Wageningen University

Marker Development for Nematode Resistance in *Hypericum*

Msc Thesis

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Abstract

Researcher develops and applies molecular markers technology for a range of purposes. These molecular markers can be used in marker assisted selection (MAS) to speed up breeding programs. Scientists are able to use molecular markers to select for desired traits in the early stages of breeding programs. Nucleotide–binding-site-leucine-rich-repeat (NBS-LRR) is conserved motif in higher plant and most of resistance gene is the member of NBS-LRR family. The objectives of this study are to explore the potential in *Hypericum* spp as well as to develop NBS marker system for nemetode in *Hypericum*. Towards these objective, This PCR-based approach (NBS profiling) were carried out to measure the level of polymorphism of difference type of crosses; interspecific and intraspecific crosses as well as genotyping F1 population of *Hypericum*. NBS profiling required digestion of DNA with restriction enzyme and the used of degenerated primers were used in PCR reactions. This technique can produce multilocus profile of the genome.

Acknowledgement

During the whole process of my master studies, I have been guided, supported and encouraged by many people. Without the help of these individuals mentioned below, I would have faced many difficulties to complete this report. I am truly grateful to all of them.
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My utmost gratitude goes to my parents, for their love, understanding, encouragement and support they have given me. To my partner, Mohd Ameen, for his affection and for constantly being a good listener to all my complaints and frustrations, and thank you for believing in me.

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1.0 Introduction

The growth of floriculture industry has taken long strides worldwide, especially in developing countries as a result of outsourcing, due to the low cost of maintenance including the labour cost. The industry must meet the demand of consumers by providing new value added , which are cost effective and unique flower characteristics; flower colour, shapes, appearances, long vase life and other qualitative traits. Even though the market of *Hypericum* is still not very big, however it is perceived as a growing niche market, therefore the demand will be increasing. Hence, it is important and practical value to continuously generated constant product quantity in the *Hypericum* industry.

Markers-assisted breeding (MAS) together with conventional breeding can contribute greatly for genetic improvement of ornamental plants and in uplifting the socio-economic benefits. In MAS, linked DNA markers are used for indirect selection for a desired trait which is not visible in early stage of the plant. Nematodes were reported to be responsible for most 5% to 10% of major crops worldwide (Haseeb et al., 1984; Stokes, 1977; Walker et al., 1994). At the same time, the spreading of the nematodes can hardly be controlled due to restriction use of effective nematicides. Breeding resistant cultivars for nematode would be in favor to resolve the address problem.

This report contains the preliminary description to implement markers assisted breeding in breeding program of *Hypericum*. The main goal is to develop nematodes resistance in cultivated *Hypericum* using NBS profiling technique.The fact that this study is the first attempt of the construction of genetic map *Hypericum* spp, various aspects need to be developed and justified. The study was also explored the background and the potential of the *Hypericum* which can benefit in future improvement of these species. However, this study was more focused to developing NBS markers for genetic mapping. Every procedures and challenges towards the main goal are also presented in this report.

2.0 Literature Reviews

2.1 The genus *Hypericum*

The *Hypericum* genus belongs to the *Clusiaceae* family, subfamily Hypericoidaea and tribe Hypericeae (Gutafsson et al., 2002). The genus *Hypericum* has 400 species in total and arranged in 30 sections with abundance of variations in their characteristics (Robson, 2006). This seed propagated and perennial herbaceous plants are widely distributed in the world. A number of the species within this genus has been identified as apomitic species for example *H. perforatum* L. The *Hypericum* flowers are bisexual (Martonf et al., 1996) and flowering in early spring. Until now, over 25% of its species has been cultivated for different purposes. For many years, *H. perforatum* L (Kirakosyan et al., 2004) and H. androsaemum (Dias et al., 2000; Valentão et al., 2002) are widely recognized for their medicinal properties in pharmacological. Study by Nahrsted & Butterweck (1997) reported H.perforatum contains hypericin and hyperforin which is most bioactive compounds to treat depression.

Simultaneously, some species of *Hypericum* were also being appreciates as an ornamental plants for example H. calycinum L, H. forrestti (Chittenden) N. Robson and H. androsaemum (Robson, 1985).

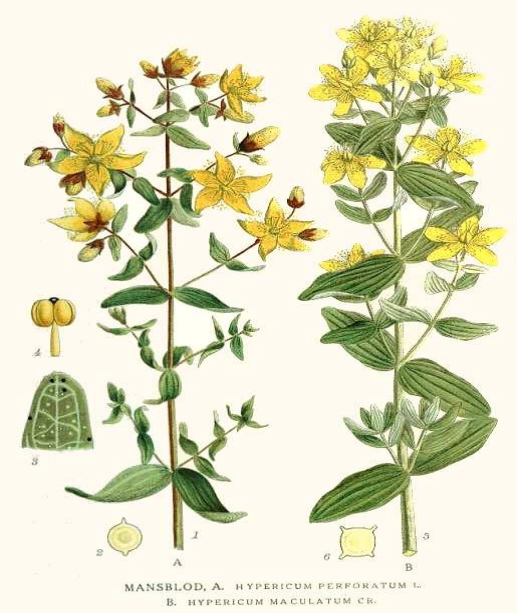

Figure 1: *Hypericum* picture (PubMed GRIN datab

2.2 Genetic aspects

The genetic aspects for this genus are not fully documented. The basic chromosome number in *Hypericum* is 12. However, Robson and Adam (1968) reported that there is a decrease in basic chromosome number due to the evolution in *Hypericum* from 12 to 7. They also described this genus as polyploid plants that have a small chromosome about 0.5 μ long at meiosis (Robson and Adams 1968). Table 1 shows number of chromosome and their ploidy level for each section in *Hypericum* genus. *Hypericum* used for ornamental was reported belong to Androsaemum and Ascyreia sections. (Robson, 1985)

Table 1: Section *Hypericum* and chromosome no and ploidy

Section No	Section Name	Number of chromosome	Ploidy Level
1	Campylosporus	12	2
2	Psorophytum	12	2
3	Ascyreia	12-9	4,6
4	Takasagoya	?	?
5	Androsaemum	10	4
6	Indora	10	4
6a	Umbraculoides	?	?
7	Roscyna	9-8	2
8	Bupleuroides	?	?
9	Hypericum	8-7	2-6
10	Olympia	9	2
11	Camylopus	8	2
12	Originifolia	9-8	2
13	Drosocarpium	8-7	2
14	Oligostema	9-8	2
15	Thasia	8	2
16	Crossphyllum	8	2
17	Hirtella	10 - 12(14)	2
18	Taeniocarpium	9	2
19	Coridium	9	2
20	Myriandra	9	2(3-4)
21	Webbia	10	4
22	Arthrophyllum	?	?
23	Triadeniodes	8	2
24	Heterophylla	9	2
25	Adenotrias	10	2
26	Humifusoideum	12, 9 - 8	2
27	Adenosepalum	10-8	2,4
28	Elodes	(10?)8	2,4
29	Brathys	12	2
30	Trigynobrathys	12,9 - 8	2,4

2.3 *Hypericum* cultivation as ornamental crop

In earlier years, this unique plant was popular as a medicinal crop as they contain various medicinal properties such as anti viral, anticancer and antidepressant. Later, the breeders started to select for its berry like fruit. This versatile and unique plant is often used as filler in floral bouquets. The berries comes in numerous colour for instance red, yellow, peach, pink and green This lovely coloured-hip *Hypericum* admired by florist as they did not stain on cloth, skin or surfaces like other berries. Gifted with this trait makes it the most successful berry producing shrub.

Market of *Hypericum* berries in ornamental purposes has been increased strongly for the past ten years. At Dutch flower auction, the figure was 57 million stems in 1995 and already risen to 199 million stem in 2000. Moreover, the growing areal for cultivated *Hypericum* berries enlarge and there has been a massive rise in their cultivation particularly in The Netherlands and Ecuador. The importing countries for this ornamental flower are Germany as well as United States.

2.4 Root knot nematode

Several of plant parasitic nematodes have been reported worldwide. The most destructive plant parasite is root knot nematode which causes a severe damage on the root system of many host plant (Kokalis-Burelle et al, 1997; Hussey and Janssen, 2001). Sikora & Fernandez (2005) reported, these endoparasitic belong to *Meloidogyne* genus include *M.incognita*, *M.javanica*, and *M.arenaria*.

These nematodes have been documented to have widely distribution to cause a great damage in ornamental crops worldwide such as in Australia (Wallace, 1969), Belgium (Coolen and Hendrickx, 1972; Stoffelen et al., 2000), Egypt (Montasser, 1995), France (De Waele and Davide, 1998), Iraq (Singh and Majeed, 1991), Ivory Coast (Adiko, 1988), Korea (Cho et al., 1996), Nigeria (Caveness and Wilson, 1977), Pakistan (Zarina and Abid, 1995), Saudi Arabia (Ibrahim and Al- Yahya, 2002), Spain (Jaizme-Vega et al., 1997).

As shows in the Figure 2 this *Meloidogyne* spp have four juvenile stages in their life cycle. In the second juvenile stage, it enters the root and migrates to the vascular cylinder. Subsequently, they formed the permanent feeding sites which cause formation of galls on the root system of the infected host plant. The nematodes stay there until they develop into the adult stage and start to produce eggs after 3 to 6 weeks after the initial infection. The

infected plants will have symptom such as wilting, reduced efficiency of fertilizer absorption and stunted growth afterwards (Williamsom & Hussey, 1996). Several studies describes the *Meloidogyne spp* able to infect wide host range as it already reported in various ornamental plant such as *Athurium andraenum* and other tropical ornamental (Bala & Hosein, 1996), *Hypericum* and *Ipex* spp (Heald, 1967), *Rosa* spp (Santo & Lear, 1976) and *Diathus caryophyllus* (Cho et al., 1996).

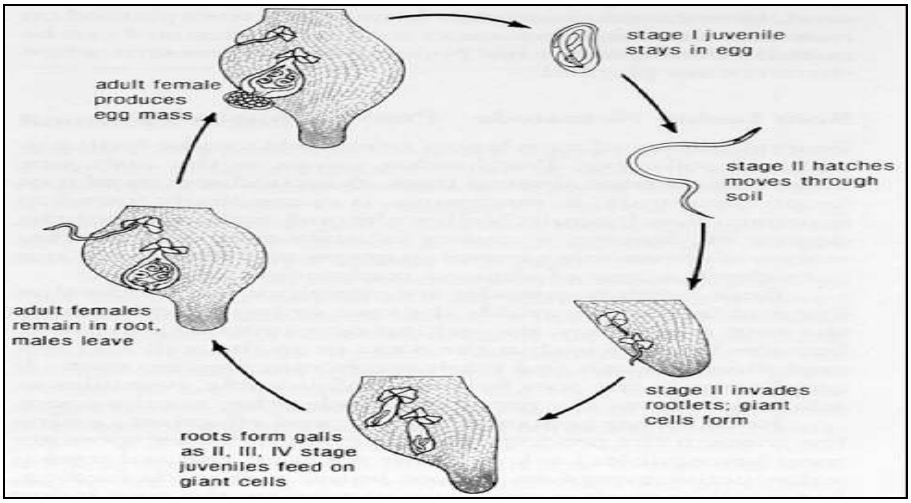

Figure 2: The life cycle of root knot nematodes (www.ctahr.hawaii.edu/nelsons/koa/koa.html)

2.5 Breeding for nematode resistance in Hypericum

Since *Hypericum* is grown in open field, an introduction of nematodes resistance variety is the best option for control the spreading of root knot nematodes. Furthermore, the introduction of nematode resistance to the cut flower industry, will guarantee the production of *Hypericum* with improved quality without the use of the dangerous nematicides and soil disinfection methods that are harmful to people and environment. Apart from that, the use of resistant cultivars will benefit growers as they can save cost for buying the nematicides and labor cost for applying the nematicides. Therefore, breeding a nematodes resistance variety to cultivated *Hypericum* is necessary to ensure the consistency in production and demand for this flower.

2.5 Marker Assisted Selection (MAS)

This approach can help breeders to simply tag desired traits which are not very straightforward to follow by using classical breeding methods. Thus, it offers breeders to do the selection in early stages even before the traits being manifested. Incorporating this approach into the conventional approach enables breeders to bring new and improved varieties into the market in short interval of time.

MAS involves the use of molecular markers such as random amplified polymorphism DNA (RAPDs), restriction fragment length polymorphism (RFLPs), simple sequence repeat (SSRs) or microsatellites and PCR based DNA markers such as amplified fragment length polymorphism (AFLP) or sequence characterized amplified region marker (SCARs). A specific location on the chromosome which can be used as a marker for genome analysis is called molecular marker (Varshney et al., 2009). Apart from marker assisted breeding, molecular markers have played an important role in linkage analysis, physical mapping, quantitative trait loci (QTL) analysis, and map based cloning (Bernatsky and Tanksley 1989; Lande and Thompson 1990; Knapp 1998) .

However, the use of marker assisted selection in *Hypericum* is still in its infancy. Previous molecular studies, only focussed on genetic diversity and phylogenetic of this species. Within this study, an initial overview on how molecular genetics study is carried out for this species will be presented. Hence, provide basic set up about this species for future works. To carry out molecular genetic study, few aspects need to be considered. This include, choosing the mapping population, markers system set-up, phenotypic evaluation of desired traits in the mapping population, construction of a genetic map based using molecular markers, mapping the QTL for the desired trait(s) as well as possible identification and cloning genes underlying the QTLs.

Nucleotide Binding Site-Leucine Rich Repeat (NBS-LRR)

Plants in their natural environment are constantly subject to a wide variety of phytopathogens. Therefore, they posses a great range of defence mechanisms such as physical barriers and defence compounds. Defence compounds that are specific determinants of effector-triggered immunity are known as resistance genes (R-genes), capable to

recognize specific avirulence proteins that being produced by the phytopathogens. This is based on the theory gene of a gene for a theory. Most R genes identified encode proteins that contain a nucleotide-binding site (NBS) and leucine-rich repeat (LRRs). NBS-LRR proteins responsible for the recognition of avirulence proteins that are thought to provide virulence function in the absence of associate R-gene (Chisholm et al., 2006). Plant NBS-LRR proteins can be distinguished into two classes; TIR and non TIR. The TIR class contains an amino-terminal domain similar to the Toll and interleukin 1 receptors. In contrast the non-TIR class is not well documented, but most contain α-helical coiled-coil-like sequences in their amino-terminal domain (Pan et al., 2000). Figure 3 shows a schematic representation of the structure of NBS-LRR sequences as described by Calenge et al (2005).

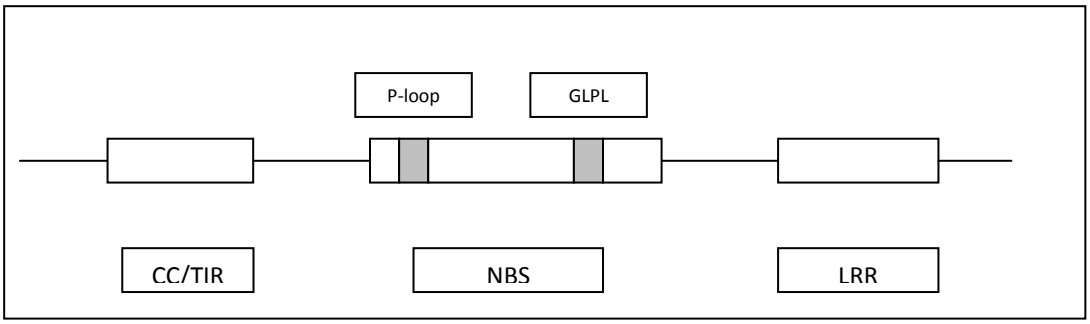

Figure 3: Schematic representation of the structure of the NBS-LRR. Exons are represented by boxes and intron by lines between boxes. Shaded boxes indicate highly conserved motifs inside the NBS-encoding region (Calenge et al., 2005)

NBS profiling-an approach to find a marker tightly linked with resistance genes

NBS profiling is a new development in markers technology that offers detection of molecular markers particularly for disease resistances based on the knowledge that most of the R-gene are the members of the cytoplasmic nucleotide-binding site-leucine-rich repeat that are highly conserved motifs in higher plants (Van der Linden et al., 2004). NBS-LRR markers are very useful as they have proven to be closely linked to important resistance genes (Syed N.H et al., 2005). This method has successfully been applied for mapping resistance gene analogues (Calenge et al., 2005), biodiversity studies (Reeves et al., 2004; Mantovani et al., 2004), as well as generating polymorphic markers with high sequence homology to RGAs in several species (Van der Linden et al., 2004). Co-localized among RGAs and QTL have also been studied with in a number of species, including bean (Geffroy et al., 2000), soybean (Kanazin et al., 1996), lettuce (Shen et al., 1998), A. thaliana (Speulman

et al.,1998), maize (Collins et al.,1998) and barley (Backes et al.,2003). Until now, this profiling method has been established in different cultivars such as potato, tomato, lettuce and barley (Van der Linden et al., 2004).

Molecular studies in *Hypericum*

Molecular studies have been known to benefit large numbers of plants. Application of molecular markers helps researchers to understand the basic genetic of the plant itself. Molecular study in *Hypericum* are mainly focus on genetic diversity studies (Matzk et al, 2001), molecular phlogeny (Park & Kim., 2003), their mode of reproductions (Mayo & Langridge., 2003) as well as to investigate the inheritance and hybridity of apomixes in *Hypericum (*Martonfi et al., 1996)

2.6 Genetic mapping & Mapping of QTLs

Linkage map from segregating markers in the mapping population helps to analysis the genetic variation of the desired trait. To construct a linkage map, all polymorphic markers are arranging in linkage group by using statistical software. Linkage relationship among markers is expressed by recombination frequency. Linkage analysis can be performed (using computer program) by calculating the odd ratio; ratio of linkage versus no linkage. The odd ratio is convey in logarithm of odds or easily called as LOD score. (Risch,1992). Lod score value > 3 (1000:1) will be used to construct a linkage map which means linkage between the markers is 1000 times more likely than no linkage. In the linkage map, all linked markers are grouped together on the chromosomes. Determination of marker order and accuracy of genetic distance are highly related to the number of individuals using in a mapping population. Young (1996) suggested that ideal number for mapping population is at least of 50 individuals.

3.0 Scope of the thesis

The main objective of this study is to establish marker assisted selection in *Hypericum*. Research has been proven, through MAS, breeders are possible to conduct many round of selection in a year. The sufficient amount of polymorphic markers is required for this purpose. Within this pilot study towards MAS, the setting up of NBS marker system is been carried out. We also exploited the NBS profiling technique to verify the progenies of interspecific crosses and to screen the diversity of population derived from infraspecific crosses and genotyping our mapping population. Analysis of the selected mapping population was also included as well as the approach for each analysis such as markers analysis, genetic similarity and linkage analysis are discussed. On top of that, steps in map construction and linkage map also been described.

4.0 Material and Methods

In this chapter, all material and methods used in this study were described.

4.1 Plant Materials

Young leaves of seedlings were supplied by the Esmeralda breeding and biotechnology company in Ecuador and were stored frozen at -80° C until DNA extraction. All the experiments were conducted using F1 populations. Details of plant materials used in each experiment as discussed below. List of all materials used as shown in Appendix 1.

Set up of the NBS marker system for Hypericum

The young leaves of these samples were taken from the plants provided by Esmeralda Company. These potted plants were grown in the tunnel. These samples consist of five F1 hybrids and six parent plants.

Verification of progenies obtained from interspecific crosses

For this purpose, nine populations of interspecific crosses were validated to confirm the progenies obtained from this crosses were true hybrids. One population consist three progenies and duplex set of parents. Total 64 individual plants were tested. The list of the populations used in this experiment as shows.

Choosing intraspecific populations for genotyping

Four populations includes eight progenies per crosses were used in this experiment. These populations are diploid and derived from intraspecific crosses of cultivated parents. Each of these populations has different levels for nematode resistance.

Genotyping the selected intraspecific population

F1 population from population 3 were used for genotyping. 94 progenies were chosen to include in this experiment. In total 96 samples were used in this experiment.

4.2 DNA isolation

Genomic DNA was isolated from fresh leaf tissue using the protocol developed by Fulton et al., (1995) with minor modifications. Approximately, 0.1 g of leaves was grounded in liquid nitrogen with mortar and pestle to a very fine powder. The leaves powder was then transferred to 2 ml eppendorf tubes. Subsequently, 750 µl microprep buffers was added to each eppendorf tube. The microprep buffers were freshly prepared just before DNA extraction as shown in Appendix 2. After the leaves powder and microprep buffers were well mixed 1 µl of RNAase was added. The mixture was incubated for at least 90 minutes in 60° C. At the end of incubation, 800 µl of chloroform:isoamyl alcohol was added to each tube. The tubes were inverted continuously to mix well. The upper viscous aqueous phase was transferred to the new tubes after centrifuge for five minutes at 15000 rpm. One times volume of cold isopropanol was added and the tubes were repeatedly inverted to let the DNA precipate. The DNA was spinned down for 5 minutes at 15000 rpm. The protocol was continuing to wash away other substances except DNA by 500 µl of 70% ethanol followed with centrifuge for 5 minutes at 15000 rpm. pellet were dried by inverting the tubes on paper towel for one hour or more if needed. The dried pellets were resuspended in 50 µl of TE^{-4} for overnight and stored at 4° C.

4.3 NBS profiling

This technique comprises three different steps which covers the digestion and ligation of genomic DNA, amplification of selected fragments and gel analysis of amplified fragment. The technique was conducted followed the protocol developed by Van der Linden et al., (2004) with some optimisation to suit the species used. In the initial steps, the DNA was digested and ligated to the adapter in one reaction. The DNA was digested using the restriction enzymes *MseI, RsaI, AluI* with a four base recognition site and ligated with the block adaptors to the end of restriction fragments. The block adaptor consist sequence similar to the adapter primers and short sequence that is blocked by an amino group at the 3' end in order to block the extension with Taq polymerase (Table 2). To facilitate ligation to blunt end fragments, the 5'end was phosphorylated. Since the restriction fragment from the *MseI* produce stick end, the short sequence was extended to match *MseI* restriction end. A total mixture of 60 µl containing 400 ng of genomic DNA, 1 µl restriction enzyme, 12 µl of restriction ligation buffer, 3 µl of 50pmol/ µl of adapter, 1 µl of T4 ligase and MQ water was incubated in a PCR block at 37° C for three hours. The reaction was terminated by heat

activation at 65 ˚ C for 15 minutes. Then, followed by two round of PCR. In the first cycle of PCR, 5 µl diluted product of restriction ligation, 2 µl of specific degenerated primer (10pmol/ µl), 2 µl adapter primer (10pmol/ µl), 1 µl dNTPs (5Mm), 2.5 µl Hotstart PCR buffer, 0.08 (5 U/ µl) of Hotstart polymerase in the reaction volume of 25 µl. The PCR program have 30 cycles of 95˚ C for 30 sec, 100 sec of annealing temperature and 2 min 7˚ C. Annealing temperature of each primers used are described in Table 3. Later, the PCR products were labelled in the same PCR condition as the first cycle. The reaction were performed in 10 µl assay containing 0.6 µl labelled 1 pmol/ µl IRD 700 adapter primer, 0.3 µl of specific degenerated primer, 0.4 dNTPs (5Mm), 1 µl Dreamtaq PCR buffer, 0.04 (5 U/ µl) of Dreamtaq polymerase.

Table 2: List of adapters and their sequences for restriction-ligation step

Adapters	Sequences
Blunt adapter long arm	5' ACTCGATTCTCAACCCGAAAGTATAGATCCA 3'
Blunt adapter short arm	3' NH_2 TTCATATCTAGGGT 5'-P
Mse1 adapter long arm	5' ACTCGATTCTCAACCCGAAAGTATAGATCCCA 3'
Mse1 adapter short arm	3' NH_2 TTCATATCTAGGGTAT 5'
Adapter primer	5' ACTCGATTCTCAACCCGAAAGTATAGATCCCA 3'

Degenerated primers

The degenerated primers used were already designed. These degenerated primers were designed based on NBS conserved region of plant disease resistance genes (Van der Linden et al., 2004). The list of degenerated primers used in this study as shows in the Table 3

Table 3: List of primers sequences and their optimal temperature used in NBS profiling

Primer	Sequences	Tm(˚C)
NBS 5A	5' YYTKRTHGTMITKGATGATGTITGG 3'	55
NBS 6	5' YYTKRTHGTMITKGATGATATITGG 3'	55
NBS GLPL	5' TGYRRAGGAYTRCCWYTAGC 3'	55
NBS 1	5' GCIARWGTWGTYTTICCYRAICC 3'	55
NBS 2	5' GTWGTYTTICCYRAICCISSCAT 3'	60
NBS 3	5' GTWGTYTTICCYRAICCISSCATICC 3'	60

Licor

The labelled PCR products were separated on polyacrylamide gels for 4 hours using the Licor DNA analyzer machine. 10 µl labelled PCR products was mixed with 10 µl loading buffer. Samples were denatured for 5 minutes at 94˚ C. Immediately put the samples on ice after the denaturing step prior the loading step.

4.4 Marker analysis

Nomenclature of markers

Markers which derived from NBS-profiling are named NBS markers followed by the primer used and the initial of the restriction enzyme used and a follow number.

Marker segregation type

Markers were score dominantly and sorted into uni-parental or bi-parental types. Uni-parental is marker that being heterozygous either in the mother (lmxll) and father (nnxnp) and bi-parental is marker that heterozygous in both parents (hkxhk).

Linkage analysis

Linkage analysis of markers obtained was performed using JoinMap software version 4 (Van Ooijen 2006). The markers order in linkage group was calculate using Chi-square value to find the best fit between markers. Linkage groups were calculated with a LOD threshold of 4.00 and the construction of map using Kosambi mapping function

Data analysis

The NBS marker obtained from the genotyping data with 15 primer and enzyme combinations was analyzed. Only polymorphic bands were scores as present and absent. The similarity analyses were performed using Jaccard's similaritity coefficient (Jaccard, 1908) done with NTSYS-pc ver.2.11 (Rohlf., 2008). The obtained similarities values were then applied for cluster analysis and relationship tree was generated. The equation for calculating similarities is showed below;

$$J = a/(n-d)$$

a=number of DNA fragments absent in both species
d= number of DNA fragments present in both species
n= total number of DNA fragments

5.0 Result

In this chapter, results of the experiments conducted are presented. First, results obtained for the marker system set up is given, followed by the verification of the progenies obtained from the interspecific crosses and relationship studies between species tested, evaluated four intraspecific crosses and choose the mapping population and later, genotyping the selected diploid population.

5.1 Set up of the NBS marker system for *Hypericum*

DNA isolation

Figure 4 shows the pictures from gel electrophoresis obtained from three methods of DNA isolation using young leaf material. The three methods of DNA isolation used are; CTAB method (Doyle & Doyle, 1990), DNeasy Plant Kit (Qiagen) and Fulton method (Fulton et al., (1995). The different methods gave different results in terms of DNA quality, overall yield and concentration. As shown in Figure 4, all samples derived from CTAB method did not give any DNA and have RNA contamination. In contrast, DNAeasy Plant Kit (Qiagen) gives DNA in several samples with some degradation observed. The best isolation method for *Hypericum* comes from Fulton method. From all samples DNA was isolated without any contamination and degradation detected. There was variation in DNA concentration but overall amounts of DNA were sufficient for downstream application.

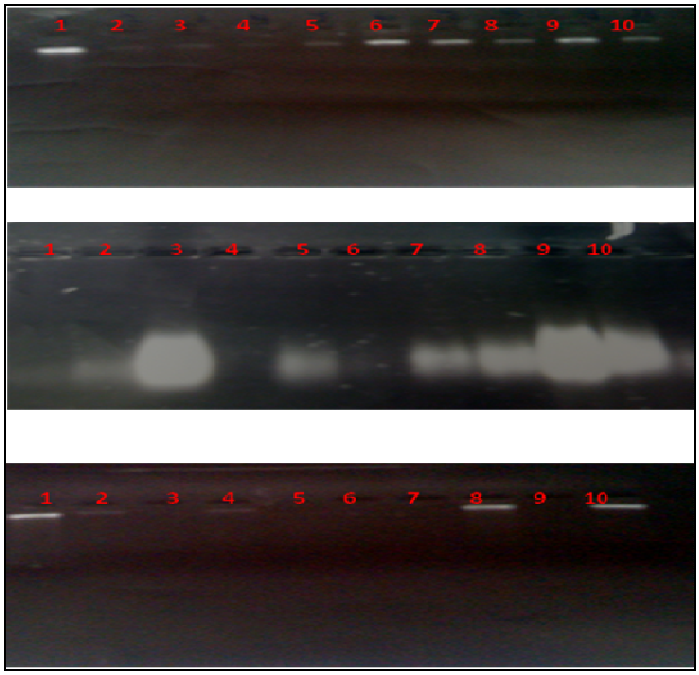

Figure 4: Comparison of three method DNA isolation used in *Hypericum* (above:Fulton method,middle: CTAB method, below: DNAeasy(Qiagen))

NBS profiling

Our plant materials were supplied by a breeding company and for several reasons they sent us matured leaves instead of young leaves. Due to this, we faced many problems to perform NBS profiling. After the first pre amplification step, we observed all samples results with no amplification at all. To find out the cause of this problem, we tested the digestion of restriction enzyme using the same set of samples and one set of control samples. The control sample is from DNA isolation of young leaves. From the agarose gel picture (Figure 5), we can clearly see the difference level` of digestion efficiency. In our samples, DNA bands can still be observed on the upper part of the gel. In contrast, "smearing effect" was observed in all samples from control set and DNA bands were not visible on the upper part of the gel. This is an indication that the DNA was digested and the smearing DNA was observed ranging from 100-500 base pairs when comparing with molecular weight lane which is as expected with four-cutter restriction enzyme.

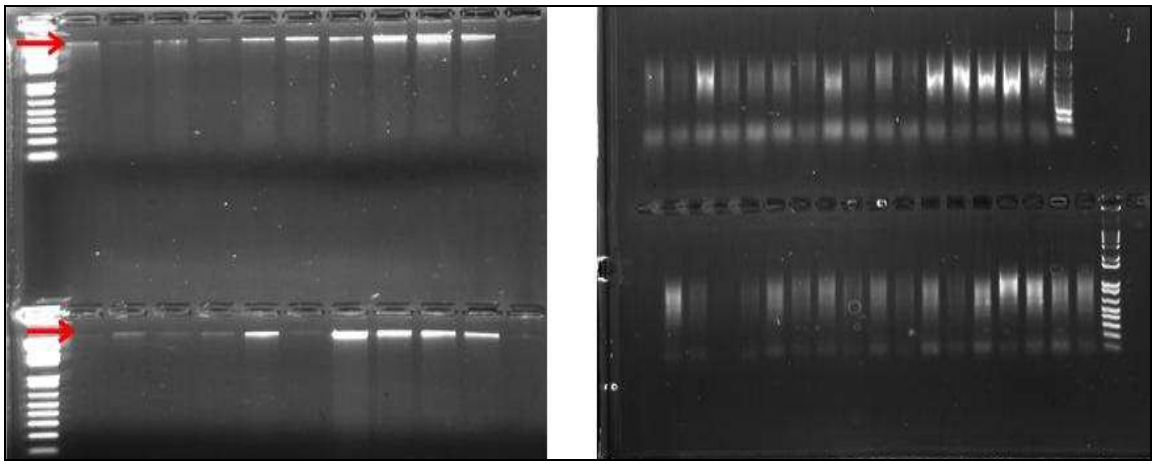

Figure 5: Gel pictures of digestion efficiency between different type of leaves used (left: matured leaves, right: young leaves)

Polymorphic marker analysis

After DNA isolation and NBS profiling have been optimised, DNA profiling of eight hybrids and six parent plants were analysed in order to observe polymorphism of the genotypes. For the initial run, three restriction enzymes (*Mse*I, *Alu*I and *Rsa*I) and four degenerated NBS primers (NBS 5A6, GLPL, NBS 1, NBS 2) were utilized. From this 12 primers-enzyme combination was obtained. In general, *Mse*I generated the highest polymorphic markers, while restriction enzyme *Rsa*I gives 145 polymorphic markers. The lowest polymorphic marker (134) was obtained from enzyme *Alu*I (Table 4). Each degenerated primer results in different numbers of polymorphic markers. Among four primers used, degenerated primer GLPL results the highest number in polymorphic markers with 135. Followed by NBS 1 (126) and NBS 2 (115) and the lowest number of polymorphic markers scored is from NBS 5A6 with only 85 markers. Within 12 enzyme-primer combinations, the best combination was NBS2_Mse1 with total 59 markers obtained.

Table 4: Number of polymorphic bands detected with each primer/enzyme combination in eight hybrids and six parent plants

	Primer				
Enzyme	NBS 5A6	NBS GLPL	NBS 1	NBS 2	Total
*Mse*I	44	44	35	59	182
Alul	17	38	44	35	134
*Rsa*I	24	53	47	21	145
Total	85	135	126	115	

5.2 Interspecific crosses

Four species and three cultivars were used in interspecific crosses made by the Esmeralda breeding company in Ecuador. Nine populations of interspecific were successful derived. However, the hybrid plants obtained were observed to have similar morphology with their mother plants. To confirm whether the hybrids derived from interspecific crosses are a true hybrids or not the verification of these hybrids were carried out.

Verification of progenies obtained from interspecific crosses

To test the applicability of interspecific crosses for mapping population progenies from nine populations and their respective parents were tested. Here, seven different parents including cultivars and species of *Hypericum* were used. Each population represent by three progenies and duplex parents which bring in total 63 individuals for each degenerated primer-enzyme combinations. The verification was carried out with three restriction enzymes (*Mse*I, *Alu*I and *Rsa*I) and four degenerated NBS primer (NBS 5A6, GLPL, NBS 1, NBS 2). DNA profiling for verification of progenies were obtained from the separation of labelled fragment using a LiCor DNA analyzer machine.

From our verification, we can clearly observed that all progenies in nine population tested have the same DNA profiling as their mother plant. This pattern happened in across 12 degenerated primers-enzyme combinations used. These results indicated the event of apomixis in nine interspecific crosses tested. Also, in a limited number of cases some bands appeared in the progenies and do not segregating. However, these bands could not be detected either it comes from the mother or the father plant.

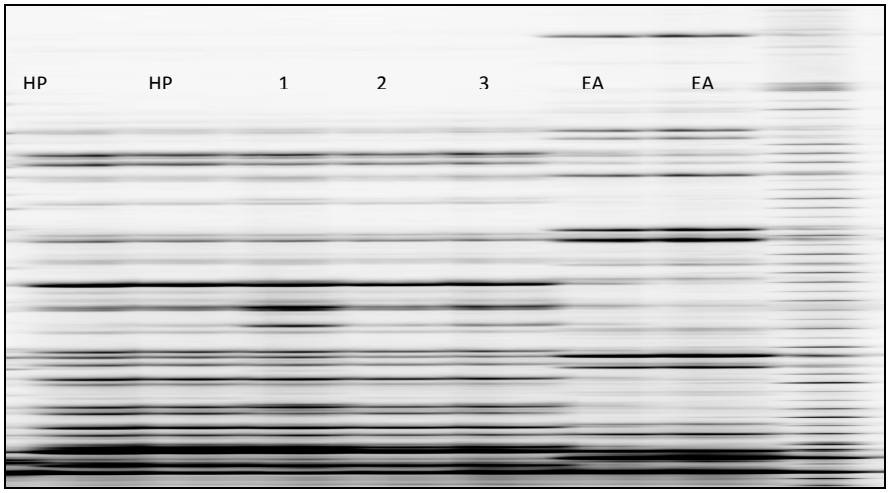

Figure 6: Gel picture after electrophoresis NBS profiling for the parents (in duplo) and three individuals belonging (1,2,3) from the H. perforatum (HP) x Elite amber (EA) cross. This pattern was obtained with Mse1 enzyme and NBS primer 5A6

Figure 6 is an example of one population that was obtained from one degenerated primer-enzyme combination in this case Mse1 enzyme and NBS primer 5A6. In this population, *H.perforatum* (P) and Elite Amber (EA) were used as the parent. As mentioned earlier,the progenies have exactly the same DNA pattern of their mother plant (*H.perforatum)* whereas none of the bands from progenies came from the father plant (Elite Amber).

Results from this verification confirmed that the interspecific crosses made by Esmeralda Breeding Company were failed in giving any hybrids in nine populations. We also detected three progenies from three different crosses that had same pattern with their father plants. Therefore, these three progenies were phenotypically checked in the field. Their phenotype result confirmed these three progenies were a father plant instead of progenies plant and were mislabelling before.

Polymorphic marker analysis from seven genotypes used in the interspecific crosses

12 degenerated NBS primer-enzyme combinations were tested in the seven genotypes used as a parent plants in interspecific crosses (Table 5). The polymorphic markers were scored dominantly. Average 175 polymorphic markers were obtained from enzyme *Rsa*I in all degenerated NBS primers used while *Alul* with 117 scored markers. 62 of average markers were scored from MseI. Within three restriction enzymes used in combination of all degenerated primers, cultivar Elite Amber (EA) gives the highest polymorphic markers with 196 in *Rsa*I, 140 markers in Alul and 73 in *Mse*I. Meanwhile, the lowest polymorphism markers scored was from *H. hidcote* (H) species with 155, 97 and 45 markers respectively. Relatively, degenerated primer NBS GLPL gives the highest polymorphic bands across three restriction enzymes, followed with primer NBS 1 and NBS 5A6 and the lowest numbers of polymorphic bands observed from primer NBS 2.

Table 5: Numbers of polymorphic bands amplified with three enzymes (Mse1, Alul, Rsa1) and primer NBS 5A6, NBS GLPL6, NBS1 and NBS2

Enzyme	Primer	Species							Total
		PA	EA	RW	P	C	H	B	
Mse1	NBS 5A6	15	15	17	24	19	3	17	110
	NBS GLPL	20	28	21	14	20	20	11	134
	NBS 1	12	13	14	6	14	16	18	93
	NBS 2	15	17	17	12	14	6	16	97
	Total	62	73	69	56	67	45	62	
Alul	NBS5A6	9	7	3	10	2	4	11	46
	NBS GLPL	17	23	25	13	10	16	11	115
	NBS 1	15	20	15	15	26	26	13	130
	NBS 2	15	17	17	12	14	6	16	97
	Total	118	140	129	106	119	97	113	
Rsa	NBS 5A6	12	10	5	16	5	9	12	69
	NBS GLPL	27	26	25	25	25	24	15	167
	NBS 1	18	17	15	15	28	16	26	135
	NBS 2	2	3	1	9	0	9	12	36
	Total	177	196	175	171	177	155	178	

Relationship study between seven genotypes used in interspecific crosses

Genetic similarities between seven genotypes that were used as parent plants in interspecific crosses were calculated by pairwise comparison using polymorphic markers that have been scored. Table 6 described genetic similarity of each genotype obtained. The scale of genetic similarity was ranged between 0 and 1. The range 0 and 1 indicated as different and identical respectively. The largest value was resulted between Red Wave (RW) cultivars and Elite Amber (EA) with 0.85. The lowest genetic similarity value was 0.15 arising from comparison between *H.perforatum* (P) and Red Wave (RW). It was observed that all cultivars (Pink attraction, Elite Amber and Red Wave) showed highest similarities between each other. On the other hands, low genetic similarities were observed f within the Hypericum species [*H.perforatum* (P) *H.calycinum* (C) *H.hidcote* (H) *H.buckleii* (B)]

Table 6: Pairwise comparison of seven genotypes of *Hypericum*

Species	PA	EA	RW	P	C	H	B
Pink attraction(PA)	-						
Elite Amber(EA)	0.764	-					
Red Wave(RW)	0.739	0.845	-				
Hypericum perforatum(P)	0.185	0.165	0.153	-			
Hypericum calycinum (C)	0.168	0.208	0.198	0.209	-		
Hypericum hidcote(H)	0.156	0.173	0.173	0.195	0.359	-	
Hypericum buckleii (B)	0.225	0.237	0.212	0.437	0.195	0.155	-

Based on genetic similarity values, a cluster analysis was performed to generate a tree that shows the relationship between the genotype studied. The length of branching that separating the different species indicates the genetic similarities values. Referring to Figure 7 three cluster groups of species can be distinguished. All cultivar were grouped together as illustrates by the figure, Pink attraction formed a cluster with Elite Amber and Red wave. However, Elite Amber and Red Wave are much closer to each other. *H.perforatum* and *H.bariloche* appeared to be in the same cluster group. The third cluster group was forming between *H.calycinum* and *H.hidcote*.

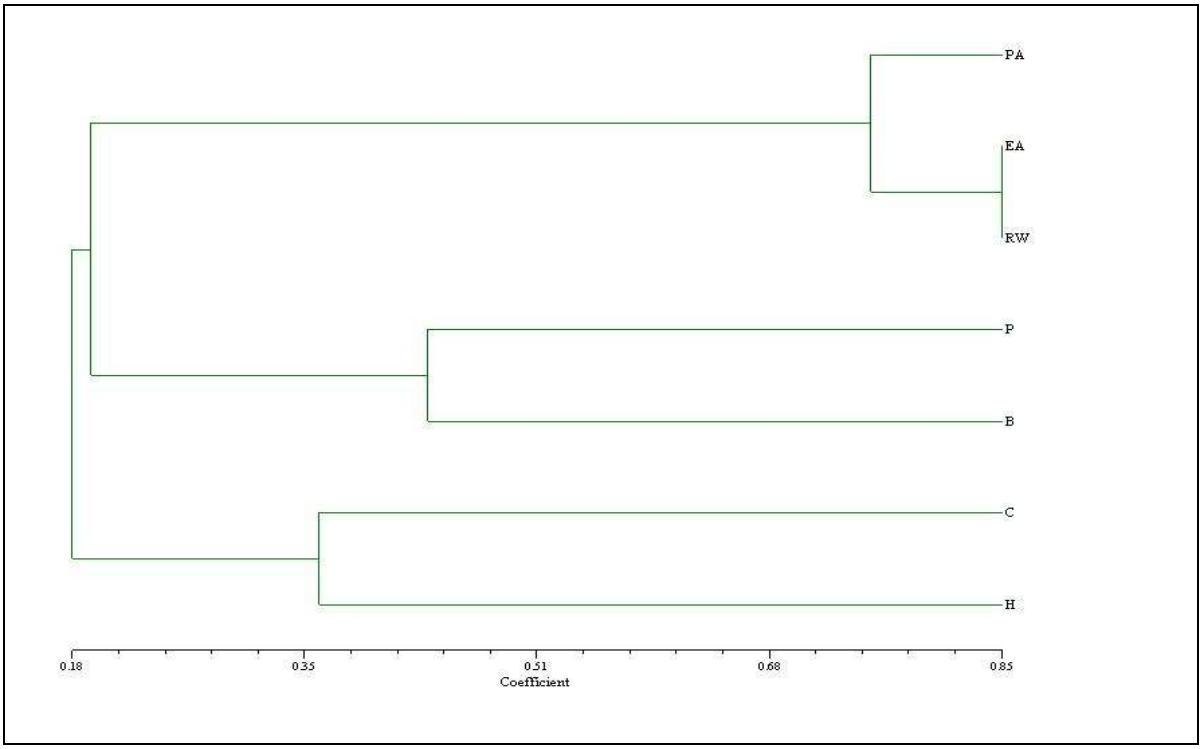

Figure 7: Relationship tree of seven different genotypes of *Hypericum*

5.3 Intraspecific crosses

In the beginning we are hoping to get a mapping population from interspecific crosses to be employ in NBS marker development study. Unfortunately, none of populations from the interspecific crosses were successfully produced hybrids because of apomixis. Hence, to precede this study, together with the supervisors we decided to change our mapping population onto intraspecific crosses that already shown different level in their resistance towards nematodes. For this purpose four intraspecific crosses were evaluated. The parent plants used in these crosses were really parent without apomixis. Since there is no disease scoring had been made yet, the mapping population was chose by observation of polymorphic markers between the parents. It need to be noted here that at that time the DNA of plant materials were not in a good quality as the plant materials arrived after being stranded in the airport for a day. The leaves of the plant materials were observed to have symptom of wilt and already started to yellowing.

Evaluation of four populations from intraspecific crosses

Numbers of polymorphic marker over four populations contain eight progeny and two parent plants from infraspecific crosses are given in Table 7. Data gained from three restriction enzymes (*Mse*I, *Alu*I and *Rsa*I) and five degenerated NBS primers (NBS 5A6, GLPL, NBS 1, NBS 2, NBS 3). Generally, from table it is noticeable that the numbers polymorphisms are very low within the population with below than 10 polymorphic markers can be scored. In this evaluation, population 3 has showed a promising result with regard to number of polymorphism obtained across 15 degenerated NBS primer-enzyme combinations with 102 scored markers. This followed by population 1 (74) and population 2 (67). The lowest polymorphism observed was from population 4 with only 60 markers scored in 15 degenerated NBS primer-enzyme combinations.

Table 7: Number of polymorphic marker over four intraspecific populations

Population	Primer-enzyme combinations															Total
	NBS 5A6			NBS GLPL6			NBS 1			NBS 2			NBS 3			
	*Mse*I	*Alu*I	*Rsa*I	*Mse*I	*Alu*I	*Rsa*I	*Mse*I	*Alu*I	*Rsa*I	*Mse*I	*Alu*I	*Rsa*I	*Mse*I	*Alu*I	*Rsa*I	
1	2	4	6	4	4	7	5	2	12	7	2	5	5	4	5	74
2	3	3	4	3	7	2	7	6	3	7	8	3	3	5	3	67
3	4	3	8	3	5	4	8	7	13	8	2	11	10	8	8	102
4	2	3	6	3	6	7	2	4	5	6	2	5	5	3	1	60

Genotyping population 3 from infraspecific crosses

Polymorphic marker analysis

94 progenies were screened with three restriction enzymes (*Mse*I, *Alu*I and *Rsa*I) and five degenerated NBS primers (NBS 5A6, GLPL, NBS 1, NBS 2. NBS 3) which bring in total 15 primer-enzyme combinations. All primer combinations were chose based on the basis of the high number polymorphism found between four population candidates and initial run of NBS system in *Hypericum*. Polymorphic markers were score present and absence dominantly. DNA profiling obtained by LiCor DNA analyzer machine showed numbers of segregating markers in progenies of population 3. However, low polymorphism can be observed in this population. The highest polymorphic markers generated from restriction enzyme, *Rsa*I with 66 polymorphic markers. Second highest was from restriction enzyme *Alul* with 49 polymorphic markers. The lowest polymorphic markers (44) were scored from enzyme *Mse*I (Table 8). Altogether 159 NBS markers were obtained from 15 primer combinations.

Table 8: Number of polymorphic marker of F1 population with three restriction enzymes (*Mse*I, *Alu*I and *Rsa*I) and five degenerated NBS primers (NBS 5A6, GLPL, NBS 1, NBS 2. NBS 3)

	Primers					
Enzyme	NBS 5A6	NBS GLPL6	NBS 1	NBS 2	NBS 3	Total
Mse1	3	7	14	12	8	44
Alul	7	11	5	6	20	49
Rsa1	19	12	11	11	13	66

The segregating marker obtained were divided into two types of markers; uni-parental markers (lm x ll / nn x np) and bi-parental markers (hkxhk) as shown in Table 9Table 9mong two types of markers, the highest types of markers were obtained from bi-parental markers with 90 markers scored. Markers segregating from mother plants and father were scored 31 and 38 respectively.

Table 9: Types of markers obtained from F1 population with three restriction enzymes (*Mse*I, *Alu*I and *Rsa*I) and five degenerated NBS primers (NBS 5A6, GLPL, NBS 1, NBS 2. NBS 3)

	Uni-parental markers		Bi-parental markers
Enzyme	lm x ll	nn x np	hk x hk
Mse1	9	12	22
Alul	11	9	29
Rsa1	11	17	39
Total	31	38	90

Linkage analysis

All 159 polymorphic markers obtained were used in constructing preliminary linkage map using Kosambi's mapping function by JoinMap 4.0 (Van Ooijen 2006). The linkage group were determined using LOD threshold of 4. The significance of segregation ratio was evaluated by Chi-square test with p value of 0.05 and degree of freedom 1. According to Mandelian fashion, the expected ratio for dominant markers was 3:1. Marker types used for mapping are uniparental (nnxnp/lmxll) type and biparental (hkxhk) type. Two markers were removed due to similarity loci calculated by the software. However, highly skewed segregation and mean Chi-square (range 1.6 to 2) of linkage group can be observed. This suggest us to re checked the scoring data. The segregations were highly skewed mainly because the missing values were entered as absence data and wrong interpretations of faint bands made by inexperienced score person. Construction genetic map was repeated using the revised data. From the Chi-square values 54 markers showed significantly fit with expected ratio meanwhile 105 markers (66%) resulted segregation distortion. We also observed that most of markers from hkxhk type showed extreme segregation distortion determined by the Chi-square value and more markers are needed to extend the map because so many markers are of the low information containing hkxhk type.

6.0 Discussion

In this chapter, all results of analyses made are discussed. Discussions start from the set up of a NBS marker system for *Hypericum*, followed by verification of progenies in interspecific crosses as well as results evaluation of four populations from infraspecific crosses. Thereafter, the results from genotyping population 3 were also discussed.

6.1 Set up the NBS marker system for Hypericum

DNA isolation and NBS profiling

Amongst three methods used for DNA isolation, the Fulton method (Fulton et al., 1995) works best in *Hypericum*. With this method we managed to obtain good quality of *Hypericum* DNA as compared to DNeasy Plant Kit (Qiagen) and CTAB method (Doyle & Doyle, 1990). DNA isolation of *Hypericum* is not easy as we encounter many problems before being able to isolate a good quality and yield of DNA. One of the problems is that the plant materials need to be grinding with liquid nitrogen with mortar. The use of bead mills causes insufficient disruption of starting material which yielding low amount of DNA. However, using liquid nitrogen and mortar cause longer handling time since the procedure need to be cautiously perform to prevent DNA loss and minimized the chance of contamination with other samples.

Another crucial problem in DNA isolation of *Hypericum* is polysaccharides contamination. The problem was observed prominent in mature *Hypericum* leaves and less prominent when young leaves are used. Studied by Crowley et al., (2003) revealed that polysaccharides contamination is common when mature samples were used. The contamination of polysaccharides in *Hypericum* DNA will reduce the efficiency of downstream reaction in NBS profiling. As described by Crowley et al., (2003) most molecular enzyme and DNA polymerase reaction will be inhibited by polysaccharides. This can caused a major problem in NBS profiling as both restriction enzyme and DNA polymerase was used in DNA digestion and in PCR amplification respectively. In addition, contamination of polysaccharides caused over–estimation of the DNA concentration as it can concentrate DNA samples by forming an extremely vicious DNA pellets (Demeke & Adam, 1992).

Nevertheless, when clear DNA was isolated from young fresh leaves, there is no major problem of NBS profiling being applied in *Hypericum*. In a number of cases when young

fresh leaves were not available and more mature leaf samples had to be used overall patterns of NBS profiling were less clear and often the longer DNA fragments got less intense and regularly not scorable. In the NBS profiling technique, we use four base cutter of restriction enzyme which means it cut the DNA every 265 base pairs. Therefore, NBS profiling is able to produce multilocus profiling. This method was able to produce high polymorphism among the *Hypericum* species. However, NBS profiling showed lower amount of polymorphism when it is employed within the *Hypericum* cultivars. This suggests the capacity of NBS profiling to generate polymorphic markers within the narrow genetic distances is depleted. Therefore, one should consider using other marker system that has high ability to generate polymorphism even within narrow genetic distances such as AFLP marker system. In the second amplification of PCR reaction, we tried to use *Dreamtaq* polymerase instead of *Supertaq* polymerase which is cheaper. Some additional bands could be scored when using *Dreamtaq* polymerase. In contrast, most of the bands present using the *Supertaq* were also present when *Dreamtaq* was used (personal communication, Koen).

6.2 Interspecific Crosses

Verification of progenies obtained from interspecific crosses

This experiment was carried out to validate the progenies derived from the interspecific crosses. Since from morphological aspects of these progenies does not has much difference visible, validation on molecular level was necessary. As was expected, DNA profiling of tested progenies achieved by the NBS profiling showed similar pattern with the mother plants. The results of this phenomenon can be described by apomixis. Band that appeared in the progenies and cannot be traced back from either parent indicates that there may be still some incomplete digestion that emerged as an artefact in the gel pictures.

What is apomixes?

As introduced by Winkler (1908) apomixis is an asexual formation of seed without meiotic reduction and fertilisation. The progeny produced by apomixis is genetically identical to the mother plant. The prevalence of apomixis is widely distributed in angiosperms. It has been described in over 300 species including representatives of 35 different plant families (Bashaw & Hanna., 1990). Apomixis composed three major element required for a viable seed set. One element is apomeoisis which is the absence or alteration of meiosis preventing reduction, followed by parthenogenesis of unreduced egg cell and the last elements is endosperm development by pseudogamy or autonomously (Spillane et al., 2001). Figure 8 shown comparison between sexual life cycle and apomictic reproduction.

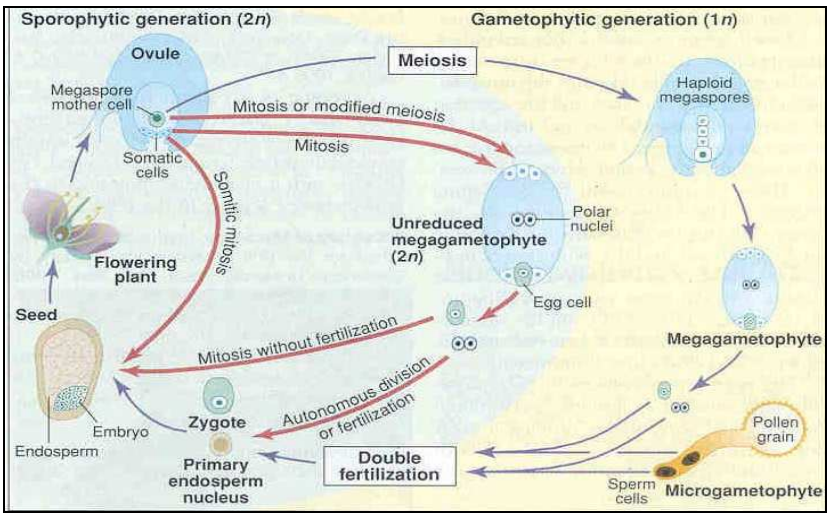

Figure 8: Comparison of sexual life cycle of flowering plant and apomictic reproduction by seed (Vielle-Calzada et al. ,1995)

Type of Apomixis

The type of apomixis can be distinguished into two; gametophytic and sporophytic. These two types of apomixis are depending on the fate of the unreduced cell. In gametophytic apomixis case, the unreduced cell gives rise to a megagametophyte. On the other hands, if unreduced cell turn out to an embryo it will become sporophytic apomixis also likely to be called adventitious embryony (Darrigues et al., 2002)

Mechanism of apomixis

Each type of apomixis has different mechanisms in order to obtain an embryo sac. Embryo arises from sporophytic apomixis which commonly occur in Citrus species is directly from the nucellus or the integument of the ovule (Koltunow., 1995). From the mitotic division of the cell nucleus, the development of the embryo is initiated as a bud-like structure (Bashaw., 1980). Information in mechanism of sporophytic apomixis is still not comparable with gametophytic apomixis. Other type of apomixis as mentioned above is gametophytic apomixis. This well studied of apomixis is divided into two mechanisms which is diplospory and apospory. Most of higher plants have apospry mechanism. The difference of these two mechanisms is the origin of unreduced cell formed eight nucleate embryo sacs (Darrigues et al., 2002). In *Hypericum* apomixis case, the embryo sac originates from somatic cells via mitosis after the degenerations of mega mother spore in meiosis. The embryo sac that generate from somatic cell is called apospry embryo sac. The structure of the embryo sac derived from unreduced cell is same as the reduced ones which rise from meiosis (Martonfi et al., 1996). Even though the egg cell is parthenogenetic, the polar nuclei must be fertilized.

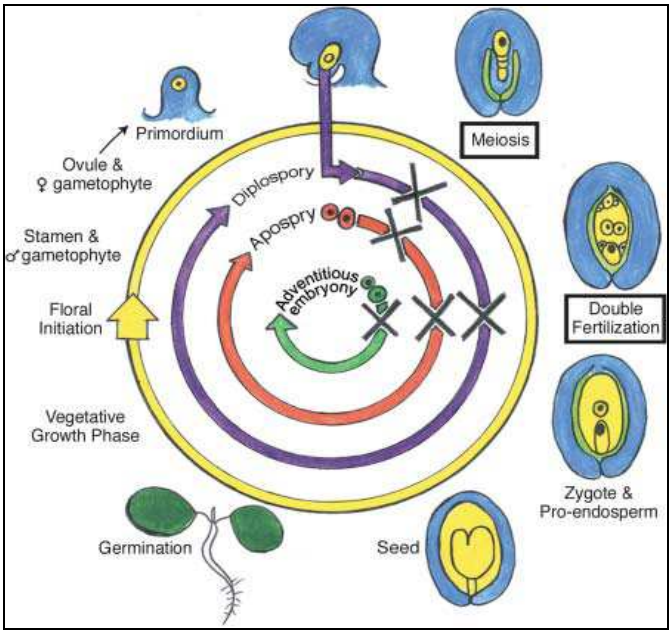

Figure 9: Mechanism of different types of apomixis as compare to sexual life cycle of angioperms

From the verification of progenies derived from interspecific crosses, four species of *Hypericum* have been identified as an apomitic plants. There are Pink Attraction, *H.perforatum*, *H.calycinum* and Elite Amber. These species were being used as a mother plant in the interspecific crosses. The progenies from those crosses were revealed to be identical to their mother plant by their individual DNA profiling. One of the species used *H.perforatum*, was described as being apomitic by Matzk et al., (2003). They identified 16 species with apomixis that include five facultative apomitic species in *Hypericum* section itself. These included *Hypericum x desetangsii* Lamotte, *H. kamtschaticum* Ledeb, *H.maculatum* spp obtusiusculum, *H.perforatum* L and *H. yezoense* Maxim.

It is also possible to have some degree of sexuality in facultative apomixes in Hypericum. Facultative apomixes plant is able to fertilized using either a reduced and unreduced egg cell. The hybrids obtained gives two different embryo sac; a reduced embryo sac and unreduced embryo cell. The facultative apomixes in *Hypericum*, normal reduced embryo sac occurred only in 3% of the ovules and 97% present in ovule with aposprous embryo sac (Noack.,1939)

Relationship of seven genotypes of *Hypericum*

From the tree plot obtained, three cluster groups can be distinguished from seven genotypes studied. Pink Attraction, Elite Amber, and Red Wave have high similarity with each other. This could be an indication that these cultivars were probably shared a common ancestors. These three cultivars are the cultivars developed by Esmeralda Breeding Company. The results showed that there is a very distant relationship between cultivars and species of *Hypericum*. The cultivars are much likely closer to each other meanwhile within the species it have more distinct relationship. This suggests that high genetic variation can be found within the species of *Hypericum*. Studies of Percifield et al., (2007) also demonstrated that there are high levels of variation between *H.perforatum* with other species from analysis of molecular variance which indicates abundance of variation at genus level.

Four species of *Hypericum* were clustered together into two groups. *H. perforatum* and *H.Buckleii* formed another cluster. *H. perforatum* has been known to be in *Hypericum* section and on the other hands; *H.Buckleii* was reported to be in Myrianda section (link from internet). These suggest that these two sections are closely related with each other. The classification of *Hypericum* genus by Noack (1939) stated that *H. calycinum* is in different section than *H.perforatum*. According to his classification, *H.calycinum* is belongs to Ascyreia section. This can be observed by the relationship tree obtained from our study. From the tree, we can clearly see that these two species were distantly related nor in the same cluster group. *H.hidcote* was clustered together with *H.calycinum* in group 3. According to the information in the internet databases, *H.hidcote* is a hybrid that comes from crosses between *H.calycinum* and *H.patatum* (link from internet). Although in our results they have the lowest similarities but they were clustered together.

6.3 Intraspecific Crosses

Evaluation of four populations from intraspecific crosses

An intraspecific cross is a mating between parents of the same species. The advantages of intraspecific over interspecific crosses are no crossing barrier from the same species, high heritability of traits and high chances to obtain favourable combination. Since genetic background of *Hypericum* used in this study seems quite narrow, low variations between the progenies were expected. Besides, the reduction on fitness of progeny cause from inbreeding depression may also occur. This is however, remained unexplained as limited evidence of inbreeding depression of progeny within populations of *H.cuminicola* were found by Trager et al., (2005).

The polymorphic bands in population three were observed to be the highest among the four crossing populations from the intraspecific crosses. Despite the problem in DNA quality of population three, we are able to score highest polymorphic markers across 15 primer and enzyme combinations. With this result, we are convinced that more markers can be scored if the quality of DNA is excellent. In addition to that, this population claimed to be one of their good crossing populations in term of their vigour in the field.

Genotyping the F1 population

Marker detection

An average of 15 polymorphic segregating markers was generated per primer and enzyme combination. Relatively, NBS profiling has been claimed to be able produce an abundance polymorphic markers. However, from the experiments beforehand, we noticed that within cultivars of *Hypericum* low polymorphism were observed. This can observed by looked at their NBS profiling as they shared plenty of common bands in the parents and minimal polymorphism can be attained. Furthermore, a large number of the segregating NBS markers are from hkxhk type which occurs with all primer and enzyme combinations used in this study. This may be an indication that the genetic background of *Hypericum* is very small rather than hypothesize that there are only few NBS-LLR genes present in *Hypericum* that also differentiate at a slow evolutionary pace. The capacity of NBS profiling to generate polymorphism within individuals with narrow genetic distances is still unclear as most species studied up to now are very heterogenous.

Linkage analysis

Segregation distortion is common in genetic mapping analysis which violated the law of segregation. This happened when observed genotype frequency was deviated from the expected genotype frequency (Lu et al., 2002). Segregation distortion may influencing the precision of genetic mapping as it can greatly affected genetic distance between markers and the order of markers on linkage groups (Lorieux et al., 1995). They also described that segregation distortion affected largely in estimation of recombination fraction between dominant markers such as NBS markers.

F1 progeny from population 3 showed large amount of segregation distortion estimated by Chi-square values. The percentage of segregation distortion showed an improvement after the markers data were rechecked. However, the percentage of segregation distortion was still considerably high in infraspecific population. In study of genetic map construction of three infraspecific (*Brassica olerace*) F2 populations and one interspecific population (*B.oleraceae x B. Insulari*) F2 population resulted only 7% showed significant segregation distortion whereas the interspecific results 59% (Kianian and Quiros, 1992). From this study, most of segregation distortions were caused by hkxhk marker type. This result were already reported by (Brummer et al., 1993; Pillen et al., 1993; Prince et al., 1993; Kesseli et al., 1994; Kalo´ et al., 2000; Song et al., 2005) in different crop species.

Error in marker genotyping can cause greatly in segregating distortion (Sibov et al., 2003). This is frequently occur in scoring process as many factors can affected the scoring process such as faint bands or contamination from other samples which can lead to misinterpretation of the absent and present of the band. Markers that caused the distortion should be traced back and improve the scoring if needed. Scoring made by inexperienced person also may differ a lot from the experience person. One can also excluded markers that obviously showed segregation distortion, however by doing this, marker coverage of the genome can be reduced and some qualitative or quantitative might be missed (Xian-Lang et al., 2006).

Apart from human and experimental error, the presence of lethal genes also leads to extremely segregating distortion. This phenomenon most prominent in double haploid (DH) population and recombinant inbred lines (RIL).The reason for segregating distortion

in DH was mainly because recessive lethal genes become homozygous and being expressed (Xian-Liang et al., 2006). Using comparative mapping can help to identify suspicious linkage and it is usually performed among different types of population from the same cross or among different populations of the same type.

Reliability scored bands

Few factors should be considered when scoring the bands in order to minimize misinterpretation of absence and presence of bands. Again, DNA quality plays the most important roles. Lower quality of DNA will results in mobility artefacts on gel pictures. The mobility artefact also will cause irregularities in the electric field which lead to another problem in gel called 'smilling effect'. The effect rise when lower electrophoretic mobility in the outer lane occur (Weising et al.,2005).

The utilization of restriction enzyme in NBS profiling also can contribute to the false impression if incompleteness restriction happened. In this study, four hours restriction and ligation were performed together in the PCR machine to maintain the optimal temperature. The restriction time used was sufficient in *Hypericum* study. This could be different if larger genome size were used.

As the detection of fluorescent labelled fragments by DNA analyzer machine is very sensitive, a spill of samples to another well could be lead misinterpret with poorly amplified fragments. However, sample spills from previous lane can be detected by careful inspection of the gel. To my experience, in order to guarantee the reliability of the scored bands, only clearly scorable bands should be included in the analysis. Apart from that, if the numbers of individuals in the population are higher than the number of samples that can be loaded, try to keep conditions as similar as possible to both subsets to make it easy to match the results of both subsets. Also, different intensity was observed in DNA profiling picture as different DNA analyzer machine could have different sensitivity and intensity.

Even though it is difficult to hundred percent sure with the reliability of scoring bands, one can still increase reliability by having a second person to do bands scoring and make a comparison. Furthermore, through standardization steps especially the in the separation DNA fragment as well as gel conditions can helps to reduce the experimental error thus scoring process will be easier and faster. If population size is too big and have different sets, we suggest using the same machine if possible. An inclusion of a molecular weight marker lane may aid to boost the reliability.

Conclusion

NBS profiling was able to generate NBS markers in *Hypericum* and DNA quality are the most important criteria in NBS profiling. In the whole process of development NBS markers, DNA quality can cause disruption in the scoring and the analysis of the markers. We also observed low polymorphism between cultivars, thus more degenerated NBS marker-enzyme combinations needed to be screened to obtain enough polymorphic markers. As mention earlier this report is a good starting point on marker development in *Hypericum*. This can facilitates research especially in marker assisted selection (MAS).

Interspecific crosses between wild species and cultivars are worthwhile to increase the genetic background of *Hypericum* varieties. One should consider exploring the possibilities to use bridge crosses in order to avoid apomixis problem as in the present study. This strategy has been employed in many species to hybridize two distant species that cannot be crossed directly or very difficult to cross (Wang et al., 2002).

For future recommendation construction of parental maps and integrated map using more informative markers such as co-dominant markers (SSR) can help to improve present linkage map. In addition, developing high throughput micro array based will be great interest. The capability of this method for scoring thousand of DNAs for a co-dominant marker on a glass slide is very useful in screening large numbers of populations for markers that linked to important trait such as resistance in nematode.

Appendices

Appendix 1: List of the samples used for each experiment

Setup NBS markers system

Sample
6H307M006
Esm H031
6H304M003-1
6H308M004
6H303M009
Green Condor
Red Wave
Red Baron
H. Buckleii
H.Calycinum Bariloche
H. Perforatum

Interspecific crosses

Crossing Number	Mother	Father
1	Pink Attraction	H Perforatum
2	Pink Attraction	H calycinum Bariloche
3	Pink Attraction	Hidcote
4	*Elite Amber*	H Perforatum
5	*Elite Amber*	H calycinum Bariloche
16	*H Perforatum*	Pink Attraction
17	*H Perforatum*	Elite Amber
23	H calycinum Bariloche	Red Wave
41	Elite Amber	*H Bukleii*

Intraspecific crosses

Population Number	Seedlings Number	Mother	Father
45	45-35	Pink Attraction	H016
45	45-102	Pink Attraction	H016
45	45-135	Pink Attraction	H016
45	45-160	Pink Attraction	H016
45	45-173	Pink Attraction	H016
45	45-207	Pink Attraction	H016
45	45-244	Pink Attraction	H016
45	45-246	Pink Attraction	H016
46	46-7	Elite Amber	H016
46	46-28	Elite Amber	H016
46	46-101	Elite Amber	H016
46	46-131	Elite Amber	H016
46	46-147	Elite Amber	H016
46	46-148	Elite Amber	H016
46	46-164	Elite Amber	H016
46	46-216	Elite Amber	H016
51	51-10	Red Wave	H021
51	51-17	Red Wave	H021
51	51-19	Red Wave	H021
51	51-33	Red Wave	H021
51	51-65	Red Wave	H021
51	51-102	Red Wave	H021
51	51-139	Red Wave	H021
51	51-190	Red Wave	H021
52	52-7	H016	H021
52	52-10	H016	H021
52	52-16	H016	H021
52	52-99	H016	H021
52	52-111	H016	H021
52	52-119	H016	H021
52	52-158	H016	H021
52	52-200	H016	H021
PA	Pink Attraction	Parental lines	
EA	Elite Amber	Parental lines	
RW	Red Wave	Parental lines	
H016	H016	Parental lines	
H021	H021	Parental lines	

Appendix 2: Fulton DNA isolation

Works well with tomato, poplar, cabbage

Starting material is lyophilized, ground leaf

All centrifugations are at maximum speed

PMB Rep. 13 (3) 1995; 207-209

⇒ Switch waterbath on at 60^0C
⇒ Prepare fresh microprep buffer 2.5 parts extraction buffer (EB), 2.5 parts lysis buffer and 1.0 part 5% Sarkosyl (w/v). Add 0.38 g sodium bisulfite/100ml buffer immediately before use.
⇒ add 750 ul microprep buffer to leaf powder, mix well
⇒ Add 1 ul RNAse (10 mg/ml)
⇒ Incubate in 60^0C waterbath for 30 - 60 min.
⇒ extract with 800 ul chloroform (mix well)
⇒ Spin 5 min.
⇒ pipet off 400 - 600 ul aqueous phase
⇒ Add equal volume cold isopropanol and invert tubes repeatedly until DNA precipitates.
⇒ Spin 5 min.
⇒ Wash with 500 ul 70% ethanol (pellet often very loose), spin 5 min. pour off.
⇒ dry pellet
⇒ resuspend DNA in TE^{-4}

Caution chloroform is carcinogenic: wear gloves. Waste chloroform in cat. 23 cans

		per litre:
Extraction buffer:	0.35 M Sorbitol, 0.1 M Tris-HCl, 5mM EDTA, pH 7.5	63.7 g 12.1 g 1.7 g
Lysis buffer:	0.2 M Tris 0.05 M EDTA 2 M NaCl 2% CTAB	

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