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# Is there a correlation between the nutrient content and variation in the *HvNAM-2* gene in *Hordeum vulgare*?

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**Sammanfattning**/Abstract:

Barley is one of the most important cereal crops in the world and a better understanding of the factors that regulates the nutrient content in the grain is of high interest. The industrial breeding during the last century has led to bigger yield but possibly a decrease in nutrient content. In wheat, the *NAM-B1* gene is a well-studied gene that affects the grain protein and micronutrient content. Two orthologue genes in barley *HvNAM-1* and *HvNAM-2* are candidate genes to play a similar role in the barley senescence process.

I have looked for a correlation between the diversity in the *HvNAM-2* gene and nutrient content in 37 Nordic barley accessions. The samples were sequenced and then aligned and analyzed for variation. I found three haplotypes which were compared in nutrient content and in micronutrient content. No significant difference between the haplotypes was found, which can be due to small sample size or that no correlation exists between the grain protein content and the *HvNAM-2* gene variation. Significant correlation was however found between the nitrogen content and the micronutrient contents that indicate that the pathways of all the nutrients' mobilizations are tightly coupled. For future research a bigger number of accessions, preferably at least 100, need to be analyzed to be able to give any conclusions. The molecular mechanisms in the cells during senescence also need further investigation.

**Nyckelord/**Keyword:

Plant genetics, barley, protein content, Fe, Zn, plant breeding

## Content

# 1. Abstract

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I have looked for a correlation between the diversity in the *HvNAM-2* gene and nutrient content in 37 Nordic barley accessions. The samples were sequenced and then aligned and analyzed for variation. I found three haplotypes which were compared in nutrient content and in micronutrient content. No significant difference between the haplotypes was found, which can be due to small sample size or that no correlation exists between grain protein content and the *HvNAM-2* gene variation. Significant correlation was however found between the nitrogen content and the micronutrient contents that indicate that the pathways of all the nutrients' mobilizations are tightly coupled. For future research a bigger number of accessions, preferably at least 100, need to be analyzed to be able to give any conclusions. The molecular mechanisms in the cells during senescence also need further investigation.

## 2. Introduction

Barley is considered to be the 4th most important cereal plant in the world after corn, rice and wheat. The world production today is almost reaching 140 million tons/year (FAOSTAT accessible at http://faostat3.fao.org/browse/Q/QC/E) and has a cultivation area of approximately 56 million HA, comparable to the size of Ukraine. Barley is used mainly for feed and malting. For malting and beer production, the protein content of the crop is an important factor determining the malting quality (Cai et al. 2013). The oldest barley cultivations are believed to be 23 000 years old and were found in today Israel (Zohary, D., Hopf, M., 2000). Today barley is cultivated in most parts of the world.

Towards the end of the 19th century the first test breedings were made in Sweden to develop new varieties of barley. The barley species that were used in the experiments were old landraces and some new foreign cultivars like ”Chevalier” (Olsson, G. 1997). Landraces are the original cultivars of specific places that are adapted for a typical environment.

The knowledge of the gene regulation and variation in barley is essential to secure the genetic diversity of the crop and to be able to cultivate for the right conditions and the specific purposes. The genetic diversity is also important to sustain to keep resistance against diseases and to secure the crop's ability to meet the consumption demands in the future (Brantestam 2005). In wheat, the industrial breeding in the latest century have overall led to higher yields but lower micronutrient content in the grains (Fan et al. 2008). So far no similar decrease in nutrients has been shown in barley.

The senescence process and its genetic regulation in plants is very complex. It is during the senescence that degradation of proteins in the leaves is activated and mobilization of nutrients to the grains is initiated. Christiansen & Gregersen (2014) studied the NAC genes in barley and their up-regulation during leaf senescence, and concluded that the NAC gene family is very likely to have big importance in regulating the senescence process. A member of the NAC gene family of transcription factors are the *NAM* genes. The wildtype *NAM-B1* gene in wheat enhances rapid senescence, high grain protein content (GPC) and micronutrients as Fe, Zn and Mn (Uauy et al. 2006; Distelfeld et al. 2014). Moreover, it has been shown that there exists a negative correlation between the GPC and the yield (Kibite and Evans, 1984). Hagenblad et al.(2012) did extensive studies mapping the frequency of the wildtype *NAM-B1* gene in wheat from a worldwide collection. They found that the *NAM-B1* gene was more common for wheat with origin from Nordic countries. A theory that the wild type *NAM-B1* is favored in short breeding seasons as in counties with shorter summer is therefore probably true.

Two orthologous genes to *NAM-B1* in barley, the *HvNAM-1* and *HvNAM-2* was studied for association to GPC by Cai et al. (2013). They studied both cultivated barley from different origin and Tibetan wild barley. Sharmila Madhavan (2011) investigated the *HvNAM-1* gene by CAPS (cleaved amplified polymorphic sequence) analysis but found no association between the genotype and the nutrient content.

In this study I have sequenced my samples to look for variation in the whole *HvNAM-2* gene, not only in single nucleotide polymorphisms (SNP's). I have looked at the allelic variation in the *HvNAM-2* gene for 37 accessions of *Hordeum vulgare* with origin from Sweden, Norway and Denmark, and compared with the phenotype data of GPC and micronutrient content.

# 3. Materials and methods

## 3.1. Extraction of plant material

Two different types of DNA samples were used, one for testing and optimizing the PCR conditions and one consisting of 40 different, already extracted, barley samples (*Table A1*.). The first test DNA was taken from two newly grown small plants of a barley variety from Algeria (accession-number IG35380). Two leafs (1.5 cm) where collected and stored in -80$^{o}$ C until used for extraction. For the extraction, the DNeasy Plant mini kit (50) from Qiagen® was utilized. The 2 samples were first placed in two Eppendorf tubes with AP1 buffer and ground with a micropestle. The protocol DNeasy plant handbook was then followed. After the extraction, the DNA quality was measured for the ratio $A_{260}/A_{280}$ with a Nanodrop 1000 Spectrophotometer. The 40 accessions of already extracted plant material (Madhavan, S., 2011) was after optimization used for the PCR amplification of *HvNAM-2*. The contents of nitrogen, iron and zink were measured (Madhavan, S., 2011) using Inductively Coupled Plasma Atomic Emission Spectrometry at Agrilab AB in Uppsala.

## 3.2. Sequencing

PCR was carried out in a S1000 Thermal cycler in 200 µl PCR tubes using a reaction mixture of 25 µl that contained 20 µl MilliQ water, 2.5 µl 10X Dreamtaq buffer, 0.5 µl dNTP mix (10 mM), 0.25 µl DreamTaq DNA polymerase (5U/µl) (ThermoScientific) and 0.25 µl of each primer (10 µM). The primers used where 5'-atgggcagctcggactcatcttcc-3', and 5'-tcagggattccagttcacgccgga-3' which was the same design as Cai et al. (2013) used. The polymerase chain reaction was initiated with a denaturation step at 90$^{o}$ C in 2.5 min followed by 38 cycles of 94$^{o}$ C for 30 s, 58$^{o}$ C for 30 s and 72$^{o}$ C for 1.5 min, followed by elongation at 72$^{o}$ C for 10 min. To amplify the samples NGB2565, NGB1480, NGB9944, NGB9554 and NGB296 a 5% DMSO concentration was added to the reaction mixture.

All 40 PCR products where cleaned from excess nucleotides and primers following an ExoTap protocol. The reaction mixture, 8 µl added to every PCR product, included 0.02 µl Exonuclease (20 U/µl), 0.2 µl Thermosensitive alkaline phosphatase (1 U/µl) and 7.78 µl MilliQ water. The tubes were incubated at 37$^{o}$ C for 30 min followed by a denaturation step at 95$^{o}$ C for 5 min, in a S1000 Thermal cycler.

To sequence the whole 1501 bp gene, two internal primers, primerF seq. '5-gcagtaaccgatctccgtattt -3' and primerR seq. '5- ggagatcggttactgcttgac -3', where used. Each of the cleaned PCR samples was divided into four tubes with 5 µl product in each tube. In the first tube 1 µl primerF was added, in the second tube, 1 µl primerR was added and in tubes three and four 1 µl of the primerF seq. and primerR seq. were added respectively. All primers had concentration 5 pmole/µl. The sequencing was carried out by Macrogen Netherlands.

## 3.3. Data analysis

The sequences were tidied up, edited and aligned using the Geneious 8.1.5 software. To measure the differences in nutrient contents between accessions with different haplotypes an ANOVA with CI = 95% was carried out using Minitab 17. For the comparison between the different time periods and the nutrient content an ANOVA was carried out. A correlation test was also carried out between the micronutrients Fe, Zn and the nutrient content, all in Minitab 17.

# 4. Results

The 40 accessions were sequenced and tidied after a visual inspection. The accessions NGB15103, NGB6925 and NGB1487 had too poor data from the sequencing to be used for further analysis and were excluded. All remaining 37 accessions were aligned and analyzed for genetic variance. The reference samples Clho15487 and Clho15856, for low and high GPC respectively, was found to be genetically different by three single nucleotide polymorphisms (SNP's) on base pairs 307, 798 and 979. One accession, the NGB296, had the exact same SNP's as Clho15487 and all the others had the same SNP's than the Clho15856 accession. The NGB296 and Clho15487 accessions were named haplotype 3 and the 31 accessions with the same SNP's as Clho15856 were named haplotype 1. The remaining four accessions NGB13022, NGB287, NGB27, NGB321 were named haplotype 2 and were genetically very different from both the haplotype 1 and 2 (see gene tree *Figure A1*) .

## 4.1. The variation of nutrient content

All the nutrient content data were measured as a part of a previous study (Sharmila, S., 2011). The nutrient content data for all the accessions were measured in nitrogen percent of the dry substance of the grains (N % of DS). The nitrogen content in the grains is comparable to the grain protein content (GPC) since most of the nitrogen is bound in proteins, and the nitrogen content can be converted to GPC through a factor. No conversion was done in this work and the GPC is referred to as the nutrient content.

In all the 37 studied accessions the nitrogen content had a mean value of 1.45 % and standard deviation 0.28 %, with nitrogen contents ranging from 1.10 % to 2.44 % of DS (Dry Substance) see *Figure 1A*. The iron concentrations were ranging from 27.23 to 53.83 mg/kg DS and the zink concentrations from 16.19 to 41.66 mg/kg DS, the mean values were 41.43 and 24.84 and standard deviations 7.31and 5.80, for iron and zink respectively *(Figure 1B,1C).*

The primary aim of this study was to investigate if there was any correlation between the grain protein content and the genetic variation in the *HvNAM-2* gene. However, no significant difference in nutrient content was detected between the haplotypes (1, 2 and 3) found (CI = 95 %, p = 0.920) see *Figure 1 D* and *Table 1*.

**Table 1**. Mean values of N % of DS for haplotype 1, haplotype 2 and haplotype 3.

| Haplotype | Number of accessions | Mean N % of DS | Standard deviation |
|---|---|---|---|
| 1 | 31 | 1.450 | 0.288 |
| 2 | 4 | 1.433 | 0.158 |
| 3 | 2 | 1.415 | 0.455 |

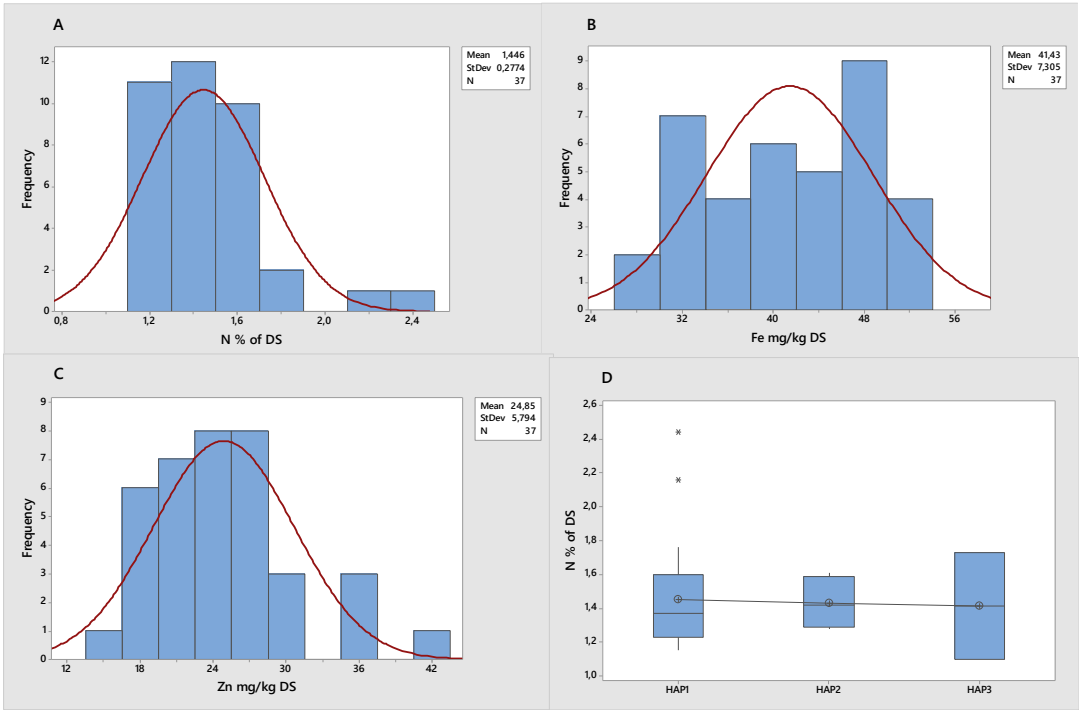


**Figure 1**. A) Distribution of N % of DS in the 37 accessions with lowest content 1.1 % and highest content 2.44 %. B) Distribution of Fe mg/kg. C) Distribution of Zn mg/kg. D) Comparison of mean N % of DS content between haplotype 1, haplotype 2 and haplotype 3.

The mean values of Zn and Fe content did not differ significantly between the haplotypes (p = 0.245 for Zn mg/kg vs haplotypes and p = 0.472 for Fe mg/kg vs haplotypes) (*Figure 2*).

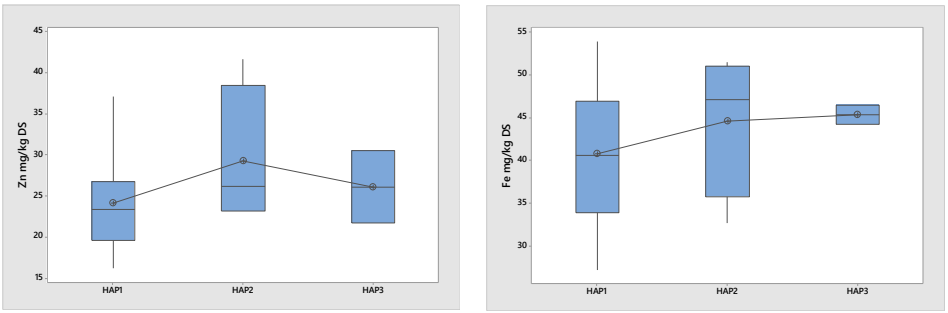


**Figure 2.** Zink and iron content mean values compared between the different haplotypes.

## 4.2. Relationships between nitrogen content, breeding period and micronutrients

The landraces' and old cultivars' mean nitrogen contents did not differ significantly compared with the new cultivars, in a one-way ANOVA (p = 0.736) (*Figure 3A*). The reference of high GPC content had lower mean nitrogen content (1.34 % of DS) than any of the different breeding periods (0.456, 0.407 and 0.478 % of DS for the landrace, old cultivars and new cultivars respectively). The Nordic barley accessions thus seem to have relatively high GPC compared to foreign accessions. No significant difference between the mean micronutrients contents from different breeding periods was observed either (p = 0.273 for Fe mg/kg, p = 0.893 for Zn mg/kg see *Figure 3 B, C*).

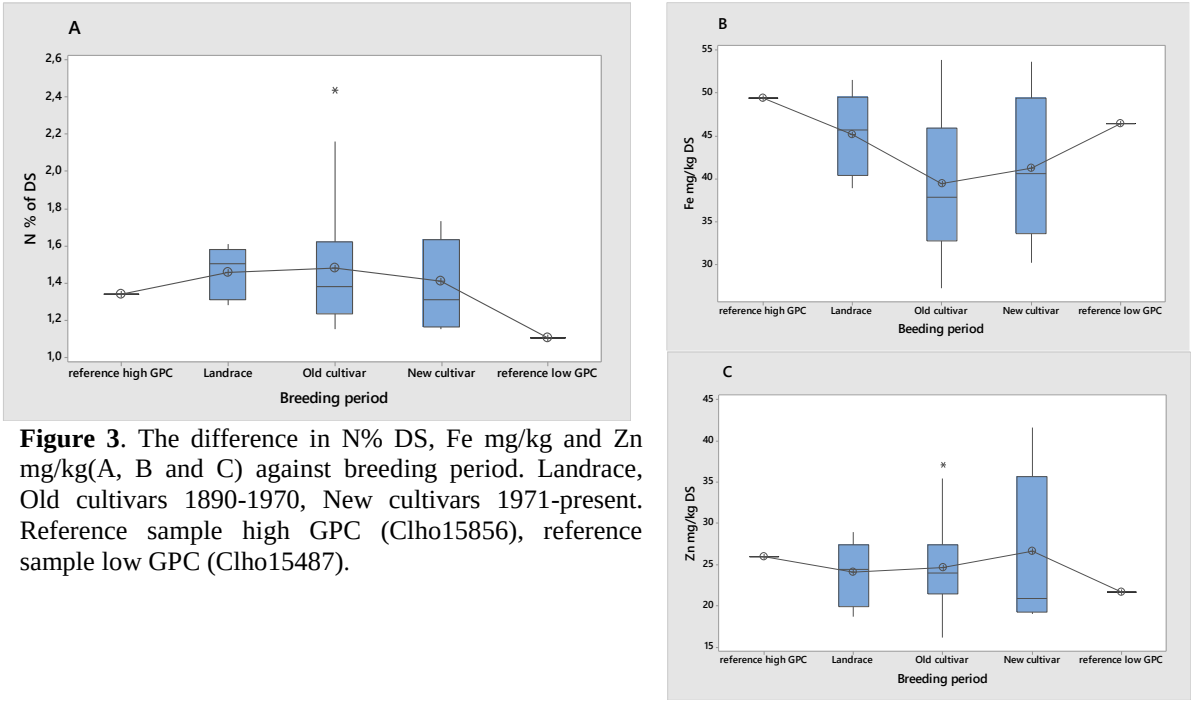


**Figure 3**. The difference in N% DS, Fe mg/kg and Zn mg/kg(A, B and C) against breeding period. Landrace, Old cultivars 1890-1970, New cultivars 1971-present. Reference sample high GPC (Clho15856), reference sample low GPC (Clho15487).

There were significant correlations between the nitrogen content and the micronutrient contents (Fe mg/kg and Zn mg/kg) in the grains ($p = 0.035$ for N % of DS vs Fe mg/kg and $p = 0.000$ for N % of DS vs Zn mg/kg, see *Figure 4*).

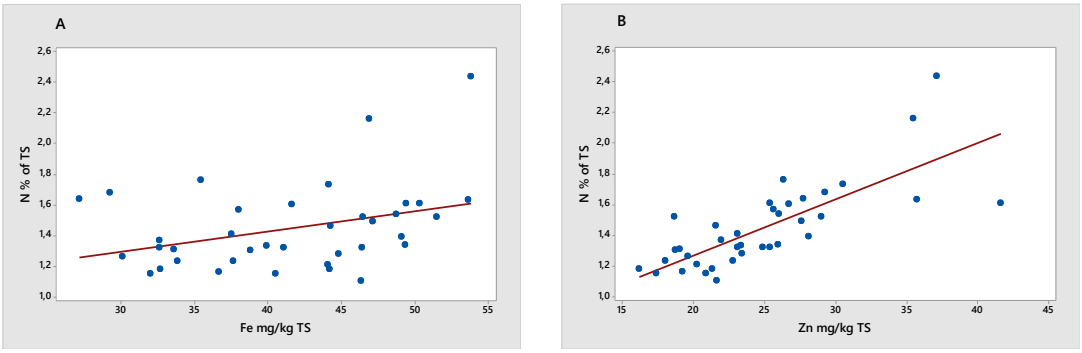


**Figure 4.** A) Positive correlation between the nitrogen content (N % of DS) and the iron (Fe mg/kg) concentration in the grains. B) Correlation between the nitrogen content and the zinc (Zn mg/kg) concentration.

## 5. Discussion

The four accessions NGB13022, NGB287, NGB27, NGB321 with haplotype 2 found, where genetically very different from haplotype 1 (see Gene tree, *Figure A1*). The reference accessions Chlo15487 and Clho15856 differed in positions 307, 798 and 979. Cai et al. 2013 also found the SNP on position 798 and that the single nucleotide polymorphism has correlation to GPC. The mutation leads to a change in amino acid from Leucine in Clho15478 to Phenylalanine in Clho15856. One of the Nordic accessions, NGB296, had the same SNP's as Clho15487.

The haplotype 3 consisting of the accessions NGB296 and Clho15487 were different in nutrient content, 1.73% N of DS and 1.10% N of DS respectively, even if they were genetically identical in the HvNAM-2 gene. Cai et al. (2013) found correlation between the GPC and different *HvNAM-2* haplotypes in barley. No such correlation was found in this

study. Since no big difference in nutrient content between the haplotypes was found it suggests that other genes are responsible for variations in the nutrient content. Moreover, mutations outside the gene, e.g. in the promoter site can be responsible for the variations in nutrient content. Finally, it is also possible that if more accessions of the haplotype 2 and 3 could be analyzed it would strengthen the effect of genetic diversity on GPC.

Interestingly the NGB15238 accession had a big 48 bp deletion of nucleotides (bp51-99). The number if nucleotides deleted is an in-frame deletion and do not affect the whole protein structure. That can be an explanation why it does not seem to affect the protein function a lot either. The nutrient content was low but not significantly low. The accession NGB466 had five extra bases from bp 95-99 but did not seem to alter the nutrient content in comparison with the other accessions. Both these results are different from those of Cai et al.(2013), that did not find any indels in the HvNAM-2 gene.

One hypothesis about the grain protein content is that it could have decreased due to selection for higher yields, in the last century of industrial breeding as it has shown in wheat for the *NAM-B1* gene (Asplund et al. 2010). The differences in nutrient content between cultivars from different breeding periods were analyzed, but no significant differences were found in this study. Neither a decrease in micronutrients over the different breeding periods were shown.

The correlations detected between the N % of DS and Fe, and N % of DS and Zn suggests that the same genes are responsible for regulating all three nutrient mobilizations and that the processes are coupled to one another. The stronger correlation measured for the N % of DS and the Zn mg/kg may be due to proteins containing Zn as cofactors. This supports the hypothesis that the mobilization of all the nutrients from the leaves to the grains could be regulated by the same factors. This confirms what Uauy et al. (2006) found when they studied the *NAM* genes in wheat by RNA interference, that the *NAM* genes seem to have close correlation to for the mobilization of both protein, Zn and Fe from the leaves. In this study all plants had been cultivated under the same conditions with equal contents of micronutrients in the soil (Madhavan, S., 2011)

The accessions Karl Clho15487 and Lewis Clho15856, with low respective higher grain protein content were used as reference samples and had origin from the United States. Both accessions had, however, low protein content compared to the analyzed Nordic material. The Nordic accessions can therefore be a good starting material for future breeding for barley with high nutrient contents.

For future research it will be important to deepen the understanding of the molecular and cellular mechanisms underlying the senescence process, as to analyze the amino acid carriers and protein degradation factors in the nitrogen and micronutrient mobilization to the grains. For example finding and analyzing other QTL (quantitative trait locus) that affects the senescence will have importance and also understand more fully how environmental factors and genes interact.

### 5.1. Civil and ethical aspects

With a bigger and bigger population on the planet we are in big need of food resources to feed everyone. A good supply of nutrients in the food is imperative for our health. The knowledge and ability to breed nutrient rich crops is thus very important. One step towards this is to get a better understanding of the genetic factors that regulate the nutrient mobilization. In this work my results can be one starting point for future research.

The ethical aspects of how this knowledge will be used, for example to develop genetically modified organisms (GMO) is a hot topic that can be discussed. We do not know the consequences on the ecosystem if these plants get to spread. Although the chance is very little that it will have adverse effects if the crops are just modified to contain more nutrients. The positive consequences, that more people can have better food and a chance to better lives are, in my opinion, overshadowing the eventual negative consequences.

## 6. Acknowledgements

I would like to thank my supervisor Jenny Hagenblad for expertise, support and great advice, Maria Lundström for all the help in the lab and Matti Leino and Per Larsson for their expertise. I am also thankful for support from my labmate Sandra Lilja, and for advice from opponent Malin Larsson.

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# 8. Appendix

### Detail description of the analysis of the PCR products

The PCR was successful and showed a result on a 0.8 % agarose gel electrophoresis. For the agarose gel electrophoresis, Sy Br safe gel stain (Invitrogen) was added to a 1/20 000 concentration of the stock solution. The agarose (Saveen Werner AB) and 0.5X TBE-buffer were mixed and prepared in a microwave on high effect for 2 min.

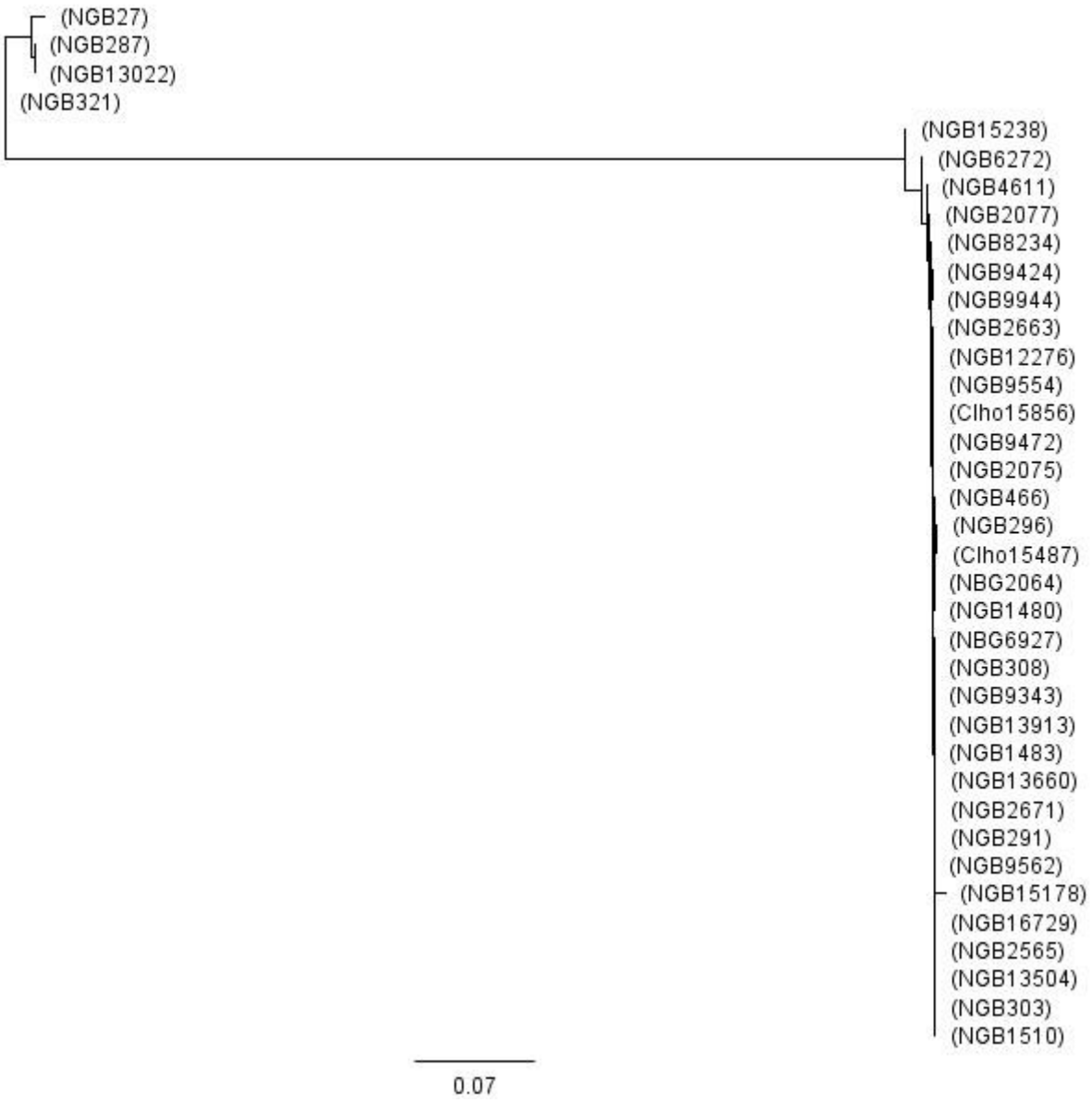


**Figure A1.** Gene tree displaying the differences between the 37 accessions. The line segment and the number ’0.07’ works as a scale for the legnth of the branches in the tree. The number 0.07 is calculated from nucleotides substituted per site divided by the sequence length.

**Table A1**. Data for the 37 accessions.

| Number | Acc. nr: | Country orgin | Subtype | Type | N % of DS | Fe mg/kg DS | Zn mg/kg DS | Haplotype |
|---|---|---|---|---|---|---|---|---|
| 10. | NGB15178 | Sweden | Landrace | 6rw | 1,61 | 50,32 | 25,42 | 1 |
| 11 | NGB2565 | Sweden | Landrace | 2rw | 1,49 | 47,15 | 27,62 | 1 |
| 12. | NGB6927 | Sweden | Landrace | 6rw | 1,30 | 38,85 | 18,76 | 1 |
| 13. | NGB9472 | Sweden | Landrace | 2rw | 1,52 | 46,51 | 18,68 | 1 |
| 14. | NGB13504 | Sweden | Landrace | 2rw | 1,60 | 41,67 | 26,73 | 1 |
| 16. | NGB27 | Finland | Landrace | 6rw | 1,28 | 44,81 | 23,45 | 2 |
| 17. | NGB308 | Finland | Landrace | 6rw | 1,33 | 39,97 | 23,36 | 1 |
| 18. | NGB321 | Finland | Landrace | 6rw | 1,52 | 51,48 | 29,00 | 2 |
| 27. | NGB2075 | Norway | Old cultivar | 6rw | 1,18 | 32,72 | 16,19 | 1 |
| 28. | NGB466 | Norway | Old cultivar | 6rw | 1,54 | 48,76 | 26,01 | 1 |
| 29. | NGB2077 | Norway | Old cultivar | 6rw | 1,57 | 38,06 | 25,64 | 1 |
| 30. | NGB2064 | Norway | Old cultivar | 6rw | 1,41 | 37,55 | 23,14 | 1 |
| 31. | NGB9424 | Sweden | Old cultivar | 2rw | 2,44 | 53,83 | 37,15 | 1 |
| 32. | NGB1483 | Sweden | Old cultivar | 2rw | 2,16 | 46,93 | 35,51 | 1 |
| 33. | NGB1480 | Sweden | Old cultivar | 2rw | 1,76 | 35,48 | 26,35 | 1 |
| 34. | NGB15238 | Sweden | Old cultivar | 6rw | 1,23 | 37,69 | 22,78 | 1 |
| 35. | NGB6272 | Sweden | Old cultivar | 6rw | 1,32 | 46,43 | 24,91 | 1 |
| 36. | NGB8234 | Finland | Old cultivar | 2rw | 1,21 | 44,07 | 20,28 | 1 |
| 37. | NGB13660 | Finland | Old cultivar | 6rw | 1,18 | 44,25 | 21,33 | 1 |
| 38. | NGB9343 | Finland | Old cultivar | 2rw | 1,15 | 32,07 | 17,38 | 1 |
| 39. | NGB9562 | Finland | Old cultivar | 2rw | 1,37 | 32,67 | 21,96 | 1 |
| 51. | NGB4611 | Sweden | Old cultivar | 2rw | 1,68 | 29,28 | 29,28 | 1 |
| 53. | NGB2671 | Sweden | Old cultivar | 2rw | 1,39 | 49,12 | 28,14 | 1 |
| 54. | NGB2663 | Sweden | Old cultivar | 2rw | 1,46 | 44,27 | 21,62 | 1 |
| 56. | NGB9554 | Finland | Old cultivar | 2rw | 1,23 | 33,86 | 18,04 | 1 |
| 57. | NGB291 | Finland | Old cultivar | 6rw | 1,32 | 41,13 | 25,40 | 1 |
| 58. | NGB303 | Finland | Old cultivar | 2rw | 1,64 | 27,23 | 27,77 | 1 |
| 59. | NGB287 | Finland | Old cultivar | 2rw | 1,32 | 32,68 | 23,11 | 2 |
| 69. | NGB13022 | Norway | New cultivar | 6rw | 1,61 | 49,39 | 41,66 | 2 |
| 70. | NGB16729 | Norway | New cultivar | 6rw | 1,63 | 53,61 | 35,72 | 1 |
| 72. | NGB1510 | Sweden | New cultivar | 2rw | 1,31 | 33,63 | 19,06 | 1 |
| 73. | NGB9944 | Sweden | New cultivar | 2rw | 1,26 | 30,14 | 19,63 | 1 |
| 74. | NGB12276 | Sweden | New cultivar | 2rw | 1,15 | 40,58 | 20,88 | 1 |
| 75. | NGB13913 | Sweden | New cultivar | 2rw | 1,16 | 36,72 | 19,23 | 1 |
| 76. | NGB296 | Finland | New cultivar | 6rw | 1,73 | 44,18 | 30,54 | 1 |
| 81. | Clho15487 | US | reference low GPC | 6rw | 1,10 | 46,39 | 21,69 | 1 |
| 82. | Clho15856 | US | reference high GPC | 2rw | 1,34 | 49,37 | 25,96 | 1 |