EVALUATION OF GENE FLOW BETWEEN CROPS AND RELATED WEEDS : RISK ASSESSMENT FOR RELEASING TRANSGENIC BARLEY (*HORDEUM VULGARE* L.) AND ALFALFA (*MEDICAGO SATIVA* L.) IN SWITZERLAND

by
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PhD THESIS

Presented in the Faculty of Science of University of Neuchâtel

Neuchâtel, December 1996

IMPRIMATUR POUR LA THESE

Evaluation of gene flow between crops and related weeds: risk assessment for releasing transgenic barley (*Hordeum vulgare)* and alfalfa *(Medicago sativa)* in Switerland

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Neuchâtel, le 7 juin 2005

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ACKNOWLEDGEMENTS

I would like to express all my gratitude to my parents, my husband and my sister's family for their lovening support and encouragement during the years of preparation of my thesis and that they have been always there for me in the difficult moments.

I thank to my supervisor prof. Philippe Küpfer for affording me the opportunuty to do my thesis in the Botanical Institute of the University of Neuchâtel, for his advises and for accepting to be a president of the jury of the thesis.

My very special gratitute is for Dr. François Felber for the useful disscussions, helpfull advises and for the help of collecting the plant material. I thank him also for the critical reading of the manuscript.

I am also grateful to prof. Klaus Ammann, Dr. Julia Keller Senften, Pia Rufener Al Mazyad from University of Bern, and Dr. Yolande Jacot for the help and the discussions we had. I thank them also for the help in collecting the plant material.

My gratitute is for Prof. J. - M. Gobat and Dr. A. - M. Chèvre for accepting to be members of the examination jury and useful counsels.

I thank to Dr. Corinne Zeroual Humbert-Droz for the help with the electrophoretic technique and many other services she rendered me, to Michele Gherardi and Dr. T. Hugo from INRA, France for the help with the DNA technique.

My gratitute is for Dr. E. Zeroual, Dr. G.-F. Zhao, K. Garcia-Herran and S. Leuba-Garcia for the interesting disscussions we had and for the various services they rendered me.

I would like to thank to M. Gauteaub for the help in collecting the plants, E. Fortis, Mr. and Mrs. Boss and Mrs. M.-A. Marguerat, who rendered me numerous services.

Thanks ever so much to Marta, Bénédicte, Abdessamad, Louis and all the colleagues from the Lab for their kindness and support. Their warmness and friendship are very important for me.

This research was carried out in the frame of the coordinated project « Gene flow between selected Swiss crops and related weeds. Risk assessment for the field release of GMO’s in Switzerland .» of the Swiss Priority Programme Biotechnology and is funded by the Swiss National Science Foundation (grant n° 5002 - 035207).

SUMMARY

The aim of the study was to evaluate the risk of gene flow, involving sexual reproduction between cultivated and wild species in Switzerland.

Two cultivated plant species - *Hordeum vulgare* (barley) and *Medicago sativa* (alfalfa) and their wild relatives present in the Swiss flora - *H. murinum* and *M. falcata*, were investigated in order to measure their potential for hybridization and the extent of introgression between them.

Barley and alfalfa have been selected, because of their contrasting characteristics. *H. vulgare* is annual, autogamous food crop, while *M. sativa* is perennial outcrossing fodder plant. In addition *M. sativa* hybridizes easily with *M. falcata*, resulting in fertile hybrids, while *H. vulgare* and *H. murinum* hybridize in a low rate, resulting in plants with low viability.

Experiments were performed on non - transgenic plants, collected in different regions of the country.

Hordeum

When studying flowering biology of *H. murinum*, excerted stamens and visible styles were observed. Thus, absence of cleistogamy and possibility for outcrossing was concluded. There was also possibility for hybridization and risk for gene flow between *H. murinum* and cultivated barley because of the overlaping flowering times of both species. Populations of *H. murinum* from different geographical regions had different flowering periods.

Pollen and seed dispersal experiments for both *H. murinum* and *H. vulgare* were carried out. Both species presented similar pattern of pollen migration, with most of it dispersed close to the plant source. The amount of pollen remained constant after 2 meters. Thus, if *H. murinum* is growing in the vicinity of plots with transgenic barley, most of the hybridization events and

higher rates of gene flow are expected within few meters. However, risk assessment must take into account the possibility of rare pollination events at longer distances.

H. murinum dispersed most seeds close to the plant source. Standard deviation of the distribution corresponded to 0.4 - 0.6 meters and at two meters we did not find any seeds. However, they could be dispersed by zoochory at larger distances. Seeds of *H. vulgare* remained on the spike (typical character of domestication), but nevertheless they can be lost during the transport, which has to be considered by the risk assessment.

In the course of this study, the first chromosome counts for *H. murinum* for Switzerland were carried out. The species was tetraploid with 2n = 4x = 28 chromosomes. *H. vulgare* was diploid with 2n = 2x = 14 chromosomes.

Isozyme markers for *H. murinum* and *H. vulgare* with glutamate oxaloacetate transaminase (GOT) and esterase (EST) were determined. Mantel test for differences between both species on isozyme level was significant. Good separation between them was performed by the Principal Coordinate Analysis (PCOORD) as well, confirming the absence of hybrid and introgressed individuals.

Significant geographical differences between *H. murinum* populations on enzyme level was also demonstrated by Mantel test.

No hybrids were obtained after experimental hybridization between *H. vulgare* and *H. murinum* in the field and greenhouse as well as no natural hybrids were detected in the investigated populations.

Thus, low risk for cultivation of transgenic barley in Switzerland was concluded, because of the hampered hybridization between *H. vulgare* and *H. murinum* and absence of evidence of gene flow between them.

Medicago

Medicago sativa and *M. falcata* are tetraploids (2n = 4x = 32) in most parts of Switzerland. Important contribution of this study was the discovery of diploid *M. falcata* (2n = 2x = 16) in Switzerland, in Unterengadin, together with Dr. F. Felber and P. Rufener Al Mazyad. This ploidy form was not known to exist in the country before. The discovery is important for the risk assessment of the gene flow between cultivated *M. sativa* and the wild relative *M. falcata*, because of the fact that the hybridization between both species is not easy between different ploidy forms. Hybrids between *M. sativa* and *M. falcata* are denominate as *M.* x*varia*.

The isozyme analysis with peroxidase (PRX), maleic enzyme (EM) and malate dehydrogenase (MDH) did not reveal specific bands for *M. sativa*, *M. falcata* or *M.* x*varia*. However, differences between diploid *M. falcata* tetraploid *M. falcata* and *M. sativa* was noticed, as some of the electrophoretic bands, existing in both *M. sativa* and tetraploid *M. falcata* were not present in the diploid *M. falcata*. Interesting enough was the presence of one MDH band, typical for the diploid *M. falcata* in the zymograms of *M. sativa* from Unterengadin, indicating that some gene flow might happen between both species although they present different ploidy levels.

PCOORD analysis separated well diploid *M. falcata* from the tetraploid species and Mantel test gave a significant difference between *M. sativa*, both ploidy forms of *M. falcata* and *M.* x*varia*. That difference remained when diploid *M. falcata* was removed from the sample.

The high rate of natural hybridization between both *Medicago* species at the tetraploid level was assessed also with the results, obtained with RAPD analysis of one population.

In addition, hybrids were detected in the F_1 progeny of the plants after experimental hybridization between *M. sativa* and tetraploid *M. falcata* in the greenhouse and in the field.

As both species hybridize freely at tetraploid level, high risk for gene flow between them was concluded.

When assessing the risk for cultivating transgenic *M. sativa*, the geographical differentiation of distribution of the both ploidy forms of *M. falcata* has to be considered. The possibility of gene transfer to tetraploid *M. falcata* in Swiss Plateau and most alpine valleys is very high, contrasting with the region of Unterengadin, where the diploid form grows.

CHAPTER 1:

OBJECTIVES OF THE THESIS

During the last fifteen years the process of conventional plant breeding has been improved by biotechnology. The new molecular and cellular biology techniques have provided opportunities to modify plants, animals and microorganisms in novel ways, to be employed in industry, agriculture and human health. The insertion of a gene from one organism into another, unrelated genetically does not occure in the nature. Therefore, genetically modified orgenisms cultivated outdoors may generate hazards for environment which must be evaluated. Investigations on risk assessment of genetically modified organisms, performed in Switzerland up to now were restricted to a review of the bibliography (Jacot 1994, Jacot and Jacot 1994) and there was a need for more detailed study of the problem as well as specific studies on the regional scale.

The purpose of this study was to evaluate the risk of gene flow involving sexual reproduction between GMO's and the wild flora (vertical gene flow).

Experiments were performed with non - transgenic plants of barley (*H. vulgare*) and alfalfa (*M. sativa*) in order to measure their potential of hybridization and introgression with *H. murinum* and *M. falcata* respectively. The two species have been chosen because of their contrasting characteristics. Barley is an annual plant, while alfalfa is perennial. *H. vulgare* is anemogamous while predominantly autogamous and *M. sativa* is entomophilous and outcrossing. *M. sativa* hybridizes easily with *M. falcata*, resulting in fertile hybrids, contrasting with *Hordeum*, which forms hybrids, but with low viability. Finally, *Hordeum vulgare* is a food crop and *Medicago sativa* is a fodder plant. Thus the expected risk of releasing transgenic plants from both species is different.

The study was realized within the framework of the Swiss Priority Programme ″Biotechnology″, in parallel with two other PhD thesis (Julia Keller Senften and Pia Rufener

Al Mazyad), including the same species, but with different approach. The present study focus on genetics, including empirical observations and experiments, while the other two thesis are based on phytogeography and morphology, mostly with the descriptive perspective.

To determine the rate of gene flow between wild and cultivated species two types of methods were used:

a) direct: seed and pollen dispersal, natural, and experimental hybridization

b) indirect: evaluation of gene flow in the hybrid zones between the crop and the wild relative, using various techniques such as karyology, isozymes and RAPD.

Based on these data, the risk for releasing transgenic plants in Switzerland was assessed. The data will contribute to the botanical files, which synthezise life history traits of the crop and the potential of gene escape to the wild flora.

CHAPTER 2:

INTRODUCTION : RISK ASSESSMENT

2.1. GENETIC MODIFICATION (Rudelsheim 1992 - 1994)

Genetic modification of plants is defined as the application of recombinant DNA technology to the production of new crop varieties. The process includes four steps:

1. Isolation of a specific gene from plants, animals or microorganisms

2. Insertion of the gene into a special « carrier » DNA molecule, called a vector and its propagation in a host organism, usually a bacterium

3. Use of such vectors to transfer the gene of interest into the plant cell

4. Regeneration of the plant from individual modified cell through tissue culture techniques. This plant then carries the transferred gene and can transmit it to its progeny.

There are several methods for producing transgenic plants. The most common is *Agrobacterium*-mediated transformation, using *Agrobacterium tumefaciens* or *A. rhizogenes*. Particle bombardment, DNA uptake into isolated protoplasts and electroporation are the other widely used methods.

The first transformed plant was tobacco. Now various species including tomato, maize, potato, alfalfa, sugar beet, oilseedrape, wheat etc. have been transformed.

Most often genetic transformation has been used to produce herbicide tolerant, pest and diseases resistant plants. Biotechnology is also capable of providing new pharmaceutical and other chemical substances, synthesized by plants, for improved nutritional properties of the plant or for resistance against environmental stress, such as drought, high salinity or cold. It is also possible to modify plant protein and oil content. All these new characteristics transferred into the crops have clear advantages. However, there are serious concerns about the containment of transgenes and their products within agricultural populations and the risks

associated with their possible escape. Genes can be transfered from genetically modified organisms (GMOs) by sexual reproduction to the wild species (vertical gene flow) or to microorganisms, aphids and viruses (horizontal gene flow). Since it is better to prevent than to cure, the precautionary approach was accepted. Before any introduction of a transgenic plant or product (experimentally or commercially) an assessment of its potential impact has to be made.

2.2. DEFINITION OF RISK ASSESSMENT (Tzotzos 1995, Levin 1995)

Risk assessment is a scientific process, an essential part of which is to define the type of the risk and its degree. Before assessing, however, it is necessary first to define what is meant by « risk ».

It is generally accepted that there are two components of risk: hazard and exposure.

2.2.1. HAZARD is defined as the potential (toxicological or ecotoxicological) harmful property of the product, encoded by the newly constructed genetic material or by the host carrying the genetic material or its product.

The hazard depends on the kinds of intrinsic characteristics that could cause undesirable effects and the intensity of their exposure.

2.2.2. EXPOSURE is the probability that susceptible populations or individuals are presented to respond to an agent of effect (stressor). It depends on the kind of the population or susceptible individuals, their number, their proximity to the stressor, the amount of the stressor and the relationships between susceptible populations and the stressor.

2.2.3. RISK is defined as the probability of the hazard occuring.

2.2.4. RISK ASSESSMENT is an analytical tool that facilitates the organization of large amounts of scientific methods, models and data with the goal of estimating the potential risk posed by a process of interest.

It is an evaluation process of possible adverse effects of living organisms on the environment and human health.

Risk assessment can be divided in two phases. In a first phase potential hazards are identified. The second phase determines the likelihood and consequence of the hazard to occur.

2.2.5. RISK MANAGEMENT is the stage following the risk assessment in which perceived risks are balanced against other criteria in order to make decisions about risk mitigation and control.

2.3. TYPES OF RISK

The essential part of the risk assessment is to determine how the transgene might change the environment, when compared to the impact of the nontransgenic crop.

When concerning transgenic plants there are four risks, recognized to be of major importance:

2.3.1. Enhanced toxicity and allergenicity of the transgenic plant

The modified plants may yield toxic substances in the product or the product itself can be harmful for non - target organisms such as birds, insects, symbions or for human health (Dale 1995, Tiedje et al. 1989). Incomplete degradation of chemicals, leading to the formation of chemical deposits or to the production of even more toxic by-products has a potential risk for the environment (Vogel and McCarty 1985).

2.3.2. Establishing of feral populations

The transgenic plant could escape due to the seed dispersal out of the cultivated field and establish feral population. These plants may therefore hybridize with the wild relatives that are geographically close, thus increasing the risk for further gene flow. According to Crawley (1987) it is difficult to predict whether a particular plant is invasive, but it is possible to determine the types of habitats that are invasible. He showed that plant communities with a high proportion of bare ground and frequent and intense soil disturbance were more likely to be invaded by alien species than the stable late - successional communities.

2.3.3. Horizontal gene flow

It is defined as a gene escape from an organism to another organism without sexual reproduction. Thus the gene transfer might occur by horizontal gene flow from plants to animals and microorganisms and vice versa. It is still unclear whether such gene flow is possible and whether the transgene could be incorporated in microorganisms genome. The assessment of the ecological risk in this case is hampered because the soil biological processes are poorly understood and deficiency in the basic monitoring procedures to enumerate the soil organisms exist (Abbott 1994).

When dealing with virus resistant plants the processes which may occur with potential environmental risk are transcapidation and template switching. In the first case when a virus attacks a transgenic plant, producing the coat protein of another virus, its nucleic acid could be capsidated with the coat protein of the original donor vector. In the second case there is a recombination between viral sequences, incerted in the plant genome and infecting viral particles. Both processes result in a « hybrid virus ». They could lead to a production of new viruses or give opportunity for mixed infection (Dale 1995).

2.3.4. Vertical gene transfer

It includes the transfer of genes by hybridization and introgression of transgenes into wild related species.

Plant breeders have long time been concerned with the gene flow between crops and wild species (Anderson 1949, De Wet 1975, Barrett 1983, Ellstrand 1988). Early studies mainly focused on the gene flow into crop strains because of fears for the purity of the seed material (Sprague 1938, Haskell 1943, Jones 1948, Hutchcroft 1955, Nieuwhof 1963). More recently the concerns regard gene flow from crops to the wild relatives via hybridization. It has been suggested that the transgenes may be transfered into natural and weed populations, creating invasive weeds or increasing the weediness itself (Ellstrand 1988, Crawley 1990, Keeler and Turner 1990, Manasse 1992). The main gene vector is the pollen. The possibility of contamination depends on the way of the dispersal and the geographic remoteness of the cultivated and wild plants. If the transgene is stable, it will be transmited in the next generations and in this way continue its diffusion. The transgene could be transmited by other vectors, such as microorganisms and viruses as well.

The elimination of wild or desirable naturalized species through competition or interference is a possible outcome in some cases.

Unwanted changes in the transformed plant may occur as well. They can include the tendency for a self - pollinated line to outcross, increasing the chance for spreading the transgene to the wild relatives.

2.4. DEGREE OF THE RISK

Different plants have different abilities to exchange genetic material with their wild relatives. Studies carried out at the national level in the Netherlands and UK have shown that the plants

could be classified into different categories according to the probability of gene flow to occur and extend.

Some plant species (potato for example) do not have wild relatives in Europe, thus the probabilities of gene transfer practically does not exist. On the contrary others (alfalfa) hybridize freely and in large extent with their wild relatives. Between these two extremes there are plants with different probabilities of gene transfer. Therefore various classifications of the espected risk were proposed.

For the Netherlands Frietema de Vries et al. (1993) have studied a herbarium collection of 42 cultivated species, present in Dutch flora. They have established Botanical files for each of the species. Each file gives information about the cultivated plant itself, its relatives, hybridization/crossing ability (gene flow by pollen) and observations on escapes from the field to nature (gene flow by diaspores). The information is summarized in codes (Dd, Dp, Df, Dpdf), indicating possible ecological effects of the cultivated plant to the wild flora:

- Dispersal by diaspores (Dd)

A scale from 0 to 4 is used to determine the probable effect of the crop by dispersal of its diaspores. The higher is the code the higher is the expected effect.

- Dispersal by pollen (Dp)

In this case it is important to know if the wild relative exists and if so, what the relationship is between it and the crop. The information is summarized in Dp codes on a scale from 0 to 5, indicating the degree of relationship with the nearest relative.

- Frequency of distribution (Df)

Df code is established on a scale from 0 to 3 in order to indicate the frequency of distribution of the nearest crop relative. The more frequent a near relative is found in the wild, the higher the chance for gene flow is.

- Ddpf codes

It is a summary of the three above mentioned codes. It implies that any, minimal or local, or substantial ecological effect should be expected. When the effect could not be assessed from what is known, the number for the code is 9, meaning that further research is necessary.
This approach has been extended to Switzerland by Jacot and Ammann (in preparation).

According to Raybould and Gray (1993) the escape of transgenes refers to the persistance of the crop`s modified genetic material in non agricultural habitats, which may occur by the crop itself invading disturbed, semi - natural or natural habitats to form feral populations, or by transfer of the transgene to the wild relatives by hybridization. In the UK they have identified crops which form feral populations and those which hybridize with wild relatives. Based on the information, obtained for crop - wild relative hybridization they have classified the crops into three groups representing increasing capacity for cross - pollination with wild species:

- group I have no wild relatives or are not sexually compatible with them (potato, tobacco, maize)
- group II - « low probability of gene flow ». Close wild relatives with limited sexual compatibility are present in the same geographical areas as the cultivated crop (oilseedrape, lettuce)
- group III - « high probability of gene flow ». Wild relatives with reproductive compatibility are present in the neighbourhood of the cultivated crop (sugarbeet, alfalfa, poplar).

A classification according to the generic trait (herbicide tolerance, disease resistance, insect and virus resistance, marker genes, stress tolerance, enhanced plant growth or survival) and to the possible effect they may have to the wild relatives if transferred, was proposed by Ahl Goy (1993):

- class I - « the minimal advantage class » - traits which are expected to confer little, if any, advantage when transfered. It includes traits which might confer disadvantages as well. These are marker genes and genes for improving or changing the quality of the crop product (e. g. delayed ripening, male sterility, changing the colour of the flower etc.)

- class II - « the low advantage class » - traits with the potential of conferring an advantage under the relevant selection pressure. This group was split into two subgroups, depending on the absence (class IIa) or presence (class IIb) of the relevant selection pressure outside agricultural systems. Class IIa contains herbicide tolerance and class IIb, resistance to insects, viruses, diseases and stress.

- class III - « the high advantage class » - traits which have the potential to confer an advantage under any conditions. It includes traits conferring enhanced growth and survival.

Further, the author defines the « potential environmental risk » as the product of the « probability of transfer » and the « consequence of transfer ». Then the assessment of the risk is a combination of both factors. She classifies the risk as minimal, low and high. The risk is minimal when the probability of transfer is high but the consequence of transfer is minimal or vice versa. A potential high risk is considered only when traits with the potential of conferring a selective advantage under any conditions are present in the crops with high capacity for transferring genes. The evaluation of the environmental risk in this way indicates that non of the field trials, conducted with transgenic plants for the period between 1986 and 1992 pose a high risk, 91% represent minimal risk and 9% - low risk (Ahl Goy 1993).

2.5. METHODS FOR MEASURING THE ESTABLISHMENT OF PLANT'S FERAL POPULATIONS AND THE VERTICAL GENE FLOW. DATA, REQUIRED FOR THE RISK ASSESSMENT

2.5.1. Methods and approaches for measuring the establishment of feral populations and the vertical gene flow

In order to obtain information on the safety of genetically modified plants, appropriate methods for monitoring the gene flow must be developed.

The gene transfer is possible through pollen (vertical gene flow) and seeds (establishing of feral populations) and that is why most of the gene flow studies monitor their movement.

Four different approaches are used to estimate the gene flow by pollen (Ellstrand 1992):

1) measuring pollen dispersal from point sources

2) measuring gene dispersal from point and block sources

3) inferring gene flow from natural population genetic structure

4) paternity analysis of progeny in sink populations

The first approach measures the pollen movement either indirectly from pollinator foraging distances (Schmitt 1980) and dispersal of pollen analogues (Campbell and Waser 1989). The second approach involves the creation of experimental populations using source plants, bearing a genetic marker, surrounded by plants fixed for alternate alleles. The progeny harvested at different distances is screened afterwards and gives information about successful fertilization and fruit set (Handel 1983, Smyth and Hamrick 1987).

The third approach measures the gene flow from existing population structure, using a number of statistical methods, based on alleles frequency distribution to estimate indirectly gene flow (Slatkin 1985, Crow 1986, Hamrick 1987).

The last approach measures the gene flow directly by paternity exclusion. First multilocus genotypes of all reproductive individuals in the population are determined. A comparison of the multilocus genotype of the maternal plant with that of its progeny allows the reconstruction of paternal gametic contribution. The gene flow rate may than be evaluated (Ellstrand and Marshall 1985, Hamrick and Schnabel 1985).

All four types of studies suggest that the gene flow can vary considerably within species, among seasons (Campbell and Waser 1989) and over a season (Palmer et al. 1988), depending on plant genotype (Tonsor 1985), plant density (Levin and Kerster 1969), ecological conditions and the way of transfer (Schmitt 1980, Peakall 1989),

The pollen dispersal will only be effective if the pollen grains land on the stigma of the related plant species. Then hybridization may occur and its rate will depend on the frequency with which the wild relative could be found and the fitness of the resulting hybrids. The potential impact of the hybrid will depend on its fertility and ability to self - fertilize or backcross to a parental species (Frietema de Vries 1993).

The seed dispersal studies are not so widely used for estimating the gene flow, because it is more difficult for monitoring and quantifying than pollen movement (Hamrick et al. 1995). The use of the paternity analysis of the progeny proved to be more suitable, but even with this method the documentation of seeds movement is difficult (Hamrick et al. 1995). There are two approaches when using it:

- to develop a set of genetic markers for maternally inherited genomes (chloroplast and mitochondria). If a sufficient number of polymorphic loci are defined the seeds or seedlings could be sampled afterwards to determine if the maternally inherited genome is coming from an adult that belongs to the population. If not, the seeds or seedlings have invaded it. However, up to date cpDNA and mDNA have not proved useful markers for such analyses.

- using nuclear genes for identifying seeds without possible parents within the study site. However it is more difficult and less probable to eliminate both parents than to exclude only the paternal individual for the progeny, whose mother is known. (Hamrick et al. 1995).

The success of the dispersal by the seeds or diaspores is related to the dispersal capacity and the fitness of the individuals, resulting from them. Among the important factors to take into account are the seed quality, and the capacity of the plant to produce viable seeds (Frietema de Vries 1993). In any case the rate of gene flow depends on the seed dispersal mechanisms. It is highest for wind - dispersed species and lower for the explosively dispersed. Species with animal - mediated dispersal are in between.

2.5.2. Techniques, providing markers for the pollen and seed dispersal studies

Pollen and seed dispersal studies have been carried out previously using markers such as flower colour (Kehr 1973). More recently isozymes were used as genetic markers (Krauss 1994, Arias and Rieseberg 1994, Ballal et al. 1994, Chèvre et al. 1995).

Although isozyme analyses have been proved useful for genetic studies and plant identification, they are limited by the small number of loci sampled by the technique. Advances in molecular biology have provided DNA - based markers (RAPD, RFLP) which resolved this problem and are neither affected by the growth environment of the plant nor of its stage of development (Tanksley 1983, Williams et al. 1990). Restriction fragment markers (RFLP) are codominantly and stabely inherited in a Mendelian fashion and they disclose unlimited polymorphic markers. The problem with RFLPs is that the procedure is time consuming, requires a large amount of DNA (2 to 10 mg) and suitable probes, and usually involves the use of radioisotopes. The Random Amplified Polymorphic DNA (RAPD) is based on the Polymerase Chain Reaction (PCR) and utilizes a single synthetic

oligonucleotide primer. In comparison with RFLP the procedure is less expensive, faster, requires a small amount of DNA (0.5 to 50 ng), does not involve the use of radioisotopes and requires less skill to operate. It has been proved useful for analyses at subgeneric level, interspecific taxonomy and classification, geographic variation, cultivar and varieties identification, genetic linkage, analyses of genetic diversity, gene flow and somaclonal variation, hybridization studies and studies of ancient DNA (Demeke and Adams 1994).

Usually when producing transgenic plants marker genes such as kanamycine resistance (*neo*), glufosinate resistance (*bar*), or GUS are transferred to the plants and used after to check if a certain transformation procedure is successful. These genes are widely utilized as markers for pollen and seed dispersal studies as well (Rudelsheim 1992 - 1994, Skogsmyr 1994, Chèvre et al. 1995, Mikkelsen et al. 1995).

2.5.3. Data, required by the EU Directives for the risk assessment

Risk assessment must be carried out at each step of the release of transgenic plants, starting from laboratory level, continuing with small - scale experiments and ending with the large - scale commercial production. Those data provide the basic information for the risk assessment.

Usually general information about the staff and institution responsible for carrying out the release is necessary. They must have a high level of knowledge and experience, and to be responsible for any field containment and monitoring.

In all cases it is essential to have information about the donor DNA organism and the recipient plant species. The last is the baseline against which the transgenic plant will be compared.

A description of the transgenic plant, including molecular data, stability of the transgene expression, changes of allergenicity, toxicity and the capacity of the plant to persist in the agricultural areas or to invade natural habitats has to be available.

For the study of vertical gene flow it is important to know the breeding system (allogamy or autogamy) of the plant and related species, as well as the pollen and seed behaviour. Allogamy promotes gene flow, while autogamy reduces it. Flowering time differentiation also decrease the potential gene flow.

Generally, when producing transgenic plants, antibiotic resistance genes are used as markers. Other DNA sequences may be used as linkage with the vector or provide other functions associated with the use of DNA - recombinant methods. It is therefore necessary to know the nature of this DNA, so that any consequences can be considered.

Ecological information on the release site is also important. This includes a survey of plant species that grow in the vicinity of the release and the dynamics of their populations in order to predict the risk in the following years. Information about the pollen and seed dissemination, the distance of successful pollination and the stay of the seeds in the soil is neccessary as well as. This information is important also to be able to The location and the type of anticipated target organisms must be specified. The target organisms are those, which the transgenes are targeted to affect. Non - target organisms are those which are not primary target of the modification and they include those that are affected unintentionally.

It is important to describe changes in the transgenic plant that may alter its invasiveness in wild habitats, because of the possibility to establish feral populations, or if the transformation changes its ability to propagate sexually or asexually.

Once plants are released in the field and if they are allowed to flower and set seeds, the transgene can move. An important part of the risk assessment is to determine the extent of which it is possible to monitor transgenes after the release and the efficiency with which it is

possible to destroy the plant if it becomes necessary. Efficient methods of identifying transgenic plants or transgenes presented in non - target species may be necessary. This may be a visual marker (B-glucuronidase), a selectable marker (antibiotic resistance) or molecular markers, detected by PCR, Southern hybridization etc.

2.6. MANAGING THE RISK (EU and USA)

The philosophy of risk assessment and regulations is that each release is considered case-by-case to build up experience and familiarity with particular crop - transgene combinations and eventually to move to a streamlining of regulatory procedures (Tepfer 1993).

The whole process of risk assessment is usually carried out by a multidisciplinary biosafety committee made up of specialists in genetics, molecular biology, environmental science, agriculture, plant pathology and other appropriate fields. It may be neccessary to include politicians - people from the local government, members of the public or local ecological groups in order to ensure public satisfaction.

The first release of a modified plant is experimental. It is at this stage that the major risk assessment must be made. The information is usually outlined by scientists, who carry out the release. Once the results of these experiments are analysed, the commercial release may be done. The release proposal is at first assessed by an Institutional biosafety commitee, where it exists. The benefit of this committee is that usually its members have a good local knowledge and good understanding of the scientific principles. It is also responsible for monitoring the site after the release. The next level are the National biosafety commitees, which are also multidisciplinary and include politicians and representatives of the society, who will carry the national responsibility. They could make further recommendations and require that the proposal be changed. Finally international biosafety commitees may also be set up (like one

established in European Union, OECD, UNIDO). At this level uniform standards for countries should be established, which needs harmonization of the requirements of the National commitees.

Between 1986 and 1994 there have been 1608 field trials with transgenic plants, conducted in more than 36 countries. Of these 858 occured in USA and Canada, 558 in Europe, 109 in Central and South America, 65 in the Pacific Rim and 18 in Middle East and Africa. GMPs from 46 different species have been field tested (Extracts from GIBiP database on field trials 1986 - 1994).

The regulations governing the use of transgenic plants vary considerably between the countries.

In 1990 the Commission of the European Community published Directives on the contained use of GMOs (CEC 1990a) and its deliberate release into the environment (CEC 1990b). These Directives are binding in all EU countries. Under the Biotechnology Action Programme (BAP) initiative, the Commission of the EU decided to support a number of independent projects for developing appropriate monitoring methods which may generally be applicable to GMPs. Later the Biotechnology Research for Innovation, Development and Growth in Europe programme (BRIDGE), built on the experience gained in the BAP, has further expanded the experimental methodology on a large scale (Rudelsheim 1992-1994). The countries which took part in this programme were Belgium, Denmark, France and United Kingdom. The plants studied were potato, oilseed rape, sugar beet, alfalfa and tobacco. During, the project methods have been developed to design the experiments and to monitor the greenhouse and field experiments.

In the USA, three agencies share the responsibility for the release of transgenic plants - the United States Environmental Protection Agency (EPA), which is responsible for environmental care; the United States Department of Agriculture (USDA), which is

concerned with the safety of crop plants and food products, and the Food and Drug Administration (FDA), which ensures efficiency and safety of food and pharmaceutical products (Levin and Strauss 1993). These agencies are responsible for achieving a balance between risks and benefits. There are numerous Acts, regulating the release of pesticides, toxic substances, food, drugs and cosmetic products when they are derived from microorganisms, plants and animals, including transgenic ones (Federal Insecticide, Fungicide and Rodenticide Act, Toxic Substances Control Act, Federal Food, Drug and Cosmetic Act, Federal Plant Pest Act and Plant Quarantine Act). In the USA the release of modified organisms may be subject of different federal laws and more than one agency may have to be consulted.

In the USA regulations are product - specific, while the EU has made the regulatory regime process - specific. It is triggered by the process by which the transgenic plant is made. Countries all around the world are adopting models related to these.

As the possibility for the movement of trangenic plants or their diaspores exists, international regulations and harmonization of standards and procedures is necessary.

The Organization for Economic Cooperation and Development (OECD) has developed guidelines for the contained use of transgenic organisms (OECD 1986, 1990, 1992).

Various international initiatives have been undertaken for developing countries through several organisations, including the United Nations Industrial Development Organisation (UNIDO) and United Nations Environment Programme (UNEP). The aim of these initiatives is to facilitate the development of common standards of biosafety. UNIDO is promoting an International Biosafety Information Network and Advisory Service (BINAS), with which Switzerland is connected. It provides data and advice from experts to the national authorities, where they do not already exist.

2.7. RESEARCH ON RISK ASSESSMENT IN SWITZERLAND

In Switzerland the commercial use of GMPs is not allowed. The experimental releases are very restricted - only two in 1991 (Ahl Goy et. al 1994).

The Swiss Interdisciplinary Commission for Biological Researches (SICBR) is the administrative body, responsible for the procedures of registration of the projects in the field of genetic engineering under the security standards. Based on the EC Directives and the German national law about genetic engineering, it prepared in 1992 a Directive for security measures for experimental use of GMOs in laboratory environment and their field release (Diggelmann and Pythoud 1993).

The Swiss Federal Council has proposed to amend the federal law on environmental protection in order to provide a legal framework to warrant a sustainable and safe development and use of genetic engineering in Switzerland. This new legislation is intended to bring the country into line with the practice of the EU.

In 1991 the Swiss working group on genetic ecology was constituted in order to put together information about risk assessment of field release of GMOs and to foster researches in the field of genetical ecology.

A study on environmental risk due to biotechnology products in agriculture has been made, based on literature researches and discussions with plant breeders, biologists, ecologists, biotechnologists etc. in the « Institut des Hautes Etudes Administratives et Politiques » of the University of Lausanne (Jaunin 1992).

In 1992 the Swiss Office of Environment, Forests and Landscape has initiated a bibliographical report on risk assessment for releasing GMOs, including a preliminary study of floristic parameters influencing the gene flow and including a plan to work out guidelines

for further risk assessment. It emphasizes the importance of developing a regional view in assessing floristic and biogeographical factors, and it stresses the lack of knowledge on this subject in Switzerland (Jacot et al. 1992).

In 1992 a Priority Programme on Biotechnology was initiated by the Swiss National Science Foundation. The programme couples basic research and development, links the activities of various Universities and Institutes, and intends to bring together science, industry and federal offices. One of its key issues was the initiation of biosafety research in Switzerland. As a core project under this programme the Agency for Biosafety Research and Assessment of Technology Impacts (BATS) was established at the begining of 1993. BATS has evaluated national and international research work, congress reports and specialised literature in the field of biosafety and technology impact assessment. It was established as a source of biosafety information for both specialists and the public.

In 1995 the Programme has externed its information activities, establishing B.I.C.S. (Biotechnology Information and Communication Service) and Biotectra technology transfer service in 1996. They were organised with the purpose to diffuse the information, provided by the research projects under the Priority Programme Biotechnology and to facilitate the technology transfer from the science to the industry. They aim to make all the information concerning biotechnology available to the publics as well.

Under the same programme a study on « Gene flow in selected Swiss crops and related weeds, risk assessment for the field release of GMOs in Switzerland » in 1993 at the University of Bern and the University of Neuchâtel was initiated, which is the first of this kind for the country. It concentrates on the dynamic biogeography of weeds and the gene flow among selected cultivated and wild species in natural conditions. The rate of natural hybridization is studied by investigating herbarium specimens and plants collected in preliminary determined test areas in different parts of the country. Experimental

hybridizations in the greenhouse and in the field are done as well in order to get more information about the degree of the gene flow. Using morphological and genetic markers the mechanism of genetic exchanges is investigated as well as pollen and seeds dispersal. Distribution maps of the investigated species are intended to be produced. In addition a bibliographical study of vertical gene flow and establishing of botanical files is carried out.

The present thesis deals with the experimental and genetical parts of that project. Two cultivated species and their close wild relatives were examined - *Hordeum vulgare* (barley) and *H. murinum* as well as *Medicago sativa* (alfalfa) and *M. falcata*.

CHAPTER 3:

INTRODUCTION : *HORDEUM*

3.1. CENTRE OF ORIGIN

Hordeum (L.) belongs to the tribe *Triticeae* of the family *Poaceae* (*Gramineae*). The tribe includes a number of important cereal crops, such as wheat (*Triticum* spp.), rye (*Secale cereale*), barley (*Hordeum vulgare* L.), the artificially synthesized triticale (*x Triticasecale*) as well as many important forage grass species. The genus *Hordeum* contents 11 species (Pedersen and Hojland 1994).

The centre of its origin is in Southwestern Asia (« The Fertile Crescent » - Iran, Turkey, Israel, Jordan) and Central Asia (Bothmer et al. 1991).

3.2. PLOIDY LEVEL

The basic chromosome number is x=7. Most of the *Hordeum* taxa are diploid (2n=2x=14). Tetraploids (2n=4x=28) and hexaploids (2n=6x=42) also exist.

Four basic quite unrelated diploid genomes occur in the genus: genome « I » (*H. vulgare*, *H. bulbosum*), genome « Y » (*H. murinum*), genome « X » (*H. marinum*) (Dewey 1984, Bothmer et al. 1983, 1985, 1986). All the other diploid species have genome « H », which is also presented in several tetraploid *Hordeum* species (Bothmer et al. 1987, 1988).

3.3. BREEDING SYSTEM

The species included in the genus are annual or perennial, the majority being perennial. The former include winter as well as spring annuals.

Autogamy predominates in the annual species, although some may be occasionally outcrossing. Most of the perennial taxa have a mixed reproductive system without any specialization. Some are mainly self - fertilizing with small anthers and stigmas and flowers which mostly do not open (*H. patagonicum*). Others are obligate outbreeders (*H. bulbosum*)

with self - incompatibility system, which largely prevents self - fertilization (Bothmer 1979). Barley does not reproduce asexually.

3.4. TRAITS TO DISTINGUISH *HORDEUM* SPECIES

The diagnostic characters of the genus are the possession of three one - flowered spikelets at each rachis node, of which the lateral spikelets are pedicellate. The spikes are identically built. The variation is in shape, size and colour of various parts. The rachis is flat with shorter or longer hairs or brislets on the edges. At each node there are three one - flowered spikelets: a so-called triplet. The central spikelet is sessile in most species, the palea is glabrous or hairy, more or less bifid in the apex. The lateral spikelets are rarely both female and male fertile, more often male fertile or completely sterile. According to the species the lemma extends into a shorter or longer awn.

The anthers also vary greately in size, the longest ones are found in outcrossing species and the smaller ones in self - pollinating species. The ovary is hairy and has two long or short feathery stigmas.

The leaf sheaths may be hairy, but the upper ones may be glabrous. The nodes are glabrous to hairy. The culms are mostly erect in a few cases nodding or even de - or procumbent. Auricles are absent in most species, but very prominent in others (Fig. 1).

3.5. CHARACTERISTICS OF *H. VULGARE* AND *H. MURINUM*

H. vulgare comprises both cultivated and wild forms that are interfertile and closely related. The cultivated forms are refered to spp. *vulgare,* whereas the wild are retained as spp. *spontaneum* (C. Koch.) Thell. The last comprises two - rowed forms. The four - and six - rowed are included in ssp. *vulgare*.

The *H. murinum* complex is autogamous, containing three taxa: *H. glaucum*, *H. murinum* (sensu stricto) and *H. leporinum* (Link) Arcong. (Giles and Lefkovitch 1986).

3.5.1. General distribution

H. vulgare has been cultivated since the Neolitic. It was among the first crops under domestication around 7000 B.C. Since early times it has been grown throughout the Mediterranean region and Ethiopia, Southwestern Asia, China and Japan. Because of its importance for both forage and brewery it is now cultivated in all temperate areas of the world. Major barley production areas are Europe, the Mediterranean fringe of Northern Africa, Ethiopia, the Near East, Russia, China, India, Canada, USA and Australia. It is rare in the tropics except in cool highlands in Mexico, the Andes and East Africa (Harlan 1976).

It occupies grassland areas, meadows as well as semi - arid regions sometimes on rather salty soils, growing from sea level in the Mediterranean area and at the Middle East up to 4500 m in the Hymalayas.

H. murinum is distributed practically on the whole territory of Europe except Scandinavia, Poland, Iceland and Ireland (Humphries 1980), from the Atlantic to the West of Asia. The original habitats of this species were probably seasides, sandy river beds and drinking places for animals. Presently it occurs in many places, disturbed by men (Bothmer et al. 1991).

3.5.2. Distribution in Switzerland

H. vulgare is cultivated in Northern, Western and Southeastern Switzerland, the region of lake of Geneva, the cantons of Valais and Tessin and the whole of the Swiss Plateau (Welten and Sutter 1982) (map 1a).

Seven wild *Hordeum* taxa are present in the Swiss flora according to Aeschimann and Burdet (1989). Among them are *H. distichon* L., *H. vulgare* ssp. *polystichon* (haller f.) Schinz and

Keller and *H. vulgare* spp. *hexastichon* (L.) Celak. *H. murinum* is a common ruderal species. *H. leporinum* is less frequent and has similar ecological requirements. Finally *H. secalium* Schreber (= *H. nodosum* L.) and *H. jubatum* L. are extremely rare in Switzerland.

H. leporinum is mentioned only in few locations of Switzerland - south of the cantons of Tessin and Graubünden, the region of Geneva, the cantons of Aargau and Valais. Herbarium data indicates its presence in Northern Switzerland.

Thus *H. murinum* is the commonest of the wild representatives of *Hordeum* in the country. It is widespread in canton Tessin, Valais and the region of lake of Geneva. It grows also in the region of the Lake of the four cantons, and is more rare in Northern, Eastern and Western Switzerland (map 1b). In all mentioned regions, where it is present, except canton Tessin and the lake Geneva region, there are sites where it had existed previously, but has not been found recently.

The locations of both *H. vulgare* and *H. murinum* overlap largely.

3.5.3. Morphological characteristics

Plants of *H. vulgare* are up to 1 m long and have usually erected culms, flat leaves, which are either scabrous or glabrous. Auricles are long prominent, linear - falcate, surrounding the culm. Spikes are longer and bigger than those of *H. murinum*, the colour varies from green over purplish to blackish. It has 10 - 30 nodes each bearing three one - flowered spikelets, of which one, two or all three are fertile viz. two - four - and six - rowed barley. The awns are normally long and straight, rachins brittle or tough. The central spikelet is sessile, glumes are flattened, lemma is glabrous or scabrous mostly near the apex, usually tightly embedding the kernel. The triplet is the functional diaspore with one central female - fertile flower and two lateral female - sterile flowers in two - rowed barley. In six - rowed all three spikelets are fertile (Reid 1985). Anthers are yellowish, rachilla is short or up to half of the length of the

palea with long or short hairs. Lateral spikelets usually are sessile when fertile and sessile to pedicellate when sterile, obtuse to acute or with an awn almost equally as well developed as the central one when fertile. Glumes are more or less flattened (Bothmer et al. 1991).

H. vulgare is a spring or winter annual mainly inbreeder. It is a diploid with 2n=14, but artificial tetraploids (2n=28) have been produced. It has a genome called « I ».

H. murinum plants are usually shorter than those of *H. vulgare* - 30 - 60 cm high. Culms are more or less erect, sometimes prostrate. Leaves are narrower, flat occasionally with involute margins. Spikes are smaller than those of *H. vulgare*, green with relatively small lateral spikelets. Central spikelet is sessile to subsessile, about the size of the lateral ones, excluding awns. Anthers of the central spikelet are of about the same length as the lateral ones. Palea of lateral spikelets is almost glabrous, rachilla is pale and thin (Bothmer et al. 1991).

H. murinum is a winter annual, mostly inbreeder. It is tetraploid with 2n=28 and contains a separate genome called « Y ».

3.5.4. Isozyme and DNA molecular markers

The technique of enzyme electrophoresis provides many genetic markers as criteria for taxonomic separation of the species. Most of them have codominant expression and variation occurs frequently. Isozymes allow thus the detection of homozygous and heterozygous individuals, facilitating the determination of genetic variability and amount of heterozygosity in natural populations.

Isozyme polymorphism allows the identification of different *Hordeum* taxa as well as barley varieties. Many isozyme investigations involved *H. vulgare* (Brown 1983, Jorgensen 1986, Nielsen and Hejgaard 1986, Powell et al. 1990, Zhang et al. 1994). A study of barley esterase (EST) for example resulted in revealing a few diagnostic locuses for *H. vulgare*. Jaaska (1992) has found diagnostic alleles for glutamate oxaloacetate transferase (GOT) of *H.*

vulgare and *H. murinum*. A substantial heterogenity in the level of polymorphism among different geographical area and agrigeographical subregions has been demonstrated as well (Zhang et al. 1994). Populations of *H. murinum* were characterized with small isozymic variability over its British, European and Mediterranean range (Giles 1984). The results indicated little genetic differentiation in enzyme characters between populations and most of them tended to be monomorphic. The most variable systems were esterase and malate dehydrogenase (MDH). However the variability between species and taxa is large thus allowing their identification and separation.

The principal difference between auto - and alloploids is the presence of fixed isozyme heterozygosity, considered to be an evidence in favour of alloploid origin. This isoenzyme variation pattern has been observed for *Hordeum* species for aspartate aminotransferase (AAT), superoxide dismutase (SOD), esterase, leucine aminopeptidase (LAP), shikimate dehydrogenase (SKD), peroxidase (PRX) (Jaaska 1987, 1992, 1994) and has been applied to distinguish the two ploidy types in various systematic groups (Ness et al. 1989, Soltis and Soltis 1989, Werth 1989).

Relatively high levels of variation have been observed both within and among *Hordeum* species using RAPD (Gonzales and Ferrer 1993) as well as within and between population diversity (Dawson et al. 1993).

Pedigree relationships in spring barley lines were examined by Tinker et al. (1993) with RAPD. Two - and six - rowed barley were separated by cluster analysis. They have found 19 primers revealing a total of 31 marker bands between 27 different lines and concluded that RAPD markers could be used to gain information about genetic similarities or differences that are not evident from pedigree information.

Finally RFLPs have been used to construct a barley linkage map (Backes et al. 1995, Laurie et al. 1993, Sorokin et al. 1994), as diagnostic markers for wild and cultivated barley (Petersen

et al. 1994) and for different barley cultivars (Pecchioni et al. 1993). Polymorphism in α-amylase genes of wild and cultivated barley was studied using PCR and RFLP (Kiribuchi et al. 1993, Weining et al. 1994).

3.5.5. Risk assessment of releasing transgenic barley

For a long time the stable transformation of barley has been a problem, because most of the varieties had a low response to tissue culturing. Although progress has been made, stably transformed barley plant were obtained only recently (Ritala et al. 1994, 1995, Wan and Lemaux 1994, Stiff et al. 1995, Harwood et al. 1995, Salmenkallio - Marttila et al. 1995).

The possibility for gene flow from barley to the related species is theoretically low, because it reproduces predominantly by self - fertilization. Nevertheless, reasonably high amounts of pollen can be released and the rate of gene flow will depend on the degree of outcrossing within the species, the successful hybridization and the fitness of the produced plants. Most of the *Hordeum* species are biologically and usually also morphologically discrete entities. With few exceptions the isolation barriers (usually the hybrid sterility) are strict between the species and there is a little genetic exchange in nature. The pollen is dispersed by wind. However, the barley seeds could be spread over considerable distances by animals, humans and transport, and they keep their germination ability for several years. Barley is generally very competitive against weeds. Intraspecific competition is also very strong.

CHAPTER 4 :

INTRODUCTION : *MEDICAGO*

4.1. CENTRE OF ORIGIN

The genus *Medicago* is very extensive, including more than 60 different species, two third of which are annual and one third perennial (Lesins and Lesins 1979).

The primary centre of the genus is found in the Caucasus, Northwestern Iran and Northeastern Turkey (Ivanov 1977).

4.2. PLOIDY LEVEL

The basic chromosome number for *Medicago* is x=8, except for the annual species *M. constricta* Dur., *M. praecox* DC., *M. polymorpha* L., *M. rigidula* (L.) All. and *M. murex* Willd., which have a genomic number of x=7 (Quiros and Bauchan 1988). Three ploidy levels are found for *Medicago* spp.: diploids 2n=2x=14 and 2n=2x=16, tetraploids 2n=4x=32, and hexaploids 2n=6x=48. The tetraploid species have probably arisen from diploids by unreduced gametes (Mariani and Veronesi 1979, McLennan et al. 1966, Vorsa and Poingham 1979).

4.3. BREEDING SYSTEM

Annual species of *Medicago* are autogamous as a result of the presence of self - tripping in their flowers. On the contrary, most of the perennials are allogamous with different degrees of self -incompatibility. As a rule they rely on insect polinators to activate their flower tripping and pollination (Lesins and Lesins 1979). Most of these species are quite polymorphic, because of their high degree of outcrossing. Their pollinating agents include numerous bee species.

4.4. TRAITS TO DISTINGUISH *MEDICAGO* SPECIES

Legume and seed characteristics are the most important traits for identification of different *Medicago* species. Growth habit and longevity, organ hairness, inflorescenses, leaves, cotyledons, pollen shape and size and the chromosome numbers are other important characters. In addition isozyme and molecular markers complete these characteristics.

Small (1981) recognized 12 groups of species in the genus, based on 75 characteristics mostly vegetative and pod traits.

4.5. CHARACTERISTICS OF *MEDICAGO SATIVA* COMPLEX

The species included in *M. sativa* complex belong to the section *Falcago,* subsection *Falcatae.* It includes *M. sativa*, *M. falcata* and *M. sativa* spp. *glutinosa*. They intercross with each other and share the same karyotype (Gillies 1972). The species in the complex are either diploid or tetraploid.

Alfalfa (*M. sativa* L.) is an essential cultivated forage crop in the world. Beyond its value as the most important protein source in animal fodder, alfalfa is able to fix atmospheric nitrogen in symbiosis with *Rhizobium meliloti*, which makes it valuable in the crop rotation.

4.5.1. General distribution

The original ranges of distribution of both diploid and tetraploid *M. sativa* cover the Mediterranean region, Near and Middle East, the Caucasus, Middle, Central and South Asia (Quiros and Bauchan 1988). Tetraploid *M. sativa* was introduced in Europe, where it has been largely cultivated. According to Vollrath (1973) it is always present as an introgressed form with *M. falcata*. The recognition of the economical value of *M. sativa* was responsible for its introduction in South America, Mexico, Canada, USA as well as Australia, New Zealand and South Africa (Freyer 1930).

Diploid *M. falcata* is present mainly in Asia (up to Siberia in the East and Caucasus in the South) and Eastern Europe, extending up to the South of Germany. It is a rare species for Denmark (Hojland and Poulsen 1994).

4.5.2. Distribution in Switzerland

Ten *Medicago* species are present in the Swiss flora (Aeschimann and Burdet 1989).

M. sativa is widespread all over the country. It grows at low altitude (colline level and montane zone) (map 2a). *M. falcata* is restricted to the Northeastern and Eastern parts of the country, the canton of Valais and the region of lake of Geneva (map 2b) (Welten and Sutter 1982). It is present from the colline level to the subalpine zone. According to the authors it has disappeared from most locations of Swiss Plateau, where it had been mentioned earlier.

M. sativa hybridizes freely with *M. falcata*. Their hybrid *M.* x*varia* Martyn is fertile and is found within the range of distribution of *M. sativa* and *M. falcata*.

4.5.3. Morphological characteristics

M. sativa and *M. falcata* differ in their morphology, mainly by the leaf's shape, the color of the flowers and the pod's shape. *M. sativa* has a deep tap root, an erected stem, rounded leaves , purple flowers and coiled pods, while *M. falcata* has a spread well - branched root system, prostrate stems, smaller narrow leaves, yellow flowers and sickle - shaped pods.

Tetraploid forms in the complex are distinguished from the diploid by the larger size of their flowers, pods and seeds. A very striking difference appears in respect to pollen size, which increases throughout with the chromosome number.

4.5.4. Isozyme and DNA molecular markers

These markers have been proved useful for the identification of the *Medicago* species and their hybrids and thus may be a helpful tool in risk assessment.

Genetic determinism in *Medicago sativa* complex was studied for peroxidase and leucine-amino peptidase (Quiros and Morgan 1981, Melchinger et al. 1991) and for glutamate oxaloacetate transaminase (Brunel 1982).

Diploid *M. falcata* has a few specific allozymes not found in *M. sativa*. Unique alleles for PRX and LAP have been found in *M. falcata* and the hybrids *M.* x*varia* and *M. hemicycla*, but not in *M. sativa*. The diploid and tetraploid forms of *M. sativa* are distinguished for the loci GOT, LAP1 and LAP2.

Molecular markers can be utilised to simplify genetic analyses applicable to alfalfa breeding. Based on RAPD and RFLP techniques, linkage maps of diploid (McCoy et al. 1991) and tetraploid alfalfa (Kiss et al. 1992, Yu and Pauls 1993a) have been produced. These techniques were successfully used for studying the structure and expression of different genes in *Medicago* (Gregerson et al. 1994) or for distinguishing populations and species within the genus (Yu and Pauls 1993b, Pupilli et al. 1995).

4.5.5. Risk assessment for releasing transgenic alfalfa

Transgenic alfalfa exists (D`Halliun et al. 1990, Larkin et al. 1990, Hill et al. 1991, Schroeder et al. 1991, Gold et al. 1991, Pereira and Erikson 1995, Austin et al. 1995) and 38 field releases have been carried out since the first one in 1988 (Extracts from GIBiP database on field trials 1986 - 1994). Most of them took place in USA and Canada (Ahl Goy et al. 1994).

According to the classification of Raybould and Gray (1993), *Medicago* belongs to the third group with a « high probability of gene flow to wild relatives ». The Dpdf code for lucerne indicates a substantial ecological effect on the flora of the Netherlands (De Vries et al. 1992).

Bibliographical research also concluded a high risk of gene flow between *M. sativa* and *M. falcata* (Jacot 1994, Jacot and Jacot 1994). *M. sativa* is an outcrossing species and asexual reproduction does not take place. Moreover it can hybridize with *M. falcata* easily but only at

the same ploidy level. Crosses between diploid and tetraploid forms are rare, because of the ploidy barrier, and they result in a low fertility (Ledingham 1940). Although seed set and dispersal are limited, many seeds are wasted during the harvesting and could remain in the areas where alfalfa has been cultivated. Due to the probable limited dispersal of the seeds, it is the extensive pollen dispersal (1600 m) and the ability of *M. sativa* to hybridize freely with *M. falcata* which exhibit the major risk of gene flow (Hojland and Poulsen 1994).

CHAPTER 5:

MATERIALS AND METHODS

5.1. SAMPLING AND CULTIVATION

The sampling sites of *Hordeum* and *Medicago* were chosen according to the present distribution of the species in Switzerland with the aim to cover the whole teritory of the country and in respect with the differece in the local conditions in each region, which could influence the risk of cultivation of GMOs.

5.1.1. *Hordeum*

Eighteen populations of *H. murinum* were sampled in Schaffhausen, Graubünden, Vaud and Valais (Table 1) in the summer of 1993 as following:

- populations close to a field of cultivated barley
- populations close to fields, previously cultivated with barley
- populations distant from cultivated fields, mainly in village surroundings.

Populations close to cultivated fields are generally linear, located in a narrow strip between the road and the field. Therefore populations were sampled along linear transects (1 or 2 by populations), by collecting all the flowering plants on a strip of variable width (Fig. 2).

For the populations close to a field of cultivated barley, *H. vulgare* was also collected.

All the plants were dried for morphological analysis and herbarium collection by the Bern team and one spike of each plant was kept separately. Two seeds of those spikes were sown in pots in the autumn of 1993 and cultivated in a cold greenhouse in the Botanical Garden of the University of Neuchâtel. After germination, only one of the plants randomly chosen, was kept. These plants - 905 in total - were used for the genetic analyses and the experiments.

5.1.2. *Medicago*

Thirteen populations were sampled in Schaffhausen, in Unterengadin and Rhine valley in Graubünden and Valais (Table 2). The plants were collected in such a way that individuals on

a one meter lattice were chosen (Fig. 3). In the cases where there was no plant on the crossing point, the closest one at less than 50 cm was collected. All the populations except the three collected in Unterengadin, included *M. falcata*, *M. sativa* and *M.* x*varia* in different degrees. In some populations, apparent spatial patterns existed with a pure zone of *M. falcata* and a pure zone of *M. sativa*, joined by a zone of intermixture. Other populations were more of hybrid types with numerous intermediary individuals. Two of the populations collected in Unterengadin consisted of *M. falcata* only and one consisted *M. falcata* and *M. sativa*,which were spatially separated.

The shoots of each collected plant were dried for herbarium collection and the morphological studies for the Bern team. The base and the rooting system were planted in pots and cultivated in the Botanical Garden of the University of Neuchâtel. These plants - 916 in total - were used for the genetic analysis and the experimental part.

5.2. FLOWERING BIOLOGY

The flowering biology of *H. murinum* has been studied by the observation of ten spikes per population under binocular. The presence or absence of cleistogamy (pollen release outside of the spike) was censused. The flowering stages were examined for all the spikes produced by each plant according to the following:

A - non visible spikes

B - spikes less than one half visible

C - spikes more than one half visible

D - spikes completely out

The flowering index (Fi) was determined as:

$$Fi = (A+2*B+3*C+4*D)/(A+B+C+D)$$

where A, B, C, D are the number of spikes at the corresponding stage.

5.3. DIRECT METHODS

5.3.1. Pollen dispersal

Pollen dispersal was studied only for *Hordeum* as for *Medicago* it had been already evaluated (Demarly 1955, Bradner et al. 1965, Rinker et al. 1988). The experiments were performed for *H. vulgare* and *H. murinum* separately. Two plants of each population were put together as pollen source, surrounded by pollen traps (Fig. 4). Traps were located on five lines regularly placed every 0.5 m up to 5 m and then every meter up to 12 m. The traps consisted of a glass plate covered with vaseline. The pollen was fixed after with Gelvatol ® (37 gr Gelvatol®, 2 gr phenol, 50 ml glycerol, 100 ml dH_2O). The experiments were performed on calm days in June 1994 and lasted 36 hours. The number of pollen grains was counted under a light microscope in a semi - quantitative way by doing five surveys along the microscope slide.

5.3.2. Seed dispersal

The seed dispersal experiments were performed in July 1994 for *Hordeum*. The experiment consisted in putting four plants surrounded by three plastic sheets of 1x2 m covered with a glue resistant to water (Soveurode®). Seeds were regularly collected and their coordinates on the plastic sheet recorded. The experiment was repeated twice for *H. vulgare* and for *H. murinum*.

Similar experiments ware carried out for *M.sativa* and *M. falcata*. However, the detection of the seeds on the plastic sheets was impossible, because of the small size of the seeds and because in most of the cases the plants shed the hole fruits but not the seeds themselves.

5.3.3. Hybridization under natural conditions

Two plants of each population of *H. murinum* were placed on the border of a cultivated field of winter *H. vulgare* in Chezard (NE). A similar experiment was performed on spring *H.*

vulgare at the Agronomic School of Cernier (NE) (photo 1). Their progenies were collected and sown in the autumn of 1994 in the Botanical garden of the University of Neuchâtel for further investigation.

Plants of *M. falcata* with non - open flowers were planted in the flowering fields of *M. sativa* at the Swiss Agronomic Research Station of Changins in July 1994 (photo 2). The seeds were collected at the end of August and sown at the Botanical Garden of the University of Bern.

Screening for hybrids was performed on the progeny.

5.3.4. Experimental hybridization

Two plants of *H. murinum* from each population were selected for crosses with *H. vulgare*. One spike from each species were put together under a pollination bag (photo 3). The experiment was performed in the greenhouse of the Botanical garden of the University of Neuchâtel in May 1994. The progeny was collected and sown in the autumn.

Experimental self - fertilization, cross - fertilization and hybridization were carried out on selected plants from *M. sativa* and *M. falcata*. Castration was performed by opening the flowers and their immersion for one minute in 55% ethanol or by removing the anthers with a vacuum. The pollination was made manually. The flowers were then covered with pollination bags. The experiments were carried out in June - July 1994. The progeny was collected and sown in the spring of 1995 in the Botanical Garden of the University of Bern for further investigations (photo 4).

5.4. INDIRECT METHODS

5.4.1. Karyology

Chromosome numbers were determined for both *Hordeum* and *Medicago* on counts of at least five individuals of each population.

5.4.1.1. *Hordeum*

The root tips were pretreated in 0.002 M 8-hydroxyquinoline for two hours at room temperature and then fixed in Carnoys solution (3:1 ethanol:acetic acid) for one night. After a hydrolysis with 1N HCL in a water bath of 60°C for 8 min, the root tips were stained in Schiff (5 gr pararosaniline, 150 ml 1N HCL, 5 gr Na-metabisulfate, 850 ml distilled H_2O) for 2 to 4 hours. Finally they were softened with a 5% pectinase - 3% cellulase solution for 3 - 4h. A somatic chromosome number was determined on metaphases.

5.4.1.2. *Medicago*

The root tips were pretreated in 0.002 M 8-hydroxyquinoline for two hours at room temperature and then fixed in Carnoy`s solution (3:1 ethanol:acetic acid). After at least one day, they were stained in 1% aceto - orcein (2 gr orcein, 100 ml acetic acid, 100 ml distilled H_2O) for two hours at room temperature, followed by heating for two minutes. Somatic chromosome number was determined on metaphases.

5.4.2. ISOZYMES

5.4.2.1. Principle of the electrophoresis

Electrophoresis is a method for separating the proteins according to their molecular weight and their charge under an electric field. Proteins of different composition and conformational properties migrate at different rates. The relative migration depends on the molecular weight

and the charge of the molecules. The charge of the proteins depends on the degree of ionization (the amount of free electrons) and the amino acids composition. Gels can be prepared from a variety of media (starch, agarose, polyacrylamide and cellulose acetate membranes) and run by various methods (horizontal, vertical, disc). Characteristics of the migration buffer and gel matrix both affect resolution. Enzymes are detected by immersing the gel into a medium containing the reaction conditions for the enzyme such that it incorporates a colored dye into the end product.

Polyacrylamide gel electrophoresis (PAGE) is required when maximum resolving power is necessary. A valuable property of PAGE is that the stringensy of molecular sieving can be varied by altering the acrylamide concentration, thus increasing flexibility in the range of protein molecular weights or dimensions that are separable (Leaback 1976). Other advantages of polyacrylamide gels include their uniformity and transparency, facilitating densitometric quantification of products, inertness of components allowing broad assay compatibility, usually rapid run time. Discontinous systems (composed of more than one gel) are used with polyacrylamide gels to create a stacking effect at the boundary between regions of large and small pore size, which improves the resolution of the bands in the second phase (Chrambach 1980, Hames and Rickwood 1981).

5.4.2.2. Enzyme extraction

All plants from *Hordeum* and *Medicago,* which had been cultivated, have been screened for isozyme markers using PAGE. Extractions were carried out on fresh leaf material. Two - three leaves were squashed in a mortar with 500 µl of extraction buffer and one point of the spatula of quartz. Two different extraction buffers were used for *Medicago* and for *Hordeum* (Appendix I). The samples were centrifugated at 10 000 rpm for 20 min and the supernatant was kept and stored at -80°C.

5.4.2.3. Protein separation

The gels were prepared according to Gasquez and Compoint (1976), modified by Lumaret (1981) and consisted of:

- separation gel - 17 cm x 7.5 cm, 2.5 mm composed of 9% acrylamide and 0.165% bis-acrylamide for *Hordeum* and peroxidase system for *Medicago*. For systems malate dehydrogenase and maleic enzyme (ME) for *Medicago* 10 % acrylamide and 0.165% bis - acrylamide were used.

- stacking gel - 17 cm x 1 cm, 2.5 mm composed of 2.5% acrylamide.

- sample gel - containing 9% acrylamide. It has the same composition as the separation gel. (Appendix I)

Forty µl of the enzyme extractions and about 20 µl of dye (1% bromophenol blue) were used. Protein migrated in Tris-glycine buffer, pH=8.6 (Appendix I) in plastic cuve. The migration was carried out at 4°C and in three stages:

- ten minutes at 600 V and 6 pps (number of the pulsations/sec) to concentrate the extracts at the end af the sample gel.

- twenty minutes at 230 V and 7 pps. At this phase extracts migrate in the stacking gel.

- between 2h30 to 3h at 600 V and 7 pps. During this phase proteins separate.

Esterase (EC 3.1.1) and glutamate oxaloacetate transaminase (or aspartate-α-ketoglutarate aminotransferase (EC 2.6.1.1) were studied for *Hordeum* and malate dehydrogenase (EC 1.1.1.37), malic enzyme (EC 1.1.40) and peroxidase (1.11.1.7.) for *Medicago*.

5.4.2.4. Revelation of the enzymes

- Glutamate oxaloacetate transaminase

This enzyme belongs to the group of aminotransferases, which catalyse the transfer of one α-amino group to α-ceto group. GOT has a key role in the reactions of nitrogen elimination

from amino acids and in the process of accumulation of α-keto acids, which participate in the cycle of Krebs. It catalyses the reaction:

aspartate + 2-oxoglutarate ⇔ oxaloacetate + glutamate

The enzyme is dissolved in the cytoplasm or is connected with the peroxisome or mitochondries. In the plants its functional form is as dimer.

Revelation: **Tris** buffer, pH = 8 - Tris 48.4 gr, distilled H_2O 2000 ml, **Substrate** - α-ketoglutaric acid 300 mg, L - Aspartic acid 600 mg. The two acids were dissolved in 100 ml of Tris buffer. One point of spatula of pyridoxal 5′P was added.

Coloring agent - Fast blue BB 200 mg, Tris buffer 100 ml.

Fifty ml of the substrat and 50 ml of the dye were poured over the gel and stained at 36°C at dark until the dye precipitates. The operation was repeated with the rest of the solutions. Finally the gel was rinsed with water.

- Esterase

This enzyme hydrolyzes a large number of compounds having different ester linkages *in vitro*, but their natural substrates are unknown in plants (Gottlieb 1981). Plant species contain high numbers of esterase isozymes in many different tissues. They may be the most variable isozymes studied to date in plant populations.

Revelation: **Tris buffer** , pH = 7 - 19.4 gr Tris, 200 ml distilled H_2O, 13 ml HCL, **Substrate** - 200 mg α-Naphtyl acetate, 150 mg β-Naphtyl acetate. They are dissolved in 20-30 ml of acetone and completed to 100 ml with Tris buffer.

Coloring agent - 200 mg Fast blue RR, 100 ml Tris buffer.

The gel was incubated for 15 min at room temperature with two thirds of the substratum. The former was poured off and the coloring agent was added for another 15 min. Finally the gel was incubated with the rest of the substrate.

- Malate dehydrogenase

At least three NAD - dependent MDH, each localized in a distinct cellular compartment (mitochondria, peroxisome, cytoplasm), are found in plant cells (Gottlieb 1981). The isozymes function in different metabolic sequences. The mitochondrial one is involved in the citric acid cycle, catalysing the reaction:

$$\text{malate} + NAD^+ \Leftrightarrow \text{oxaloacetate} + NADH + H^+$$

The soluble one is involved in non - autotrophic CO_2 fixation to form malate in the reaction:

$$\text{Pyruvic acid} + NADPH + H^+ + CO_2 \Leftrightarrow \text{malate} + NADP^+$$

The enzymes in the peroxisomes take part in the photorespiration and the glyoxylate cycle:

$$\text{malate} + NAD^+ \Leftrightarrow \text{oxaloacetate} + NADH + H^+$$

The isoenzymes are dimeric and are under independent nuclear gene control.

Revelation: **Substrate**: 5 ml 1M Tris buffer, pH = 9.1, 10 ml 2M Malic acid pH = 7, 50 ml distilled H_2O, 20 mg NAD, 1 ml PMS 0.2% (phenazine methosulfate), 1 ml NBT 1% (nitro blue tetrazolium).

Preparation of 2M Malic acid: 134.1 gr DL Malic acid, 80 gr NaOH and distilled H_2O up to 500 ml, pH = 7. The preparation was done on ice as the reaction of disolving NaOH is exothermic.

The dyes were added just before the revelation. The gel was incubated with the substrate at 37°C for about 30 min.

- Maleic enzyme

It is a NADP - dependent enzyme, belonging to the group of MDH and localised in the chloroplasts. It catalyses the reaction of transformation of malic acid to pyruvate:

$$\text{malic acid} + NADP^+ \Leftrightarrow \text{pyruvic acid} + NADPH + H^+$$

Revelation: **Substrate**: 10 ml 2M Malic acid, pH = 7, 5 ml 1M Tris buffer pH = 8, 50 ml distilled H_2O, 100 mg $MgCL_2$, 10 mg NADP, 1 ml NBT 1%, 1 ml MTT 1%, 1 ml PMS 0.2%.

The dyes were added just before the revelation. The gel was incubated with the substrate at 37°C for about 20 min.

- Peroxidase

Peroxidases utilize H_2O_2 to oxidize a very large number of hydrogen donors including phenolic substances, amines, leuco-dyes, ascorbic acid, indoles and certain inorganic ions such as iodides. All higher plants examined have a large number of them. The complexity of the electrophoretic patterns of the peroxidases is matched by the complexity of their presence in different tissues and developmental variability (Gottlieb 1981).

The peroxidase catalyses the reaction:

$$RCH_2 + H_2O_2 \Leftrightarrow R + 2H_2O$$

The enzyme is present in most of the tissues, but it is often linked with one organel or specific tissue as well such as endoplasmatic reticulum, Golgi apparat, ribosomes and vacuoles. Peroxidases are glycoproteins.

Revelation: **Acetate buffer** - Solution a: 115 ml 0.2M Acetic acid, 100 ml distilled H_2O. Solution b: 0.2M Sodium Acetate 16.4 gr/l.

72 ml of Solution a, 176 ml of Solution b and 750 ml distilled H_2O were used for the preparation of 1000 ml 0.05M Acetate buffer, pH = 5.

Solution 1: 25 mg 3-amino-9-ethylcarbazole, 2.5 ml NN-dimethyl formamide

Solution 2: 45 ml 0.05M Acetate buffer, pH = 5, 1 ml guaiacol, 1 ml 0.1M $CaCL_2$, 1 ml 3% H_2O_2 .

Before the revelation solutions 1 and 2 were mixed and poured over the gel. It was incubated at room temperature for about 20 min.

5.4.3. DNA MARKERS

Random Amplified Polymorphic DNA (RAPD) was used to reveal DNA markers for each of the *Medicago* species studied and to detect the putative hybrids.

It is a method which applies the Polymerase Chain Reaction (PCR). PCR is an *in vitro* technique for amplification of short specific DNA sequences. It is based on DNA denaturation after heating followed by anniling of short DNA sequences (primers) to each separate DNA chain. The primers act as a starting points for the DNA synthesis with the help of the DNA - Polymerase. DNA - Polymerase is a thermostable enzyme, isolated from *Thermophilus aquaticus*, and it satisfies the condition to be resistant to the high temperature, use for the DNA denaturation, without loosing its activity.

The first step of the PCR is a heating to 92 - 95°C during which the separation of the two DNA chains happens. The next step is the attachment of the primers at 40 - 60°C. This temperature depends on the sequence of the primers and the target DNA. Finally the probes are heated at 72°C to allow the DNA - Polymerase to synthesize the new chains. This process must be repeated 20 - 40 times and after each cycle the DNA doubles, getting an amount which allows it to be visualised on agarose gel (Fig. 5).

RAPD uses randomly constructed oligonucleotides as primers, permitting the detection of polymorphisms as different PCR - products. As this technique uses arbitrary primers to amplify random segments of genomic DNA, showing polymorphism these markers are called

RAPDs. The primer attaches at the places with sufficient complementarity of its nucleotide sequence. If the frequency of these sequences in the target DNA are relatively high, there will be a chance that they will be close to each other. Based on this, PCR will amplify fragments which could be different in size. The polymorphism observed may result from point mutations, insertions, deletions and invertions. RAPDs are usually dominant markers and are inherited in a single Mendelian fashion.

5.4.3.1. DNA extraction

Leaf material was stored at -80°C. Between 100 and 200 mg of it was squashed in mortar with liquid nitrogen. The powder was transfered to an eppendorf tube with 1 ml of solution - 1 (10 ml 1M Tris, 20 ml 0.5M EDTA, 2.92 gr NaCl, 30 gr sucrose, up to 200 ml distilled H_2O). The samples were centrifuged for 5 min at 5000 rpm and 300 µl of solution - 2 (4 ml 1N Tris, pH=8, 4 ml 0.5M EDTA, 192 ml distilled H_2O) and 20 µl of 20% sodium dodecyl sulfat (SDS) were added to the pellet, which consists of the nuclei. The tubes were incubated at 70°C for 15 min. To the former mix 150 µl of 7.5 M ammonium acetate was added and the tubes were cooled on an ice bath for 30 min. The ammonium acetate was sedimented by centrifuging for 5 min at 14 000 rpm. The supernatant was transfered to a new microfuge tube and 700 µl isopropanol was added. The samples were left for 15 min followed by centrifugation for 5 min at high speed. The supernatant was decanted and the pellet was washed with 1 ml cold 75% ethanol. Finally the samples were microfuged again for 5 min and ethanol was poured out. After the tubes were dried, DNA was resuspended in 1 ml TE buffer, pH=8 (1 ml 1M Tris, pH=7.4, 0.2 ml 0.5M EDTA, pH= 8, 98.8 ml distilled H_2O). The quality of the isolated DNA was checked in 1.6% agarose gel, run in 0.5 x TBE buffer (Appendix II) at 50 V for about 40 min. The quantity of DNA was measured with TKO 100

mini - fluorometer. When it was necessary the extractions were diluted so that final concentration of 30 ng/µl DNA was obtained.

5.4.3.2. PCR conditions

Each PCR - tube contained 19.3 µl sterile distilled H_2O, 2.5 µl incubation buffer (Appligene), 1 µl 5mM dNTP mix (Boehringer Mannheim), 1 µl primer (30 ng/µl), Taq - Polymerase (Appligene) and 1 µl genomic DNA (30 ng/µl). The PCR was performed in Perkin Elmer Gene Amp PCR System 2400 as follows:

4 min at 94°C

1 min at 93°C 1 min at 45°C 1 min at 72°C	38 cycles

5 min at 72°C

∞ 4°C

Nine µl of each PCR - product were mixed with 4 µl loading dye (10 gr sucrose, 5 ml 0.5M EDTA, 1.25 ml bromphenol blue - 1%, 1.25 mg SDS and distilled H_2O up to 25 ml) and run in 1.6% agarose gel in 0.5 x TBE at 50 V for about 40 min. The RAPDs were observed under UV light.

A screening of 44 ten - mer primers - kits OPB, OPL and OPP (OPERON Technologies, Inc.) was carried out. Three primers (OPP - 05, OPP - 06 and OPP - 08) provide specific bands for *M. sativa* and *M. falcata* and a RAPD with 90 plants from one selected population (4A, 4B), consisting of the both species and their hybrid was carried out.

5.4.4. Data analysis

5.4.4.1. Isozymes

Principal coordonate analysis (PCOORD) was computed with the enzyme data for *H. vulgare* and *H. murinum* in order to get an overview of the genetic variation in the studied populations and to determine if both species are separated from each other. The same analysis was performed only for *H. murinum* to investigate if there was differences between the populations on enzyme level, according to the collection site. PCOORD is based on the matrix of similarity or of distance. It consists in locating the objects in a space of lowered dimension size, which preserves as well as possible their relative distances. The matrices may be calculated with several coefficients. Similarity matrix was calculated with the asymetrical Jaccard's coefficient. It has the advantage of considering the absence of electrophoretic band as an absence of information, rather then an element of similarity.

A Mantel test (Mantel 1967) for significance of differences between *H. murinum* and *H. vulgare*, based on the isozyme data was computed. A matrix of similarities of the dataset was compared with another one of species belonging. The test is based on calculating a value, expressing the similarity between both matrices - standarized r - score, which is a standarized form of z statistics (Smouse et al. 1986) and is equivalent to Pearson`s linear correlation.

The same test was performed to compare the populations of *H. murinum* according to their geographical location, where similarity matrix with the isozyme data was compared with a matrix of geographical distances.

PCOORD was performed with the enzyme data for *Medicago* species. A Mantel test, based on the same isozyme data for significance between *M. sativa*, tetraploid and diploid *M. falcata* and *M.* x*varia,* or only between the species on tetraploid level was also carried out.

5.4.4.2. RAPD

A PCOORD was made to get an overview of the genetic variation in the studied population and to determine if *M. sativa, M. falcata* and *M.* x*varia* are separated from each other as well as Mantel test.

CHAPTER 6:

RESULTS

6.1. FLOWERING BIOLOGY

Ten spikes/population of *Hordeum murinum* were examined in May 1994. We observed regularly excerted stamens and visible styles (photo 5).

The comparative analysis, including score of the flowering stages of all tillers of the examined plants showed differentiation in the flowering phenology between the population of *H. murinum*. Those of Valais flowered before those from Swiss Plateau and the last flowering populations were from Graubünden (Table 3).

The flowering periods of both cultivated and wild barley overlaped.

6.2. DIRECT METHODS

6.2.1. Pollen dispersal

Both *H. murinum* and *H. vulgare* presented similar pattern of dispersal. The frequency of the pollen decreased with increasing distance (Fig. 6). Most of the pollen dispersed was closed to the plant source. After two meters, the amount of the pollen was more or less constant.

Standard deviation of the distribution (87% of the disseminated pollen) corresponded to 5 - 6 meters for *H. murinum* and 5 -7 meters for *H. vulgare* (Fig. 6). Although the proportion of dispersed pollen decline at twelve meters, there was still some amount of it at this distance.

6.2.2. Seed dispersal

This experiment evaluated the dispersal of the seeds by the wind.

H. murinum dispersed most of the seeds, close to the source. Standard deviation of the distribution corresponded to 0.4 - 0.6 meters. The biggest number of seeds were dispersed at 0.4 - 0.5m. At 2.0 m we practically did not find any seeds (Fig. 7).

H. vulgare did not shed seeds.

6.3. INDIRECT METHODS

6.3.1. Karyology

6.3.1.1. *Hordeum*

The plants of all populations of *H. murinum* were tetraploids with 2n=4x=28 chromosomes (Fig. 8). The finding of 2n=28 chromosomes was in agreement with former reports, performed on plants from other regions in Europe (Morrison 1958, Rajhathy and Morrison 1962, Strid 1971, Van Loon and de Jong 1978, Linde-Laursen et al. 1992). Two pairs of SAT - chromosomes were observed. These are the first counts for the species for Switzerland (Felber and Savova 1994).

H. vulgare was diploid with 2n=2x=14.

6.3.1.2. *Medicago*

M. sativa was always tetraploid with 2n=4x=32 (Fig. 9a, Table 4).

M. falcata was tetraploid (2n=4x=32) in Valais, Schaffhausen and Rhine valley of Graubünden. All populations from Unterengadin were diploid (2n=2x=16) (Fig. 9b, Table 4).

M. xvaria was always tetraploid (2n=4x=32) and was present in all populations except those from Unterengadin (Savova et al. 1996).

6.3.2. Isozymes

The aim of the experiment was to define specific marker electrophoretic bands for each of the species studied, in order to be used as indication for outcrossing and presence of interspecies hybrids after experimental hybridization.

6.3.2.1. *Hordeum*

From 18 studied enzyme systems on polyacrylamide and starch gel for *H. murinum* and *H. vulgare*, eleven of the examined enzyme systems revealed electrophoretic products, two revealed rather spots than clear bands, while five did not revealed any products. From them nine were monomorphic for both species and two - GOT and EST - on PAGE established specific electrophoretic bands for *H. murinum* and *H. vulgare*. All *Hordeum* plants were examined with these two systems.

Glutamate oxaloacetate transaminase

All 18 populations of *H. murinum* showed identical enzyme pattern (Fig. 10). The zymogram consisted of five electromorphs, two of which monomorphous (0.75 and 1.00) and three (1.12, 1.20, 1.29) polymorphous.

The six populations of *H. vulgare* were divided into three groups (Fig. 10). In total they revealed eleven electromorphs, three of which (1.12, 1.20, 1.29) were common for all the populations and were also present in *H. murinum*. Five electrophoretic bands were present only in population 2 and three of them (0.56, 0.60, 0.69) were rare (found only in few plants). *H. murinum* had one unique band (0.75) not present in *H. vulgare*, which on the other hand had two specific bands, present in three of the populations (0.79, 0.92) and one present in the other three populations (0.90). Those bands were used as markers to detect the putative hybrids between both species.

Esterase

For the EST inter - as well as intrapopulation variation for both *H. murinum* and *H. vulgare* was observed. *H. murinum* was divided in seven groups and *H. vulgare* in five. The

zymograms showed 8 electromorphs in total for *H. murinum* and 12 for *H. vulgare* (Fig. 11). There were two specific electrophoretic bands (0.59, 1.93), present in all populations of *H. vulgare*. Two specific bands (0.62, 1.00) was found for *H. murinum* as well. Bands 0.59 and 0.62 were present in all the populations of *H. vulgare* and *H. murinum* respectively, but not in all of the plants. The band at 0.78 was the only common band for both species. The other bands were typical only for *H. vulgare* (0.32, 0.49, 0.66, 0.86, 0.88, 1.41, 1.52, 1.60, 1.74) or *H. murinum* (0.67, 1.19, 1.89, 1.96, 2.04), but they were present only in some of the populations of each species and not always in all individuals.

Interpopulation differentiation in distribution of electrophoretic bands for GOT and EST for *H. murinum* was evident from Table 5, where the percent of presence of the bands per population were given. Bands, such as GOT A, GOT B, GOT E, EST B, EST C, EST E, EST F, EST G (Table 6) were the more frequently present for *H. murinum*.

The Principle Coordinate Analysis was computed with all the data from isozyme analysis from all plants of *H. murinum* and *H. vulgare* and showed clear separation between both species when Factors 1 and 2 (Fig. 12) as well as when Factors 1 and 3 were plotted together (Fig. 12a). When Factor 1 and 2 were plotted together a separation between the populations of *H. vulgare* (populations 2, 6 and 7 from one hand , and 12, 13 and 14 from the other hand were grouped together) was observed as well. No clear separation according to the geographical distribution was observed, when the same analysis was performed only for *H. murinum* plants (Fig. 13, 13a), although there was a tendency for the populations from Vaud to separate appart, when Factors 1 and 2 are plotted together (Fig. 13). However, still one third of the samples from Vaud were grouped together with those of the other regions.

Mantel test for differences of samples in regard to their species affiliation and geographical areas was made as well. For the first case the test for difference between the species gave r =

0.89, t = 42,8, prob(t) = 0.00000. The r value indicates the correlation between the similarity matrix, calculated with the enzyme data and that of the species. Probability (prob(t)) value indicates highly significant differences between both *Hordeum* species according to their isozyme profiles.

For the second case, Mantel test was computed with the enzyme data for *H. murinum* and a matrix of distances between populations, derived from the coordinates of the test areas in the different regions of Switzerland, where the plants were collected. Data from three of the sampling regions (Graubünden, Vaud and Swiss Plateau) was considered only, as plants from Valais had a very restricted number. The following results were obtained:

r = 0.17, t = 15.1, prob(t) = 0.00000

The r value is lower, but prob(t) indicates highly significant difference between the populations of *H. murinum* from different regions of Switzerland.

6.3.2.2. *Medicago*

From 19 enzyme systems studied on polyacrylamide and starch gels, three did not reveal electrophoretic bands, nine gave rather spots, but not clear bands and seven revealed clear products. From them four enzyme systems were monomorphous for all plants and three systems - PRX, EM and MDH on PAGE were selected, as they showed polymorphism between individuals.

Peroxidase

Eight electromorphs were revealed for PRX for *M. sativa* and tetraploid *M. falcata*, and six for diploid *M. falcata* (Fig. 14). They had the same migration distance for both ploidy levels. All the bands, except that at 0.37 were present in all examined plants.

Maleic Enzyme

Six distinct electromorphs were detected for EM for both tetraploid species and five for the diploid *M. falcata*. They all migrated at the same distance for the different species and were present in all examined individuals (Fig. 14). In addition bands in 1.00 zone existed for all species, but unfortunately they were unclear and it was impossible to determine their number. Therefore their presence was not considered.

Malate dehydrogenase

Six clear electromorphs were observed for diploid *M. falcata* and five for tetraploid *M. falcata* and *M. sativa*. They migrated at the same distance for both ploidy levels (Fig. 14). Again at 1.00 there were unclear bands, which number was difficult to determine.

M. sativa and *M. falcata* considered as a whole did not present species specific electrophoretic bands with none of the studied enzymes. There was difference in the zymograms only between the individuals. However, distinction between diploid *M. falcata*, and both tetraploid species was possible.

Thus, for PRX the eight isozymes were more or less equally present in all species, except band PRX 4 (0.37), which was not present in the tetraploid *M. falcata* and band PRX 5 (0.42), not present in the diploid *M. falcata* (Table 7). PRX 1 (0.20) was very rare for the diploids.

For EM all seven isozymes were present rather equally in both species, except EM 6 (0.69), which was missing in the diploids. EM 1 (0.22) was much more rare for them as well (Table 7).

More variability between the two ploidy levels in *M. falcata* was observed in the MDH profiles. Three of the bands - MDH 4 (0.55), MDH 5 (0.63) and MDH 6 (0.74) were not

present in the diploid *M. falcata*. It was interesting to observe that MDH 1 (0.04) was present only in diploid *M. falcata* and in *M. sativa* from Unterengadin.

All electrophoretic bands were present in *M.* x*varia* as well, except MDH 1.

Table 8 gives the presence of all bands of PRX, EM and MDH for the plants of population 4. This population was chosen as an example, because it includes the three taxa present at tetraploid level. Some electromorphs, are more frequent for either *M. falcata* or *M. sativa*, such as PRX 2, PRX 4, EM 1, MDH 2, MDH 3.

PCOORD (Fig. 15 and 15a) clearly set apart the diploid *M. falcata* from the tetraploid *Medicago* species, which are grouped together according to their isozyme profiles.

Mantel test for differences between *M. sativa*, diploid and tetraploid *M. falcata* and *M* x*varia* for enzymes was made. The following result was obtained:

r = 0.22, t = 42.0, prob(t) = 0.00000.

Although r value is not high, prob(t) gives significant differences between the species according to their enzymes.

The same test, performed only for tetraploid plants (tetraploid *M. falcata*, *M. sativa* and *M.* x*varia*) gave r = 0.04, t = 6.80 and p(t) = 0.00000. The r value is very low, but there is still significant differences between the species.

6.3.3. DNA analysis

A preliminary screening of 44 primers was performed with genomic DNA on ten samples of each *M. sativa*, tetraploid *M. falcata* and *M.* x*varia* for species specific RAPD markers. Banding patterns were directly scored from the gel for presence or absence of each amplification product. Eleven primers did not amplify detectable products. Thirty three primers were effective in initiating PCR amplification of DNA and three of them (OPP - 05,

OPP - 06 and OPP - 08) gave banding patterns that were polymorphic for *M. sativa* and *M. falcata*. Primer OPP - 5 amplified a 850 bp band, specific for *M. sativa*, OPP - 6 amplified a 800 bp band and OPP - 8 amplified a 700 bp band, specific for *M. falcata*. Those bands were present in the hybrid *M.* x*varia*. Only those fragments that could be clearly scored and were repeatable were taken into consideration.

Occasionally amplification products were observed in the control, that did not had the template DNA. However, these bands were always absent, when template DNA was included.

An analysis of 90 plants (including 18 plants *M. sativa*, 35 plants *M.* x*varia* and 37 plants *M. falcata*) from population 4 was carried out with those three selected markers.

A total of 24 amplified products were observed in patterns of *Medicago* from that population with the three primers (primer OPP - 05: 9 bands, primer OPP - 06: 7 bands and primer OPP - 08: 8 bands). The size of the fragments was from 517 to 2036 bp. The number of amplification products varied for the different species: for *M. sativa* from 1 to 6; for *M. falcata* from 0 to 7; for *M.* x*varia* from 1 to 7.

Although during the preliminary screening on ten samples/species specific bands were determined, when performed on the large sample (90 individuals), there was not any more specificity, present by the primers. However, some bands were mostly presented either in *M. sativa* (P5 D, P5 I, P6 C, P6 D) or *M. falcata* (P5 A, P6 B, P8 F) (Table 9).

The Principal Coordinate Analysis based on this data did not show separation between the three species and they tended to group together (Fig. 16).

Mantel test, calculated with RAPD data gave the following result:

r = 0.07, t = 3.68, Prob(t) = 0.00012.

The r value is low, but the difference between species, based on RAPD is significant.

6.4. Hybridization experiments

6.4.1. Hybridization under natural conditions

6.4.1.1. *Hordeum*

The plants of *H. murinum*, used for the hybridization experiments in the field with winter barley in Chézard had died.

Those, used for the hybridization with spring barley in Cernier (30 plants), produced 646 seeds in total, which were sown in the Botanical garden of Neuchâtel. They resulted in 588 plants (91% of the seeds germinated) (Table 10).

From these plants only ten/parent, randomly chosen were used for further investigations with isozymes. When the plants were less then ten, enzyme analysis was done on all of them (272 plants in total).

All produced plants have been checked also morphologically for the presence of hybrids. No hybrids have been detected. No evidence of hybrids has been observed with the isozymes as well (Table 11). All the plants repeated the isozyme profile of GOT and EST of the female parent. The only difference were in the relative intensity of the bands.

6.4.1.2. *Medicago*

For the field experiments in Changins 21 plants were used. From them 17 have produced fruits, which were collected (Appendix III). All the seeds were sown in the Botanical garden of the University of Bern, but only seeds of 11 mother plants (65%) germinated and formed seedlings. However, only seven of them have survived and yielded 30 progenies. They were examined for presence of hybrids with isozymes. For 29 of the plants there was evidence for

presence of hybrids (Table 16), detected with the three studied enzyme systems, as the progeny represent bands, from both parents.

6.4.2. Experimental hybridization

6.4.2.1. *Hordeum*

Thirty one flowering plants of each *H. vulgare* and *H. murinum* were used for the experimental hybridization in the greenhouse. All produced seeds - 1251, were sown. From them 1223 (98%) gave plants (Table 12). Twenty progeny plants per hybridization were randomly chose and used for isozyme analysis (or all of them when less then 20).

In addition, all the plants have been screened morphologically for the presence of hybrids.

No evidence of hybrids was detected in all 933 analysed plants with the isozymes (Table 13)**.** All examined putative hybrids showed isozyme profiles for GOT and EST, which repeated those of the female parent, with only differences in the relative intensity of the bands.

Among the examined plants there were few, with unusual morphological characteristics. The chromosome number of those plants was checked. The following results were obtained:

13A 3 x 2A 815/36 - 20 2n=14
7A 17 x 7A 244/282 - 24 2n=14
7A 9 x 16A 910/118 - 19 2n=15
7A 9 x 16A 910/118 - 23 2n=14
2A 29 x 18A 173/56 - 17 2n=14
7A 2 x 5B 710 - 20 2n=14

One of the plants was aneuploid with 2n=15 chromosomes and all the rest were diploids with 2n=2x=14, as their female parent. Aneuploidy does not necessarly relate to hybridization.

6.4.2.2. *Medicago*

With *Medicago*, experimental hybridization between *M. sativa* and *M. falcata* as well as self - fertilization and cross - fertilization experiments were performed.

For *M. sativa* as the female parent, 32 hybridizations, 32 self - fertilizations and 30 cross - fertilizations were made.

When *M. falcata* was the female parent 28 of each hybridizations, self - fertilizations and cross - fertilizations were carried out.

Self - and cross - fertilizations were performed on the same plants, used as female parents in the hybridization experiments.

From those 60 plants, 46 (77%) produced fruits. From them 44 (98%) individuals produced ripe seeds and the seeds of 39 (87%) germinated. Seedlings from 20 plants have survived producing 92 plants in total (Table 14).

Those plants were tested with isozymes. The progeny, obtained after self - fertilization (28 plants) represent the same enzyme profile as their parent for 7 of the plants and the rest 21 were contaminated (Table 15). There was evidence for hybrids, produced after cross - fertilization and hybridization experiments, as specific bands for both parents were present in the progeny. From the cross - fertilization experiment 10 plants were obtained, from which 5 intermediate individuals, one plant as result of self - fertilization and 4 contaminated plants (Table 17). From the experimental hybridizations 54 plants were obtained, from which 27 with evidance to be hybrids.

In all types of crosses, bands, which did not exist in the parents zymogram were detected.

CHAPTER 7:

DISCUSSION

7.1. *Hordeum*

7.1.1. Flowering biology

Flowering of *Hordeum* plants usually begins in the florets in the middle of the ear and spreads up and down, taking about four days to complete. Ears on the different tillers may mature at different times (Briggs 1978).

Hordeum species are usually reported as autogamous (Hammer 1984). Although *H. murinum* is self - fertilizing, when studying flowering biology we observed excerted stamens and visible styles. That proved the absence of cleistogamy and the potential for outcrossing.

Thus, the hybridization and the risk for gene flow between *H. murinum* and cultivated barley exists. It was also possible, because of the fact that their flowering periods overlap.

Intrapopulation differentiation of the flowering stage of *H. murinum* from different regions of the country was observed in experimental garden. Earliest flowering populations were from Valais, followed by those from Swiss Plateau. The last plants to flower were from Graubünden. The interpopulation differentiation of the flowering time for *H. murinum* has been already observed earlier (Giles 1984). The study of flowering time of several populations from Europe, Egypt, Israel and New Zealand indicated intra - and interpopulation heritable variability. The causes of these differences are however unknown, but the possible reason could be the adaptation to the local climate and daylength requirements. The existence of geographical difference in the flowering periods might be important for risk assessment, in case some populations of *H. murinum* would flower together with *H. vulgare*, but some other would have different flowering times. As said before, this was not the case for our investigated populations.

7.1.2. Pollen and seed dispersal

Both *H. vulgare* and *H. murinum* presented a similar pattern of pollen dispersal. Most of pollen was found within few meters of the plant source, remaining constant after certain distance. This pattern is classical for the pollen dispersal and has been already observed for example for *Raphanus sativus* by Klinger et al. (1991) and for *Solanum tuberosum* by Skogsmyr (1994). It is interesting to note that those species are insect pollinated, while *H. murinum* and *H. vulgare* are wind pollinated.

Data on pollination is important for determining the isolation distances between plots with transgenic plants and other sexual compatible plants, growing in the vicinity. Therefore, according to our results, if *H. murinum* is growing close to the field with transgenic barley, most of the hybridization events and higher rate of gene flow is expected at a distance up to 7 meters.

Nevertheless, the majority of pollen migration occured within few meters of the pollen donor, the fact that at 12 meters we still found certain amount of it, indicates that the isolation distances between both species, when cultivating transgenic barley must be larger. Especially some studies on pollen dispersal on *H. vulgare* indicate that outcrossing may occur up to 21meters (Doll, 1987) and 60 meters (Wagner and Allard, 1991). Even the isolation stretches are determined, it is never possible to guarantee that the pollen grain will not fertilize another plant at a great distance (Dale 1992). Thus it is important that the risk assessment must take into account this possibility for rare pollination events and focus on the consequences of potential hybridization.

It seems that the species does not play an important role for the pattern of pollen dispersal in our case as both species have more or less the same type of pollen movement.

Moreover, the environmental conditions may influence the gene flow rate between the transgenic and non - transgenic plants of the same species (Scheffler et al. 1993) and between the populations of the same species (Arias and Rieseberg 1994).

The standard deviation of the distances at which the seeds of *H. murinum* are spread by the wind was also not large - 0.5 - 0.6 meters - without any seeds found at two meters limits of the plastic sheet. However, it is important to mention that barley seeds as well as those of *H. murinum* have sticky ends and hairs on the glumes, which make them adhere to the furs of the animals, bird feathers and human clothes, then may be thus dispersed at large distances. In addition seeds of *H. vulgare* could be dispersed by the seed transport. Thereby, the risk for the establishment of subspontaneous populations increases.

7.1.3. Genetic differentiation between *H. murinum* and *H. vulgare*

A number of well - defined characteristics are necessary to describe and identify different crop species or varieties. The traits used to distinguish and identify different *Hordeum* species are mostly morphological features. However, some useful morphological traits can only be measured at later stages of plants development. Biochemical markers, i. e. isozymes and molecular markers, are valuable supplements or alternatives to the morphological characters. The use of isozymes for identifying barley varieties or *Hordeum* species have been described by several authors (Nielsen and Johansen 1986, Jaaska 1992, Jaaska 1994).

Our results proved that isozymes were suitable genetic markers for distinguishing cultivated and wild barley *H. murinum*. With both studied enzyme systems - GOT and EST - specific electrophoretic bands were determined for the two species. Principal coordinate analysis separated well both species, according to their enzyme structure (Fig. 12, 12a). Mantel test confirmed the significant genetic difference between them.

This is in accordance with the results from the PCA analysis with the morphological characteristics, obtained by Julia Keller Senften (1996), performed on the same plants, which clearly separated *H. vulgare* and *H. murinum*.

Genetic structure of populations of *H. murinum* was typical for that of inbreeding plants, with a low intrapopulation variability, joined with genetic differentiation between populations (Hamrick et al. 1979).

Jaaska (1992) obtained a GOT zymogram for *H. murinum* similar to ours with two monomorphous bands and one heteromorphous triplet. All the studied populations of *H. murinum* had the same enzyme profile. The two slower migrating bands were present in the zymograms of all the populations at similar frequencies. Variation in frequency was found for the three faster electromorphs (Table 5). This variation determined separation of the population according to their geographical regions as demonstrated by the Mantel test.

However, no intrapopulation differences according to the geographical distribution, were demonstrated by PCOORD (Fig.13, 13a).

The results of the PCA according to the morphology of the plants tended to separate the populations from Graubünden (Keller Senften 1996), which was not observed in our case.

A total of 20 esterase alleles for both *H. vulgare* (12 bands) and *H. murinum* (9 bands) were observed. These numbers are similar to those reported by Nielsen and Johansen (1986) for *H. vulgare* (11 bands) and Jaaska (1994) for *H. marinum* (11 bands), but lower then that reported by Zhang et al. (1994) (20 bands) for cultivated barley from Tibet.

The six studied populations of *H. vulgare* presented different zymograms for EST and GOT (except populations 6 and 7, which were grouped together according their EST and GOT profiles and populations 12, 13 and 14, which had the same GOT pofile). It demonstrates that probably different varieties of cultivated barley were sampled, although it was not possible to

verify this information. This grouping of the barley populations according to the GOT zymogram is the reason for the separation of populations 2, 6 and 7 from populations 12, 13 and 14 of *H. vulgare* by the PCOORD, when Factors 1 and 2 were plotted together (Fig. 12a). Only one common EST band out of 20 and 4 GOT bands (one monomorphous band and one triplet) out of 13, was shared by *H. vulgare* and *H. murinum*, indicating that they are not genetically very close. This fact is not surprising, as *H. murinum* is not considered as one of the wild progenitors of cultivated barley.

Good correspondance between the results was obtained with isozyme and morphological analysis for *Hordeum*. The two types of markers clearly distinguished both species, indicating relatively low similarity between them, which leads to the conclusion that low gene flow take place between *H. vulgare* and *H. murinum* in natural conditions. Moreover, no intermediate individuals have been detected in the collected populations of both species.

7.1.4. Hybridization ability

It is obvious from our results that the hybridization between *H. murinum* and *H. vulgare* is extremely difficult, if possible at all, without using special *in vitro* techniques. We did not obtain any hybrids, neither under natural conditions nor in the greenhouse. The results were independant of species type of the female or the male parent.

The fact that no hybridization has occured was expected, as no spontaneous hybrids between both species have been reported.

When studying the zymograms of the progeny, there was no evidence of hybrids. In most of the cases (especially for GOT) they strictly repeated the isozyme profile of their female

parents, proving that self - pollination had taken part. The results from biochemical investigations were confirmed by the study with morphological characters, performed on the same plants by Julia Keller Senften (1996).

In the zymograms of certain crosses, there are bands, present for parent plants, but missing in the progeny, which is explained by the segregation of the alleles in the different gametes.

Although *H. vulgare* could outcross and despite the absence of cleistogamy for *H. murinum*, the hybridization between the both species is hampered by the incompatibility of their genomes. *H. vulgare* has an I genome, while *H. murinum* has Y.

Bothmer et al. (1991) divided the *Hordeum* species into three categories with respect to their relation to cultivated barley and how accessible they are as gene donors to the crop:

- primary gene pool (*H. spontaneum* and old landraces) - material closely related to the crop that have no or very weak biological barriers

- secondary gene pool (*H. bulbosom*) - all wild or weedy forms that have a comparatively good crossability with the cultivated species, but in which certain sterility factors are operating

- tertiary genome - these species (*H. murinum*), possess genomes other than cultivated barley and the hybrids with barley are highly sterile. With these species interspecific hybrids could be established only by embryo rescue.

The authors suggest that there are no chromosomal homology among the polyploids of *H. murinum* complex (subsp. *murinum*, 4x and subsp. *leporinum*, 4x and 6x) and other *Hordeum* species, including *H. vulgare*. Natural hybridization occurs very rarely, resulting in sterile offspring.

Bothmer et al. (1983) have been carried out experimental crosses between wild and cultivated barley and showed that the percent of seed set were very low, when *H. vulgare* was used as a female. It was significantly higher when barley was the male parent. However, the obtained

seeds developed very few hybrids. There were less hybrids when *H. vulgare* was the female as well. This has been verified also by the other reports (Morrison and Rajhathy 1959). They proved the existence of strong barriers for seedling establishment in hybrid combination. In tetra - and hexaploid combinations the regeneration of the plants were much higher. The crosses with tetraploid *H. murinum* were the only one from which plants were not obtained, although seeds were set.

The absence of natural hybrids between *H. vulgare* and *H. murinum* and the results of our experimental crosses allow us to conclude a low risk for gene flow between both species in Switzerland.

7.2. *Medicago*

7.2.1 The two ploidy forms of *M. falcata* in Switzerland

The fact that diploid *M. sativa* in Switzerland does not exist was expected as there was no report for its presence in Europe. All previous records indicated that *M. sativa* in Europe is tetraploid (Small and Bauchan 1984, Majovsky et al. 1974, Strid and Franzen 1981, Bijok et al. 1971).

Few chromosome counts of *M. falcata* have been reported for Europe.

Diploid *M. falcata* is present mainly in Asia and Eastern Europe, extending westwards to South of Germany. According to Quiros and Bauchan (1988) the distribution area of tetraploid *M. falcata* concides somehow with that of the diploid form. However, it shows more western and northern distribution (from Portugal and France to Sweden) in Europe in contrast to the diploids. The presence of tetraploid form in most parts of Switzerland is consistent with previously reports from Portugal and Balkans (Fernandes and Quiros 1978, Small and Bauchan 1984, Van Loon and Kieft 1980).

The Western distribution boundary of the diploid *M. falcata* in Europe runs through the Balkans and Eastern Europe to the South of Germany. The populations in Unterengadin lie in the margin of its range. Diploid *M. falcata* is an additional example of the affinity of floristic elements of the Unterengadin with Eastern Europe, where this cytotype is also present. At the begining of the postglacial period (ca. 10 000 years BP) the climate favoured the expansion of the steppes. Unterengadin was probably invaded through the Inn valley, where other relicts from that period have subsisted e.g. *Dorycnium germanicum* (Gremli) Rikli (Landolt 1986). Contrasting with that, Swiss tetraploid *M. falcata* have migrated either from the Balkans or from Western and Northern Europe, according to its present distribution. However, it is not ruled out that *Medicago* colonization of Switzerland originates from different regions.

Both cytotypes present in Switzerland are probably geographically separated, but this has to be proved by more detailed study.

M. sativa and *M. falcata* hybridize readily at tetraploid level (Ledingham 1940), but crosses between different ploidy levels are hampered. **Thus the geographical differentiation according to the ploidy levels is important from the point of view of the risk for releasing transgenic *M. sativa* in Switzerland. The possibility for transfer of modified genes from cultivated alfalfa to tetraploid *M. falcata* is higher in the Swiss Plateau and most alpine valleys. In the contrary the risk will be lower in the region of Unterengadin where diploid *M. falcata* grows. Thus the genetics of the species may vary geographically and may influence the ability of hybridization with other species, influencing the risk of the vertical gene flow.**

As most of the populations, collected in Valais, Schaffhausen and Rhine valley in Graubünden consisted of *M.* x*varia*, there is strong evidence for intensive genetic erosion of the native tetraploid *M. falcata*. The phenomenon is not recent as Schinz and Keller mentioned already in 1923 that the hybrid *M.* x*varia* could outcompete *M. falcata*. The

present decline of habitats as a result of intensive land use is an additional danger for the conservation of tetraploid *M. falcata.*

Landolt (1991) describes *M. falcata* as endangered in the Plateau and extremely endangered for the western part of the Jura and western part of the Northern Alps. We believe that except for Unterengadin, where the diploid type grows, there is a danger of genetic erosion suppressing *M. falcata* in the whole country. Therefore, protection of this indigenous species as a part of Swiss flora and as potential genetic ressource for the improvement of cultivated *M. sativa* is urgent. Protection measures could consist in the prescription of a minimal distance between cultures of *M. sativa* and present populations of *M. falcata* or more often rotation of the cultured species.

7.2.2. Genetic differentiation between *M. sativa* and *M. falcata*

7.2.2.1. Isozymes

The results, obtained with the three studied enzyme systems allowed us to compare *M. sativa, M. falcata* and *M.* x*varia* as well as the two ploidy levels of *M. falcata.*

No specific bands for *M. sativa* or *M. falcata* were found with the three studied enzyme systems, and the electrophoretic bands were more or less equally present in both species at tetraploid level (Table 7), presuming that they belong to the same gene pool. Nevertheless, some of the bands, such as PRX 5, MDH 2, MDH 3, were more rare for *M. falcata*, which determined the significant difference between both species, revealed by the Mantel test. In spite of this, *M. sativa*, tetraploid *M. falcata* and *M.* x*varia* tended to group together and no clear separation was observed between them with PCOORD (Fig. 15 and 15a).

Genetic proximity between *M. sativa* and *M. falcata* at the same ploidy level is an evidence for extensive gene flow between them, caused by the free hybridization between the species.

Genetically more distant are *M. sativa* and diploid *M. falcata*. There was more similarity between both species at the same ploidy level, then between both ploidy forms of *M. falcata* as well. Bands PRX 5, EM 6, MDH 5 and MDH 6, which are present in *M. sativa* and tetraploid *M. falcata* are missing in the diploid form. This genetic remoteness between the diploid *M. falcata* and the tetraploid species was demonstrated clearly by the PCOORD as well (Fig. 15 and 15a), where they form a clearly separated group. **This confirms the existence of the ploidy barrier, which hampers the hybridization beween the tetraploid *M. sativa* and the diploid *M. falcata,* determining lower gene exchange between both species.**

Interesting enough is the presence of band MDH 1, specific for the diploid *M. falcata* in the zymogram of *M. sativa* from Unterengadin. That band determined the presence of the plants of *M. sativa* from Unterengadin in the group of the diploids after PCOORD. **It indicates that some gene flow happens at different ploidy levels in natural conditions as well, probably by 2n gametes.** However, this case is rare, as we did not observed any *M.* x*varia* or triploid plants (which could be the result of such crosses) in the populations from Unterengadin and according to the literature.

7.2.2.2. RAPD

The Principal Coordinate Analysis based on RAPD data did not show separation between the three species and they tended to group together (Fig. 16).

This could be explained with the high hybridization and introgression rates of *M. sativa* and *M. falcata* and confirms the high rate of gene flow between both species at the tetraploid level in natural conditions. Because *Medicago* is an outcrossing species and each population is actually a heterogeneous mixture of genotypes our results indicate that with RAPD, we could identify specific individuals within *Medicago* populations, but not differentiate the species,

which is in accordance with the conclusions of Barcaccia et al. (1994) with alfalfa. Thus another DNA technique, such as RFLP, AFLP, microsatellites etc. should be used in order to obtain more specific and stable DNA markers for both *Medicago* species.

Mantel test, calculated with RAPD data for differences between the three species was still significant, although the r value was very low.

7.2.3 Hybridization ability

It is known that the genomes of *M. sativa* and *M. falcata* are homologous and can pair. They cross easily at the same ploidy level. Our observations of natural populations and experimental hybridizations are in accordance with that fact.

Because no specific isozyme bands were determined for the studied *Medicago* species, we have used for the hybridization experiments plants of *M. sativa* and *M. falcata*, which differed in their zymograms for one, two or all three enzyme systems.

Hybrids were detected in the progeny of the plants after hybridization in natural conditions (Table 16) and in the greenhouse (Table 18) as they had zymograms intermediate between those of both parents or bands not typical for the female parent in the case of hybridization in the field.

For some crosses, it was possible to detect the hybrids with all three enzyme systems, while in the other cases there was evidence only with one or two of them. One reason for this was the same profile for both parents for one or two of the enzymes.

In total five individuals were proved to be the result of cross - fertilization and 27 were obtained after hybridization in the greenhouse. More hybrids - 29 were produced after natural hybridization. The lower number of hybrids, obtained in the greenhouse could be explained by imperfections of the technique, used for the artificial hybridization and the relatively big number of the contaminations during the pollination procedure. The contaminations were

manifested by the presence of « alien » bands (not typical for any of the parents) in the zymograms of the progeny and were in the progeny after experimental hybridization, self - and cross - fertilizations. However, most contaminations were detected in the progeny of the plants after self - fertilizations, where eight from twelve crosses, which regenerate plants consists of non - typical bands. In the rest three crosses zymograms of the progeny was as expected, repeating parents profiles.

A big part of the plants after the experimental crosses failed to form fruits. In addition only 41% of those, which produce seeds (after hybridization and cross - fertilization) regenerated hybrids. This is in accordance with the observation of Quiros and Morgan (1981). Their experiments resulted in low seed production and reduced fitness of the plants in many progenies from selfed or sibling crosses in *Medicago,* considered as consequence of homosygosity, determined by the predominantly outcrossing nature of the genus. The seeds from such crosses generaly failed to germinate or produce abnormal seedlings.

Finally, the progeny, obtained after the experimental crosses, performed by Pia Rufener Al Mazyad from University of Bern were also examined with isozymes for evidence of hybrids. One cross *M. sativa* x *M. falcata,* five crosses between *M. falcata* and *M*. x*varia*, eight between *M. sativa* and *M*. x*varia*, as well as two self - fertilizations (*M. sativa* x *M. sativa* and *M*. x*varia* x *M*. x*varia*) and one cross -fertilization (*M. sativa* x *M. sativa*) was carried out by her.

Only 41% of all performed crosses produced ripe seeds and only 28% of the plants produced seedlings. In total 38 plants were regenerated after these crosses. Evidence for 19 hybrids after hybridizations *M. falcata* x *M*. x*varia, M. sativa* x *M*. x*varia* and *M*. x*varia* x *M*. *sativa* existed according to their isozyme profiles (Table 19). Thus *M*. x*varia* could easily backcross

with *M. sativa* and *M. falcata*. Once again relatively low seed production and regeneration ability after experimental hybridization was observed similarly with the results, obtained in Neuchâtel. Unfortunately, it was not possible to determine if the only regenerated plant after the cross *M. sativa* x *M. falcata* is a hybrid (not present in table 19), because of the absence of the zymograms of PRX, EM and MDH for the male parent.

The number of plants, producing seeds after hybridization when *M. sativa* was the female parent, was approximately the same of that when *M. falcata* was the female. However, more successful hybridizations and more hybrids was scored when *M. falcata* was used as female parent.

Similar was the situation when self - and cross - fertilizations were carried out. In these cases for the seed production and plant regeneration it was of importance whether they were performed with *M. falcata* or *M. sativa*. With *M. falcata* more seeds and more regenerated plants were obtained, compared to *M. sativa*.

The literature data (Ledingham 1940) and our experimental hybridization confirm that at the tetraploid level *M. sativa* hybridizes freely with *M. falcata.* Our observations of natural populations as well support the presence of extensive gene exchange between both tetraploid species. Some of the collected population consisted exclusively of intermediate individuals between both species (*M. xvaria*). Thus high risk for gene flow was concluded.

Diploid *M. falcata* was discovered too late to carry the experimental crosses. Nevertheless literature indicates that crosses between diploid *M. falcata* and tetraploid *M. sativa* are hampered, due to the ploidy barrier. Crosses between the diploid and tetraploid forms results in low fertility (Ledingham 1940, Lesins 1952). If diploid *M. falcata* is the female parent, the ovules abort, while the ovary may develop a well - formed pod. When *M. sativa* is the female,

fertilization delaies and the development of the endosperm and of the embryo usually stops. The offspring are either sterile triploids or rarely tetraploids. These tetraploids are highly fertile and have morphological characters intermediate between both parental species (Ledingham 1940). **In the populations from Unterengadin neither triploid plants nor *M.* x*varia* were observed, which confirms the existence of the ploidy barrier, thus determining the lower risk of growing transgenic alfalfa in the regions, where the diploid form of *M. falcata* grows.**

CONCLUSIONS

The purpose of this study was to evaluate the risk for vertical gene flow in the case of two cultivated species : *Hordeum vulgare* and *Medicago sativa* to their wild relatives *Hordeum murinum* and *Medicago falcata* respectively.

The necessity of doing risk assessment studies case by case and moreover on regional scale was demonstrated by the study. The different plant biology, behaviour and distribution of both group of species determine the specificity of the gene transfer and the different degree of the risks that appear.

Hordeum murinum* - *Hordeum vulgare

We concluded low risk for gene flow between cultivated barley and his wild relative *H. murinum* in Switzerland. No hybrids were detected, when examining the populations of *H. murinum*, collected at the proximity with cultivated barley. No evidence of hybridization between *H. vulgare* and *H. murinum* was found after our experimental crosses in the field and in the greenhouse. It was caused probably by the different genomes of both species.

However, for the risk assessment should be considered that although *H. vulgare* and *H. murinum* reproduce predominantly by self - fertilization, some outcrossing has been reported. The preconditions for hybridization between the two species exist, because of their overlaping flowering periods and the absence of cleistogamy for *H. murinum* in Switzerland. The dispersal of the genes by pollen and seeds has to be considered as well: the majority of their migration occurs within few meters from the donor plant. Nevertheless, pollen migration of

H. vulgare has been noticed up to 60 meters according to the literature. Moreover, long distance dispersal of seeds of *H. murinum* by zoochory may occur, as well as dispersion of *H. vulgare* during transport. These types of dispersion may increase considerably the risk for gene flow.

Medicago falcata - Medicago sativa

We concluded that high rate of gene flow occurs between *M. sativa* and *M. falcata* at tetraploid level, determined by the high rate of outcrossing and free hybridization between both species. They have a relatively low seed and fruit sets and more or less limited seed dispersal, but extensive pollen dispersal (1600 meters). This together with outcrossing exhibits the major risk for gene flow.

In course of this study the existence of diploid *M. falcata* in Switzerland was discovered. As the hybridization between tetraploid *M. sativa* and diploid *M. falcata* is very restricted due to the ploidy barrier, the geographical distribution of the two ploidy forms of *M. falcata* is important from the point of view of the risk for cultivation of transgenic alfalfa in Switzerland. The possibility of gene transfer to tetraploid *M. falcata* is higher in Swiss Plateau and most alpine valleys, contrasting with the region of Unterengadin, where the diploid form grows.

Our results indicate that risk assessment must be carried out at a regional scale according to the genetic differentiation of species, which may occurs on small scale.

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APPENDIX I

1. Composition of the enzyme extraction buffer for *Hordeum* (for 100 ml)

0.60 gr Tris, 0.36 gr EDTA, pH = 7.5

2. Composition of the enzyme extraction buffer for *Medicago* (for 100 ml)

1.21 gr Tris, 1.86 gr KCL, 1 gr PEG (20 000), 0.04 gr Na -Thioglycolate, pH = 8

One point of the spatule of Polyvinylpolypyrolidon was added in the mortar as well.

3. Composition of the Polyacrylamide gel

Stock solutions:

A

0.4 ml N,N,N′,N′ - tetramethyl - ethylenediamine (TEMED), 36.6 gr Tris, 48 ml HCL 1N, bidistilled H_2O up to 100 ml, pH = 8.9

B

0.5 ml TEMED, 5.98 gr Tris, bidistilled H_2O up to 100 ml, pH = 8.9

C

80 gr Acrylamide, 1.47 gr N,N′-methylene-bis-Acrylamide, bidistilled H_2O up to 200 ml

D

10 gr Acrylamide, 2.5 gr N,N′-methylene-bis-Acrylamide, bidistilled H_2O up to 100 ml

E

0.004 gr Riboflavin, bidistilled H_2O 100 ml

F

40 gr Sucrose, bidistilled H_2O 100 ml

G (to be prepared the same day)

0.185 gr Ammonium peroxydisulfate, bidistilled H_2O 200 ml

Composition of the gels:

		9%	10.5%
- separation gel:	sol. A	6.5 ml	6.5 ml
	sol. C	11.7 ml	13.65 ml
	sol. G	33.8 ml	31.85 ml
- stacking gel:	sol. B	1 ml	1 ml
	sol. D	2 ml	2 ml
	sol. E	1 ml	1 ml
	sol. F	4 ml	4 ml
- sample gel:	sol. A	0.62 ml	0.62 ml
	sol. C	1.12 ml	1.12 ml
	sol. G	3.25 ml	3. 25 ml

4. Electrode buffer

Stock solution (for 1000 ml)

15.26 gr Tris, 135.18 gr Glycine, pH = 8.6

The final buffer was prepared by mixing 200 ml of the stock solution and 3600 ml dH_2O.

APPENDIX II

1. Composition of 10 x TBE buffer:

154.4 gr Tris, 26.2 gr Boric acid, 9 gr EDTA, 810 ml bidistilled H_2O

2. Nucleotide sequence of the primers, tested for RAPD analyses

kit B

OPB - 01 GTTTCGCTCC	OPB - 11 GTAGACCCGT
OPB - 02 TGATCCCTGG	OPB - 12 CCTTGACGCA
OPB - 03 CATCCCCCTG	OPB - 13 TTCCCCCGCT
OPB - 04 GGACTGGAGT	OPB - 14 TCCGCTCTGG
OPB - 05 TGCGCCCTTC	OPB - 15 GGAGGGTGTT
OPB - 06 TGCTCTGCCC	OPB - 16 TTTGCCCGGA
OPB - 07 GGTGACGCAG	OPB - 17 AGGGAACGAG
OPB - 08 GTCCACACGG	OPB - 18 CCACAGCAGT
OPB - 09 TGGGGGACTC	OPB - 19 ACCCCCGAAG
OPB - 10 CTGCTGGGAC	OPB - 20 GGACCCTTAC

kit L

OPL - 02 TGGGCGTCAA	OPL - 08 AGCAGGTGGA
OPL - 04 GACTGCACAC	OPL - 12 GGGCGGTACT

kit P

OPP - 01	GTAGCACTCC	OPL - 11	AACGCGTCGG
OPP - 02	TCGGCACGCA	OPL - 12	AAGGGCGAGT
OPP - 03	CTGATACGCC	OPL - 13	GGAGTGCCTC
OPP - 04	GTGTCTCAGG	OPL - 14	CCAGCCGAAC
OPP - 05	CCCCGGTAAC	OPL - 15	GGAAGCCAAC
OPP - 06	GTGGGCTGAC	OPL - 16	CCAAGCTGCC
OPP - 07	GTCCATGCCA	OPL - 17	TGACCCGCCT
OPP - 08	ACATCGCCCA	OPL - 18	GGCTTGGCCT
OPP - 09	GTGGTCCGCA	OPL - 19	GGGAAGGACA
OPP - 10	TCCCGCCTAC	OPL - 20	GACCCTAGTC

APPENDIX III

List of *Medicago* plants, which produced fruits after experimental hybridization in the field, with the number of F_1 individuals, which have survived:

1A 220/0800
4B 310/01 (4 F_1 plants)
4B 400/03 (11 F_1 plants)
4B 110/12 (1 F_1 plant)
4B 395/11
4B 500/0170
4B 780/02
4B 400/13
5A 500/0850
7A 440/1470 (7 F_1 plants)
7A 330/0930 (2 F_1 plants)
7A 290/0820
7A 410/3420
7A 390/171
7A 290/18
8B 270/05 (4 F_1 plants)
8B 300/07 (1 F_1 plant)

ACKNOWLEDGEMENTS

I would like to express all my gratitude to my parents, my husband and my sister's family for their lovening support and encouragement during the years of preparation of my thesis and that they have been always there for me in the difficult moments.

I thank to my supervisor prof. Philippe Küpfer for affording me the opportunuty to do my thesis in the Botanical Institute of the University of Neuchâtel, for his advises and for accepting to be a president of the jury of the thesis.

My very special gratitute is for Dr. François Felber for the useful disscussions, helpfull advises and for the help of collecting the plant material. I thank him also for the critical reading of the manuscript.

I am also grateful to prof. Klaus Ammann, Dr. Julia Keller Senften, Pia Rufener Al Mazyad from University of Bern, and Dr. Yolande Jacot for the help and the discussions we had. I thank them also for the help in collecting the plant material.

My gratitute is for Prof. J. - M. Gobat and Dr. A. - M. Chèvre for accepting to be members of the examination jury and useful counsels.

I thank to Dr. Corinne Zeroual Humbert-Droz for the help with the electrophoretic technique and many other services she rendered me, to Michele Gherardi and Dr. T. Hugo from INRA, France for the help with the DNA technique.

My gratitute is for Dr. E. Zeroual, Dr. G.-F. Zhao, K. Garcia-Herran and S. Leuba-Garcia for the interesting disscussions we had and for the various services they rendered me.

I would like to thank to M. Gauteaub for the help in collecting the plants, E. Fortis, Mr. and Mrs. Boss and Mrs. M.-A. Marguerat, who rendered me numerous services.

Thanks ever so much to Marta, Bénédicte, Abdessamad, Louis and all the colleagues from the Lab for their kindness and support. Their warmness and friendship are very important for me.

This research was carried out in the frame of the coordinated project « Gene flow between selected Swiss crops and related weeds. Risk assessment for the field release of GMO's in Switzerland .» of the Swiss Priority Programme Biotechnology and is funded by the Swiss National Science Foundation (grant n° 5002 - 035207).

SUMMARY

The aim of the study was to evaluate the risk of gene flow, involving sexual reproduction between cultivated and wild species in Switzerland.

Two cultivated plant species - *Hordeum vulgare* (barley) and *Medicago sativa* (alfalfa) and their wild relatives present in the Swiss flora - *H. murinum* and *M. falcata*, were investigated in order to measure their potential for hybridization and the extent of introgression between them.

Barley and alfalfa have been selected, because of their contrasting characteristics. *H. vulgare* is annual, autogamous food crop, while *M. sativa* is perennial outcrossing fodder plant. In addition *M. sativa* hybridizes easily with *M. falcata*, resulting in fertile hybrids, while *H. vulgare* and *H. murinum* hybridize in a low rate, resulting in plants with low viability.

Experiments were performed on non - transgenic plants, collected in different regions of the country.

Hordeum

When studying flowering biology of *H. murinum*, excerted stamens and visible styles were observed. Thus, absence of cleistogamy and possibility for outcrossing was concluded. There was also possibility for hybridization and risk for gene flow between *H. murinum* and cultivated barley because of the overlaping flowering times of both species. Populations of *H. murinum* from different geographical regions had different flowering periods.

Pollen and seed dispersal experiments for both *H. murinum* and *H. vulgare* were carried out. Both species presented similar pattern of pollen migration, with most of it dispersed close to the plant source. The amount of pollen remained constant after 2 meters. Thus, if *H. murinum* is growing in the vicinity of plots with transgenic barley, most of the hybridization events and

higher rates of gene flow are expected within few meters. However, risk assessment must take into account the possibility of rare pollination events at longer distances.

H. murinum dispersed most seeds close to the plant source. Standard deviation of the distribution corresponded to 0.4 - 0.6 meters and at two meters we did not find any seeds. However, they could be dispersed by zoochory at larger distances. Seeds of *H. vulgare* remained on the spike (typical character of domestication), but nevertheless they can be lost during the transport, which has to be considered by the risk assessment.

In the course of this study, the first chromosome counts for *H. murinum* for Switzerland were carried out. The species was tetraploid with 2n = 4x = 28 chromosomes. *H. vulgare* was diploid with 2n = 2x = 14 chromosomes.

Isozyme markers for *H. murinum* and *H. vulgare* with glutamate oxaloacetate transaminase (GOT) and esterase (EST) were determined. Mantel test for differences between both species on isozyme level was significant. Good separation between them was performed by the Principal Coordinate Analysis (PCOORD) as well, confirming the absence of hybrid and introgressed individuals.

Significant geographical differences between *H. murinum* populations on enzyme level was also demonstrated by Mantel test.

No hybrids were obtained after experimental hybridization between *H. vulgare* and *H. murinum* in the field and greenhouse as well as no natural hybrids were detected in the investigated populations.

Thus, low risk for cultivation of transgenic barley in Switzerland was concluded, because of the hampered hybridization between *H. vulgare* and *H. murinum* and absence of evidence of gene flow between them.

Medicago

Medicago sativa and *M. falcata* are tetraploids (2n = 4x = 32) in most parts of Switzerland.

Important contribution of this study was the discovery of diploid *M. falcata* (2n = 2x = 16) in Switzerland, in Unterengadin, together with Dr. F. Felber and P. Rufener Al Mazyad. This ploidy form was not known to exist in the country before. The discovery is important for the risk assessment of the gene flow between cultivated *M. sativa* and the wild relative *M. falcata,* because of the fact that the hybridization between both species is not easy between different ploidy forms. Hybrids between *M. sativa* and *M. falcata* are denominate as *M.* x*varia*.

The isozyme analysis with peroxidase (PRX), maleic enzyme (EM) and malate dehydrogenase (MDH) did not reveal specific bands for *M. sativa*, *M. falcata* or *M.* x*varia*. However, differences between diploid *M. falcata* tetraploid *M. falcata* and *M. sativa* was noticed, as some of the electrophoretic bands, existing in both *M. sativa* and tetraploid *M. falcata* were not present in the diploid *M. falcata*. Interesting enough was the presence of one MDH band, typical for the diploid *M. falcata* in the zymograms of *M. sativa* from Unterengadin, indicating that some gene flow might happen between both species although they present different ploidy levels.

PCOORD analysis separated well diploid *M. falcata* from the tetraploid species and Mantel test gave a significant difference between *M. sativa*, both ploidy forms of *M. falcata* and *M.* x*varia.* That difference remained when diploid *M. falcata* was removed from the sample.

The high rate of natural hybridization between both *Medicago* species at the tetraploid level was assessed also with the results, obtained with RAPD analysis of one population.

In addition, hybrids were detected in the F_1 progeny of the plants after experimental hybridization between *M. sativa* and tetraploid *M. falcata* in the greenhouse and in the field.

As both species hybridize freely at tetraploid level, high risk for gene flow between them was concluded.

When assessing the risk for cultivating transgenic *M. sativa*, the geographical differentiation of distribution of the both ploidy forms of *M. falcata* has to be considered. The possibility of

gene transfer to tetraploid *M. falcata* in Swiss Plateau and most alpine valleys is very high, contrasting with the region of Unterengadin, where the diploid form grows.

CHAPTER 1:

OBJECTIVES OF THE THESIS

During the last fifteen years the process of conventional plant breeding has been improved by biotechnology. The new molecular and cellular biology techniques have provided opportunities to modify plants, animals and microorganisms in novel ways, to be employed in industry, agriculture and human health. The insertion of a gene from one organism into another, unrelated genetically does not occure in the nature. Therefore, genetically modified orgenisms cultivated outdoors may generate hazards for environment which must be evaluated. Investigations on risk assessment of genetically modified organisms, performed in Switzerland up to now were restricted to a review of the bibliography (Jacot 1994, Jacot and Jacot 1994) and there was a need for more detailed study of the problem as well as specific studies on the regional scale.

The purpose of this study was to evaluate the risk of gene flow involving sexual reproduction between GMO's and the wild flora (vertical gene flow).

Experiments were performed with non - transgenic plants of barley (*H. vulgare*) and alfalfa (*M. sativa*) in order to measure their potential of hybridization and introgression with *H. murinum* and *M. falcata* respectively. The two species have been chosen because of their contrasting characteristics. Barley is an annual plant, while alfalfa is perennial. *H. vulgare* is anemogamous while predominantly autogamous and *M. sativa* is entomophilous and outcrossing. *M. sativa* hybridizes easily with *M. falcata*, resulting in fertile hybrids, contrasting with *Hordeum,* which forms hybrids, but with low viability. Finally, *Hordeum vulgare* is a food crop and *Medicago sativa* is a fodder plant. Thus the expected risk of releasing transgenic plants from both species is different.

The study was realized within the framework of the Swiss Priority Programme ″Biotechnology″, in parallel with two other PhD thesis (Julia Keller Senften and Pia Rufener Al Mazyad), including the same species, but with different approach. The present study focus

on genetics, including empirical observations and experiments, while the other two thesis are based on phytogeography and morphology, mostly with the descriptive perspective.

To determine the rate of gene flow between wild and cultivated species two types of methods were used:

a) direct: seed and pollen dispersal, natural, and experimental hybridization

b) indirect: evaluation of gene flow in the hybrid zones between the crop and the wild relative, using various techniques such as karyology, isozymes and RAPD.

Based on these data, the risk for releasing transgenic plants in Switzerland was assessed. The data will contribute to the botanical files, which synthezise life history traits of the crop and the potential of gene escape to the wild flora.

CHAPTER 2:

INTRODUCTION : RISK ASSESSMENT

2.1. GENETIC MODIFICATION (Rudelsheim 1992 - 1994)

Genetic modification of plants is defined as the application of recombinant DNA technology to the production of new crop varieties. The process includes four steps:

1. Isolation of a specific gene from plants, animals or microorganisms

2. Insertion of the gene into a special « carrier » DNA molecule, called a vector and its propagation in a host organism, usually a bacterium

3. Use of such vectors to transfer the gene of interest into the plant cell

4. Regeneration of the plant from individual modified cell through tissue culture techniques. This plant then carries the transferred gene and can transmit it to its progeny.

There are several methods for producing transgenic plants. The most common is *Agrobacterium*-mediated transformation, using *Agrobacterium tumefaciens* or *A. rhizogenes*. Particle bombardment, DNA uptake into isolated protoplasts and electroporation are the other widely used methods.

The first transformed plant was tobacco. Now various species including tomato, maize, potato, alfalfa, sugar beet, oilseedrape, wheat etc. have been transformed.

Most often genetic transformation has been used to produce herbicide tolerant, pest and diseases resistant plants. Biotechnology is also capable of providing new pharmaceutical and other chemical substances, synthesized by plants, for improved nutritional properties of the plant or for resistance against environmental stress, such as drought, high salinity or cold. It is also possible to modify plant protein and oil content. All these new characteristics transferred into the crops have clear advantages. However, there are serious concerns about the containment of transgenes and their products within agricultural populations and the risks associated with their possible escape. Genes can be transfered from genetically modified

organisms (GMOs) by sexual reproduction to the wild species (vertical gene flow) or to microorganisms, aphids and viruses (horizontal gene flow). Since it is better to prevent than to cure, the precautionary approach was accepted. Before any introduction of a transgenic plant or product (experimentally or commercially) an assessment of its potential impact has to be made.

2.2. DEFINITION OF RISK ASSESSMENT (Tzotzos 1995, Levin 1995)

Risk assessment is a scientific process, an essential part of which is to define the type of the risk and its degree. Before assessing, however, it is necessary first to define what is meant by « risk ».

It is generally accepted that there are two components of risk: hazard and exposure.

2.2.1. HAZARD is defined as the potential (toxicological or ecotoxicological) harmful property of the product, encoded by the newly constructed genetic material or by the host carrying the genetic material or its product.

The hazard depends on the kinds of intrinsic characteristics that could cause undesirable effects and the intensity of their exposure.

2.2.2. EXPOSURE is the probability that susceptible populations or individuals are presented to respond to an agent of effect (stressor). It depends on the kind of the population or susceptible individuals, their number, their proximity to the stressor, the amount of the stressor and the relationships between susceptible populations and the stressor.

2.2.3. RISK is defined as the probability of the hazard occuring.

2.2.4. RISK ASSESSMENT is an analytical tool that facilitates the organization of large amounts of scientific methods, models and data with the goal of estimating the potential risk posed by a process of interest.

It is an evaluation process of possible adverse effects of living organisms on the environment and human health.

Risk assessment can be divided in two phases. In a first phase potential hazards are identified. The second phase determines the likelihood and consequence of the hazard to occur.

2.2.5. RISK MANAGEMENT is the stage following the risk assessment in which perceived risks are balanced against other criteria in order to make decisions about risk mitigation and control.

2.3. TYPES OF RISK

The essential part of the risk assessment is to determine how the transgene might change the environment, when compared to the impact of the nontransgenic crop.

When concerning transgenic plants there are four risks, recognized to be of major importance:

2.3.1. Enhanced toxicity and allergenicity of the transgenic plant

The modified plants may yield toxic substances in the product or the product itself can be harmful for non - target organisms such as birds, insects, symbions or for human health (Dale 1995, Tiedje et al. 1989). Incomplete degradation of chemicals, leading to the formation of chemical deposits or to the production of even more toxic by-products has a potential risk for the environment (Vogel and McCarty 1985).

2.3.2. Establishing of feral populations

The transgenic plant could escape due to the seed dispersal out of the cultivated field and establish feral population. These plants may therefore hybridize with the wild relatives that are geographically close, thus increasing the risk for further gene flow. According to Crawley (1987) it is difficult to predict whether a particular plant is invasive, but it is possible to determine the types of habitats that are invasible. He showed that plant communities with a high proportion of bare ground and frequent and intense soil disturbance were more likely to be invaded by alien species than the stable late - successional communities.

2.3.3. Horizontal gene flow

It is defined as a gene escape from an organism to another organism without sexual reproduction. Thus the gene transfer might occur by horizontal gene flow from plants to animals and microorganisms and vice versa. It is still unclear whether such gene flow is possible and whether the transgene could be incorporated in microorganisms genome. The assessment of the ecological risk in this case is hampered because the soil biological processes are poorly understood and deficiency in the basic monitoring procedures to enumerate the soil organisms exist (Abbott 1994).

When dealing with virus resistant plants the processes which may occur with potential environmental risk are transcapidation and template switching. In the first case when a virus attacks a transgenic plant, producing the coat protein of another virus, its nucleic acid could be capsidated with the coat protein of the original donor vector. In the second case there is a recombination between viral sequences, incerted in the plant genome and infecting viral particles. Both processes result in a « hybrid virus ». They could lead to a production of new viruses or give opportunity for mixed infection (Dale 1995).

2.3.4. Vertical gene transfer

It includes the transfer of genes by hybridization and introgression of transgenes into wild related species.

Plant breeders have long time been concerned with the gene flow between crops and wild species (Anderson 1949, De Wet 1975, Barrett 1983, Ellstrand 1988). Early studies mainly focused on the gene flow into crop strains because of fears for the purity of the seed material (Sprague 1938, Haskell 1943, Jones 1948, Hutchcroft 1955, Nieuwhof 1963). More recently the concerns regard gene flow from crops to the wild relatives via hybridization. It has been suggested that the transgenes may be transfered into natural and weed populations, creating invasive weeds or increasing the weediness itself (Ellstrand 1988, Crawley 1990, Keeler and Turner 1990, Manasse 1992). The main gene vector is the pollen. The possibility of contamination depends on the way of the dispersal and the geographic remoteness of the cultivated and wild plants. If the transgene is stable, it will be transmited in the next generations and in this way continue its diffusion. The transgene could be transmited by other vectors, such as microorganisms and viruses as well.

The elimination of wild or desirable naturalized species through competition or interference is a possible outcome in some cases.

Unwanted changes in the transformed plant may occur as well. They can include the tendency for a self - pollinated line to outcross, increasing the chance for spreading the transgene to the wild relatives.

2.4. DEGREE OF THE RISK

Different plants have different abilities to exchange genetic material with their wild relatives. Studies carried out at the national level in the Netherlands and UK have shown that the plants

could be classified into different categories according to the probability of gene flow to occur and extend.

Some plant species (potato for example) do not have wild relatives in Europe, thus the probabilities of gene transfer practically does not exist. On the contrary others (alfalfa) hybridize freely and in large extent with their wild relatives. Between these two extremes there are plants with different probabilities of gene transfer. Therefore various classifications of the espected risk were proposed.

For the Netherlands Frietema de Vries et al. (1993) have studied a herbarium collection of 42 cultivated species, present in Dutch flora. They have established Botanical files for each of the species. Each file gives information about the cultivated plant itself, its relatives, hybridization/crossing ability (gene flow by pollen) and observations on escapes from the field to nature (gene flow by diaspores). The information is summarized in codes (Dd, Dp, Df, Dpdf), indicating possible ecological effects of the cultivated plant to the wild flora:

- Dispersal by diaspores (Dd)

A scale from 0 to 4 is used to determine the probable effect of the crop by dispersal of its diaspores. The higher is the code the higher is the expected effect.

- Dispersal by pollen (Dp)

In this case it is important to know if the wild relative exists and if so, what the relationship is between it and the crop. The information is summarized in Dp codes on a scale from 0 to 5, indicating the degree of relationship with the nearest relative.

- Frequency of distribution (Df)

Df code is established on a scale from 0 to 3 in order to indicate the frequency of distribution of the nearest crop relative. The more frequent a near relative is found in the wild, the higher the chance for gene flow is.

- Ddpf codes

It is a summary of the three above mentioned codes. It implies that any, minimal or local, or substantial ecological effect should be expected. When the effect could not be assessed from what is known, the number for the code is 9, meaning that further research is necessary.
This approach has been extended to Switzerland by Jacot and Ammann (in preparation).

According to Raybould and Gray (1993) the escape of transgenes refers to the persistance of the crop`s modified genetic material in non agricultural habitats, which may occur by the crop itself invading disturbed, semi - natural or natural habitats to form feral populations, or by transfer of the transgene to the wild relatives by hybridization. In the UK they have identified crops which form feral populations and those which hybridize with wild relatives. Based on the information, obtained for crop - wild relative hybridization they have classified the crops into three groups representing increasing capacity for cross - pollination with wild species:

- group I have no wild relatives or are not sexually compatible with them (potato, tobacco, maize)
- group II - « low probability of gene flow ». Close wild relatives with limited sexual compatibility are present in the same geographical areas as the cultivated crop (oilseedrape, lettuce)
- group III - « high probability of gene flow ». Wild relatives with reproductive compatibility are present in the neighbourhood of the cultivated crop (sugarbeet, alfalfa, poplar).

A classification according to the generic trait (herbicide tolerance, disease resistance, insect and virus resistance, marker genes, stress tolerance, enhanced plant growth or survival) and to the possible effect they may have to the wild relatives if transferred, was proposed by Ahl Goy (1993):

- class I - « the minimal advantage class » - traits which are expected to confer little, if any, advantage when transfered. It includes traits which might confer disadvantages as well. These are marker genes and genes for improving or changing the quality of the crop product (e. g. delayed ripening, male sterility, changing the colour of the flower etc.)

- class II - « the low advantage class » - traits with the potential of conferring an advantage under the relevant selection pressure. This group was split into two subgroups, depending on the absence (class IIa) or presence (class IIb) of the relevant selection pressure outside agricultural systems. Class IIa contains herbicide tolerance and class IIb, resistance to insects, viruses, diseases and stress.

- class III - « the high advantage class » - traits which have the potential to confer an advantage under any conditions. It includes traits conferring enhanced growth and survival.

Further, the author defines the « potential environmental risk » as the product of the « probability of transfer » and the « consequence of transfer ». Then the assessment of the risk is a combination of both factors. She classifies the risk as minimal, low and high. The risk is minimal when the probability of transfer is high but the consequence of transfer is minimal or vice versa. A potential high risk is considered only when traits with the potential of conferring a selective advantage under any conditions are present in the crops with high capacity for transferring genes. The evaluation of the environmental risk in this way indicates that non of the field trials, conducted with transgenic plants for the period between 1986 and 1992 pose a high risk, 91% represent minimal risk and 9% - low risk (Ahl Goy 1993).

2.5. METHODS FOR MEASURING THE ESTABLISHMENT OF PLANT'S FERAL POPULATIONS AND THE VERTICAL GENE FLOW. DATA, REQUIRED FOR THE RISK ASSESSMENT

2.5.1. Methods and approaches for measuring the establishment of feral populations and the vertical gene flow

In order to obtain information on the safety of genetically modified plants, appropriate methods for monitoring the gene flow must be developed.

The gene transfer is possible through pollen (vertical gene flow) and seeds (establishing of feral populations) and that is why most of the gene flow studies monitor their movement.

Four different approaches are used to estimate the gene flow by pollen (Ellstrand 1992):

1) measuring pollen dispersal from point sources

2) measuring gene dispersal from point and block sources

3) inferring gene flow from natural population genetic structure

4) paternity analysis of progeny in sink populations

The first approach measures the pollen movement either indirectly from pollinator foraging distances (Schmitt 1980) and dispersal of pollen analogues (Campbell and Waser 1989). The second approach involves the creation of experimental populations using source plants, bearing a genetic marker, surrounded by plants fixed for alternate alleles. The progeny harvested at different distances is screened afterwards and gives information about successful fertilization and fruit set (Handel 1983, Smyth and Hamrick 1987).

The third approach measures the gene flow from existing population structure, using a number of statistical methods, based on alleles frequency distribution to estimate indirectly gene flow (Slatkin 1985, Crow 1986, Hamrick 1987).

The last approach measures the gene flow directly by paternity exclusion. First multilocus genotypes of all reproductive individuals in the population are determined. A comparison of the multilocus genotype of the maternal plant with that of its progeny allows the reconstruction of paternal gametic contribution. The gene flow rate may than be evaluated (Ellstrand and Marshall 1985, Hamrick and Schnabel 1985).

All four types of studies suggest that the gene flow can vary considerably within species, among seasons (Campbell and Waser 1989) and over a season (Palmer et al. 1988), depending on plant genotype (Tonsor 1985), plant density (Levin and Kerster 1969), ecological conditions and the way of transfer (Schmitt 1980, Peakall 1989),

The pollen dispersal will only be effective if the pollen grains land on the stigma of the related plant species. Then hybridization may occur and its rate will depend on the frequency with which the wild relative could be found and the fitness of the resulting hybrids. The potential impact of the hybrid will depend on its fertility and ability to self - fertilize or backcross to a parental species (Frietema de Vries 1993).

The seed dispersal studies are not so widely used for estimating the gene flow, because it is more difficult for monitoring and quantifying than pollen movement (Hamrick et al. 1995). The use of the paternity analysis of the progeny proved to be more suitable, but even with this method the documentation of seeds movement is difficult (Hamrick et al. 1995). There are two approaches when using it:

- to develop a set of genetic markers for maternally inherited genomes (chloroplast and mitochondria). If a sufficient number of polymorphic loci are defined the seeds or seedlings could be sampled afterwards to determine if the maternally inherited genome is coming from an adult that belongs to the population. If not, the seeds or seedlings have invaded it. However, up to date cpDNA and mDNA have not proved useful markers for such analyses.

- using nuclear genes for identifying seeds without possible parents within the study site. However it is more difficult and less probable to eliminate both parents than to exclude only the paternal individual for the progeny, whose mother is known. (Hamrick et al. 1995).

The success of the dispersal by the seeds or diaspores is related to the dispersal capacity and the fitness of the individuals, resulting from them. Among the important factors to take into account are the seed quality, and the capacity of the plant to produce viable seeds (Frietema de Vries 1993). In any case the rate of gene flow depends on the seed dispersal mechanisms. It is highest for wind - dispersed species and lower for the explosively dispersed. Species with animal - mediated dispersal are in between.

2.5.2. Techniques, providing markers for the pollen and seed dispersal studies

Pollen and seed dispersal studies have been carried out previously using markers such as flower colour (Kehr 1973). More recently isozymes were used as genetic markers (Krauss 1994, Arias and Rieseberg 1994, Ballal et al. 1994, Chèvre et al. 1995).

Although isozyme analyses have been proved useful for genetic studies and plant identification, they are limited by the small number of loci sampled by the technique. Advances in molecular biology have provided DNA - based markers (RAPD, RFLP) which resolved this problem and are neither affected by the growth environment of the plant nor of its stage of development (Tanksley 1983, Williams et al. 1990). Restriction fragment markers (RFLP) are codominantly and stabely inherited in a Mendelian fashion and they disclose unlimited polymorphic markers. The problem with RFLPs is that the procedure is time consuming, requires a large amount of DNA (2 to 10 mg) and suitable probes, and usually involves the use of radioisotopes. The Random Amplified Polymorphic DNA (RAPD) is based on the Polymerase Chain Reaction (PCR) and utilizes a single synthetic

oligonucleotide primer. In comparison with RFLP the procedure is less expensive, faster, requires a small amount of DNA (0.5 to 50 ng), does not involve the use of radioisotopes and requires less skill to operate. It has been proved useful for analyses at subgeneric level, interspecific taxonomy and classification, geographic variation, cultivar and varieties identification, genetic linkage, analyses of genetic diversity, gene flow and somaclonal variation, hybridization studies and studies of ancient DNA (Demeke and Adams 1994).

Usually when producing transgenic plants marker genes such as kanamycine resistance (*neo*), glufosinate resistance (*bar*), or GUS are transferred to the plants and used after to check if a certain transformation procedure is successful. These genes are widely utilized as markers for pollen and seed dispersal studies as well (Rudelsheim 1992 - 1994, Skogsmyr 1994, Chèvre et al. 1995, Mikkelsen et al. 1995).

2.5.3. Data, required by the EU Directives for the risk assessment

Risk assessment must be carried out at each step of the release of transgenic plants, starting from laboratory level, continuing with small - scale experiments and ending with the large - scale commercial production. Those data provide the basic information for the risk assessment.

Usually general information about the staff and institution responsible for carrying out the release is necessary. They must have a high level of knowledge and experience, and to be responsible for any field containment and monitoring.

In all cases it is essential to have information about the donor DNA organism and the recipient plant species. The last is the baseline against which the transgenic plant will be compared.

A description of the transgenic plant, including molecular data, stability of the transgene expression, changes of allergenicity, toxicity and the capacity of the plant to persist in the agricultural areas or to invade natural habitats has to be available.

For the study of vertical gene flow it is important to know the breeding system (allogamy or autogamy) of the plant and related species, as well as the pollen and seed behaviour. Allogamy promotes gene flow, while autogamy reduces it. Flowering time differentiation also decrease the potential gene flow.

Generally, when producing transgenic plants, antibiotic resistance genes are used as markers. Other DNA sequences may be used as linkage with the vector or provide other functions associated with the use of DNA - recombinant methods. It is therefore necessary to know the nature of this DNA, so that any consequences can be considered.

Ecological information on the release site is also important. This includes a survey of plant species that grow in the vicinity of the release and the dynamics of their populations in order to predict the risk in the following years. Information about the pollen and seed dissemination, the distance of successful pollination and the stay of the seeds in the soil is neccessary as well as. This information is important also to be able to The location and the type of anticipated target organisms must be specified. The target organisms are those, which the transgenes are targeted to affect. Non - target organisms are those which are not primary target of the modification and they include those that are affected unintentionally.

It is important to describe changes in the transgenic plant that may alter its invasiveness in wild habitats, because of the possibility to establish feral populations, or if the transformation changes its ability to propagate sexually or asexually.

Once plants are released in the field and if they are allowed to flower and set seeds, the transgene can move. An important part of the risk assessment is to determine the extent of which it is possible to monitor transgenes after the release and the efficiency with which it is

possible to destroy the plant if it becomes necessary. Efficient methods of identifying transgenic plants or transgenes presented in non - target species may be necessary. This may be a visual marker (B-glucuronidase), a selectable marker (antibiotic resistance) or molecular markers, detected by PCR, Southern hybridization etc.

2.6. MANAGING THE RISK (EU and USA)

The philosophy of risk assessment and regulations is that each release is considered case-by-case to build up experience and familiarity with particular crop - transgene combinations and eventually to move to a streamlining of regulatory procedures (Tepfer 1993).

The whole process of risk assessment is usually carried out by a multidisciplinary biosafety committee made up of specialists in genetics, molecular biology, environmental science, agriculture, plant pathology and other appropriate fields. It may be neccessary to include politicians - people from the local government, members of the public or local ecological groups in order to ensure public satisfaction.

The first release of a modified plant is experimental. It is at this stage that the major risk assessment must be made. The information is usually outlined by scientists, who carry out the release. Once the results of these experiments are analysed, the commercial release may be done. The release proposal is at first assessed by an Institutional biosafety commitee, where it exists. The benefit of this committee is that usually its members have a good local knowledge and good understanding of the scientific principles. It is also responsible for monitoring the site after the release. The next level are the National biosafety commitees, which are also multidisciplinary and include politicians and representatives of the society, who will carry the national responsibility. They could make further recommendations and require that the proposal be changed. Finally international biosafety commitees may also be set up (like one

established in European Union, OECD, UNIDO). At this level uniform standards for countries should be established, which needs harmonization of the requirements of the National commitees.

Between 1986 and 1994 there have been 1608 field trials with transgenic plants, conducted in more than 36 countries. Of these 858 occured in USA and Canada, 558 in Europe, 109 in Central and South America, 65 in the Pacific Rim and 18 in Middle East and Africa. GMPs from 46 different species have been field tested (Extracts from GIBiP database on field trials 1986 - 1994).

The regulations governing the use of transgenic plants vary considerably between the countries.

In 1990 the Commission of the European Community published Directives on the contained use of GMOs (CEC 1990a) and its deliberate release into the environment (CEC 1990b). These Directives are binding in all EU countries. Under the Biotechnology Action Programme (BAP) initiative, the Commission of the EU decided to support a number of independent projects for developing appropriate monitoring methods which may generally be applicable to GMPs. Later the Biotechnology Research for Innovation, Development and Growth in Europe programme (BRIDGE), built on the experience gained in the BAP, has further expanded the experimental methodology on a large scale (Rudelsheim 1992-1994). The countries which took part in this programme were Belgium, Denmark, France and United Kingdom. The plants studied were potato, oilseed rape, sugar beet, alfalfa and tobacco. During, the project methods have been developed to design the experiments and to monitor the greenhouse and field experiments.

In the USA, three agencies share the responsibility for the release of transgenic plants - the United States Environmental Protection Agency (EPA), which is responsible for environmental care; the United States Department of Agriculture (USDA), which is

concerned with the safety of crop plants and food products, and the Food and Drug Administration (FDA), which ensures efficiency and safety of food and pharmaceutical products (Levin and Strauss 1993). These agencies are responsible for achieving a balance between risks and benefits. There are numerous Acts, regulating the release of pesticides, toxic substances, food, drugs and cosmetic products when they are derived from microorganisms, plants and animals, including transgenic ones (Federal Insecticide, Fungicide and Rodenticide Act, Toxic Substances Control Act, Federal Food, Drug and Cosmetic Act, Federal Plant Pest Act and Plant Quarantine Act). In the USA the release of modified organisms may be subject of different federal laws and more than one agency may have to be consulted.

In the USA regulations are product - specific, while the EU has made the regulatory regime process - specific. It is triggered by the process by which the transgenic plant is made. Countries all around the world are adopting models related to these.

As the possibility for the movement of trangenic plants or their diaspores exists, international regulations and harmonization of standards and procedures is necessary.

The Organization for Economic Cooperation and Development (OECD) has developed guidelines for the contained use of transgenic organisms (OECD 1986, 1990, 1992).

Various international initiatives have been undertaken for developing countries through several organisations, including the United Nations Industrial Development Organisation (UNIDO) and United Nations Environment Programme (UNEP). The aim of these initiatives is to facilitate the development of common standards of biosafety. UNIDO is promoting an International Biosafety Information Network and Advisory Service (BINAS), with which Switzerland is connected. It provides data and advice from experts to the national authorities, where they do not already exist.

2.7. RESEARCH ON RISK ASSESSMENT IN SWITZERLAND

In Switzerland the commercial use of GMPs is not allowed. The experimental releases are very restricted - only two in 1991 (Ahl Goy et. al 1994).

The Swiss Interdisciplinary Commission for Biological Researches (SICBR) is the administrative body, responsible for the procedures of registration of the projects in the field of genetic engineering under the security standards. Based on the EC Directives and the German national law about genetic engineering, it prepared in 1992 a Directive for security measures for experimental use of GMOs in laboratory environment and their field release (Diggelmann and Pythoud 1993).

The Swiss Federal Council has proposed to amend the federal law on environmental protection in order to provide a legal framework to warrant a sustainable and safe development and use of genetic engineering in Switzerland. This new legislation is intended to bring the country into line with the practice of the EU.

In 1991 the Swiss working group on genetic ecology was constituted in order to put together information about risk assessment of field release of GMOs and to foster researches in the field of genetical ecology.

A study on environmental risk due to biotechnology products in agriculture has been made, based on literature researches and discussions with plant breeders, biologists, ecologists, biotechnologists etc. in the « Institut des Hautes Etudes Administratives et Politiques » of the University of Lausanne (Jaunin 1992).

In 1992 the Swiss Office of Environment, Forests and Landscape has initiated a bibliographical report on risk assessment for releasing GMOs, including a preliminary study of floristic parameters influencing the gene flow and including a plan to work out guidelines for further risk assessment. It emphasizes the importance of developing a regional view in

assessing floristic and biogeographical factors, and it stresses the lack of knowledge on this subject in Switzerland (Jacot et al. 1992).

In 1992 a Priority Programme on Biotechnology was initiated by the Swiss National Science Foundation. The programme couples basic research and development, links the activities of various Universities and Institutes, and intends to bring together science, industry and federal offices. One of its key issues was the initiation of biosafety research in Switzerland. As a core project under this programme the Agency for Biosafety Research and Assessment of Technology Impacts (BATS) was established at the begining of 1993. BATS has evaluated national and international research work, congress reports and specialised literature in the field of biosafety and technology impact assessment. It was established as a source of biosafety information for both specialists and the public.

In 1995 the Programme has externed its information activities, establishing B.I.C.S. (Biotechnology Information and Communication Service) and Biotectra technology transfer service in 1996. They were organised with the purpose to diffuse the information, provided by the research projects under the Priority Programme Biotechnology and to facilitate the technology transfer from the science to the industry. They aim to make all the information concerning biotechnology available to the publics as well.

Under the same programme a study on « Gene flow in selected Swiss crops and related weeds, risk assessment for the field release of GMOs in Switzerland » in 1993 at the University of Bern and the University of Neuchâtel was initiated, which is the first of this kind for the country. It concentrates on the dynamic biogeography of weeds and the gene flow among selected cultivated and wild species in natural conditions. The rate of natural hybridization is studied by investigating herbarium specimens and plants collected in preliminary determined test areas in different parts of the country. Experimental hybridizations in the greenhouse and in the field are done as well in order to get more

information about the degree of the gene flow. Using morphological and genetic markers the mechanism of genetic exchanges is investigated as well as pollen and seeds dispersal. Distribution maps of the investigated species are intended to be produced. In addition a bibliographical study of vertical gene flow and establishing of botanical files is carried out.

The present thesis deals with the experimental and genetical parts of that project. Two cultivated species and their close wild relatives were examined - *Hordeum vulgare* (barley) and *H. murinum* as well as *Medicago sativa* (alfalfa) and *M. falcata*.

CHAPTER 3:

INTRODUCTION : *HORDEUM*

3.1. CENTRE OF ORIGIN

Hordeum (L.) belongs to the tribe *Triticeae* of the family *Poaceae* (*Gramineae*). The tribe includes a number of important cereal crops, such as wheat (*Triticum* spp.), rye (*Secale cereale*), barley (*Hordeum vulgare* L.), the artificially synthesized triticale (*x Triticasecale*) as well as many important forage grass species. The genus *Hordeum* contents 11 species (Pedersen and Hojland 1994).

The centre of its origin is in Southwestern Asia (« The Fertile Crescent » - Iran, Turkey, Israel, Jordan) and Central Asia (Bothmer et al. 1991).

3.2. PLOIDY LEVEL

The basic chromosome number is x=7. Most of the *Hordeum* taxa are diploid (2n=2x=14). Tetraploids (2n=4x=28) and hexaploids (2n=6x=42) also exist.

Four basic quite unrelated diploid genomes occur in the genus: genome « I » (*H. vulgare*, *H. bulbosum*), genome « Y » (*H. murinum*), genome « X » (*H. marinum*) (Dewey 1984, Bothmer et al. 1983, 1985, 1986). All the other diploid species have genome « H », which is also presented in several tetraploid *Hordeum* species (Bothmer et al. 1987, 1988).

3.3. BREEDING SYSTEM

The species included in the genus are annual or perennial, the majority being perennial. The former include winter as well as spring annuals.

Autogamy predominates in the annual species, although some may be occasionally outcrossing. Most of the perennial taxa have a mixed reproductive system without any specialization. Some are mainly self - fertilizing with small anthers and stigmas and flowers which mostly do not open (*H. patagonicum*). Others are obligate outbreeders (*H. bulbosum*)

with self - incompatibility system, which largely prevents self - fertilization (Bothmer 1979). Barley does not reproduce asexually.

3.4. TRAITS TO DISTINGUISH *HORDEUM* SPECIES

The diagnostic characters of the genus are the possession of three one - flowered spikelets at each rachis node, of which the lateral spikelets are pedicellate. The spikes are identically built. The variation is in shape, size and colour of various parts. The rachis is flat with shorter or longer hairs or brislets on the edges. At each node there are three one - flowered spikelets: a so-called triplet. The central spikelet is sessile in most species, the palea is glabrous or hairy, more or less bifid in the apex. The lateral spikelets are rarely both female and male fertile, more often male fertile or completely sterile. According to the species the lemma extends into a shorter or longer awn.

The anthers also vary greately in size, the longest ones are found in outcrossing species and the smaller ones in self - pollinating species. The ovary is hairy and has two long or short feathery stigmas.

The leaf sheaths may be hairy, but the upper ones may be glabrous. The nodes are glabrous to hairy. The culms are mostly erect in a few cases nodding or even de - or procumbent. Auricles are absent in most species, but very prominent in others (Fig. 1).

3.5. CHARACTERISTICS OF *H. VULGARE* AND *H. MURINUM*

H. vulgare comprises both cultivated and wild forms that are interfertile and closely related. The cultivated forms are refered to spp. *vulgare,* whereas the wild are retained as spp. *spontaneum* (C. Koch.) Thell. The last comprises two - rowed forms. The four - and six - rowed are included in ssp. *vulgare*.

The *H. murinum* complex is autogamous, containing three taxa: *H. glaucum*, *H. murinum* (sensu stricto) and *H. leporinum* (Link) Arcong. (Giles and Lefkovitch 1986).

3.5.1. General distribution

H. vulgare has been cultivated since the Neolitic. It was among the first crops under domestication around 7000 B.C. Since early times it has been grown throughout the Mediterranean region and Ethiopia, Southwestern Asia, China and Japan. Because of its importance for both forage and brewery it is now cultivated in all temperate areas of the world. Major barley production areas are Europe, the Mediterranean fringe of Northern Africa, Ethiopia, the Near East, Russia, China, India, Canada, USA and Australia. It is rare in the tropics except in cool highlands in Mexico, the Andes and East Africa (Harlan 1976).

It occupies grassland areas, meadows as well as semi - arid regions sometimes on rather salty soils, growing from sea level in the Mediterranean area and at the Middle East up to 4500 m in the Hymalayas.

H. murinum is distributed practically on the whole territory of Europe except Scandinavia, Poland, Iceland and Ireland (Humphries 1980), from the Atlantic to the West of Asia. The original habitats of this species were probably seasides, sandy river beds and drinking places for animals. Presently it occurs in many places, disturbed by men (Bothmer et al. 1991).

3.5.2. Distribution in Switzerland

H. vulgare is cultivated in Northern, Western and Southeastern Switzerland, the region of lake of Geneva, the cantons of Valais and Tessin and the whole of the Swiss Plateau (Welten and Sutter 1982) (map 1a).

Seven wild *Hordeum* taxa are present in the Swiss flora according to Aeschimann and Burdet (1989). Among them are *H. distichon* L., *H. vulgare* ssp. *polystichon* (haller f.) Schinz and

Keller and *H. vulgare* spp. *hexastichon* (L.) Celak. *H. murinum* is a common ruderal species. *H. leporinum* is less frequent and has similar ecological requirements. Finally *H. secalium* Schreber (= *H. nodosum* L.) and *H. jubatum* L. are extremely rare in Switzerland.

H. leporinum is mentioned only in few locations of Switzerland - south of the cantons of Tessin and Graubünden, the region of Geneva, the cantons of Aargau and Valais. Herbarium data indicates its presence in Northern Switzerland.

Thus *H. murinum* is the commonest of the wild representatives of *Hordeum* in the country. It is widespread in canton Tessin, Valais and the region of lake of Geneva. It grows also in the region of the Lake of the four cantons, and is more rare in Northern, Eastern and Western Switzerland (map 1b). In all mentioned regions, where it is present, except canton Tessin and the lake Geneva region, there are sites where it had existed previously, but has not been found recently.

The locations of both *H. vulgare* and *H. murinum* overlap largely.

3.5.3. Morphological characteristics

Plants of *H. vulgare* are up to 1 m long and have usually erected culms, flat leaves, which are either scabrous or glabrous. Auricles are long prominent, linear - falcate, surrounding the culm. Spikes are longer and bigger than those of *H. murinum*, the colour varies from green over purplish to blackish. It has 10 - 30 nodes each bearing three one - flowered spikelets, of which one, two or all three are fertile viz. two - four - and six - rowed barley. The awns are normally long and straight, rachins brittle or tough. The central spikelet is sessile, glumes are flattened, lemma is glabrous or scabrous mostly near the apex, usually tightly embedding the kernel. The triplet is the functional diaspore with one central female - fertile flower and two lateral female - sterile flowers in two - rowed barley. In six - rowed all three spikelets are fertile (Reid 1985). Anthers are yellowish, rachilla is short or up to half of the length of the

palea with long or short hairs. Lateral spikelets usually are sessile when fertile and sessile to pedicellate when sterile, obtuse to acute or with an awn almost equally as well developed as the central one when fertile. Glumes are more or less flattened (Bothmer et al. 1991).

H. vulgare is a spring or winter annual mainly inbreeder. It is a diploid with 2n=14, but artificial tetraploids (2n=28) have been produced. It has a genome called « I ».

H. murinum plants are usually shorter than those of *H. vulgare* - 30 - 60 cm high. Culms are more or less erect, sometimes prostrate. Leaves are narrower, flat occasionally with involute margins. Spikes are smaller than those of *H. vulgare*, green with relatively small lateral spikelets. Central spikelet is sessile to subsessile, about the size of the lateral ones, excluding awns. Anthers of the central spikelet are of about the same length as the lateral ones. Palea of lateral spikelets is almost glabrous, rachilla is pale and thin (Bothmer et al. 1991).

H. murinum is a winter annual, mostly inbreeder. It is tetraploid with 2n=28 and contains a separate genome called « Y ».

3.5.4. Isozyme and DNA molecular markers

The technique of enzyme electrophoresis provides many genetic markers as criteria for taxonomic separation of the species. Most of them have codominant expression and variation occurs frequently. Isozymes allow thus the detection of homozygous and heterozygous individuals, facilitating the determination of genetic variability and amount of heterozygosity in natural populations.

Isozyme polymorphism allows the identification of different *Hordeum* taxa as well as barley varieties. Many isozyme investigations involved *H. vulgare* (Brown 1983, Jorgensen 1986, Nielsen and Hejgaard 1986, Powell et al. 1990, Zhang et al. 1994). A study of barley esterase (EST) for example resulted in revealing a few diagnostic locuses for *H. vulgare*. Jaaska (1992) has found diagnostic alleles for glutamate oxaloacetate transferase (GOT) of *H.*

vulgare and *H. murinum*. A substantial heterogenity in the level of polymorphism among different geographical area and agrigeographical subregions has been demonstrated as well (Zhang et al. 1994). Populations of *H. murinum* were characterized with small isozymic variability over its British, European and Mediterranean range (Giles 1984). The results indicated little genetic differentiation in enzyme characters between populations and most of them tended to be monomorphic. The most variable systems were esterase and malate dehydrogenase (MDH). However the variability between species and taxa is large thus allowing their identification and separation.

The principal difference between auto - and alloploids is the presence of fixed isozyme heterozygosity, considered to be an evidence in favour of alloploid origin. This isoenzyme variation pattern has been observed for *Hordeum* species for aspartate aminotransferase (AAT), superoxide dismutase (SOD), esterase, leucine aminopeptidase (LAP), shikimate dehydrogenase (SKD), peroxidase (PRX) (Jaaska 1987, 1992, 1994) and has been applied to distinguish the two ploidy types in various systematic groups (Ness et al. 1989, Soltis and Soltis 1989, Werth 1989).

Relatively high levels of variation have been observed both within and among *Hordeum* species using RAPD (Gonzales and Ferrer 1993) as well as within and between population diversity (Dawson et al. 1993).

Pedigree relationships in spring barley lines were examined by Tinker et al. (1993) with RAPD. Two - and six - rowed barley were separated by cluster analysis. They have found 19 primers revealing a total of 31 marker bands between 27 different lines and concluded that RAPD markers could be used to gain information about genetic similarities or differences that are not evident from pedigree information.

Finally RFLPs have been used to construct a barley linkage map (Backes et al. 1995, Laurie et al. 1993, Sorokin et al. 1994), as diagnostic markers for wild and cultivated barley (Petersen

et al. 1994) and for different barley cultivars (Pecchioni et al. 1993). Polymorphism in α-amylase genes of wild and cultivated barley was studied using PCR and RFLP (Kiribuchi et al. 1993, Weining et al. 1994).

3.5.5. Risk assessment of releasing transgenic barley

For a long time the stable transformation of barley has been a problem, because most of the varieties had a low response to tissue culturing. Although progress has been made, stably transformed barley plant were obtained only recently (Ritala et al. 1994, 1995, Wan and Lemaux 1994, Stiff et al. 1995, Harwood et al. 1995, Salmenkallio - Marttila et al. 1995).

The possibility for gene flow from barley to the related species is theoretically low, because it reproduces predominantly by self - fertilization. Nevertheless, reasonably high amounts of pollen can be released and the rate of gene flow will depend on the degree of outcrossing within the species, the successful hybridization and the fitness of the produced plants. Most of the *Hordeum* species are biologically and usually also morphologically discrete entities. With few exceptions the isolation barriers (usually the hybrid sterility) are strict between the species and there is a little genetic exchange in nature. The pollen is dispersed by wind. However, the barley seeds could be spread over considerable distances by animals, humans and transport, and they keep their germination ability for several years. Barley is generally very competitive against weeds. Intraspecific competition is also very strong.

CHAPTER 4 :

INTRODUCTION : *MEDICAGO*

4.1. CENTRE OF ORIGIN

The genus *Medicago* is very extensive, including more than 60 different species, two third of which are annual and one third perennial (Lesins and Lesins 1979).

The primary centre of the genus is found in the Caucasus, Northwestern Iran and Northeastern Turkey (Ivanov 1977).

4.2. PLOIDY LEVEL

The basic chromosome number for *Medicago* is x=8, except for the annual species *M. constricta* Dur., *M. praecox* DC., *M. polymorpha* L., *M. rigidula* (L.) All. and *M. murex* Willd., which have a genomic number of x=7 (Quiros and Bauchan 1988). Three ploidy levels are found for *Medicago* spp.: diploids 2n=2x=14 and 2n=2x=16, tetraploids 2n=4x=32, and hexaploids 2n=6x=48. The tetraploid species have probably arisen from diploids by unreduced gametes (Mariani and Veronesi 1979, McLennan et al. 1966, Vorsa and Poingham 1979).

4.3. BREEDING SYSTEM

Annual species of *Medicago* are autogamous as a result of the presence of self - tripping in their flowers. On the contrary, most of the perennials are allogamous with different degrees of self -incompatibility. As a rule they rely on insect polinators to activate their flower tripping and pollination (Lesins and Lesins 1979). Most of these species are quite polymorphic, because of their high degree of outcrossing. Their pollinating agents include numerous bee species.

4.4. TRAITS TO DISTINGUISH *MEDICAGO* SPECIES

Legume and seed characteristics are the most important traits for identification of different *Medicago* species. Growth habit and longevity, organ hairness, inflorescenses, leaves, cotyledons, pollen shape and size and the chromosome numbers are other important characters. In addition isozyme and molecular markers complete these characteristics.

Small (1981) recognized 12 groups of species in the genus, based on 75 characteristics mostly vegetative and pod traits.

4.5. CHARACTERISTICS OF *MEDICAGO SATIVA* COMPLEX

The species included in *M. sativa* complex belong to the section *Falcago,* subsection *Falcatae.* It includes *M. sativa*, *M. falcata* and *M. sativa* spp. *glutinosa*. They intercross with each other and share the same karyotype (Gillies 1972). The species in the complex are either diploid or tetraploid.

Alfalfa (*M. sativa* L.) is an essential cultivated forage crop in the world. Beyond its value as the most important protein source in animal fodder, alfalfa is able to fix atmospheric nitrogen in symbiosis with *Rhizobium meliloti*, which makes it valuable in the crop rotation.

4.5.1. General distribution

The original ranges of distribution of both diploid and tetraploid *M. sativa* cover the Mediterranean region, Near and Middle East, the Caucasus, Middle, Central and South Asia (Quiros and Bauchan 1988). Tetraploid *M. sativa* was introduced in Europe, where it has been largely cultivated. According to Vollrath (1973) it is always present as an introgressed form with *M. falcata*. The recognition of the economical value of *M. sativa* was responsible for its introduction in South America, Mexico, Canada, USA as well as Australia, New Zealand and South Africa (Freyer 1930).

Diploid *M. falcata* is present mainly in Asia (up to Siberia in the East and Caucasus in the South) and Eastern Europe, extending up to the South of Germany. It is a rare species for Denmark (Hojland and Poulsen 1994).

4.5.2. Distribution in Switzerland

Ten *Medicago* species are present in the Swiss flora (Aeschimann and Burdet 1989).

M. sativa is widespread all over the country. It grows at low altitude (colline level and montane zone) (map 2a). *M. falcata* is restricted to the Northeastern and Eastern parts of the country, the canton of Valais and the region of lake of Geneva (map 2b) (Welten and Sutter 1982). It is present from the colline level to the subalpine zone. According to the authors it has disappeared from most locations of Swiss Plateau, where it had been mentioned earlier.

M. sativa hybridizes freely with *M. falcata*. Their hybrid *M.* x*varia* Martyn is fertile and is found within the range of distribution of *M. sativa* and *M. falcata*.

4.5.3. Morphological characteristics

M. sativa and *M. falcata* differ in their morphology, mainly by the leaf's shape, the color of the flowers and the pod's shape. *M. sativa* has a deep tap root, an erected stem, rounded leaves , purple flowers and coiled pods, while *M. falcata* has a spread well - branched root system, prostrate stems, smaller narrow leaves, yellow flowers and sickle - shaped pods.

Tetraploid forms in the complex are distinguished from the diploid by the larger size of their flowers, pods and seeds. A very striking difference appears in respect to pollen size, which increases throughout with the chromosome number.

4.5.4. Isozyme and DNA molecular markers

These markers have been proved useful for the identification of the *Medicago* species and their hybrids and thus may be a helpful tool in risk assessment.

Genetic determinism in *Medicago sativa* complex was studied for peroxidase and leucine-amino peptidase (Quiros and Morgan 1981, Melchinger et al. 1991) and for glutamate oxaloacetate transaminase (Brunel 1982).

Diploid *M. falcata* has a few specific allozymes not found in *M. sativa*. Unique alleles for PRX and LAP have been found in *M. falcata* and the hybrids *M.* x*varia* and *M. hemicycla*, but not in *M. sativa*. The diploid and tetraploid forms of *M. sativa* are distinguished for the loci GOT, LAP1 and LAP2.

Molecular markers can be utilised to simplify genetic analyses applicable to alfalfa breeding. Based on RAPD and RFLP techniques, linkage maps of diploid (McCoy et al. 1991) and tetraploid alfalfa (Kiss et al. 1992, Yu and Pauls 1993a) have been produced. These techniques were successfully used for studying the structure and expression of different genes in *Medicago* (Gregerson et al. 1994) or for distinguishing populations and species within the genus (Yu and Pauls 1993b, Pupilli et al. 1995).

4.5.5. Risk assessment for releasing transgenic alfalfa

Transgenic alfalfa exists (D`Halliun et al. 1990, Larkin et al. 1990, Hill et al. 1991, Schroeder et al. 1991, Gold et al. 1991, Pereira and Erikson 1995, Austin et al. 1995) and 38 field releases have been carried out since the first one in 1988 (Extracts from GIBiP database on field trials 1986 - 1994). Most of them took place in USA and Canada (Ahl Goy et al. 1994).

According to the classification of Raybould and Gray (1993), *Medicago* belongs to the third group with a « high probability of gene flow to wild relatives ». The Dpdf code for lucerne indicates a substantial ecological effect on the flora of the Netherlands (De Vries et al. 1992).

Bibliographical research also concluded a high risk of gene flow between *M. sativa* and *M. falcata* (Jacot 1994, Jacot and Jacot 1994). *M. sativa* is an outcrossing species and asexual reproduction does not take place. Moreover it can hybridize with *M. falcata* easily but only at

the same ploidy level. Crosses between diploid and tetraploid forms are rare, because of the ploidy barrier, and they result in a low fertility (Ledingham 1940). Although seed set and dispersal are limited, many seeds are wasted during the harvesting and could remain in the areas where alfalfa has been cultivated. Due to the probable limited dispersal of the seeds, it is the extensive pollen dispersal (1600 m) and the ability of *M. sativa* to hybridize freely with *M. falcata* which exhibit the major risk of gene flow (Hojland and Poulsen 1994).

CHAPTER 5:

MATERIALS AND METHODS

5.1. SAMPLING AND CULTIVATION

The sampling sites of *Hordeum* and *Medicago* were chosen according to the present distribution of the species in Switzerland with the aim to cover the whole teritory of the country and in respect with the differece in the local conditions in each region, which could influence the risk of cultivation of GMOs.

5.1.1. *Hordeum*

Eighteen populations of *H. murinum* were sampled in Schaffhausen, Graubünden, Vaud and Valais (Table 1) in the summer of 1993 as following:

- populations close to a field of cultivated barley
- populations close to fields, previously cultivated with barley
- populations distant from cultivated fields, mainly in village surroundings.

Populations close to cultivated fields are generally linear, located in a narrow strip between the road and the field. Therefore populations were sampled along linear transects (1 or 2 by populations), by collecting all the flowering plants on a strip of variable width (Fig. 2).

For the populations close to a field of cultivated barley, *H. vulgare* was also collected.

All the plants were dried for morphological analysis and herbarium collection by the Bern team and one spike of each plant was kept separately. Two seeds of those spikes were sown in pots in the autumn of 1993 and cultivated in a cold greenhouse in the Botanical Garden of the University of Neuchâtel. After germination, only one of the plants randomly chosen, was kept. These plants - 905 in total - were used for the genetic analyses and the experiments.

5.1.2. *Medicago*

Thirteen populations were sampled in Schaffhausen, in Unterengadin and Rhine valley in Graubünden and Valais (Table 2). The plants were collected in such a way that individuals on

a one meter lattice were chosen (Fig. 3). In the cases where there was no plant on the crossing point, the closest one at less than 50 cm was collected. All the populations except the three collected in Unterengadin, included *M. falcata*, *M. sativa* and *M.* x*varia* in different degrees. In some populations, apparent spatial patterns existed with a pure zone of *M. falcata* and a pure zone of *M. sativa*, joined by a zone of intermixture. Other populations were more of hybrid types with numerous intermediary individuals. Two of the populations collected in Unterengadin consisted of *M. falcata* only and one consisted *M. falcata* and *M. sativa*,which were spatially separated.

The shoots of each collected plant were dried for herbarium collection and the morphological studies for the Bern team. The base and the rooting system were planted in pots and cultivated in the Botanical Garden of the University of Neuchâtel. These plants - 916 in total - were used for the genetic analysis and the experimental part.

5.2. FLOWERING BIOLOGY

The flowering biology of *H. murinum* has been studied by the observation of ten spikes per population under binocular. The presence or absence of cleistogamy (pollen release outside of the spike) was censused. The flowering stages were examined for all the spikes produced by each plant according to the following:

A - non visible spikes

B - spikes less than one half visible

C - spikes more than one half visible

D - spikes completely out

The flowering index (Fi) was determined as:

$$Fi = (A+2*B+3*C+4*D)/(A+B+C+D)$$

where A, B, C, D are the number of spikes at the corresponding stage.

5.3. DIRECT METHODS

5.3.1. Pollen dispersal

Pollen dispersal was studied only for *Hordeum* as for *Medicago* it had been already evaluated (Demarly 1955, Bradner et al. 1965, Rinker et al. 1988). The experiments were performed for *H. vulgare* and *H. murinum* separately. Two plants of each population were put together as pollen source, surrounded by pollen traps (Fig. 4). Traps were located on five lines regularly placed every 0.5 m up to 5 m and then every meter up to 12 m. The traps consisted of a glass plate covered with vaseline. The pollen was fixed after with Gelvatol ® (37 gr Gelvatol®, 2 gr phenol, 50 ml glycerol, 100 ml dH_2O). The experiments were performed on calm days in June 1994 and lasted 36 hours. The number of pollen grains was counted under a light microscope in a semi - quantitative way by doing five surveys along the microscope slide.

5.3.2. Seed dispersal

The seed dispersal experiments were performed in July 1994 for *Hordeum*. The experiment consisted in putting four plants surrounded by three plastic sheets of 1x2 m covered with a glue resistant to water (Soveurode®). Seeds were regularly collected and their coordinates on the plastic sheet recorded. The experiment was repeated twice for *H. vulgare* and for *H. murinum*.

Similar experiments ware carried out for *M.sativa* and *M. falcata*. However, the detection of the seeds on the plastic sheets was impossible, because of the small size of the seeds and because in most of the cases the plants shed the hole fruits but not the seeds themselves.

5.3.3. Hybridization under natural conditions

Two plants of each population of *H. murinum* were placed on the border of a cultivated field of winter *H. vulgare* in Chezard (NE). A similar experiment was performed on spring *H.*

vulgare at the Agronomic School of Cernier (NE) (photo 1). Their progenies were collected and sown in the autumn of 1994 in the Botanical garden of the University of Neuchâtel for further investigation.

Plants of *M. falcata* with non - open flowers were planted in the flowering fields of *M. sativa* at the Swiss Agronomic Research Station of Changins in July 1994 (photo 2). The seeds were collected at the end of August and sown at the Botanical Garden of the University of Bern.

Screening for hybrids was performed on the progeny.

5.3.4. Experimental hybridization

Two plants of *H. murinum* from each population were selected for crosses with *H. vulgare*. One spike from each species were put together under a pollination bag (photo 3). The experiment was performed in the greenhouse of the Botanical garden of the University of Neuchâtel in May 1994. The progeny was collected and sown in the autumn.

Experimental self - fertilization, cross - fertilization and hybridization were carried out on selected plants from *M. sativa* and *M. falcata*. Castration was performed by opening the flowers and their immersion for one minute in 55% ethanol or by removing the anthers with a vacuum. The pollination was made manually. The flowers were then covered with pollination bags. The experiments were carried out in June - July 1994. The progeny was collected and sown in the spring of 1995 in the Botanical Garden of the University of Bern for further investigations (photo 4).

Photo 1. Experimental hybridization of *Hordeum murinum* and *Hordeum vulgare* in the field.

Photo 2. Experimental hybridization of *M. falcata* and *M. sativa* in the field

Photo 3. Experimental hybridization of *H. murinum* and *H. vulgare* in the greenhouse

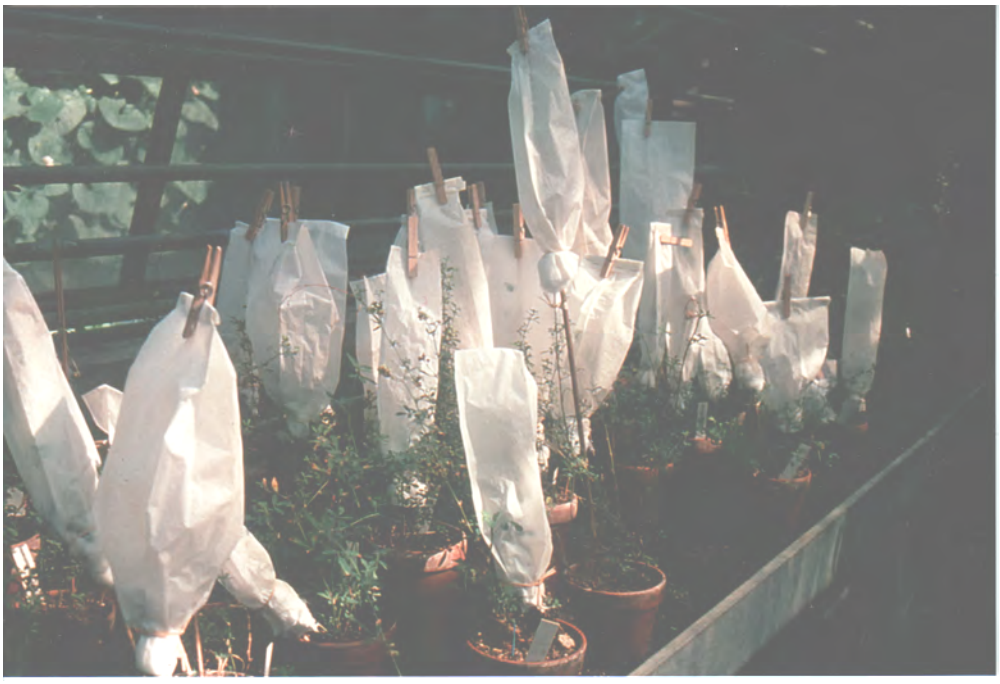

Photo 4. Experimental hybridization of *M. falcata* and *M. sativa* in the greenhouse

5.4. INDIRECT METHODS

5.4.1. Karyology

Chromosome numbers were determined for both *Hordeum* and *Medicago* on counts of at least five individuals of each population.

5.4.1.1. *Hordeum*

The root tips were pretreated in 0.002 M 8-hydroxyquinoline for two hours at room temperature and then fixed in Carnoys solution (3:1 ethanol:acetic acid) for one night. After a hydrolysis with 1N HCL in a water bath of 60°C for 8 min, the root tips were stained in Schiff (5 gr pararosaniline, 150 ml 1N HCL, 5 gr Na-metabisulfate, 850 ml distilled H_2O) for 2 to 4 hours. Finally they were softened with a 5% pectinase - 3% cellulase solution for 3 - 4h. A somatic chromosome number was determined on metaphases.

5.4.1.2. *Medicago*

The root tips were pretreated in 0.002 M 8-hydroxyquinoline for two hours at room temperature and then fixed in Carnoy`s solution (3:1 ethanol:acetic acid). After at least one day, they were stained in 1% aceto - orcein (2 gr orcein, 100 ml acetic acid, 100 ml distilled H_2O) for two hours at room temperature, followed by heating for two minutes. Somatic chromosome number was determined on metaphases.

5.4.2. ISOZYMES

5.4.2.1. Principle of the electrophoresis

Electrophoresis is a method for separating the proteins according to their molecular weight and their charge under an electric field. Proteins of different composition and conformational properties migrate at different rates. The relative migration depends on the molecular weight

and the charge of the molecules. The charge of the proteins depends on the degree of ionization (the amount of free electrons) and the amino acids composition. Gels can be prepared from a variety of media (starch, agarose, polyacrylamide and cellulose acetate membranes) and run by various methods (horizontal, vertical, disc). Characteristics of the migration buffer and gel matrix both affect resolution. Enzymes are detected by immersing the gel into a medium containing the reaction conditions for the enzyme such that it incorporates a colored dye into the end product.

Polyacrylamide gel electrophoresis (PAGE) is required when maximum resolving power is necessary. A valuable property of PAGE is that the stringensy of molecular sieving can be varied by altering the acrylamide concentration, thus increasing flexibility in the range of protein molecular weights or dimensions that are separable (Leaback 1976). Other advantages of polyacrylamide gels include their uniformity and transparency, facilitating densitometric quantification of products, inertness of components allowing broad assay compatibility, usually rapid run time. Discontinous systems (composed of more than one gel) are used with polyacrylamide gels to create a stacking effect at the boundary between regions of large and small pore size, which improves the resolution of the bands in the second phase (Chrambach 1980, Hames and Rickwood 1981).

5.4.2.2. Enzyme extraction

All plants from *Hordeum* and *Medicago,* which had been cultivated, have been screened for isozyme markers using PAGE. Extractions were carried out on fresh leaf material. Two - three leaves were squashed in a mortar with 500 µl of extraction buffer and one point of the spatula of quartz. Two different extraction buffers were used for *Medicago* and for *Hordeum* (Appendix I). The samples were centrifugated at 10 000 rpm for 20 min and the supernatant was kept and stored at -80°C.

5.4.2.3. Protein separation

The gels were prepared according to Gasquez and Compoint (1976), modified by Lumaret (1981) and consisted of:

- separation gel - 17 cm x 7.5 cm, 2.5 mm composed of 9% acrylamide and 0.165% bis-acrylamide for *Hordeum* and peroxidase system for *Medicago*. For systems malate dehydrogenase and maleic enzyme (ME) for *Medicago* 10 % acrylamide and 0.165% bis - acrylamide were used.

- stacking gel - 17 cm x 1 cm, 2.5 mm composed of 2.5% acrylamide.

- sample gel - containing 9% acrylamide. It has the same composition as the separation gel. (Appendix I)

Forty µl of the enzyme extractions and about 20 µl of dye (1% bromophenol blue) were used. Protein migrated in Tris-glycine buffer, pH=8.6 (Appendix I) in plastic cuve. The migration was carried out at 4°C and in three stages:

- ten minutes at 600 V and 6 pps (number of the pulsations/sec) to concentrate the extracts at the end af the sample gel.

- twenty minutes at 230 V and 7 pps. At this phase extracts migrate in the stacking gel.

- between 2h30 to 3h at 600 V and 7 pps. During this phase proteins separate.

Esterase (EC 3.1.1) and glutamate oxaloacetate transaminase (or aspartate-α-ketoglutarate aminotransferase (EC 2.6.1.1) were studied for *Hordeum* and malate dehydrogenase (EC 1.1.1.37), malic enzyme (EC 1.1.40) and peroxidase (1.11.1.7.) for *Medicago*.

5.4.2.4. Revelation of the enzymes

- Glutamate oxaloacetate transaminase

This enzyme belongs to the group of aminotransferases, which catalyse the transfer of one α-amino group to α-ceto group. GOT has a key role in the reactions of nitrogen elimination

from amino acids and in the process of accumulation of α-keto acids, which participate in the cycle of Krebs. It catalyses the reaction:

aspartate + 2-oxoglutarate ⇔ oxaloacetate + glutamate

The enzyme is dissolved in the cytoplasm or is connected with the peroxisome or mitochondries. In the plants its functional form is as dimer.

Revelation: **Tris** buffer, pH = 8 - Tris 48.4 gr, distilled H_2O 2000 ml, **Substrate** - α-ketoglutaric acid 300 mg, L - Aspartic acid 600 mg. The two acids were dissolved in 100 ml of Tris buffer. One point of spatula of pyridoxal 5′P was added.

Coloring agent - Fast blue BB 200 mg, Tris buffer 100 ml.

Fifty ml of the substrat and 50 ml of the dye were poured over the gel and stained at 36°C at dark until the dye precipitates. The operation was repeated with the rest of the solutions. Finally the gel was rinsed with water.

- Esterase

This enzyme hydrolyzes a large number of compounds having different ester linkages *in vitro*, but their natural substrates are unknown in plants (Gottlieb 1981). Plant species contain high numbers of esterase isozymes in many different tissues. They may be the most variable isozymes studied to date in plant populations.

Revelation: **Tris buffer** , pH = 7 - 19.4 gr Tris, 200 ml distilled H_2O, 13 ml HCL, **Substrate** - 200 mg α-Naphtyl acetate, 150 mg β-Naphtyl acetate. They are dissolved in 20-30 ml of acetone and completed to 100 ml with Tris buffer.

Coloring agent - 200 mg Fast blue RR, 100 ml Tris buffer.

The gel was incubated for 15 min at room temperature with two thirds of the substratum. The former was poured off and the coloring agent was added for another 15 min. Finally the gel was incubated with the rest of the substrate.

- Malate dehydrogenase

At least three NAD - dependent MDH, each localized in a distinct cellular compartment (mitochondria, peroxisome, cytoplasm), are found in plant cells (Gottlieb 1981). The isozymes function in different metabolic sequences. The mitochondrial one is involved in the citric acid cycle, catalysing the reaction:

$$\text{malate} + NAD^+ \Leftrightarrow \text{oxaloacetate} + NADH + H^+$$

The soluble one is involved in non - autotrophic CO_2 fixation to form malate in the reaction:

$$\text{Pyruvic acid} + NADPH + H^+ + CO_2 \Leftrightarrow \text{malate} + NADP^+$$

The enzymes in the peroxisomes take part in the photorespiration and the glyoxylate cycle:

$$\text{malate} + NAD^+ \Leftrightarrow \text{oxaloacetate} + NADH + H^+$$

The isoenzymes are dimeric and are under independent nuclear gene control.

Revelation: **Substrate**: 5 ml 1M Tris buffer, pH = 9.1, 10 ml 2M Malic acid pH = 7, 50 ml distilled H_2O, 20 mg NAD, 1 ml PMS 0.2% (phenazine methosulfate), 1 ml NBT 1% (nitro blue tetrazolium).

Preparation of 2M Malic acid: 134.1 gr DL Malic acid, 80 gr NaOH and distilled H_2O up to 500 ml, pH = 7. The preparation was done on ice as the reaction of disolving NaOH is exothermic.

The dyes were added just before the revelation. The gel was incubated with the substrate at 37°C for about 30 min.

- Maleic enzyme

It is a NADP - dependent enzyme, belonging to the group of MDH and localised in the chloroplasts. It catalyses the reaction of transformation of malic acid to pyruvate:

$$\text{malic acid} + NADP^+ \Leftrightarrow \text{pyruvic acid} + NADPH + H^+$$

Revelation: **Substrate**: 10 ml 2M Malic acid, pH = 7, 5 ml 1M Tris buffer pH = 8, 50 ml distilled H_2O, 100 mg $MgCL_2$, 10 mg NADP, 1 ml NBT 1%, 1 ml MTT 1%, 1 ml PMS 0.2%.

The dyes were added just before the revelation. The gel was incubated with the substrate at 37°C for about 20 min.

- Peroxidase

Peroxidases utilize H_2O_2 to oxidize a very large number of hydrogen donors including phenolic substances, amines, leuco-dyes, ascorbic acid, indoles and certain inorganic ions such as iodides. All higher plants examined have a large number of them. The complexity of the electrophoretic patterns of the peroxidases is matched by the complexity of their presence in different tissues and developmental variability (Gottlieb 1981).

The peroxidase catalyses the reaction:

$$RCH_2 + H_2O_2 \Leftrightarrow R + 2H_2O$$

The enzyme is present in most of the tissues, but it is often linked with one organel or specific tissue as well such as endoplasmatic reticulum, Golgi apparat, ribosomes and vacuoles. Peroxidases are glycoproteins.

Revelation: **Acetate buffer** - Solution a: 115 ml 0.2M Acetic acid, 100 ml distilled H_2O. Solution b: 0.2M Sodium Acetate 16.4 gr/l.

72 ml of Solution a, 176 ml of Solution b and 750 ml distilled H_2O were used for the preparation of 1000 ml 0.05M Acetate buffer, pH = 5.

Solution 1: 25 mg 3-amino-9-ethylcarbazole, 2.5 ml NN-dimethyl formamide

Solution 2: 45 ml 0.05M Acetate buffer, pH = 5, 1 ml guaiacol, 1 ml 0.1M $CaCL_2$, 1 ml 3% H_2O_2 .

Before the revelation solutions 1 and 2 were mixed and poured over the gel. It was incubated at room temperature for about 20 min.

5.4.3. DNA MARKERS

Random Amplified Polymorphic DNA (RAPD) was used to reveal DNA markers for each of the *Medicago* species studied and to detect the putative hybrids.

It is a method which applies the Polymerase Chain Reaction (PCR). PCR is an *in vitro* technique for amplification of short specific DNA sequences. It is based on DNA denaturation after heating followed by anniling of short DNA sequences (primers) to each separate DNA chain. The primers act as a starting points for the DNA synthesis with the help of the DNA - Polymerase. DNA - Polymerase is a thermostable enzyme, isolated from *Thermophilus aquaticus*, and it satisfies the condition to be resistant to the high temperature, use for the DNA denaturation, without loosing its activity.

The first step of the PCR is a heating to 92 - 95°C during which the separation of the two DNA chains happens. The next step is the attachment of the primers at 40 - 60°C. This temperature depends on the sequence of the primers and the target DNA. Finally the probes are heated at 72°C to allow the DNA - Polymerase to synthesize the new chains. This process must be repeated 20 - 40 times and after each cycle the DNA doubles, getting an amount which allows it to be visualised on agarose gel (Fig. 5).

RAPD uses randomly constructed oligonucleotides as primers, permitting the detection of polymorphisms as different PCR - products. As this technique uses arbitrary primers to amplify random segments of genomic DNA, showing polymorphism these markers are called RAPDs. The primer attaches at the places with sufficient complementarity of its nucleotide

sequence. If the frequency of these sequences in the target DNA are relatively high, there will be a chance that they will be close to each other. Based on this, PCR will amplify fragments which could be different in size. The polymorphism observed may result from point mutations, insertions, deletions and invertions. RAPDs are usually dominant markers and are inherited in a single Mendelian fashion.

5.4.3.1. DNA extraction

Leaf material was stored at -80°C. Between 100 and 200 mg of it was squashed in mortar with liquid nitrogen. The powder was transfered to an eppendorf tube with 1 ml of solution - 1 (10 ml 1M Tris, 20 ml 0.5M EDTA, 2.92 gr NaCl, 30 gr sucrose, up to 200 ml distilled H_2O). The samples were centrifuged for 5 min at 5000 rpm and 300 µl of solution - 2 (4 ml 1N Tris, pH=8, 4 ml 0.5M EDTA, 192 ml distilled H_2O) and 20 µl of 20% sodium dodecyl sulfat (SDS) were added to the pellet, which consists of the nuclei. The tubes were incubated at 70°C for 15 min. To the former mix 150 µl of 7.5 M ammonium acetate was added and the tubes were cooled on an ice bath for 30 min. The ammonium acetate was sedimented by centrifuging for 5 min at 14 000 rpm. The supernatant was transfered to a new microfuge tube and 700 µl isopropanol was added. The samples were left for 15 min followed by centrifugation for 5 min at high speed. The supernatant was decanted and the pellet was washed with 1 ml cold 75% ethanol. Finally the samples were microfuged again for 5 min and ethanol was poured out. After the tubes were dried, DNA was resuspended in 1 ml TE buffer, pH=8 (1 ml 1M Tris, pH=7.4, 0.2 ml 0.5M EDTA, pH= 8, 98.8 ml distilled H_2O). The quality of the isolated DNA was checked in 1.6% agarose gel, run in 0.5 x TBE buffer (Appendix II) at 50 V for about 40 min. The quantity of DNA was measured with TKO 100 mini - fluorometer. When it was necessary the extractions were diluted so that final concentration of 30 ng/µl DNA was obtained.

5.4.3.2. PCR conditions

Each PCR - tube contained 19.3 µl sterile distilled H_2O, 2.5 µl incubation buffer (Appligene), 1 µl 5mM dNTP mix (Boehringer Mannheim), 1 µl primer (30 ng/µl), Taq - Polymerase (Appligene) and 1 µl genomic DNA (30 ng/µl). The PCR was performed in Perkin Elmer Gene Amp PCR System 2400 as follows:

4 min at 94°C

1 min at 93°C
1 min at 45°C — 38 cycles
1 min at 72°C

5 min at 72°C

∞ 4°C

Nine µl of each PCR - product were mixed with 4 µl loading dye (10 gr sucrose, 5 ml 0.5M EDTA, 1.25 ml bromphenol blue - 1%, 1.25 mg SDS and distilled H_2O up to 25 ml) and run in 1.6% agarose gel in 0.5 x TBE at 50 V for about 40 min. The RAPDs were observed under UV light.

A screening of 44 ten - mer primers - kits OPB, OPL and OPP (OPERON Technologies, Inc.) was carried out. Three primers (OPP - 05, OPP - 06 and OPP - 08) provide specific bands for *M. sativa* and *M. falcata* and a RAPD with 90 plants from one selected population (4A, 4B), consisting of the both species and their hybrid was carried out.

5.4.4. Data analysis

5.4.4.1. Isozymes

Principal coordonate analysis (PCOORD) was computed with the enzyme data for *H. vulgare* and *H. murinum* in order to get an overview of the genetic variation in the studied populations and to determine if both species are separated from each other. The same analysis was performed only for *H. murinum* to investigate if there was differences between the populations on enzyme level, according to the collection site. PCOORD is based on the matrix of similarity or of distance. It consists in locating the objects in a space of lowered dimension size, which preserves as well as possible their relative distances. The matrices may be calculated with several coefficients. Similarity matrix was calculated with the asymetrical Jaccard's coefficient. It has the advantage of considering the absence of electrophoretic band as an absence of information, rather then an element of similarity.

A Mantel test (Mantel 1967) for significance of differences between *H. murinum* and *H. vulgare*, based on the isozyme data was computed. A matrix of similarities of the dataset was compared with another one of species belonging. The test is based on calculating a value, expressing the similarity between both matrices - standarized r - score, which is a standarized form of z statistics (Smouse et al. 1986) and is equivalent to Pearson`s linear correlation.

The same test was performed to compare the populations of *H. murinum* according to their geographical location, where similarity matrix with the isozyme data was compared with a matrix of geographical distances.

PCOORD was performed with the enzyme data for *Medicago* species. A Mantel test, based on the same isozyme data for significance between *M. sativa*, tetraploid and diploid *M. falcata* and *M.* x*varia,* or only between the species on tetraploid level was also carried out.

5.4.4.2. RAPD

A PCOORD was made to get an overview of the genetic variation in the studied population and to determine if *M. sativa*, *M. falcata* and *M.* x*varia* are separated from each other as well as Mantel test.

CHAPTER 6:

RESULTS

6.1. FLOWERING BIOLOGY

Ten spikes/population of *Hordeum murinum* were examined in May 1994. We observed regularly excerted stamens and visible styles (photo 5).

The comparative analysis, including score of the flowering stages of all tillers of the examined plants showed differentiation in the flowering phenology between the population of *H. murinum*. Those of Valais flowered before those from Swiss Plateau and the last flowering populations were from Graubünden (Table 3).

The flowering periods of both cultivated and wild barley overlaped.

6.2. DIRECT METHODS

6.2.1. Pollen dispersal

Both *H. murinum* and *H. vulgare* presented similar pattern of dispersal. The frequency of the pollen decreased with increasing distance (Fig. 6). Most of the pollen dispersed was closed to the plant source. After two meters, the amount of the pollen was more or less constant.

Standard deviation of the distribution (87% of the disseminated pollen) corresponded to 5 - 6 meters for *H. murinum* and 5 -7 meters for *H. vulgare* (Fig. 6). Although the proportion of dispersed pollen decline at twelve meters, there was still some amount of it at this distance.

6.2.2. Seed dispersal

This experiment evaluated the dispersal of the seeds by the wind.

H. murinum dispersed most of the seeds, close to the source. Standard deviation of the distribution corresponded to 0.4 - 0.6 meters. The biggest number of seeds were dispersed at 0.4 - 0.5m. At 2.0 m we practically did not find any seeds (Fig. 7).

H. vulgare did not shed seeds.

Photo 5. Flowering biology of *H. murinum*

6.3. INDIRECT METHODS

6.3.1. Karyology

6.3.1.1. *Hordeum*

The plants of all populations of *H. murinum* were tetraploids with 2n=4x=28 chromosomes (Fig. 8). The finding of 2n=28 chromosomes was in agreement with former reports, performed on plants from other regions in Europe (Morrison 1958, Rajhathy and Morrison 1962, Strid 1971, Van Loon and de Jong 1978, Linde-Laursen et al. 1992). Two pairs of SAT - chromosomes were observed. These are the first counts for the species for Switzerland (Felber and Savova 1994).

H. vulgare was diploid with 2n=2x=14.

6.3.1.2. *Medicago*

M. sativa was always tetraploid with 2n=4x=32 (Fig. 9a, Table 4).

M. falcata was tetraploid (2n=4x=32) in Valais, Schaffhausen and Rhine valley of Graubünden. All populations from Unterengadin were diploid (2n=2x=16) (Fig. 9b, Table 4).

M. x*varia* was always tetraploid (2n=4x=32) and was present in all populations except those from Unterengadin (Savova et al. 1996).

6.3.2. Isozymes

The aim of the experiment was to define specific marker electrophoretic bands for each of the species studied, in order to be used as indication for outcrossing and presence of interspecies hybrids after experimental hybridization.

6.3.2.1. *Hordeum*

From 18 studied enzyme systems on polyacrylamide and starch gel for *H. murinum* and *H. vulgare*, eleven of the examined enzyme systems revealed electrophoretic products, two revealed rather spots than clear bands, while five did not revealed any products. From them nine were monomorphic for both species and two - GOT and EST - on PAGE established specific electrophoretic bands for *H. murinum* and *H. vulgare*. All *Hordeum* plants were examined with these two systems.

Glutamate oxaloacetate transaminase

All 18 populations of *H. murinum* showed identical enzyme pattern (Fig. 10). The zymogram consisted of five electromorphs, two of which monomorphous (0.75 and 1.00) and three (1.12, 1.20, 1.29) polymorphous.

The six populations of *H. vulgare* were divided into three groups (Fig. 10). In total they revealed eleven electromorphs, three of which (1.12, 1.20, 1.29) were common for all the populations and were also present in *H. murinum*. Five electrophoretic bands were present only in population 2 and three of them (0.56, 0.60, 0.69) were rare (found only in few plants).

H. murinum had one unique band (0.75) not present in *H. vulgare*, which on the other hand had two specific bands, present in three of the populations (0.79, 0.92) and one present in the other three populations (0.90). Those bands were used as markers to detect the putative hybrids between both species.

Esterase

For the EST inter - as well as intrapopulation variation for both *H. murinum* and *H. vulgare* was observed. *H. murinum* was divided in seven groups and *H. vulgare* in five. The zymograms showed 8 electromorphs in total for *H. murinum* and 12 for *H. vulgare* (Fig. 11).

There were two specific electrophoretic bands (0.59, 1.93), present in all populations of *H. vulgare*. Two specific bands (0.62, 1.00) was found for *H. murinum* as well. Bands 0.59 and 0.62 were present in all the populations of *H. vulgare* and *H. murinum* respectively, but not in all of the plants. The band at 0.78 was the only common band for both species. The other bands were typical only for *H. vulgare* (0.32, 0.49, 0.66, 0.86, 0.88, 1.41, 1.52, 1.60, 1.74) or *H. murinum* (0.67, 1.19, 1.89, 1.96, 2.04), but they were present only in some of the populations of each species and not always in all individuals.

Interpopulation differentiation in distribution of electrophoretic bands for GOT and EST for *H. murinum* was evident from Table 5, where the percent of presence of the bands per population were given. Bands, such as GOT A, GOT B, GOT E, EST B, EST C, EST E, EST F, EST G (Table 6) were the more frequently present for *H. murinum*.

The Principle Coordinate Analysis was computed with all the data from isozyme analysis from all plants of *H. murinum* and *H. vulgare* and showed clear separation between both species when Factors 1 and 2 (Fig. 12) as well as when Factors 1 and 3 were plotted together (Fig. 12a). When Factor 1 and 2 were plotted together a separation between the populations of *H. vulgare* (populations 2, 6 and 7 from one hand , and 12, 13 and 14 from the other hand were grouped together) was observed as well. No clear separation according to the geographical distribution was observed, when the same analysis was performed only for *H. murinum* plants (Fig. 13, 13a), although there was a tendency for the populations from Vaud to separate appart, when Factors 1 and 2 are plotted together (Fig. 13). However, still one third of the samples from Vaud were grouped together with those of the other regions.

Mantel test for differences of samples in regard to their species affiliation and geographical areas was made as well. For the first case the test for difference between the species gave r = 0.89, t = 42,8, prob(t) = 0.00000. The r value indicates the correlation between the similarity

matrix, calculated with the enzyme data and that of the species. Probability (prob(t)) value indicates highly significant differences between both *Hordeum* species according to their isozyme profiles.

For the second case, Mantel test was computed with the enzyme data for *H. murinum* and a matrix of distances between populations, derived from the coordinates of the test areas in the different regions of Switzerland, where the plants were collected. Data from three of the sampling regions (Graubünden, Vaud and Swiss Plateau) was considered only, as plants from Valais had a very restricted number. The following results were obtained:

r = 0.17, t = 15.1, prob(t) = 0.00000

The r value is lower, but prob(t) indicates highly significant difference between the populations of *H. murinum* from different regions of Switzerland.

6.3.2.2. *Medicago*

From 19 enzyme systems studied on polyacrylamide and starch gels, three did not reveal electrophoretic bands, nine gave rather spots, but not clear bands and seven revealed clear products. From them four enzyme systems were monomorphous for all plants and three systems - PRX, EM and MDH on PAGE were selected, as they showed polymorphism between individuals.

Peroxidase

Eight electromorphs were revealed for PRX for *M. sativa* and tetraploid *M. falcata*, and six for diploid *M. falcata* (Fig. 14). They had the same migration distance for both ploidy levels. All the bands, except that at 0.37 were present in all examined plants.

Maleic Enzyme

Six distinct electromorphs were detected for EM for both tetraploid species and five for the diploid *M. falcata*. They all migrated at the same distance for the different species and were present in all examined individuals (Fig. 14). In addition bands in 1.00 zone existed for all species, but unfortunately they were unclear and it was impossible to determine their number. Therefore their presence was not considered.

Malate dehydrogenase

Six clear electromorphs were observed for diploid *M. falcata* and five for tetraploid *M. falcata* and *M. sativa*. They migrated at the same distance for both ploidy levels (Fig. 14). Again at 1.00 there were unclear bands, which number was difficult to determine.

M. sativa and *M. falcata* considered as a whole did not present species specific electrophoretic bands with none of the studied enzymes. There was difference in the zymograms only between the individuals. However, distinction between diploid *M. falcata*, and both tetraploid species was possible.

Thus, for PRX the eight isozymes were more or less equally present in all species, except band PRX 4 (0.37), which was not present in the tetraploid *M. falcata* and band PRX 5 (0.42), not present in the diploid *M. falcata* (Table 7). PRX 1 (0.20) was very rare for the diploids.

For EM all seven isozymes were present rather equally in both species, except EM 6 (0.69), which was missing in the diploids. EM 1 (0.22) was much more rare for them as well (Table 7).

More variability between the two ploidy levels in *M. falcata* was observed in the MDH profiles. Three of the bands - MDH 4 (0.55), MDH 5 (0.63) and MDH 6 (0.74) were not present in the diploid *M. falcata*. It was interesting to observe that MDH 1 (0.04) was present only in diploid *M. falcata* and in *M. sativa* from Unterengadin.

a) b)

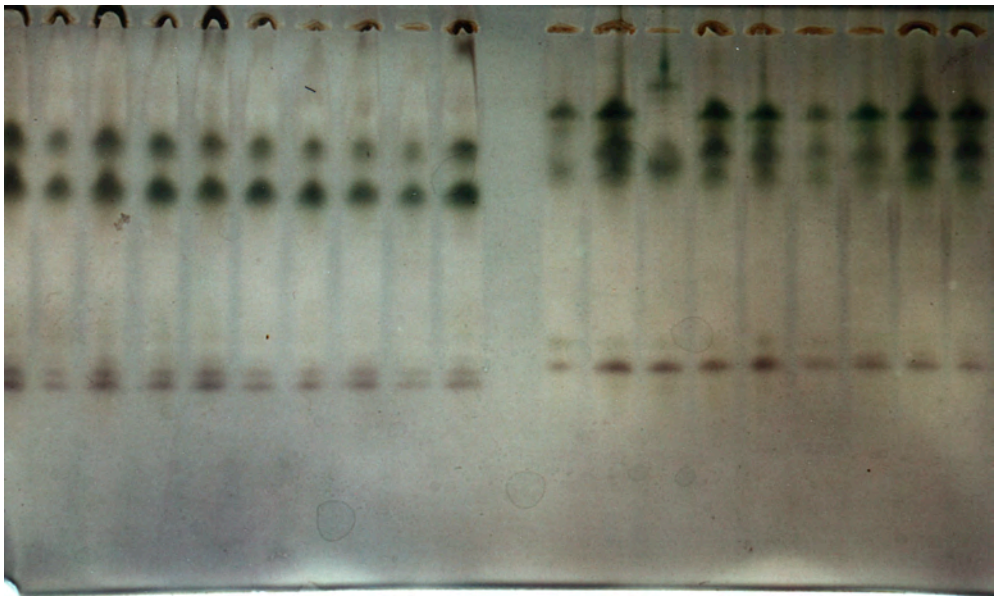

Photo 6. Isozyme polymorphism for esterase between *H. murinum* a) and *H. vulgare* b).

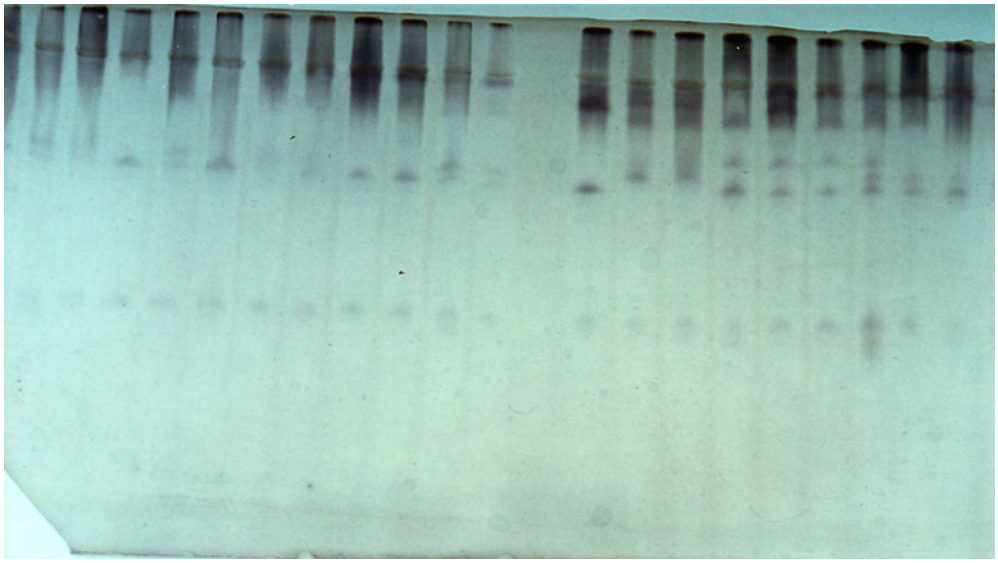

Photo 7. Isozyme polymorphism for peroxidase of different *Medicago* individuals.

All electrophoretic bands were present in *M.* x*varia* as well, except MDH 1.

Table 8 gives the presence of all bands of PRX, EM and MDH for the plants of population 4. This population was chosen as an example, because it includes the three taxa present at tetraploid level. Some electromorphs, are more frequent for either *M. falcata* or *M. sativa*, such as PRX 2, PRX 4, EM 1, MDH 2, MDH 3.

PCOORD (Fig. 15 and 15a) clearly set apart the diploid *M. falcata* from the tetraploid *Medicago* species, which are grouped together according to their isozyme profiles.

Mantel test for differences between *M. sativa*, diploid and tetraploid *M. falcata* and *M* x*varia* for enzymes was made. The following result was obtained:

r = 0.22, t = 42.0, prob(t) = 0.00000.

Although r value is not high, prob(t) gives significant differences between the species according to their enzymes.

The same test, performed only for tetraploid plants (tetraploid *M. falcata*, *M. sativa* and *M.* x*varia*) gave r = 0.04, t = 6.80 and p(t) = 0.00000. The r value is very low, but there is still significant differences between the species.

6.3.3. DNA analysis

A preliminary screening of 44 primers was performed with genomic DNA on ten samples of each *M. sativa*, tetraploid *M. falcata* and *M.* x*varia* for species specific RAPD markers. Banding patterns were directly scored from the gel for presence or absence of each amplification product. Eleven primers did not amplify detectable products. Thirty three primers were effective in initiating PCR amplification of DNA and three of them (OPP - 05, OPP - 06 and OPP - 08) gave banding patterns that were polymorphic for *M. sativa* and *M. falcata*. Primer OPP - 5 amplified a 850 bp band, specific for *M. sativa*, OPP - 6 amplified a

800 bp band and OPP - 8 amplified a 700 bp band, specific for *M. falcata*. Those bands were present in the hybrid *M.* x*varia*. Only those fragments that could be clearly scored and were repeatable were taken into consideration.

Occasionally amplification products were observed in the control, that did not had the template DNA. However, these bands were always absent, when template DNA was included.

An analysis of 90 plants (including 18 plants *M. sativa,* 35 plants *M.* x*varia* and 37 plants *M. falcata*) from population 4 was carried out with those three selected markers.

A total of 24 amplified products were observed in patterns of *Medicago* from that population with the three primers (primer OPP - 05: 9 bands, primer OPP - 06: 7 bands and primer OPP - 08: 8 bands). The size of the fragments was from 517 to 2036 bp. The number of amplification products varied for the different species: for *M. sativa* from 1 to 6; for *M. falcata* from 0 to 7; for *M.* x*varia* from 1 to 7.

Although during the preliminary screening on ten samples/species specific bands were determined, when performed on the large sample (90 individuals), there was not any more specificity, present by the primers. However, some bands were mostly presented either in *M. sativa* (P5 D, P5 I, P6 C, P6 D) or *M. falcata* (P5 A, P6 B, P8 F) (Table 9).

The Principal Coordinate Analysis based on this data did not show separation between the three species and they tended to group together (Fig. 16).

Mantel test, calculated with RAPD data gave the following result:

r = 0.07, t = 3.68, Prob(t) = 0.00012.

The r value is low, but the difference between species, based on RAPD is significant.

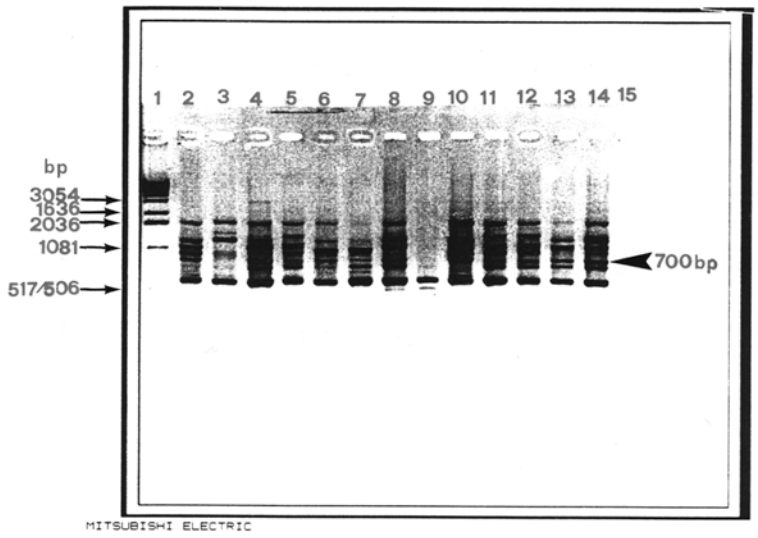

Photo 8. RAPD profiles for primer OPP - 8 of *Medicago sativa*, *M. falcata* and *M.* x*varia* (population 4). Line 1: λ-X DNA molecular size marker, line 2: *M.* x*varia*, line 3: *M. sativa*, line 4: *M. falcata*, line 5: *M.* x*varia*, line 6: *M. falcata*, lines 7-10: *M.* x*varia*, lines 11-12: *M. falcata*, lines 13-14 *M.* x*varia*, line 15: control without DNA. This primer amplified 700 bp band, specific for *M. falcata*.

6.4. Hybridization experiments

6.4.1. Hybridization under natural conditions

6.4.1.1. *Hordeum*

The plants of *H. murinum*, used for the hybridization experiments in the field with winter barley in Chézard had died.

Those, used for the hybridization with spring barley in Cernier (30 plants), produced 646 seeds in total, which were sown in the Botanical garden of Neuchâtel. They resulted in 588 plants (91% of the seeds germinated) (Table 10).

From these plants only ten/parent, randomly chosen were used for further investigations with isozymes. When the plants were less then ten, enzyme analysis was done on all of them (272 plants in total).

All produced plants have been checked also morphologically for the presence of hybrids. No hybrids have been detected. No evidence of hybrids has been observed with the isozymes as well (Table 11). All the plants repeated the isozyme profile of GOT and EST of the female parent. The only difference were in the relative intensity of the bands.

6.4.1.2. *Medicago*

For the field experiments in Changins 21 plants were used. From them 17 have produced fruits, which were collected (Appendix III). All the seeds were sown in the Botanical garden of the University of Bern, but only seeds of 11 mother plants (65%) germinated and formed seedlings. However, only seven of them have survived and yielded 30 progenies. They were examined for presence of hybrids with isozymes. For 29 of the plants there was evidence for presence of hybrids (Table 16), detected with the three studied enzyme systems, as the progeny represent bands, from both parents.

6.4.2. Experimental hybridization

6.4.2.1. *Hordeum*

Thirty one flowering plants of each *H. vulgare* and *H. murinum* were used for the experimental hybridization in the greenhouse. All produced seeds - 1251, were sown. From them 1223 (98%) gave plants (Table 12). Twenty progeny plants per hybridization were randomly chose and used for isozyme analysis (or all of them when less then 20).

In addition, all the plants have been screened morphologically for the presence of hybrids.

No evidence of hybrids was detected in all 933 analysed plants with the isozymes (Table 13)**.** All examined putative hybrids showed isozyme profiles for GOT and EST, which repeated those of the female parent, with only differences in the relative intensity of the bands.

Among the examined plants there were few, with unusual morphological characteristics. The chromosome number of those plants was checked. The following results were obtained:

13A 3 x 2A 815/36 - 20 2n=14
7A 17 x 7A 244/282 - 24 2n=14
7A 9 x 16A 910/118 - 19 2n=15
7A 9 x 16A 910/118 - 23 2n=14
2A 29 x 18A 173/56 - 17 2n=14
7A 2 x 5B 710 - 20 2n=14

One of the plants was aneuploid with 2n=15 chromosomes and all the rest were diploids with 2n=2x=14, as their female parent. Aneuploidy does not necessarly relate to hybridization.

6.4.2.2. *Medicago*

With *Medicago*, experimental hybridization between *M. sativa* and *M. falcata* as well as self - fertilization and cross - fertilization experiments were performed.

For *M. sativa* as the female parent, 32 hybridizations, 32 self - fertilizations and 30 cross - fertilizations were made.

When *M. falcata* was the female parent 28 of each hybridizations, self - fertilizations and cross - fertilizations were carried out.

Self - and cross - fertilizations were performed on the same plants, used as female parents in the hybridization experiments.

From those 60 plants, 46 (77%) produced fruits. From them 44 (98%) individuals produced ripe seeds and the seeds of 39 (87%) germinated. Seedlings from 20 plants have survived producing 92 plants in total (Table 14).

Those plants were tested with isozymes. The progeny, obtained after self - fertilization (28 plants) represent the same enzyme profile as their parent for 7 of the plants and the rest 21 were contaminated (Table 15). There was evidence for hybrids, produced after cross - fertilization and hybridization experiments, as specific bands for both parents were present in the progeny. From the cross - fertilization experiment 10 plants were obtained, from which 5 intermediate individuals, one plant as result of self - fertilization and 4 contaminated plants (Table 17). From the experimental hybridizations 54 plants were obtained, from which 27 with evidance to be hybrids.

In all types of crosses, bands, which did not exist in the parents zymogram were detected.

CHAPTER 7:

DISCUSSION

7.1. *Hordeum*

7.1.1. Flowering biology

Flowering of *Hordeum* plants usually begins in the florets in the middle of the ear and spreads up and down, taking about four days to complete. Ears on the different tillers may mature at different times (Briggs 1978).

Hordeum species are usually reported as autogamous (Hammer 1984). Although *H. murinum* is self - fertilizing, when studying flowering biology we observed excerted stamens and visible styles. That proved the absence of cleistogamy and the potential for outcrossing.

Thus, the hybridization and the risk for gene flow between *H. murinum* and cultivated barley exists. It was also possible, because of the fact that their flowering periods overlap.

Intrapopulation differentiation of the flowering stage of *H. murinum* from different regions of the country was observed in experimental garden. Earliest flowering populations were from Valais, followed by those from Swiss Plateau. The last plants to flower were from Graubünden. The interpopulation differentiation of the flowering time for *H. murinum* has been already observed earlier (Giles 1984). The study of flowering time of several populations from Europe, Egypt, Israel and New Zealand indicated intra - and interpopulation heritable variability. The causes of these differences are however unknown, but the possible reason could be the adaptation to the local climate and daylength requirements. The existence of geographical difference in the flowering periods might be important for risk assessment, in case some populations of *H. murinum* would flower together with *H. vulgare*, but some other would have different flowering times. As said before, this was not the case for our investigated populations.

7.1.2. Pollen and seed dispersal

Both *H. vulgare* and *H. murinum* presented a similar pattern of pollen dispersal. Most of pollen was found within few meters of the plant source, remaining constant after certain distance. This pattern is classical for the pollen dispersal and has been already observed for example for *Raphanus sativus* by Klinger et al. (1991) and for *Solanum tuberosum* by Skogsmyr (1994). It is interesting to note that those species are insect pollinated, while *H. murinum* and *H. vulgare* are wind pollinated.

Data on pollination is important for determining the isolation distances between plots with transgenic plants and other sexual compatible plants, growing in the vicinity. Therefore, according to our results, if *H. murinum* is growing close to the field with transgenic barley, most of the hybridization events and higher rate of gene flow is expected at a distance up to 7 meters.

Nevertheless, the majority of pollen migration occured within few meters of the pollen donor, the fact that at 12 meters we still found certain amount of it, indicates that the isolation distances between both species, when cultivating transgenic barley must be larger. Especially some studies on pollen dispersal on *H. vulgare* indicate that outcrossing may occur up to 21meters (Doll, 1987) and 60 meters (Wagner and Allard, 1991). Even the isolation stretches are determined, it is never possible to guarantee that the pollen grain will not fertilize another plant at a great distance (Dale 1992). Thus it is important that the risk assessment must take into account this possibility for rare pollination events and focus on the consequences of potential hybridization.

It seems that the species does not play an important role for the pattern of pollen dispersal in our case as both species have more or less the same type of pollen movement.

Moreover, the environmental conditions may influence the gene flow rate between the transgenic and non - transgenic plants of the same species (Scheffler et al. 1993) and between the populations of the same species (Arias and Rieseberg 1994).

The standard deviation of the distances at which the seeds of *H. murinum* are spread by the wind was also not large - 0.5 - 0.6 meters - without any seeds found at two meters limits of the plastic sheet. However, it is important to mention that barley seeds as well as those of *H. murinum* have sticky ends and hairs on the glumes, which make them adhere to the furs of the animals, bird feathers and human clothes, then may be thus dispersed at large distances. In addition seeds of *H. vulgare* could be dispersed by the seed transport. Thereby, the risk for the establishment of subspontaneous populations increases.

7.1.3. Genetic differentiation between *H. murinum* and *H. vulgare*

A number of well - defined characteristics are necessary to describe and identify different crop species or varieties. The traits used to distinguish and identify different *Hordeum* species are mostly morphological features. However, some useful morphological traits can only be measured at later stages of plants development. Biochemical markers, i. e. isozymes and molecular markers, are valuable supplements or alternatives to the morphological characters. The use of isozymes for identifying barley varieties or *Hordeum* species have been described by several authors (Nielsen and Johansen 1986, Jaaska 1992, Jaaska 1994).

Our results proved that isozymes were suitable genetic markers for distinguishing cultivated and wild barley *H. murinum*. With both studied enzyme systems - GOT and EST - specific electrophoretic bands were determined for the two species. Principal coordinate analysis separated well both species, according to their enzyme structure (Fig. 12, 12a). Mantel test confirmed the significant genetic difference between them.

This is in accordance with the results from the PCA analysis with the morphological characteristics, obtained by Julia Keller Senften (1996), performed on the same plants, which clearly separated *H. vulgare* and *H. murinum*.

Genetic structure of populations of *H. murinum* was typical for that of inbreeding plants, with a low intrapopulation variability, joined with genetic differentiation between populations (Hamrick et al. 1979).

Jaaska (1992) obtained a GOT zymogram for *H. murinum* similar to ours with two monomorphous bands and one heteromorphous triplet. All the studied populations of *H. murinum* had the same enzyme profile. The two slower migrating bands were present in the zymograms of all the populations at similar frequencies. Variation in frequency was found for the three faster electromorphs (Table 5). This variation determined separation of the population according to their geographical regions as demonstrated by the Mantel test.

However, no intrapopulation differences according to the geographical distribution, were demonstrated by PCOORD (Fig.13, 13a).

The results of the PCA according to the morphology of the plants tended to separate the populations from Graubünden (Keller Senften 1996), which was not observed in our case.

A total of 20 esterase alleles for both *H. vulgare* (12 bands) and *H. murinum* (9 bands) were observed. These numbers are similar to those reported by Nielsen and Johansen (1986) for *H. vulgare* (11 bands) and Jaaska (1994) for *H. marinum* (11 bands), but lower then that reported by Zhang et al. (1994) (20 bands) for cultivated barley from Tibet.

The six studied populations of *H. vulgare* presented different zymograms for EST and GOT (except populations 6 and 7, which were grouped together according their EST and GOT profiles and populations 12, 13 and 14, which had the same GOT pofile). It demonstrates that probably different varieties of cultivated barley were sampled, although it was not possible to

verify this information. This grouping of the barley populations according to the GOT zymogram is the reason for the separation of populations 2, 6 and 7 from populations 12, 13 and 14 of *H. vulgare* by the PCOORD, when Factors 1 and 2 were plotted together (Fig. 12a). Only one common EST band out of 20 and 4 GOT bands (one monomorphous band and one triplet) out of 13, was shared by *H. vulgare* and *H. murinum*, indicating that they are not genetically very close. This fact is not surprising, as *H. murinum* is not considered as one of the wild progenitors of cultivated barley.

Good correspondance between the results was obtained with isozyme and morphological analysis for *Hordeum*. The two types of markers clearly distinguished both species, indicating relatively low similarity between them, which leads to the conclusion that low gene flow take place between *H. vulgare* and *H. murinum* in natural conditions. Moreover, no intermediate individuals have been detected in the collected populations of both species.

7.1.4. Hybridization ability

It is obvious from our results that the hybridization between *H. murinum* and *H. vulgare* is extremely difficult, if possible at all, without using special *in vitro* techniques. We did not obtain any hybrids, neither under natural conditions nor in the greenhouse. The results were independant of species type of the female or the male parent.

The fact that no hybridization has occured was expected, as no spontaneous hybrids between both species have been reported.

When studying the zymograms of the progeny, there was no evidence of hybrids. In most of the cases (especially for GOT) they strictly repeated the isozyme profile of their female parents, proving that self - pollination had taken part. The results from biochemical

investigations were confirmed by the study with morphological characters, performed on the same plants by Julia Keller Senften (1996).

In the zymograms of certain crosses, there are bands, present for parent plants, but missing in the progeny, which is explained by the segregation of the alleles in the different gametes.

Although *H. vulgare* could outcross and despite the absence of cleistogamy for *H. murinum*, the hybridization between the both species is hampered by the incompatibility of their genomes. *H. vulgare* has an I genome, while *H. murinum* has Y.

Bothmer et al. (1991) divided the *Hordeum* species into three categories with respect to their relation to cultivated barley and how accessible they are as gene donors to the crop:

- primary gene pool (*H. spontaneum* and old landraces) - material closely related to the crop that have no or very weak biological barriers

- secondary gene pool (*H. bulbosom*) - all wild or weedy forms that have a comparatively good crossability with the cultivated species, but in which certain sterility factors are operating

- tertiary genome - these species (*H. murinum*), possess genomes other than cultivated barley and the hybrids with barley are highly sterile. With these species interspecific hybrids could be established only by embryo rescue.

The authors suggest that there are no chromosomal homology among the polyploids of *H. murinum* complex (subsp. *murinum*, 4x and subsp. *leporinum*, 4x and 6x) and other *Hordeum* species, including *H. vulgare*. Natural hybridization occurs very rarely, resulting in sterile offspring.

Bothmer et al. (1983) have been carried out experimental crosses between wild and cultivated barley and showed that the percent of seed set were very low, when *H. vulgare* was used as a female. It was significantly higher when barley was the male parent. However, the obtained seeds developed very few hybrids. There were less hybrids when *H. vulgare* was the female

as well. This has been verified also by the other reports (Morrison and Rajhathy 1959). They proved the existence of strong barriers for seedling establishment in hybrid combination. In tetra - and hexaploid combinations the regeneration of the plants were much higher. The crosses with tetraploid *H. murinum* were the only one from which plants were not obtained, although seeds were set.

The absence of natural hybrids between *H. vulgare* and *H. murinum* and the results of our experimental crosses allow us to conclude a low risk for gene flow between both species in Switzerland.

7.2. *Medicago*

7.2.1 The two ploidy forms of *M. falcata* in Switzerland

The fact that diploid *M. sativa* in Switzerland does not exist was expected as there was no report for its presence in Europe. All previous records indicated that *M. sativa* in Europe is tetraploid (Small and Bauchan 1984, Majovsky et al. 1974, Strid and Franzen 1981, Bijok et al. 1971).

Few chromosome counts of *M. falcata* have been reported for Europe.

Diploid *M. falcata* is present mainly in Asia and Eastern Europe, extending westwards to South of Germany. According to Quiros and Bauchan (1988) the distribution area of tetraploid *M. falcata* concides somehow with that of the diploid form. However, it shows more western and northern distribution (from Portugal and France to Sweden) in Europe in contrast to the diploids. The presence of tetraploid form in most parts of Switzerland is consistent with previously reports from Portugal and Balkans (Fernandes and Quiros 1978, Small and Bauchan 1984, Van Loon and Kieft 1980).

The Western distribution boundary of the diploid *M. falcata* in Europe runs through the Balkans and Eastern Europe to the South of Germany. The populations in Unterengadin lie in

the margin of its range. Diploid *M. falcata* is an additional example of the affinity of floristic elements of the Unterengadin with Eastern Europe, where this cytotype is also present. At the begining of the postglacial period (ca. 10 000 years BP) the climate favoured the expansion of the steppes. Unterengadin was probably invaded through the Inn valley, where other relicts from that period have subsisted e.g. *Dorycnium germanicum* (Gremli) Rikli (Landolt 1986). Contrasting with that, Swiss tetraploid *M. falcata* have migrated either from the Balkans or from Western and Northern Europe, according to its present distribution. However, it is not ruled out that *Medicago* colonization of Switzerland originates from different regions.

Both cytotypes present in Switzerland are probably geographically separated, but this has to be proved by more detailed study.

M. sativa and *M. falcata* hybridize readily at tetraploid level (Ledingham 1940), but crosses between different ploidy levels are hampered. **Thus the geographical differentiation according to the ploidy levels is important from the point of view of the risk for releasing transgenic *M. sativa* in Switzerland. The possibility for transfer of modified genes from cultivated alfalfa to tetraploid *M. falcata* is higher in the Swiss Plateau and most alpine valleys. In the contrary the risk will be lower in the region of Unterengadin where diploid *M. falcata* grows. Thus the genetics of the species may vary geographically and may influence the ability of hybridization with other species, influencing the risk of the vertical gene flow.**

As most of the populations, collected in Valais, Schaffhausen and Rhine valley in Graubünden consisted of *M.* x*varia*, there is strong evidence for intensive genetic erosion of the native tetraploid *M. falcata*. The phenomenon is not recent as Schinz and Keller mentioned already in 1923 that the hybrid *M.* x*varia* could outcompete *M. falcata*. The present decline of habitats as a result of intensive land use is an additional danger for the conservation of tetraploid *M. falcata.*

Landolt (1991) describes *M. falcata* as endangered in the Plateau and extremely endangered for the western part of the Jura and western part of the Northern Alps. We believe that except for Unterengadin, where the diploid type grows, there is a danger of genetic erosion suppressing *M. falcata* in the whole country. Therefore, protection of this indigenous species as a part of Swiss flora and as potential genetic ressource for the improvement of cultivated *M. sativa* is urgent. Protection measures could consist in the prescription of a minimal distance between cultures of *M. sativa* and present populations of *M. falcata* or more often rotation of the cultured species.

7.2.2. Genetic differentiation between *M. sativa* and *M. falcata*

7.2.2.1. Isozymes

The results, obtained with the three studied enzyme systems allowed us to compare *M. sativa, M. falcata* and *M.* x*varia* as well as the two ploidy levels of *M. falcata.*

No specific bands for *M. sativa* or *M. falcata* were found with the three studied enzyme systems, and the electrophoretic bands were more or less equally present in both species at tetraploid level (Table 7), presuming that they belong to the same gene pool. Nevertheless, some of the bands, such as PRX 5, MDH 2, MDH 3, were more rare for *M. falcata*, which determined the significant difference between both species, revealed by the Mantel test. In spite of this, *M. sativa*, tetraploid *M. falcata* and *M.* x*varia* tended to group together and no clear separation was observed between them with PCOORD (Fig. 15 and 15a).

Genetic proximity between *M. sativa* and *M. falcata* at the same ploidy level is an evidence for extensive gene flow between them, caused by the free hybridization between the species.

Genetically more distant are *M. sativa* and diploid *M. falcata*. There was more similarity between both species at the same ploidy level, then between both ploidy forms of *M. falcata* as well. Bands PRX 5, EM 6, MDH 5 and MDH 6, which are present in *M. sativa* and tetraploid *M. falcata* are missing in the diploid form. This genetic remoteness between the diploid *M. falcata* and the tetraploid species was demonstrated clearly by the PCOORD as well (Fig. 15 and 15a), where they form a clearly separated group. **This confirms the existence of the ploidy barrier, which hampers the hybridization beween the tetraploid *M. sativa* and the diploid *M. falcata,* determining lower gene exchange between both species.**

Interesting enough is the presence of band MDH 1, specific for the diploid *M. falcata* in the zymogram of *M. sativa* from Unterengadin. That band determined the presence of the plants of *M. sativa* from Unterengadin in the group of the diploids after PCOORD. **It indicates that some gene flow happens at different ploidy levels in natural conditions as well, probably by 2n gametes.** However, this case is rare, as we did not observed any *M.* x*varia* or triploid plants (which could be the result of such crosses) in the populations from Unterengadin and according to the literature.

7.2.2.2. RAPD

The Principal Coordinate Analysis based on RAPD data did not show separation between the three species and they tended to group together (Fig. 16).

This could be explained with the high hybridization and introgression rates of *M. sativa* and *M. falcata* and confirms the high rate of gene flow between both species at the tetraploid level in natural conditions. Because *Medicago* is an outcrossing species and each population is actually a heterogeneous mixture of genotypes our results indicate that with RAPD, we could identify specific individuals within *Medicago* populations, but not differentiate the species,

which is in accordance with the conclusions of Barcaccia et al. (1994) with alfalfa. Thus another DNA technique, such as RFLP, AFLP, microsatellites etc. should be used in order to obtain more specific and stable DNA markers for both *Medicago* species.

Mantel test, calculated with RAPD data for differences between the three species was still significant, although the r value was very low.

7.2.3 Hybridization ability

It is known that the genomes of *M. sativa* and *M. falcata* are homologous and can pair. They cross easily at the same ploidy level. Our observations of natural populations and experimental hybridizations are in accordance with that fact.

Because no specific isozyme bands were determined for the studied *Medicago* species, we have used for the hybridization experiments plants of *M. sativa* and *M. falcata*, which differed in their zymograms for one, two or all three enzyme systems.

Hybrids were detected in the progeny of the plants after hybridization in natural conditions (Table 16) and in the greenhouse (Table 18) as they had zymograms intermediate between those of both parents or bands not typical for the female parent in the case of hybridization in the field.

For some crosses, it was possible to detect the hybrids with all three enzyme systems, while in the other cases there was evidence only with one or two of them. One reason for this was the same profile for both parents for one or two of the enzymes.

In total five individuals were proved to be the result of cross - fertilization and 27 were obtained after hybridization in the greenhouse. More hybrids - 29 were produced after natural hybridization. The lower number of hybrids, obtained in the greenhouse could be explained by imperfections of the technique, used for the artificial hybridization and the relatively big number of the contaminations during the pollination procedure. The contaminations were

manifested by the presence of « alien » bands (not typical for any of the parents) in the zymograms of the progeny and were in the progeny after experimental hybridization, self - and cross - fertilizations. However, most contaminations were detected in the progeny of the plants after self - fertilizations, where eight from twelve crosses, which regenerate plants consists of non - typical bands. In the rest three crosses zymograms of the progeny was as expected, repeating parents profiles.

A big part of the plants after the experimental crosses failed to form fruits. In addition only 41% of those, which produce seeds (after hybridization and cross - fertilization) regenerated hybrids. This is in accordance with the observation of Quiros and Morgan (1981). Their experiments resulted in low seed production and reduced fitness of the plants in many progenies from selfed or sibling crosses in *Medicago,* considered as consequence of homosygosity, determined by the predominantly outcrossing nature of the genus. The seeds from such crosses generaly failed to germinate or produce abnormal seedlings.

Finally, the progeny, obtained after the experimental crosses, performed by Pia Rufener Al Mazyad from University of Bern were also examined with isozymes for evidence of hybrids. One cross *M. sativa* x *M. falcata,* five crosses between *M. falcata* and *M*. x*varia*, eight between *M. sativa* and *M*. x*varia*, as well as two self - fertilizations (*M. sativa* x *M. sativa* and *M*. x*varia* x *M*. x*varia*) and one cross -fertilization (*M. sativa* x *M. sativa*) was carried out by her.

Only 41% of all performed crosses produced ripe seeds and only 28% of the plants produced seedlings. In total 38 plants were regenerated after these crosses. Evidence for 19 hybrids after hybridizations *M. falcata* x *M*. x*varia, M. sativa* x *M*. x*varia* and *M*. x*varia* x *M*. *sativa* existed according to their isozyme profiles (Table 19). Thus *M*. x*varia* could easily backcross

with *M. sativa* and *M. falcata*. Once again relatively low seed production and regeneration ability after experimental hybridization was observed similarly with the results, obtained in Neuchâtel. Unfortunately, it was not possible to determine if the only regenerated plant after the cross *M. sativa* x *M. falcata* is a hybrid (not present in table 19), because of the absence of the zymograms of PRX, EM and MDH for the male parent.

The number of plants, producing seeds after hybridization when *M. sativa* was the female parent, was approximately the same of that when *M. falcata* was the female. However, more successful hybridizations and more hybrids was scored when *M. falcata* was used as female parent.

Similar was the situation when self - and cross - fertilizations were carried out. In these cases for the seed production and plant regeneration it was of importance whether they were performed with *M. falcata* or *M. sativa*. With *M. falcata* more seeds and more regenerated plants were obtained, compared to *M. sativa*.

The literature data (Ledingham 1940) and our experimental hybridization confirm that at the tetraploid level *M. sativa* hybridizes freely with *M. falcata.* Our observations of natural populations as well support the presence of extensive gene exchange between both tetraploid species. Some of the collected population consisted exclusively of intermediate individuals between both species (*M. xvaria*). Thus high risk for gene flow was concluded.

Diploid *M. falcata* was discovered too late to carry the experimental crosses. Nevertheless literature indicates that crosses between diploid *M. falcata* and tetraploid *M. sativa* are hampered, due to the ploidy barrier. Crosses between the diploid and tetraploid forms results in low fertility (Ledingham 1940, Lesins 1952). If diploid *M. falcata* is the female parent, the ovules abort, while the ovary may develop a well - formed pod. When *M. sativa* is the female,

fertilization delaies and the development of the endosperm and of the embryo usually stops. The offspring are either sterile triploids or rarely tetraploids. These tetraploids are highly fertile and have morphological characters intermediate between both parental species (Ledingham 1940). **In the populations from Unterengadin neither triploid plants nor *M.* x*varia* were observed, which confirms the existence of the ploidy barrier, thus determining the lower risk of growing transgenic alfalfa in the regions, where the diploid form of *M. falcata* grows.**

CONCLUSIONS

The purpose of this study was to evaluate the risk for vertical gene flow in the case of two cultivated species : *Hordeum vulgare* and *Medicago sativa* to their wild relatives *Hordeum murinum* and *Medicago falcata* respectively.

The necessity of doing risk assessment studies case by case and moreover on regional scale was demonstrated by the study. The different plant biology, behaviour and distribution of both group of species determine the specificity of the gene transfer and the different degree of the risks that appear.

Hordeum murinum - Hordeum vulgare

We concluded low risk for gene flow between cultivated barley and his wild relative *H. murinum* in Switzerland. No hybrids were detected, when examining the populations of *H. murinum*, collected at the proximity with cultivated barley. No evidence of hybridization between *H. vulgare* and *H. murinum* was found after our experimental crosses in the field and in the greenhouse. It was caused probably by the different genomes of both species.

However, for the risk assessment should be considered that although *H. vulgare* and *H. murinum* reproduce predominantly by self - fertilization, some outcrossing has been reported. The preconditions for hybridization between the two species exist, because of their overlaping flowering periods and the absence of cleistogamy for *H. murinum* in Switzerland. The dispersal of the genes by pollen and seeds has to be considered as well: the majority of their migration occurs within few meters from the donor plant. Nevertheless, pollen migration of *H. vulgare* has been noticed up to 60 meters according to the literature. Moreover, long distance dispersal of seeds of *H. murinum* by zoochory may occur, as well as dispersion of *H.*

vulgare during transport. These types of dispersion may increase considerably the risk for gene flow.

Medicago falcata - Medicago sativa

We concluded that high rate of gene flow occurs between *M. sativa* and *M. falcata* at tetraploid level, determined by the high rate of outcrossing and free hybridization between both species. They have a relatively low seed and fruit sets and more or less limited seed dispersal, but extensive pollen dispersal (1600 meters). This together with outcrossing exhibits the major risk for gene flow.

In course of this study the existence of diploid *M. falcata* in Switzerland was discovered. As the hybridization between tetraploid *M. sativa* and diploid *M. falcata* is very restricted due to the ploidy barrier, the geographical distribution of the two ploidy forms of *M. falcata* is important from the point of view of the risk for cultivation of transgenic alfalfa in Switzerland. The possibility of gene transfer to tetraploid *M. falcata* is higher in Swiss Plateau and most alpine valleys, contrasting with the region of Unterengadin, where the diploid form grows.

Our results indicate that risk assessment must be carried out at a regional scale according to the genetic differentiation of species, which may occurs on small scale.

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APPENDIX I

1. Composition of the enzyme extraction buffer for *Hordeum* (for 100 ml)

0.60 gr Tris, 0.36 gr EDTA, pH = 7.5

2. Composition of the enzyme extraction buffer for *Medicago* (for 100 ml)

1.21 gr Tris, 1.86 gr KCL, 1 gr PEG (20 000), 0.04 gr Na -Thioglycolate, pH = 8

One point of the spatule of Polyvinylpolypyrolidon was added in the mortar as well.

3. Composition of the Polyacrylamide gel

Stock solutions:

A

0.4 ml N,N,N′,N′ - tetramethyl - ethylenediamine (TEMED), 36.6 gr Tris, 48 ml HCL 1N, bidistilled H_2O up to 100 ml, pH = 8.9

B

0.5 ml TEMED, 5.98 gr Tris, bidistilled H_2O up to 100 ml, pH = 8.9

C

80 gr Acrylamide, 1.47 gr N,N′-methylene-bis-Acrylamide, bidistilled H_2O up to 200 ml

D

10 gr Acrylamide, 2.5 gr N,N′-methylene-bis-Acrylamide, bidistilled H_2O up to 100 ml

E

0.004 gr Riboflavin, bidistilled H_2O 100 ml

F

40 gr Sucrose, bidistilled H_2O 100 ml

G (to be prepared the same day)

0.185 gr Ammonium peroxydisulfate, bidistilled H_2O 200 ml

Composition of the gels:

		9%	10.5%
- separation gel:	sol. A	6.5 ml	6.5 ml
	sol. C	11.7 ml	13.65 ml
	sol. G	33.8 ml	31.85 ml
- stacking gel:	sol. B	1 ml	1 ml
	sol. D	2 ml	2 ml
	sol. E	1 ml	1 ml
	sol. F	4 ml	4 ml
- sample gel:	sol. A	0.62 ml	0.62 ml
	sol. C	1.12 ml	1.12 ml
	sol. G	3.25 ml	3. 25 ml

4. Electrode buffer

Stock solution (for 1000 ml)

15.26 gr Tris, 135.18 gr Glycine, pH = 8.6

The final buffer was prepared by mixing 200 ml of the stock solution and 3600 ml dH_2O.

APPENDIX II

1. Composition of 10 x TBE buffer:

154.4 gr Tris, 26.2 gr Boric acid, 9 gr EDTA, 810 ml bidistilled H_2O

2. Nucleotide sequence of the primers, tested for RAPD analyses

kit B

OPB - 01 GTTTCGCTCC	OPB - 11 GTAGACCCGT
OPB - 02 TGATCCCTGG	OPB - 12 CCTTGACGCA
OPB - 03 CATCCCCCTG	OPB - 13 TTCCCCCGCT
OPB - 04 GGACTGGAGT	OPB - 14 TCCGCTCTGG
OPB - 05 TGCGCCCTTC	OPB - 15 GGAGGGTGTT
OPB - 06 TGCTCTGCCC	OPB - 16 TTTGCCCGGA
OPB - 07 GGTGACGCAG	OPB - 17 AGGGAACGAG
OPB - 08 GTCCACACGG	OPB - 18 CCACAGCAGT
OPB - 09 TGGGGGACTC	OPB - 19 ACCCCCGAAG
OPB - 10 CTGCTGGGAC	OPB - 20 GGACCCTTAC

kit L

OPL - 02 TGGGCGTCAA	OPL - 08 AGCAGGTGGA
OPL - 04 GACTGCACAC	OPL - 12 GGGCGGTACT

kit P

OPP - 01 GTAGCACTCC	OPL - 11 AACGCGTCGG
OPP - 02 TCGGCACGCA	OPL - 12 AAGGGCGAGT

OPP - 03	CTGATACGCC	OPL - 13	GGAGTGCCTC
OPP - 04	GTGTCTCAGG	OPL - 14	CCAGCCGAAC
OPP - 05	CCCCGGTAAC	OPL - 15	GGAAGCCAAC
OPP - 06	GTGGGCTGAC	OPL - 16	CCAAGCTGCC
OPP - 07	GTCCATGCCA	OPL - 17	TGACCCGCCT
OPP - 08	ACATCGCCCA	OPL - 18	GGCTTGGCCT
OPP - 09	GTGGTCCGCA	OPL - 19	GGGAAGGACA
OPP - 10	TCCCGCCTAC	OPL - 20	GACCCTAGTC

APPENDIX III

List of *Medicago* plants, which produced fruits after experimental hybridization in the field, with the number of F_1 individuals, which have survived:

1A 220/0800

4B 310/01 (4 F_1 plants)

4B 400/03 (11 F_1 plants)

4B 110/12 (1 F_1 plant)

4B 395/11

4B 500/0170

4B 780/02

4B 400/13

5A 500/0850

7A 440/1470 (7 F_1 plants)

7A 330/0930 (2 F_1 plants)

7A 290/0820

7A 410/3420

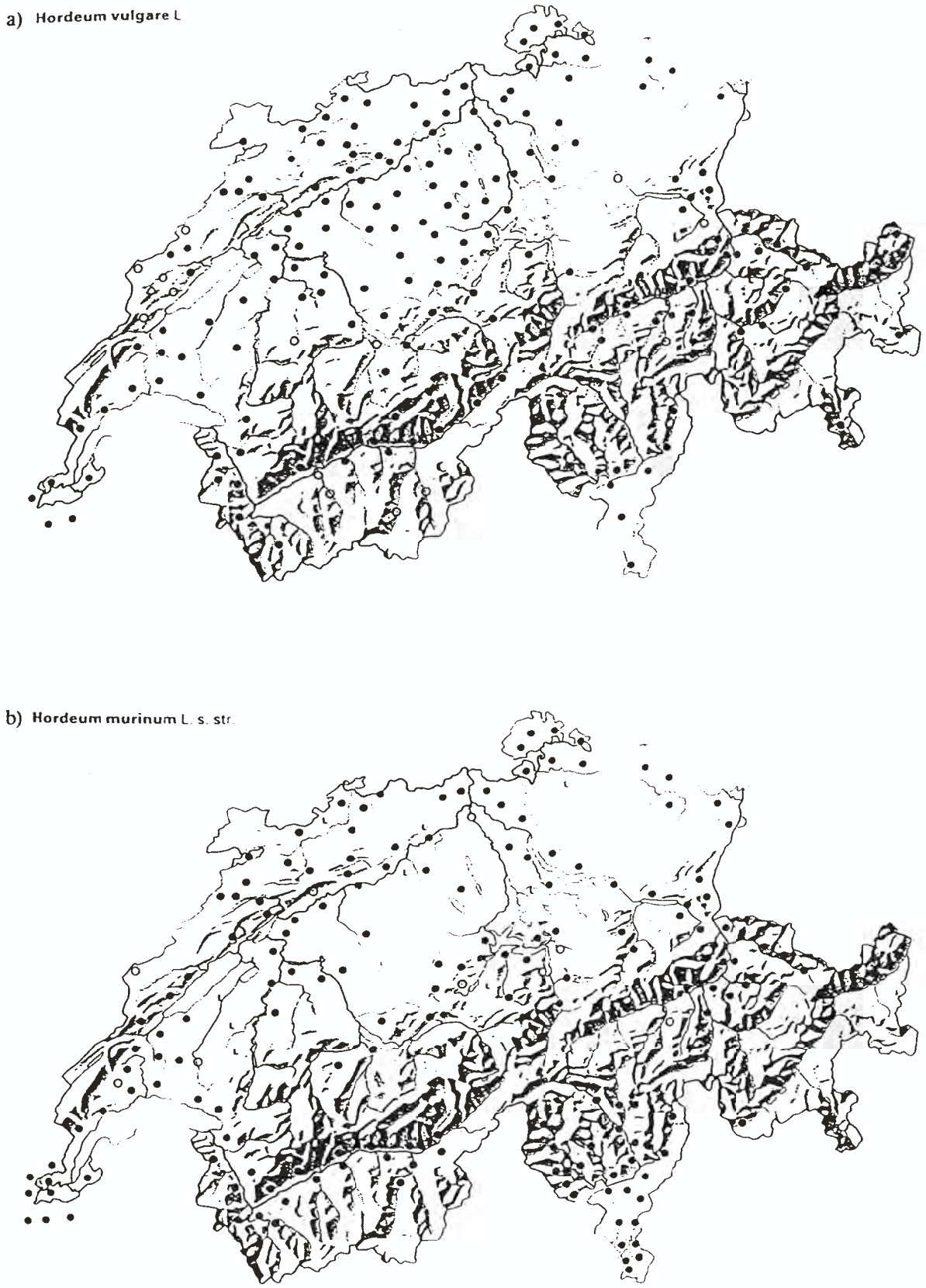
7A 390/171

7A 290/18

8B 270/05 (4 F_1 plants)

8B 300/07 (1 F_1 plant)

ANNEX

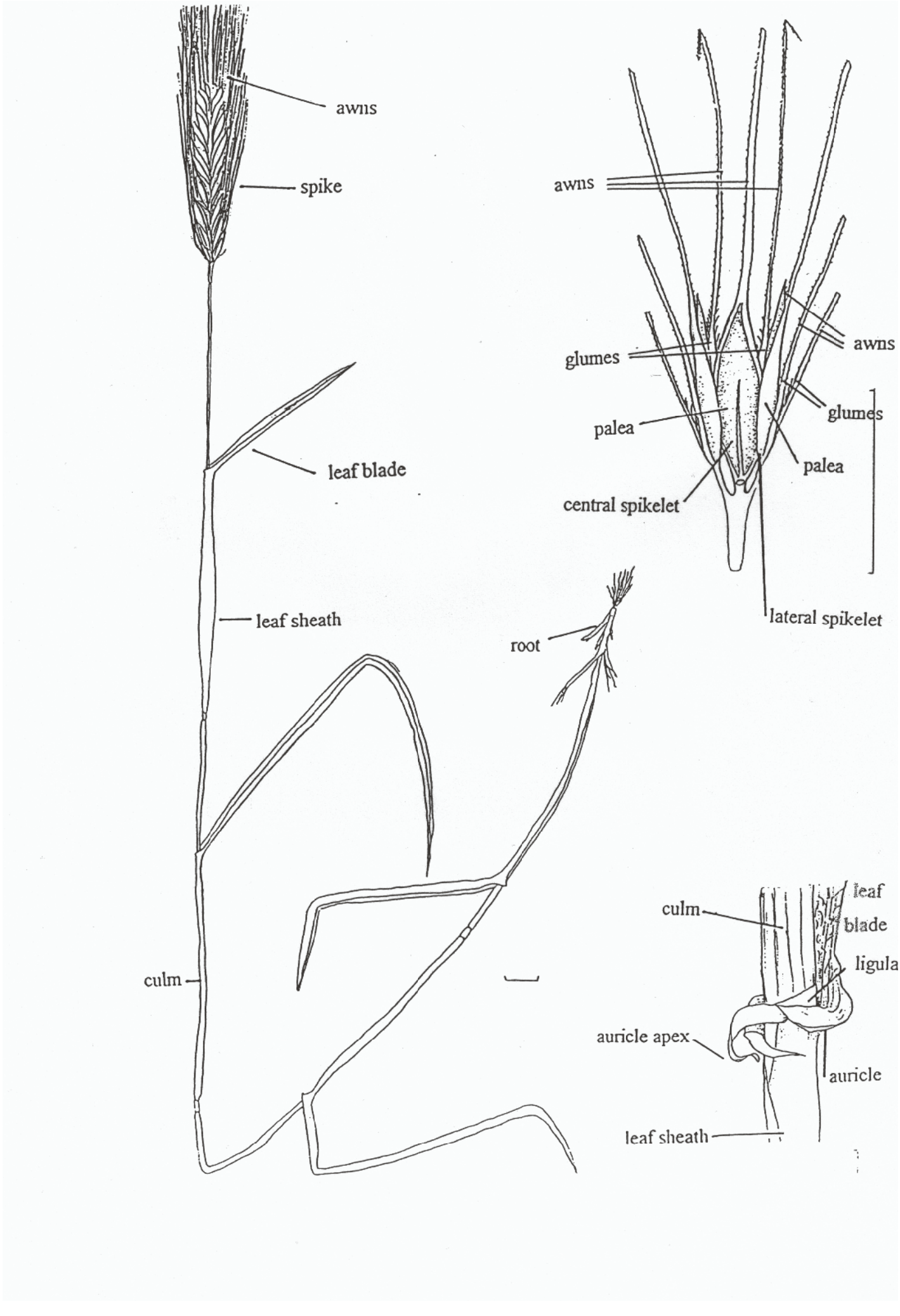


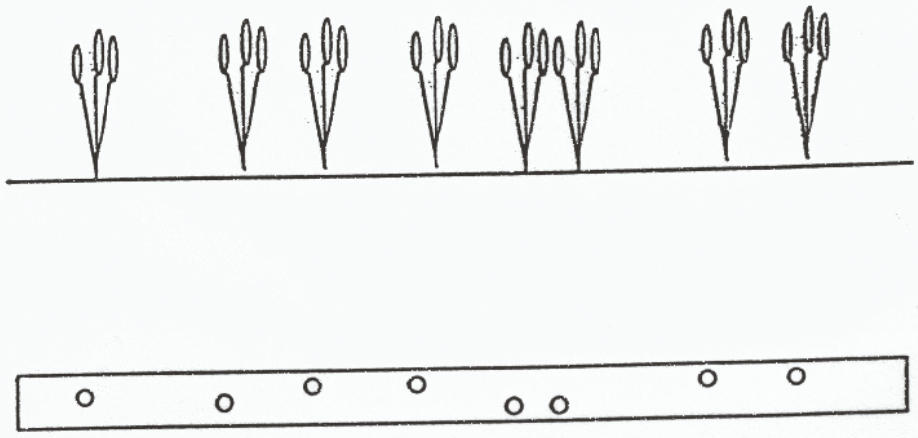
Map 1 (a, b). Distribution of *Hordeum vulgare* and *H. murinum* in Switzerland (from Welten and Sutter 1982).

a) **Medicago sativa** L.

b) **Medicago falcata** L.

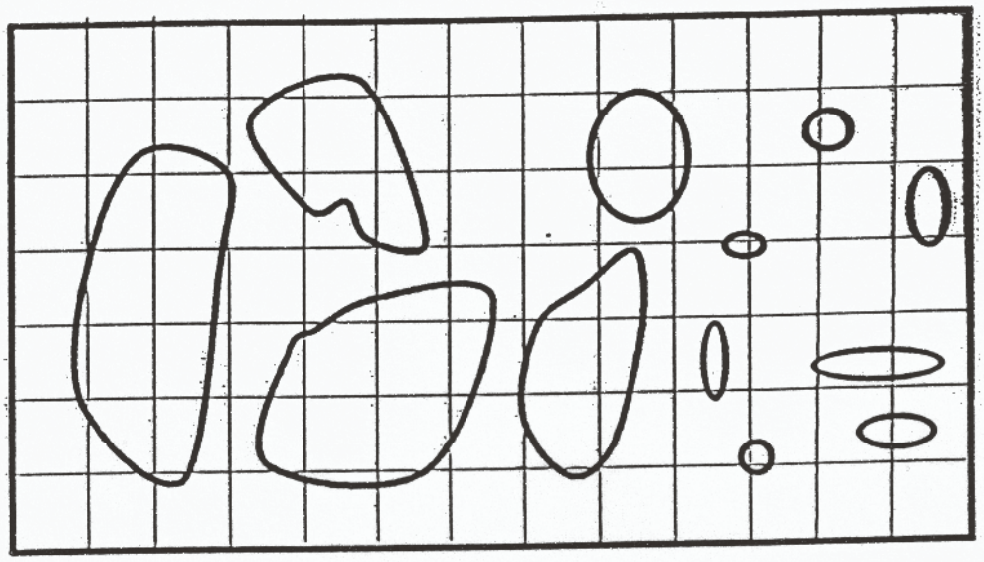
Map 2 (a, b). Distribution of *Medicago sativa* and *M. falcata* in Switzerland (from Welten and Sutter


awns
spike
leaf blade
leaf sheath
root
culm
awns
glumes
palea
central spikelet
awns
glumes
palea
lateral spikelet
culm
leaf
blade
ligula
auricle apex
auricle
leaf sheath



All the flowering plants are collected on a strip

Fig. 2. Sampling of *Hordeum*.



Sampling of the closest ramet on a grid of one meter by one meter.

Fig. 3. Sampling of *Medicago*.

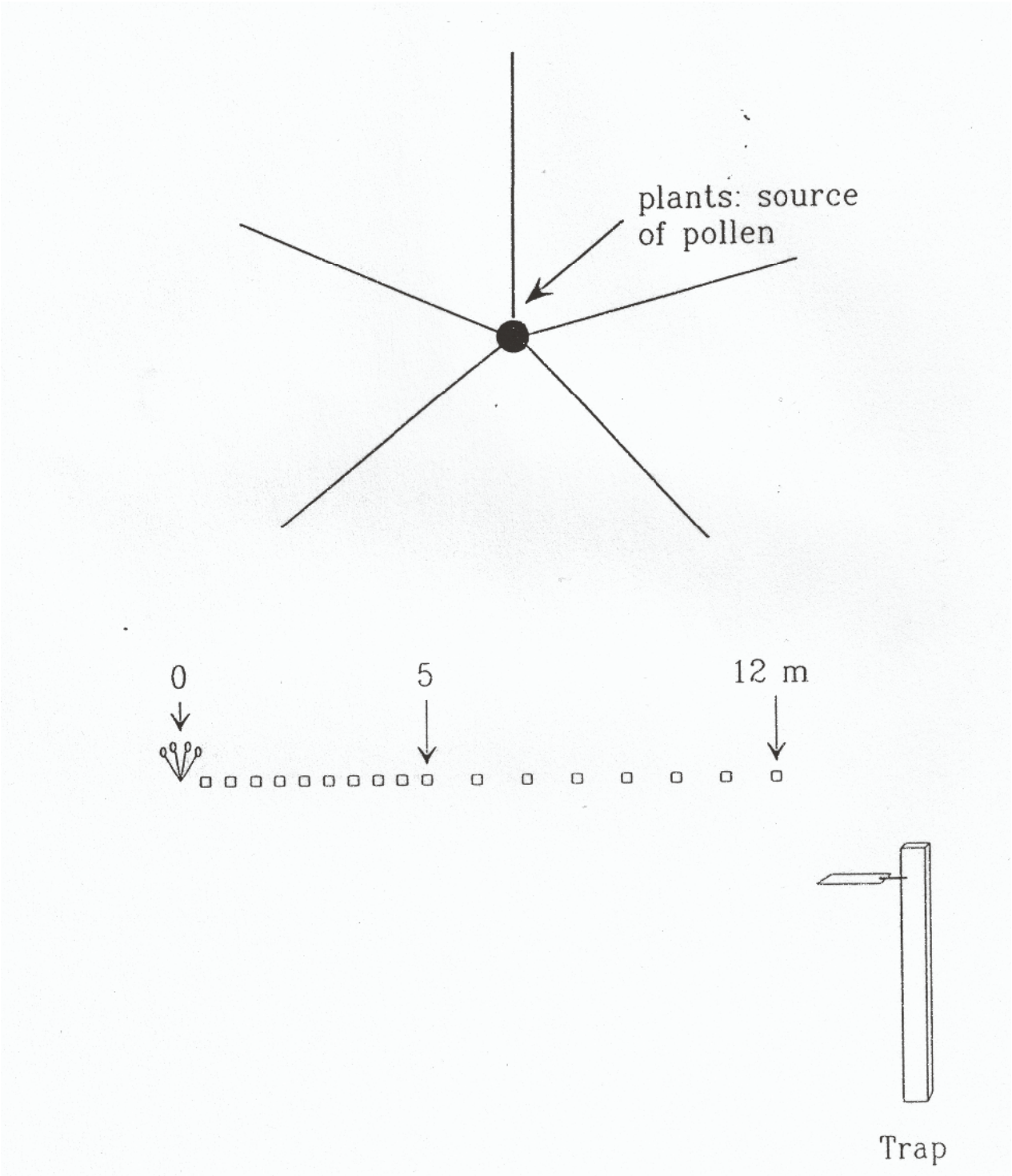


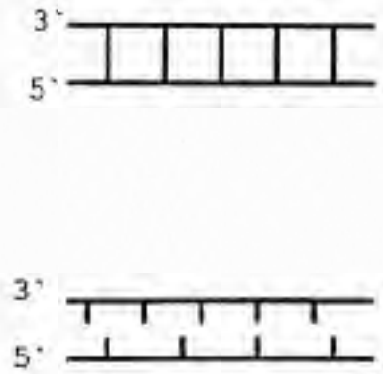
Fig. 4. Design of the experiment for measuring pollen dispersal of *Hordeum vulgare* and *H. murinum*. The pollen traps were placed on five lines up to 12 m from the plant source (on every 0.5 m up to 5 m and on every 1 m after the fifth meter up to 12 m).

PCR

1. Initial denaturation

94-97°C for 1-5 min

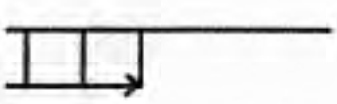
(Separating of the strands)



2. Annealing

40-70°C for 30s-5 min

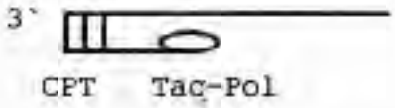
(annealing of the primer)



3. Extension

70-75°C for 3s-2 min

(amplification of target DNA)



4. Denaturation

90-95°C fo 20s-1 min

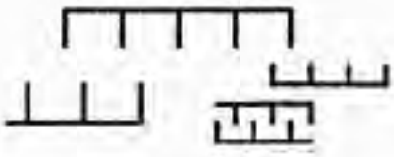
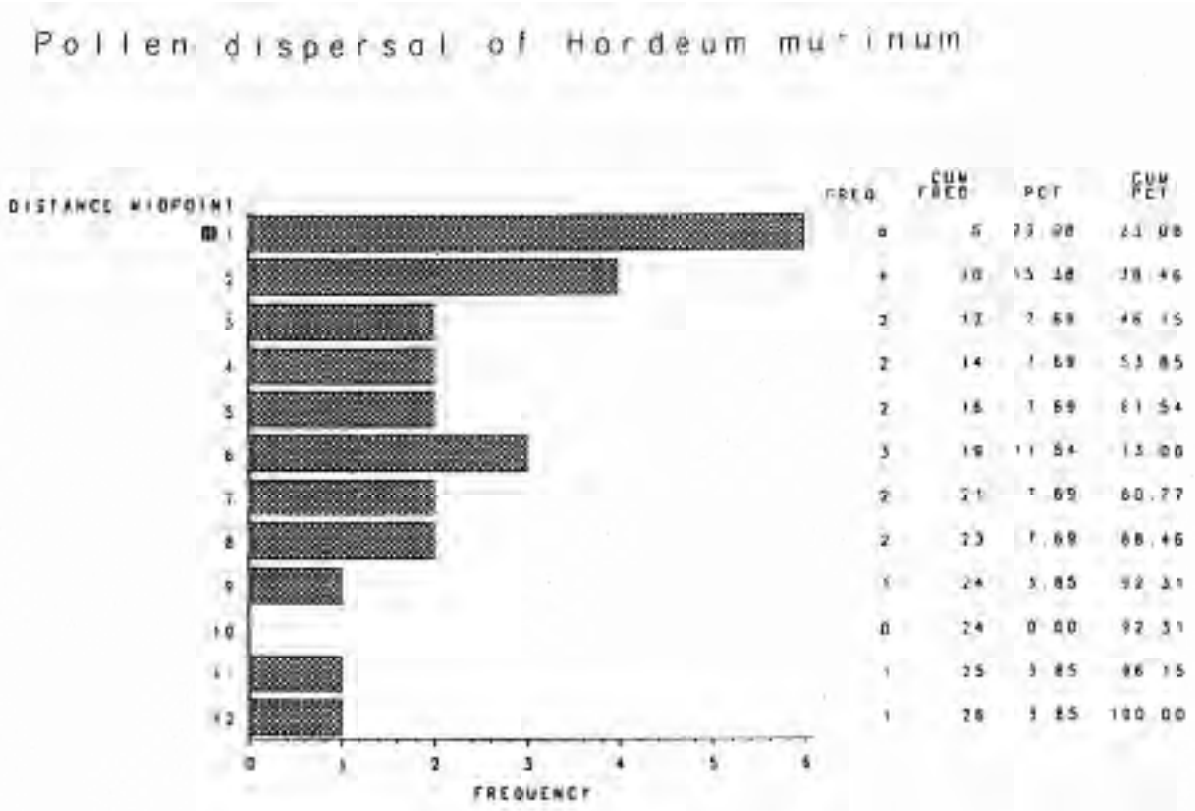


Fig. 5. Main steps of the Polymerase chain reaction (PCR)



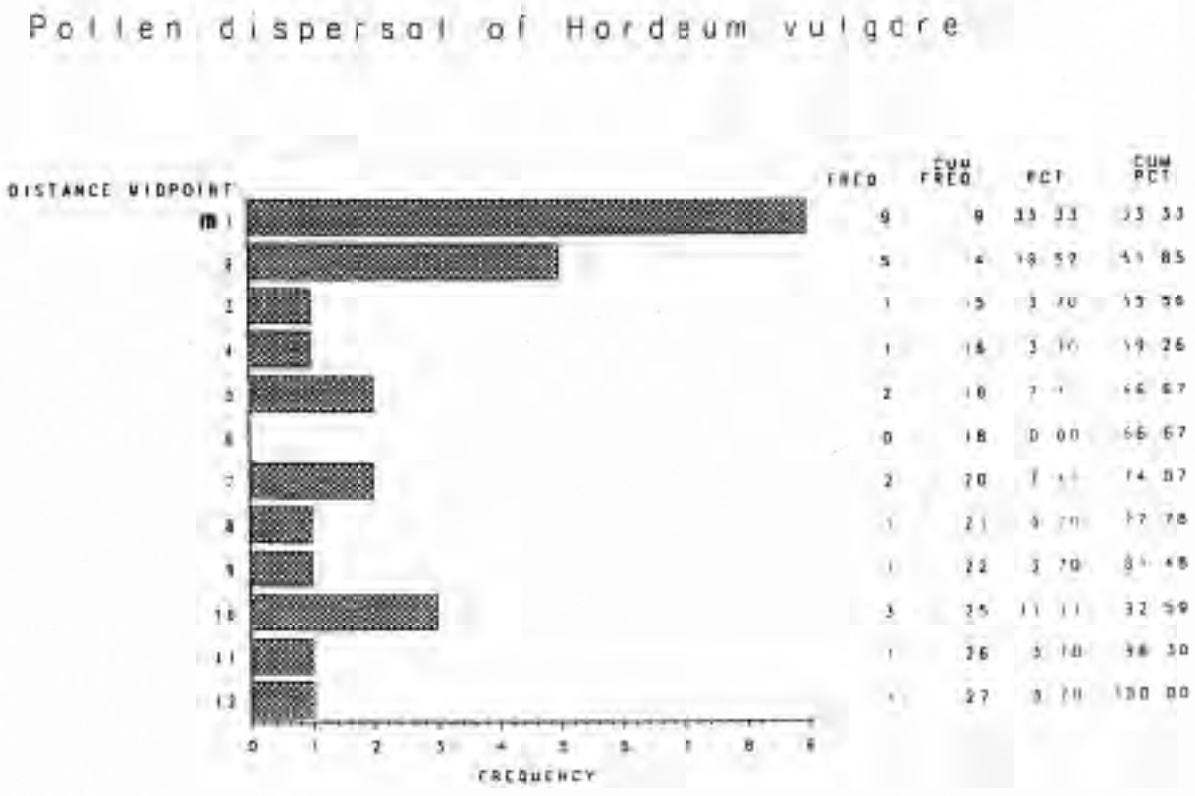


Fig. 6. Results from pollen dispersal experiment for *Hordeum murinum* and *H. vulgare*. Standard deviation of the distribution correspond to 5-6 m for *H. murinum* and 5-7 m for *H. vulgare*.

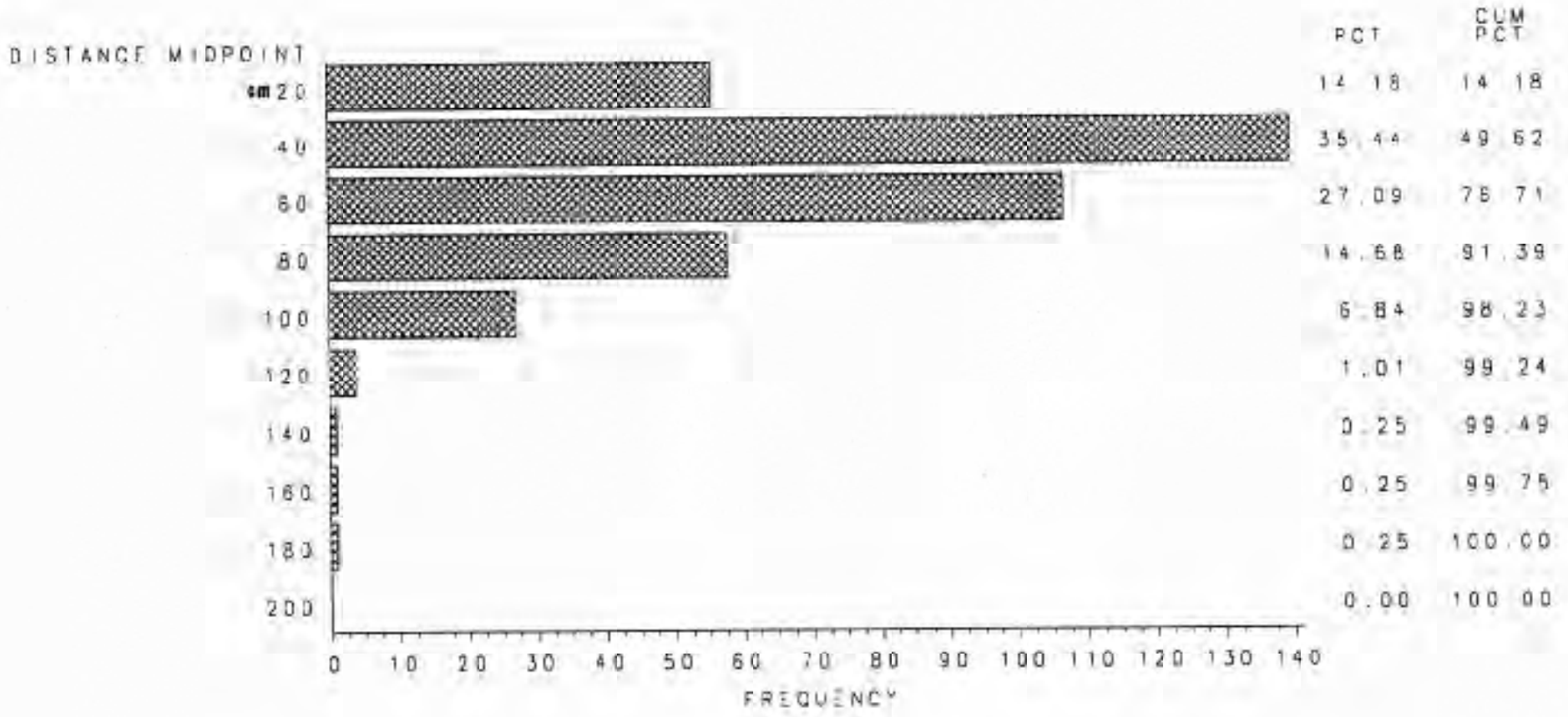


Fig. 7. Results from seed dispersal experiment for *Hordeum marinum*. Standard deviation of the distribution correspond to 40 - 60 cm.

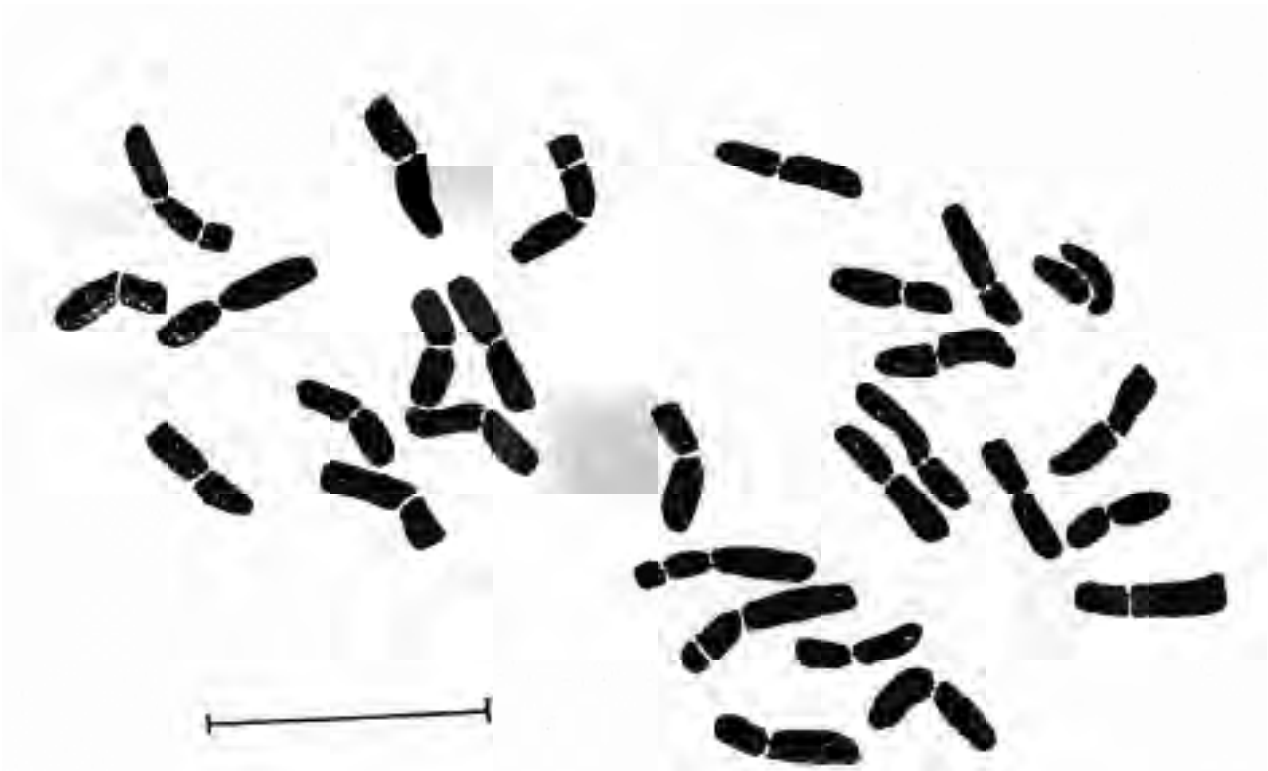


Fig. 8. *Hordeum murinum*, 2n = 28 somatic metaphase from germinating seeds, sampled in Zürich, Wasserfangen. Scale bar = 10µm.

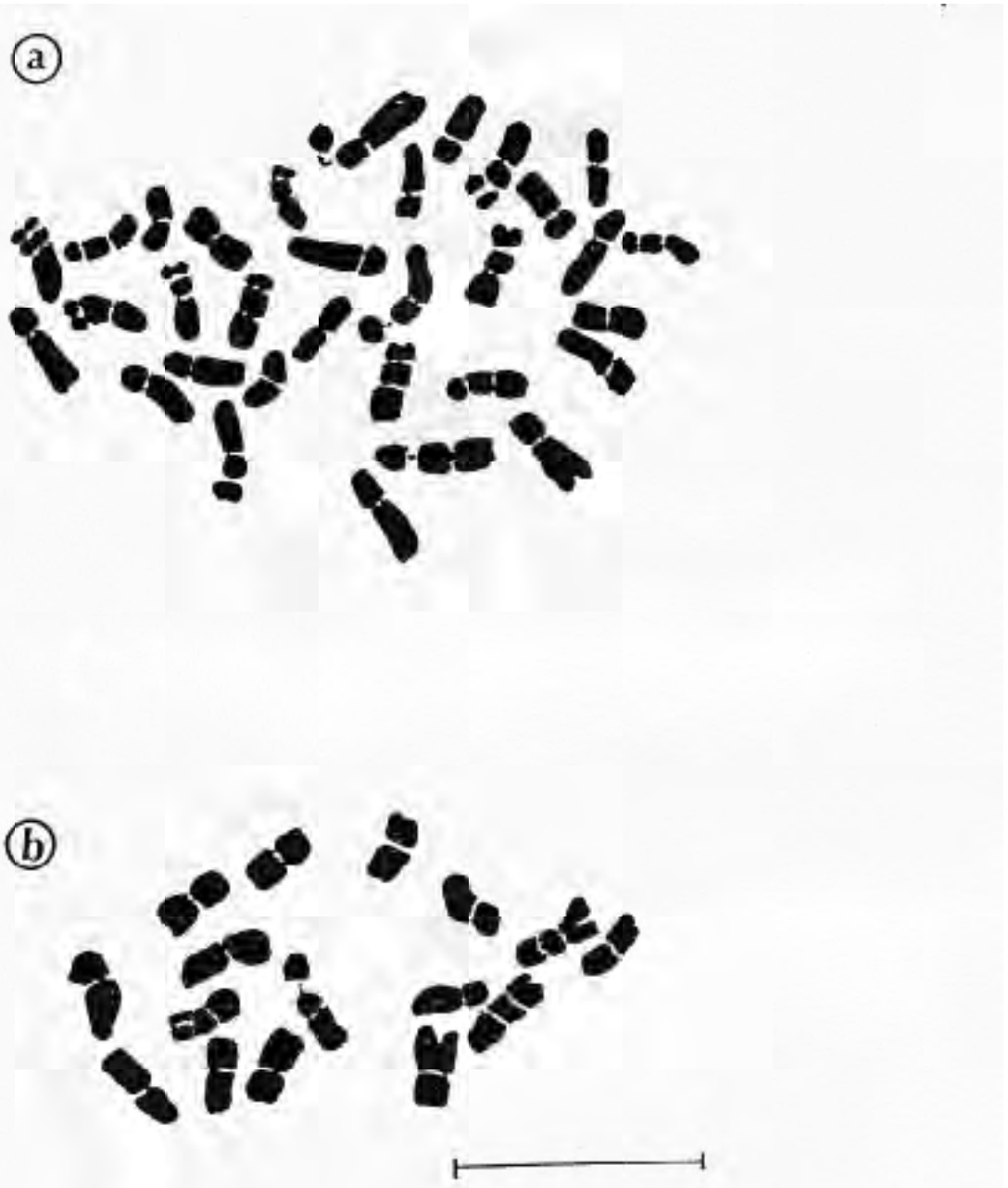


Fig. 9 Somatic metaphases from root tips: a) tetraploid *Medicago sativa*, 2n = 32 (Graubünden, Guarda, 13A 2/0); b) diploid *M. falcata*, 2n = 16 (Graubünden, Guarda, 13A 14.9/1.34). Scale bar = 10µm.

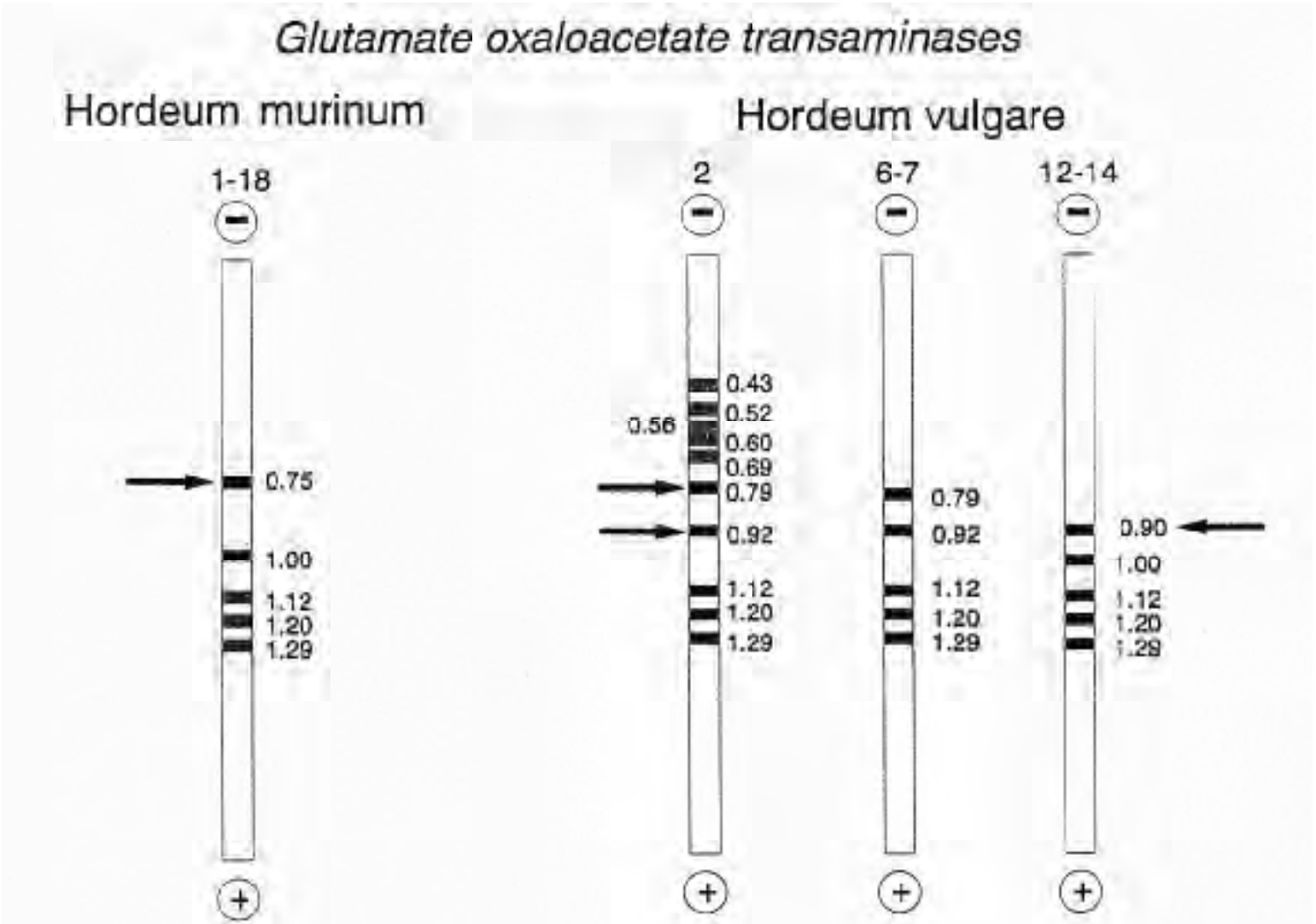


Fig. 10. Zymograms of glutamate oxaloacetate transaminase for *Hordeum murinum* and *H. vulgare*. Arrows show the specific bands for both species. Dark bands are the bands, present in all of the individuals of the population and the lighter ones are the bands, present only in some of the individuals.

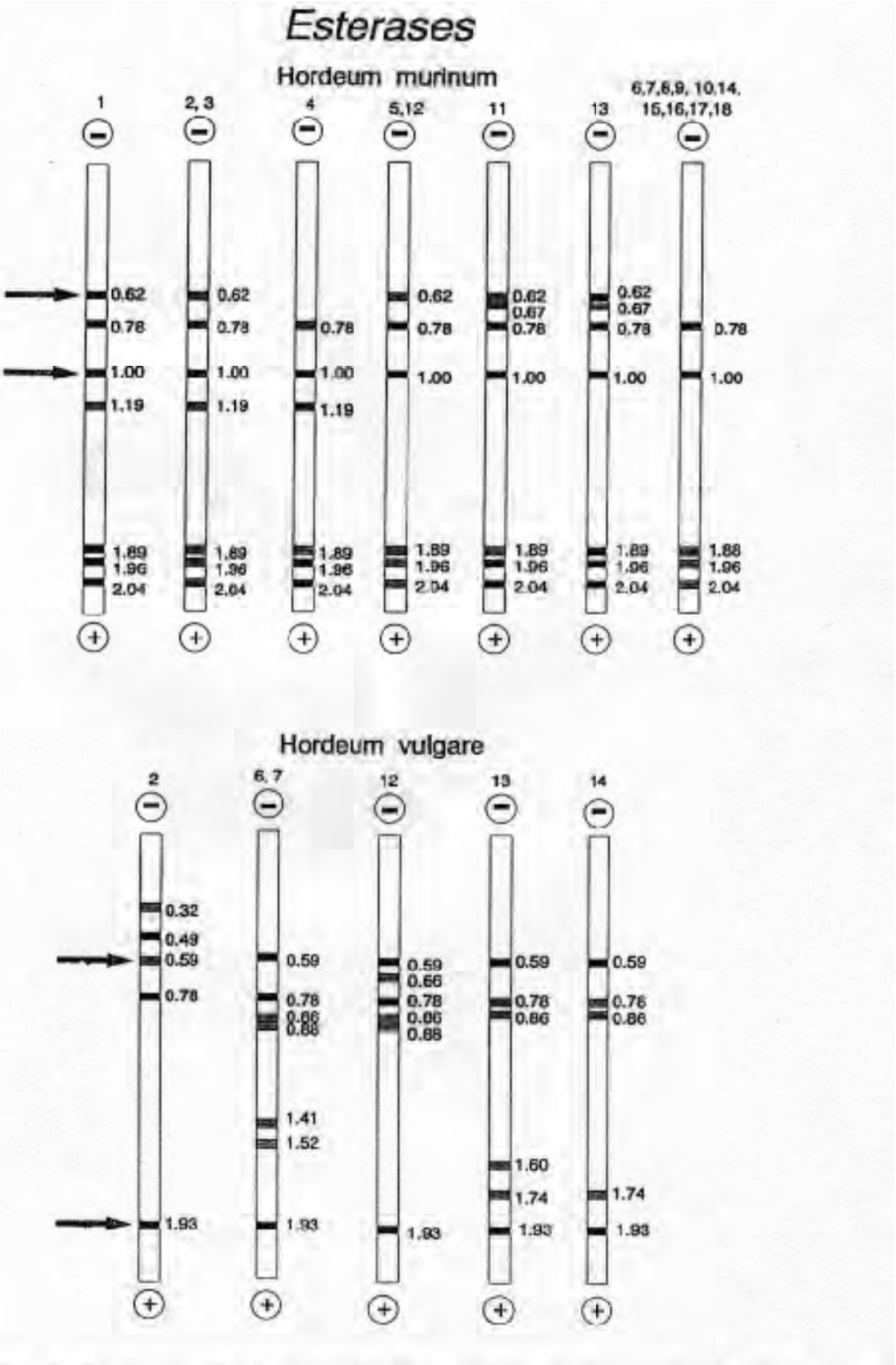


Fig. 11. Zymograms of *Hordeum murinum* and *H. vulgare* for esterases. Arrows show the specific bands for both species. Dark bands are the bands, present in all of the individuals of the population and the lighter ones are the bands, present only in some of the individuals.

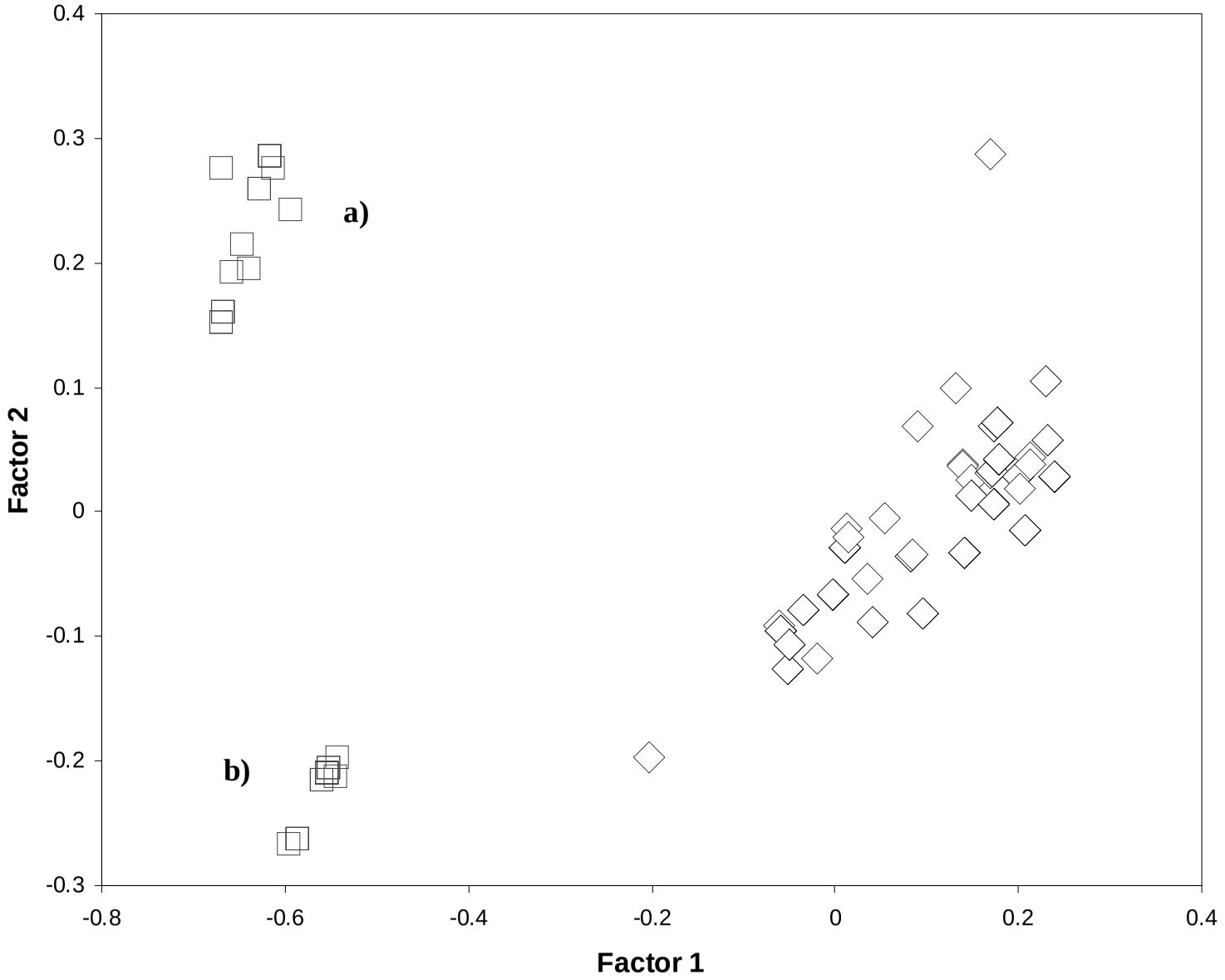


Fig. 12. Principal coordinate analysis for *H. murinum* (◊) and *H. vulgare* (□) with isozymes.
a) H. vulgare-pop. 2,6,7 ; b) H. vulgare-pop. 12, 13, 14.

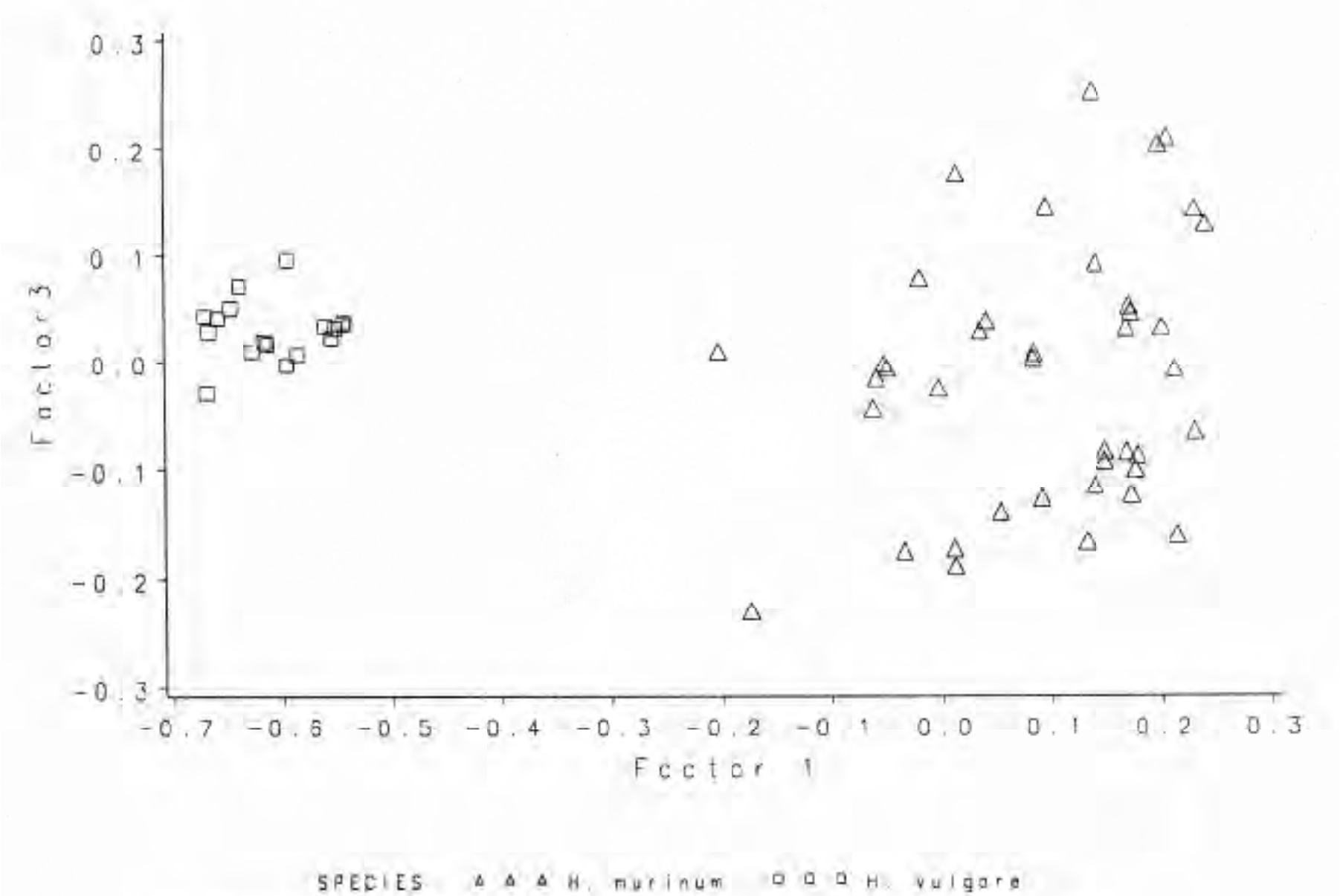


Fig. 12a. Principle Coordonate Analysis for *Hordeum murinum* and *Hordeum vulgare* with isozymes.

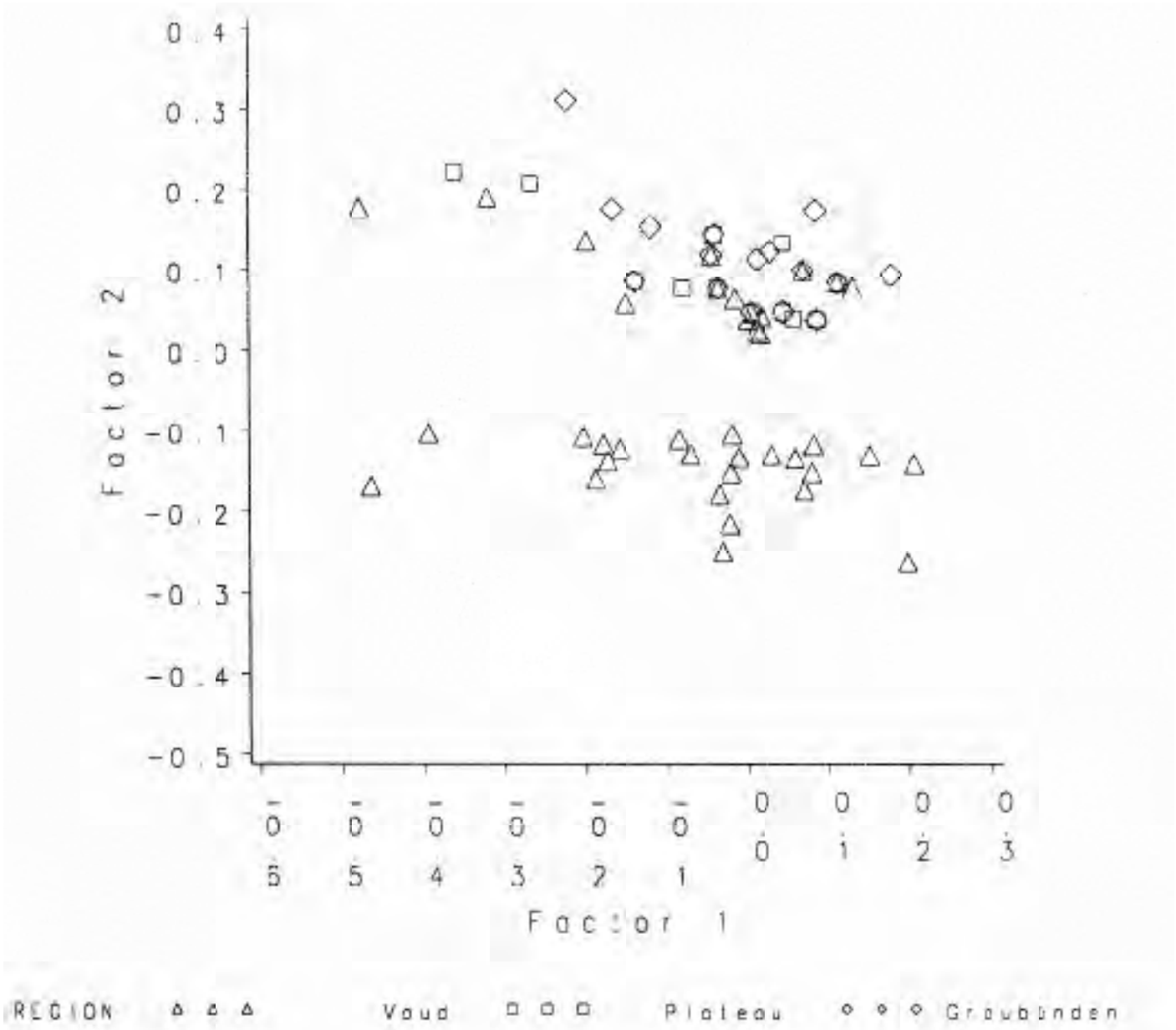


Fig. 13. Principal Coordinate Analysis for *Hordeum murinum* with isozymes, according to the sampling region.

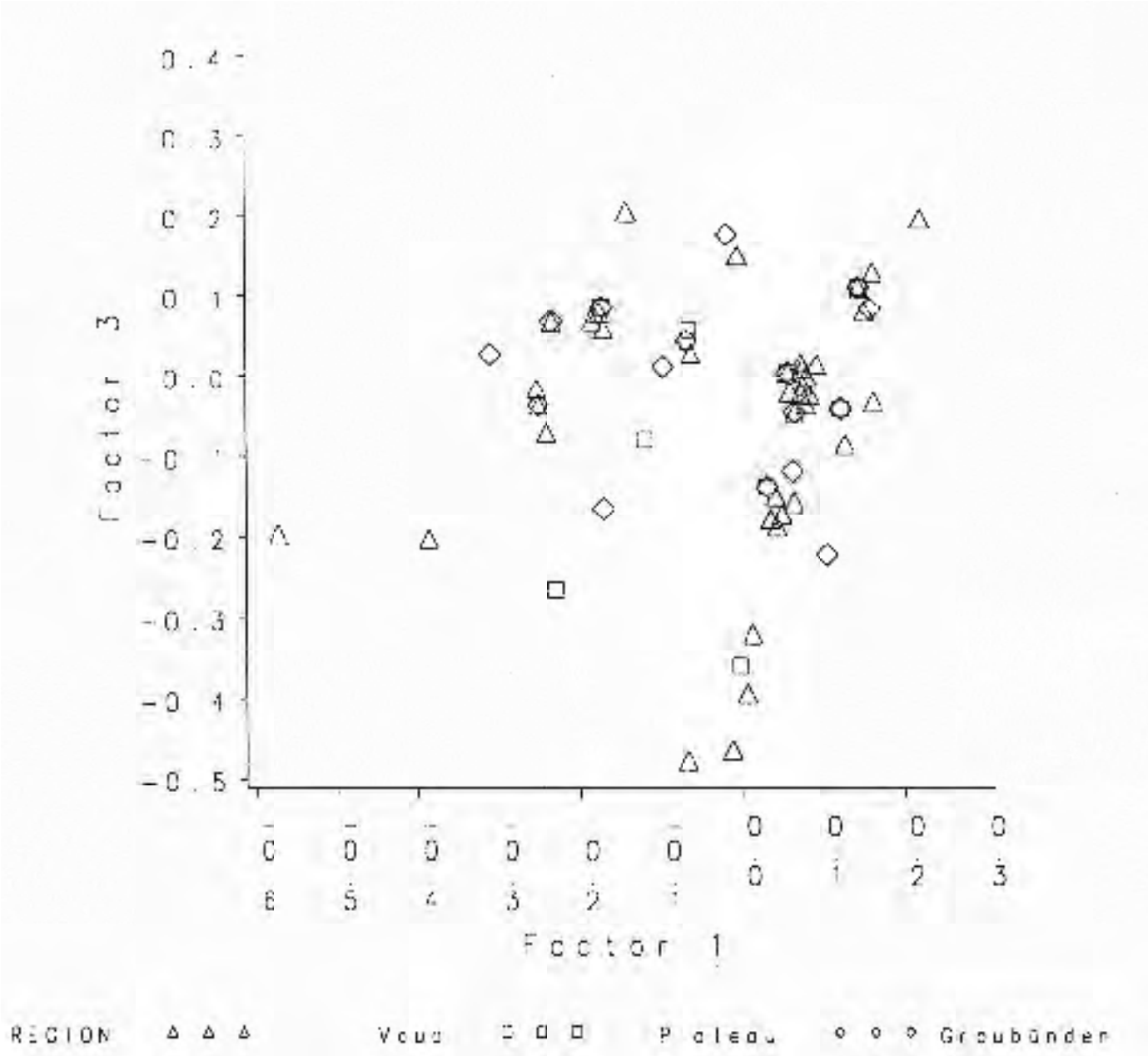


Fig. 13a. Principal Coordinate Analysis for *Hordeum marinum* with isozymes, according to the sampling region.

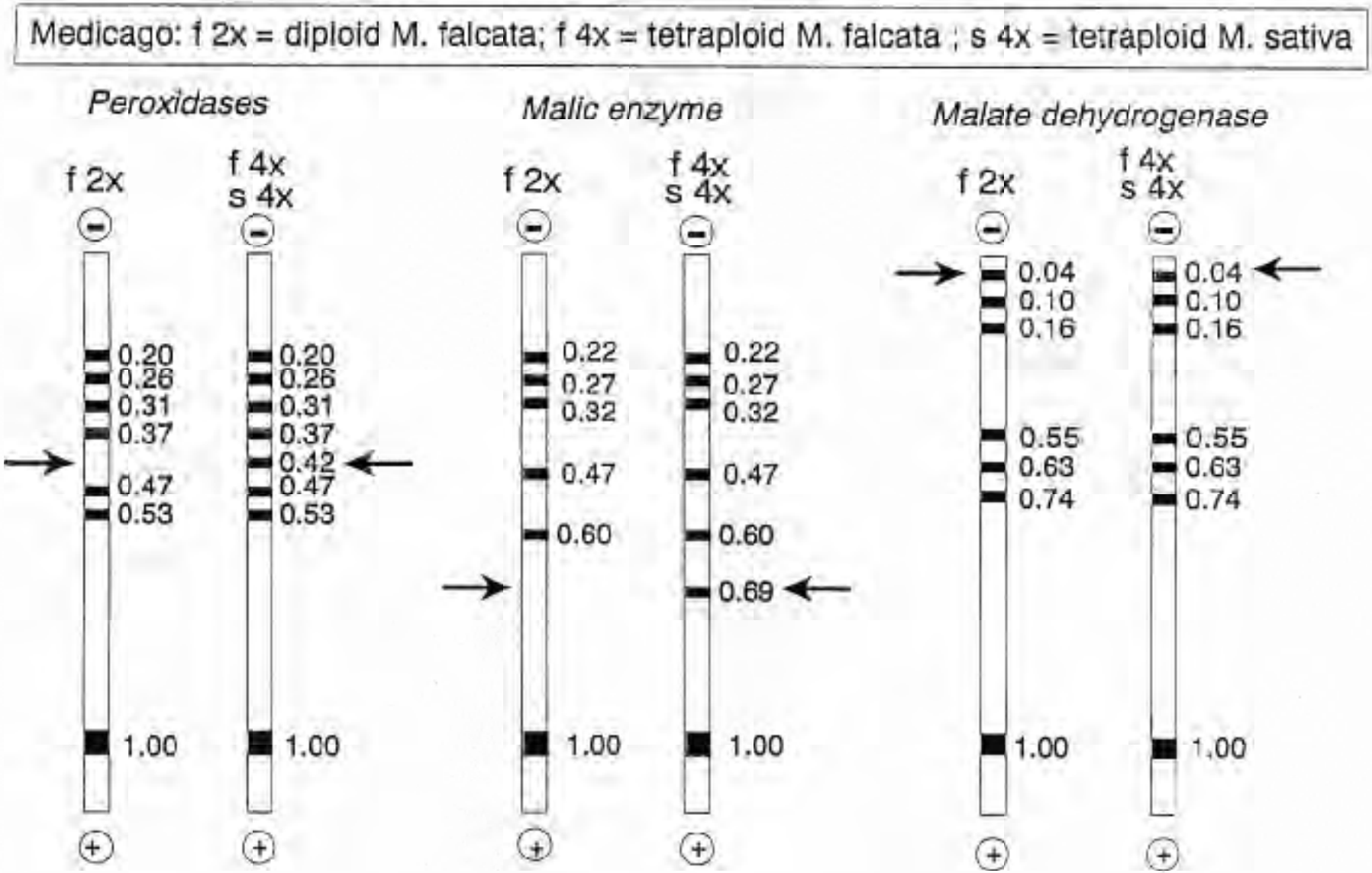


Fig. 14. Zymograms of peroxidase, maleic enzyme and malate dehydrogenase for *Medicago sativa* (s 4x), diploid (f 2x) and tetraploid (f 4x) *M. falcata*, and *M. xvaria* (together with s 4x and f 4x). The arrows show the specific bands for the both ploidy levels. The band at 0.04 for MDH is present only in the diploid *M. falcata* and plants of *M. sativa* from Unterengadin

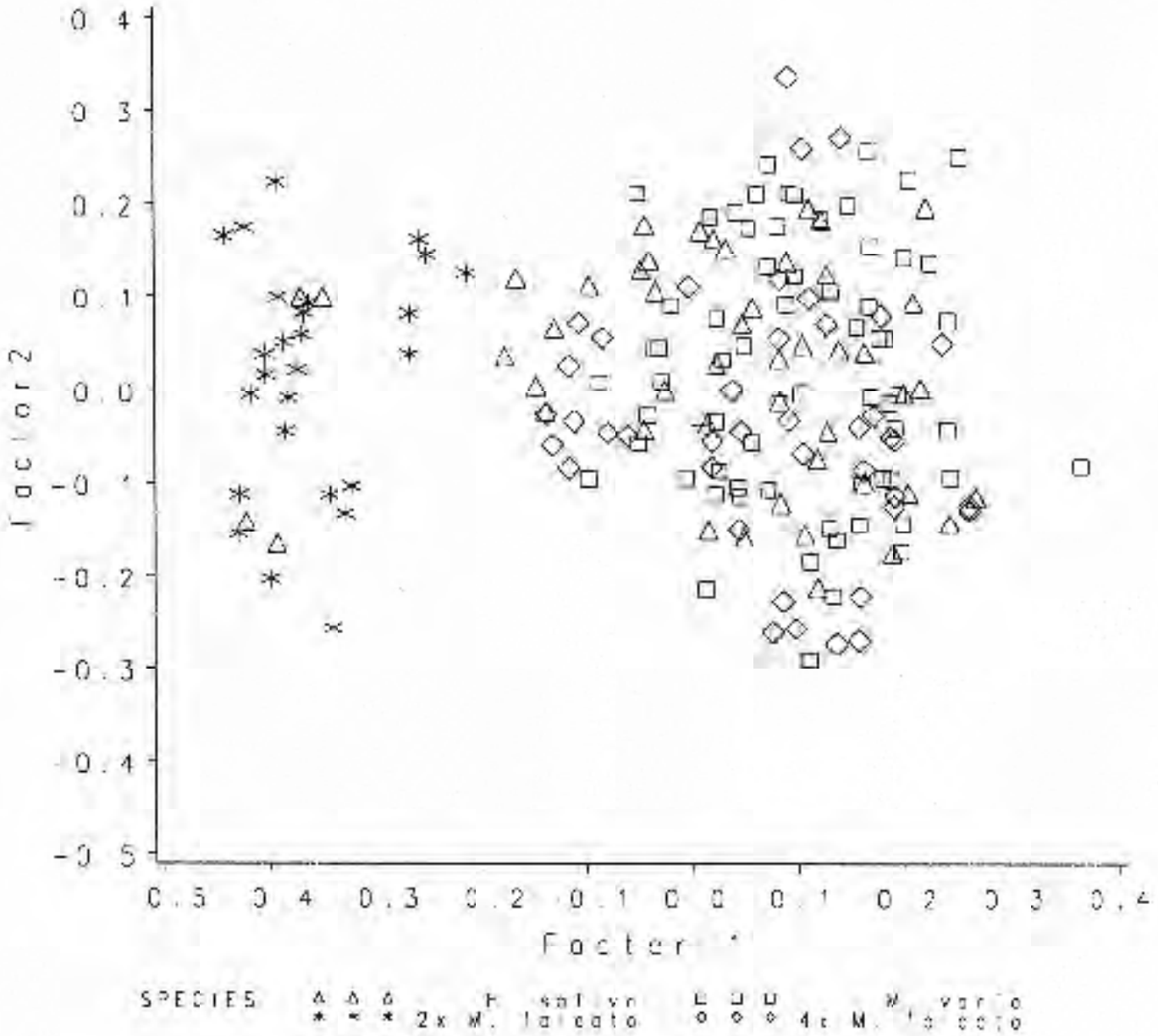


Fig. 15. Principle Coordinate Analysis for *Medicago sativa*, diploid and tetraploid *M. falcata* and *M. xvaria* with isozymes. Diploid *M. falcata* and *M. sativa* plants from Unterengadin are grouped separately from the tetraploid species.

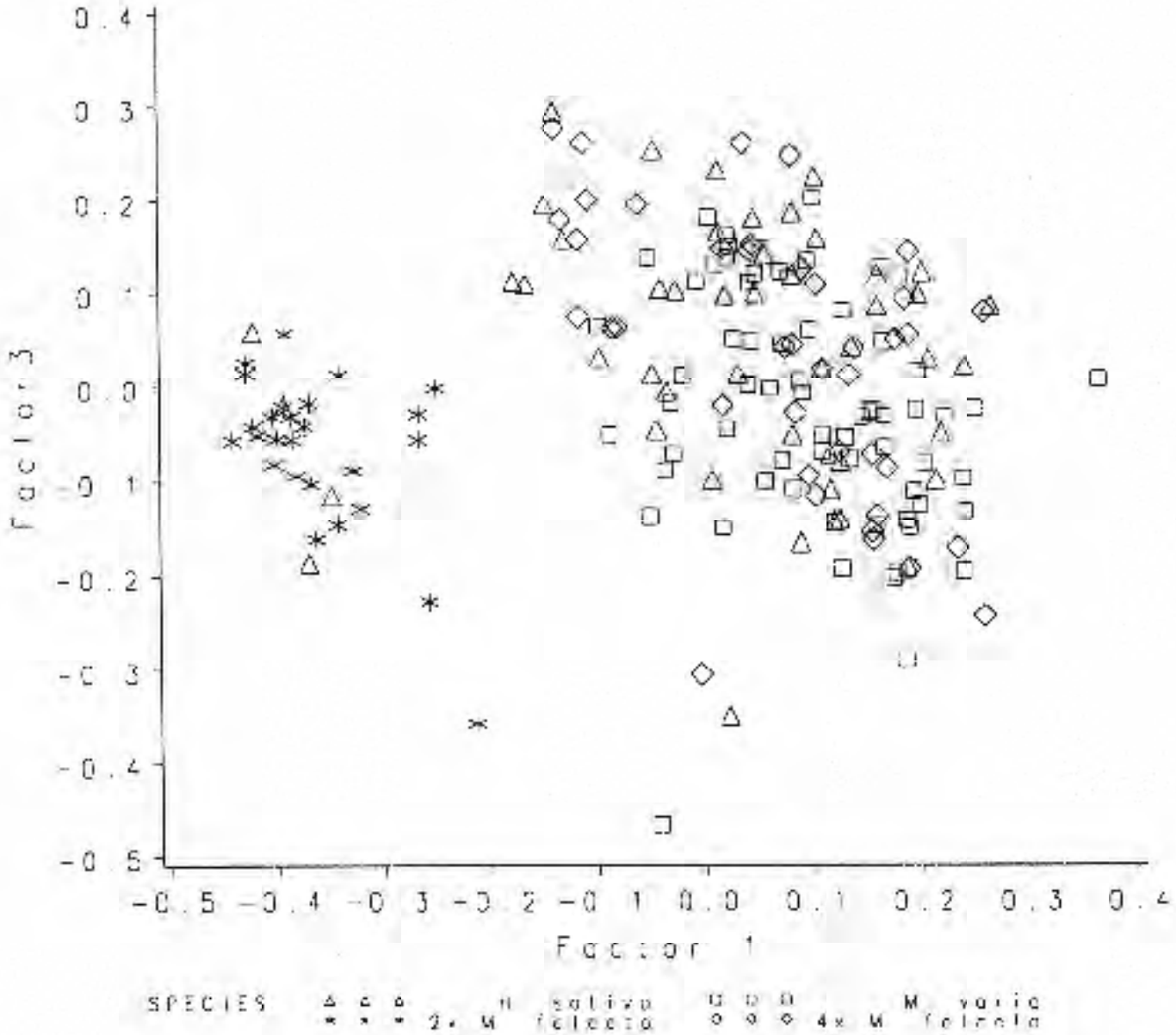


Fig. 15a. Principle Coordinate Analysis for *Medicago sativa*, diploid and tetraploid *M. falcata* and *M. xvaria* with isozymes. Diploid *M. falcata* and *M. sativa* plants from Unterengadin are grouped separately from the tetraploid species.

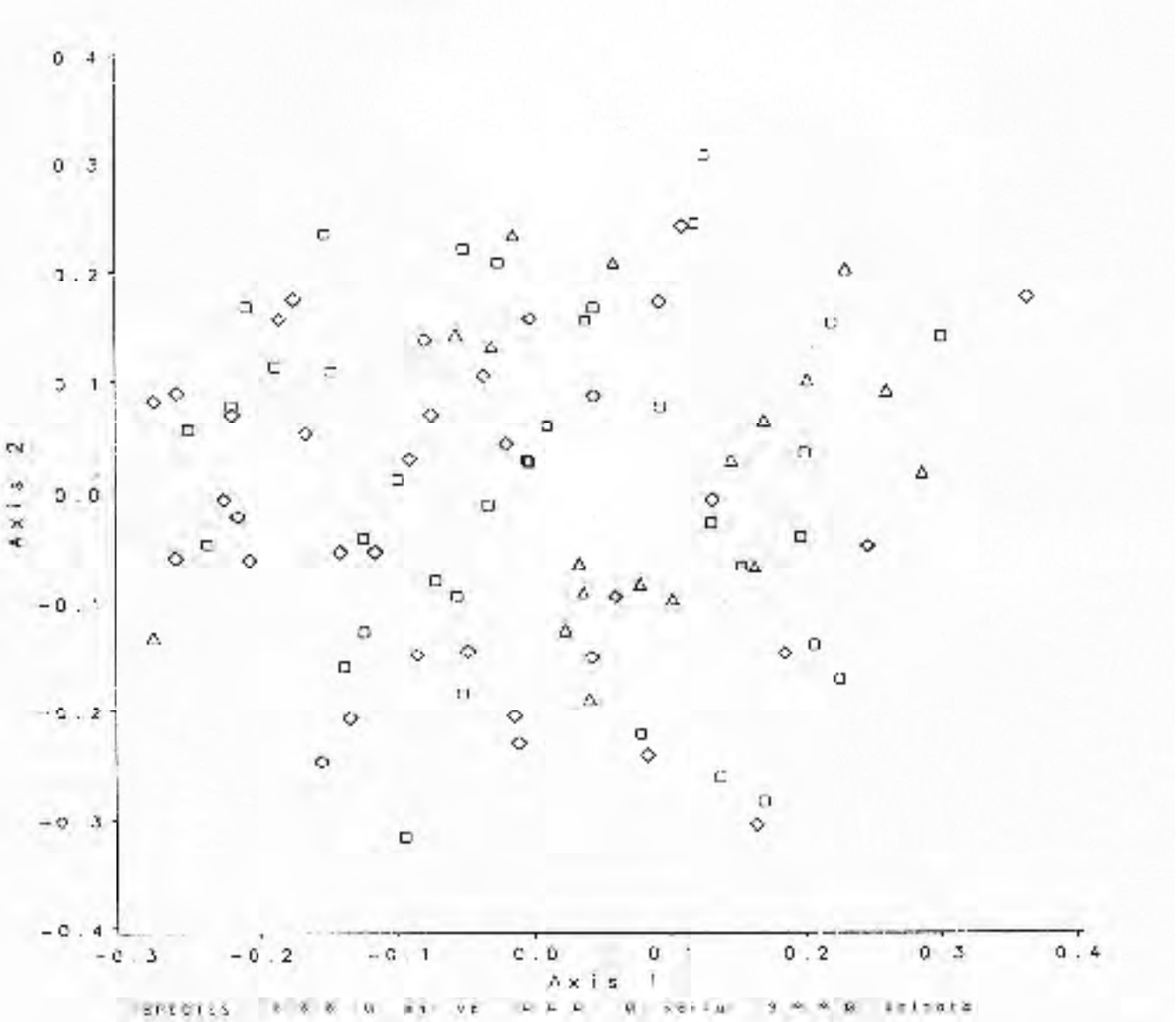


Fig. 16. Principal Coordinate Analysis for *M. sativa*, tetraploid *M. falcata* and *M. xvaria* (population 4), calculated with the RAPD data (with Primers OPP - 05, OPP - 06 and OPP - 08).

Table 1. Sampling locations of *Hordeum*.

POPULATION	LOCATION
1A	VAUD, Bonvillars, La Fuselle, 46°50`7 ``N, 6°39`52``E, pasture, 250 m
2A	VAUD, between Champagne and Fiez, sur Biolex, 46°50`0``N, 6°39`5``E, meadow between the road and a field with cultivated barley, 480 m
3AB	VAUD, Ferreyres, 46°39`34``N, 6°29`1``E, pasture in the vicinity of a field with cultivated barley, 560 m
4AB	VAUD, Ferreyres, 46°39`39``N, 6°29`15``E, in the village, 560 m
5AB	VAUD, Arnex, 46°41`40``N, 6°31`5``E, in the village, at the edge of an orchard, 540 m
6A	ZURICH, Wasterkingen, (D. Bülach), 47°35`24``N, 8°28`14``E, edge of the road, close to the field with cultivated barley, 390 m
7A	ZURICH, Ellikon am Rein (D. Andelfingen), 47°36`8``N, 8°36`17``E, in the village, edge of the road, 350 m
8AB	ZURICH, Truttikon, (D. Andelfingen), 47°37`47``N, 8°43`46``E, in the village, edge of the road, 480 m
9A	SCHAFFHAUSEN, Buch, Unterwiesen, 47°43`2``N, 8°47`33``E, edge of the road, 420 m
10A	THURGAU, Uesslingen, Aelikerberg, 47°35`5``N, 8°49`21``E, edge of the vineyard, 380 m
11A	VAUD, Champagne, 46°49`54``N, 6°39`20``E, edge of the village, between vineyard and a mown meadow, 450 m
12AB	VAUD, Grandson, en Bru, 46°49`33``N, 6°40`15``E, between the road and the field with cultivated barley, 450 m
13A	VALAIS, Leuk, Brentjong, 46°19`14``N, 7°39`4``E, close to a field with cultivated barley, 950 m
14A	GRAUBUNDEN, Domat/Ems, Plarenga, 46°50`21``N, 9°28`43``E, close to a filed with cultivated barley, 580 m
15A	GRAUBUNDEN, Domat/Ems, Vegnader, 46°50`3``N, 9°28`42``E, field margin, 600 m
16AB	GRAUBUNDEN, Jenins, Eichholz, 46°59`47``N, 9°32`57``E, vineyard, 520 m
17AB	GRAUBUNDEN, Maienfeld, Müllager, 46°59`53``N, 9°32`48``E, vineyard, 540 m
18A	GRAUBUNDEN, Jenins, 47°00`3``N, 9°33`37``E, in the village, 630 m

Table 2. Sampling locations of *Medicago*

POPULATION	LOCATION
1AB	VALAIS, Orsières, Cenaire, 577.700/97.000, 1000 m
1C	VALAIS, Orsières, Cernaire, 577.700/96.700, 1000 m
2A	VALAIS, Orsières, Cernaire, 577.775/96.675, 1000 m
3A	VALAIS, Orsières, Fin, 576.650/97.025, 1020 m
4AB	GRAUBUNDEN, Bonaduz, Tadi, 749.490/186.050, 560 m
5AB	GRAUBUNDEN, Malans, Bau, 763.300/205.250, 570 m
6A	GRAUBUNDEN, Seewis, station Seewis-Valzeina, 766.775/205.175, 595 m
7AB	ZURICH, Flaach, Thurhus, 687.800/271.575, 350 m
8AB	SCHAFFHAUSEN, Stetten, Rotacker, 691.875/288.650, 580 m
9A	VALAIS, Stalden, Ribe, 634.500/125.600, 780 m
10AB	VALAIS, Martigny, channel of the Drance, 571.660/106.300, 465 m
11A	GRAUBUNDEN, Zernez, Sosa., 802.700/175.700, 1460 m
12A	GRAUBUNDEN, Ramosch, Chants., 825.550/191.750, 1400 m
12B	GRAUBUNDEN, Ramosch, Chants., 825.225/191.725, 1350 m
13A	GRAUBUNDEN, Guarda, Suot la Sassa, 807.225/183.650, 1450 m

Table 3. Flowering phenology of *Hordeum murinum*: score.
Populations from Valais flower before those of Swiss Plateau and the last flowering populations are from Graubünden. G - Graubünden, M - Swiss Plateau, VS - Valais.

	pop	n	mean	P25	P50	P75
G	17	10	2.21	1.75	2.10	2.50
G	14	10	2.34	2.00	2.32	2.60
M	12	10	2.37	1.83	2.60	2.83
G	18	10	2.39	1.86	2.28	3.00
G	15	10	2.43	2.00	2.45	2.80
G	16	10	2.48	2.17	2.38	2.80
M	9	10	2.75	2.42	2.58	3.25
M	11	9	2.86	2.50	2.67	3.14
M	7	10	2.91	2.67	2.78	3.25
M	2	10	3.00	2.60	2.94	3.38
M	5	10	3.01	2.78	3.00	3.40
M	4	10	3.02	2.78	3.00	3.43
M	6	10	3.05	2.71	3.00	3.20
M	8	10	3.08	2.60	3.00	3.33
M	10	10	3.10	3.00	3.29	3.40
M	3	10	3.31	2.86	3.25	3.75
M	1	9	3.34	3.00	3.43	3.67
VS	13	2	3.40	3.00	3.40	3.80

Table 4. Description of localities and results of the chromosome counts

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Graubünden, Bonaduz, Tadi, 560 m, 749.490/186.050; tetraploid *M. falcata*, 2n=32 (4a 26/1, 4a 0.2/14, 4a 22/1.5, 4a 13.1/1, 4b 5/2.7); tetraploid *M. sativa*, 2n=32 (4a 17/0, 4a 6.90/0.10, 4a 19.60/0); tetraploid *M. xvaria*, 2n=32 (4a 21.2/3, 4a 11.0/1, 4b 3.9/7.1, 4b 10/7.1)

Graubünden, Malans, Bau, 570 m, 763.300/205.250; tetraploid *M. falcata*, 2n=32 (5a 3.2/7.4); tetraploid *M. sativa*, 2n=32 (5a 0.2/6.8, 5a 3/11, 5a 1.7/1, 5a 3.65/0.20, 5a 6.20/1.60, 5a 0.9/4, 5a 0.1/2.9)

Graubünden, Seewis, station Seewis-Valzeina, 595 m, 766.775/205.175; tetraploid *M. falcata*, 2n=32 (6a 1.6/16); tetraploid *M. sativa*, 2n=32 (6a 1.5/24.4, 6a 2.4/3.6)

Graubünden, Zernez, Sosa., 1460 m, 802.700/175.700; diploid *M. falcata*, 2n=16 (11a 8.95/3.3, 11a 19.6/4.70, 11a 16.7/3.25, 11a 10/5, 11a 3.9/2.3, 11a 4.65/2.37, 11a 4.68-3.27, 11a 18.6/4.7);

Graubünden, Ramosch, Chants., 1400 m, 825.550/191.750; diploid *M. falcata*, 2n=16 (12a 2, 12a 4, 12a 8)

Graubünden, Ramosch, Chants., 1350 m, 825.225/191.725; diploid *M. falcata*, 2n=16 (12b 2, 12b 3, 12b 6, 12b 7, 12b 8, 12b 9, 12b 10)

Graubünden, Guarda, Suot la Sassa, 1450 m, 807.225/183.650, diploid *M. falcata*, 2n=32 (13a 13.2/2.05, 13a 4.3/42, 13a 15.2/3.55, 13a 15.6/2.4, 13a 16.05/1.2, 13a 8/2.7, 13a 2.8/2.54, 13a 4.3/4.25, 3a 18.55/2.3, 13a 19.3/0.8, 13a 13.4/1.2, 13a 17.6/2.85, 13a 12/26, 13a 12.8/2.2, 13a 13.8/2.2, 13a 14.9/1.34, 13a 18/1.15, 13a 4.6/1.4, 13a 15.5/2.4, 13a 0.7/1.3, 13a 13.8/2.2), tetraploid *M. sativa*, 2n=32 (13a 0/0, 13a 2.0/0, 13a 2.8/0, 13a 5/0, 13a 6/0, 13a 9/0, 13a 11/0, 13a 12/0, 13a 13/0, 13a 16/0, 13a 17/0, 13a 19/0, 13a 20/0, 13a 28/0)

Table 4. (continued)

Valais, Martigny, channel of the Drance, 465 m, 571.660/106.300, tetraploid *M. falcata*, 2n=32 (10a 1.0/14.0, 10a 4.3/4.4); tetraploid *M. sativa*, 2n=32 (10a 1.0/0, 10b 1/1); tetraploid *M. xvaria*, 2n=32 (10a 1.1/9.2, 10a 3.1/4.1, 10a 1.1/1)

Valais, Orsières, Cenaire, 1000 m, 577.700/97.000; tetraploid *M. falcata*, 2n=32 (1a 3/10.9, 1a 1.7/12, 1a 1.7/12, 1a 3/8, 1a 2.2/8)

Valais, Orsières, Cenaire, 1000 m, 577.700/96.700; tetraploid *M. sativa*, 2n=32 (1c 4/1.6, 1c 9/3, 1c 4.7/6.3)

Valais, Orsières, Cenaire, 1000 m, 577.775/96.675; tetraploid *M. falcata*, 2n=32 (2a 0.5/10.6, 2a 17.8/5.86, 2a 11.7/7.2, 2a 16.35/5.5); tetraploid *M. sativa*, 2n=32 (2a 4.1/6.9); tetraploid *M. xvaria*, 2n=32 (2a 15.7/6.8, 2a 17.8/5.6)

Valais, Orsières, Fin, 1020 m, 576.650/97.025, tetraploid *M. falcata*, 2n=32 (3a 0/3.88, 3a 1.6/3.8); tetraploid *M. sativa*, 2n=32 (3a 1.2/0.5, 3a 4/7, 3a 31.80/20.0, 3a 1.2/0.5, 3a 7.7/9.3); Valais, Orsières, Fin, 1020 m, 576.650/97.025, tetraploid *M. xvaria*, 2n=32 (3a 7.9/8.3)

Valais, Stalden, Ribe, 780 m, 634.500/125.600, tetraploid *M. falcata*, 2n=32 (9a 0.75/2.2, 9a 4.3/20.2); tetraploid *M. sativa*, 2n=32 (9a 6.3/9)

Schaffhausen, Stetten, Rotacker, 580 m, 691.875/288.650, tetraploid *M. falcata*, 2n=32 (8b 3.0/8.3, 8b 2.7/5.0); tetraploid *M. sativa*, 2n=32 (8a 0.4/11.3, 8a 1.2/1, 8a 1/8); tetraploid *M. xvaria*, 2n=32 (8a 1.9/11.0)

Table 4. (continued)

Zürich, Flaach, Thurhus, 350 m, 687.800/271.575, tetraploid *M. falcata*, 2n=32 (7a 1/8, 7a 3.7/1.3, 7a 3.65/8.2); tetraploid *M. sativa*, 2n=32 (7b 32.30/2); tetraploid *M. xvaria*, 2n=32 (7a 39/50)

Table 5. Presence of the isozyme bands (in %)/population for *H. murinum*. VD -Vaud, VS - Valais, SH - Schaffhausen, GR - Graubünden, TH - Thurgau, ZH - Zürich. GOT - glutamate oxaloacetate transminase, EST - esterase.

BAND POP	GOT A	GOT B	GOT C	GOT D	GOT E	EST A	EST AA	EST B	EST C	EST D	EST E	EST F	EST G
1 VD	98.2	98.2	0	0	96.4	100	0	87.5	100	16.1	100	100	100
2 VD	98.5	98.5	31.8	19.7	80.3	97	0	98.5	100	27.3	92.4	95.5	95.5
3 VD	98.3	98.31	13.6	13.6	89.8	74.6	0	96.6	98.3	27.1	94.9	96.6	96.6
4 VD	100	100	13.8	13.8	79.3	0	0	93.1	98.3	98.3	79.3	96.6	96.6
5 VD	100	100	67.2	67.2	98.3	94.8	0	94.8	94.9	0	55.2	91.4	91.4
6 ZH	100	100	33.3	33.3	95.8	0	0	100	100	0	66.7	91.7	100
7 ZH	100	100	2.6	12.8	97.4	0	0	100	100	0	38.5	97.4	97.4
8 ZH	100	100	39.7	38.1	87.3	0	0	100	100	0	61.9	98.4	98.4
9 SH	100	100	60.7	7.1	71.4	0	0	100	100	3.6	64.3	100	100
10 TH	100	100	14.1	15.6	92.2	0	0	100	100	0	90.6	95.3	95.3
11 VD	100	100	14.6	14.6	100	85.4	63.4	100	97.6	0	78.1	97.6	97.6
12 VD	100	100	25.5	25.5	87.2	34	0	100	100	0	85.1	100	74.5
13 VS	100	100	0	0	0	100	66.7	100	100	0	100	100	100
14 GR	100	100	31.6	36.8	73.7	0	0	100	100	0	100	68.4	0
15 GR	100	100	0	37.5	46.9	0	0	100	100	3.1	78.1	93.8	21.9
16 GR	100	100	57.1	57.1	71.4	0	0	100	100	0	92.9	100	100
17 GR	100	100	16.1	19.4	35.5	0	0	100	100	0	96.8	100	100
18 GR	100	100	50	50	65.6	0	0	100	100	0	84.4	100	100

Table 6. Frequency of the isozyme bands (in %) for all populations of *Hordeum murinum.*
GOT - glutamate oxaloacetate transaminase; EST - esterase.
'+' - present '-' - absent

BAND	GOT A	GOT B	GOT C	GOT D	GOT E
_	0.4	0.4	73.8	74.4	17.3
+	99.6	99.6	26.2	25.6	82.7

BAND	EST A	EST AA	EST B	EST C	EST D	EST E	EST F	EST G
_	63.3	96	2.3	0.8	86.4	19.8	3.7	9.9
+	36.8	4	97.7	99.2	13.6	80.2	96.3	90.2

Table 7. Frequency of the isoenzyme bands/species (in %) for all populations of *Medicago.* S- *M. sativa,* V - *M.* x*varia,* F 4x - tetraploid *M. falcata,* F 2x - diploid *M. falcata.* PX - peroxidase, EM - malic enzyme MDH - malate dehydrogenase.

band spec.	**PX1**	**PX2**	**PX3**	**PX4**	**PX5**	**PX6**	**PX7**	**PX8**	**EM1**	**EM2**	**EM3**
S	68.1	19.7	22.3	0.5	16	49.5	62.2	98.4	17.6	60.6	64.9
V	67.5	23.1	19.9	0.4	13.4	45.5	80.1	94.6	22.7	50.2	51.6
F 4x	65.1	34.9	10.3	0	8	27.4	78.3	96	24.6	51.4	57.7
F 2x	0.8	42.5	40.8	6.7	0	62.5	76.7	99.2	3.3	68.3	71.7

band spec.	**EM4**	**EM5**	**EM6**	**EM7**	**MDH 1**	**MDH 2**	**MDH 3**	**MDH4**	**MDH 5**	**MDH 6**	**MDH 7**
S	30.3	39.4	77.1	99.5	8	13.8	49.5	63.3	38.8	82.5	99.5
V	19.9	26.4	75.1	99	0	4	26.7	67.5	35	89.5	98.2
F 4x	29.7	33.7	70.3	98.3	0	5.1	24.6	57.7	46.3	85.1	93.1
F 2x	70.8	87.5	0	99.2	17.5	46.7	90.8	0	0	0	100

Table 8. Frequency of the isozyme bands/species (in %) for population 4 of *Medicago*. S - *M. sativa*, V - *M.* x*varia*, F 4x - tetraploid *M. falcata*. PX - peroxidase, EM - malic enzyme, MDH - malate dehydrogenase.

BAND SPEC.	**PX 1**	**PX 2**	**PX 3**	**PX 4**	**PX 5**	**PX 6**	**PX 7**	**PX 8**	**EM 1**	**EM 2**	**EM 3**
S	52.4	4.8	9.5	0	14.3	61.9	57.1	95.2	4.8	52.4	71.4
V	52.2	8.7	10.9	0	4.4	50	47.8	91.3	13	21.7	60.9
F 4x	60.7	26.8	23.2	0	3.6	32.1	66.1	94.6	14.3	48.2	80.4

BAND SPEC.	**EM 4**	**EM 5**	**EM 6**	**EM 7**	**MDH 1**	**MDH 2**	**MDH 3**	**MDH 4**	**MDH 5**	**MDH 6**	**MDH 7**
S	33.3	33.3	76.2	95.2	0	0	23.8	38.1	61.9	100	100
V	10.9	10.9	84.8	100	0	6.5	17.4	23.9	30.4	97.8	97.8
F4x	14.3	26.8	73.2	100	0	5.4	35.7	41.1	58.9	92.9	96.4

Table 9. Frequency of RAPD bands/species (in %) for population 4 of *Medicago.* S - *M. sativa*, V - *M.* x*varia*, F 4x - tetraploid *M. falcata*.
P5 - primer OPP-5, P6 - primer OPP-6, P8 - primer OPP-8.

band spec.	**P5A**	**P5B**	**P5C**	**P5D**	**P5E**	**P5F**	**P5G**	**P5H**	**P5I**	**P6A**	**P6B**	**P6C**
S	16.7	100	11.1	83.3	27.8	38.9	72.2	33.3	5.6	88.9	11.1	38.9
V	51.4	97.3	8.1	78.4	18.9	40.5	70.3	35.1	18.9	91.9	46	40.5
F 4x	40	100	8.8	51.4	48.6	25.7	51.4	37.1	25.7	91.4	74.3	11.4

band spec.	**P6D**	**P6E**	**P6F**	**P6G**	**P8A**	**P8B**	**P8C**	**P8D**	**P8E**	**P8F**	**P8G**	**P8H**
S	55.6	66.7	100	11.1	100	11.1	27.8	50	44.4	38.9	16.7	44.4
V	24.3	78.4	86.5	2.7	100	13.5	32.4	46	54.1	51.4	46	29.7
F 4x	20	57.1	82.9	2.9	97.1	28.6	42.9	48.6	42.9	51.4	42.9	40

Table 10. Plants of *Hordeum*, used for the experimental hybridization in the field, with the number of produced seeds, regenerated plants and percent of germination.

PLANT	N° OF SEEDS	N° OF REGENERATED PLANTS	% OF GERMINATION
1A 1420	12	11	91.6%
2A 180/53	36	35	97.2%
2A 260/1613	9	9	100%
3B 450/3	23	21	91.3%
4B 340	4	1	25%
6A 1065/25	14	14	100%
6A 758/24	33	31	93.9%
6A 817/15	31	28	90.3%
7A 50/46	52	45	86.5%
7A 22/82	2	1	50%
8A 0/18	40	39	97.5%
8B 100	23	17	73.9%
9A 3935/25	15	15	100%
9A 2120/28	21	19	90.5%
10A 260/103	16	16	100%
10A 244/44	18	18	100%
11A 355	19	18	94.7%
11A 1580	9	8	88.9%
12A 1765	31	27	87.1%
12A 1485	19	19	100%
14A 170/23	32	30	93.8%
14B 723/80	30	28	93.3%
15A 110/0	19	18	94.7%
15A 65/0	5	5	100%
16A 4170/77	15	15	100%
16A 4442/7	14	8	57.1%
17A 18/18	18	18	100%
17B 520/210	27	23	85.2%
18A 99/20	24	21	87.5%
18A 25/0	35	30	85.7%
TOTAL:	646	588	91%

Table 11. Number of hybrids of *Hordeum* progeny, obtained after hybridization on the field of Cernier, according to their isozyme profiles for GOT and EST. All plants resulted from self - fertilization.

PARENT	N° of analysed plants	N° of hybrids
1A 1420	10	0
2A 180/55	10	0
2A 260/1613	9	0
3B 450/3	10	0
4B 340	1	0
6A 1065/25	10	0
6A 817/15	10	0
6A 758/24	10	0
7A 50/46	10	0
7A 22/82	1	0
8A 0/18	10	0
8B 100	10	0
9A 2120/28	10	0
9A 3935/25	10	0
10A 260/103	10	0
10A 244/44	10	0
11A 1580	8	0
11A 355	10	0
12A 1485	10	0
12A 1765	10	0
14A 170/23	10	0
14B 723/80	10	0
15A 110/0	10	0
15A 65/0	5	0
16A 4170/77	10	0
16A 4442/7	8	0

Table 11 (continued)

17A 18/18	10	0
17B 520/210	10	0
18A 25/0	10	0
18A 99/20	10	0

crosses	N° of seeds	N° of plants	% of germination
1A 418 x 12A 18	9	9	100%
12A 18 x 1A 418	18	18	100%
1A 453 x 6A 7	17	16	94.1%
6A 7 x 1A 453	22	21	95.5%
2A 815/36 x 13A 3	10	10	100%
13A 3 x 2A 815/36	22	22	100%
2A 238/68 x 2A 27	10	10	100%
2A 27 x 2A 238/68	33	24	72.7%
3A 815/+20 x 14AB 5	6	5	83.3%
14AB 5 x 3A 815/+20	25	25	100%
3B 1668/45 x 2A 24	0	0	-
2A 24 x 3B 1668/45	42	41	97.6%
4B 975 x 12A 26	9	9	100%
12A 26 x 4B 975	22	22	100%
5B 710 x 7A 24	8	8	100%
7A 24 x 5B 710	21	21	100%
5A 710 x 13A 6	17	16	94.1%
13A 6 x 5A 710	29	29	100%
6A 1065/25 x 6A 11	21	21	100%
6A 11 x 6A 1065/25	39	39	100%
7A 244/282 x 7A 17	10	10	100%
7A 17 x 7A 244/282	28	28	100%
7A 86/137 x 14AB 15	5	5	100%
14AB 15 x 7A 86/137	13	13	100%
8B 20 x 12AB 11	15	15	100%
12AB 11 x 8B 20	23	23	100%
8A 36/0 x 6A 19	0	0	-
6A 19 x 8A 36/0	0	0	-
9A 12/0 x 6A 10	9	9	100%
6A 10 x 9A 12/0	27	27	100%
9A 1140/6 x 14AB 1	5	5	100%
14AB 1 x 9A 1140/6	25	25	100%
10A 260/103 x 2A 5	13	12	92.3%
2A 5 x 10A 260/103	29	29	100%
11A 255 x 7A 26	14	14	100%
7A 26 x 11A 255	35	34	94.1%
11A 190 x 12A 20	8	8	100%
12A 20 x 11A 190	13	13	100%
12B 1075 x 12A 22	23	23	100%
12A 22 x 12B 1075	44	44	100%

Table 12. List of the experimental crosses with *Hordeum* with number of seeds, produced/cross, number produced plants with percent of germination.

Table 12 (continued)

crosses	N° of seeds	N°of plants	% of germination
12A 1745 x 7A 23	15	15	100%
7A 23 x 12A 1745	26	26	100%
13A 200/60 x 13A 26	19	18	94.7%
13A 26 x 13A 200/60	30	30	100%
13A 335/12 x 6A 2	21	20	95.2%
6A 2 x 13A 335/12	26	26	100%
14A 281/37 x 2A 28	10	10	100%
2A 28 x 14A 281/37	54	53	98.1%
14B 674/63 x 14AB 17	12	11	91.7%
14AB 17 x 14B 674/63	28	28	100%
15A 40/25 x 14AB 22	20	20	100%
14AB 22 x 15A 40/25	31	27	87.1%
15A 110/52 x 7A 7	14	14	100%
7A 7x x15A 110/52	37	37	100%
16A 910/118 x 7A 9	13	12	92.3%
7A 9 x 16A 910/118	42	41	97.6%
16A 50/172 x 13A 4	14	14	100%
13A 4 x 16A 50/172	36	35	97.2%
17A 18/9 x 13A 5	15	15	100%
13A 5 x 17A 18/9	7	7	100%
18A 173/56 x 2A 29	12	11	91.7%
2A 29 x 18A 173/56	50	50	100%
Total:	1251	1223	97.76%

Table 13. Number of hybrids and number of plants as a result of self-fertilization, obtained after experimental hybridization with *Hordeum* in the greenhouse, according to their isozyme profiles for GOT and EST. F - female parent, M - male parent.

CROSS F x M	N°of analysed plants	N° of hybrids
2A5 x 10A260/130	20	0
10A260/130 x 2A5	12	0
2A24 x 3B1668/45	20	0
3B1668/45 x 2A24	0	0
2A27 x 2A238/68	20	0
2A238/68 x 2A27	9	0
2A28 x 14A281/37	20	0
14A281/37 x 2A28	10	0
2A29 x 18A173/56	20	0
18A173/56 x 2A29	9	0
6A11 x 6A106/25	20	0
6A106/25 x 6A11	20	0
6A2 x 13A335/12	20	0
13A335/12 x 6A2	19	0
6A7 x 1A453	21	0
1A453 x 6A7	16	0
6A10 x 9A12/0	20	0
9A12/0 x 6A10	9	0
7A7 x 15A110/52	18	0
15A110/52 x 7A7	12	0
7A9 x 16A910/118	22	0
16A910/118 x 7A9	12	0
7A17 x 7A244/282	21	0
7A244/282 x 7A17	10	0
7A23 x 12A1745	20	0
12A1745 x 7A23	15	0

Table 13 (continued)

CROSS F x M	N° of analysed plants	N° of hybrids
7A24 x 5B710	21	0
5B710 x 7A24	8	0
7A26 x 11A255	20	0
11A255 x 7A26	14	0
12AB11x 8B20	20	0
8B20 x 12AB11	15	0
12A18 x 1A418	18	0
1A418 x 12A18	9	0
12A20 x 11A190	13	0
11A190 x 12A20	7	0
12A22 x 12B1075	20	0
12B1075 x 12A22	18	0
12A26 x 4B975	20	0
4B975 x 12A26	9	0
13A3 x 2A218/36	20	0
2A218/36 x 13A3	10	0
13A4 x 16A50/172	20	0
16A50/172 x 13A4	14	0
13A5 x 17A18/9	7	0
17A18/9 x 13A5	15	0
13A6 x 5A710	20	0
5A710 x 13A6	15	0
13A26 x 13A200/60	20	0
13A200/60 x 13A26	18	0
14AB1 x 9A1140	20	0
9A1140 x 14AB1	12	0

Table 13 (continued)

CROSS F x M	N° of analysed plants	N° of hybrids
14AB5 x 3A815/20	20	0
3A815/20 x 14AB5	5	0
14AB15 x 7A86	13	0
7A86 x 14AB15	5	0
14AB17 x 14AB674/63	20	0
14AB674/63 x 14AB17	12	0
14AB22 x 15A40/25	20	0
15A40/25 x 14AB22	20	0

Table 14. Results from experimental crosses with *Medicago.*

TYPE OF THE CROSSES	N° OF CROSSES	N° OF CROSSES, PRODUCING SEEDS	N° OF REGENERATED PLANTS
HYBRIDIZATION			
M. falcata x *M. sativa*	28	8	23
M. sativa x *M. falcata*	32	9	31
CROSS - FERTILIZATION			
M. falcata x *M. falcata*	28	7	9
M. sativa x *M. sativa*	30	1	1
SELF - FERTILIZATION			
M. falcata	28	13	22
M. sativa	32	6	6
TOTAL:	178	44	92

Table 15. Results from the isozyme analysis (PRX, EM, MDH) of the individuals, obtained after experimental self - fertilization in the greenhouse with *Medicago*. s - *M. sativa*, f - *M. falcata*.

PARENT		N° of analysed individuals	N° of indivuduals as result of self-fertilization	N° of contaminated plants
3A 2.45/9.1	s.	2	0	2
8A 2/10	s.	4	0	4
1A 2.6/12	f.	6	2	4
1A 3.8/7	f.	2	0	2
1A 1/11	f.	3	3	0
4A 30/20	f.	1	1	0
4A 12.1/1.8	f.	1	1	0
4B 0.2/14.0	f.	1	0	1
4B 6/1.6	f.	1	0	1
7A 2/6	f.	3	0	3
7A 4.5/7.9	f.	1	0	1
7A 4.2/35	f.	3	0	3

Table 16. Number of hybrids and number of plants as a result of self - fertilization, produced after experimental hybridization in the field (Changin) of *Medicago falcata* (female) with *Medicago sativa* as an evidence of their zymograms for PRX, EM and MDH.

PARENT as	N° of analysed plants	N° of hybrids	N° of plants result of self-fertilization
4B 3.1/1	4	4	0
4B 4/3	11	11	0
4B 4/13	1	0	1
7A 4.4/14.7	7	7	0
7A 3.3/9.3	2	2	0
8B 2.7/5	4	4	0
8B 3/7	1	1	0

Table 17. Number of individuals from the progeny, obtained as result of the experimental cross - fertilization with *Medicago* in the greenhouse, with evidence of cross - fertilization and self - fertilization, according to the isozyme profiles of PRX, EM and MDH. CF - cross - fertilization, SF - self - fertilization. F - female parent, M - male parent. s - *M. sativa*, f - *M. falcata*.

CROSS	N° of analysed plants	N° of plants as result of CF	N° of plants as result of SF	N° of contaminated plants
F 7A 2/6 f. x M 7A 2.9/8.2 f.	1	1	0	0
F 1A 2.6/12 f. x M4B 290/1200 f.	1	0	1	0
F 7A 4.6/32 f. x M 4B 10/12 f.	2	0	0	2
F 4A 12.1/1.8 f. x M 1A 3.6/7.7 f.	1	1	0	0
F 1A 1/11 f. x M 1B 7.8/5.6 f.	1	0	0	1
F 7A 0/10 f. x M 7A 4.4/14.7 f.	3	3	0	0
F 5A 0.2/2 s. x M 5A 0.4/10 s.	1	0	0	1

Table 18. Number of obtained hybrids after the experimental hybridization with *Medicago* in the greenhouse as an evidence of their isozyme profiles for PRX, EM and MDH. F - female parent, M - male parent. s - *M. sativa*, f - *M. falcata*.

CROSS	N° of analysed plants	N° of hybrids	N° of plants as result of self-fertilization	N° contaminated plants
F 3A 7.2/3.1s. x M 4B 510/1200 f.	3	3	0	0
F 3A 18.4/10.1s x M 4B 400/130 f.	8	7	0	0
F 3A 2.45/9.1 s. x M 4B 13.10/1 f.	2	2	0	0
F 5A 0.2/2 s. x M 4B 7.40/2 f.	5	1	0	4
F 4A 25/0 s. x M 6A 690/45.0 f.	2	2	0	0
F 5A 1.2/3 s. x M 4B 10/12 f.	3	0	3	0
F 8A 2/10 s. x M 7A 4.4/14.7 f.	4	0	0	4
F 9A 5.8/10 s. x M 4B 4/3 f.	4	2	0	2
F 1A 3.6/7.7 f. x M 5A 410/1010 s.	1	0	1	0
F 1A 2.6/12 f. x M 5A 0.2/6.8 s.	8	7	0	1
F 1A 1/11 f. x M 3A 2010/1.91 s.	3	0	2	1
F 1A 3.8/7 f. x M 9A 5.8/10.8 s.	7	0	0	7
F 1A 2.6/4.4 f. x M 5A 0.0.1/ s.	1	0	0	1
F 4A 12.1/1.8 f. x M 5A 365/020 s.	1	1	0	0
F 7A 4.2/35 f. x M 9A 6.3/7 s.	2	2	0	0

Table 19. Results of the isozyme analysis (PRX, EM, MDH) of the individuals, obtained after the experimental crosses (hybridization, cross - and self - fertilization), performed in Bern. F - female parent, M - male parent. s - *M. sativa*, f - *M. falcata*, v - *M.* x*varia*. SF - self - fertilization, CF - cross - fertilization.

CROSS	N° of analysed plants	N° of hybrids	N° of plants as result of CF	N° of plants as result of SF	N° contaminated plants
F 6A 0/0.1 v. x M 6A 1.4/49 f.	1	0	0	1	0
F 6A 4/12 v. x M 6A 1.3/16.7 f.	2	0	0	2	0
F 6A 1.4/49 f. x M 6A 0/0.1 v.	2	2	0	0	0
F 6A 1.6/16.7 f. x M 6A 4/12 v.	1	1	0	0	0
F 6A 6/16 f. x M 6A 1.9/42 v.	1	1	0	0	0
F 6A 1.9/42 v. x M 6A 1.5/24.4 s.	2	0	0	0	2
F 6A 0/0.1 v. x M 6A 2.4/36 s.	1	0	0	1	0
F 6A 4.4/1 v. x M 6A 1.5/24.4 s.	3	1	0	0	2
F 4A 17.7/3 v. x M 4A 6/1 s.	4	4	0	0	0
F 4A 6/1 s. x M 4A 28/2 v.	3	3	0	0	0
F 6A 9/33 s. x M 6A 0/1 v.	1	0	0	0	1
F 4A 6/1 s. x M 4A 17.7/3 v.	8	7	0	0	1
F 4A 13/0.2 s. x M 4A 28/2 v.	2	0	0	0	2
F 6A 0/0.1 v. x M6A 0/1 v.	1	0	0	1	0
F 4A 6/1 s. x M4A 6/1 s.	3	0	0	3	0
F 4A 2.9/1.9 s. x M 4A 22.9/3 s.	2	0	0	0	2