Confirmation and fine-mapping of seed quality QTLs in tomato

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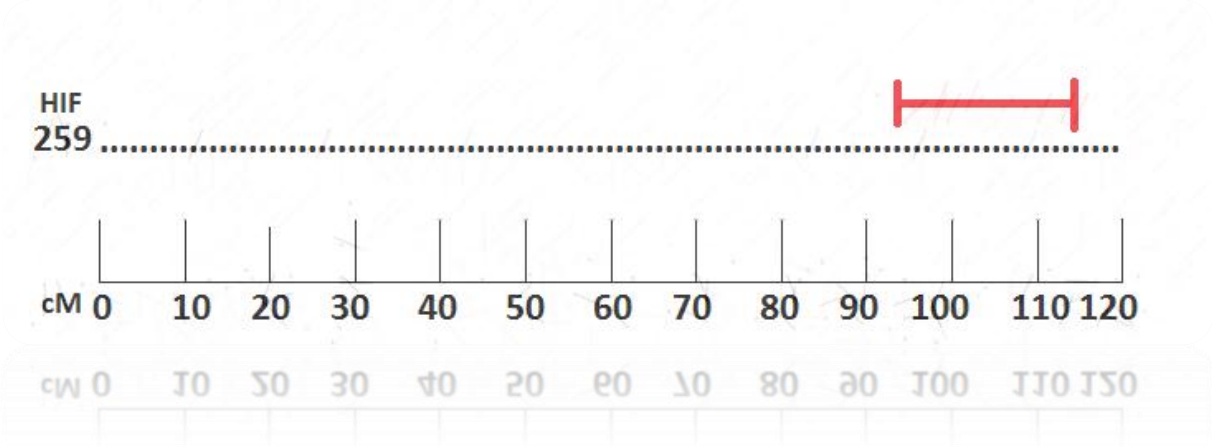

Confirmation and fine-mapping of seed quality QTLs in tomato

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Abstract

Seed quality is a complex trait that has attracted the interest of different people, from farmers till researchers for many decades. Everyone has a different field of interest concerning seed quality but everyone has a common purpose, to obtain the highest quality concerning yield, uniformity and different other quality traits. The researchers have tried to accomplish their goal by exploring the loci that control seed quality traits. They discovered that there are multiple loci which can control only one characteristic and to give it the different phenotypes. These genomic regions are called Quantitative Trait Loci (QTLs). After many years of research QTL mapping was achieved. By using molecular markers the visualization of the QTLs positions throughout the genome of different plants (e.g. Arabidopsis thaliana, tomato, rice etc.) was attained, offering an essential tool in the hands of researchers. This achievement gave the motivation for further studies as the fine-mapping of the QTLs and the complete understanding of the function of the genes causal for these QTLs. Until now a great effort has been done and interesting results have been published. This project is based on the findings of previous projects. The aim is to confirm specific QTLs of seed quality traits of tomato that have been identified on chromosome 9 by using the approach of Heterogeneous Inbred Families (HIFs). HIFs are progenies of a specific population of Recombinant Inbred Lines (RILs) and segregate together with the examined QTLs that control the desirable characteristics. As a second part of this project, it was managed to develop new molecular markers in order to identify the recombinations at the QTL region. Additionally, a new method for the “SEED LAB” the High Resolution Melt Analysis was used for genotyping. With the achievement of good results we made a step further that consequently will result in the discovery of the molecular mechanism that underlie the complex trait of seed quality.

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Acknowledgment

Six months of a master student life is dedicated in researching, experimenting and finally writing the master thesis. On the one hand this period of time is one of the most stressing, difficult and time-taking procedure of the whole master session. On the other hand if you are lucky enough you will experience situations of great pleasure.

For the reason above, I would like to thank the whole group of “Seed Lab” for their resonant presence in my student life. They equipped me not only with their positive feelings but also with their positive look of life that was really inspirational.

Furthermore, I would like to thank my supervisors Wilco Ligterink and Leo Willems whose help was a valuable “weapon” for confronting every difficulty that I came across during my thesis period and of course to thank them for the great patience that they showed. Without them it would be impossible to finish my thesis and consequently to get my master degree. However, the most important is the knowledge that offered to me and the opportunity to broad my horizons of thinking. Special thanks to Plant Breeding department that provided me with the appropriate equipment for the execution of my experiments.

Last but not least I would like to thank all of my friends that were patiently listening and consulting me, even if sometimes I was more grouchy than a baby having its first tooth, but mostly I would like to thank my family that gave me the opportunity to come to Wageningen University and to be part of this community and they provided me all these years with many useful competences for my future life.

1. Introduction

Feeding the continually growing human world is one of the main concerns of humanity nowadays. Hence, the crop production in great scale can be a solution to this problem. Since the first time that the people have established their first crop cultivation they knew the importance of the seed for a satisfying, good quality yield. Seed is the beginning of the life cycle of a plant and is consisted of three basic parts: an embryo, the endosperm and the testa. The embryo is the fertilized ovule, consisting of cotyledons, hypocotyls and the embryonic root (radicle) (Booth B.D. *et a*l., 2003). The quality of the seed has attracted the attention of people that belongs to different working fields. For example the seed companies are interested in the quality of the seeds focusing on breeding attempts, harvest and post –harvest treatments (Ligterink *et al*., 2012). Furthermore, the seed companies as well as the researchers are also interested in the functions of the genes and their effect on seed quality. Thus, we can understand that the seed quality is a complex term that attracts the interest of many different sides and has to be examined "in depth".

Seed quality is a complex, quantitatively inherited trait. This trait can be described as the ability of seed to germinate under different biotic and abiotic conditions and to produce 'usable plants' (Kazmi *et al*., 2012; Ligterink *et al*., 2012). There are many different characteristics that can together be used to describe seed quality. Seed and seedling vigor, dormancy, germination, viability and uniformity are some of them (Dickson 1980; Hilhorst & Toorop 1997). All these characteristics are controlled by different genes or in many occasions by numerous of genes. The regions on genome that contain these genes that are associated with quantitative traits are called Quantitative Trait Loci (QTL). Hence QTL analysis is a powerful tool to elucidate the behavior of complex traits.

Quantitative Trait Loci analysis has been conducted in previous researches. In the genome of many different plants QTLs were identified that control different seed quality attributes such as in *Arabidopsis thaliana*, QTLs for dormancy (Bentsink *et al*., 2010) and many different germination characteristics (Joosen *et al*. ,2010). Many QTLs controlling seed and seedling quality characteristics that concern the seed size and weight or germination under stress have also been described for tomato (Foolad *et al*., 2003, 2008; Doganlar *et al*., 2000). In the present research follow –up on a QTL analysis that has been performed for seed quality traits in a RIL population that was the product of a cross between *Solanum lycopersicum* (cv. Moneymaker) a superior breeding line of tomato and the wild most closely related species with *S. lycopersicum*, *S. pimpinellifolium* G1.1554 (Kazmi *et al*., 2012; Khan *et al*., 2012; Voorips *et al*., 2000). After linkage analysis of the 100 RILs at F_8 with the help of SNPs markers, 12 linkage groups were observed. Moreover, the genomic locations of QTLs for seed and seedlings traits were mapped with the use of LOD threshold of 2.0. The heat map is a tool to help in visualization of the locations of the QTLs throughout the 12 chromosomes of

tomato (Figure 1). The QTL mapping analysis has exposed the existence of a total 120 important QTLs for germination traits under different conditions (Kazmi *et al*. and Khan *et al*., 2012). Co-localizations of QTLs were observed for different seed and seedling traits on the bottom of the chromosomes 1, 4, 6, 9 and 11.

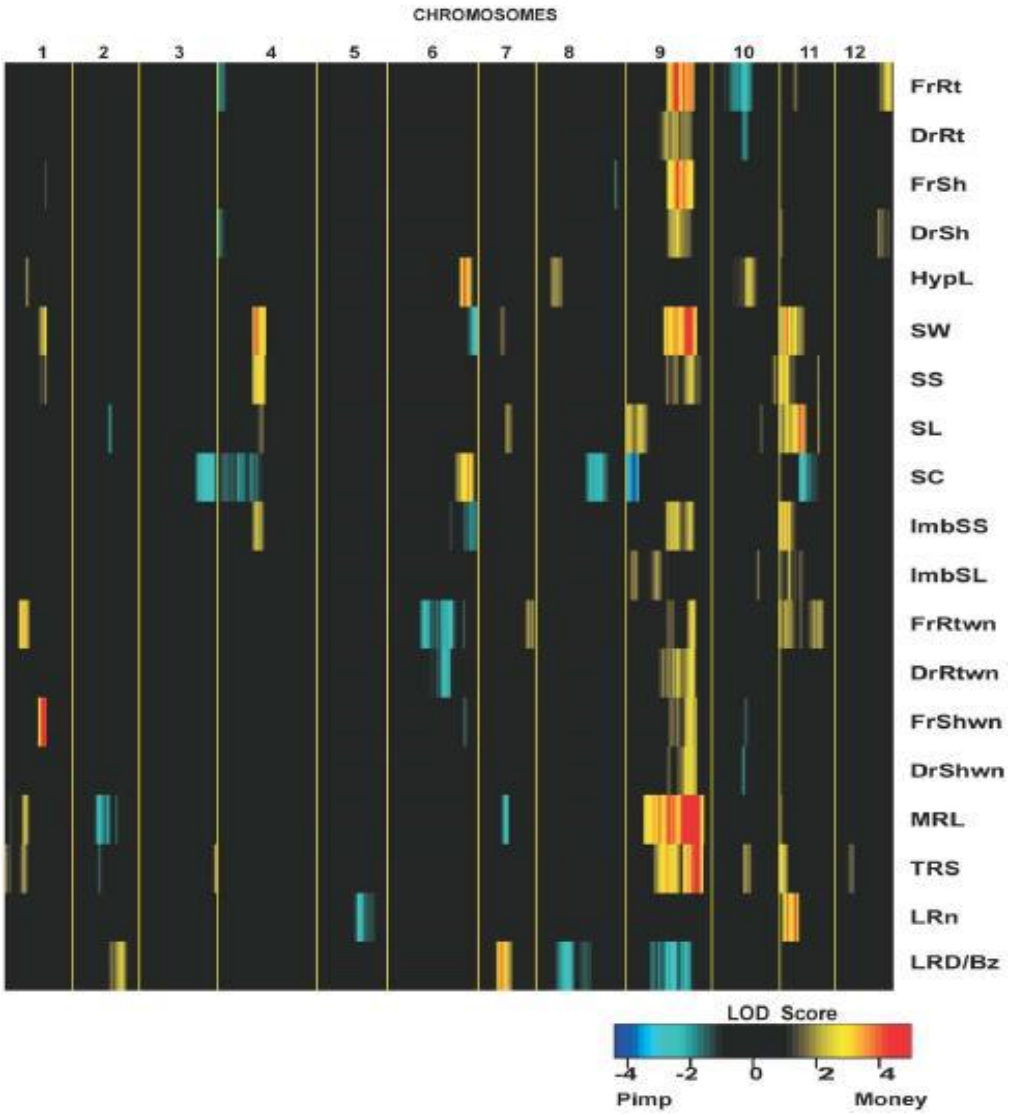

Figure 1: Heatmap of QTLs identified for seed and seedling quality traits across 12 chromosomes of tomato from Khan et al. (2012). Traits are abbreviated as: SW = Seed Weight, SS = Seed Size SL = Seed Length. FrRt = Fresh Root weight, DrRt = Dry Root weight, FrSh = Fresh Shoot weight, DrSh Dry Shoot weight. Similar traits measured in nutrient deprived condition are abbreviated as FrShwn, DrShwn, FrRtwn and DrRtwn respectively. MRL = Main Root Length, TRS = Total Root Size, LRn = Lateral Root number per main root, LRD/Bz = Lateral Root Density per branched zone. Colors indicate QTLs significant at P = 0.002 in multiple QTL mapping models (1-LOD intervals). Blue and light blue colors indicate larger effect of the trait in S. pimpinellifolium, and yellow and red in S. lycopersicum.

Although QTL analysis of RIL populations with help of molecular markers has been a great tool for identifying QTLs, the region that is spanned by QTL is in general still very big and comprises of at least hundreds of genes. Fine-mapping is the extra step that has to be done in order to narrow down the knowledge gap that we have for the seed quality traits of tomato. A specific tool for fine-mapping and confirming of QTLs are Hetorogeneous Inbred

Families (HIFs) (Tuinstra *et al*., 1997). HIFs make use of the last residual heterozygosity present in RIL populations on the specific QTLs. Two homozygous parents are crossed. After 7 generations of selfing and segregating of the specific trait, at F_8 the lines are almost homozygous except only from small regions (around 5% of the total genome). Progenies of the lines with these heterozygous regions conquering with the concerned QTL can be segregate only for the heterozygous region creating homozygous lines with different genotypes at the QTL region in a single generation. Thus, HIFs offer the advantage of comparison of the trait of interest for that specific region for both parental genotypes in an isogenic background (Kooke *et al*., 2012). By selfing of RI Lines with heterozygosity on the specific loci, NILs are created for the regions of heterozygosity. HIFs only differ for flanking markers to the QTL of interest and they are a specific type of NILs (Figure 2). With the development of more molecular markers and the convenience that the HIFs give us we can zoom in on the causal gene for each QTL and ultimately decipher its function. These steps will give breeders the opportunity to transfer the most important genes for seed quality immediately to the plants by genetic modification or by normal breeding as it would be easier for the specific genes to be identified, making the selection of the desired characteristic faster and easier.

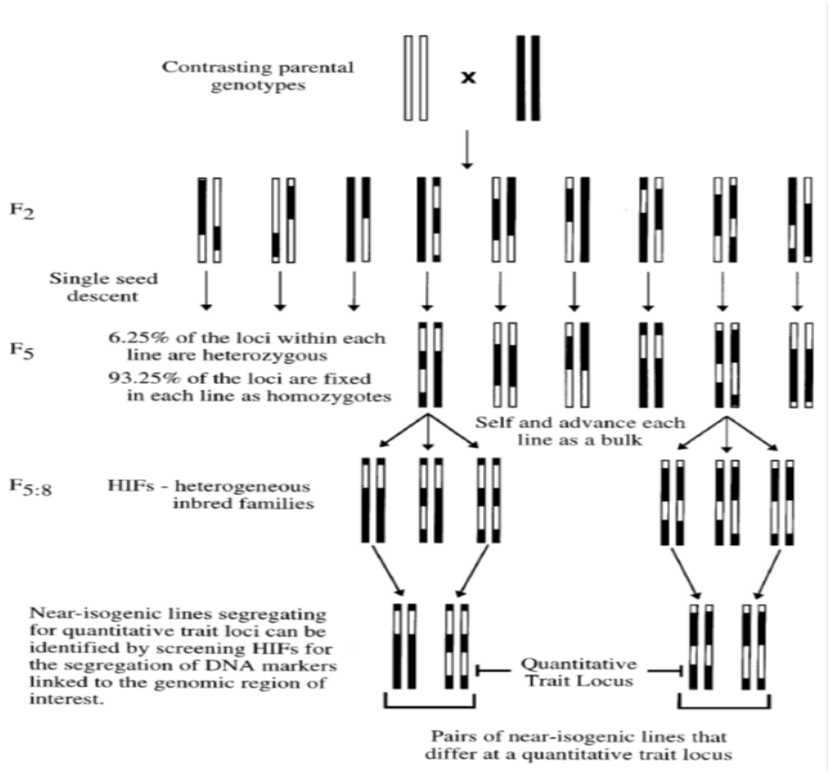

Figure 2: Generating heterogeneous inbred families from recombinant inbred lines and deriving NILs (Tuinstra et al., 1997). Selfing the F1 obtained from two homozygous parents with single seed decent approach for five generations results in 93.25% of the loci fixed homozygous and 6.25% loci heterozygous within each line. After continues selfing and advancing each line as a bulk can provide heterozygous inbred families (HIFs). HIFs that are heterozygous at the regions of interest (QTL) are selected from NILs by using segregating markers.

Molecular markers

The invention of DNA molecular markers in the 1980s gave a great advantage to breeders, making their breeding efforts more efficient and effective. Molecular markers belong to the wider category of Genetic Markers that are characterized as the "signs" of specific genes because they can be situated in nearby region of the analysed gene or QTLs and they can segregate together, nevertheless DNA molecular markers are not always in or nearby genes providing no information for the analysed gene. Molecular markers have the advantage of not being affected by environmental factors or the developmental stage of the plants (Winter and Kahl, 1995). They are depicting the possible variation in sequences in DNA among individuals and are products of variation of nucleotide sequences caused by substitution mutations or rearrangements of bigger regions (insertions , deletions) or even errors in replication of consecutive repeated DNA (Paterson, 1996a). The most common variations in the nucleotide sequence are Single Nucleotide Polymorphism (SNPs) and Simple Sequence Repeats (SSRs). By using the SNPs genome variation is effective to design primers only by ascertaining the nucleotide substitutions between the parental genomes. There are different methods to detect the variation of the genome such as Cleaved Amplified Polymorphic Sequence (CAPs) and many more. In the specific project we focused on SNP identification by means of CAPs and HRM analysis methods.

Cleaved Amplified Polymorphic Sequence (CAPs) Method

Cleaved Amplified Polymorphic Sequence is a technique for analysis of the genetic markers that involves amplification of a target DNA through PCR, followed by digesting with restriction enzymes. CAPS technique is a combination of Restriction Fragment Length Polymorphism and PCR. As in RFLP, CAPS technique based on the fact that genetic differences between individuals can create or nullify endonuclease restriction sites, and that these differences can be detected in the resulting DNA fragment length after digestion (Konieczny A. *et al*., 1993).

High Resolution Melt Analysis Genotyping Method

For many decades the scientific community is interested in discovering a more elucidated and easy way in identifying genetic mutations and genotyping of the genome of different organisms. Recently, a new quantitative method came to replace the already known insufficient way of Low Resolution Melt Analysis. This method is called High Resolution Melt Analysis and has proven to be a low-cost, easily used, non-destructive technique with high proportion of accurate results (Fisher *et al*. 2010; Taylor *et. al*, 2010). Low Resolution Melt Analysis is also a post –PCR melt curve analysis based on the same principles as the High Resolution Melt Analysis with the only difference in the intercalating dye and the temperature increments. For HRM the difference of the temperature between two consecutive measurements of fluorescence is commonly 0.008 - 0.2^{o}C compare to the LRMA that is 0.5^{o}C. In addition, the use of improved dsDNA intercalating dyes in HRM enhanced the reliability of the results providing more details about the melting behaviour.

The technique of HRM analysis is based on the ability of the nucleotide sequence as also of the complementarity of the double-stranded chain to change the melting temperature (Tm) of double stranded DNA. Accurate results can be attained with the aid of specific

intercalating dye which binds in the double stranded DNA without inhibiting DNA polymerases or altering the Tm of the product and which promotes the visualization of the fluctuation of the fluorescence of the samples. In addition to that the appropriate software is also important.

As it is referred above the main tools for succeeding in producing the results are the intercalating dye and the appropriate software. The software has to fulfil certain prerequisites which are indicated in the appendices. Secondly, the intercalating dye is the most important tool in order to analyse the melting behaviour of the samples.

There are three types of intercalating dyes; the non-saturating, the saturating and the release on–demand dyes. These double stranded DNA dyes have different properties concerning the binding targets and the changes of the melting profile of the PCR products (KAPABIOSYSTEMS, 2010). In the present project the saturating type was used. Compared to the other dyes it is the only one which is not relocating among the bases of the DNA strands giving more reliable results concerning the monitoring of the melting temperature (Tm) of the products.

Several other important issues, besides using suitable software and the appropriate dye, have to be considered for a successful HRM analyses. An important starting step for the HRM analysis is the DNA which is going to be used for the PCR. In first place the quality of the DNA and the concentration have to be examined. Secondly the primers that are going to be used have to be checked beforehand in a gel for their function. Furthermore the length of the DNA amplicon is preferable not longer than 50base pairs. PCR protocol optimization is an equally important issue that has to be adapted beforehand. For attaining correct results optimization of the PCR protocol by experimenting with the annealing temperature is compulsory.

In our project HRM analysis was used to check if the primers that were designed for the fine-mapping of the specific QTL could successfully recognise the two different parental alleles (Money and Pimp).

Figure 3: 96 wells plate and LightScanner Instrument and Analysis Software

To conclude, the aim of this research will be to confirm the QTLs for seed traits on chromosome 9 by analyzing the results of seed weight, length and area. Furthermore, this project aims to fine-map the seed quality QTLs with the clearest phenotype by developing markers in order to detect the possible recombination inside this region. In addition more markers will be developed to determine the recombination points as precise possible.

2. Materials and Methods

2.1 Plant material

The starting plant material for our experiment is a tomato Recombinant Inbred Line (RIL) population derived from a cross between *Solanum lycopersicum (cv Moneymaker)* and a wild tomato, *Solanum pimpinellifolium (G1.1554)* (Khan *et* al., 2012 and Kazmi *et* al., 2012). After genotyping of the lines and subsequent linkage analysis, 12 chromosomes were identified in tomato and many QTLs that are responsible for seed and seedlings traits were identified. For our experiment we will use the Heterozygous Inbred Families (HIFs: 233, 239, 241, 259, 266, 288) that were obtained from the residual heterozygosity in the RILs on specific loci on chromosomes 6 and 9. These HIFs were part of the F8 RILs. After cultivation of these HIFs in the greenhouse (Wageningen UR, The Netherlands) under long day conditions (16hr light/ 8hr dark) and approximately 25^{o}C day and 15^{o}C night temperature, seeds from the second harvest and from 20 plants of each HIF were collected and genotyped with specific flanking markers of the region of interest. The region of the seed quality QTL on chromosome 9 spans from 54.142 to 105.399 cM. Seeds from the homozygous parental plants were also collected to use them in our data analysis as control. In total we have seeds from 120 lines, 20 per HIF. The heterozygous lines of HIF259 were used for further fine-mapping. The heterozygous region of HIF259 spans from 94.46cM to112.29 cM.

2.2 Seed phenotyping

After selection of the HIFs (233, 239, 241, 259, 266 and 288) seeds of the second harvest were weighted. In HIF line 259 also seeds of the first harvest were weighted for validation of the results of the second harvest. Approximately 100mg of seeds were weighted and transferred to trays. Seeds were carefully placed on a white filter apart from each other in order to have accurate results after the analysis of the photographs with the Image-J software to count the number of seeds. Images were taken by Nikon D80 camera, using Nikon camera control pro software version 2.0. Fifteen ml of demi water were added to every tray to also measure imbibed seed size. The trays were placed in piles at 24^{o}C in the dark in a closed, plastic bag. Twelve hours later photographs for measuring the area and length of the imbibed seeds were taken and analysed. Counting the seeds that have been germinated after 4 days in the dark, for having an estimation about germination capability, was the last part of the phenotyping (Figure 4).

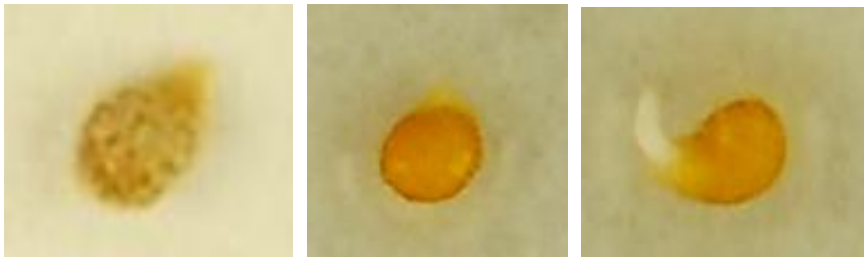

Figure 4: Not imbibed seed image the 1st day, imbibed seed after 12h and germinated seed at the 4th day.

The pictures were analysed by using the public domain Java image processing and analysis programme Image-J which can measure the total size and the pixels of every particle of the selected picture by using colour thresholds (http: //rsbweb.nih.gov/ij/). Additionally the

computer programme outliner for the rejection of the incongruous images of seeds, was used for more accurate results.

2.3 DNA isolation and concentration measuring

Small leaves from the *S.lycopersicum* and *S.pimpinellifolium* plants (10 leaves per parent) were deducted from the tomato plants by using a tong. They were separately placed in eppendorf tubes and transferred to the lab in an ice-bucket. The detailed procedure of DNA isolation is described in the appendices. The concentration of the DNA in the samples was measured with the ND 1000 Nanodrop. The samples with high quantities of DNA were further diluted to end with a DNA concentration of 10 ng / µl.

2.4 Designing of primers

The SOL Genomics Network (http://solgenomics.net) was used for identifying the chromosome sequence of the target regions of *S.lycopersicum* and furthermore for blasting the target region of *S.lycopersicum* against the sequence of *S.pimpinellifolium.* The identified sequences were used for alignment analysis and primers design. For HRM SNP genotyping primers were designed by CLC-Bio (Version 6.0; CLC-Bio, Katrinebjerg, Denmark). The primers were designed with specific parameters (55-62^{o}Cmelting temperature and 18-22bp long). Primers were made by Integrated DNA Technologies (IDT; Leuven, Belgium) and dissolved with milliQ-water to a final the concentration of 100 µM. SNPs were used also for genotyping with the method of Cleaved Amplified Polymorphic Sequences (CAPS) after conducting a restriction site analysis between the aligned sequences. Primer mixes were prepared by combining 10 µl of each of the two primers (forward and reverse) and 80 µl of milliQ-water ending with a volume of 100 µl of solution.

2.5 FIREPol PCR

Amplification of specific regions of the genome was obtained by PCR with FIREPol DNA polymerase**.** One µl DNA of each of the 20 samples of the two parents was added in separate PCR micro tubes. The master mix was prepared using the quantities and components of table 1. We used the primer mix as described in 2.4. In total we tested 12 primer mixes (appendices) for their function with the examined DNA. Fourteen µl of the master mix were added in each well to obtain a volume of 15 µl. Before transfer to the PCR machine (PTC-200, MJ Research) the tubes were centrifuged to spin down all components.

Table 1: Master Mix content for FIREPol PCR

Component	Volume 1x (µl)
Primer mix (10µM)	0.4
dNTPs (10mM)	0.3
mgCl (25mM)	1.5
Buffer B (10x)	1.5
FirePol	0.15
MQ	10.15
DNA (5-10ng)	1
Total Volume	15

The flow diagram of the PCR that we used is depicted below.

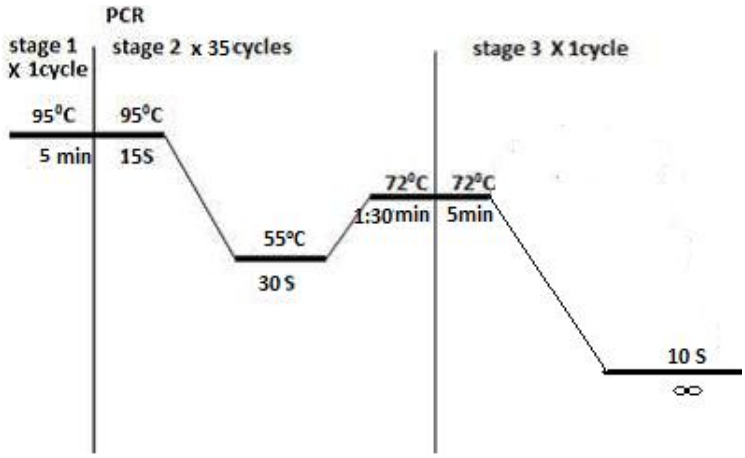

Figure 5: Diagram of FIREPol PCR reaction flow

To check for the function of the primers, the PCR products were loaded on a 1.5% agarose gel for electrophoresis (150ml TAE IX and 1.5g agarose) and ran at 80 volts for 80 min. Before loading the wells with the PCR product we added the loading buffer (12.5µl) to each tube. Moreover the Ladder marker, in which 1.25 µl of the GelRed Nucleic Acid Stain was added previously, was loaded on the gel.

2.6 Genotyping methods

a. High Resolution Melt Analysis (LightScanner)

One µl DNA of all the samples of the two parents was added in separate wells of the 96 well plates (Figure 3). Secondly the master mix was prepared using the quantities and components described in table 2. For the primers we used the primer mix that was described in 2.4. In total we genotyped our samples with 12 primer mixes (appendices). Eight and half µl of the master mix were added in each well to obtain a volume of 9.5 µl. Spinning down of the mix followed and in the end 20 µl of mineral oil was added on top. The mineral oil was used to avoid vapor production due to the high temperature which can cause malfunction of the scanning camera of the LightScanner. The plate was transferred to a PCR machine (My Cycler™ Personal Thermal Cycler, BioRad) and the PCR program was set at 30 sec at 98^{o}C, then 50 cycles of 10 sec at 98^{o}C, 30 sec at 55^{o}C, 2 min at 72^{o}C and a final elongation step for 5 min at 72^{o}C. In the particular project the software that was used for analysing the results was the LightScanner Instrument and Analysis Software (Idaho Technology Ink.).

Table 2: Master Mix content used for PCR reactions for LightScanner

Component	Volume 1x (µl)
5xPhire enzyme	0.1
Reaction buffer for Phire (5x)	2
LC-green	1
dNTPs	0.4
Primer mix	0.25
MQ	4.75
DNA (5-10ng)	1
Total Volume	9.5
Mineral oil	20

The most important change was the increase of the cycles from 40 to 50 cycles. Moreover the elongation time was increased to 5 min at 72°C.

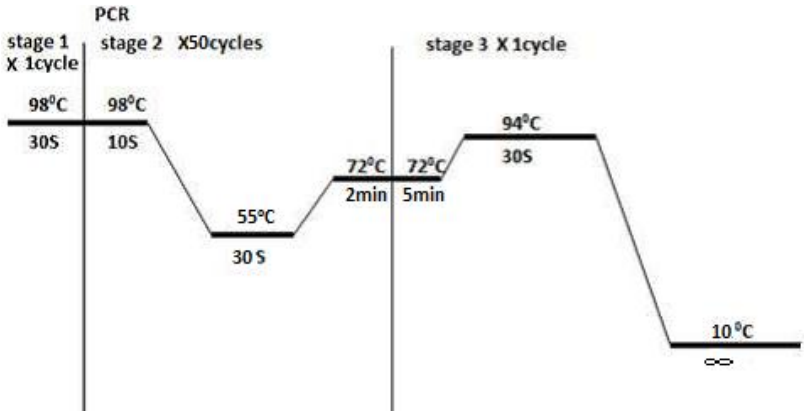

Figure 6: Diagram of PCR reaction flow for LightScanner

b. CAPS markers

To run the CAPS markers, 1µl of the examined DNA was pippeted in separate micro tubes, following by adding 14µl of the master-mix (table 1) in each tube. The FIREPol PCR was used for amplification (Figure 5). After the termination of the PCR the products were examined on an agarose gel. Continuing with the digestion procedure by the appropriate enzymes (appendices) we followed the table 3 for making the master-mix for the restriction enzymes. Five µl of each of the products of the FIREPol PCR were transferred to new micro-tubes. In the end 20 µl were in each micro-tube. For the restriction the PCR samples were incubated at 37°C for 4 or 6 hours and the reaction was stopped by incubation at 65/80°C (depending on the restriction enzyme) for 20 minutes. The products were loaded in a 1.5 % agarose gel for visualisation of the results.

Table 3: Master Mix content used for digestion of PCR samples

Component	Volume 1x (µl)
Enzyme	0.2
Buffer for the enzyme	2
BSA (optional)	2
MQ	12.8
DNA (5-10ng)	1
Total Volume	15

To verify the adequacy of the primers a first trial on DNA of 20 seedlings was performed. Twenty seeds of the heterozygous lines (B3 and B4) of the HIF259 were placed in a tray following the same procedure as described in the seed phenotyping subchapter in order to germinate. After radical protrusion (3 days) the germinated seeds were transferred on the Copenhagen table for further development (Figure 7).

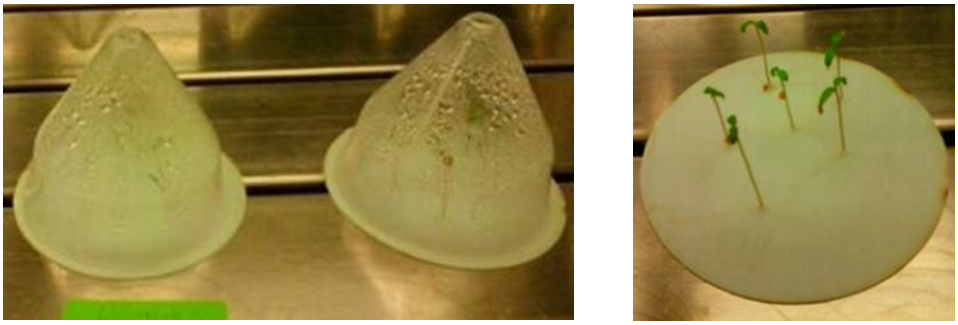

Figure 7: Germinated tomato seeds in Copenhagen table and seeds with their cotyledons

After 8 days the cotyledons were deducted and their DNA was extracted. The evaluation of the primers was done in the same way as described previously. Later a larger scale trial in the greenhouse with 200 heterozygous tomato plants followed. The used genotyping procedures both HRM and CAPS markers were the same.

Figure 8: Seedlings of one of the four trays. Fifty four heterozygous tomato seeds were planted. Genotyping of every seedling was conducted.

3. Results

3.1 Confirmation of seed quality QTLs

The confirmation of QTLs is a combination of phenotyping and analysis of the results using statistical formulas. As it is described in the materials and methods different HIFs were genotyped in a previous project for the position of the markers shown in the figures. In this project 6 seed quality traits (seed weight, dry and imbibed seed area, dry and imbibed seed length and seed germination) of HIFs for chromosome 9 were measured and analysed. Phenotyping of parents was also applied. For all the results standard errors were calculated and the significant differences with student's t-test are depicted with asterisks.

a. Seed weight

In the figure below seeds from HIFs 233, 239, 241, 259, 266 and 288 as well as from the two parents Money and Pimp were used for phenotyping. The average weight of every HIF was calculated by dividing the total mg of seeds by the total number of seeds that were used. The different colours in the columns indicate the genetic background (Money, Pimp or Heterozygous) of the seeds at the studied interval. Genotyping of these lines was the result of previous project.

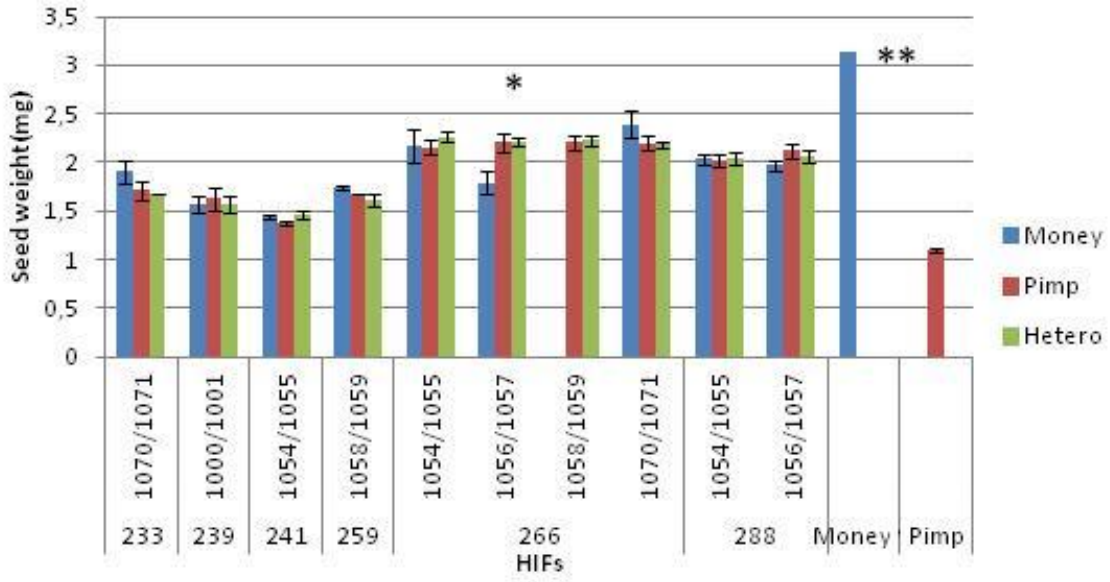

*Figure 9: Average seed weight (mg/seed) results from HIFs on chromosome 9. The standard error of a minimum of 2 biological replicates is represented by the error bars. On the x-axis the HIFs, parents and the flanking markers are depicted. *= significant difference (p<0.05) **= significant difference (p<0.01)*

As shown in figure 9, there is significant difference between the seed weight of the two parents with Money seeds being twice as big as Pimp seeds. A significant difference is also observed in the 266HIFs at the location of the 1056/1057 marker providing statistical evidence for the confirmation of QTL. Additionally for HIFs 233, 241, 266 (markers

1054/1055, 1070/1071) and 288 (marker1054/1055) there is no statistical significant difference. Moreover, the values of heterozygous lines are at the same levels of the lines with the parental backgrounds.

b. Seed area

The seed area of the dry seeds was calculated by the image J software. By using the outliner the seeds having extreme values either high or low were excluded. The difference in seed area between the two parents is significant with p values < 0.01 (figure10). Additionally, significant difference is also observed for the HIF 241 with p<0.05. The rest of the HIFs are showing no significant differences.

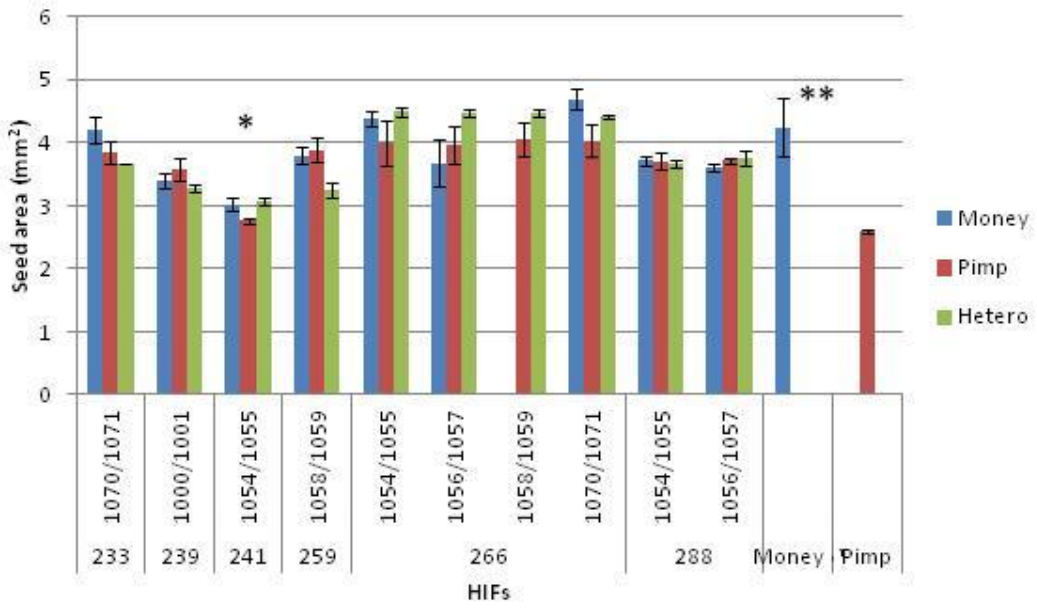

*Figure 10: Average seed area (mm2) results from HIFs on chromosome 9. The standard error of a minimum of 2 biological replicates is represented by the error bars. On the x-axis the HIFs, parents and the flanking markers are depicted. *= significant (p<0.05) **= significant difference (p<0.01)*

c. Seed length

Seed length was also calculated using the Image-J software. Besides the parents also two different HIFs showed significant differences for seed length being, HIFs 241 and 266 at the marker locus 1070/1071. These results confirm the seed length QTL found at this locus. In the rest of the HIFs the values are approximately the same.

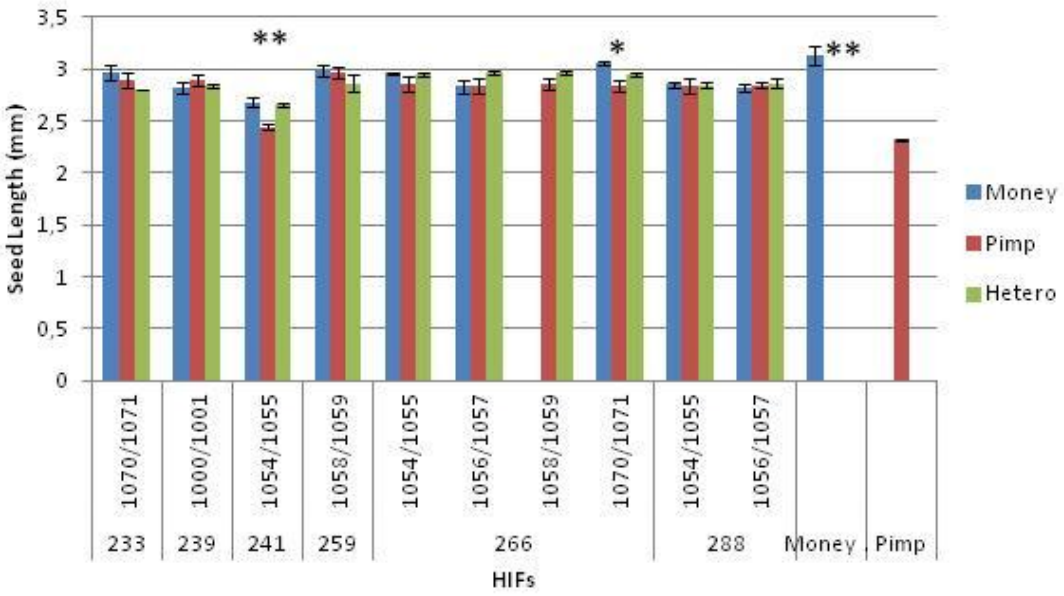

*Figure 11: Average seed length (mm) results from HIFs on chromosome 9. The standard error of a minimum of 2 biological replicates is represented by the error bars. On the x-axis the HIFs, parents and the flanking markers are depicted. *= significant (p<0.05) **= significant difference (p<0.01)*

d. Imbibed seed area

After 12 hours of imbibition the values of the seed area were scored using the Image-J software. In general the imbibed seed area values are higher as compared to the seed area without imbibition. This is the result of the absorption of water and the expansion of the seeds, giving a higher area. The two parents Money and Pimp are again showing a significant difference ($p<0.01$) (figure 12). The same pattern is not followed by any HIF. The values of the heterozygous lines seem to be higher than the lines of the same HIFs with homozygous backgrounds in most of the cases, although these differences are not significant.

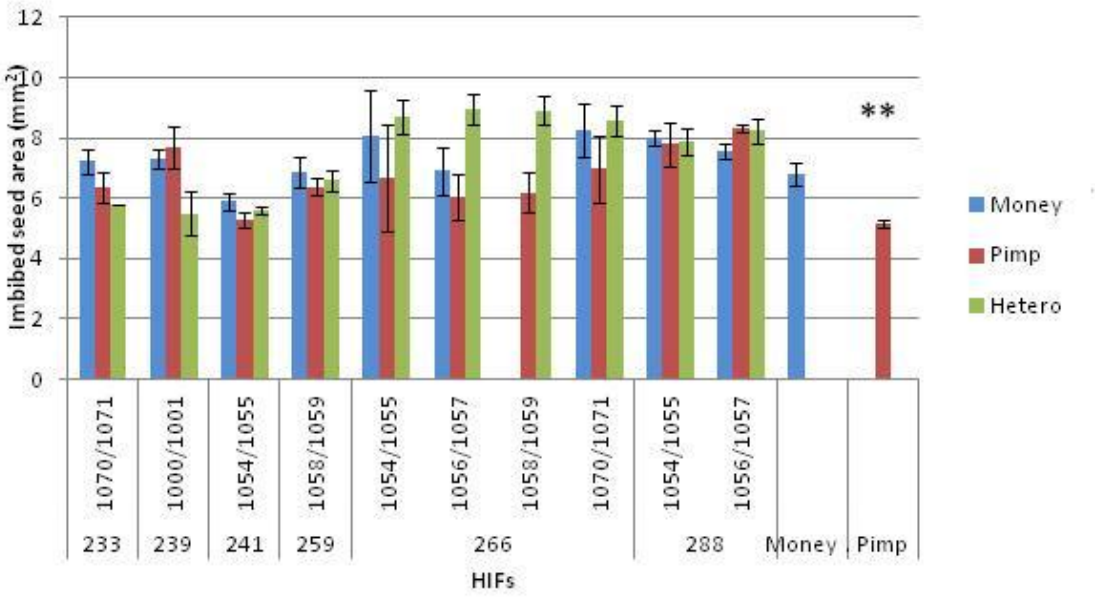

*Figure 12: Average imbibed seed area (mm^2) results from HIFs on chromosome 9. The standard error of a minimum of 2 biological replicates is represented by the error bars. On the x-axis the HIFs, parents and the flanking markers are depicted. **=significant difference (p<0.01)*

e. Imbibed seed length

Besides significant differences of the parents for imbibed seed length (p<0.05), also HIF 241 showed significant differences between the lines with different genetic background (figure 13)

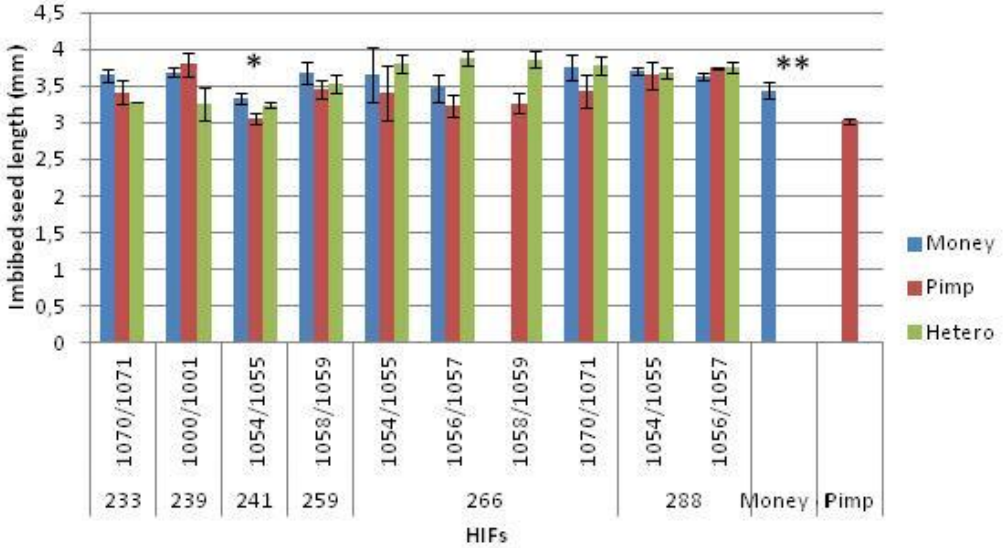

*Figure 13: Average imbibed seed length (mm) results from HIFs on chromosome 9. The standard error of a minimum of 2 biological replicates is represented by the error bars. On the x-axis the HIFs, parents and the flanking markers are depicted. *= significant (p<0.05) **= significant difference (p<0.01)*

f. Germination percentage

After five days at 25°C, in the dark, the germinated seeds were scored (figure 14). The germination percentage is the only trait from the 6 that were analysed for which, the Pimp parent has higher values than Money (significant at $p<0.05$). Furthermore, also HIF 241 show significant differences ($p<0.05$) and thereby is confirming the QTL for seed germination found on this locus. Remarkable is the fact that HIF 288 showed very low germination percentages as compared to the other HIFs.

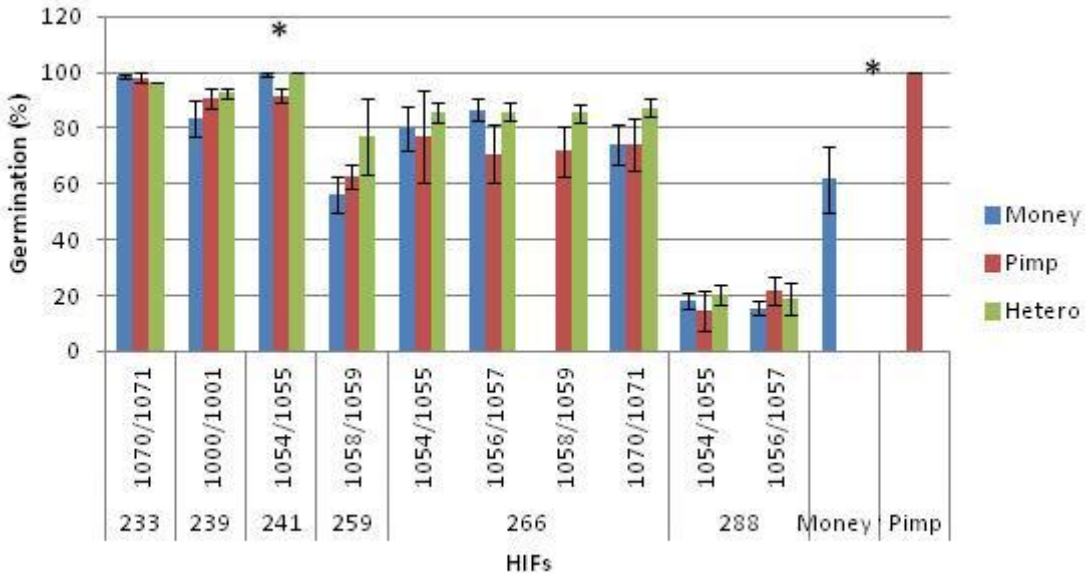

*Figure 14: Average germination percentage from HIFs on chromosome 9. The standard error of a minimum of 2 biological replicates is represented by the error bars. On the x-axis the HIFs, parents and the flanking markers are depicted. *= significant (p<0.05) **= significant difference (p<0.01)*

3.2 Fine mapping of seed quality QTL

Two genotyping methods for identifying markers in the heterozygous region of HIF 259 were applied. For both methods the primers were designed (with different procedures as described) in the two borders of the heterozygous region. The results of the genotyping methods that were conducted are depicted in two different ways. In the High Resolution Melt Analysis the results are shown in a figure of fluorescent curves while the results of the CAPS markers technique are visualized by a picture of the agarose gel. In both cases the assay is first shown among parental plants and subsequently for the confirmation within the heterozygous lines.

a. High Resolution Melt Analysis

Since HRM analysis was the main genotyping method used in this project many primer mixes were tested. Only one (651-652) gave clear results for the two parents (figure 15). The grey line depicts the DNA samples of the Pimp parent and the red one depicts the samples of the Money parent. For the unsuccessful assays, the melting curves don't show clear differences between the parents (appendix 7). The following results obtained after base lining of the

data with values before and after the change of the melt behaviour of the samples. Normalization and clustering to unique thermal profiles gave the figures 15 and 16.

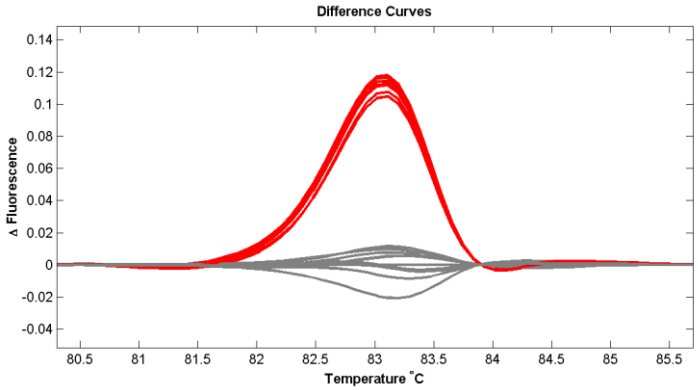

Figure 15: Difference of melt curves of Money and Pimp tomato samples. In the x-axis are depicting the various temperature increments and in the y-axis the fluorescence intensity of the curves. Red lines are the samples of Money parental plants and the grey the Pimp samples.

The genotyping results of the two heterozygous lines, 259B3 and 259B4 show less clear separations as compared to the two parents (figure 16 versus figure 15), but the observed differences are still adequate for successful genotyping (figure 16).

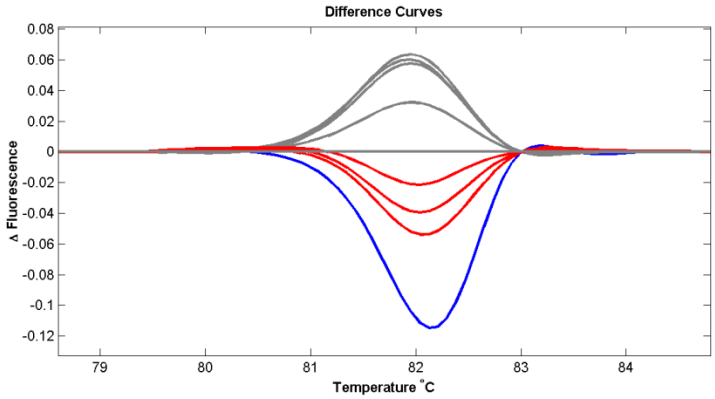

Figure 16: Difference of melt curves of heterozygous tomato seeds. The grey lines depicting the Pimp background seeds, the red lines depict the samples of heterozygous genotype and the blue the Money samples.

Although this technique is used with great success, in the specific project it was partially unsuccessful causing to follow the technique of CAPs markers

b. Cleaved Amplified Polymorphic Sequence (CAPS)

Samples of Money and Pimp parents and the B3 and B4 lines of HIF 259 were genotyped with the help of CAPS marker 665-666. DNA was amplified by FIREPol PCR and amplification was confirmed on an agarose gel (Figure 17A, B).

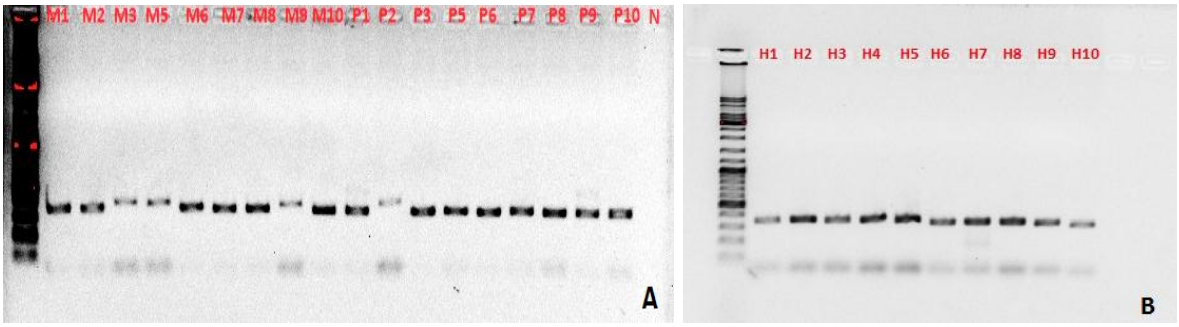

Figure 17: PCR samples tested on 1.5% agarose gel. A: parental samples Money (M) and Pimp (P) and a negative control (N).The quality of DNA in M3, M5, M9 and M10 is low. B: Picture of heterozygous lines.

Later, PCR products were cut with NHeI and followed by analyses on an agarose gel (Figure 18A, B). In figure A. digestion was only provided to 6 of the previous amplified samples. Additionally in this picture the double bands at the left side (Pimp) is the same product divided in two pieces because of the imbalance between the quantity of the enzyme and the amplified product. In the picture B the H1, H4, H7, H9 have Pimp background and the rest are heterozygous lines.

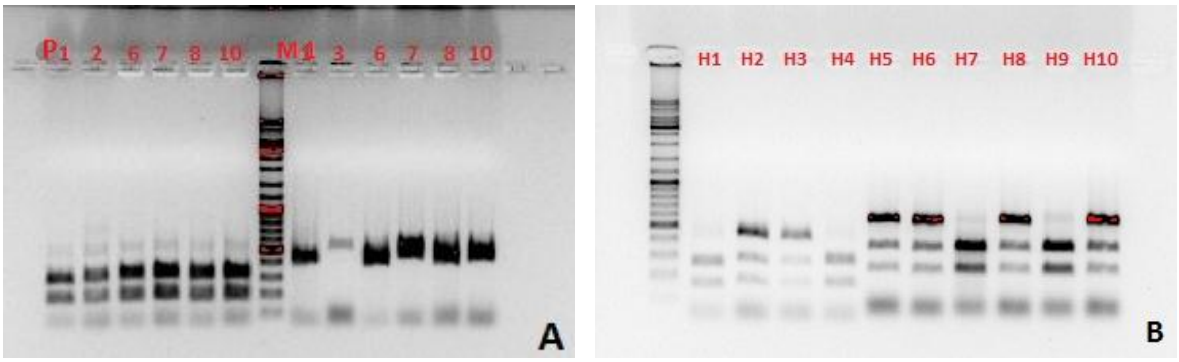

Figure 18: Digestion results on 1.5% agarose gel. A: Parental samples Money (M) and Pimp (P). The DNA fragment of the Money parent is longer than this of the Pimp. B: Samples of heterozygous lines.

For the verification of the functionality of the developed primer mixes, we tested them in a bigger population of approximately 200 seedlings. Furthermore by genotyping these heterozygous lines we determined their genetic background for use in future projects.

Unfortunately the results that we obtained from this last experiment were not as clear as we expected. The assay was confined to only 96 samples because of the inability to amplify the rest of the samples. This is probably due to either a malfunction of the PCR machine or an error with the master mix since the DNA quantity and quality was sufficient for further analysis. Unfortunately, restriction analysis of the 96 amplified samples with NheI failed.

4. Discussion

4.1 Confirmation

In previous studies of Kazmi *et* al. and Khan *et* al., multiple QTLs were identified throughout the genome of tomato. Especially on chromosome 9 QTLs that controls specific seeds and seedlings traits were identified. The seed dimension related traits were tested in 6 HIFs in an attempt to confirm their QTL. The percentage of germination was also analyzed in order to prevent any abnormalities caused by malfunctioned seeds. As it is observed and verified by previous researches the seeds of the wild *Solanum pimpinellifolium* parent have a significant higher percentage of germination comparing to seeds of Moneymaker (Kazmi et al.2012). However the seed size and weight are not significantly correlated to germination, with Pimp having smaller seeds compared to Money (Khan et al., 2012). In our germination results the different backgrounds of HIF 241 show significant differences. However this result may not be trustworthy because of lack of notable amount of progenies for the HIF 241.

Concerning the seed weight Moneymaker has significant higher values compared to Pimp. As we can see in the rest of the HIFs, the values of Moneymaker background are also higher in most cases but not significant higher. The average for the heterozygous lines was as high as the money lines, implying that the allele coming from Moneymaker is dominant for seed weight.

Khan et al. (2012) was the first to measure the seed size as seed area and seed length in a genetic population. As expected, it was shown that the seed dimension traits are correlated with the seed weight trait. However, the seed area and the seed length are assumed to be controlled by different genetic loci than these of seed weight based on the results that we obtained. In our project dry and imbibed seed size and also dry and imbibed seed length were scored for confirmation of the findings by Khan et al., 2012.

In both figures of dry and imbibed seed area the parents differ significantly with Money having higher values compared to Pimp. Among the HIFs the differences of progenies carrying different parental alleles are obvious, with HIF 241 segregating for marker STW 1054/1055 to stand out in the dry seed area calculations, by having significant different values between the parental alleles, indicating the association of this locus with seed size trait. The same pattern is not followed in the imbibed seed area even though the p-value is not much higher than 0.05 which is the threshold for being significantly different. As it is written in Khan et al.2012 the dimension traits as well as the seed weight trait are complicated traits and thus they can be affected by the environmental conditions resulting in the incorrect identification, location and number of QTLS. In general, higher values of seed area are observed in progenies of HIFs which have Money background.

The results of seed length don't differ from the seed area results. The difference between the two parents is significant; the breeding line of Money appears to have higher values of

seed length than those of Pimp for both the dry and imbibed seeds. Additionally, the trait of seed length it is assumed to be inherited from the Money parent. Significant difference between the lines with different alleles has been identified in HIF 241 for both dry and imbibed seed length, indicating the consistency of the QTL.

In previous projects HIF 259 segregating for the marker STW 1058/1059 had also shown significant difference between the lines of different genomic background for all the above mentioned seed traits, suggesting the existence of the QTL in this locus. However, in this project was not possible to identify the consistency of the QTL on HIF259. This may be due to the fact we chose specific values among the total number of the values attained, by excluding the extreme ones (outliner). In previous projects metabolite analysis was also conducted verifying the previous results of the existence of the QTL at this genomic locus.

However in this project, seedling traits were not analysed. As a result, it was impossible to confirm the findings of Khan *et al.* who found that the seed dimension related traits are correlated with seedling traits. In addition we could not confirm the possibility of the pleiotropic effect of this locus from the identification of the co-location of the traits above.

4.2 Fine –mapping

Only in two cases among the 6 HIFs, those of 241 and 259 were we successful in confirming the QTL on chromosome 9 responsible for seed quality traits. We decided to proceed with fine-mapping in HIF 259 because of the strong statistical evidence of the existence of the QTL at this HIF line.

The objective of this report was to develop molecular markers in order to narrow down as much as possible the QTL heterozygous region of HIF259 on chromosome 9. By designing many primers with the SNP method we succeeded in finding two markers positioned in the two borders of the heterozygous region of the QTL (65062496- 6656161219bp or 94.48-112.29 cM). At the left side of the region the marker that was developed is the 665/666 which was identified in the position of 95cM and at the right side the marker is the 651/652 which was identified in distance 112.2 cM. Both of them were tested in multiple samples providing considerate results.

High Resolution Melting Analysis method

The High Resolution Melt Analysis is a high-throughput genotyping method and it is an easy, fast and reliable way to genotype large amounts of samples in few assays. However, in order to succeed in having notable results there are some bottlenecks that should be taken care. Firstly, the DNA of our samples could not be amplified. After many trials of changing the annealing temperature or designing primers with different characteristics, it was found that the problem was in the PCR reaction flow diagram. The number of cycles that the procedure of denaturation and hybridization has to be imitated increased to 50 instead of 30 (Fisher *et* al., 2010). Moreover the time of reheating to 72^{o}C was also changed from 30 sec to 2 and 5 minutes respectively (Figure 6). It is supposed that the protocol found in the literature was

not successful because of incompatibility between the Cycler PCR machine and the LightScanner. As it has already been mentioned the compatible PCR instrumentations with the LightScanner are compulsory for the correct HRM analysis. Furthermore the quantity and the quality of the DNA of the samples is a crucial point to be checked carefully in order to obtain high quality results. Controlling the DNA with the Nanodrop and diluting it if necessary, as well as checking beforehand the existence of it, can result in a successful procedure.

In general, when comparing the results of the two genotyping methods we used, HRM and CAPS, we can see that the samples depicted to have Money background with the CAPS method may show different background using the HRM genotyping method. This can be explained by the existence of recombination between the two markers loci. As it was described earlier the two markers that were found to be close to the researched region were designed in the two sides of the heterozygous region of the HIF 259. As the recombination can take place in every part of the heterozygous region and especially in case of large heterozygous region (1498723bp or 17, 75 cM), it is reasonable to have differences in the background of our lines concluding in different results.

Another issue that concerns both the genotyping methods is the designing of the primers. Many primers have been designed and were tested. However, only two primers mix one for each method, were functional. The procedure of primer's designing is a difficult procedure and especially in the specific occasion that the sequence of the exact accession of *S. pimpinellifolium* parent was not provided in the SOL Genomics Network for blasting. The primers were designed based on Single Nucleotide Polymorphisms. While the sequence was not the appropriate one it is understandable that the primers could be designed at the wrong position throughout the genome and consequently that they were ineffective.

5. Conclusions and recommendations

Having performed the High Resolution Melt Analysis several times during this project, it was indeed an easier and faster way of genotyping compared to CAPS markers technique. The possibility that this technique offers to analyse at the same time 96 different genotypes, is less time consuming for the researcher. However, in case of fine mapping in an unknown genome can be quite a difficult task to manage. Moreover, many bottlenecks starting from the simplest one as obtaining the appropriate DNA quality and quantity and concluding with the most troublesome as the correct designing of the primers have to be deciphered beforehand. Fine-mapping of QTLs concerning seed quality is a time-consuming procedure and most of the times unsuccessful in finding the right markers. On the other hand it is compulsory to be continued in order to have a clearer image of the genes that are responsible for the trait of seed quality in tomato. Furthermore the molecular mechanisms of the identified genes should be researched. Finally, confirmation of the already existing QTLs is a process to validate the results of previous projects. I n this specific project the QTL in HIF259 could not be confirmed by using the common procedure. However, with metabolite analysis, confirmation was achieved. The existence of QTL was also confirmed in HIF241. Supplementary production of HIF progenies for confirmation is mandatory for our results to be statistically correct. Additionally, the designing of the primers for further fine-mapping has to be based on the DNA sequence of the exact accession of *S. pimpinellifolium* that we are interested in.

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7. Appendices

1. Lines of HIFs that were used for confirmation and their background genotype at specific marker loci

Table 3: Showing HIFs 233, 239, 241, 259, 266 and 288. The A and B lines are progenies of the same HIF but produced by different parents. The lines were genotyped and the results are depicting with blue for Money (M), red for Pimp (P) and green for Heterozygous (H) at the specific marker loci.

cM	98.2	105.5		54.1	65		83	93		
bp	65492233	66097878		58369026	60987757		63638559	64993126		
Line nr.	STW1058/1059	STW1070/1071	Line nr.	STW998/999	STW1000/1001 leo	Line nr.	STW1054/1055	STW1056/1057		
233A1	M	M	*239A1*	P	P	*241A1*	P	P		
233A2	M	M	*239A2*	P	P	*241A2*	P	P		
233A3	M	M	*239A3*	P	P	*241A3*	P	P		
233A4	M	M	*239A4*	P	P	*241A4*	P	P		
233A5	M	M	*239A5*	P	P	*241A5*	P	P		
233B2	M	H	*239B6*	P	M	*241B1*	M	P		
233B8	M	P	*239B1*	P	H	*241B13*	P	P		
233B9	M	?	*239B2*	P	M	*241B15*	M	P		
233B13	M	M	*239B5*	P	M	*241B18*	M	P		
233B14	M	M	*239B9*	P	M	*241B19*	M	P		
233B15	M	M	*239B13*	P	P	*241B2*	H	P		
233B17	M	P	*239B16*	P	P	*241B3*	H	P		
233B18	M	M	*239B7*	P	P	*241B4*	H	P		
233B20	M	P	*239B8*	P	P	*241B7*	H	P		
			239B3	P	H					
			239B4	P	H					
			239B18	P	M					
			239B19	P	M					
			239B11	P	P					

cM	98.2	105.5		83	93	98.2	105.5		83	93
bp	65492233	66097878		63638559	64993126	65492233	66097878		63638559	64993126
Line nr.	STW1058/1059	STW1070/1071	Line nr.	STW1054/105	STW1056/1057	STW1058/	STW1070/1071	Line nr.	STW1054/	STW1056/1057
259A1	P	P	*266A4*	M	H	P	P	*288B5*	H	H
259A2	P	P	*266A5*	H	H	P	P	*288B11*	M	H
259A3	P	P	*266A9*	H	H	P	P	*288B18*	M	H
259A4	P	P	*266A18*	M	M	P	P	*288B2*	P	H
259A5	P	P	*266A2*	M	M	P	P	*288B4*	H	M
259B4	H	H	*266A20*	?	M	P	P	*288B1*	M	M
259B7	H	H	*266A6*	?	M	P	P	*288B10*	M	M
259B3	H/P	H	*266A8*	M	M	P	P	*288B19*	M	M
259B10	M	M	*266A1*	P	P	P	P	*288B3*	M	M
259B17	M	M	*266A13*	P	P	P	P	*288B7*	M	M
259B18	M	M	*266A15*	P	P	P	P	*288A2*	H	P
259B2	M	M	*266A16*	P	P	P	P	*288A5*	H	P
259B5	M	M	*266A17*	?	P	P	P	*288B12*	H	P
259B12	P	P	*266A7*	P	P	P	P	*288B8*	H	P
259B15	P	P	*266B10*	H	H	H	H	*288A1*	M	P
259B8	P	P	*266B11*	H	H	H	H	*288A10*	M	P
259B9	P	P	*266B12*	P	H	H	H	*288A11*	M	P
259B1	P/H	P/H	*266B14*	H	H/P	H/P	H	*288A3*	M	P
			266B18	M	M	H	H	*288A6*	M	P
			266B19	H	H	H	H	*288A15*	P	P
			266B3	M	H	H	H	*288A4*	P	P
			266B4	?	P	P	H			
			266B6	H	H	H	H			
			266B7	P	H	H	H			
			266B17	H	H	H	M			
			266B5	H	H	H	M			
			266B9	H	H	H	M			
			266B1	?	H	P	P			
			266B15	H	H	H	P			
			266B16/1	H	P	P	P			
			266B16/2	H	P	P	P			
			266B2	?	P	P	P			
			266B20	H	P	P	P			
			266B8	P	P	P	P			

2. HRM-Compatible Software (Taylor et al., 2010)

- Compare and combine data from multiple experiments by combining run results into a single melt study
- Display a plate view for easy identification of sample genotypes
- Share analysis settings among experiments
- Analyse multiple experiments from a single plate
- View all charts in a single window for simplified data analysis and interpretation

3. Quick DNA extraction protocol (Cheung WY et al., 1993)

Before the extraction starts, an ice –bucket for storing the eppendorf tubes with the collected leaves has to be aside.

Steps that have to be followed:

a. Put 1 leaf (preferably small) in 1ml tube.
b. Add one stainless steel bullet, 150µl extraction buffer* and then grind the mixture for 1min in 30Hz.
c. Put the tubes in 60^{o}C for 60 min.
d. Spin them for 10 min in 1700g.
e. Take 75 µl supernatant to a new tube (microtiter plate).
f. Add 75µl iso-propanol and 30 µl 10M NH4Ac.
g. Mix the commixture and leave it for 15 min in room temperature.
h. Spin it for20 min in 2400 RPM.
i. Discard the supernatant.
j. Wash the pellet with 70% ethanol (the quantity is around 250µl).
k. Spin for 5 min in 2400RPM.
l. Then remove ethanol and dry for 5 min at room temperature.
m. Dissolve pellet in 50 µl MQ.

The DNA can be stored in the refrigerator for future use (4^{o}C).

*Extraction Buffer: 2M NaCl, 200mM Tris-HCL pH 8, 70mM EDTA, 20mM $Na_2S_2O_5$

4. Example calculation of significant difference between Pimp and Money lines of HIFs.

Table 4: Calculations of the averages of the lines of 241 HIF. Standard error and Student t-test are also shown. Standard error is calculated by dividing the sample's standard deviation with the square root of the sample size. When t-test is <0.05 then the difference between the compared samples is significant.

	Average	SE	T-TEST
Money	3.018157	0.100086	0.032363
Pimp	2.769134	0.043958	
Heterozygous	3.065387	0.063804	

5. The heterozygous region of 259 HIF on chromosome 9 which was used for fine-mapping

bp	6506246	65152660	65285997	65468989	65685493	65814934	65923428
cM	94.46	94.984	96.32	98.225	98.748	102.145	103.206

bp	66074223	66164836	66260125	66398300	66518095	66561219
cM	105.399	106.738	106.998	107.521	111.228	112.29

6. List of primers for HRM analysis and CAPS markers on chromosome 9 of HIF259.

Table 5: List of primers for HRM in chromosome 9 of HIF259. The primer mix 651-652 was effective in fine-mapping of the heterozygous region.

Number	Length	Database entry	Sequence
641	22	WSL641_HRM1-Fw	TTTTCTGTTTTAATGTTGTCCT
642	21	WSL642_HRM1_Rev	ACTTTTCAATTTCAGCTTTTC
643	21	WSL643_HRM2-Fw	AGGTTAACTGAAAAGGAAAGA
644	22	WSL644_HRM2_Rev	AACTCCTTTTAAAACTTGTGAT
645	18	WSL645_HRM3-Fw	CGCGTAAAGATAAAAGGA
646	20	WSL646_HRM3_Rev	ACTAAAGCTATTCAATTCGG
647	20	WSL647_HRM4-Fw	ATGGACAAGCAATTTTGGAG
648	20	WSL648_HRM4_Rev	AGCACTACCATCCCAAAAAC
649	18	WSL649_HRM5-Fw	TTTCCATCAGGCAGAGCA
650	18	WSL650_HRM5_Rev	GACTCCATCCTCCACCTT
651	**18**	**WSL651_HRM6-Fw**	**TGTTTTGCTCCACTGGTT**
652	**20**	**WSL652_HRM6_Rev**	**TTTTGTGTAGTTCCCTGCTT**
653	20	WSL653_HRM7-Fw	GAAGCTAAGGAGAAGAAAGA
654	20	WSL654_HRM7_Rev	CCACTTAACAAAACTCCTAC
655	22	WSL655_HRM8-Fw	AAAGCGGAATGGATATAGAGGA
656	21	WSL656_HRM8_Rev	AAAGAAAGCTAGCAGGGAGGA
657	19	WSL657_HRM9-Fw	TGAGTGGCATTGCTGTAGT
658	21	WSL658_HRM9_Rev	CAAGTGAGCAAGAAAAGGAGT
659	19	WSL659_HRM10-Fw	GCCAGCAAGCTAAACCAAA
660	20	WSL660_HRM10_Rev	ACGTGCCAAAACAGCTTAAT
661	21	WSL661_HRM11-Fw	ACCTTTTCTCACTTTACCCCT

662	20	WSL662_HRM11_Rev	CTTTTGGTCTGCCTCTTCCT
663	18	WSL663_HRM12-Fw	TGTCAGTGGCGATGTAGC
664	20	WSL664_HRM12_Rev	GAACAAAGACAGGCGTACAA

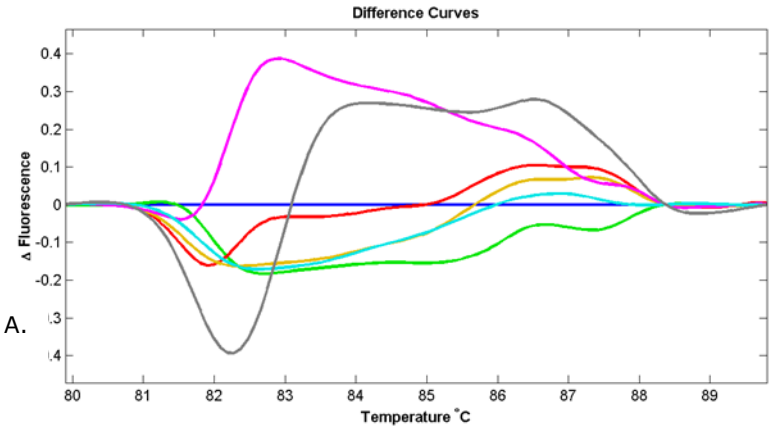
Name	bp	Primers	Sequence	Comments
665	6265(65150000-65160000)	665-666	GTCTTTCAACATCATGCAC	MONEY
			ACTAATCACACCTCCATCT	
667	1505(65160000-65170000)	667-668	CCAGAGACGGAATCAATAA	PIMP
			ACCACAAGATGAAAGGAC	

Table 6: List of CAPS markers for chromosome 9 of HIF259. The primer mix 665-666 was effective in fine-mapping of the heterozygous region.

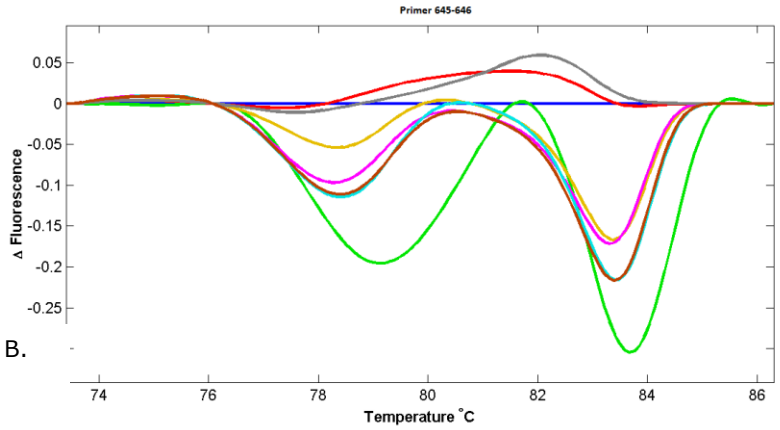
7. Examples of difference between melt curves of Money and Pimp samples with ineffective assays of different primer mixes

Table 7: Difference of melt curves of Money and Pimp samples. In the x-axis are depicting the various temperature increments and in the y-axis the fluorescence intensity of the curves. A. The graph corresponds to the primer mix assay 661-662 B. primer mix of 645-646. In the first picture pink, grey and blue lines have Pimp background and the rest Money. In the second picture red, grey and blue lines have Pimp background and the rest Money.

A.



B.


Primer 645-646
0.05
0
-0.05
-0.1
-0.15
-0.2
-0.25
Δ Fluorescence
74
76
78
80
82
84
86
Temperature ˚C

C.