comparison. Other demes (P1 and P5) were sampled to assess mutation rates under the influence of radiation in pines, but not used for this study (Kuchma et al., in prep.) In summary, material used for this study was harvested from 166 trees. All samples were stored in silica gel until DNA extraction and genotyping.

The radiation exposure of the plant material was quantified by measuring the radiation dose on site and by calculation of the absorbed radiation doses from the main doseforming radionuclides in pine tissues. The present radiation dose in “Red forest” is 0.9 – 4.4 mR/h, about 100 – 400-folds over background levels measured for the control plot (0,005-0,008 mR/h). The accumulated dose was 9.8 Gy/year for the “Red forest” and $3x10^{-6}$ Gy/year for the control plot, respectively.

**DNA isolation and AFLP genotyping**

Genomic DNA was extracted from dried needles using the DNeasy 96 Plant Kit protocol of QIAGEN (QIAGEN GmbH, Hilden, Germany).

AFLP analysis was performed according to Vos et al. (1995) with minor modifications. Total genomic DNA (4 µl) was digested with the restriction enzymes EcoRI/MseI. Double-stranded *Mse*I and *Eco*RI adaptors were ligated to the ends of the restriction fragments to generate template DNA for polymerase chain reaction (PCR) amplification. The restriction/ligation reaction was performed at room temperature overnight. Diluted ligation mix was used for preamplification reactions with the preselective primer pairs E01/M03 and the selective nucleotide A and G, respectively. The reaction started at 72°C for 2 min, followed by 20 cycles each consisting of 94°C for 10 sec, 56°C for 30 sec, and 72°C for 2 min, and a final step at 60°C for 30 min. Selective PCR were carried out using diluted DNA from the preamplification reaction with the primer combination *Eco*RI-ACA and *Mse*I-GAAC (nomenclature according to Keygene N. V.). Primer *Mse*I-GAAC was labeled with the fluorescent dye FAM. Cycling conditions for the selective PCR were as follows: start at 94°C for 2 min, 10 cycles, each consisting of 10 sec at 94°C, 30 sec at 65°C and 2 min at 72°C. The 65°C annealing temperature of the first cycle is subsequently reduced by 1°C for the next 10 cycles and continued at 56°C for 30 sec for the remaining 23 cycles, and ends with a final extension step at 60°C for 30 min. This primer combination was selected from a test of 16 primer combinations in 8 samples of *P. sylvestris* based on the production of polymorphic, reproducible, clear and easily scorable fragments. At least two samples were run on each plate to test repeatability of the fragments. A total of 222 fragments with 100% reproducibility in size range from 75 bp to 324 bp were selected for further analyses. All PCR reactions were conducted in a Peltier Thermal Cycler (PTC-200 version 4.0, MJ Research). The fragments were separated electrophoretically on an ABI Genetic Analyzer 3100 together with the internal size standard GeneScan 500 ROX

(fluorescent dye ROX) from Applied Biosystems. The size of the AFLP fragments was determined with the software packages GeneScan 3.7 and Genotyper 3.7 (Applied Biosystems Inc.). AFLP markers were scored according to the absence/presence of peaks, i.e. as dominant markers and transformed to a 1/0 matrix. Each fragment was controlled and edited manually.

**Data analysis**

The software AFLP-SURV version 1.0 (Vekemans et al. 2002; available at http://www.ulb.ac.be/sciences/lagev/) was used to estimate allelic frequencies at AFLP loci from the observed fragment frequencies according to a Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky 1999) and assuming Hardy-Weinberg proportion. The genetic diversity for each population was estimated by the percentage of polymorphic loci (*PPL*) at the 5% level (i.e. loci with allelic frequencies lying within the range 0.05 to 0.95) and expected heterozygosity ($H_e$).

A $\chi 2$-test was performed to test for significant deviation between observed and expected frequencies of band presence at each locus in pairwise and global population comparisons. A Bonferroni correction for the 99% confidence level was applied to avoid detection of false positive (Rice 1989).

Identification of outlier loci was performed using the program DFDIST (available at http://www.rdg.ac.uk/~mab/stuff/), which is based on the approach developed by Beaumont and Nichols (1996). It was modified from Beaumont and Balding (2004) to analyze dominant data. Allele frequencies are estimated in DFDIST based on Zhivotovsky (1999), and $F_{ST}$ (Weir and Cockerham 1984) is calculated for each locus in the sample. The program follows a hierarchical Bayesian approach to compute $F_{ST}$ values conditional on heterozygosity in a subdivided population, using coalescent simulations to generate thousands of loci evolving under a neutral model of symmetrical islands (Wright 1951) with a mean $F_{ST}$ close to the empirical $F_{ST}$. Single locus $P$ values are calculated for the empirical distribution with the subroutine PV2 (DFDIST package). Those loci with $F_{ST}$ values significantly greater ($P < 0.05$ and $P < 0.01$) than the simulated distribution are considered as outliers (5% outliers and 1% outliers, respectively) for divergent selection. For this study, DFDIST was run for each possible combination of populations (3 single analyses) with two demes (i.e., populations). A global analysis was also done using all

three demes together. The simulations were carried out generating 50,000 loci using a model with two islands. The maximum allowable allele frequency was 0.99, and *Ne* was set to 1000, as indicated by Kuchma et al. (in prep.). For the empirical $F_{ST}$ used in the simulations, a trimmed mean $F_{ST}$ was calculated, where the 30% of the loci with the highest $F_{ST}$ and the 30% with the lowest were removed and the mean $F_{ST}$ was computed. Trimming the $F_{ST}$ removes the effects of the outlier loci on the mean $F_{ST}$ value; its use has been supported by simulation studies (Beaumont and Balding 2004; Caballero et al. 2008). We chose the 0.995 value or 0.05% critical probability to define an area within which 99% of the data points are expected to lie and reduce the false positive rate as recommended by Caballero et al. (2008). All loci occurring outside this limit were regarded as outliers potentially under selection.

## Results

### Genetic diversity and overall differentiation

For the 164 individuals analysed with the AFLP technique, 222 bands were scored in total. Mean heterozygosity over all samples was 0.231. Overall genetic diversity is high in all populations; only minor differences were observed (Table 1). An analysis of population differentiation after performing comparison of demes with DFDIST is presented in Table 2. The global $F_{ST}$ between demes was 0.0454. In pairwise analyses, trimmed $F_{ST}$ values (0.0013-0.0234) indicated low differentiation between all populations, while untrimmed values showed moderate level of differentiation between P2/P4 and P3/P4 with $F_{ST}$ 0.0562 and 0.0619, respectively. Genetic differentiation was very low between the population exposed to high radiation with or without exhibiting strong symptoms of damage: $F_{ST}$ between the pair P2/P3 is only 0.0187.

### Outlier loci detection

Results of significance test with Bonferroni correction are presented in Table 2. In total, 23 loci out of 222 were identified as significant with a significance threshold set to 4.5E-5 (corresponding to a 99% confidence level including Bonferroni correction). No significant differences between loci were detected in pairwise comparisons between P2-P3, while the numbers of loci with significant differentiation were 7 and 17 for P2-P4 and P3-P4

comparisons, respectively. A global analysis of all demes (P2-P3-P4) revealed that 19 loci significantly deviated after Bonferroni correction.

The number of outlier loci after pairwise analyses of population differentiation and for the overall data set of three populations are given in Table 3. $F_{ST}$ is plotted against heterozygosity in Figure 1. The pairwise population comparisons performed with DFDIST resulted in a total of 15 different loci showing higher differentiation than expected at the 99% confidence level. All these loci are good candidates to be outliers, but some of them deserve special attention due to big differences in allele frequencies between populations, because the number of recessive phenotypes (band absence) is crucial for their estimation.

Significant differences of allele frequencies in pair comparisons were indicated by (a), (b) and (c) for P2/P3, P2/P4 and P3/P4, respectively, in Figure 2. A global analysis on all three demes revealed eight loci above the 99% confidence line (Table 3). Loci 82, 94, 318 were identified as outliers in two independent pairwise comparisons as well as in the global analysis. Five loci (83, 92, 121, 201, 317) were outlier loci in one of the pairwise comparisons and in global analyses. Seven loci (90, 93, 97, 122, 170, 183, 235) showed significant values only in one of the pairwise comparisons, but were above the 95% confidence level in the global analysis, as well; they still can be considered as “true” outliers. Most of the identified loci can be divided into two groups: the dominant allele is either overrepresented in irradiated populations or in the control population. Large differences in allele frequencies, with variation from 19% to 92% between compared demes, show locus 97, detected as an outlier in pair comparison P2/P3, as well as loci 121, 183, 201, 317 in pair comparison P2/P4, and loci 90, 92, 93, 235 in pair comparison P3/P4. An allele responsible for a fragment of size 97 bp was estimated with a frequency of 0.3 in the irradiated ‘healthy’ deme (P3) and a frequency of 0.06 in the irradiated ‘damaged’ deme (P2); the frequency in the control (P4) was only 0.01. On the other hand, the frequency of an allele causing a fragment at position 317 bp was 0.44 in the control, but the allele was not observed in the deme P2 and with a frequency of 0.16 in the deme P3. These two markers were identified as significant after applying of Bonferroni correction and are obvious candidates for an involvement in selective responses to high radiation. The number of loci that showed significant deviation after DFDIST analysis and Bonferroni correction in at least one pair comparison are presented in Table 3. Five loci in comparison P2-P4, seven loci in P3-P4 pair and eight loci in the global analysis were detected as highly significant with both tests.

The percentage of outlier loci detected between demes in pairwise comparisons and in the global analysis was above 3%. In total, 6% of the observed loci were identified as candidate loci under the influence of selection in at least one comparison.

**Table 1** Genetic diversity within demes of *Pinus sylvestris*

| Deme | Sample size | Polymorphic loci | *PPL* | *He* |
|---|---|---|---|---|
| P2 | 48 | 165 | 74.3 | 0.230 |
| P3 | 50 | 169 | 76.1 | 0.224 |
| P4 | 66 | 173 | 77.9 | 0.239 |

*PPL*: percentage of polymorphic loci; *He*: expected heterozygosity within demes

**Table 2** Results of $\chi 2$-test with Bonferroni correction at $P < 0.01$

| | Deme comparison | | | |
|---|---|---|---|---|
| | P2/P3 | P2/P4 | P3/P4 | P2/P3/P4 |
| No loci | - | 7 | 17 | 19 |
| % | - | 3.2 | 7.2 | 8.6 |

No loci: number of loci with significant differences in allele frequencies at the 99% confidence level; %: percent of loci detected as significant among 222 analysed loci

**Table 3** Deme differentiation of *Pinus sylvestris* and results of the outlier detection for 222 AFLP loci

| | Deme comparison | | | |
|---|---|---|---|---|
| | P2/P3 | P2/P4 | P3/P4 | P2/P3/P4 |
| Outlier locus, bp | $F_{ST}$ | | | |
| 82 | -0.010116 | $0.363863_a^{**}$ | $0.400109_a^{**}$ | $0.283293_a^{**}$ |
| 83 | $0.071718^{*}$ | 0.092195 | $0.307967_a^{**}$ | $0.165845_a^{**}$ |
| 90 | $0.082264^{*}$ | 0.089400 | $0.294899_a^{**}$ | $0.164750^{*}$ |
| 92 | -0.009456 | $0.300444^{*}$ | $0.326778_a^{**}$ | $0.257511_a^{**}$ |
| 93 | $0.110836^{*}$ | 0.037866 | $0.260162_a^{**}$ | $0.152526^{*}$ |
| 94 | $0.298685^{**}$ | $0.311777^{**}$ | n/v | $0.272011_a^{**}$ |
| 97 | $0.081566^{**}$ | 0.004603 | $0.132470^{*}$ | $0.095025^{*}$ |
| 121 | 0.002027 | $0.223091_a^{**}$ | $0.164690^{*}$ | $0.172096_a^{**}$ |
| 122 | $0.152756^{**}$ | -0.018842 | $0.197818^{*}$ | $0.126135^{*}$ |
| 170 | -0.003446 | $0.181343^{*}$ | $0.289632_a^{**}$ | $0.176399^{*}$ |
| 183 | 0.013240 | $0.303380_a^{**}$ | 0.181564 | $0.165130^{*}$ |
| 201 | 0.108086 | $0.351322_a^{**}$ | 0.096242 | $0.198765_a^{**}$ |
| 235 | 0.028512 | 0.088401 | $0.208839_a^{**}$ | $0.123180^{*}$ |
| 317 | 0.069188 | $0.239654_a^{**}$ | 0.086069 | $0.144972_a^{**}$ |
| 318 | 0.069188 | $0.333831^{**}$ | $0.335881^{**}$ | $0.298274_a^{**}$ |
| | $No_{DFDIST}$ (%) | | | |
| | 3 (1.4) | 7 (3.2) | 8 (3.6) | 8 (3.6) |
| | $No_{Bonfer}$ | | | |
| | - | 5 | 7 | 8 |
| | Mean outlier $F_{ST}$ | | | |
| | 0.0187 | 0.0562 | 0.0619 | 0.0454 |
| | Mean trimmed $F_{ST}$ | | | |
| | 0.0013 | 0.0234 | 0.0222 | 0.0208 |

a: loci whose deviation was still significant after Bonferroni correction; n/v: no value; P value: $P<0.05^{*}$, $P<0.01^{**}$; $No_{DFDIST}$ (%): the number and percent of outlier loci detected with DFDIST at the 99% confidence level; $No_{Bonfer}$: the number of common loci identified as significant between DFDIDT and $\chi 2$-test at $P<0.01$; $F_{ST}$: proportion of total genetic diversity partitioned among demes; mean outlier $F_{ST}$: values, calculated for the whole set of loci; mean trimmed $F_{ST}$: values, calculated after removing 30% of loci with the highest and with the lowest $F_{ST}$

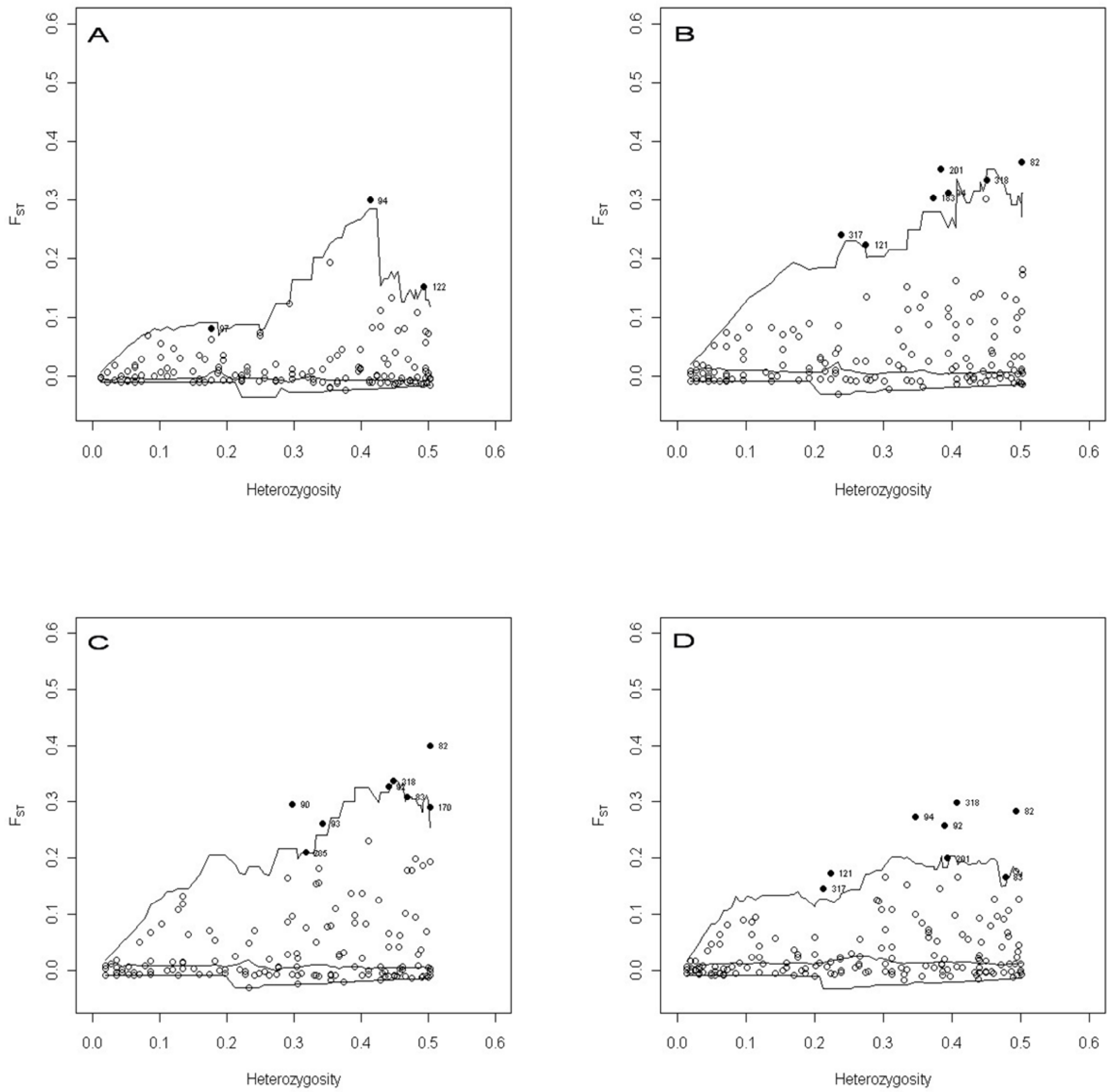


**Fig. 1** Results of the simulations performed with DFDIST. Plots of $F_{ST}$ values against heterozygosity estimated for the deme pairs: P2/P3 (A), P2/P4 (B), P3/P4 (C) and global analysis P2/P3/P4 (D). Each dot indicates AFLP marker; black dots referred by numbers are significant (P<0.01) outliers. The lower, intermediate and higher lines represent the 5%, 50% and 99% confidence intervals, respectively.

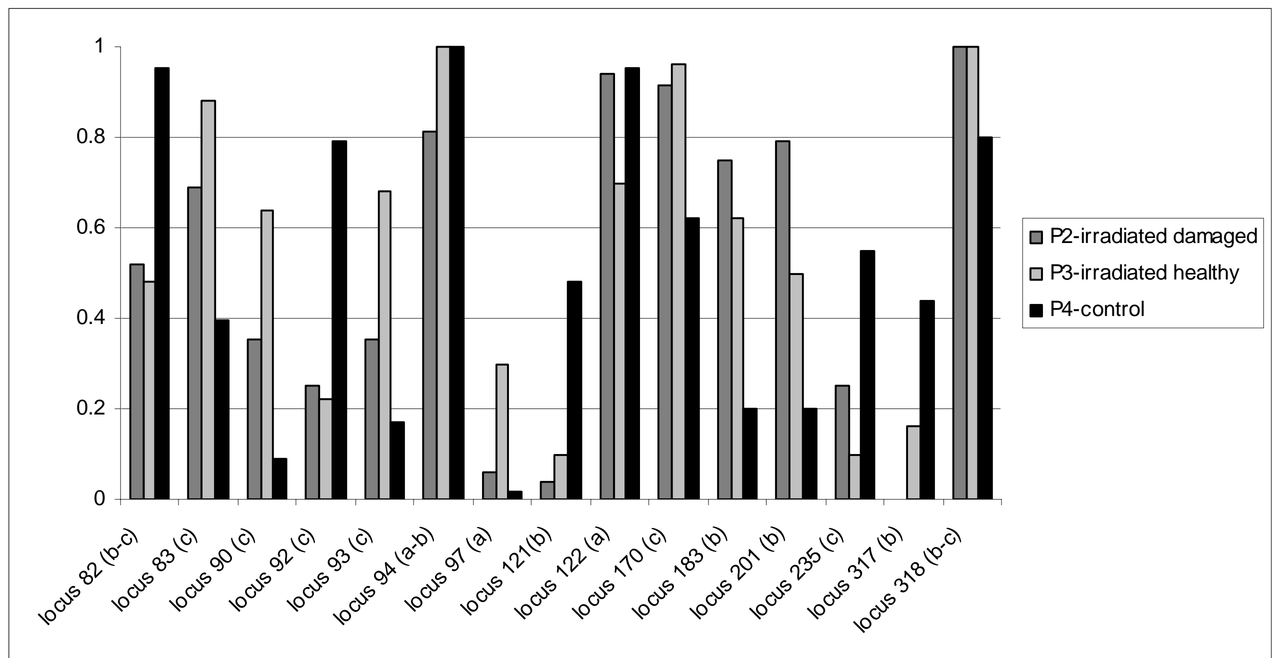


**Fig. 2** Frequencies of the dominant alleles at AFLP loci revealed as outliers by DFDIST between compared deme pairs. Significant differences in pair comparisons are indicated by (a) for P2/P3, (b) for P2/P4 and (c) for P3/P4.

## Discussion

Plants are immobile and, accordingly, need to adapt to even extreme environmental change at their respective growing sites for survival. Evolutionary adaptations by changes of genetic structures due to selection are believed to be one of the main responses of populations to environmental stress. Although a mechanistic understanding of selective responses to rapid environmental change has rarely been achieved (Nielsen 2005), numerous experimental studies suggest adaptation by selective changes of genetic structures in plant populations including the trees *Betula pendula* (Kelly et al. 2003), *Pinus edulis* (Cobb et al. 1994; Mitton et al. 1998; Mitton and Duran 2004), *Picea abies* (Bozhko et al. 2003; Longauer et al. 2004) and *Pinus sylvestris* (Dukharev et al. 1992; Prus-Glowacki et al. 1999).

Pre-existing or “standing” genetic variation is a requirement for evolutionary adaptations to rapid environmental change. *P. sylvestris* is known to be a highly variable species (Neale and Savolainen 2004), and our results confirm high levels of polymorphism in pines planted in the Chernobyl Exclusion Zone and control stands. Previously neutral genetic variation may become adaptive if selection favours certain alleles or genotypes at

particular gene loci. However, strong viability selection is unlikely to simultaneously occur at many loci due to the drastic reduction in population size. Strong changes of allelic or genotypic structures as a result of viability selection are expected to be limited to a minor part of the genome.

The accident of the Chernobyl nuclear power plant in April 1986 created unique conditions for investigating the influence of radiation as a strong and long-lasting stress factor on plants. Selection in response to radiation several orders of magnitude above natural levels seems likely. Ionizing radiation is known to have general adverse effects on plant growth and development (Holst and Nagel 1997), activating universal mechanisms of stress response. Experimental data suggest that low-level irradiation induces general stress response (Real et al. 2004) that makes radiation an interesting factor to study plant adaptation to extreme environments (Kovalchuk et al. 2004; Zelena et al. 2005).

Experiments on adaptation of *Pinus sylvestris* to high radiation started in the early 1960s (Sparrow and Woodwell 1962) demonstrating very low radiotolerance and similar radiosensitivity between pine and human cells. Scots pine became one of the main natural objects for ecological genetic monitoring (Prus-Glowacki et al. 1999; Micieta et al. 1998) and after the Chernobyl accident one of the main species for investigation of radiation effects on organisms. Different types of morphological abnormalities (Arkhipov et al. 1994), mutagenesis of enzyme loci (Kal'chenko et al. 1995) replacement of radiosensitive cells by more radioresistant cells (Kal'chenko and Fedotov 2001), epigenetic variability, i.e. changes in the spectrum of functionally active genes (Zelena et al. 2005), increase in the cytogenetic aberration rate (Geras'kin et al. 2005), and hypermethylation of the DNA (Kovalchuk et al., 2003) have been reported for pines irradiated after the Chernobyl accident. Even though the phenotypic appearance of many pines suggests that the plants suffer from high irradiation, adaptation is obviously possible. Here we provide evidence that selection plays a complementary role to the mechanisms described before in the adaptive response of pines exposed to high radiation.

We used the anonymous AFLP technique to identify genomic regions putatively under selection, and found strong evidence that selection in response to high radiation changed the genetic structures at several markers. We did not further characterize the fragments showing contrasting frequencies between the different demes. It is quite likely that the fragments are not parts of selective genes, but are associated to polymorphisms responsible for different adaptive responses. However, rapid decay of linkage disequilibrium has been

found in most trees (Savolainen and Pyhäjärvi 2007) including pines (Neale and Savolainen 2004). Accordingly, the observation of several outlier loci indicating selective responses was not expected.

We compared genetic structures of trees demonstrating strong phenotypic differences under comparable irradiation conditions and revealed loci which can be responsible for such contrasting characteristics. Summary estimates (sum of outlier loci observed in any of the pairwise comparisons) indicated that more than 6% of detected loci are candidates for the adaptation response between compared populations, whereas pairwise estimates (outlier loci in a single specific pairwise comparison) were 1.4-3.6% (Figure 1, Table 3). In other studies the percentage of candidate loci in multiple population comparisons was mostly similar, varying between 1% and 8% (e.g. Campbell and Bernatchez 2004; Emelianov et al. 2004; Acheré et al. 2005; Beaumont 2005; Nielsen 2005; Storz 2005; Bonin et al. 2006; Jump et al. 2006; Mealor and Hild 2006; Nosil et al. 2008; fully reviewed in Galindo et al. 2009) and only a few studies showed higher percentage, but with comparing different species (12% in Scotti-Saintagne et al. 2004; 16% in Papa et al. 2007; 15% in Egan et al. 2008). Most of the loci identified as outliers by DFDIST were pointed as significant also with Bonferroni correction (Table 3). Few outlier loci which were not detected by significance test have quite small differences in allele frequencies (19 – 21%). Possibly, it is hard to reveal them by a homogeneity test, but they can be identified by locus to locus comparison provided in DFDIST analysis where even small difference in allele frequencies may be significant. Results of both tests complement each other and suggest that a relevant part of genome is affected by the adaptation process.

We cannot rule out that some of the putatively selective loci are in fact neutral, but we used stringent condition for the detection of outliers. The investigated populations are strictly comparable since they have a common origin from a seed crop of the same clonal seed orchard from the same year. Only strictly repeatable loci were analyzed, positive and negative controls were included in all analyzes, and all fragments were manually scored. Most importantly, we followed the suggestions by Caballero (2008) for the detection of candidate loci for selection using the $F_{ST}$ outlier approach to minimize the likelihood to include a large number of false positives: a low significance threshold ($< 0.01$), a large selection coefficient affecting the loci and the use of a trimmed $F_{ST}$.

The trimmed $F_{ST}$ represents an estimate of the average “neutral” $F_{ST}$ value uninfluenced by outlier loci. Our estimate of $F_{ST} = 0.021$ on average indicates low differentiation

between all populations as expected for pines. This observation also confirms the common origin of the investigated stands. However, the untrimmed $F_{ST}$ indicates a moderate level of overall differentiation, if populations planted on irradiated sites (P2 and P3) are compared to the control (P4) (Table 3). Thus, the large number of outliers influenced overall differentiation between these populations. Low differentiation was observed between the two irradiated demes with contrasting phenotypes (strong and weak symptoms of stress, respectively), and only few outlier loci did not strongly elevate the overall differentiation. Thus, the investigated markers are unlikely to be directly involved in the specific symptoms used to assess the health status of the sampled plants, but may have been responsible for the survival of both 'damaged' and 'healthy' plants planted under high radiation.

Pairwise comparisons revealed that up to 8 out of 222 investigated loci are outliers showing unexpectedly strong differentiation between different demes. In total, our genome scan pointed out 15 loci potentially involved in adaptation to the influence of radiation. Even if it is very unlikely that most or even all of these makers are within functioning genes involved in adaptation, our results point towards complex selective responses to radiation involving numerous genomic regions.

Our results prove the usefulness of the outlier locus methods to study adaptation of long-living organisms like forest trees to rapid and extreme environmental change. Long-term effects of radiation can be studied after the analyzed populations start to reproduce, since fertility selection is expected to cause further changes of genetic structures. Sequencing of AFLP fragments showing strong frequency differences between the irradiated demes and the control will potentially allow a functional assessment of the investigated fragments. However, most fragments are expected in non-coding regions in view of the large genome size of pines (Ahuja et al. 2005). Thus, only the availability of a genome sequence of a conifer, ideally a pine, will facilitate the identification of the genome region of most sequenced AFLP fragments. Comparative sequencing of candidate genes for adaptation to high radiation is another valid approach to further study selection in pines from Chernobyl. Sequencing and SNP genotyping in candidate genes are enhanced by recent technological advances, in particular high throughput sequencing methods. Our results indicate that selection in response to high radiation is observable in pines from the Chernobyl exclusion zone. Accordingly, these trees are ideal models to further analyze rapid adaptation of long-

living plants to extreme environmental change, and to take selection causing evolutionary adaptations into consideration in this context.

## II. Mutation rates in Scots pine (*Pinus sylvestris* L.) from the Chernobyl exclusion zone evaluated with AFLP and microsatellite markers

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### Abstract

Radiation is a strong mutageneous factor; accordingly, elevated mutation rates are expected in plants exposed to high chronic or acute radiation after the Chernobyl accident in 1986. The absence of a germline in plants implies that somatic mutations may be transferred to gametes and progenies. We observed somatic mutations in pines (*Pinus sylvestris* L.) planted before and after the accident and control material of the same origin planted in control sites with natural radiation. We collected plant tissue from different main branches of single trees and interpreted intraindividual variation as an indication of a mutation event. Microsatellites (SSRs) and Amplified Fragment Length Polymorphisms (AFLPs) were investigated. The mutation rates for microsatellites were estimated as $2.8 \times 10^{-4}$ to $7.1 \times 10^{-4}$ per locus for different irradiated tree populations; no mutations were detected in the control. In case of AFLPs, the observed mutation rates were $3.74 \times 10^{-3}$-$3.99 \times 10^{-3}$ and $1.06 \times 10^{-3}$ per locus for contaminated and control areas, respectively. A statistically highly significant three fold increase in number of mutations was found by the use of AFLP markers. Our data suggest that radiation causes strong DNA damage across the entire genome and AFLPs may be used as molecular marker for investigation of mutation rates under the influence of radiation.

Key words: Mutation rate, radiation, *Pinus sylvestris*, microsatellites, AFLP, Chernobyl

### Introduction

The accident on 26 April 1986 at the 4th unit of the Chernobyl Nuclear Power Plant (ChNPP) resulted in the largest reported accidental release of radioactive materials. The level of background gamma-irradiation in many parts of the contaminated zone was as

high as 1-20 mR/h. The accident contaminated over 145 000 $km^2$ of the territory of the Ukraine, the Republic of Belarus and the Russian Federation, the density of radioactive pollution exceeding 37 $kBq/m^2$ ($10^3$ Ci/ $km^2$) in those areas. Besides these three countries most affected, the radioactive cloud after the explosion at the ChNPP also affected Sweden, Norway, Poland, the United Kingdom, Austria, Germany, Finland and Switzerland (National Report of Ukraine 2006). An area of more than 200 000 $km^2$ in Europe was contaminated with radioactive fallout (Environmental consequences of the Chernobyl accident 2006).

All organisms on the territory of the 30-km ChNPP exclusion zone were exposed to high doses of acute radiation in the first post-accident days and continued to be exposed to chronic radiation to the present days. However, the doses of radiation received by biota vary widely – from exposures that are lethal at least for the most radiosensitive species to exposures similar to background levels of natural radioactivity. The most sensitive ecosystem to radiation exposure was the pine forest around ChNPP (National Report of Ukraine 2006). Even 20 years after the accident, trees show various symptoms of strong radioactive damage.

Ionizing radiation was the first identified mutagen and causes a wide range of intragenic and intergenic mutative changes (Evans and DeMarini 1999). The range of mutation events caused by radiation, vary from simple base substitutions to single and double-strand breaks of DNA in all living organisms (Grosovsky et al. 1988). An increase of mutation rates is part of the complex reactions of an organism to exposure to ionizing radiation. Natural mutation processes rarely affect the genome organization, but a severe damaging factor such as high radiation may cause significant changes in the structure and functioning of the genome. During the years after the Chernobyl accident a number of studies were conducted that investigated changes in mutation rates around Chernobyl, often compared to control areas. Most of these studies were conducted on humans or animal species. Investigation of germline mutations showed significant increases in mutation rates (Ellegren et al. 1997; Dubrova 1998; Weinberg et al. 2001; Dubrova et al.2002), while changes in rates of somatic mutations mostly were not significant. Only few studies were conducted on plant species about changes of mutation rates; studied plant species included *Pinus sylvestris* (Shevchenko et al. 1996), *Arabidopsis thaliana* (Abramov et al. 1992), and *Triticum sativum* (Kovalchuk et al. 2000). Plants from the Chernobyl area often showed increased levels of somatic mutations compared to control plants. The number of lethal and

chlorophyll mutations in all studied populations of *Arabidopsis taliana* in the 30-km exclusion zone was up to 8-fold higher than the spontaneous level (Abramov et al. 1992). The frequency of microsatellite mutations in *Triticum sativum* was 6-fold higher in the contaminated territories (Kovalchuk et al. 2000). The average frequency of mutations in *Pinus sylvestris* in the 30-km zone was 10-fold higher than in control locations at enzyme loci (Shevchencko et al. 1996; Kal'chenko and Fedotov 2001). Unfortunatelly, the knowledge of changes of mutation rates in plants under the influence of radiation is still small, though the absence of a predetermined germline in plants implies that somatic mutations can be inherited to the next generation. This makes plants excellent object for an investigation of mutation processes.

In the present study, we analysed different trees of *P. sylvestris* which grow in the Chernobyl exclusion zone and control plants. All trees in the exclusion were exposed to chronic irradiation. One population experienced high acute radiation during the accident. Two different types of molecular markers were used.

Genomic microsatellites (Simple Sequence Repeats; SSRs) are tandem repetitions of one to six base nucleotide motifs, which are found in the DNA of all investigated higher organisms. Microsatellites show a high degree of polymorphism caused by a difference in the number of repeat units between alleles (Weising et al. 2005). Estimates of mutation rates for SSRs are very high ($10^{-2}$-$10^{-6}$ events per locus per generation) as compared to the rates of point mutation at coding gene loci. The rate of mutations at SSR loci is affected by various factors including allele size, repeated motif, GC content in DNA, cell division (mitotic or meiotic process), chromosome position, genotype and sex (Ellegren 2004). There is increasing evidence that SSR structure may have a functional importance for gene translation, transcription, recombination, DNA replication, chromatin organization, and the cell cycle (Li et al. 2002) although the variation of repeat motifs in SSRs is usually regarded as selectively neutral. An over-representation of SSR repeats was demonstrated in stress response genes (Rocha et al. 2002) suggesting the importance of SSR motifs for adaptation. Due to the functional importance of some microsatellites, selection is expected to act against change of SSR size at those loci. All these characteristics make microsatellites useful tools for the investigation of the influence of radiation on changes in mutation rates. Significant increases in microsatellite mutations have been already observed in *Triticum sativum* (Kovalchuk et al. 2000), *Hirundo rustica* (Ellegren et al.

1997) and in a pilot study on *Pinus sylvestris* (Vornam et al. 2004) from the highly contaminated areas of the Chernobyl exclusion zone.

The amplified fragment length polymorphism (AFLP; Vos *et al*. 1995) technique allows the scoring of a large number of loci which are assumed to be randomly distributed within the genome of the analysed individuals. Mutation rates for AFLPs were estimated as $10^{-5}$-$10^{-6}$ per locus per generation (Mariette et al. 2001; Kropf et al. 2009). In AFLPs, mutations can occur at restriction sites, primer elongation binding sites or due to insertion/deletions (indels) in the amplified region, resulting in the loss and/or an appearance of a 'new' AFLP bands (Ke Li 2007; Kropf et al. 2009).

The AFLP technique allows the amplification of genomic fragments without any knowledge of sequence information, and is expected to cover the genome better than SSRs, since many loci are simultaneously scored. Mutation mechanisms differ between both marker types, which may also differ with regard to mutation rates. In view of these contrasting properties we used both marker types for the same plant material to evaluate the influence of radiation on different parts of the genome.

In the present study, we analyzed pine trees of different age and irradiation conditions from the Chernobyl exclusion zone and control areas. *Pinus sylvestris* (Scots pine) is one from the main forest-forming species, native to Europe and Asia, from Norway to Spain, and to eastern Siberia. High level of genetic diversity, strong stress response and a long history of ecological genetic research (Morgenstern 1996; Neale and Savolainen 2004; Guevara et al. 2005) made pines a good model to study genetic effects in alternating environment. With a large genome size as compared to most other plant species (24,600 Mb; Ahuja et al. 2005), *P. sylvestris* exhibits one of the highest sensitivities to radiation ($LD_{50}$ is 5–20 Gy) among various investigated species (Sarapul'tsev et al. 1993). Experiments on radiation-exposed *P. sylvestris* plants started in early 1960s (Sparrow and Woodwell 1962) and demonstrated very low radiotolerance and similar radiosensitivity between pine and human cells. Scots pine became one of the main natural test objects for ecological genetic monitoring (Micieta et al. 1998; Prus-Glowacki et al. 1999) and one of the most frequently used species for investigations of radiation effects on organism in the Chernobyl exclusion zone (Arkhipov et al. 1994; Kal'chenko et al. 1995; Kal'chenko and Fedotov 2001; Kovalchuk et al. 2003; Geras'kin et al. 2005).

We used microsatellites and AFLP markers in order to detect somatic mutation events in different regions of the tree genome and to evaluate changes in mutation rates between

exposed and control pines. This is the first attempt to combine the two different types of DNA markers for comparative analyses of mutation procesess in pines of different age and irradiation conditions growing in the Chernobyl exclusion zone.

## Materials and methods

### Plant material and sampling strategy

Fresh needles were collected from pine trees (*Pinus sylvestris*) of different age and irradiation conditions (Table 1). The trees were devided in 5 different groups (demes). Deme P1 (Prypiat) is represented by about 50 years old trees growing on heavily radiation-exposed sites close to the nuclear power plant. In the age of 30 years they have been exposed to acute high radiation during the accident in 1986, followed by chronic irradiation until now.

Deme P2 and deme P3 are composed of trees derived from pine plantations established after the accident on heavily contaminated soils close to the nuclear power plant. Material was collected from 20 years old trees which have been planted after the accident and were exposed to chronic irradiation during the whole time. Needles were samples from directly neighboring pairs of trees with contrasting phenotypic appearance: one tree with no or minor damage and one tree with strong damage symptoms (abnormalities in growth, morphogenesis, bud and shoot formation, etc.). Fifty pairs of trees were sampled in the so called “Red forest” area. Deme P2 consists of trees with strong damage (irradiated damaged) and deme P3 consists of trees with no or only minor directly visible damage symptoms (irradiated healthy).

Deme P4 and deme P5 are represented by trees which were randomly selected in the plantations established in the area with low radiation exposure. These pines were planted in the same years as the trees in the Chernobyl exclusion zone (see above) and have the same age and origin. Thus, demes P5 and P4 serve as controls for demes P1 and P2/P3, respectively. In order to exclude the influence of other factors than radiation on mutation rates, from each tree, needles were harvested from four branches with different orientation (North, East, South, West). In total, needles were sampled from 264 trees (Table 1). All samples were stored in silica gel until DNA extraction and genotyping.

The radiation exposure of the plant material was quantified by measuring the radiation dose on site and by calculation of the absorbed radiation doses from the main dose-forming radionuclides (Table 1). The present radiation dose measured for Chernobyl plots was about 60 – 400-times higher than the background levels measured for the control plots.

**Table 1** Number of samples for each plot and radiation measurements

| Deme | No of trees (samples) | Radiation exposure (µR/h) | Absorbed dose (Gy/year) |
|---|---|---|---|
| P1 | 50 (197) | 0.6 – 1.1 | 5.7 |
| P2 | 48 (156) | 0.9 – 4.4 | 10.6 |
| P3 | 50 (167) | 0.9 – 4.4 | 9.0 |
| P4 | 66 (210) | 0.005 – 0.008 | $3x10^{-6}$ |
| P5 | 50 (113) | 0.005 – 0.008 | $2.3x10^{-6}$ |

**DNA isolation, microsatellite and AFLP genotyping**

Genomic DNA was extracted from dried needles using the DNeasy 96 Plant Kit protocol QIAGEN (QIAGEN GmbH, Hilden, Germany).

A total of 843 (all demes) samples were genotyped for nine microsatellite loci (Table 2). Amplification of genomic DNA with SPAC primers (Soranzo et al. 1998) was done in a 15 µl volume containing 1.5 µl of template DNA, 7.5 µl of HotStarTaq Master Mix (10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 0.5 U of Taq polymerase) (Qiagen, Hilden, Germany), 2 µl of each forward and reverse primer (5 pmol/µl) and 3 µl of HPLC $H_2O$. The PCR reaction for LOP and PtTX primers (Liewlaksaneeyanawin et al.2004; González-Martínez et al.2004) was performed with a volume of 15 µl containing 3 µl of template DNA, 3.55 µl of HPLC $H_2O$, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 1 U of Taq polymerase (Solid BioDyne, Estonia), 3 µl of Q-solution (Qiagen), 0.2 mM of each dNTP, 0.75 µl of each forward and reverse primer (5 pmol/µl). The forward primers were labeled with the fluorescent dyes 6-FAM or HEX. All amplifications were performed in a Peltier thermal cycler (PTC-0200 version 4.0, MJ Research) with a heated lid under the following conditions: SPAC primers: an initial denaturation step of 94°C for 15 min, followed by 35 cycles of 94°C for 1 min 30 sec (denaturation), $T_a$ for 1 min 30 sec (annealing), 72°C for 1 min 30 sec (extension) and a final extension step of 72°C for 10 min. LOP primers and PtTX 2146: 15 min initial

denaturation at 95°C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at $T_a$, 1 min extension at 72°C and a final extension step of 72°C for 10 min. For PtTX 3107 a touchdown PCR was applied: denaturation at 95°C for 15 min, 10 cycles at 94°C for 1 min, 60°C for 1 min (-1°C per cycle) and 72°C for 3 min, followed by 20 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 3 min and a final extension step of 72°C for 10 min.

A subset of these DNA samples were used for the AFLP analyses; 533 samples from demes P2, P3 and P4 were successfully amplified. AFLP analysis was performed according to Vos et al. (1995) with minor modifications. Total genomic DNA (4 µl) was digested with the restriction enzymes EcoRI/MseI. Double-stranded *Mse*I and *Eco*RI adaptors were ligated to the ends of the restriction fragments to generate template DNA for polymerase chain reaction (PCR) amplification. The restriction/ligation reaction was performed at room temperature overnight. Diluted ligation mix was used for preamplification reactions and the preselective primer pairs E01/M03 (nomenclature according to Keygene N. V.) with the selective nucleotides A and G, respectively. The reactions were programmed to start at 72°C for 2 min, 20 cycles each consisting at 94°C for 10 sec, at 56°C for 30 sec, at 72°C for 2 min, and then at 60°C for 30 min. Selective PCR were carried out using diluted DNA from the preamplification reaction with the primer combination E35 (5'-GAC TGC GTA CCA ATT CAC A-3') and M63-C (5'-GAT GAG TCC TGA GTA AGA AC-3'). Primer *Mse*I-GAAC was labeled with the fluorescent dye FAM. Cycling conditions for selective PCR were as follows: start at 94°C for 2 min, 10 cycles, each consisting of 10s at 94°C, 30 sec at 65°C and 2 min at 72°C. The 65°C annealing temperature of the first cycle is subsequently reduced by 1°C for the next 10 cycles and continued at 56°C for 30 sec for the remaining 23 cycles, and ends with a final extension step at 60°C for 30 min. This primer combination was selected from a test of 16 primer combinations in 8 samples of *P. sylvestris* based on the production of polymorphic, reproducible, clear and easily scorable fragments. At least two negative controls were run on each plate to test repeatability of the fragments. A total of 222 fragments with 100% reproducibility in size range from 75 bp to324 bp were selected for further analyses.

The fragments were separated electrophoretically on the ABI Prism Genetic Analyzer 3100 together with the internal size standard GeneScan 500 ROX (fluorescent dye ROX) from Applied Biosystems. The size of the microsatellites and AFLP fragments was determined with the software packages GeneScan 3.7 and Genotyper 3.7 (Applied Biosystems Inc.).

AFLP markers were scored according to the absence (0) or presence (1) of bands, i.e. as dominant markers, and entered into a binary matrix representing the AFLP profile of each sample. Each fragment was controlled and edited manually.

**Cloning and sequencing of microsatellites**

All alleles with mutations in microsatellites were cloned and sequenced to confirm the occurrence of mutations and to determine the molecular basis for size variation. The selected sequences were analyzed in at least five clones and compared to the non-mutated type from the same tree.

Fragments were amplified with unlabelled primers and PCR products were purified from the gel using the QIAquick Gel Extraction kit (Qiagen®, Germany) following the manufacturer's instructions. The purified PCR products were cloned in a pCR2.1 vector using a TOPO TA cloning® kit (Invitrogen, Carlsbad, CA). The ligated vector fragments were transformed into *Escherichia coli* XL1-Blue strain bacterial cells and plated onto LB agar medium. After proliferation, the white colonies were additionally incubated overnight in liquid LB medium to obtain more copies of plasmids. The plasmids were extracted and purified using the QuickLyse Miniprep Kit (Qiagen®, Germany). Purified plasmids were used for the sequencing reactions based on the dideoxy-mediated chain termination method (Sanger *et al.*, 1977). Sequencing was carried out with the Big Dye® Terminator v.3.1. Cycle Sequencing Kit (Applied Biosystems). The sequencing reaction (in 10 µl total volume) consisted of 4.8 µl of HPLC $H_2O$, 1 µl of ready reaction premix (Big Dye vers.3.1), 1.5 µl of sequencing buffer (SB3.1), 0.7 µl of forward or reverse M13 primer (5 pmol/µl) and 2 µl of purified plasmids. The PCR protocol for sequencing reaction consisted of an initial denaturation step of 96°C for 1 min, followed by 35 cycles of 96°C for 10 sec (denaturation), 45°C for 10 sec (annealing) and 60°C for 4 min (extension). Products of the reaction were purified with ethanol precipitation and run on the ABI Prism Genetic Analyzer 3100.

For editing and visual inspection of the sequences, the sequences were aligned using Codon Code Aligner (CodonCode cooperation, www.condoncode.com) and BioEdit version 7.0.0 (Hall 1999) using ClustalW multiple alignment (Thompson *et al*., 1994).

### Mutation analysis

Microsatellite and AFLP mutations were identified by locus-wise comparisons of genotypes of the same tree. A polymorphism within the four samples analyzed for a tree was regarded as an indication of mutation. Mutations were manifested as fragment length polymorphisms or as presence/absence of a particular amplification product (fragment of particular size). To confirm theobservation of mutations, PCR amplification and mutation analyses were carried out at least twice for mutated and control samples from the same tree. Mutation rates were calculated as the number of mutations per locus per individual. Statistical significance was confirmed by the Chi-square test (Pearson 1900).

**Table 2** Description of nuclear microsatellites used in the present study

| Gene locus | Repeat motif | Forvard and reverse primer sequences (5' – 3') | $T_a$ (°C) | Allele size range (bp) | No alleles |
|---|---|---|---|---|---|
| SPAC11.4[a] | (AT)5(GT)19 | TCACAAAACACGTGATTCACA<br>GAAAATAGCCCTGTGTGAGACA | 62 | 112-168 | 19 |
| SPAC11.8[a] | (TG)16 | AGGGAGATCAATAGATCATGG<br>CAGCCAAGACATCAAAAATG | 55 | 121-174 | 22 |
| SPAC12.5[a] | (GT)20(GA)10 | CTTCTTCACTAGTTTCCTTTGG<br>TTGGTTATAGGCATAGATTGC | 57 | 113-207 | 35 |
| SPAC7.14[a] | (TG)17(AG)21 | TTCGTAGGACTAAAAATGTGTG<br>CAAAGTGGATTTTGACCG | 59 | 173-246 | 36 |
| LOP1[b] | $(TA)_{10}$ | GGCTAATGGCCGGCCAGTGCT<br>GCGATTACAGGGTTGCAGCCT | 60 | 151-186 | 14 |
| LOP3[b] | $(TA)_9$ | GTCTCCAGCCAGTTCACCTGC<br>CAGTGGATCTGTCACCTCCTC | 57 | 206-232 | 5 |
| LOP5[b] | $(TA)_{33}$ | AGCCGTAAAAGCTATCTTGTG<br>GGCATACTTACATTTTAATAA | 48 | 140-220 | 28 |
| PtTX2146[b] | $(GCT)_4GCC(GCT)_7GCC(GCT)_8$ | CCTGGGGATTTGGATTGGGTATTTG<br>ATATTTTCCTTGCCCCTTCCAGACA | 60 | 170-261 | 10 |
| PtTX3107[c] | $(CAT)_{14}$ | AAACAAGCCCACATCGTCAATC<br>TCCCCTGGATCTGAGGA | 60↓50[d] | 150-182 | 22 |

$T_a$ Annealing temperature; [a] Soranzo et al. 1998; [b] Liewlaksaneeyanawin et al.2004; [c] González-Martínez et al.2004; [d] Starting and final temperatures of the touchdown PCR

**Results**

Mutations and their nature detected at microsatellite and AFLP loci are summarized in Table 3 and Table 6. Only four mutation events were detected in demes P1, P2 and P3 and no mutations were found in the control plots (P4 and P5) at microsatellites. The difference in the number of mutations between two irradiated demes from the "Red forest" (P2 and P3) was not significant. The mutations were found in the repeat motifs of three different microsatellite loci (LOP5, PtTX3107 and PtTX2146) and represent deletions. For example, the loss of twenty nucleotides at LOP 5 was detected in one tree of deme P1 (Fig.1). In total, one mutation was found at the pine stand under the combined influence of acute/chronic exposure and three mutations were observed at the pine stand growing in conditions of only chronic exposure. The level of radiation exposure in the "Red forest" (P2-P3) is 3 times higher than in "Prypiat" (P1) and was 2.65 µR/h, while in "Prypiat" it was 0.85 µR/h. The difference in absorbed dose between these two stands is 1.7 times (Table 1).

Twelve mutations were detected in the trees of the control plot P4, and 34 and 32 mutation events in the trees of deme P2 and P3, respectively, at AFLP loci. Here the mutations were represented by both the deletion of whole fragments and the appearance of new fragments and only in some samples one nucleotide insertions/deletions were detected. Quite often, two mutations were observed within the same tree but in different branches and, in some cases, even more mutations were detected in the same tree. The difference in number of mutations between irradiated damaged (P2) and irradiated healthy (P3) trees was not significant.

Mutation rates for microsatellite and AFLP loci are summarized in Table 4 and Table 5, respectively. For microsatellite loci, the mutation rates in irradiated groups are $2.8 \times 10^{-4}$ (1/3546) for deme P1, $7.1 \times 10^{-4}$ (2/2808) and $3.3 \times 10^{-4}$ (1/3006) for demes P2 and P3, respectively. The comparison of demes P1-P5 and demes P3-P4 showed no significant statistical differences. A highly significant statistical difference ($P<0.0001$) was only found in the P2-P4 group, representing strongly damaged trees of the "Red forest" area which were planted after the accident in heavily contaminated soils and the corresponding control group of trees.

For AFLP loci, the estimates of mutation rates are $1.06 \times 10^{-3}$ (12/23668) for control (deme P4), $3.99 \times 10^{-3}$ (34/17483) and $3.74 \times 10^{-3}$ (32/17699) for demes P2 and P3, respectively. A

highly significant statistical difference ($P<0.0001$) was found for the demes P2 and P3 compared to the control group P4. In these cases the observed mutation rate was 3.8- and 3.5 times higher than in the control group.

**Table 3** Microsatellite and AFLP mutations in irradiated and control samples of *P.sylvestris*

| Primer combination | Un-exposed control groups (P4, P5) | | Exposed groups (P1, P2, P3) | |
|---|---|---|---|---|
| | No. of mutations | Size change (bp)[a] of mutations | No. of mutations | Size change (bp)[a] of mutations |
| Microsatellites | | | | |
| LOP5 | 0 | | 1 | (–20) |
| PtTX2146 | 0 | | 2 | (–1)<br>loss |
| PtTX3107 | 0 | | 1 | (–9) |
| AFLP | | | | |
| E35/ M63-C | 12 | 4 new[b]<br>6 loss[c]<br>2 (+1) | 66 | 35 new<br>22 loss<br>4 (+1)<br>5 (–1) |

[a] +, increased; –, decreased; [b] Additional fragment was detected; [c] Fragment was lost

**Table 4** Microsatellite mutation rates in exposed and control groups

| | No of mutations | No of alleles | Mutation rate | $\chi2$-test |
|---|---|---|---|---|
| P1 | 1 | 3546 | $2.8x10^{-4}$ | |
| P2 | 2 | 2808 | $7.1x10^{-4}$ | |
| P3 | 1 | 3006 | $3.3x10^{-4}$ | |
| P4 | 0 | 3780 | 0 | |
| P5 | 0 | 2034 | 0 | |
| P1-P5 | | | | 0.574 |
| P2-P3 | | | | 0.405 |
| P2-P4 | | | | 363.378*** |
| P3-P4 | | | | 1.257 |

*P* values: *P*<0.0001***

**Table 5** AFLP mutation rates in exposed and control groups

| | No of mutations | No of loci | Mutation rate | Ratio | $\chi2$-test |
|---|---|---|---|---|---|
| P2 | 34 | 17483 | $3.99x10^{-3}$ | | |
| P3 | 32 | 17699 | $3.74x10^{-3}$ | | |
| P4 | 12 | 23668 | $1.06x10^{-3}$ | | |
| P2-P3 | | | | 1.06 | 0.767 |
| P2-P4 | | | | 3.78 | 19.004*** |
| P3-P4 | | | | 3.53 | 17.491*** |

*P* values: *P*<0.0001***

**Table 6:** Mutations detected with AFLP markers for *Pinus sylvestris* populations from the Chernobyl exclusion zone

| Locus | Deme | | | Locus | Deme | | |
|---|---|---|---|---|---|---|---|
| | P2 | P3 | P4 | | P2 | P3 | P4 |
| 83 | | | n | 205 | l | | |
| 87 | n | | | 209 | n | n | |
| 90 | n | | | 211 | n/3l/2(-1) | 2n | |
| 95 | | l | | 228 | l | l | |
| 96 | | n | | 235 | | l | |
| 97 | | -1/l | | 239 | n | | |
| 110 | | | l | 240 | | -1 | +1 |
| 112 | n | 2n | | 245 | | n | |
| 113 | | n | | 247 | | | n |
| 114 | n /l | -1 | | 251 | | +1 | |
| 115 | n/2l/+1 | | | 252 | 2(+1) | | |
| 144 | | n | | 254 | | n | |
| 145 | | | l | 257 | | 2n | |
| 147 | | l | | 264 | | l | |
| 150 | 2n | n | | 265 | n | | |
| 154 | n | | | 278 | l | | |
| 156 | | | l | 283 | n | | |
| 157 | | n | | 288 | | n | |
| 168 | n/l | | | 292 | | | +1 |
| 183 | l | | | 294 | n | | |
| 185 | | l | | 300 | | | n |
| 193 | n | | | 311 | | | n |
| 195 | | n | | 312 | | l | |
| 196 | | l | | 313 | n | | |
| 201 | l | n | | 318 | | | l |
| 204 | | | l | 322 | | n/l | |

+1: one nucleotide insertion; -1: one nucleotide deletion; n (new): additional fragment was detected; l (loss): fragment was lost

```
                           10        20        30        40        50        60        70
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sample 11-194     GCCGTAAAAGCTATCTTGTGAAACAGGAGATGTCATATTGCCATACATGCATTATACTAACTATGGCGGA
Sample 12-174     GCCGTAAAAGCTATCTTGTGAAACAGGAGATGTCATATTGCCATACATGCATTATACTAACTATGGCGGA
Sample 12-194     GCCGTAAAAGCTATCTTGTGAAACAGGAGATGTCATATTGCCATACATGCATTATACTAACTATGGCGGA

                           80        90       100       110       120       130       140
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sample 11-194     TGTATCTTTCCCATGAATGTACAGTAATACCTTGAAGGTGAACACTTATATATATATATATATATATATA
Sample 12-174     TGTATCTTTCCCATGAATGTACAGTAATACCTTGAAGGTGAACACTT--------------------ATA
Sample 12-194     TGTATCTTTCCCATGAATGTACAGTAATACCTTGAAGGTGAACACTTATATATATATATATATATATATA

                          150       160       170       180       190
                  ....|....|....|....|....|....|....|....|....|....|....
Sample 11-194     TATATATATATATATATATCAAATTACCTACTATTATTAAAATGTAAGTATGCC
Sample 12-174     TATATATATATATATATATCAAATTACCTACTATTATTAAAATGTAAGTATGCC
Sample 12-194     TATATATATATATATATATCAAATTACCTACTATTATTAAAATGTAAGTATGCC
```

**Fig. 1** Sequences of three alleles at the LOP5 locus. Sample 11 and 12 belong to the same tree. Sample 11 represents the non-mutated type, which was observed in three needle samples; it is homozygous for the 194 bp allele. Sample 12 shows a mutation – a loss of 20 nucleotides (indicated by oval); the sample appears to be heterozygous (174bp/194bp).

## Discussion

Plants, especially long-lived and sessile organisms like forest trees, which could not escape contaminated areas, are sensitive to the influence of chronic radiation. They cannot avoid harmful environmental effects and had to adapt to live in unfavorable conditions. During the years after the Chernobyl accident various effects and responses of plants to acute and chronic irradiation were observed, such as death of radiosensitive plants and radiomorphoses (Arkhipov et al., 1994), an increased frequency of chlorophyll and embryonic lethal mutations (Abramov et al. 1992; Bubryak et al. 1992; Shevchencko et al. 1996; Kovalchuk et al. 2000), an increased frequency of chromosome aberrations (Kal'chenko and Fedotov 2001; Oudalova et al. 2005), changes in levels of DNA methylation (Kovalchuk et al. 2003) and gene expression (Zelena et al. 2005), etc. Another useful and interesting characteristic of plants is the absence of a predetermined germline. In the process of plant development, the germ cells will be produced from somatic cells and parts of mutations can be inherited (Walbot 1985; Ledig 1986).

In our study, the mutation rates for microsatellites in the irradiated groups are about $10^{-4}$ that corresponds with general estimations of mutation frequencies of SSRs. We observed very low number of mutations in irradiated material and no mutations in control samples, however a highly significant difference ($P<0.0001$) was detected in the mutation rate between the most radiation-damaged samples and the contol (P2-P4 comparison). A

comparison of pines with different age showed no difference in the number of mutations between deme P1 and demes P2-P3. The detected number of mutations at microsatellites was lower as expected in such environmental conditions. According to literature, there are a lot of factors that can influence the mutation rates in microsatellites, such as chromosome position, allele size, repeated motif, GC content in DNA, cell division (mitotic or meiotic process), genotype and sex (Li et al. 2002). In contrast to a former pilot study (Vornam et al. 2004), we found exclusively deletions within the repeat motifs of microsatellite loci; the flanking regions of these loci were not affected. In studies of humans, mutation rates were analysed by microsatellites and minisatellites after the Chernobyl accident (Dubrova et al. 2002; Dubrova 2003; Furitsu et al. 2005). High levels of spontaneous mutations were found, but only a slight increase in the number of mutations was observed. Investigations of *Betula verrucosa* and *Oenothera biennis* from the Chernobyl exclusion zone demonstrated an improved DNA repair capacity in irradiated material (Boubriak et al. 2008). This might be one of the possible explanations for the low number of mutations in microsatellites, if some of the investigated SSR loci are linked to the actively expressed DNA regions where reparation process is very substantial.

Only few data on mutation rates at AFLP markers are yet available. According to some estimations, the mutation rates for AFLP are$10^{-5}$-$10^{-6}$ per locus per generation (Mariette et al. 2001; Kropf et al. 2009). AFLPs screening covers not only repetitive DNA, but also functionally active genes and can provide information about mutation process in the whole genome. The results, obtained with AFLP markers, showed very high level of spontaneous mutations ($10^{-3}$ per locus per generation) which can be compared with mutation rates of microsatellites. Possibly, a high amount of repetitive DNA in the conifer genome (Ahuja and Neale 2005) is the reason for high mutation rates revealed with AFLPs. The difference in the number of mutations between the control and exposed groups was more than 3-fold and highly significant ($P<0.0001$) indicating a strong DNA damage caused by radiation. Thus, AFLP markers are a useful tool for investigating changes of mutation rates under the influence of radiation.

Comparisons of the number of mutations between irradiated demes from the "Red forest" (P2 and P3) was not significant with use of both types of markers, microsatellites and AFLPs. Thus, the phenotypic differences between the investigated demes (P2 and P3) are probably not due to mutations at the investigated regons, and may not be caused by mutations alone.

Our results correspond with results of other studies (Kovalchuk et al. 2000; Kovalchuk et al. 2004; Geras'kin et al. 2005; Møller and Mousseau 2006) showing that change of mutation rates in the Chernobyl exclusion zone is one of the reactions of organisms to irradiation. The application of different kinds of molecular markers, such as microsatellites and AFLPs, allows a better view on mutations and factors controlling mutation rates than studies restricted to a single marker type. To understand consequences of such changes, other molecular techniques should be additionally used including analysis of gene expression profiles and sequence variation of genes involved in stress response and adaptation. The use of high-throughput sequencing methods will facilitate the analysis of mutation events at the level of DNA sequences.

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## 9. Appendices:

**Appendix 1:** Sample size and radiation measurements for populations of *Pinus sylvestris* sampled in the Chernobyl exclusion zone and control areas
**Appendix 2:** Laboratory protocols
**Appendix 3:** Genetic diversity and differentiation among populations of *Pinus sylvestris* for microsatellite markers
**Appendix 4:** Mutations detected with microsatellite markers for *Pinus sylvestris* populations from the Chernobyl exclusion zone
**Appendix 5:** Sequences, originated from three microsatellite loci of *Pinus sylvestris* with detected mutation events

**Appendix 1:** Sample size and radiation measurements for populations of *Pinus sylvestris* sampled in the Chernobyl exclusion zone and control areas

**P1 (Prypiat)**

| Tree № | Branches | Radiation level, mR/h | Absorbed dose, Gy/y | Tree № | Branches | Radiation level, mR/h | Absorbed dose, Gy/y |
|---|---|---|---|---|---|---|---|
| 1 | S, W, E, N | 0,81 | 13.12 | 26 | N, W, E, S | 0,86 | 14.93 |
| 2 | S, W, E, N | 1,11 | 12.76 | 27 | N, W, S, E | 0,81 | 35.01 |
| 3 | S, E, W, N | 1,13 | 21.06 | 28 | W, S, E, N | 0,76 | 11.97 |
| 4 | E, W, N, S | 0,77 | 10.47 | 29 | W, E, N, S | 0,81 | 41.58 |
| 5 | S, W, E, N | 0,91 | 2.41 | 30 | N, E, S, W | 0,74 | 14.62 |
| 6 | S, W, E, N | 0,88 | 12.50 | 31 | W, N, S, E | 0,79 | 3.63 |
| 7 | N, S, W, E | 0,87 | 10.27 | 32 | S, W, E, N | 0,74 | 8.69 |
| 8 | S, W, E, N | 0,81 | 15.40 | 33 | N, W, E, S | 0,74 | 3.20 |
| 9 | E, S, W, N | 0,71 | 3.23 | 34 | S, E, W, N | 0,78 | 4.49 |
| 10 | S, W, E, N | 0,74 | 8.16 | 35 | E, S, W, N | 0,81 | 19.68 |
| 11 | W, N, S, E | 0,78 | 12.48 | 36 | E, S, W, N | 0,81 | 13.97 |
| 12 | W, E, N, S | 0,76 | 6.04 | 37 | E, W, N, S | 0,78 | 8.98 |
| 13 | S, E, W, N | 0,86 | 6.97 | 38 | N, W, E, S | 0,75 | 5.14 |
| 14 | W, N, S, E | 0,83 | 14.37 | 39 | W, E, S, N | 0,84 | 15.06 |
| 15 | W, S, N, E | 0,87 | 6.47 | 40 | W, S, E, N | 0,79 | 7.79 |
| 16 | W, E, S, N | 0,86 | 8.79 | 41 | W, E, N, S | 0,72 | 8.11 |
| 17 | E, W, S, N | 0,81 | 1.46 | 42 | W, E, S, N | 0,76 | 9.72 |
| 18 | N, W, E, S | 0,84 | 0.55 | 43 | W, E, S, N | 0,86 | 2.28 |
| 19 | S, W, E, N | 0,81 | 6.88 | 44 | E, N, W, S | 0,78 | 17.95 |
| 20 | S, W, N, E | 0,89 | 17.83 | 45 | S, E, W | 0,76 | 11.36 |
| 21 | N, E, S, W | 0,91 | 13.51 | 46 | W, S, E, | 0,78 | 7.68 |
| 22 | S, W, E, N | 0,84 | 5.96 | 47 | S, W, E, | 0,77 | 5.72 |
| 23 | S, E, W, N | 0,82 | 10.32 | 48 | W, E, S | 0,67 | 13.09 |
| 24 | E, N, W, S | 0,86 | 12.50 | 49 | W, S, E | 0,71 | 14.09 |
| 25 | E, W, N, S | 0,61 | 9.98 | 50 | S, W, E, N | 0,68 | 29.44 |

**P2 (Red forest)**

| Tree № | Branches | Radiation level, mR/h | Absorbed dose, Gy/y | Tree № | Branches | Radiation level, mR/h | Absorbed dose, Gy/y |
|---|---|---|---|---|---|---|---|
| 1 | W, S, E, N | 1,9 | 33.83 | 26 | S, E, W | 1,4 | 5.92 |
| 2 | S, W, N, E | 3,1 | 23.00 | 27 | S, E, W | 1,6 | 46.16 |
| 3 | N, E, S | 1,5 | 12.79 | 28 | N, E, S | 1,1 | 24.63 |
| 4 | N, E | 1,4 | 22.39 | 29 | E, S, W, N | 2,4 | 38.41 |
| 5 | N, E, S, W | 1,9 | 46.10 | 30 | N, E, S | 2 | 12.76 |
| 6 | N, W, E, S | 1,1 | 19.48 | 31 | E, N, S, W | 1,5 | 34.81 |
| 7 | E, W, S, N | 1,1 | 19.66 | 32 | E, N | 2,4 | 11.11 |
| 8 | S, W, E, N | 1,6 | 40.64 | 33 | N, W, E | 1,9 | 9.34 |
| 9 | S, W, N, E | 1,1 | 14.85 | 34 | E, S, W | 1,6 | 11.64 |
| 10 | S, W, N | 1,9 | 10.71 | 35 | W, N, E | 2,5 | 15.89 |
| 11 | W, N | 1,8 | 24.62 | 36 | W, S, E | 2,3 | 30.73 |
| 12 | W, S, N | 1,6 | 33.45 | 37 | S, W, N, E | 2,4 | 11.25 |
| 13 | N, W, E | 1 | 8.08 | 38 | N, W, E, S | 1,8 | 28.23 |
| 14 | W, S | 1,1 | 15.72 | 39 | W, S, N | 2,2 | 19.38 |
| 15 | W, S, N, E | 1,8 | 19.65 | 40 | W, S, N | 1,7 | 19.25 |
| 16 | W, S | 3 | 37.41 | 41 | N, W, S, E | 1,7 | 18.39 |
| 17 | E, W, S, N | 1,9 | 18.13 | 42 | W, E, N | 1,9 | 21.32 |
| 18 | E, W, N | 0,9 | 12.71 | 43 | W, E, N, S | 1,7 | 8.05 |
| 19 | E, W, N, S | 1,2 | 24.36 | 44 | E, N, W, S | 1,8 | 20.46 |
| 20 | N, E | 1,3 | 26.57 | 45 | E, N, S, W | 3,2 | 25.96 |
| 21 | W, N, E | 1,2 | 17.74 | 46 | W, E, S, N | 2 | 13.66 |
| 22 | E, N, S | 1,9 | 16.33 | 47 | E, W | 1,5 | 16.29 |
| 23 | W, S, E, N | 2,5 | 30.47 | 48 | W, E, S, N | 2,1 | 25.87 |
| 24 | N, W, E | 1,6 | 13.23 | 49 | N, W, E | 4,4 | 13.58 |
| 25 | E, N, S | 2,5 | 19.64 | 50 | S, W, E, N | 2,2 | 13.95 |

**P3 (Red forest)**

| Tree № | Branches | Radiation level, mR/h | Absorbed dose, Gy/y | Tree № | Branches | Radiation level, mR/h | Absorbed dose, Gy/y |
|---|---|---|---|---|---|---|---|
| 1 | S, W ,N, E | 1,9 | 26.55 | 26 | S, W, E | 1,4 | 9.73 |
| 2 | W, N, S, E | 3,1 | 36.89 | 27 | E, N, W | 1,6 | 18.38 |
| 3 | E, S | 1,5 | 10.71 | 28 | E, N, S | 1,1 | 28.17 |
| 4 | S, E, N, W | 1,4 | 23.56 | 29 | E, S, N, W | 2,4 | 25.41 |
| 5 | E, N, S, W | 1,9 | 36.32 | 30 | E, S, N | 2 | 10.20 |
| 6 | N, W, E, S | 1,1 | 13.86 | 31 | N, S, E | 1,5 | 30.07 |
| 7 | N, E | 1,1 | 15.41 | 32 | E, N, W, S | 2,4 | 17.42 |
| 8 | E, S, N, W | 1,6 | 32.91 | 33 | E, N, W, S | 1,9 | 13.92 |
| 9 | E, S, W, N | 1,1 | 16.31 | 34 | E | 1,6 | 5.50 |
| 10 | W, N, S | 1,9 | 12.31 | 35 | W, N, S, E | 2,5 | 25.95 |
| 11 | W, S, E, N | 1,8 | 19.52 | 36 | W, N | 2,3 | 22.53 |
| 12 | W, E, N, S | 1,6 | 24.20 | 37 | N, W, S, E | 2,4 | 8.51 |
| 13 | W, E, S, N | 1 | 10.71 | 38 | N, W, E, E | 1,8 | 40.74 |
| 14 | W, S | 1,1 | 9.32 | 39 | W, E, S, N | 2,2 | 25.07 |
| 15 | N, S, W | 1,8 | 35.64 | 40 | N, S, E, W | 1,7 | 9.94 |
| 16 | W, N, S | 3 | 10.66 | 41 | W, S, N, E | 1,7 | 10.66 |
| 17 | W, S | 1,9 | 9.75 | 42 | W, S, N, E | 1,9 | 17.94 |
| 18 | N, W, E, S | 0,9 | 8.80 | 43 | W, E, S, N | 1,7 | 14.41 |
| 19 | E, N | 1,2 | 10.65 | 44 | E, N | 1,8 | 25.95 |
| 20 | E, N, W, S | 1,3 | 41.17 | 45 | S, N, E, W | 3,2 | 9.73 |
| 21 | W, N, S | 1,2 | 9.53 | 46 | N, E, W | 2 | 14.53 |
| 22 | E, W | 1,9 | 13.90 | 47 | E, N | 1,5 | 9.22 |
| 23 | W, N, S, E | 2,5 | 9.74 | 48 | N, S | 2,1 | 15.04 |
| 24 | E, S, N | 1,6 | 5.97 | 49 | N, E, S | 4,4 | 7.76 |
| 25 | E, S, N | 2,5 | 10.63 | 50 | E, N, W, S | 2,2 | 26.55 |

**P4 (control)**

| Tree № | Branches | Absorbed dose, Gy/y | Tree № | Branches | Absorbed dose, Gy/y |
|---|---|---|---|---|---|
| 1 | E, S | 5.02E-06 | 34 | E, N, W, S | 3.75E-06 |
| 2 | S, W, N | 1.03E-05 | 35 | W, N, S, E | 6.32E-06 |
| 3 | N, E, W | 4.64E-06 | 36 | S, E, W, N | 6.57E-06 |
| 4 | E, N | 4.82E-06 | 37 | N, E | 3.80E-06 |
| 5 | N, E, S | 5.00E-06 | 38 | E, W, N, S | 7.23E-06 |
| 6 | E, N | 5.83E-06 | 39 | W, E, S, N | 5.97E-06 |
| 7 | S, W, N, E | 6.82E-06 | 40 | N, S, E, W | 4.59E-06 |
| 8 | E, S, N, W | 4.95E-06 | 41 | W, S, N | 7.20E-06 |
| 9 | E, S, W, N | 4.62E-06 | 42 | S, N, E | 5.40E-06 |
| 10 | W, N | 8.13E-06 | 43 | W, E, S, N | 6.73E-06 |
| 11 | W, S | 6.90E-06 | 44 | S, W, N | 6.33E-06 |
| 12 | W, E, N | 4.25E-06 | 45 | N, W, S | 7.96E-06 |
| 13 | W, E, N | 6.84E-06 | 46 | S, E | 4.63E-06 |
| 14 | N, E | 6.64E-06 | 47 | E, N, S, W | 3.64E-06 |
| 15 | N, S, W, E | 5.66E-06 | 48 | N, S, E, W | 5.51E-06 |
| 16 | N, W, S | 6.19E-06 | 49 | N, E, S, W | 4.34E-06 |
| 17 | N, W | 3.51E-06 | 50 | N, W, S | 4.42E-06 |
| 18 | N, W, E, S | 5.11E-06 | 51 | N, E, S | 4.65E-06 |
| 19 | S, E, N | 7.93E-06 | 52 | S, N, E | 5.42E-06 |
| 20 | S, N, W | 5.46E-06 | 53 | S, N, W, E | 6.31E-06 |
| 21 | N, S, E | 6.78E-06 | 54 | N, W, S, E | 7.75E-06 |
| 22 | E, N, S | 7.56E-06 | 55 | N, W, S, E | 1.54E-05 |
| 23 | W, N, S, E | 8.90E-06 | 56 | N, W, E, S | 4.60E-06 |
| 24 | W, S, N | 7.29E-06 | 57 | N, W, E, S | 6.51E-06 |
| 25 | W, S, N | 3.81E-06 | 58 | N, W, E, S | 4.46E-06 |
| 26 | S, W, N | 7.35E-06 | 59 | N, W, E, S | 5.53E-06 |
| 27 | E, N, W, S | 4.16E-06 | 60 | N, W, E, S | 3.47E-06 |
| 28 | W, N, S | 5.37E-06 | 61 | N, W, E, S | 6.41E-06 |
| 29 | E, S, N, W | 7.11E-06 | 62 | N, S, E | 7.49E-06 |
| 30 | S, N | 6.48E-06 | 63 | N, W, E | 6.71E-06 |
| 31 | N, S | 4.52E-06 | 64 | N, W, E | 7.42E-06 |
| 32 | S, W, E | 5.36E-06 | 65 | N, W, E | 4.05E-06 |
| 33 | E, N, W, E | 4.73E-06 | 66 | N, S, W | 7.69E-06 |

**P5 (control)**

| Tree № | Branches | Absorbed dose, Gy/y | Tree № | Branches | Absorbed dose, Gy/y |
|---|---|---|---|---|---|
| 1 | S, E, N | 5.73E-06 | 26 | S, E | 4.98E-06 |
| 2 | S, W, N | 1.34E-05 | 27 | E, S | 2.62E-06 |
| 3 | N, E | 5.23E-06 | 28 | S, W | 5.66E-06 |
| 4 | S, S | 4.97E-06 | 29 | S, E | 1.43E-05 |
| 5 | W, S | 5.87E-06 | 30 | S, W | 4.82E-06 |
| 6 | E, S | 3.24E-06 | 31 | E, W, S | 1.22E-06 |
| 7 | S, E, W | 5.17E-06 | 32 | S, W | 7.71E-06 |
| 8 | E, S | 3.96E-07 | 33 | E, S | 6.24E-06 |
| 9 | S, S | 3.39E-06 | 34 | W, E | 5.92E-06 |
| 10 | E, S, W | 7.44E-06 | 35 | E, S | 5.11E-07 |
| 11 | E, N | 4.18E-06 | 36 | E, S | 8.14E-07 |
| 12 | E, W, S | 4.28E-06 | 37 | E, W | 3.53E-06 |
| 13 | N, E | 4.89E-06 | 38 | W, S | 4.76E-06 |
| 14 | E, S | 3.54E-06 | 39 | E, S | 5.84E-06 |
| 15 | N, E, S | 5.44E-06 | 40 | E, S | 1.23E-06 |
| 16 | N, E | 3.28E-06 | 41 | S, E | 4.19E-06 |
| 17 | E, N | 3.94E-06 | 42 | E, W, S | 2.11E-06 |
| 18 | E, S, N, W | 3.68E-06 | 43 | S, W | 4.99E-06 |
| 19 | S, E | 2.19E-06 | 44 | E, S | 5.49E-06 |
| 20 | S, E ,N | 4.29E-06 | 45 | E, S, W | 3.23E-06 |
| 21 | E, S | 4.89E-06 | 46 | S, W | 5.80E-06 |
| 22 | E, S | 4.14E-06 | 47 | S, E, W | 7.21E-06 |
| 23 | S, E | 4.15E-06 | 48 | N, E | 4.92E-06 |
| 24 | E, S | 5.79E-06 | 49 | S, W | 3.11E-06 |
| 25 | E, S | 9.19E-07 | 50 | S, W, E, N | 2.60E-06 |

## Appendix 2: Laboratory protocols

### A. Microsatellites

*SPAC primers*

**PCR mix**

Prepare 15-µl mix for each sample as follows:

7.5 µl HotStarTaq Master Mix (10 mM Tris-HCl (pH 9.0)

2 µl each forward and reverse primer (5 pmol/µl)

3 µl HPLC $H_2O$

1.5 µl template DNA

**PCR programme**

Initiation at 94°C for 15 min.

35-cycles denaturation at 94°C for 1 min 30 sec, annealing at $T_a$ for 1 min 30 sec (details are given at Manuscript II) and extension at 72°C for 1 min 30 sec.

Final extension at 72°C for 10 min.

***LOP and PtTX primers***

**PCR mix**

Prepare 15-µl mix for each sample as follows:

1.5 µl PCR buffer (10x)

1.5 µl mM $MgCl_2$ (25 mM)

0.75 µl each dNTP (10 mM)

0.75 µl each forward and reverse primer (5 pmol/µl)

0.2 µl Taq polymerase (Solid BioDyne, Estonia), (5 U/ µl)

3 µl Q-solution (Qiagen)

3.55 µl HPLC $H_2O$

3 µl template DNA

**PCR programme for LOP primers and PtTX 2146**

Initiation at 95°C for 15 min.

30-cycles denaturation at 94°C for1 min, annealing at $T_a$ for 1 min (details are given at Manuscript II) and extension at 72°C for 1 min.

Final extension at 72°C for 10 min.

**PCR programme for PtTX 3107**

Initial denaturation at 95°C for 15 min.

10-cycles denaturation at 94°C for 1 min, annealing at 60°C for 1 min (-1°C per cycle) and extension at 72°C for 3 min.

20-cycles denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 3 min.

Final extension at 72°C for 10 min.

Analyze the length of PRC products using ABI Prism 3100 Genetic Analyzer.

**B. Cloning**

Amplify fragments with unlabelled primers.

Purify PCR products from gel using the QIAquick Gel Extraction kit (Qiagen®, Germany).

**Ligation**

Prepare ligation mix as follows:

7 µl Fresh purified PCR product

1 µl Salt solution

1 µl TOPO® vector

Mix gently and incubate for 30 min at room temperature.

**Bacterial transformation**

Thaw chemically competent cells (*Escherichia coli* XL1-Blue strain) on ice.

Add 2.5 µl of ligated reaction to 30 µl of cells.

Mix carefully by tapping at tube and incubate on ice for 30 min.

Heat shock cells in water bath at exactly 42°C for 45 sec, then put cells back on ice for 2 min.

Add 200 µl of SOB media to cells.

Shake cells at 37°C for 1 hour.

Centrifuge briefly.

Pipette reaction (150 µl) in one plate containing antibiotic (100 µg/ml Kanamycin sulphate) and XGal (80 µg/ml) for blue-white screening.

Incubate plates at 37°C overnight.

**Colony PCR**

Prepare 15-µl mix as follows:

7.5 µl Master Mix

2 µl Vector F primer (BF) (5 pmol/ µl)

2 µl Vector R primer (BR) (5 pmol/ µl)

1.5 µl HPLC $H_2O$

Pick white colony with pipeting tip and dip the tip into the reaction.

**PCR programme**

Initiation at 95°C for 15 min.

35-cycles denaturation at 94°C for1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min.

Final extension at 72°C for 10 min.

**C. Sequencing**

**Sequencing mix**

Prepare 10-µl mix as follows:

1 µl Ready reaction premix (Big Dye vers.3.1)

1.5 µl Sequencing buffer (SB3.1) (5X)

4.8 µl HPLC $H_2O$

0.7 µl Forward or reverse M13 primer (5 pmol/µl)

2 µl Purified plasmids

**PCR programme**

Initiation at 96°C for 1 min.

35-cycles denaturation at 96°C for10 sec, annealing at 55°C for 10 sec and extension at 60°C for 4 min.

**Ethanol precipitation**

Add 40 µl HPLC $H_2O$, 5 µl NaAc (pH 6.0), 150 µl ethanol absolute.

Centrifuge 25 min at maximal speed (14,000 rpm).

Add 250 µl 70% ethanol and centrifuge 10 min.

Dry pellet at 37°C for 1 hour.

Elute DNA with 15 µl high formamide.

Use eluted DNA for capillary electrophoresis.

Sequencing was performed using ABI Prism 3100 Genetic Analyzer.

**D. AFLP**

**Sequences of primer pairs (5' – 3')**

*Eco*RI adapter CTC GTA GAC TGC GTA CC

CAT CTG ACG CAT GGT TAA

*Mse*I adapter GAC GAT GAG TCC TGA G

TA CTC AGG ACT CAT

E01 GAC TGC GTA CCA ATT CA

M03 GAT GAG TCC TGA GTA AG

E35 GAC TGC GTA CCA ATT CAC A

M63-C GAT GAG TCC TGA GTA AGA AC

**Restriction-Ligation-Mix RLM (for 10 probes)**

2 µl T4-DNA Ligase buffer

2 µl NaCl 0.5M

1 µl BSA (1 mg/ml)

0.8 µl *Mse*I (10 U/ µl)

4 µl *Eco*RI (10 U/ µl)

1.9 µl T4-DNA Ligase (4 U/ µl)

8.3 µl HPLC $H_2O$

**Restriction-Ligation-Reaction RLR (for 10 probes)**

10 µl T4-DNA Ligase buffer

10 µl NaCl 0.5M

5 µl BSA (1 mg/ml)

6 µl *Mse*I adapter pair (5 pmol/ µl)

6 µl *Eco*RI adapter pair (5 pmol/ µl)

23 µl HPLC $H_2O$

Incubate the Restriction-Ligation-Mix (4 µl DNA + 6 µl RLR + 2 µl RLM) at room temperature overnight.

***Pre-selective amplification***

**PCR mix**

Prepare a 25-µl mix using the following components:

0.38 µl dNTPs (10mM)

0.25 µl E01 primer (5 pmol/ µl)

0.25 µl M03 primer (5 pmol/ µl)

0.1 µl *Taq* polymerase (Qiagen) (5 U/ µl)

2.5 µl PCR-buffer 10x

13.5 µl HPLC $H_2O$

0.8 µl DNA (restriction-ligation)

**PCR programme**

Initiation at 72°C for 2 min.

20-cycles denaturation at 94°C for 10 sec, annealing at 56°C for 30 sec and extension at 72°C for 2 min.

Final extention at 60°C for 30 min.

***Selective amplification***

**PCR mix**

Prepare a 25-µl mix using the following components:

1.67 µl PCR-buffer 10x

0.25 µl dNTPs (10mM)

0.25 µl E35 Cy-5 labelled primer (5 pmol/ µl)

0.25 µl M63-C (5 pmol/ µl)

0.0675 µl *Taq* polymerase (Qiagen) (5 U/ µl)

9.25 µl HPLC $H_2O$

4.0 µl DNA (pre-selective amplification)

**PCR programme**

Initiation at 94°C for 2 min.

10-cycles denaturation at 94°C for 10sec, annealing at 65°C (-1 per cycle) for 30 sec and extension at 72°C for 2 min.

24-cycles denaturation at 94°C for 10sec, annealing at 56°C for 30 sec and extension at 72°C for 2 min.

Final extension at 60°C for 30 min.

Analyze the length of PRC products using ABI Prism 3100 Genetic Analyzer.

**Appendix 3:** Genetic diversity and differentiation among populations of *Pinus sylvestris* for microsatellite markers

| | Deme comparison | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | P1-P5 | | | | P2-P3-P4 | | | |
| Locus | Na | *Ho* | *He* | $F_{ST}$ | Na | *Ho* | *He* | $F_{ST}$ |
| SPAC11.4 | 13 | 0.808 | 0.867 | 0.004 | 14.3 | 0.804 | 0.854 | 0.003 |
| SPAC11.8 | 11.5 | 0.330 | 0.762 | 0.007 | 12.7 | 0.441 | 0.783 | 0.015 |
| SPAC12.5 | 27.5 | 0.920 | 0.936 | 0.007 | 29 | 0.916 | 0.941 | 0.011 |
| SPAC7.14 | 28 | 0.715 | 0.938 | 0.020 | 30.3 | 0.684 | 0.945 | 0.009 |
| LOP1 | 11.5 | 0.727 | 0.738 | 0.009 | 7.7 | 0.622 | 0.675 | 0.007 |
| LOP3 | 3.5 | 0.162 | 0.242 | 0.050 | 4 | 0.127 | 0.129 | 0.007 |
| LOP5 | 17.5 | 0.576 | 0.813 | 0.004 | 19.7 | 0.729 | 0.852 | 0.009 |
| PtTX2146 | 12 | 0.728 | 0.740 | 0.003 | 11.3 | 0.779 | 0.754 | 0.009 |
| PtTX3107 | 7 | 0.434 | 0.766 | 0.005 | 7.7 | 0.346 | 0.808 | 0.013 |
| Mean | 14.5 | 0.600 | 0.756 | 0.012 | 15.2 | 0.605 | 0.749 | 0.009 |

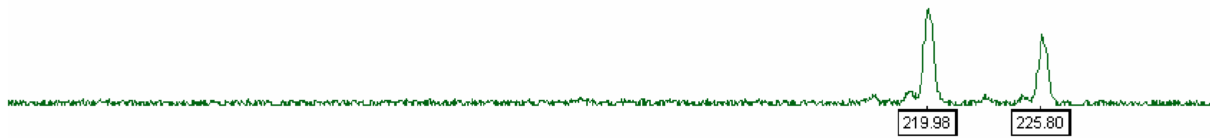
Na: number of alleles; *Ho*: observed heterozygosity; *He*: expected heterozygosity; $F_{ST}$: fixation index

**Appendix 4:** Mutations detected with microsatellite markers for *Pinus sylvestris* populations from the Chernobyl exclusion zone

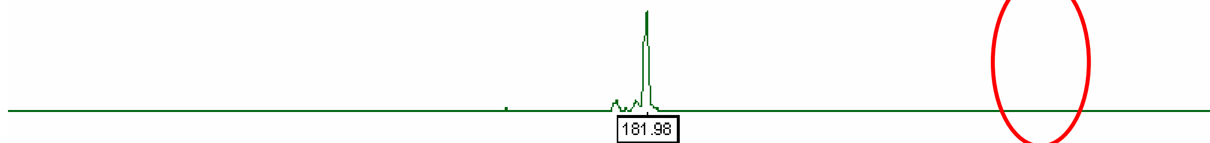
| Deme | Microsatellite locus | | |
|---|---|---|---|
| | LOP5 | PtTX3107 | PtTX2146 |
| P1 | 174 194 | | |
| | 194 194 | | |
| P2 | | | 182 |
| | | | 182 220 |
| | | | 220 225 |
| | | | 220 226 |
| P3 | | 156 162 | |
| | | 162 165 | |

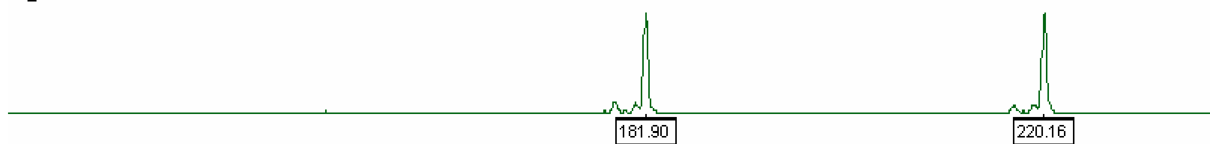
Mutations indicated with red color

**PtTX2146 –** one nucleotide deletion

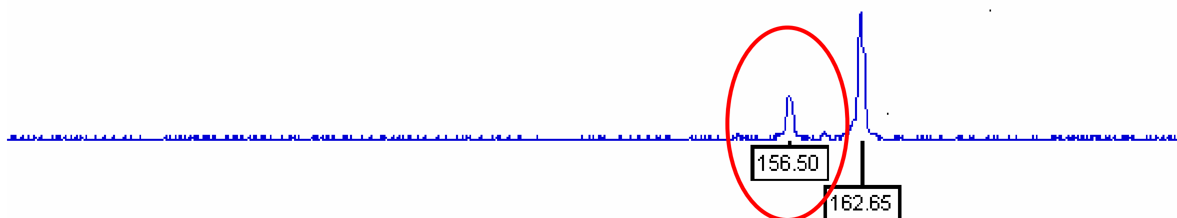


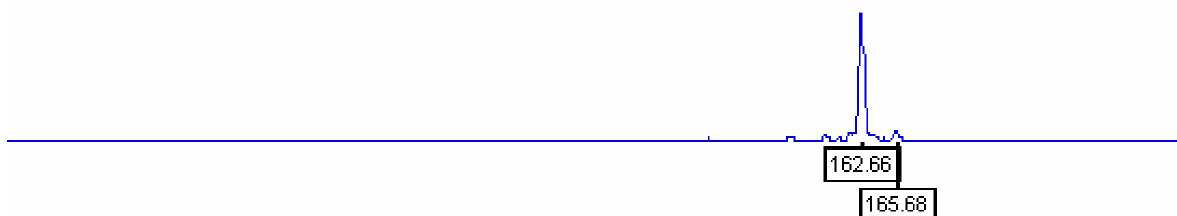
**PtTX2146 –** null allele



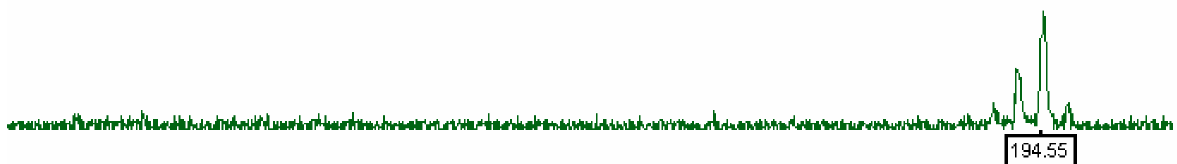



**PtTX3107 –** deletion of nine nucleotides fragment




**LOP5 –** deletion of twenty nucleotides fragment



**Appendix 5:** Sequences, originated from three microsatellite loci of *Pinus sylvestris* with detected mutation events (mutations indicated by blue circle)

**PtTX2146 –** one nucleotide deletion

```
                        10        20        30        40        50        60        70
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sample 23-220  TATATTTTCCTTGCCCCTTCCAGACAACCCAAATGAAGGAAAAACTGTGTGTGCAGTTTTTACGGTTAGA
Sample 23-226  TATATTTTCCTTGCCCCTTCCAGACAACCCAAATGAAGGAAAAACTGTGTGTGCAGCTTTTACGGTTAGA
Sample 24-220  TATATTTTCCTTGCCCCTTCCAGACAACCCAAATGAAGGAAAAACTGTGTGTGCAGCTTTTACGGTTAGA
Sample 24-225  TATATTTTCCTTGCCCCTTCCAGACAACCCAAATGAAGGAAAAACTGTGTGTGCAGCTTTTACGGTTAGA

                        80        90       100       110       120       130       140
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sample 23-220  GGAGGAGGAGGAGAAGGGGGTGCAGTTCAGCAGCAGCAGCAGCAGCA------ACGGCAGCAGCAGCAGC
Sample 23-226  GGAGGAGGAGGAGAAGGGGGTGCAGTTCAGCAGCAGCAGCAGCAGCAGCAGCAACGGCAGCAGCAGCAGC
Sample 24-220  GGAGGAGGAGGAGAAGGGGGTGCAGTTCAGCAGCAGCAGCAGCAGCA------ACGGCAGCAGCAGCAGC
Sample 24-225  GGAGGAGGAGGAGAAGGGGGT-CAGTTCAGCAGCAGCAGCAGCAGCAGCAGCAACGGCAGCAGCAGCAGC

                       150       160       170       180       190       200       210
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sample 23-220  GGCAGCAGCAGCAGGAGCAGCAGCAGCGGCAGCGGCAGCAGGAGCAGCAGCAGCGGCAGCAGCAAATACC
Sample 23-226  GGCAGCAGCAGCAGGAGCAGCAGCAGCGGCAGCAGCAGCAGGAGCAGCAGCAGCGGCAGCAGCAAATACC
Sample 24-220  GGCAGCAGCAGCAGGAGCAGCAGCAGCGGCAGCAGCAGCAGGAGCAGCAGCAGCGGCAGCAGCAAATACC
Sample 24-225  GGCAGCAGCAGCAGGAGCAGCAGCAGCGGCAGCAGCAGCAGGAGCAGCAGCAGCGGCAGCAGCAAATACC

                       220
               ....|....|....|..
Sample 23-220  CAATCCAAATCCCCAGG
Sample 23-226  CAATCCAAATCCCCAGG
Sample 24-220  CAATCCAAATCCCCAGG
Sample 24-225  CAATCCAAATCCCCAGG
```

**PtTX2146 –** null allele

```
                        10        20        30        40        50        60        70
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sample 54-220  ATATTTTCCTTGCCCCTTCCAGACAACCCAAATGAAGGAAAAACTGTGTGTGCAGCTTTTACGGTTAGAG
Sample 54-182  ATATTTTCCTTGCCCCTTCCAGACAACCCAAATGAAGGAAAAACTGTGTGTGCAGCTTTTACGGTTAGAG
Sample 53-182  ATATTTTCCTTGCCCCTTCCAGACAACCCAAATGAAGGAAAAACTGTGTGTGCAGCTTTTACGGTTAGAG

                        80        90       100       110       120       130       140
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sample 54-220  GAGGAGGAGGAGAA---GGGGGTGCAGTTCAGCAGCAGCAGCAGCAGCAACGGCAGCAGCAGCAGCGGCA
Sample 54-182  GAGGAGGAGGAGGAGGAGGGGGTGCAGTTCAGCAGCAGCAGCAACAGCAACGGCAGCAGCAGCAGCAGCA
Sample 53-182  GAGGAGGAGGAGGAGGAGGGGGTGCAGTTCAGCAGCAGCAGCAACAGCAACGGCAGCAGCAGCAGCAGCA

                       150       160       170       180       190       200       210
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sample 54-220  GCAGCAGCAGGAGCAGCAGCAGCGGCAGCAGCAGCAGGAGCAGCAGCAGCGGCAGCAGCAAATACCCAAT
Sample 54-182  GCAGCGGCAGCAGCAG------------------------------------------CAAATACCCAAT
Sample 53-182  GCAGCGGCAGCAGCAG------------------------------------------CAAATACCCAAT

                       220
               ....|....|..
Sample 54-220  CCAAATCCCCAG
Sample 54-182  CCAAATCCCCAG
Sample 53-182  CCAAATCCCCAG
```

**PtTX3107 –** deletion of nine nucleotides fragment

```
                           10        20        30        40        50        60
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Sample 57-156      AAAACAAGCCCACATCGTCAATCTAGGCCTCATGAAGGGTATGCCATGGAGTTAGGGCAT
Sample 57-162      AAAACAAGCCCACATCGTCAATCTAGGCCTCATGAAGGGTATGCCATGGAGCTAGGGCAT
Sample 58-162      AAAACAAGCCCACATCGTCAATCTAGGCCTCATGAAGGGTATGCCATGGAGCTAGGGCAT
Sample 58-165      AAAACAAGCCCACATCGTCAATCTAGGCCTCATGAAGGGTATGCCATGGAGCTAGGGCAT

                           70        80        90       100       110       120
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Sample 57-156      GATGTCATCATGCTAAGAAAGTACCATCCCCTCCAAAGTGGTCACATGTCATCACCATCA
Sample 57-162      GATGTCGTCATGCTAAGAAAGTACCATCCCCTCCAAAGTGGTCACATGTCATCACCATCA
Sample 58-162      GATGTCGTCATGCTAAGAAAGTACCATCCCCTCCAAAGTGGTCACATGTCATCACCATCA
Sample 58-165      GATGTCGTCATGCTAAGAAAGTACCATCCCCTCCAAAGTGGTCACATGTCATCACCATCA

                          130       140       150       160
                   ....|....|....|....|....|....|....|....|....|
Sample 57-156      TCATCATCATCATC---------AAATCTCCTCAGATCCAGGGGA
Sample 57-162      TCATCATCATCATCATCATC---AAATCTCCTCAGATCCAGGGGA
Sample 58-162      TCATCATCATCATCATCATC---AAATCTCCTCAGATCCAGGGGA
Sample 58-165      TCATCATCATCATCATCATCATCAAATCTCCTCAGATCCAGGGGA
```

**LOP5 –** deletion of twenty nucleotides fragment

```
                           10        20        30        40        50        60        70
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sample 11-194      GCCGTAAAAGCTATCTTGTGAAACAGGAGATGTCATATTGCCATACATGCATTATACTAACTATGGCGGA
Sample 12-174      GCCGTAAAAGCTATCTTGTGAAACAGGAGATGTCATATTGCCATACATGCATTATACTAACTATGGCGGA
Sample 12-194      GCCGTAAAAGCTATCTTGTGAAACAGGAGATGTCATATTGCCATACATGCATTATACTAACTATGGCGGA

                           80        90       100       110       120       130       140
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sample 11-194      TGTATCTTTCCCATGAATGTACAGTAATACCTTGAAGGTGAACACTTATATATATATATATATATATATA
Sample 12-174      TGTATCTTTCCCATGAATGTACAGTAATACCTTGAAGGTGAACACTT--------------------ATA
Sample 12-194      TGTATCTTTCCCATGAATGTACAGTAATACCTTGAAGGTGAACACTTATATATATATATATATATATATA

                          150       160       170       180       190
                   ....|....|....|....|....|....|....|....|....|....|....
Sample 11-194      TATATATATATATATATATCAAATTACCTACTATTATTAAAATGTAAGTATGCC
Sample 12-174      TATATATATATATATATATCAAATTACCTACTATTATTAAAATGTAAGTATGCC
Sample 12-194      TATATATATATATATATATCAAATTACCTACTATTATTAAAATGTAAGTATGCC
```

## Curriculum Vitae

**Oleksandra Kuchma**
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**Personal Data**

Date of birth: 17 May 1982

Sex: Female

Nationality: Ukrainian

**Education and Professional Experience**

2007 – 2010 PhD-student, Department of Forest Genetics and Forest Tree Breeding, Büsgen Institute, Georg-August-University, Göttingen

2004 – 2006 PhD-student, Institute of Cell Biology and Genetic Engineering National Academy of Sciences of Ukraine, Laboratory of cell radiobiology; suspended

2002 – 2004 Technician, Institute of Cell Biology and Genetic Engineering National Academy of Sciences of Ukraine, Laboratory of cell radiobiology

1999 – 2004 Kyiv National Taras Shevchenko University, Dept. of Biology, Kyiv, Ukraine - Specialist's degree in Biology, Qualification - Radiobiology

**Awards**

2006 – 2008 Scholarship of National Academy of Sciences of Ukraine for Young Scientists

**Research Interests**

- Molecular genetics of plants
- Interactions between plants and other organisms
- Mechanisms of response to biotic and abiotic stresses in plants
- Expression of genes in plants under the stress factors influence
- Changes in DNA under the influence of different factors
- Application of plant technologies for clean up of soils contaminated with pollutants

**Research Technique Experience**

Isolation and purification of DNA and RNA, agarose gel electrophoresis, PCR (Polymerase Chain Reaction): RAPD (Randomly Amplified Polymorphic DNA), ISSR (Inter-Simple Sequence Repeat), SSRs (Simple Sequence Repeats), AFLP (Amplified Fragment Length Polymorphism), light microscopy, cytogenetical studies, phytoremediation technology, work with plant cell culture, cloning, sequencing.

**Participation in Research Projects**

- The use of mycorrhizal fungi in phytoremediation projects (Project EC MYCOREM)
- Current state of GM crops in Ukraine and their possible influence on biodiversity (British Council)
- Application of foreign markers for evaluation of foreign genes' transference (Ministry of Sciences of Ukraine)
- Introduction of methods for content evaluation of genetically modified components in foodstuffs, feeds and cosmetic products (Ministry of Ecology of Ukraine)
- Mutation rates in Scots pine (*Pinus sylvestris* L.) after the explosion on the Chernobyl Nuclear Power Plant (DFG, Germany, 436 UKR 113/65/0-1)
- Genetic processes in Scots pine (*Pinus sylvestris* L.) in the Chernobyl exclusion zone (DFG, Germany, FI 569 /11-1)

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Russia, 25 – 28 October 2010. Book of abstracts. Peoples' Friendship University of Russia (PFUR). Pp. 267. ISBN: 978-5-209-03884-9.

**International Language Skills**

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**References**

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