# „A study on the possible use of isoenzyme characteristics for the assessment of distinctness, uniformity and stability in *Calluna vulgaris* L.“

## Final report

## Agreement number CPV.4937


## Summary

It has been assessed whether isoenzyme characteristics are useful to supplement DUS testing of *Calluna vulgaris* L. Existing electrophoresis techniques were adapted for the application in *Calluna*. In total, 25 varieties have been investigated with 15 single plants each. Four of the isoenzymes revealed polymorphic and clear banding patterns. As a result, isoenzymes are suited to characterise varieties and could be appropriate to contribute to the identification of varieties. Options were developed to apply isoenzymes characteristics in the assessment of distinctness in addition to traditional morphological characteristics. Further, the use of isoenzymes can support the examination of uniformity and may contribute to reduce duration of testing.


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## 1. Introduction

In the last 10 years, substantial progress has taken place within the group of bud bloomers of the species *Calluna*. Breeding efforts are less intensive in the group of open flowering types. Initially, only two breeders worked on the new type of bud bloomers and applied for plant breeders' rights (PBR). Later, several breeders from Germany, France and the Netherlands concentrated also on bud bloomers instead of open flowering types and developed a number of new varieties by crossing varieties or by selection of mutants.

Breeding progress started with a first white bud bloomer and continued with pink flowering types. Bud bloomers are highly competitive on the market and propagation by cuttings is possible with low energy costs. As a consequence, the types of bud bloomers were improved and varieties with white, pink, blue-pink, red, blue and violet flower colour were developed. Variations in colour of leaves or shoot tips were other breeding purposes. Types with white or pink buds and yellow-green to yellow leaves are popular now.

The German reference collection of *Calluna* consists of 307 varieties, including 60 open flowering types. The relation of number of bud bloomers to open flowering types is wider for the PBR applications. November 1st 2005, a total of 66 candidates were grown in the PBR testing plot, but only two candidates were open flowering types. All in all, 81 varieties are protected on the national level, and Community plant variety rights (CPVR) are granted for 37 varieties. At present, five varieties are applied for CPVR.

The increasing number of varieties led to a decrease of phenotypic differences and increased efforts in DUS testing are necessary now. Within the scope of this project, the possibility to complement DUS testing with assessments of isoenzyme characteristics was investigated. As a first step, existing electrophoreses techniques were adapted for the application in *Calluna*. Second, banding patterns and polymorphisms were described and classified. The variation of characteristics within and between varieties has been assessed in a larger set of varieties to appraise the possibility to use this new type of characteristics in *Calluna*. Further, relations between results from the field trials and the laboratory work have been analysed.

## 2. Materials and methods

Research within period 2004 – 2005

Plant material

The plant material consisted of reference varieties and candidates of the species *Calluna vulgaris* L. of the DUS testing in 2004 and 2005 (Table 1). Varieties have been chosen considering a wide genetic variability of the set of bud bloomers varieties. In the DUS test, each variety is represented by 30 single plants. For isoenzyme analysis, ten shoot tips of each of 15 plants were cut between August and October 2004 and 2005 (Figure 2, page 12).

Isoenzyme analyses

Isolation protocols of six different isoenzymes in *Calluna vulgaris* L. have been optimised. Proteins have been isolated for each single plant and isoenzymes have been separated by starch gel electrophoresis (Malatdehydrogenase (MDH), Isocitratdehydrogenase (IDH); Phosphoglucomutase (PGM); Diaphorase (DIA); Phosphoglucose-Isomerase (PGI) und Shikimat-Dehydrogenase (ShDH)).

Field characteristics

Twenty two characteristics have been observed for each variety according to the technical protocol CPVO TP/94 from 6.11.2003 and DUS has been assessed in accordance with the guidelines.

Preliminary investigations and further evaluations

Four single plants per variety have been assessed in earlier testing periods. In addition to the set of varieties within this project, further varieties have been assessed for other purposes. Results will be discussed if they touch on the current topic.

Table 1: Plant material for isoenzyme analyses

| Typ[1] | Code | Derived from mutations [2] | No. of single plants | | Remark |
|---|---|---|---|---|---|
| | | | 2004 | 2005 | |
| R | 1 | a | 15 | 15 | |
| R | 2 | | 5 | 3 | |
| R | 3 | | 15 | 15 | |
| R | 4 | | 5 | 11 | |
| R | 5 | | 15 | 15 | |
| K | 6 | | 15 | 15 | |
| K | 7 | | 15 | 15 | |
| K | 8 | | 15 | 15 | |
| K | 9 | | 15 | 15 | |
| R | 10 | b | 15 | 15 | |
| R | 11 | b | 15 | 15 | |
| K | 12 | | 15 | 15 | |
| K | 13 | | 14 | 15 | |
| K | 14 | | 15 | 15 | |
| K | 15 | | 15 | 15 | |
| K | 16 | | 15 | 15 | |
| K | 17 | | 15 | 15 | |
| K | 18 | | 15 | 15 | Not uniform in field characteristics |
| K | 19 | | 15 | 15 | |
| K | 20 | c | 15 | 15 | |
| K | 21 | a | 15 | 15 | |
| K | 22 | c | 15 | 15 | |
| K | 23 | | 15 | 15 | |
| R | 24 | | 15 | 15 | |
| R | 25 | | 15 | 15 | |

[1] In growing period 2004, 2005, Reference = R, Candidate = K

[2] Varieties with the same letter have a common genetic background apart from the mutation (according to breeder's information)

## 3. Results

Isolation of isoenzymes

The protocols optimised for isolation, separation and scoring of isoenzyme patterns are attached (Annex).

Four loci of the isoenzymes were monomorphic within the set of varieties of the preliminary and the current study. Five loci showed clear differentiation between varieties which is reflected in the frequencies of genotypes (Table 2). Genotype frequencies of the preliminary set and the current set of varieties were in concordance. Therefore, similar degree of discrimination in isoenzyme patterns can be expected in future assessments. Banding patterns of each locus were reliable.

Small differences in the intensity between bands of two varieties did not effect scoring and were considered as a result of differences in growing stage of plant material. No conclusions from the banding pattern of DIA could be drawn regarding type of enzyme configuration. It is considered, that locus Dia1 might consist of two loci, which could not be scored separately in this project. Nevertheless, different genotypes can be identified.

Table 2 : Overview isoenzymes

| Isoenzyme | Locus | Genotype values | Frequency of genotypes | Type |
|---|---|---|---|---|
| Malatdehydrogenase (MDH) | Mdh1, | A | monomorph | Dimer |
| | Mdh2 | A, AB, B | 8%, 48%, 44% | Dimer |
| Isocitratdehydrogenase (IDH) | Idh | A, AB, B | 0%, 68%, 32% | Dimer |
| Phosphoglucomutase (PGM) | Pgm | A, AB, B | 24%, 40%, 36% | Monomer |
| Diaphorase (DIA) | Dia1, | A ,B, D, E | 40%, 52%, 4%, 4% | - [1]) |
| | Dia2 | A, B, D | 52%, 44%,4% | - [1]) |
| Phosphoglucose-Isomerase | Pgi | A | monomorph | Dimer |
| (PGI) | Pgd | A | monomorph | Dimer |
| Shikimat-Dehydrogenase (ShDH) | Shdh | A | monomorph | Dimer |

[1]) no conclusion possible based on the banding patterns

Results based on different harvesting dates of plant material and isolation of enzymes were in concordance for all single plants of the respective variety. Summarising, isoenzymes revealed consistent and reliable results and showed to be robust in occurrence and scoring quality.

Differences between varieties in the expression of isoenzyme characteristics

In addition to the 25 varieties, further genotypes have been assessed, because of problems in distinctness based on morphological characteristics (R26, R29, R30) or due to identical isoenzyme patterns (R28, R29, R30). Therefore, data for a total of 435 pairwise comparisons are available for field characteristics and isoenzymes (Figure 1).

| | |
|---|---|
| Number of pairs assessed | 435 |
| No. of pairs without differences in isoenzymes characteristics | 29 |
| No. of pairs without clear differences of field characteristics | 4 |
| No. of pairs without clear differences neither in field nor in isoenzyme characteristics | 3 |

Figure 1 Lack of distinctness (X) in field characteristics (above diagonal) and in isoenzymes (below diagonal) of 435 pairs of varieties.

| Mut- | Type | | R | R | R | R | R | K | K | K | K | R | R | K | K | K | K | K | K | K | K | K | K | K | K | R | K | R | R | R | R | R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ants | Code | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| a[1] | R | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | R | 2 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | R | 3 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | R | 4 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | R | 5 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | K | 6 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | K | 7 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | K | 8 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | K | 9 | | | | | | X | | | | | | | | | | | | | | | | | | | | | | | | |
| b | R | 10 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| b | R | 11 | | | | | | | | | | X | | | | | | | | | | | | | | | | | | | | |
| | K | 12 | | | | | | | | | | X | | | | | | | | | | | | | | | | | | | | |
| | K | 13 | | | | | | | | | | | | | | | X | | | | | | | | | | | | | | | |
| | K | 14 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | K | 15 | | | | | | | | | | | | | | X | | | | | | | | | | | | | | | | |
| | K | 16 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | K | 17 | | X | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | K | 18 | | | X | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | K | 19 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| c | K | 20 | | | | | | | | | | X | X | X | | | | | | | | | | | | | | | | | | |
| a | K | 21 | X | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| c | K | 22 | | | | | | | | | | | | | X | | | | | | | | | | | | | | | | | X |
| | K | 23 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | R | 24 | | | | | | | | | | X | X | X | | | | | | | | X | | | | | | | | | | |
| d | R | 25 | | | X | | | | | | | | | | | | | | | X | | | | | | | | X | | | | |
| d | K | 26 | | | X | | | | | | | | | | | | | | | X | | | | | | | X | | | | | |
| | R | 27 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | R | 28 | | | | | | | | | | | | | X | | | | | | | | | X | | | | | | | X | |
| | R | 29 | | | | | | | | | | | | | X | | | | | | | | | X | | | | | | X | | |
| | R | 30 | | | | | | | | | | | | | X | | | | | | | | | X | | | | | X | X | X | |

[1] same letter indicate a common genetic background due to a mutation

field characteristics
isoenzyme characteristics

The investigations revealed that the 22 field characteristics and the 5 polymorphic isoenzyme characteristics were not related.

Examples for combinations of different results in the field and in the laboratory are presented in Table 3. Mutants with the same genetic background are not expected to show differences in the isoenzyme characteristics. Four pairs of varieties with a common origin by mutation are included in the variety set. Two of these pairs showed differences in field characteristics, but no differences in isoenzyme characteristics (Figure 1, R1/K21 und R10/R11). On of the pairs could not be differentiated in the field nor by isoenzymes (R25/R26). The remaining pair showed differences in the field and the isoenzyme characteristics (Table 3, Case 6). It can be assumed, that this pair was not deviated by mutation from a common ancestor. Within the scope of DUS testing, there was no need to verify the origin quoted by the breeder and no further examination followed.

One pair of varieties without clear differences in the field characteristics showed differences in isoenzyme patterns (K13/K14).

Table 3: Examples of pairs of varieties regarding results of differentiation of the field and the isoenzyme characteristics

| Case | Typ | Code | Genotype in | | | | | Remark |
|---|---|---|---|---|---|---|---|---|
| | | | Mdh2 | Idh | Pgm | Dia 1 | Dia 2 | |
| 1 | R | 10 | B | B | B | B | B | Identity of isoenzyme characteristics |
| | R | 11 | B | B | B | B | B | and distinct in field characteristics |
| | R | 31 | B | B | B | B | B | (colour of flowers and shoots) |
| 2 | R | 27 | AB | AB | A | A | A | Identitiy of isoenzyme characteristics |
| | K | 7 | AB | AB | A | A | A | and distinct in field characteristics (flowering shoot, colour of shoot tip) |
| 3 | K | 22 | AB | AB | AB | A | A | Identity of isoenzyme and field |
| | R | 30 | AB | AB | AB | A | A | characteristics |
| 4 | K | 26 | AB | AB | A | B | A | Identity of isoenzyme and field |
| | R | 25 | AB | AB | A | B | A | characteristics |
| 5 | K | 13 | AB | AB | AB | A | A | Differences in two isoenzyme |
| | K | 14 | B | B | AB | A | A | characteristics and identity in field characteristics |
| 6 | K | 20 | B | B | B | B | B | Differences in isoenzyme and field |
| | K | 22 | AB | AB | AB | A | A | characteristics; Originating from a third variety by mutations. |

Additional test on varieties with lack of uniformity in field characteristics

Several submissions of plant material were available for two varieties. Within some of these samples, small differences in field characteristics were observed but it was not possible to clearly identify off-types. The analysis of isoenzymes confirmed, that the small differences were not caused by environment (Table 4, one example). Isoenzymes characteristics revealed several of clear differences between the varieties and the supposed off-types.

Table 4: Example for non-uniform sample of variety from different origins

| Sample | Remarks | Result of isoenzyme analyses Genotype | | | | |
|---|---|---|---|---|---|---|
| | | Mdh 2 | Idh | Pgm | Dia 1 | Dia 2 |
| 1 | Type 1 with 4 of 4 plants (no off-types) | AB | AB | AB | A | A |
| 2 | Type 1 with 5 plant and | AB | AB | AB | A | A |
| | Type 2 with 3 off-types | B | AB | A | A | A |
| 3 | Type 1 with 3 plants and | AB | AB | AB | A | A |
| | Type 2 with 1 off-types | B | AB | A | A | A |

Uniformity of isoenzyme characteristics within the varieties

All single plants of each variety showed uniformity in isoenzyme patterns. One candidate variety (K18) was not uniform in field characteristics but uniform in isoenzyme characteristics. Finally, this variety was rejected because of the lack of uniformity.

Uniformity problems as observed in some varieties during the preliminary study have not been found in the current set of varieties. Optimising all steps of isoenzyme analyses might be a reason for the different situation regarding uniformity in the preliminary and the current study.

## 4. Conclusions

The method to assess isoenzyme characteristics is robust. The banding expressions are stable and can be clearly described. The variation of the isoezyme patterns has a high potential to discriminate varieties. In addition, the level of uniformity is comparable to that of field characteristics. Therefore, a supplement of isoenzyme

characteristics in DUS testing of this vegetatively propagated species would not increase requirements for uniformity. In conclusion, isoenzyme characteristics are suitable to characterise varieties and may contribute to identification of varieties.

Breeding activity in ornamental plants are mainly focused on morphological characteristics. Therefore, every new variety should be clearly different to all varieties of common knowledge. Isoenzyme characteristics should not be used alone to establish distinctness. In case of differences in the isoenzyme patterns, the requirement for the size of differences in the field characteristic might be reduced. Nevertheless, a small difference in the field characteristics should be necessary at least. A suggestion how to combine both types of characteristics is shown in Table 5.

The use of isoenzymes as additional characteristics could sometimes reduce the duration of the test. If only small differences are observed in morphological characteristics in a second growing cycle would be necessary to confirm their stability. Additional differences in the isoenzyme patterns could provide the possibility for a positive decision at the end of the first growing cycle (Table 5).

If two varieties have the same origin by mutation, it is not expected to observe differences in isoenzyme characteristics even if the varieties are clearly distinct in field characteristics. In contrary, in case of differences in the isoenzyme patterns, a pair of morphologically distinct varieties was probably not derived by mutations.

In some cases, *Calluna* plants might not develop typically in the first growing season. Thus, the majority of characteristics can be examined reliably in the following season only. Isoenzyme characteristics could contribute to differentiate these problems in development from true off-types.

Table 5: Possible implementation of isoenzyme characteristics in the DUS testing of *Calluna*

**Use of field and isoenzyme characteristics for the assessment of distinctness under the condition that uniformity and stability criteria are fulfilled for all characteristics**

| Field characteristic | Isoenzyme characteristic | | Decision |
|---|---|---|---|
| clear difference | no consideration | ➡ | positive decision (one growing cycle) |
| small difference | clear difference | ➡ | positive decision (one growing cycle) |
| small difference | no difference | ➡ | second growing cycle |
| no differences | no consideration | ➡ | negative decision (one growing cycle) |

## 5. Cost statement

| | 11/03-12/04 | | | 1/05-11/05 | | | 11/03-11/05 |
|---|---|---|---|---|---|---|---|
| | hours | € per hour[1]) | Total | hours | € per hour[1]) | Total | |
| Sampling | 13,8 | 35,59 | 491,14 € | 25,8 | 23,63 | 608,47 € | 1.099,61 € |
| Protein extraction | 317,2 | 26,02 | 8.253,80 € | 238,5 | 26,75 | 6.379,88 € | 14.633,68 € |
| Elektrophorese | 44,0 | 24,96 | 1.098,74 € | 33,1 | 23,63 | 782,15 € | 1.880,89 € |
| Interpretation of results | 37,1 | 47,46 | 1.760,77 € | 5,5 | 45,91 | 252,51 € | 2.013,27 € |
| Establishment of protocols | | | | 20,5 | 45,91 | 941,16 € | 941,16 € |
| Report of results | | | | 25,5 | 45,91 | 1.170,71 € | 1.170,71 € |
| Project-organisation | 10,7 | 47,46 | 507,82 € | | | | 507,82 € |
| Chemicals | | | 1.218,72 € | | | 1.218,72 € | 2.437,44 € |
| Total | 422,8 | | 12.112,27 € | 348,9 | | 10.134,87 € | 24.684,58 € |

[1]) cost per hour includes 7% overhead

Totals cost: 24 685 EUR

BSA cost: 11 335 EUR

CPVO cost: 13 350 EUR

Figure 2: Cutting of shoot tips

Attachment: Protocols

# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Preparation of plant material

### 1.) Chemicals

Ascorbic acid, sodium salt
Bovine serum albumin
Dry ice: $CO_2$, solid
Dithioerythritol (DTE)
Hydrochloric acid (HCl), 25%
ß-Mercaptoethanol
Polyvinylpyrrolidone 40 (PVP-40)
Sodium metabisulfite
di-Sodium tetraborate decahydrate
Sucrose
Tris (Hydroxymethyl) aminomethane (Tris)

### 2.) Security advices

Potential risks of the used chemicals:
**Dry ice and ß-mercaptoethanol cause burns.**
**ß-Mercaptoethanol is toxic in contact with the skin.**
The remaining chemicals may be harmful also and can irritate eyes, respiratory organs and skin.

**Wear suitable clothing, gloves and eye protection during the handling of the above-mentioned chemicals.**

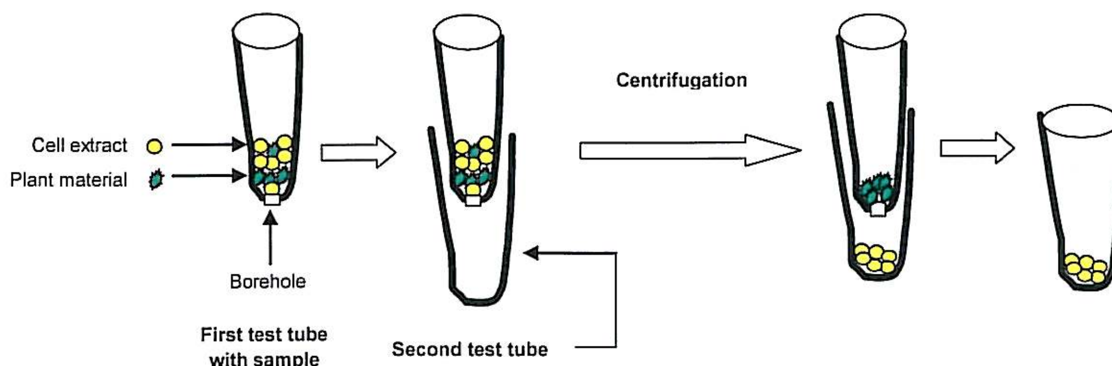
In the case of accident:
In case of contact with skin wash immediately with plenty of water and soap.
In case of contact with eyes rinse immediately with plenty of water for at least 15 min and seek medical advice.

### 3.) Production of extraction solution

0.6 g Tris are dissolved in 25 ml 20% PVP40 solution and adjusted mit 25% HCl ad pH 7.5. 3.5 g sucrose, 2.48 g ascorbic acid sodium salt, 0.19 g sodium metasulfite, 3.8 g di-sodium tetraborate decahydrate and 0.5 g bovine serum albumin are added and dissolved by gently heating. This solution is diluted with de-ionised water ad 50 ml and stored in portions à 10 ml in a deep-freezer.
Immediately prior use a portion à 10 ml is thawed and 10 µl ß-Mercaptoethanol plus 0.02 g DTE are added. This solution cannot be stored more than one day.

### 4.) Performance of extraction

Ten shoot tips of each of 15 plants were cut and grinded with dry ice in a mortar. Together with the dry ice the grinded sample is transferred in a 1.5 ml test tube. .After the evaporation of the dry ice 150 µl extraction solution is added. Plant material and extraction solution are mixed carefully by use of a small spatula and incubated for 30 min at room temperature. Then the test tube is bored from the bottom and placed in a second test tube. Both test tubes are centrigated together at 4000 rpm for 10 min. The cell extract collected in the lower test tube is tranferred in a microtiter plate. Until starting the electrophoresis the microtiter plate is stored in a deep-freezer.



# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Production of starch gels

### 1.) Chemicals

Citric acid monohydrate
L-Histidine
Starch hydrolysed, for electrophoresis

### 2.) Security advices

Potential risks of the used chemicals:
**Citric acid causes burns and irritates eyes, respiratory organs and skin**

**Wear suitable clothing, gloves and eye protection during the handling of citric acid.**

In the case of accident:

In case of contact with skin wash immediately with plenty of water and soap.
In case of contact with eyes rinse immediately with plenty of water for at least 15 min and seek medical advice.

### 3.) Production of gel buffer pH 6.5

100.9 g L-Histidine and 16.4 g Citric acid monohydrate are dissolved in de-ionised water ad 2 l.

### 4.) Production of starch gel

60 g starch*) are mixed in a 1 l Büchner flask with 32 ml gel buffer and 450 ml de-ionised water. This suspension is heated on a heating plate with a capacity of at least 450°C. During the heating phase the starch suspension are stirred powerfully by use of a motor-driven, bladed stirrer. In this time the gel holder is assembled, according to the design of the equipment used.

After a heating time of round about 20 min the starch suspension is boiling. It forms then a transparent gel solution. The Büchner flask with the boiling starch gel solution is removed from the heater and connected with a water jet aspirator. The starch gel solution is de-gased carefully until all the air bubbles have disappeared. The de-gased gel solution is filled in the assembled gel holder. The gel is covered by a glase plate. The formation of air bubbles between gel surface and glas plate must be avoided.

The gel needs round about 5 h for becoming solid and is stored over night in the refrigerator at 13°C or over the weekend at 17°C.

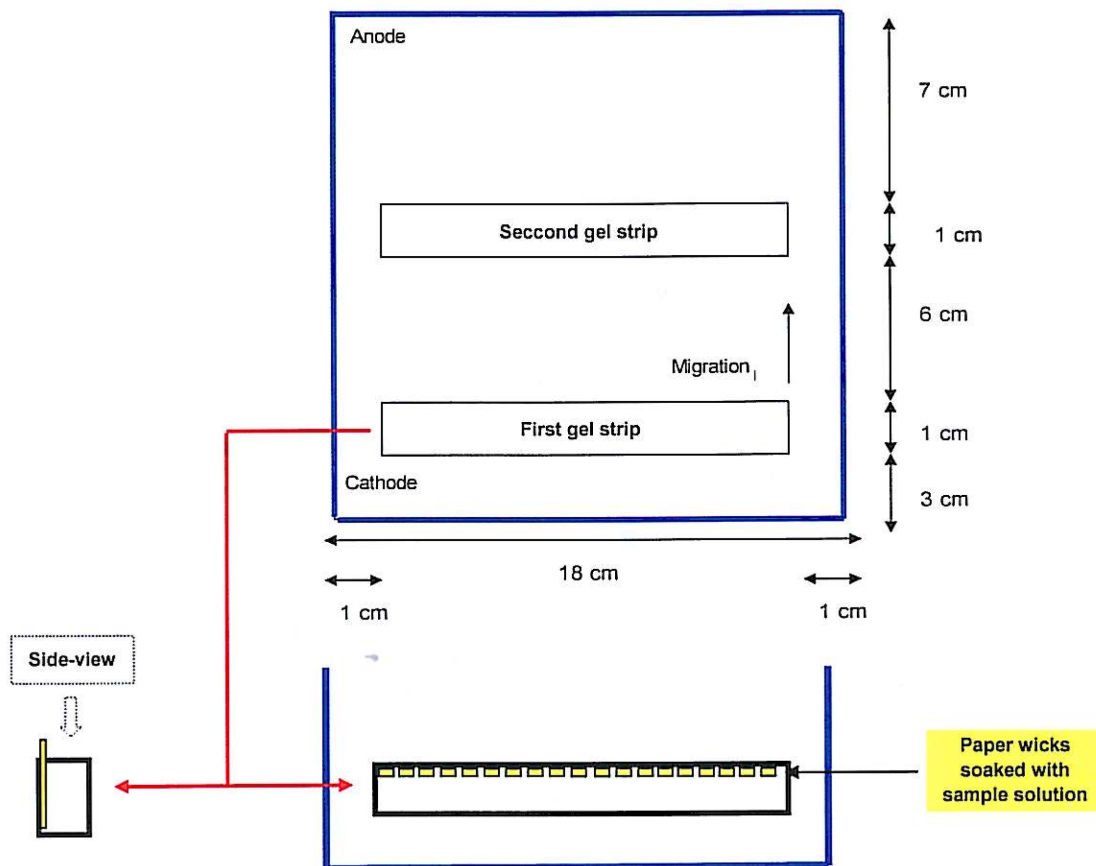
*): The optimal starch concentration can be varied between 60 und 70 g dependent from the individual batch of starch.

## Observation of isoenzyme polymorphisms in Calluna by use of SGE

### Sample loading

Filter paper wicks with the size 4 x 12 mm are dipped into the wells of the microtiterplate containing the cell extracts. Later on the wicks will be used for sample loading. Now the gel is prepared in the following way:

The holder with the gel is taken away from the gel mould. The upper glas plate and the spacers are removed. A 1 cm broad and 15 cm long gel strip is cut from in a distance of 3 cm to the cathodic side as demonstrated in the figure. In a distance of 6 cm to the first gel strip a second strip is cut in the same way as before. Both gel strips are stored separately. Now two gaps are in the gel.




The paper wicks soaked with the sample solutions are taken away from the microtiter plate using forceps and pressed against the anodic wall of the gap. The distances between the wicks should be 2-3 mm. The wicks come out a little bit outside from the gel. Therefore they can be removed after pre-electrophoresis with any trouble. Each of the two gaps are loaded with 19 samples: 15 samples of the variety under test and two samples of two reference varieties. On the left or right side a wick soaked with bromphenol blue solution is placed. This is the marker for the migration distance.

The two gel strips are replaced in the gel. The formation of any empty spaces between gel and gel strip and displacing of the wicks should be avoided. Then the electrophoresis can be started.

# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Performence of electrophoresis

### 1.) Chemicals

Citric acid monohydrate
L-Histidine

### 2.) Security advices

Potential risks of the used chemicals:
**Citric acid causes burns and irritates eyes, respiratory organs and skin**

**Wear suitable clothing, gloves and eye protection during the handling of citric acid.**

In the case of accident:

In case of contact with skin wash immediately with plenty of water and soap.
In case of contact with eyes rinse immediately with plenty of water for at least 15 min and seek medical advice.

### 3.) Production of electrophoresis buffer pH 6.5

100.9 g L-Histidine and 16.4 g Citric acid monohydrate are dissolved in de-ionised water ad 2 l. 400 ml of the stock solution are diluted with de-ionised water ad 2 l.

### 4.) Performence of electrophoresis

The electrophoresis chamber is filled with electrophoresis buffer up to the upper side of the cooling plate. The gel holder with the gel is set in the chamber. Electrophoresis buffer is refilled in the chamber up to the level of the gel. The gel is cooled during the electrophoresis to 10°C. Now a pre-electrophoresis is performed for 20 min. The conditions for the pre-electrophoresis are the following:

180 V
max. 110 mA
max. 100 W

Migration direction: Cathode → Anode

After the pre-electrophoresis the paper wicks are removed from the gel. If necessary, the chamber is re-filled with elctrophoresis buffer. The buffer level must be a little bit lower than the upper surface of the gel. Now the main-electrophoresis is started with the following conditions:

200 bis 220 V
max. 110 mA
max. 100 W

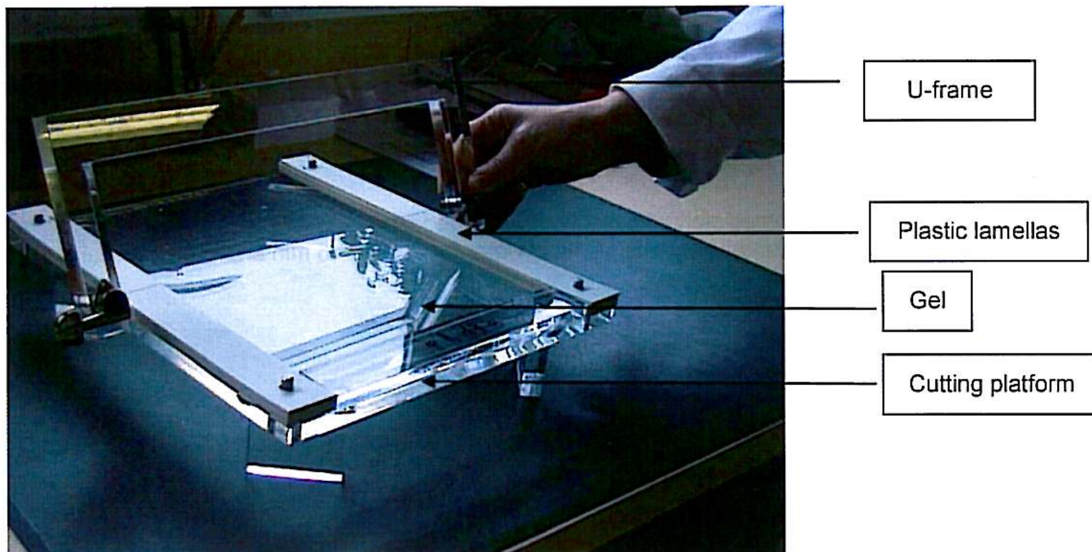
Migration direction: Cathode → Anode
Migration time: circa 4,5 h
Migration distance: circa 14 cm ( Bromphenol blue)

CPV. 4937
Laboratory protocol

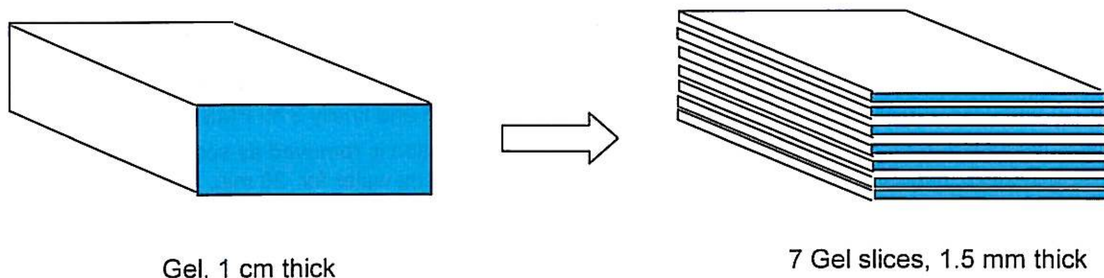
# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Preparation of gel slices for isoenzyme staining

The electrophoresis is finished as soon as the marker dye arrives a migration distance of 14 cm. Gel strips 0.5 cm broad are cut away on both longitudinal gel sides and discarded. The first part of the gel until the sample positions is cut away and discarded also. Now the gel is transferred from the gel gel holder to the cutting platform.The gel is over-layed with a polyethylene sheet and a glas plate.
A depot of 1.5 mm thick plastic lamellas are placed on the cutting platform on left and right side of the gel. The lamelleas are setted in pins and can be removed individually.




The gel is cut in slices 1.5 mm thick of use of a very fine steel wire ( guitar string: high e string). The steel wire is (clamped in an U-formed frame) is set horizontally on the lamellas and is pulled then carefully through the gel. It must be observed strictly, that the steel wire is accurately clean. Dirty steel wire causes rough cuts. After the first cut the first lamella on the left side and on the right side is removed and the procedure of cutting is repeated. Each of the gel slices is used for an individual isoenzyme staining. But the first (the upper) gel slice and the last gel slice (on the bottom) are discarded.



Gel, 1 cm thick

7 Gel slices, 1.5 mm thick

CPV. 4937
Laboratory protocol

# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Visualisation of malate dehydrogenase (MDH)

### 1.) Chemicals

Dimethylthiazol diphenyl tetrazolium (MTT)
ß-Nicotinamide adenine dinucleotide (NAD)
Hydrochloric acid (HCl), 25%
Malic acid
Phenazine methosulfate (PMS)
Tris (Hydroxymethyl) aminomethane (Tris)

### 2.) Security advices

Potential risks of the used chemicals:
**25% HCl causes burns.**
**PMS and TRIS irritate eyes, respiratory organs and skin.**

**Wear suitable clothing, gloves and eye protection during the handling of the above-mentioned chemicals.**

In the case of accident:

In case of contact with skin wash immediately with plenty of water and soap.
In case of contact with eyes rinse immediately with plenty of water for at least 15 min and seek medical advice.

### 3.) Solutions

Staining buffer pH 9.0

121.1 g Tris are dissolved in circa 500 ml de-ionised water, adjusted with 1 N HCl ad pH 9.0 and maked up to 1000 ml with de-ionised water.

Malic acid solution

5 g malic acid are dissolved in 100 ml de-ionised water ad 100 ml.

MTT solution

0.9 g MTT are dissolved in 100 ml de-ionised water.

PMS solution

0.2 g PMS are dissolved in 100 ml de-ionised water.

### 4.) Procedure

A staining container is filled with 20 ml staining buffer pH 9.0 and 180 ml de-ionised water. If a heating chamber is avaible, the filled staining container is made warm ad 37°C. Now the gel is transferred in the staining container. The following chemicals or solutions are added successively to the staining solution in the container: 9 ml malic acid solution, 0.086 g NAD (dissolved in a little bit water), 4 ml MTT solution and finally 3 ml PMS solution.

The gel is incubated at 37°C for round about 30 min. The staining solution is removed as soon as clear banding patterns are visible in the gel. Then the gel is washed two times in 200 ml water for 20 min. After this the gel conservation is performed.

CPV. 4937
Laboratory protocol

# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Visualisation of isocitrate dehydrogenase (IDH)

### 1.) Chemicals

Dimethylthiazol diphenyl tetrazolium (MTT)
Hydrochloric acid (HCl), 25%
Isocitric acid, trisodiumsalt
Magnesium chloride hexahydrate ($MgCl_2$ x 6 $H_2O$)
ß-Nicotinamide adenine dinucleotide phosphate (NADP)
Phenazine methosulfate (PMS)
Tris (Hydroxymethyl) aminomethane (Tris)

### 2.) Security advices

Potential risks of the used chemicals:
**25% HCl causes burns.**
**PMS and Tris irritate eyes, respiratory organs and skin.**

**Wear suitable clothing, gloves and eye protection during the handling of the above-mentioned chemicals.**

In the case of accident:

In case of contact with skin wash immediately with plenty of water and soap.
In case of contact with eyes rinse immediately with plenty of water for at least 15 min and seek medical advice.

### 3.) Solutions

Staining buffer pH 8.0

121.1 g Tris are dissolved in circa 500 ml de-ionised water, adjusted with 1 N HCl ad pH 8.0 and maked up to 1000 ml with de-ionised water.

MgCl solution

21.4 g $MgCl_2$ x 6 $H_2O$ are dissolved in 100 ml de-ionised water.

MTT solution

0.9 g MTT are dissolved in 100 ml de-ionised water.

PMS solution

0.2 g PMS are dissolved in 100 ml de-ionised water.

### 4.) Procedure

A staining container is filled with 20 ml staining buffer pH 8.0,10 ml MgCl solution and 180 ml de-ionised water. If a heating chamber is avaible, the filled staining container is made warm ad 37°C. Now the gel is transferred in the staining container. The following chemicals or solutions are added successively to the staining solution in the container: 0.45 g isocitric acid trisodiumsalt (dissolved in a small quantity of water), 0.06 g NADP (dissolved in a little bit water), 4 ml MTT solution and finally 3 ml PMS solution.
The gel is incubated at 37°C for round about 30 min. The staining solution is removed as soon as clear banding patterns are visible in the gel. Then the gel is washed two times in 200 ml water for 20 min. After this the gel conservation is performed.

CPV. 4937
Laboratory protocol

# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Visualisation of phosphoglucomutase (PGM)

### 1.) Chemicals

Dimethylthiazol diphenyl tetrazolium (MTT)
Glucose 1-phosphate
Glucose 6-phosphate dehydrogenase
Hydrochloric acid (HCl), 25%
Magnesium chloride hexahydrate ($MgCl_2$ x 6 $H_2O$)
ß-Nicotinamide adenine dinucleotide phosphate (NADF
Phenazine methosulfate (PMS)
Tris (Hydroxymethyl) aminomethane (Tris)

### 2.) Security advices

Potential risks of the used chemicals:
**25% HCl causes burns.**
**PMS and Tris irritate eyes, respiratory organs and skin.**

**Wear suitable clothing, gloves and eye protection during the handling of the above-mentioned chemicals.**

In the case of accident:

In case of contact with skin wash immediately with plenty of water and soap.
In case of contact with eyes rinse immediately with plenty of water for at least 15 min and seek medical advice.

### 3.) Solutions

Staining buffer pH 8.0

121.1 g Tris are dissolved in circa 500 ml de-ionised water, adjusted with 1 N HCl ad pH 8.0 and maked up to 1000 ml with de-ionised water.

MgCl solution

21.4 g $MgCl_2$ x 6 $H_2O$ are dissolved in 100 ml de-ionised water.

MTT solution

0.9 g MTT are dissolved in 100 ml de-ionised water.

PMS solution

0.2 g PMS are dissolved in 100 ml de-ionised water.

### 4.) Procedure

A staining container is filled with 20 ml staining buffer pH 8.0, 2 ml MgCl solution and 180 ml de-ionised water. If a heating chamber is avaible, the filled staining container is made warm ad 37°C. Now the gel is transferred in the staining container. The following chemicals or solutions are added successively to the staining solution in the container: 0,8 g glucose 1-phosphate (dissolved in a small quantity of water), 0.02 g NADP (dissolved in a little bit water), 110 units glucose-6-phosphate dehydrogenase, 4 ml MTT solution and finally 3 ml PMS solution.

The gel is incubated at 37°C for round about 30 min. The staining solution is removed as soon as clear banding patterns are visible in the gel. Then the gel is washed two times in 200 ml water for 20 min. After this the gel conservation is performed.

# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Visualisation of diaphorase (DIA)

### 1.) Chemicals

Dichlorphenolindophenol (DCPIP)
Dimethylthiazol diphenyl tetrazolium (MTT)
Hydrochloric acid (HCl), 25%
ß-Nicotinamide adenine dinucleotide, reduced type (NADH)
Tris (Hydroxymethyl) aminomethane (Tris)

### 2.) Security advices

Potential risks of the used chemicals:

**Dichlorphenolindophenol (DCPIP) is probable mutagen and cancerogen.**
**25% HCl and Tris cause burns and irritate eyes, respiratory organs and skin**

**Wear suitable clothing, gloves and eye protection**
**during the handling of the above-mentioned chemicals.**

In the case of accident:

In case of contact with skin wash immediately with plenty of water and soap.
In case of contact with eyes rinse immediately with plenty of water for at least 15 min and seek medical advice.

### 3.) Solutions

Staining buffer pH 9.0

121.1 g Tris are dissolved in circa 500 ml de-ionised water, adjusted with 1 N HCl ad pH 9.0 and maked up to 1000 ml with de-ionised water.

DCPIP solution

0.1 g DCPIP are dissolved in de-ionised water ad 25 ml.

MTT solution

0.9 g MTT are dissolved in 100 ml de-ionised water.

### 4.) Procedure

A staining container is filled with 20 ml staining buffer pH 9.0, 180 ml de-ionised water and 5 ml DCPIP solution. The filled staining container is made warm ad 37°C. Now the gel is transferred in the staining container and 9 ml MTT solution is added to the staining buffer in the container. The gel is incubated on rocking platform shaker at 40° C for round about 90 min. Now the staining solution is removed and preheated water is added to the gel. The gel is is incubated on rocking platform shaker at 40° C. The washing procedure is repeated until the gel is showing a white coloured background. After this the gel conservation is performed.

CPV. 4937
Laboratory protocol

# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Visualisation of phosphoglucose isomerase (PGI)

### 1.) Chemicals

Dimethylthiazol diphenyl tetrazolium (MTT)
Fructose 6-phosphate disodiumsalt
Glucose-6-phosphate dehydrogenase
Hydrochloric acid (HCl), 25%
Magnesium chloride hexahydrate ($MgCl_2$ x 6 $H_2O$)
ß-Nicotinamide adenine dinucleotide phosphate (NADP)
Phenazine methosulfate (PMS)
6-Phosphogluconic acid, trisodium salt, trihydrate
Tris (Hydroxymethyl) aminomethane (Tris)

### 2.) Security advices

Potential risks of the used chemicals:
**25% HCl causes burns.**
**PMS and Tris irritate eyes, respiratory organs and skin.**

**Wear suitable clothing, gloves and eye protection during the handling of the above-mentioned chemicals.**

In the case of accident:

In case of contact with skin wash immediately with plenty of water and soap.
In case of contact with eyes rinse immediately with plenty of water for at least 15 min and seek medical advice.

### 3.) Solutions

Staining buffer pH 8.0

121.1 g Tris are dissolved in circa 500 ml de-ionised water, adjusted with 1 N HCl ad pH 8.0 and maked up to 1000 ml with de-ionised water.

MgCl solution

21.4 g $MgCl_2$ x 6 $H_2O$ are dissolved in 100 ml de-ionised water.

MTT solution

0.9 g MTT are dissolved in 100 ml de-ionised water.

PMS solution

0.2 g PMS are dissolved in 100 ml de-ionised water.

### 4.) Procedure

A staining container is filled with 20 ml staining buffer pH 8.0, 2 ml MgCl solution and 180 ml de-ionised water. If a heating chamber is avaible, the filled staining container is made warm ad 37°C. Now the gel is transferred in the staining container. The following chemicals or solutions are added successively to the staining solution in the container: 0.2 g fructose 6-phosphate disodium salt (dissolved in a small quantity of water), 0.02 g NADP (dissolved in a little bit water), 110 units Glucose 6-phosphate dehydrogenase, 4 ml MTT solution and finally 3 ml PMS solution.
The gel is incubated at 37°C for round about 30 min. The staining solution is removed as soon as clear banding patterns are visible in the gel. Then the gel is washed two times in 200 ml water for 20 min. After this the gel conservation is performed.

# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Visualisation of phosphogluconate dehydrogenase (PGD)

### 1.) Chemicals

Dimethylthiazol diphenyl tetrazolium (MTT)
Hydrochloric acid (HCl), 25%
Magnesium chloride hexahydrate ($MgCl_2$ x 6 $H_2O$)
ß-Nicotinamide adenine dinucleotide phosphate (NADP)
Phenazine methosulfate (PMS)
6-Phosphogluconic acid trisodium salt trihydrate
Tris (Hydroxymethyl) aminomethane (Tris)

### 2.) Security advices

Potential risks of the used chemicals:
**25% HCl causes burns.**
**PMS and Tris irritate eyes, respiratory organs and skin.**

**Wear suitable clothing, gloves and eye protection during the handling of the above-mentioned chemicals.**

In the case of accident:

In case of contact with skin wash immediately with plenty of water and soap.
In case of contact with eyes rinse immediately with plenty of water for at least 15 min and seek medical advice.

### 3.) Solutions

Staining buffer pH 8.0

121.1 g Tris are dissolved in circa 500 ml de-ionised water, adjusted with 1 N HCl ad pH 8.0 and maked up to 1000 ml with de-ionised water.

MgCl solution

21.4 g $MgCl_2$ x 6 $H_2O$ are dissolved in 100 ml de-ionised water.

MTT solution

0.9 g MTT are dissolved in 100 ml de-ionised water.

PMS solution

0.,2 g PMS are dissolved in 100 ml de-ionised water.

### 4.) Procedure

A staining container is filled with 20 ml staining buffer pH 8.0, 10 ml MgCl solution and 180 ml de-ionised water. If a heating chamber is avaible, the filled staining container is made warm ad 37°C. Now the gel is transferred in the staining container. The following chemicals or solutions are added successively to the staining solution in the container: 0.08 g 6-Phosphogluconic acid trisodium salt trihydrate (dissolved in a small quantity of water), 0.02 g NADP (dissolved in a little bit water), 4 ml MTT solution and finally 3 ml PMS solution.
The gel is incubated at 37°C for round about 30 min. The staining solution is removed as soon as clear banding patterns are visible in the gel. Then the gel is washed two times in 200 ml water for 20 min. After this the gel conservation is performed.

CPV. 4937
Laboratory protocol

# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Visualisation of shikimate dehydrogenase (ShDH)

### 1.) Chemicals

Dimethylthiazol diphenyl tetrazolium (MTT)
Hydrochloric acid (HCl), 25%
Magnesium chloride hexahydrate ($MgCl_2$ x 6 $H_2O$)
ß-Nicotinamide adenine dinucleotide phosphate (NADP)
Phenazine methosulfate (PMS)
Shikimic acid
Tris (Hydroxymethyl) aminomethane (Tris)

### 2.) Security advices

Potential risks of the used chemicals:
**25% HCl causes burns.**
**PMS and Tris irritate eyes, respiratory organs and skin.**

**Wear suitable clothing, gloves and eye protection during the handling of the above-mentioned chemicals.**

In the case of accident:

In case of contact with skin wash immediately with plenty of water and soap.
In case of contact with eyes rinse immediately with plenty of water for at least 15 min and seek medical advice.

### 3.) Solutions

Staining buffer pH 9.0

121.1 g Tris are dissolved in circa 500 ml de-ionised water, adjusted with 1 N HCl ad pH 9.0 and maked up to 1000 ml with de-ionised water.

MgCl solution

21.4 g $MgCl_2$ x 6 $H_2O$ are dissolved in 100 ml de-ionised water.

MTT solution

0.,9 g MTT are dissolved in 100 ml de-ionised water.

PMS solution

0.,2 g PMS are dissolved in 100 ml de-ionised water.

### 4.) Procedure

A staining container is filled with 20 ml staining buffer pH 9.0, 2 ml MgCl solution and 180 ml de-ionised water. If a heating chamber is avaible, the filled staining container is made warm ad 37°C. Now the gel is transferred in the staining container. The following chemicals or solutions are added successively to the staining solution in the container: 0.25 g shikimic acid (dissolved in a small quantity of water), then 0.02 g NADP (dissolved in a little bit water), 4 ml MTT solution and finally 3 ml PMS solution.

The gel is incubated at 37°C for round about 30 min. The staining solution is removed as soon as clear banding patterns are visible in the gel. Then the gel is washed two times in 200 ml water for 20 min. After this the gel conservation is performed.

# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Conservation of gels

### 1.) Chemicals

Glycerol

### 2.) Security advices

Dichlorphenolindophenol (DCPIP) is used for staining of DIA
**Dichlorphenolindophenol (DCPIP) is probable mutagen and cancerogen.**
Therefore:

**Wear suitable protective gloves for handling the gels.**

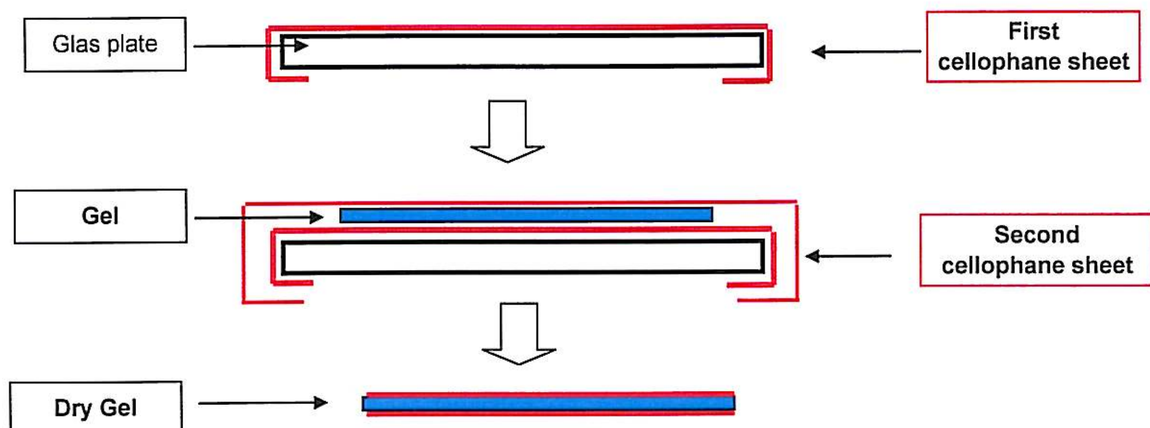
### 3.) Solutions

Glycerol solution

Glycerol are diluted in de-ionised water ad 1000 ml dependent from the room temperature and humidity:
At low room temperature and high humidity: 40 g Glycerol in 1000 ml
At high room temperature and lowh humidity: 80 g Glycerol in 1000 ml

### 4.) Procedure

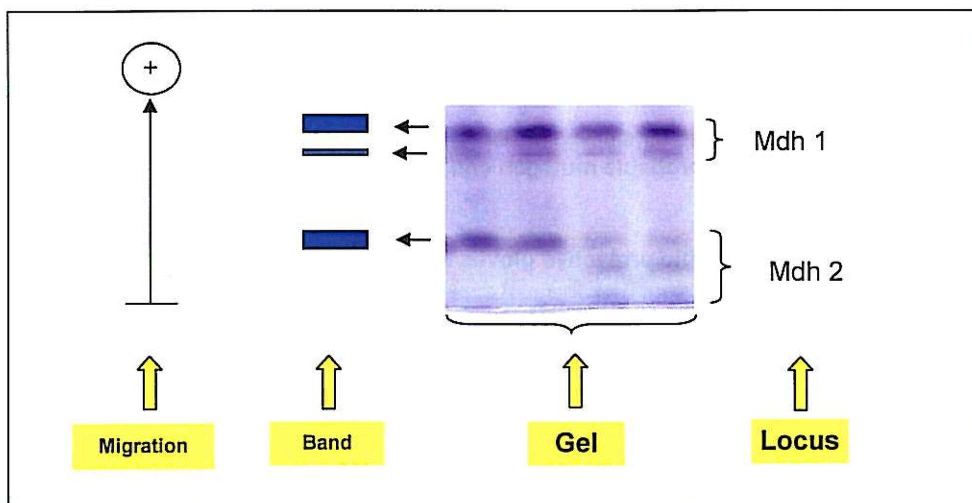
The gel is incubated in 200 ml glycerol solution for 20 min. A cellophane sheet soaked in the glycerol solution is fixed on a glas plate. The gel is put down on this cellophane sheet and covered by a second cellophane sheet pre-soaked in glycerol solution. This procedure is demonstrated in the figure. The package "Glas plate - cellophane sheet - gel - cellophane sheet" is dryed at room temperature for 1 - 2 days. During drying the gel combines with the cellophane sheets. Finally the dryed gel (including the cellophane sheets) is removed from the glas plate and cut to size.



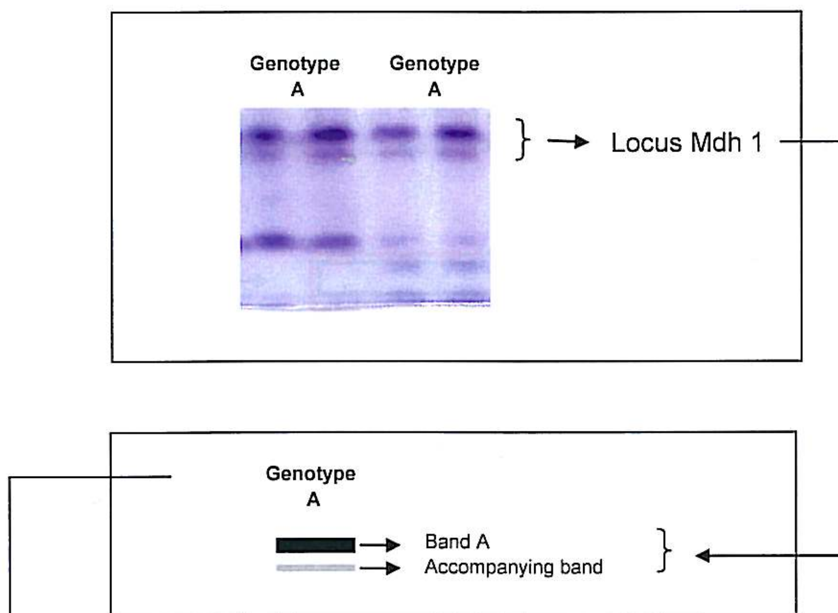
## Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Interpretation of malate dehydrogenase polymorphisms (MDH)

### 1.) Description of MDH loci

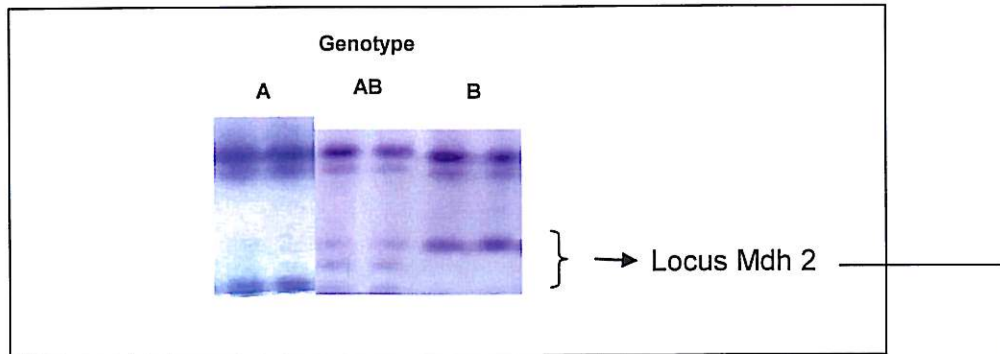


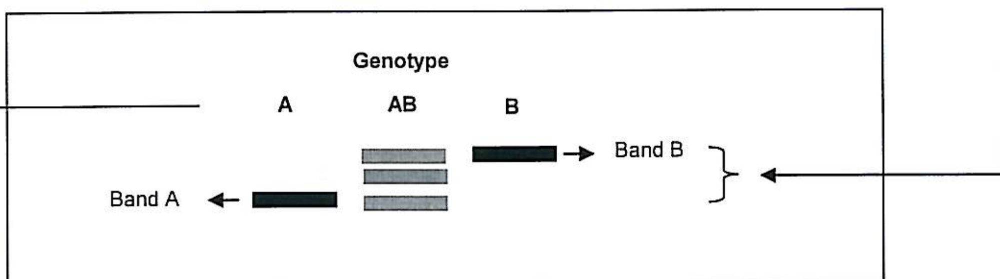

### 2.) Analysis of locus Mdh 1




**For the time being the locus Mdh 1 is monomorphic.**
**There are not any differences betwee the varieties.**
**Therefore, the locus Mdh 1 doesn't use for testing the distinctness.**

## 3.) Analysis of locus Mdh 2



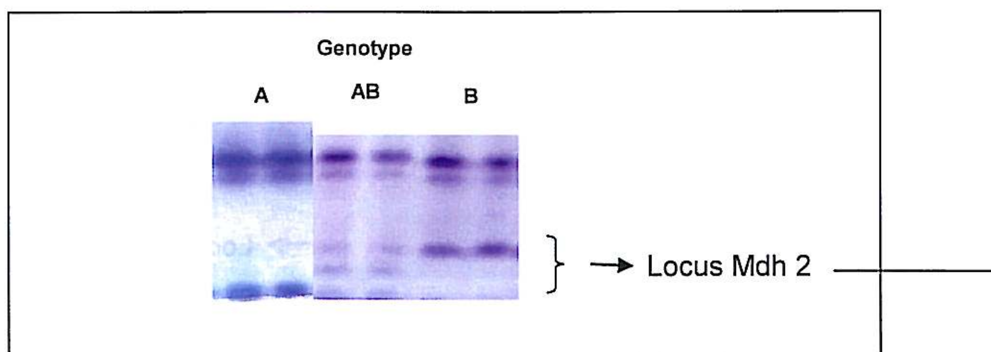



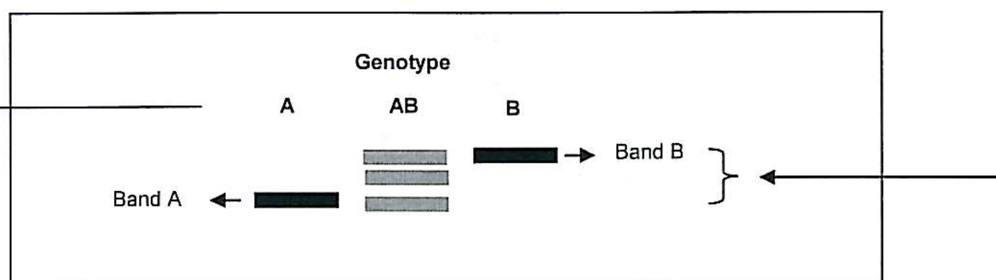

Malate dehydrogenase (MDH) is dimeric. The heterozygous genotype AB contains the homodimeric bands A and B with decreased intensities and additionally the heterodimeric band AB located between

***Characteristic: Expression at locus Mdh 2***

| Genotype | Example variety | Note |
|---|---|---|
| A | | 1 |
| B | | 2 |
| AB | Melanie | 3 |

## 3.) Analysis of locus Mdh 2




**Malate dehydrogenase (MDH) is dimeric. The heterozygous genotype AB contains the homodimeric bands A and B with decreased intensities and additionally the heterodimeric band AB located between**

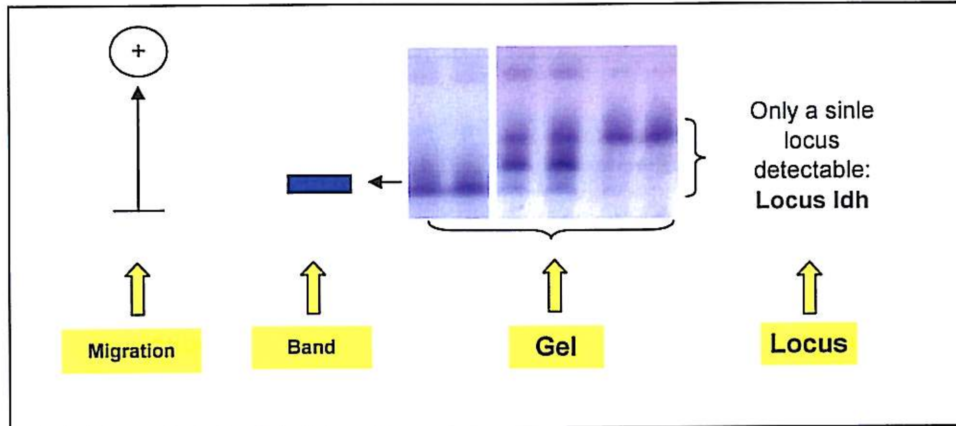
***Characteristic: Expression at locus Mdh 2***

| Genotype | Example variety | Note |
|---|---|---|
| A | | 1 |
| B | | 2 |
| AB | | 3 |

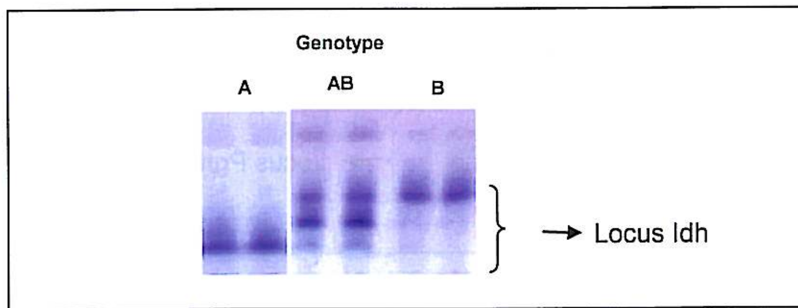
## Observation of isoenzyme polymorphisms in Calluna by use of SGE

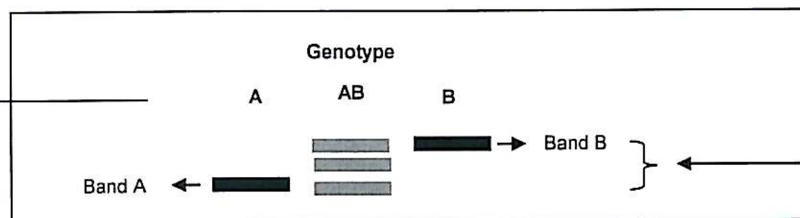
## Interpretation of isocitrate dehydrogenase polymorphisms (IDH)

### 1.) Description of IDH loci




### 2.) Analysis of locus Idh




**Isocitrate dehydrogenase (IDH) ist dimeric. The heterozygous genotype AB contains the homodimeric bands A and B with decreased intensities and additionally the heterodimeric band AB located between the homodimeric bands.**

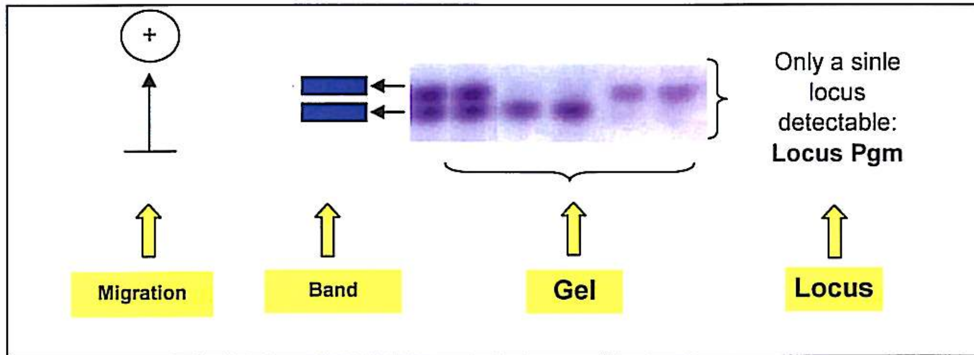
***Characteristic: Expression at locus Idh***

| Genotype | Example variety | Note |
|---|---|---|
| A | | 1 |
| B | | 2 |
| AB | | 3 |

# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Interpretation of phosphoglucomutase polymorphisms (PGM)

### 1.) Description of PGM loci




### 2.) Analysis of locus Pgm

Genotype

AB A B

Locus Pgm

Genotype

AB A B

Band B

Band A

**Phosphoglucomutase (PGM) is monomeric. Therefore, the heterozygous genotype AB contains both bands A and B of the homozygous genotypes A and B.**

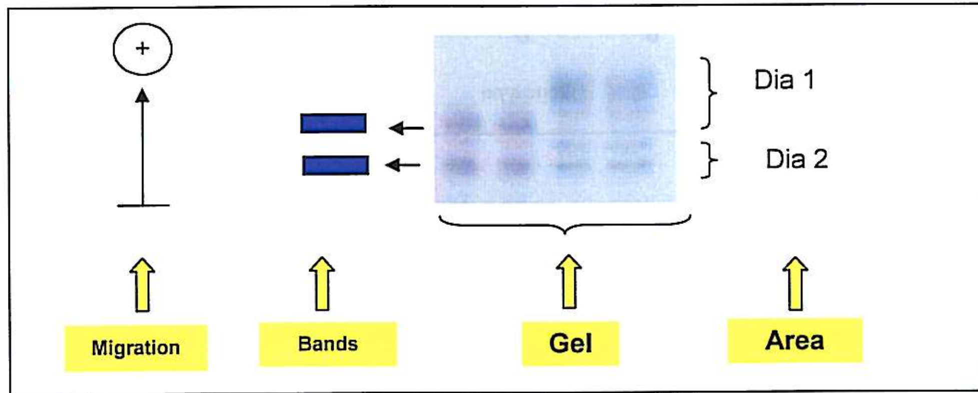
***Characteristic: Expression at locus Pgm***

| Genotype | Example variety | Note |
|---|---|---|
| A | | 1 |
| B | | 2 |
| AB | | 3 |

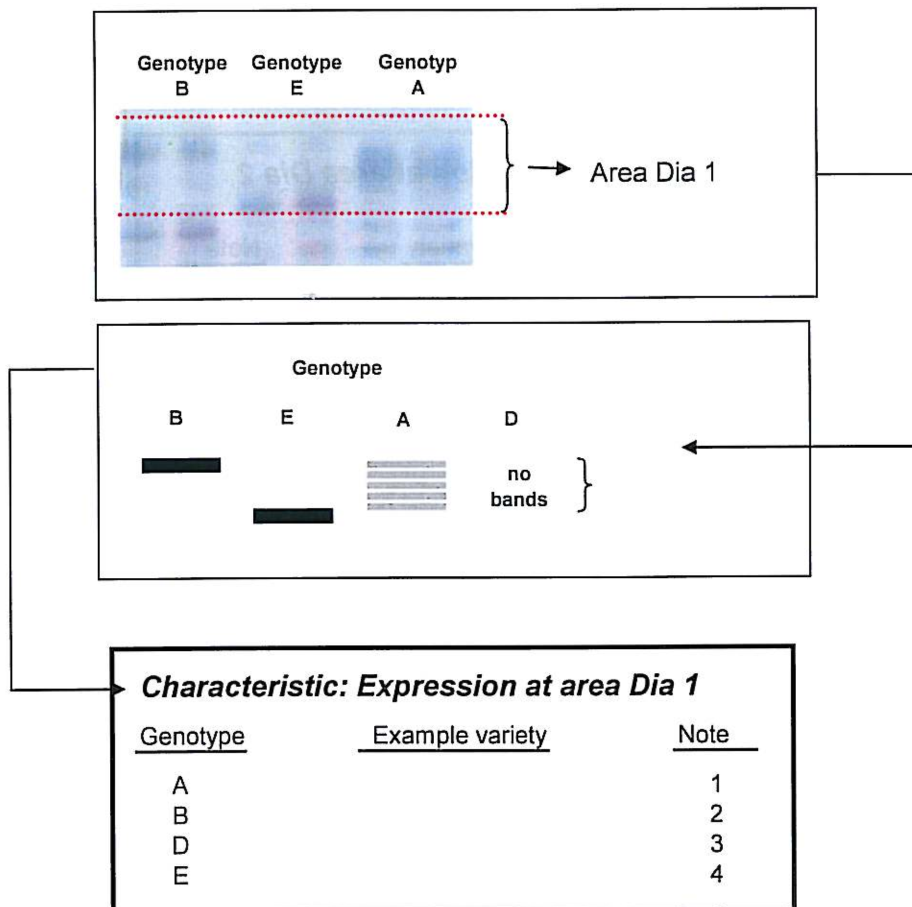
## Observation of isoenzyme polymorphisms in Calluna by use of SGE

### Interpretation of diaphorase polymorphisms (DIA)

#### 1.) Description of DIA areas



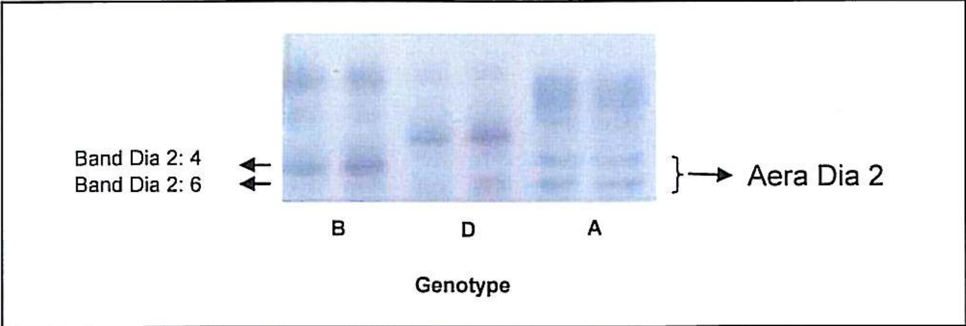

#### 2.) Analysis of area Dia 1

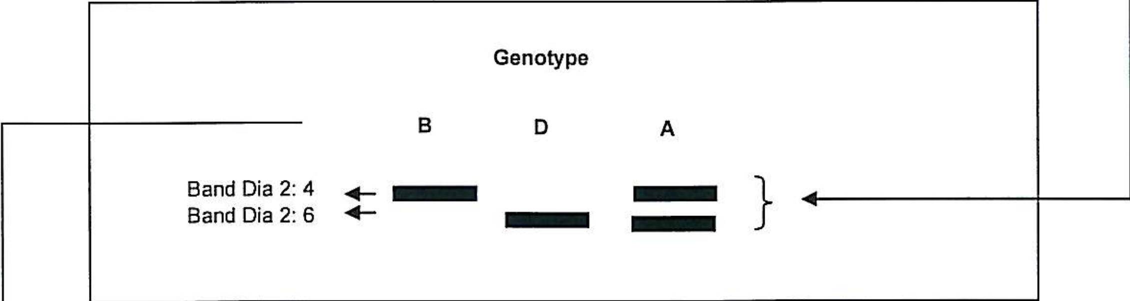



***Characteristic: Expression at area Dia 1***

| Genotype | Example variety | Note |
|---|---|---|
| A | | 1 |
| B | | 2 |
| D | | 3 |
| E | | 4 |

### 3.) Analysis of area Dia 2



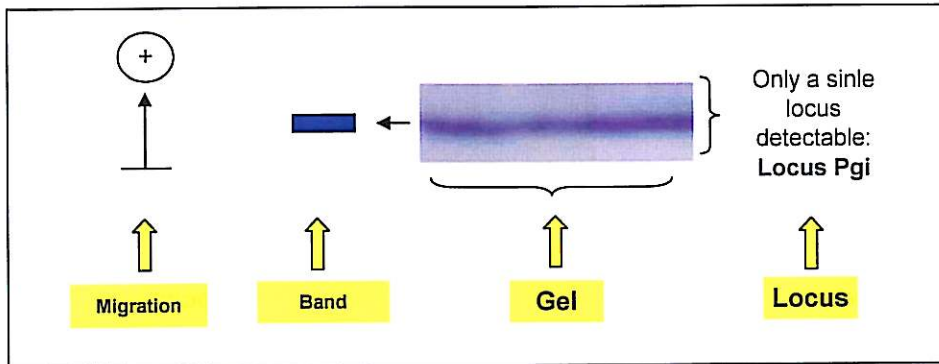

***Characteristic: Expression at area Dia 2***

| Genotype | Example variety | Note |
|---|---|---|
| A | | 1 |
| B | | 2 |
| D | | 3 |

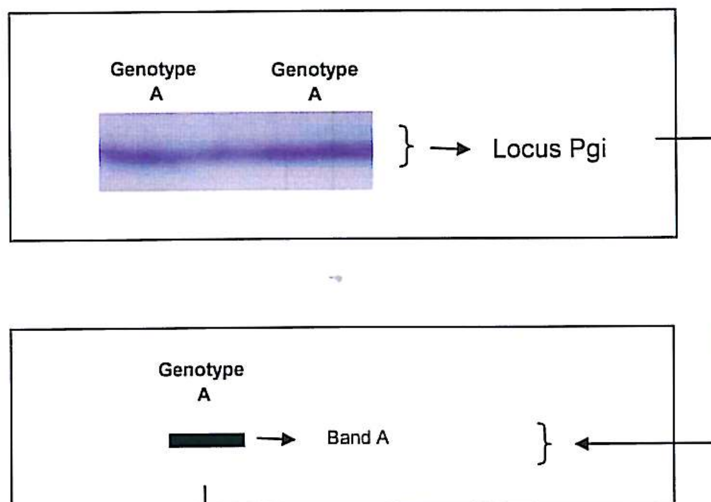
## Observation of isoenzyme polymorphisms in Calluna by use of SGE

### Interpretation of phosphoglucose isomerase polymorphisms (PGI)

#### 1.) Description of PGI loci




#### 2.) Analysis of locus Pgi

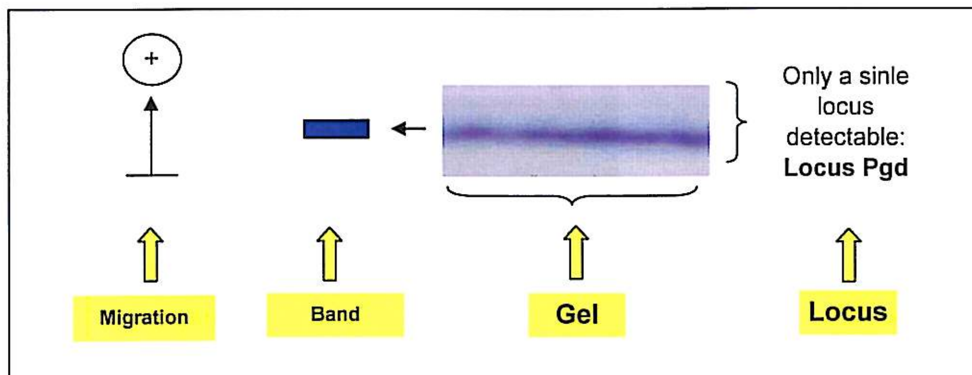



For the time being the locus Pgi is monomorphic.
There are not any differences betwee the varieties.
Therefore, the locus Pgi doesn't use for testing the distinctness.

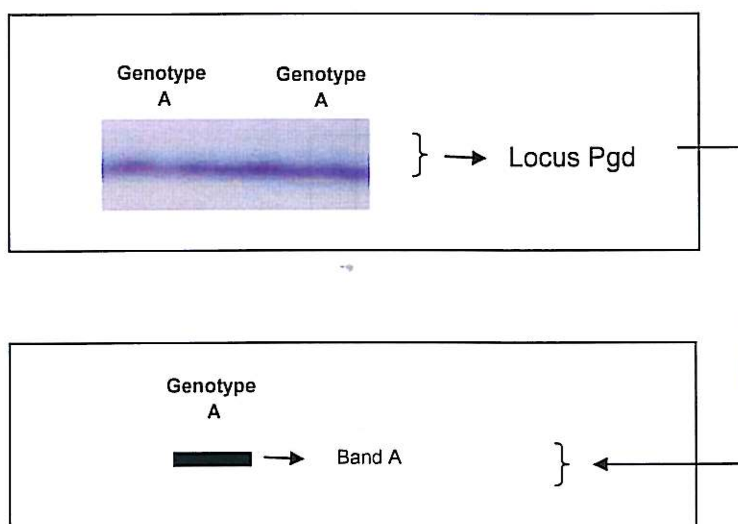
## Observation of isoenzyme polymorphisms in Calluna by use of SGE

### Interpretation of phosphogluconate dehydrogenase polymorphisms (PGD)

### 1.) Description of the loci




### 2.) Analysis of the Pgd

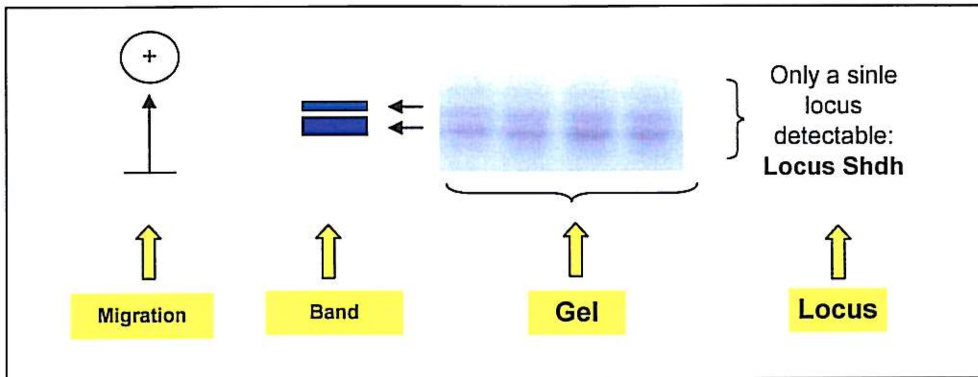



For the time being the locus Pgd is monomorphic.
There are not any differences betwee the varieties.
Therefore, the locus Pgd doesn't use for testing the distinctness.

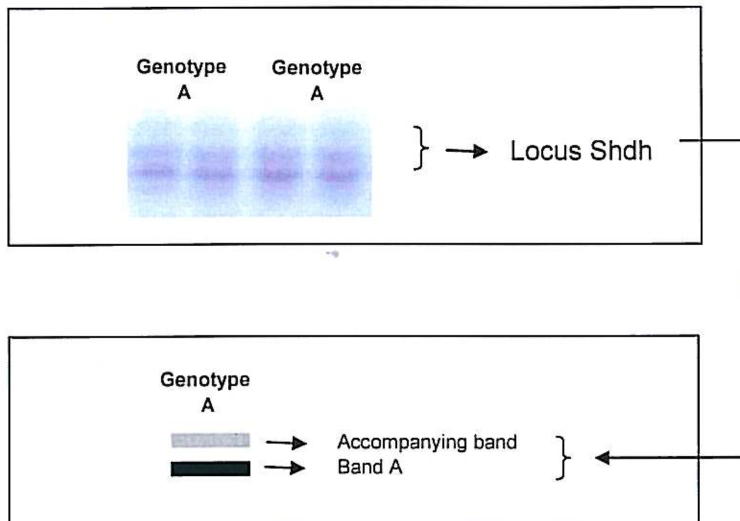
# Observation of isoenzyme polymorphisms in Calluna by use of SGE

## Interpretation of shikimate dehydrogenase polymorphisms (ShDH)

### 1.) Description of ShDH loci




### 2.) Analysis of the ShDH




For the time being the locus Shdh is monomorphic.
There are not any differences betwee the varieties.
Therefore, the locus Shdh doesn't use for testing the distinctness.