DEVELOPMENT OF SSR MARKERS IN POPPY
(*Papaver somniferum L.)*

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ABSTRACT

DEVELOPMENT OF SSR MARKERS IN POPPY
(*Papaver somniferum L.*)

The opium poppy (*Papaver somniferum L.*) belongs to the family *Papaveraceae*. Opium poppy is an important agronomic plant due to its production of more than 80 different alkaloids. In addition, poppy oil and seeds are used in food. The number of molecular markers for opium poppy is limited. To date, molecular characterization of opium poppy has been done using general marker systems such as AFLP, RAPD, EST-SSR and ISSR. However, development of other molecular markers systems is essential for more comprehensive analysis of the opium poppy genome. A genomic library was constructed from opium poppy DNA. Genomic DNA was sequenced by Roche 454 Sequencing™ platform. Genomics reads were clustered by MIRA software. Di-, tri-, tetra-, penta- and hexanucleotide SSR repeats were identified and flanking primers were designed by Batchprimer3 software. A total of 1399 contigs containing 3284 SSRs were identified. A total of 1820 primer pairs which fulfilled the criteria for primer design were designed from flanking sequence of SSRs. A total of 100 SSR primers were tested in six *Papaver somniferum* accessions. No polymorphism was found among the *Papaver somniferum* accessions. Transferability of the markers was tested in seven *Papaver* species. SpSSR-6, spSSR-8 and spSSR-23 detected polymorphic alleles in these species. These markers are the first set of opium poppy-specific SSR markers derived from genomic sequence of this crop. These markers can be used for assessment of genetic diversity, mapping and marker assisted selection in opium poppy.

ÖZET

HAŞHAŞ (*Papaver somniferum L.*)’TA SSR MARKÖRLERİNİN GELİŞTİRİLMESİ

Haşhaş içeriğinde bulunan 80’den fazla alkaloidden dolayı önemli bir sanayi bitkisidir. Buna ek olarak haşhaş yağı ve tohumu besin olarak kullanılır. Haşhaşta geliştirilmiş sınırlı sayıda markör vardır. Haşhaşta genetik çeşitlilik çalışmaları AFLP, RAPD, EST-SSR and ISSR markörleriyle yapılmıştır. Bundan dolayı haşhaşta kapsamlı genom analizleri için markörler geliştirmek gereklidir. Haşhaş genomik DNA fragmentleri ikinci nesil Roche 454 FLX dizileme platformunda dizilenmiştir. Genomik diziler MIRA montajlama programı kullanılarak daha büyük diziler (contigs) haline getirilmiştir. Montaj edilen (biraraya getirilen) diziler kullanılarak SSR primerleri dizayn edilmiştir. Genomik diziler BatchPrimer3 programı kullanılarak SSR’lar bakımından taranmıştır. Bu program herbir diziden SSR primerlerinin dizaynını da sağlamıştır**.** Contiglerden 3284 SSR tekrarı belirlenmiştir. Bu tekrarlar arasında dinükleotid, trinükleotid, tetranükleotid, pentanükleotid ve heksanükleotid gibi değişik motifler yer almıştır. Toplam 100 SSR markör altı Türk haşhaş hattında test edilmiştir. Bu haşhaş hatlarında polimorfizm gözlenmemiştir. Ayrıca 100 SSR markör yedi farklı *Papaver* türünde test edilmiş ve üç polimorfik markör (spSSR-6, spSSR-8 and spSSR-23) bulunmuştur. Bu çalışmada geliştirilen markörler haşhaş genomik DNA’sından geliştirilen ilk markörlerdir ve haşhaşta genetik çeşitlilik ve haritalama çalışmalarında kullanılabilir.

TABLE OF CONTENTS

LIST OF FIGURES……….………………………..viii

LIST OF TABLES...ix

CHAPTER 1. INTRODUCTION……………………………………….......................... 1

1.1. Opium Poppy Cultivation.. 1

1.2. Biology of Opium Poppy.. .1

1.3. Diversity Analysis...…3

1.4. SSR (Simple Sequence Repeats or Microsatellites) ……….......….....4

1.5. Source of SSR markers...…….…..... 4

1.6. In silico processing of EST and Genomic Sequences………………......5

1.7. SSR Primer Design………….. 5

1.8 . Functional Annotation of EST and Genomic Sequences……….........6

1.9. Goals……………………………………………………….... ……..7

CHAPTER 2. MATERIALS AND METHODS……………………………….…....... 8

2.1. Material..8

2.1.1. Plant Materials……………………………………………….…..... 8

2.2. Methods.. 9

2.2.1. DNA Extraction.. 9

2.2.2. Genomic SSR Marker Design..…………………………….............9

2.2.3. SSR Analysis…………………………………………………….…... 10

CHAPTER 3. RESULTS AND DISCUSSION……………………………….…......11

3.1. Shotgun Assembly of Genomic Reads of *Papaver somniferum*...........11

3.2. Development and Distribution of SSR Markers Derived from Genomic Sequence of *Papaver somniferum*…………………............12

3.2.1. Mining of Genomic Sequence-Derived SSRs in *Papaver somniferum*……………………………………………. 12

3.2.2. Distribution of Repeat Motif Types………………………….....….15

3.2.3. SSR Primer Design ……………………....................................... 16

3.3. Amplification of Genomic SSR Markers…………………………….....17

3.4. Transferability of Genomic SSRs……………………………........ 22

CHAPTER 4. CONCLUSION…………………………………………………….........28

REFERENCES 29

APPENDIX A. SSR PRIMERS …………………………………………………..........33

LIST OF FIGURES

Figure **Page**

Figure 1.1. Opium poppy production in the word..2

Figure 1.2. World morphine production...2

Figure 2.1. SSR marker development flowchart……………………………………... 10

Figure 3.1. Distribution of SSR motif types……………………………………...... ….13

Figure 3.2. Number of repeat units of di-nucleotide……………………………....... 13

Figure 3.3. Number of repeat units of tri-nucleotide……………………………....... 14

Figure 3.4. Number of repeat units of tetra-nucleotide…………………………........14

Figure 3.5. Number of repeat units of penta-nucleotide……………………….......…14

Figure 3.6. Number of repeat units of hexa-nucleotide……………………………...... 15

Figure 3.7. Distribution of dinucleotide motif types……………………………......... ..16

Figure 3.8. Distribution of SSR primers……………………….………………........ …17

Figure 3.9. Survey of spSSR-25, spssr-26, spSSR-27,
spSSR-28 and spSSR-29 in six *Papaver somniferum* accessions…...........…18

Figure 3.10. Number of SSR markers transferred to *Papaver* species……………........23

Figure 3.11. SpSSR-23 marker polymorphism…………………………………........24

Figure 3.12. SpSSR-6 marker polymorphism…………………………………….......24

Figure 3.13. SpSSR-8 marker polymorphism……………………………….........…24

LIST OF TABLES

Table **Page**

Table 2.1. Opium poppy accessions, sources and locations……………………….....….8

Table 3.1. Genomic sequence reads statistics……………………………………….....11

Table 3.2. Shotgun assembly statistics of genomic sequence reads…………….. .....…11

Table 3.3. Average motif length of SSR motifs in nucleotides…………………... 13

Table 3.4. Distribution of motif types……………………………………….........…16

Table 3.5. Tested spSSR markers in *Papaver somniferum* accessions……………........19

Table 3.6. Transferability of spSSR markers in different *Papaver* species……….....25

CHAPTER 1

INTRODUCTION

1.1. Opium Poppy Cultivation

The opium poppy (2n = 22, *Papaver somniferum L.*) belongs to the family *Papaveraceae* (Acharya et al. 2009). *Argemonidium, Carinata, Glauca, Horrida, Meconella, Miltantha*, *Oxytona, Papaver, Pilosa,* and *Rhoeadium* are the sections of the *Papaver* genus. All the sections except for *Horrida* and *Scapiflorae* exist in Turkey with 35 species found in this country (Sariyar et al. 2002).

World production, trade, export and import of narcotic drugs are controlled by the Single Convention on Narcotic Drugs which was signed in 1961. Turkey, India, Australia, France, Spain, and Hungary are considered the main opium poppy producers by the United Nations (UN). Among these countries, Turkey and Hungary are traditionally opium poppy producers. Opium poppy is also illegally cultivated in Afghanistan, Pakistan, Myanmar, Colombia, and Mexico (Reuter et al. 2001). Because of its long history of cultivation, poppy is now distributed worldwide. However, the origin of opium poppy is southern Europe (Dittbrenner et al. 2008) and poppy was first cultivated in the Neolithic (B.C 8000-5500). Although Turkey currently ranks first (48%) in world production area of opium poppy (Figure 1.1), the country ranks second in world morphine production with 150 tons (18% of the world total) (Figure 1.2) (Soil Product Office, 2009). This is because the morphine content of opium poppy in Turkey is very low. Growth of low production cultivars is due to lack of characterization of poppy germplasm and inefficient poppy breeding methods because of limited molecular research on opium poppy (Gümüşçü et al. 2008).

1.2. Biology of Opium Poppy

Poppies are self-pollinating angiosperms; however, outcrossing (10 – 37 %) can occur in the presence of insects (Patra et al. 1992). Opium poppy is an important

agronomic plant due to its production of more than 80 different alkaloids. In addition, poppy oil and seeds are used in food (Schulz et al. 2004). There are two groups of alkaloids in opium poppy; benzylisoquinoline alkaloids (BIAs) and monoterpenoid indole alkaloids (MIAs). Approximately 2500 natural product structures are found in the benzylisoquinoline alkaloid (BIA) group. The analgesic and narcotic drug morphine, the cough suppressant codeine, the muscle relaxant papaverine, the anti-microbial agents sanguinarine and berberine are benzylisoquinoline alkaloids (BIA) and are extracted from *Catharanthus roseus* and *P. somniferum* (Facchini et al. 2004). MIAs have approximately 3000 structures. Due to their strong biological activity they are used as drugs such as reserpine, vinblastine, vincristine and yohimbine (Ziegler et al. 2009).

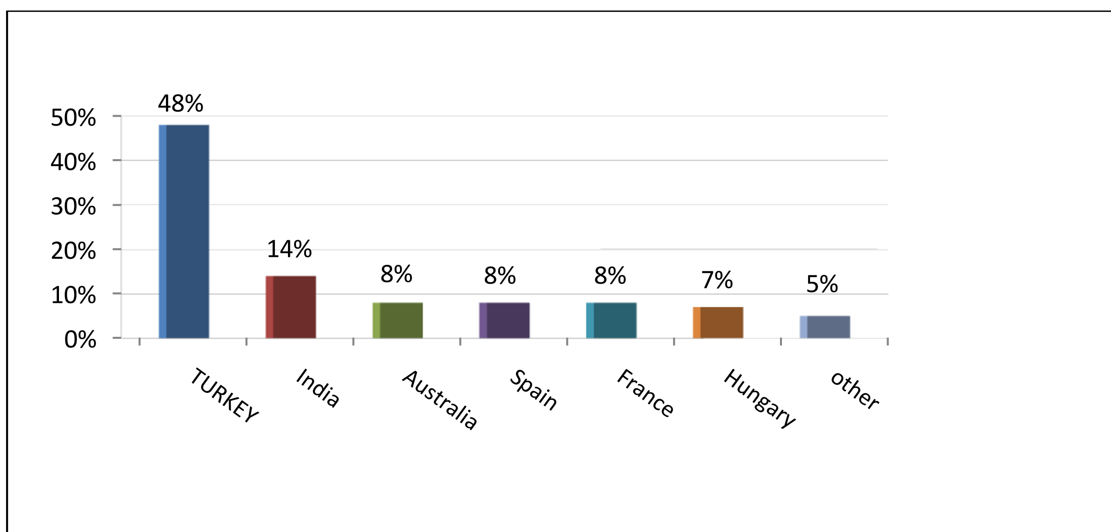

Figure 1.1. Opium poppy production in the world.
(Source: Opium poppy report, Soil Product Office, 2009)

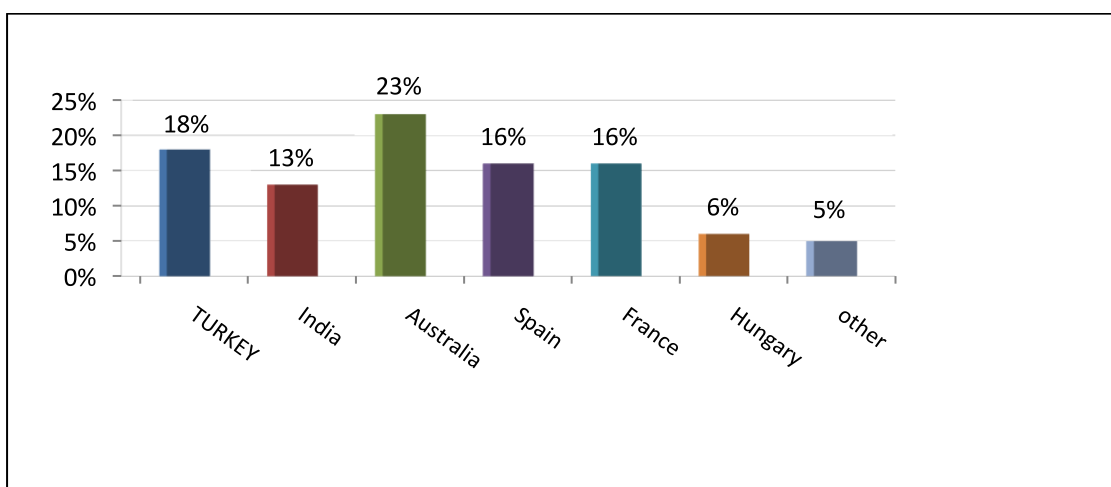

Figure 1.2. World morphine production.
(Source: Opium poppy report, Soil Product Office, 2009)

1.3. Diversity Analysis

In the literature there are very few examples of research on *P. somniferum* diversity. James A. Saunders identified and characterized 40 accessions of opium poppy and two control genera (*P. bracteatum Lindley* and *P. setigerum DC.*) from a commercial breeding collection held in Tasmania, Australia using AFLP (amplified restriction fragment length polymorphism) markers (Saunders et al. 2001). Saunders proved that AFLP is an efficient and reliable marker system to identify opium poppy varieties. In other research the morphological, molecular and chemical characteristics of 53 line selections from three different species of the *Oxytona* section were analyzed (Parmaksız 2004). In this research, 20 morphological and chemical characters were analyzed and 15 different RAPD primers were used for molecular characterization. In total, 84.3% of the bands generated by RAPD primers were polymorphic. A dendrogram of the 53 plants was constructed. In the dendrogram there were two clusters. One of these clusters had 12 *P. bracteatum* varieties and one east Anatolian variety. The other cluster had one variety of *P. bracteatum*, six varieties of *P. orientale* and eight varieties of *P. pseudo-orientale* species. Other molecular characterization of opium poppy was done by Hari Shanker Acharya (Acharya et al. 2009). In this research, a dendrogram of 24 germplasm accessions cultivated in the USA was constructed based on RAPD and ISSR markers. Based on RAPD marker analysis, the germplasms clustered in one major group A and one minor group B. The ISSR marker-based dendrogram had the same clusters as the RAPD-based dendrogram. Combined RAPD and ISSR analysis resulted in a dendrogram which had one major group A and three minor groups. The most comprehensive analysis of genetic diversity of *P. somniferum* was done by A. Dittbrenner (Dittbrenner et al. 2008). In this work, 300 accessions of the German gene bank collected from all over the world were characterized by AFLP markers. Results showed that all tetraploid *P. somniferum* subsp. *setigerum* accessions formed one cluster while the diploid accessions from subsp. *somniferum* and subsp. *songaricum* were intermixed. Also major alkaloids such as morphine, codeine, thebaine, papaverine, and noscapine were analyzed by HPLC. The amount of these alkaloids was highly variable.

1.4. SSR (Simple Sequence Repeats or Microsatellites)

An SSR is an iteration of one to six-nucleotide motifs. They show a high level of polymorphism due to replication slippage and unequal crossover. SSR loci can be neutral or can affect processes such as chromatin organization, regulation of gene activity, recombination, DNA replication, cell cycle, and mismatch repair system (Li et al. 2002).

SSR markers are valuable marker system which are commonly used in plant genomic research because of their multi allelic nature, high polymorphism content, locus specificity, reproducibility, and inter-lab transferability. SSRs are hot spots for recombination and as a result, polymorphism is high. Although SSR primers usually amplify only one locus, they are multi-allelic. Application of SSR markers is easy. A small amount of DNA is enough for PCR reactions and SSR markers can be easily automated. Also the cost of assaying SSR markers is less than CAPS and AFLP because there is no enzyme digestion. SSR primers are species-specific so although SSR primers can give a PCR product in plants from the same genus, SSR primers may not give PCR product in plants from a different genus. To design SSR markers, sequence information is needed. After SSR motifs are identified in the sequence, flanking PCR primers (forward and reverse) are used to amplify the SSR locus (Jones et al. 2009).

The number of molecular markers for genomic analysis of opium poppy is limited. As previously mentioned, molecular characterization of opium poppy has been restricted to general marker systems such as EST-SSR, AFLP, RAPD and ISSR. Moreover, there is only one genetic linkage map which was constructed with just 77 AFLP and 48 RAPD markers in an F2 mapping population (Straka et al. 2002). Therefore, developing other molecular marker systems is essential for more comprehensive analyses of the opium poppy genome.

1.5. Source of SSR markers

SSR markers can be designed from EST sequences or DNA sequences. In the literature, SSR markers designed from ESTs are generally considered less polymorphic than SSR markers designed from genomic sequences (Varshney et al. 2005). Jifeng Tang et al. designed SSR primers from EST sequences of potato, tomato, rice,

Arabidopsis, *Brassica* and chicken and showed that SSR markers from the 5' UTR of mRNA are more polymorphic (Tang et al. 2008). Although long SSRs are often considered more polymorphic than short SSRs (long SSRs have at least ten repeats of a repeat motif for di-nucleotide SSR, six repeats for tri-nucleotide SSR, and five repeats for a tetra, penta or hexanucleotide SSR, Tang et al. showed that short SSRs are more polymorphic.

1.6. In Silico Processing of EST and Genomic Sequences

Assembly of EST sequences and genomic reads is similar but there are some differences. EST sequences of *P. somniferum* are available in the NCBI EST database but there is no publicly available genomic sequence of *P. somniferum*. The first step in processing sequences is vector and adaptor trimming. Software such as vecscreen and vmatch (Sczyrba et al. 2005) are used to Blast sequences against vector databases. If there are significant strong matches between your sequence and vector sequences (Expect one random match in 1,000,000 queries of length 350 kb, terminal match with Score ≥ 24, internal match with Score ≥ 30), the matched sequences are deleted from reads. Vector trimming prevents misassembling of reads (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen_docs.html). Due to alternative splicing and high copy number of genes such as housekeeping genes, one gene can have more than one EST sequence. These EST sequences must be clustered. In genomic assembly, reads are aligned and merged to form longer sequences which are called contigs. Other sequences which cannot be merged are called singlets. In EST sequences contig and singlet sequences are called unigenes. Contig and singlet sequences of EST and genomic sequences can be used to search for SSR motifs and to design primers (Huang et al. 1999).

1.7. SSR Primer Design

For SSR primer design, first SSR motifs such as di-, tri-, tetra-, penta- and hexa-nucleotide are determined. Because longer SSRs are more useful, more than six repeats of dinucleotides, more than four repeats of tri-nucleotides and more than three repeats of tetra, penta and hexa nucleotides are identified in the sequences (You et al. 2008).

Some of the criteria that must be considered for design of reliable forward and reverse primers to amplify SSR loci are melting temperature (Tm) of forward and reverse primers, size of PCR product, GC content and size of primers. If the Tms of forward and reverse primers are very different, the SSR motif cannot be amplified. PCR products which are between 100 and 300 nt can be easily produced by SSR primers. The GC content of PCR primers should be at least 50%. Primer size must be more than 18 nt for specific attachment of primers (Tang et al. 2008). Primers whose size is less than 18 nt can make nonspecific attachment. Also primer sequences which cause secondary structure formation can prevent the binding of the primer to genomic DNA. There are many open source software programs available to design primers such as FastPCR, Primer3 and Batchprimer3 (You et al. 2008).

Locus specificity of SSR primers is important because if SSR primers amplify more than one locus, PCR products of different loci are considered as alleles. This causes false positive errors. One reason for multiple locus priming may be paralogous genes in the genome. To eliminate this problem, PCR primers which give more than four PCR products are excluded and these primers are not used for diversity analysis (Tang et al. 2008).

1.8. Functional Annotation of EST and Genomic Sequences

EST and genomic sequences are annotated to assign SSR markers to functional categories. Plant EST or genomic sequences can be blasted against *Arabidopsis thaliana* protein or genome databases. For functional annotation, protein databases make more significant alignments than genome databases because proteins are more conserved than DNA sequences. Thus, conserved domains of proteins can be determined. For this reason, BLASTX translates DNA sequences to amino acid sequences and blasts amino acid sequences against protein databases. If there is significant similarity between the query sequence and a subject sequence of known function, the function of the query sequence is considered to be the same as the subject sequence (Gupta et al. 2010).

1.9. Goals

The aim of this study is analyzing SSR motifs in the *Papaver* genome and development of SSR markers derived from *Papaver* genomic sequences. These SSR markers are a valuable marker system that can be used to construct a genetic linkage map and to perform quantitative trait loci (QTLs) mapping of agronomic traits of *P. somniferum* in the future. Another goal of the research was to show transferability of genomic SSR markers to *P. bracteatum, P. umbonatum, P. arenarium, P. armeniacum, P. orientale, P. rhoeas* and *P. nudicaule* species. These results indicate that these markers can be used to detect polymorphism in accessions of different *Papaver* species.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Material

Six opium poppy accessions were obtained from Anatolian Agricultural Research Institute, Eskişehir. In addition, two accessions of *P. bracteatum, P. orientale, P. rhoeas* and one accession of *P. nudicaule, P. arenarium, P. armeniacum* were obtained from USDA-ARS Plant Germplasm Inspection Station, Beltsville, Maryland, USA. All accessions which were used in this study are shown in Table 2.1.

For genomic DNA isolation, each accession was planted in seedling plates. Plants were grown in greenhouse (24-25 °C approximately 33% humidity).

Table 2.1. Opium poppy accessions, sources and locations.

Accession name	Source	Location
P .somniferum L. cv. kemerkaya	AARI	Turkey
P. somniferum L. cv. 1259-1	AARI	Turkey
P. somniferum L. cv. 1065	AARI	Turkey
P. somniferum L. cv. 1061-7	AARI	Turkey
P. somniferum L. cv. aday1	AARI	Turkey
P. somniferum L. cv. 1290	AARI	Turkey
P. bracteatum L.	USDA	Turkey
P. bracteatum L.	USDA	Turkey
P. umbonatum L.	USDA	Turkey
P. umbonatum L.	USDA	Turkey
P. arenarium L.	USDA	Armenia
P. armeniacum L.	USDA	Armenia
P. nudicaule L.	USDA	Mongolia
P. rhoeas L.	USDA	Bulgaria
P. orientale L.	USDA	Iran
P. rhoeas L.	USDA	Bulgaria
P. orientale L.	USDA	Iran

2.2. Methods

2.2.1. DNA Extraction

For generation of opium poppy sequences, genomic DNA of Kemerkaya accession was isolated by Promega Wizard magnetic 96 DNA kit according to manufacturer's instructions using Beckman Coulter Biomek NX^p workstation. Quantification of DNAs was performed with a Nanodrop ND-1000 spectrophotometer. A genomic library was constructed from opium poppy genomic DNA. After emulsion-based clonal amplification of the DNA library fragments, they were sequenced by Roche FLX 454 Sequencing™ platform.

For SSR marker analysis, DNA extraction was performed from fresh leaf tissues of opium poppy accessions by Promega CTAB genomic DNA isolation kit according to manufacturer's instructions. Quantification of DNAs was performed with a Nanodrop ND-1000 spectrophotometer and DNA samples were stored at -20 °C in TE buffer.

2.2.2. Genomic SSR Marker Design

The sequence data that was generated from the 454 FLX Sequencing™ platform was used to design SSR primers. Sff. format which is the default file format of 454 FLX Sequencing™ platform was converted to fasta format to assemble the sequences with MIRA software (Chevreux et al. 1999). After several calculations of minimum value of unassembled reads and maximum number of contigs, the best result was used for SSR screening. Di-, tri-, tetra-, penta- and hexanucleotide SSR repeats in contig sequences were screened and flanking primers were designed by Batchprimer3 software using the following criteria: expected amplified fragment size between 100 and 300 nt, primer size between 18 and 25 bases, primer GC% content between 40 and 60 and primer melting temperature (Tm) between 55 and 60°C. Batchprimer3 was installed to our local server to run large files. Singlet sequences were not analyzed for SSRs (You et al. 2008). The primers which we designed have many properties such as the contigs that had been used while designing each primer, SSR motif, product size, etc. A script was developed to display these properties of the primers when needed. Also forward and

reverse primer sequences were blasted against contig sequences to check the uniqueness of the SSR primers.

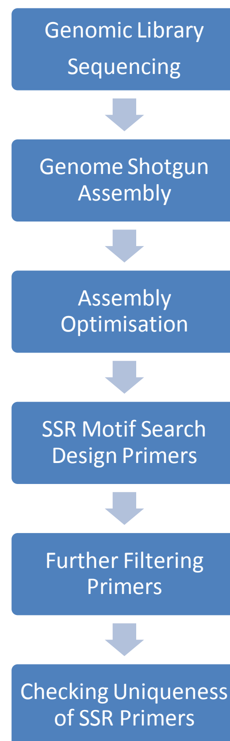

Figure 2.1. SSR marker development flowchart

2.2.3. SSR Analysis

A total of 100 SSR primer pairs were tested in Kemerkaya, 1259-1, 1065, 1061-7, aday1 and 1290 opium poppy accessions. Also 100 SSR primer pairs were tested in two accessions of *P. bracteatum, P. orientale,* and *P. rhoeas* and one accession of *P. nudicaule, P. arenarium,* and *P. armeniacum* to check transferability of SSR primers. DNA amplification was carried out in a 25 µL reaction mixture containing 1X PCR buffer, 3 mM MgCl, 0.125 nM deoxyribo-nucleotide triphosphates (dNTPs), 1 U Taq Polymerase, 2 pmol forward and reverse primers and 80 ng template DNA. After one step of 5 min at 94°C, 35 cycles were performed with 45 sec at 94°C, 1 min at 55 °C as annealing temperature, 1 min at 72°C and a final extension step of 5 min at 72°C. PCR products were separated on 3% agarose gels in 1X TAE buffer. They were visualized under UV light.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Shotgun Assembly of Genomic Reads of *Papaver somniferum*

A total of 1,244,412 reads, which were 52 to 1,201 bases long, were sequenced by the Roche 454 FLX Sequencing™ platform (Table 3.1). Median read length was 549 bases. Read sequences totaled 695,339,138 bases. A total of 599,603 reads (48.18%) were assembled into 210,720 contigs which were 40 to 10,640 bases long (Table 3.2). Median contig length was 342 bases with the assembled sequences totaling 82,412,704 bases. A total of 644,809 reads could not be assembled. Only contigs were used for SSR primer design.

Table 3.1. Genomic sequence reads statistics.

Reads available for assembly	1,244,412
Total cumulative read length (nt)	695,339,138
Median length of reads (nt)	549
Unassembled reads	644,809

Table 3.2. Shotgun assembly statistics of genomic sequence reads.

Reads used for the assembly	599,603
Cumulative contig length (nt)	82,412,704
Number of contigs	210,720
Min contig length (nt)	40
Median length of contigs (nt)	342
Max contig length (nt)	10,604

3.2. Development and Distribution of SSR Markers Derived from Genomic Sequence of *Papaver somniferum*

3.2.1. Mining of Genomic Sequence-Derived SSRs in *Papaver somniferum*

A total of 1399 contigs containing 3284 SSRs were identified. The SSRs were compromised of 212 (6.5%) di-, 1983 (60.4%) tri-, 758 (23.1%) tetra-, 198 (6%) penta- and 133 (4%) hexanucleotide repeats (Figure 3.1). Trinucleotide repeat was the most common repeat motif and there was an inverse relationship between motif length and frequency except for dinucleotide repeats. Although this result has also been observed in studies of genic SSRs in *Papaver somniferum* and other species such as eggplant, barley, maize, oats, rice, rye and wheat (Tümbilen et al. 2011; Lee et al. 2011; Varshney et al. 2002), the dinucleotide motif was most common in genomic SSRs in *Brassica oleracea*, *Jatropha curcas*, robusta coffee, papaya, rubber tree, and pigeonpea (Iniguez-Luy et al. 2008; Wen et al. 2010; Hendre et al. 2008; Eustice et al. 2007; Yu et al. 2011; Bohra et al. 2011). This unexpected result might be explained if our genomic sequences contained more coding sequences than noncoding sequences. The prevalence of trinucleotide SSR motifs in coding genes is expected because they do not lead to frameshift mutations in the genome and, therefore, may not be subject to negative selection (Li et al. 2002). This unexpected result might also be explained by our research criteria, which ignored dinucleotide repeats less than six repeats, because such short dinucleotide SSRs would not be useful to detect polymorphism. Average motif length was 13.85 nucleotides for di-, 13.30 for tri-, 12.36 for tetra-, and 15.56 for hexanucleotide repeats (Table 3.3) Also there was an inverse relationship between repeat type and number of repeat units in all SSR motif types. Six repeats in di-, four repeats in tri- and three repeats in tetra-, penta- and hexanucleotides were most common (Figure 3.2, Figure 3.3, Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7).

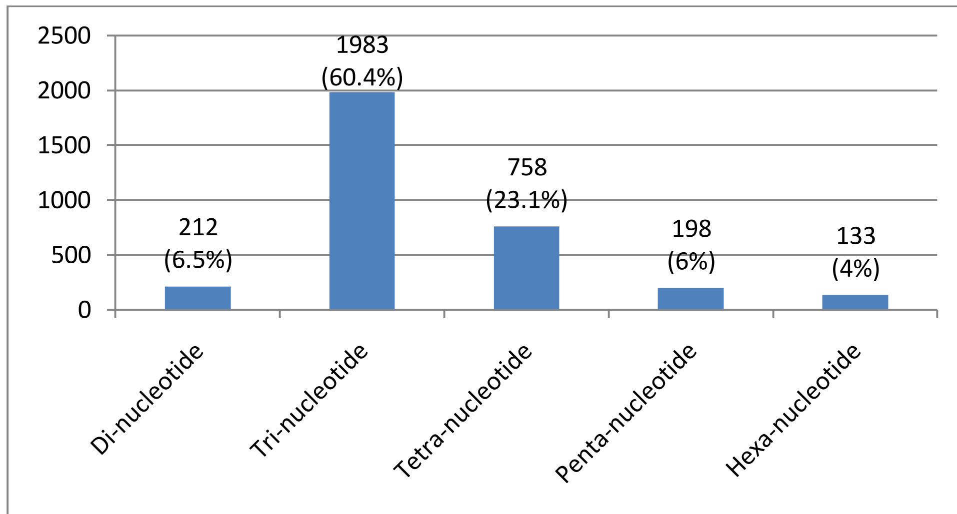

Figure 3.1. Distribution of SSR motif types.

Table 3.3. Average motif length of SSR motifs in nucleotides.

SSR motifs	Average motif length (nt)
Di-nucleotide	13.85
Tri-nucleotide	13.30
Tetra-nucleotide	12.36
Penta-nucleotide	15.56
Hexa-nucleotide	18.99

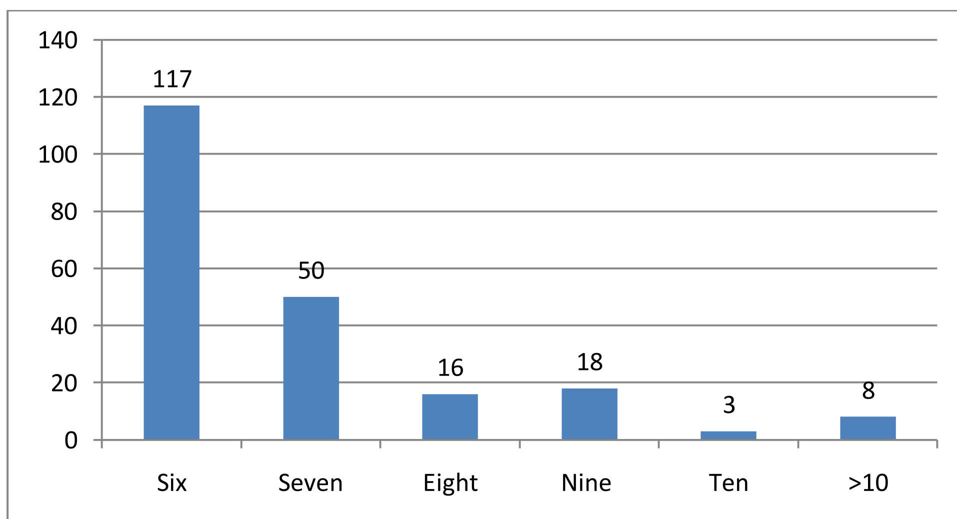

Figure 3.2. Number of repeat units of di-nucleotide.

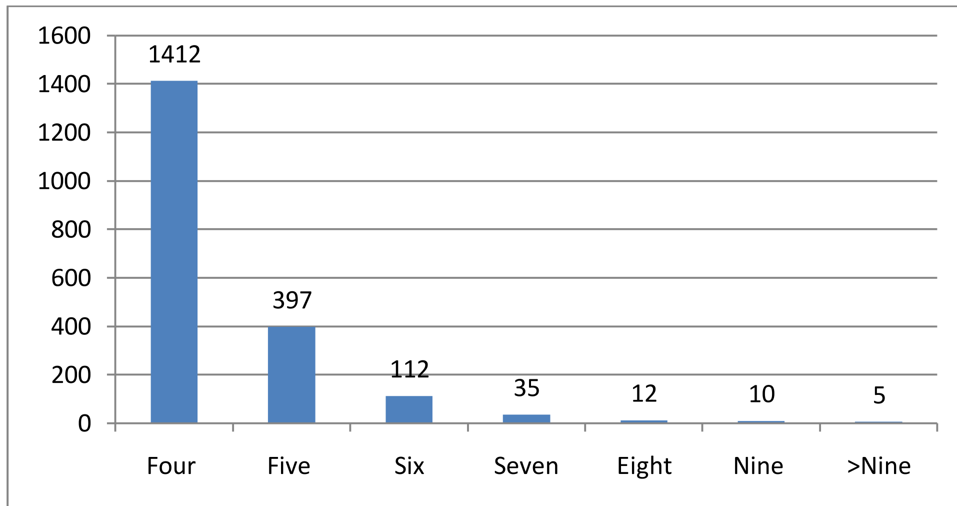

Figure 3.3. Number of repeat units of tri-nucleotide.

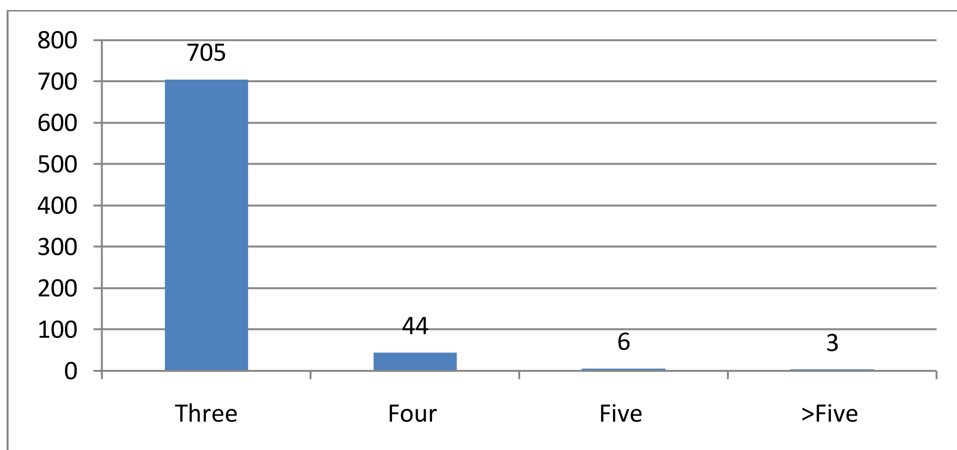

Figure 3.4. Number of repeat units of tetra-nucleotide.

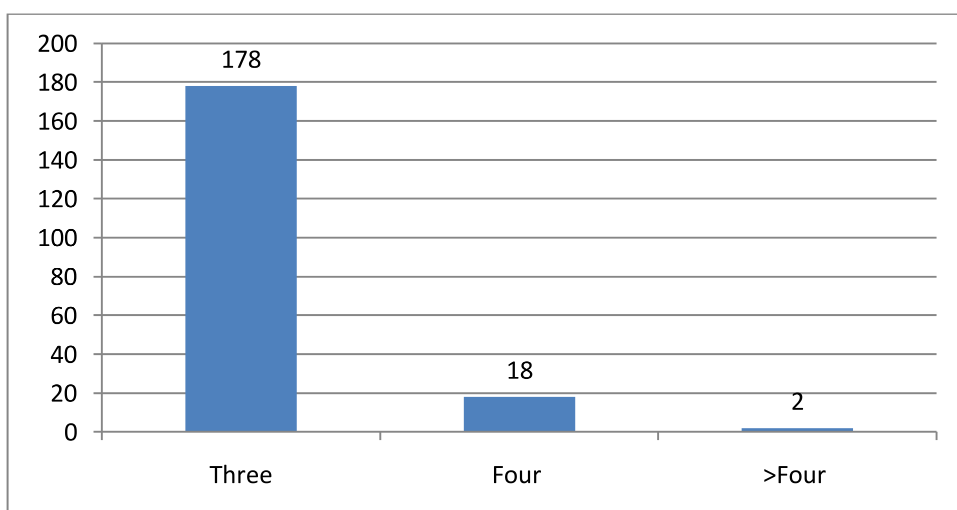

Figure 3.5. Number of repeat units of penta-nucleotide.

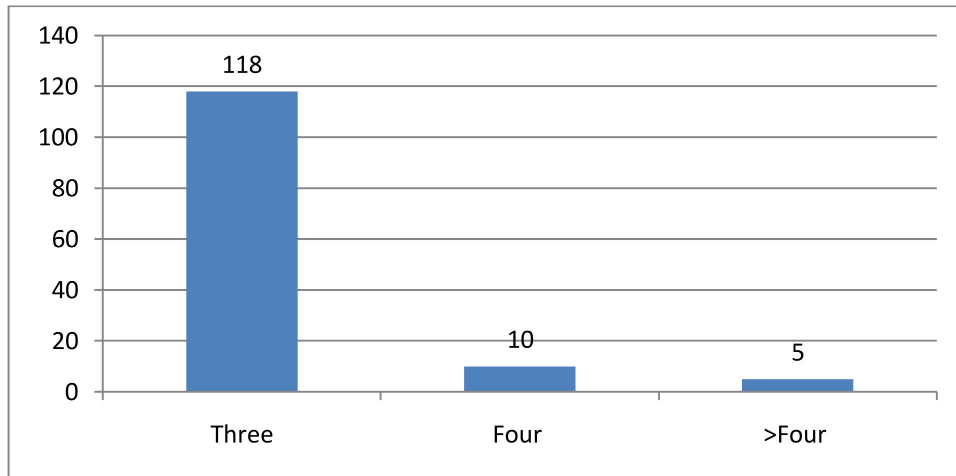

Figure 3.6. Number of repeat units of hexa-nucleotide.

3.2.2. Distribution of Repeat Motif Types

A total of 400 repeat motif types were identified in 3284 SSRs (Table 3.4). The most abundant repeat motifs were: $(AT/TA)_n$ (27.8%) and $(TA/AT)_n$ (21.7%) in dinucleotides (Figure 3.7); $(CTG/GAC)_n$ (9%) in trinucleotides; $(ATTT/TAAA)_n$ (4.6%), $(GGCC/CCGG)_n$ (3.8%) and $(AAAT/TTTA)_n$ (3.4%) in tetranucleotides; $(TTATT/AATAA)_n$ (6.5%) and $(AAAAT/TTTTA)_n$ (5%) in pentanucleotides; and $(AAAAAT/TTTTTA)_n$, $(ATTTTT/TAAAAA)_n$ and $(GGGTGC/CCCACG)_n$ (3% each) in hexanucleotides. Prevalence of the $(AT/TA)_n$ repeat was expected because this repeat has also been frequently found in genic SSRs in *Papaver somniferum* and genomic SSRs in other species such as coffee, papaya, and *Brassica napus* (Lee et al. 2011; Hendre et al. 2008; Eustice et al. 2007; Cheng et al. 2009). The prevalence of the $(CTG/GAC)_n$ motif was an unexpected result because the $(GAA/CTT)_n$ motif was most common in genic SSRs in *Papaver somniferum* (Lee et al. 2011). This result can be explained by different distribution of SSR loci in genomic DNA and ESTs. The $(ATT/TAA)_n$ and $(AAG/TTC)_n$ motifs were most common in papaya and *Brassica napus* genomic SSRs, respectively (Eustice et al. 2007; Cheng et al. 2009).

Table 3.4. Distribution of motif types.

	Number of motif types	Frequency
Di-nucleotide	11	2.75%
Tri-nucleotide	52	13%
Tetra-nucleotide	139	34.75%
Penta-nucleotide	100	25%
Hexa-nucleotide	98	24.50%

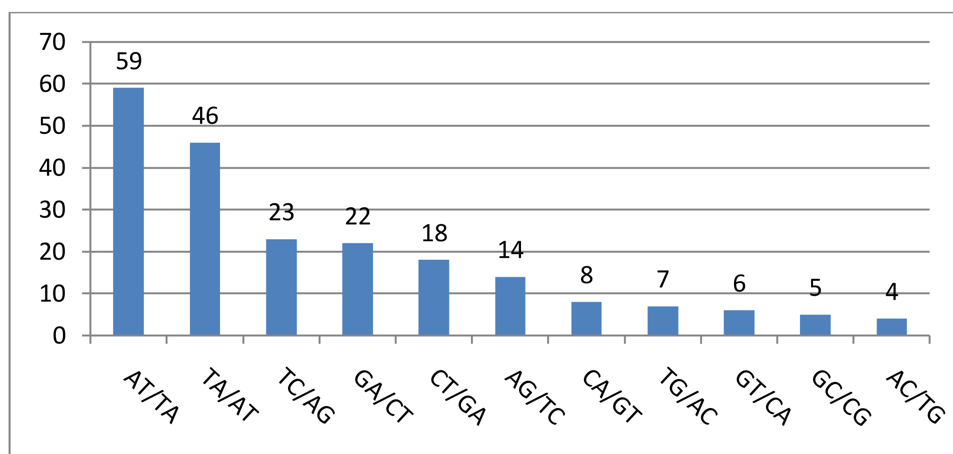

Figure 3.7. Distribution of dinucleotide motif types.

3.2.3. SSR Primer Design

A total of 1820 primer pairs (for 54.4% of total SSR motifs) which fulfilled the criteria for primer design were designed from flanking sequence of SSRs. Primer pairs flanking tri- (41.2%) and tetra- (39.4%) repeats were the most abundant (Figure 3.8). It was not possible to design SSR primers for all SSR motifs because some of the SSRs were at the termini of contig sequences. Primers were blasted to contig sequences. For each pair of primers, forward and reverse sequences did not match to any other contig sequences with 100% identity indicating that they should be single copy. Therefore, all SSR markers were expected to be unique.

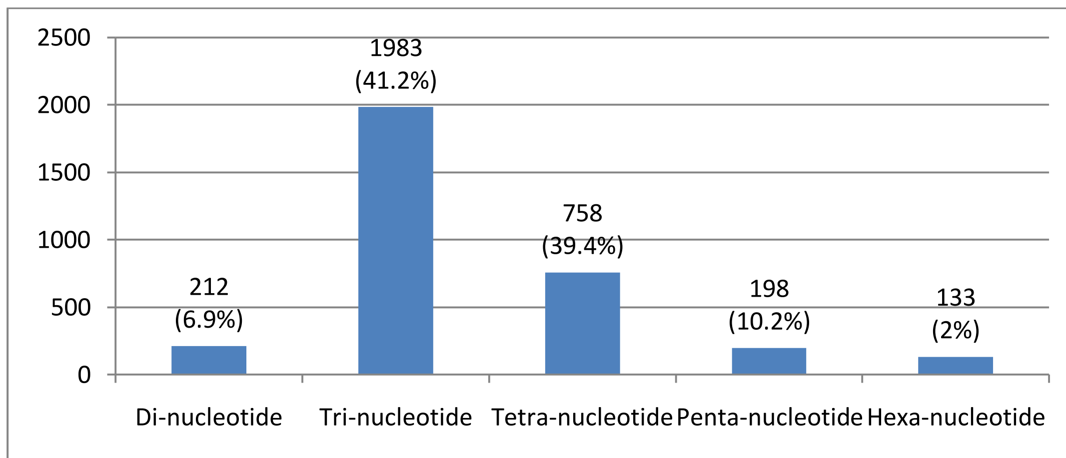

Figure 3.8. Distribution of SSR primers.

3.3. Amplification of Genomic SSR Markers

A total of 100 opium poppy-specific SSR markers was tested in Kemerkaya, 1259-1, 1065, 1061-7, aday1 and 1290 opium poppy accessions. Of these primers, 96 (96%) had amplified products in the six *P. somniferum* accessions (Figure 3.9, Table 3.5). For 89 primer pairs, the size of the PCR product was expected, however, seven primer pairs (sp-SSR-5, 24, 41, 52, 53, 69, 87) gave amplified fragments which were longer than expected. Single PCR products were generated by 85 primer pairs. Two primer pairs had three amplified fragments and eight primer pairs had two amplified fragments. Multiple PCR products can be due to paralogous genes in the *Papaver* genome or may be alleles in heterozygous germplasm. A high rate of successful amplification (96%) was expected because such high rates of positive PCR amplification were achieved with 524 (89%) of 587 SSRs in *Brassica oleracea*, 830 (88.4%) of the 938 SSRs in papaya, and 591 (94.3%) of 627 SSRs in *Brassica napus* (Iniguez-Luy et al. 2008; Eustice et al. 2007; Cheng et al. 2009). No polymorphism was found among the six *P. somniferum* accessions. This may be due to the limitation of the separation methods used. Agarose gel electrophoresis is less effective than capillary electrophoresis to detect SSR marker polymorphism (Vemireddy et al. 2007) so future experiments should use capillary electrophoresis to detect polymorphism. In another study six EST-SSR markers were tested in 135 *P. somniferum* accessions and this study showed the low genetic differentiation power of the EST-SSR markers (Lee et al. 2011). Although these initial findings suggest that SSR markers were less efficient than AFLP, RAPD and ISSR for assessment of genetic diversity in opium poppy (Saunders et al.

2001; Acharya et al. 2009; Parmaksız et al. 2011), SSR markers are potentially more applicable and useful for opium poppy genetics and breeding than AFLP. AFLP markers are dominant, costly and not suitable for quick screening. Moreover, AFLPs need to be converted into sequence-specific markers for marker-assisted selection in breeding programs (Lisbona et al. 2008). On the other hand, SSR markers are commonly used because of their multi allelic nature and locus specificity. In addition, codominant SSR markers are more informative than dominant markers. Because of these advantages, opium poppy specific SSR markers can be used for genomic mapping and marker assisted selection in opium poppy breeding programs.

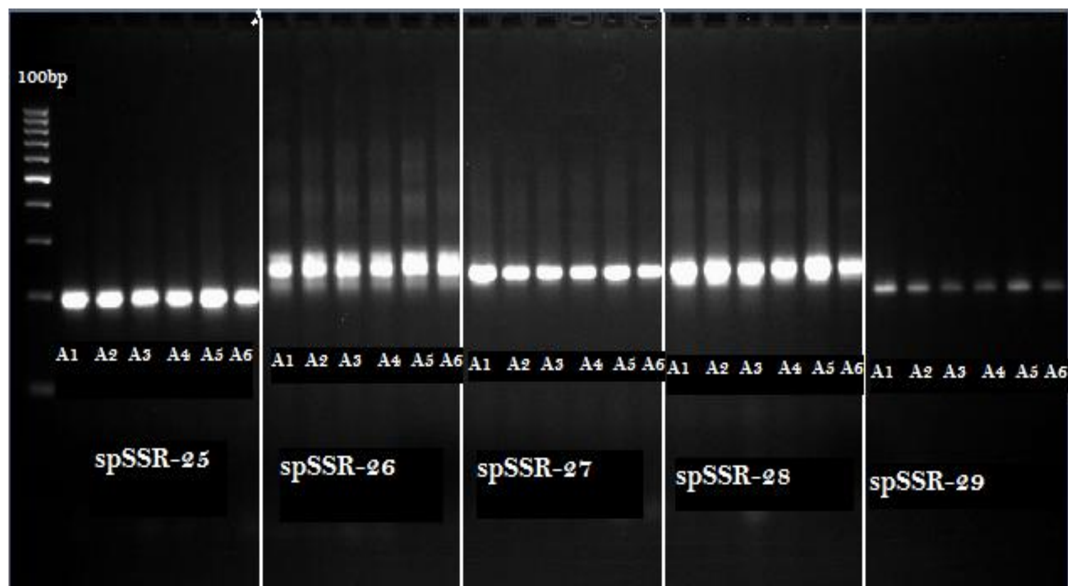

Figure 3.9. Survey of spSSR-25, spssr-26, spSSR27, spSSR-28 and spSSR 29 in six *Papaver somniferum* accessions. 100bp: 100bp size standard DNA ladder. A1: Kemerkaya, A2: 1259-1, A3: 1065, A4: 1061-7, A5: aday1 A6: 1290

Table 3.5. Tested spSSR markers in *P. somniferum* accessions.
NP: Non polymorphic, NA: No amplification.

		PCR product (bp)						
SSR Marker	**SSR Motif**	***Kemerkaya***	***1259-1***	***1065***	***1061-7***	***aday1***	***1290***	**Result**
spSSR-1	$(CTT/GAA)_4$	210	210	210	210	210	210	NP.
spSSR-2	$(ATG/TAC)_4$	200	200	200	200	200	200	NP.
spSSR-3	$(GAA/CTT)_5$	190	190	190	190	190	190	NP.
spSSR-4	$(GAT/CTA)_4$	200	200	200	200	200	200	NP.
spSSR-5	$(CATCTG/GTAGC)_3$	190	190	190	190	190	190	NP.
spSSR-6	$(AACA/TTGT)_3$	210	210	210	210	210	210	NP.
spSSR-7	$(CCA/GGT)_4$	190	190	190	190	190	190	NP.
spSSR-8	$(AAG/TTC)_8$	210	210	210	210	210	210	NP.
spSSR-9	$(CTG/GAC)_4$	180/210/ 250	180/210 /250	180/210/ 250	180/210/ 250	180/210/ 250	180/210/ 250	NP.
spSSR-10	$(CATT/GTAA)_3$	220	220	220	220	220	220	NP.
spSSR-11	$(TTTTC/AAAAG)_3$	150	150	150	150	150	150	NP.
spSSR-12	$(TTTA/AAAT)_3$	220	220	220	220	220	220	NP.
spSSR-13	$(ATA/TAT)_4$	200	200	200	200	200	200	NP.
spSSR-14	$(AGC/TCG)_4$	210	210	210	210	210	210	NP.
spSSR-15	$(CAG/GTC)_4$	220	220	220	220	220	220	NP.
spSSR-16	$(AGGA/TCCT)_3$	190	190	190	190	190	190	NP.
spSSR-17	$(TGG/ACC)_6$	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-18	$(GAT/CTA)_4$	220	220	220	220	220	220	NP.
spSSR-19	$(AGA/TCT)_5$	210	210	210	210	210	210	NP.
spSSR-20	$(CTT/GAA)_5$	210	210	210	210	210	210	NP.
spSSR-21	$(TA/AT)_6$	210	210	210	210	210	210	NP.
spSSR-22	$(TGG/ACC)_4$	120	120	120	120	120	120	NP.
spSSR-23	$(TGTCA/ACAGT)_3$	210/220	210/220	210/220	210/220	210/220	210/220	NP.
spSSR-24	$(TTC/AAG)_6$	210	210	210	210	210	210	NP.
spSSR-25	(AATA/TTAT)	200	200	200	200	200	200	NP.
spSSR-26	$(TGG/ACC)_4$	220	220	220	220	220	220	NP.
spSSR-27	$(TA/AT)_6$	210	210	210	210	210	210	NP.
spSSR-28	$(TGG/ACC)_4$	210	210	210	210	210	210	NP.
spSSR-29	$(TCAT/AGTA)_3$	230	230	230	230	230	230	NP.
spSSR-30	$(AACA/TTGT)_3$	230/240	230/240	230/240	230/240	230/240	230/240	NP.
spSSR-31	$(ACA/TGT)_4$	210	210	210	210	210	210	NP.
spSSR-32	$(TATT/ATAA)_3$	210	210	210	210	210	210	NP.

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Table 3.5. (cont.)

SSR Marker	SSR Motif	*Kemerkaya*	*1259-1*	*1065*	*1061-7*	*aday1*	*1290*	Result
spSSR-33	$(CAAA/GTTT)_3$	210	210	210	210	210	210	NP.
spSSR-34	$(TGG/ACC)_4$	210	210	210	210	210	210	NP.
spSSR-35	$(AG/TC)_6$	200	200	200	200	200	200	NP.
spSSR-36	$(CCAA/GGTT)_3$	210	210	210	210	210	210	NP.
spSSR-37	$(GAA/CTT)_{10}$	200	200	200	200	200	200	NP.
spSSR-38	$(TGAT/ACTA)_3$	210/220	210/220	210/220	210/220	210/220	210/220	NP.
spSSR-39	(ACAAC/TGTTG)	210	210	210	210	210	210	NP.
spSSR-40	$(TGT/ACA)_4$	190/210/ 600	190/210/ 600	190/210/ 600	190/210/ 600	190/210/ 600	190/210/ 600	NP.
spSSR-41	$(TCTTA/AGAAT)_3$	200	200	200	200	200	200	NP.
spSSR-42	$(TTCA/AAGT)_4$	230	230	230	230	230	230	NP.
spSSR-43	$(AAT/TTA)_4$	220	220	220	220	220	220	NP.
spSSR-44	$(TGA/ACT)_4$	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-45	$(TCTAT/AGATA)_3$	210	210	210	210	210	210	NP.
spSSR-46	$(TGAT/ACTA)_3$	230	230	230	230	230	230	NP.
spSSR-47	$(AGA/TCT)_4$	210	210	210	210	210	210	NP.
spSSR-48	$(CTG/GAC)_7$	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-49	$(TCT/AGA)_4$	210	210	210	210	210	210	NP.
spSSR-50	$(GAA/CTT)_4$	210	210	210	210	210	210	NP.
spSSR-51	$(TGAT/ACTA)_3$	210/220	210/220	210/220	210/220	210/220	210/220	NP.
spSSR-52	$(TTTTG/AAAAC)_5$	200	200	200	200	200	200	NP.
spSSR-53	$(TA/AT)_7$	200	200	200	200	200	200	NP.
spSSR-54	$(TCGT/AGCA)_3$	220	220	220	220	220	220	NP.
spSSR-55	$(TC/AG)_7$	200	200	200	200	200	200	NP.
spSSR-56	$(GAA/CTT)_4$	200	200	200	200	200	200	NP.
spSSR-57	$(AAATTA/TTTAAT)_3$	210/220	210/220	210/220	210/220	210/220	210/220	NP.
spSSR-58	$(AATA/TTAT)_3$	200	200	200	200	200	200	NP.
spSSR-59	$(AAAT/TTTA)_3$	200	200	200	200	200	200	NP.
spSSR-60	$(TCT/AGA)_4$	220	220	220	220	220	220	NP.
spSSR-61	$(AAAT/TTTA)_3$	210	210	210	210	210	210	NP.
spSSR-62	$(AGAC/TCTG)_3$	210	210	210	210	210	210	NP.
spSSR-63	$(AAGGGG/TTCCCC)_3$	200	200	200	200	200	200	NP.
spSSR-64	$(ATA/TAT)_4$	200	200	200	200	200	200	NP.
spSSR-65	$(TGGA/ACCT)_3$	210	210	210	210	210	210	NP.
spSSR-66	$(TTA/AAT)_4$	210	210	210	210	210	210	NP.

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Table 3.5. (cont.)

SSR Marker	SSR Motif	*Kemerkaya*	*1259-1*	*1065*	*1061-7*	*aday1*	*1290*	Result
spSSR-67	$(TTCT/AAGA)_3$	210	210	210	210	210	210	NP.
spSSR-68	$(TTCT/AAGA)_3$	210	210	210	210	210	210	NP.
spSSR-69	$(CAAT/GTTA)_4$	210	210	210	210	210	210	NP.
spSSR-70	$(ATT/TAA)_5$	220	220	220	220	220	220	NP.
spSSR-71	$(TATTC/ATAAG)_3$	140/150	140/150	140/150	140/150	140/150	140/150	NP.
spSSR-72	$(GAG/CTC)_4$	210	210	210	210	210	210	NP.
spSSR-73	$(ACAA/TGTT)_3$	210	210	210	210	210	210	NP.
spSSR-74	$(GTTT/CAAA)_3$	220	220	220	220	220	220	NP.
spSSR-75	$(ACT/TGA)_4$	210/200	210/200	210/200	210/200	210/200	210/200	NP.
spSSR-76	$(AATG/TTAC)_3$	210	210	210	210	210	210	NP.
spSSR-77	$(ATC/TAG)_4$	210	210	210	210	210	210	NP.
spSSR-78	$(GTATT/CATAA)_3$	210	210	210	210	210	210	NP.
spSSR-79	$(GGAA/CCTT)_3$	220	220	220	220	220	220	NP.
spSSR-80	$(GGAA/CCTT)_3$	210/310	210/310	210/310	210/310	210/310	210/310	NP.
spSSR-81	$(GAA/CTT)_4$	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-82	$(AAT/TTA)_4$	220	220	220	220	220	220	NP.
spSSR-83	$(CA/GT)_6$	200	200	200	200	200	200	NP.
spSSR-84	$(CCAA/GGTT)_3$	160	160	160	160	160	160	NP.
spSSR-85	$(ATTT/TAAA)_3$	210	210	210	210	210	210	NP.
spSSR-86	$(TTGA/AACT)_3$	200	200	200	200	200	200	NP.
spSSR-87	$(TTCT/AAGA)_3$	210	210	210	210	210	210	NP.
spSSR-88	$(GA/CT)_8$	200	200	200	200	200	200	NP.
spSSR-89	$(ACGA/TGCT)_3$	200	200	200	200	200	200	NP.
spSSR-90	$(TGT/ACA)_4$	210	210	210	210	210	210	NP.
spSSR-91	$(ATCA/TAGT)_3$	210	210	210	210	210	210	NP.
spSSR-92	$(AT/TA)_8$	210	210	210	210	210	210	NP.
spSSR-93	$(AAT/TTA)_4$	200	200	200	200	200	200	NP.
spSSR-94	$(GCA/CGT)_4$	200	200	200	200	200	200	NP.
spSSR-95	$(AATA/TTAT)_3$	200	200	200	200	200	200	NP.
spSSR-96	$(CT/GA)_6$	190	190	190	190	190	190	NP.
spSSR-97	$(CATC/GTAG)_3$	200	200	200	200	200	200	NP.
spSSR-98	$(TGTTG/ACAAC)_3$	210	210	210	210	210	210	NP.
spSSR-99	$(ATC/TAG)_4$	200	200	200	200	200	200	NP.
spSSR-100	$(GTG/CAC)_4$	200	200	200	200	200	200	NP.

3.4. Transferability of Genomic SSRs

The 100 SSR primers were used to amplify *P. bracteatum, P. umbonatum, P. arenarium, P. armeniacum, P. orientale, P. rhoeas* and *P. nudicaule* DNA. Amplification was successful for 27 primers in *P. bracteatum,* 50 primers in *P. umbonatum,* 62 primers in *P. arenarium,* 62 primers in *P. armeniacum,* 51 primers in *P. nudicaule,* 58 primers in *P. orientale,* and 45 primers in *P. rhoeas* (Table 3.6, Figure 3.10). Transferability of opium poppy genomic SSR was expected because transferability of genomic SSR in other species is between 35% and 92%, depending on the report. For example, 58 coffee genomic SSR markers were tested on 14 related species of coffee and showed ~92% transferability (*C. congensis, C. excels, C. liberica, C. abeokuteae, C. dewevrei, C. arnoldiana, C. aruwemiensis, C. eugenioides, C. racemosa, C. racemosa, C. kapakata, C. stenophylla, P. wightiana P. bengalenis*) (Hendre et al. 2008). In another study 38% of the tall fescue genomic SSRs were transferred to five species (meadow fescue, ryegrass, rice and wheat) (Saha et al. 2006). Moreover, 20 cucumber SSR markers were tested on melon, watermelon, pumpkin and bitter gourd. PCR products could be generated with 13 primers (65%) in melon, 11 primers (55%) in bitter gourd, 10 primers (50%) in watermelon and seven primers (35%) in pumpkin (Watcharawongpaiboon et al. 2008). Most primers gave single fragment amplification as expected. However, some markers gave more than one fragment. For example, two polymorphic fragments (210/220 bp) were generated by spSSR-23 marker in two accessions of *P. umbonatum* (Figure 3.11) Three polymorphic fragments (210/250/280 bp) were generated by spSSR-6 marker in P. *umbonatum, P. arenarium*, *P. armeniacum*, *P. orientale*, and *P. rhoeas* (Figure 3.12). SpSSR-8 detected two polymorphic alleles (210/250 bp) in two accessions of *P. orientale*. Also three polymorphic fragments (210/250/290 bp) were detected by spSSR-8 in two accessions of *P. rhoeas* (Figure 3.13). These three SSR markers can be used for assessment of genetic diversity in opium poppy.

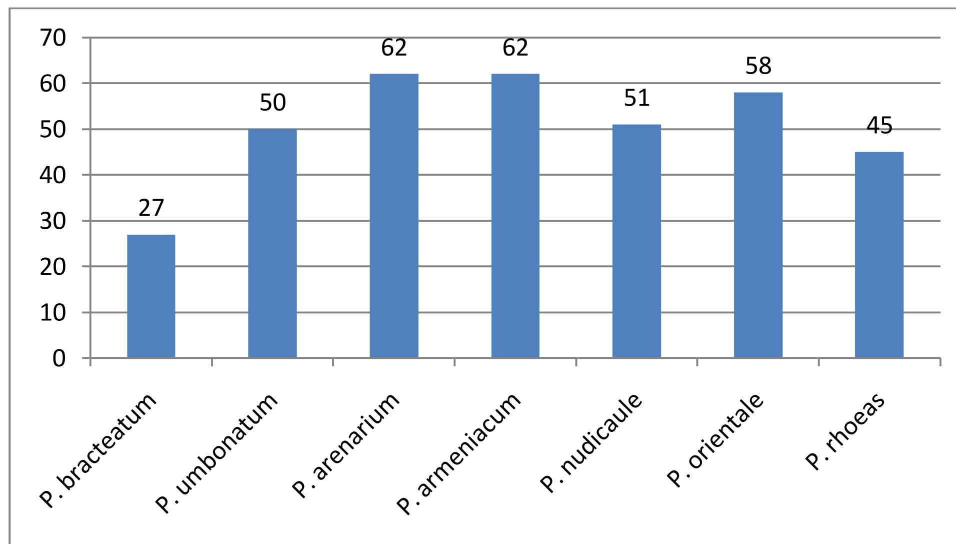

Figure 3.10. Number of SSR markers transferred to *Papaver* species.

Three main lineages (clade 1, 2 and 3) were identified in phylogenetic analysis of nuclear ribosomal ITS and plastid trnL–F nucleotide sequences of *Papaver* and related genera (Carolan et al. 2006). *P. bracteatum, P. orientale, P. armeniacum* and *P. somniferum* belonged to clade 2 and only *P. nudicaule* belonged to clade 1. *P. bracteatum* and *P. orientale* belonged to the Oxytona section in clade 2. Although the numbers of transferred markers in *P. bracteatum* and *P. orientale* were expected to be equal, fewer SSR markers were successfully transferred to *P. bracteatum* than *P. orientale*. The reason for this can be that *P. bracteatum* has fewer chromosomes than *P. orientale.* Although *P. nudicaule* belongs to a different clade, there was no significant differences in number of transfered SSR markers for *P. umbonatum, P. arenarium, P. armeniacum, P. orientale, P. rhoeas* and *P. nudicaule*. Overall, the results show that the SSR loci were conserved in a diverse array of *Papaver* species.

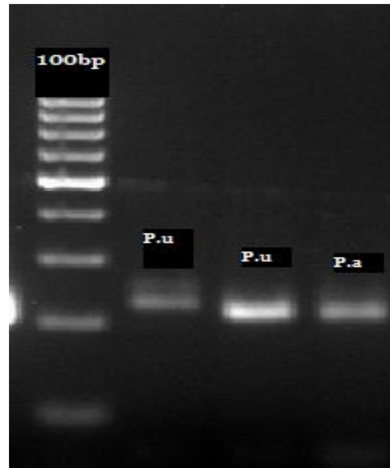

Figure 3.11. SpSSR-23 marker polymorphism.100bp: 100bp size standard DNA ladder. P.u: *P. umbonatum*, P.a : *P. arenarium.*

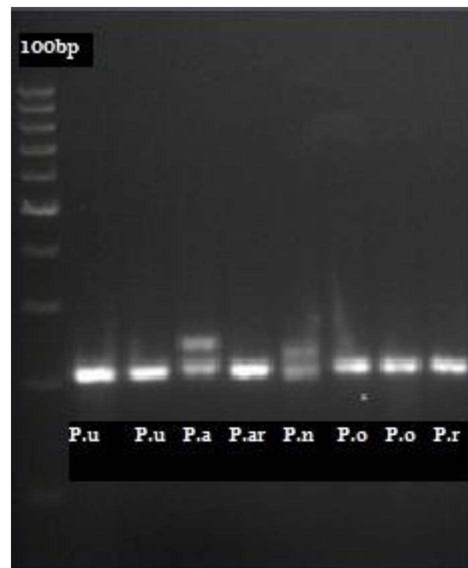

Figure 3.12. SpSSR-6 marker polymorphism.100bp: 100bp size standard DNA ladder. P.u: *P. umbonatum*, P.a : *P. arenarium*, P.ar: *P. armeniacum*, P.o: *P. orientale*, P.r: *P. rhoeas.*

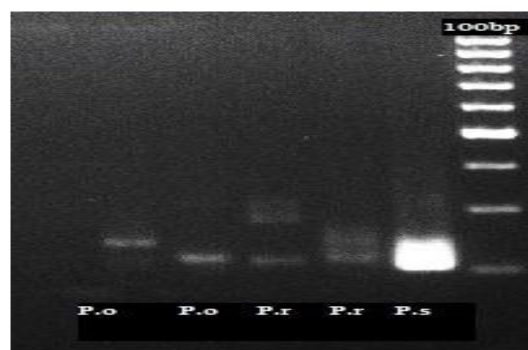

Figure 3.13. SpSSR-8 marker polymorphism.100bp: 100bp size standard DNA ladder. P.o: *P. orientale*, P.r: *P. rhoeas*, P.s: *Papaver somniferum.*

Table 3.6. Transferability of spSSR markers in different *Papaver* species. NP: Non polymorphic, NA: No amplification.

	Product Size (bp)						
SSR marker	**P. bracteatum**	***P. umbonatum***	***P. arenarium***	***P. armeniacum***	***P. nudicaule***	***P. orientale***	***P. rhoeas***
spSSR-1	210	210	210	210	210	210	210
spSSR-2	NA.	200	200	200	200	200	NA.
spSSR-3	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-4	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-5	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-6	210	210	210/280	210	210/250	210	210
spSSR-7	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-8	210	210	NA.	NA.	NA.	210/250	210/ 250/ 290
spSSR-9	NA.	NA.	400	NA.	NA.	NA.	NA.
spSSR-10	NA.	220	220	220	220	220	220
spSSR-11	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-12	220	220	220	220	220	220	220
spSSR-13	NA.	NA.	200	200	NA.	200	NA.
spSSR-14	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-15	NA.	220	220	220	220	220	220
spSSR-16	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-17	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-18	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-19	210	NA.	210	210	210	210	210
spSSR-20	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-21	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-22	120	120	120	120	120	120	120
spSSR-23	210	210	210/220	210	210	210	210
spSSR-24	210	210	210	210	210	210	210
spSSR-25	NA.	200	200	200	200	200	200
spSSR-26	NA.	NA.	NA.	220	220	220	220
spSSR-27	210	210	210	210	210	210	210
spSSR-28	NA.	NA.	210	210	NA.	NA.	NA.
spSSR-29	NA.	230/290	230/290	230	230	230	NA.
spSSR-30	NA.	230	230	230	230	230	230
spSSR-31	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-32	NA.	NA.	210	210	NA.	210	210
spSSR-33	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-34	NA.	NA.	210	210	210	210	210
spSSR-35	NA.	210/300	210	210	210	210	210

(cont. on next page)

Table 3.6. (cont.)

SSR marker	P. bracteatum	*P. umbonatum*	*P. arenarium*	*P armeniacum.*	*P. nudicaule*	*P. orientale*	*P. rhoeas*
spSSR-36	NA.	210	210	210	210	210	210
spSSR-37	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-38	NA.	210	210	210	210	210	NA.
spSSR-39	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-40	200	200	200	200	200	200	200
spSSR-41	NA.	NA.	200	200	200	200	NA.
spSSR-42	NA.	230	230	230	230	230	230
spSSR-43	NA.	230	230	230	NA.	NA.	NA.
spSSR-44	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-45	NA.	220	220	220	220	220	220
spSSR-46	NA.	NA.	230	230	NA.	230	NA.
spSSR-47	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-48	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-49	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-50	NA.	210	210	210	NA.	210	210
spSSR-51	NA.	220	220	220	220	220	220
spSSR-52	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-53	NA.	200	200	200	200	200	200
spSSR-54	NA.	NA.	230	230	NA.	230	NA.
spSSR-55	NA.	NA.	230	230	230	230	NA.
spSSR-56	NA.	NA.	NA.	210	210	210	210
spSSR-57	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-58	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-59	NA.	210	210	210	210	210	NA.
spSSR-60	NA.	NA.	220	220	220	220	NA.
spSSR-61	NA.	NA.	210	210	210	210	NA.
spSSR-62	NA.	210	210	210	NA.	210	NA.
spSSR-63	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-64	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-65	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-66	NA.	NA.	210	210	NA.	NA.	NA.
spSSR-67	NA.	NA.	230	210	NA.	NA.	NA.
spSSR-68	210	210	210	210	210	210	210
spSSR-69	190	190	190	NA.	NA.	210	210
spSSR-70	220	200	220	220	220	220	220
spSSR-71	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-72	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-73	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-74	200	200	200	200	200	200	200
spSSR-75	NA.	NA.	NA.	NA.	NA.	NA.	NA.

(cont. on next page)

Table 3.6. (cont.)

SSR marker	P. bracteatum	*P. umbonatum*	*P. arenarium*	*P armeniacum.*	*P. nudicaule*	*P. orientale*	*P. rhoeas*
spSSR-76	210	210	210	210	210	210	210
spSSR-77	210	210	210	210	210	210	210
spSSR-78	210	210	210	210	210	210	210
spSSR-79	220	210	220	220	220	220	220
spSSR-80	200	200	200	210/310	200	210/310	210/ 310
spSSR-81	NA.	NA.	NA.	NA.	NA.	NA.NA.	NA.
spSSR-82	220	210	210	NA.	210	210	210
spSSR-83	NA.	220	NA.	NA.	NA.	NA.	NA.
spSSR-84	NA.	NA.	160	160	NA.	160	NA.
spSSR-85	210	210	210	210	210	210	210
spSSR-86	NA.	NA.	200/250	NA.	NA.	NA.	NA.
spSSR-87	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-88	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-89	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-90	210	210	210	210	210	210	210
spSSR-91	NA.	210	210	210	210	210	210
spSSR-92	210	210	210	210	210	210	210
spSSR-93	NA.	200	200	200	200	NA.	200
spSSR-94	200	200	200	200	200	200	NA.
spSSR-95	NA.	NA.	NA.	NA.	NA.	NA.	NA.
spSSR-96	190	190	190	190	190	190	190
spSSR-97	NA.	200	200	200	200	NA.	200
spSSR-98	210	210	210	210	210	210	210
spSSR-99	210	210	210	210	210	210	210
spSSR-100	210	210	210/200	210	210	210	210

CHAPTER 4

CONCLUSION

In this thesis, genomic DNA of opium poppy was sequenced by Roche 454 FLX Sequencing™ platform. In total 41.8% of reads were clustered by MIRA software. From these reads, 400 different SSR motifs were identified and analyzed. A total of 1820 primers derived from genomic sequence were designed and 100 SSR markers were tested in six *Papaver somniferum* accessions. No polymorphism was found among the six *P. somniferum* accessions. These initial findings suggest low levels of DNA polymorphism in this species. Transferability of spSSR markers was tested in *P. bracteatum, P. umbonatum, P. arenarium, P. armeniacum, P. orientale, P. rhoeas* and *P. nudicaule* and transferable SSR markers were determined. SpSSR-6, spSSR-8 and spSSR-23 detected polymorphic alleles in transferred species. These markers will enrich the limited marker resources in opium poppy. They are the first set of opium poppy-specific SSR markers derived from genomic sequence of this crop. These markers can be used for assessment of genetic diversity, mapping and marker assisted selection in opium poppy.

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APPENDIX A

SSR PRIMERS

Table A.1. Tested SSR primers with their Tm, sequence and product size.

SSR primers	Tm (°C)	Sequence	Product size (nt)
psSSR-1 –F	55.35	TAACCATGCAGGAGTAGCTTA	203
psSSR-1 –R	54.91	AACTTAGCAGCAGCTTACAAA	
psSSR-2 –F	54.57	AAATCCATTTGTTCCTTCTCT	193
psSSR-2 –R	54.38	TTTGTTGAAGGTAATCTGGTC	
psSSR-3 –F	54.64	AGTGATGGGTTAGTGATTTGA	183
psSSR-3 -R	54.67	ACAACACCCAAATCTATTTCA	
psSSR-4 -F	54.37	GACAACGATGAAGAGAACAGT	196
psSSR-4 -R	53.83	CCGTTACCTAAAAGCTACTTG	
psSSR-5 -F	54.91	TGTTTTAGCTGCTTTAGCTGT	158
psSSR-5 -R	54.97	AAATGGAAATGGAACAGATG	
psSSR-6 -F	55.23	AATCCCTCTCTTTCCTTACCT	187
psSSR-6 -R	55.22	GGGGATATTTTTATGTCTTGG	
psSSR-7 -F	55.39	CATTTCACCACCACCAAC	177
psSSR-7 -R	54.85	TTTTGGATGAACTTCAGTTGT	
psSSR-8 -F	54.63	TCCAGTATTACACGATTCAAAG	190
psSSR-8 -R	55.35	CACTACCATTCATGATTCACC	
psSSR-9 -F	55.5	GCCACTTCTTCTCCTGCT	209
psSSR-9 -R	55.74	CTGAGGCTAACAACAGTGCT	
psSSR-10 -F	55.09	CGCGAGAAAAATAGACAACTA	194
psSSR-10 -R	55.38	AAGGGTAGCTTCATCAGAAAG	
psSSR-11 -F	54.53	TTGTGTTGTTGGTGTATCTTG	138
psSSR-11 -R	55.14	GAAAAATTCCAAACGGGTA	
psSSR-12 -F	54.67	ACAACACAAGGATGGAATTTA	199
psSSR-12 -R	54.11	GTGGCTGTGAGATATAAAAGG	
psSSR-13 -F	54.95	TTTGGAGTTCTAGGTTGATGA	152
psSSR-13 -R	54.94	ACCCCATTACATATTAGCTGTC	
psSSR-14 -F	54.92	GGTTTCACTGAGTTGAACTTG	212
psSSR-14 -R	55.45	TTCTCTGCTACAGTTGCTGTT	
psSSR-15 -F	55.25	CAATGACAAAATAAGACTGCTG	202
psSSR-15 -R	54.6	GAGAGCCAAAGAAGAAGAAAT	
psSSR-16 -F	54.89	TCAGGGACAAAATAATCAAGA	167
psSSR-16 -R	54.88	TTTCTCTCTCAGTCTTTCACG	
psSSR-17 -F	56.7	CAAAATCGGTTTCATCGTATT	206
psSSR-17 -R	57.33	CCTACCATCCAGCACCAC	
psSSR-18 -F	55.12	GTCGAACAACAACAGATCATT	216

(cont. on next page)

Table A.1. (cont.)

SSR primers	Tm (°C)	Sequence	Product size (nt)
psSSR-18 -R	55.2	AATTTGAAGGAAGAAGACTGG	
psSSR-19 -F	54.93	ATCAGCAAAGCATGACATATT	191
psSSR-19 -R	55.35	CCCTAGCTCTAGCTACTTTGG	
psSSR-20 -F	55.5	GGTTCTCGTACGTTTTAATCC	198
psSSR-20 -R	54.97	CCAAAACAATTGAGAATTGAG	
psSSR-21 -F	54.41	AGGACCAGGATCTACAGATTT	197
psSSR-21 -R	54.75	GTATTGTTTGTGGGTTGAAAG	
psSSR-22 -F	55.57	TGTCACCAACTTCTATGAAGAAT	110
psSSR-22 -R	54.24	AACTTTCTGGTAGGGCATC	
psSSR-23 -F	54.97	CTGATTGAGTACCTTTCATGC	198
psSSR-23 -R	55.09	GAGCCTAATGAGTATTTGAAGC	
psSSR-24 -F	55.51	TGTATTGGCGTTACAGAGTTC	171
psSSR-24 -R	55.49	GAGCAACTTCTCTGCTTCTG	
psSSR-25 -F	55.51	AACATGCTTGAGATAACAACG	216
psSSR-25 -R	55.18	ACTTACAACCTTTGGGATCAT	
psSSR-26 -F	54.99	CTCTTCCATCACTGTCTCTTG	205
psSSR-26 -R	55.87	ACCCACAATCTCAACCATTAT	
psSSR-27 -F	55.44	TACGTCTCGAAATACCAAATG	203
psSSR-27 -R	55.88	CTCAAGGACTTCATGGAGATT	
psSSR-28 -F	54.81	TTTTACTTTTGCACCAACTTC	200
psSSR-28 -R	55.36	ATTTTCATGGACATACCACAA	
psSSR-29 -F	54.89	TAATGAGGAACTGGAAATTGA	213
psSSR-29 -R	54.99	AGACCATAAATGTCTGCAAAA	
psSSR-30 -F	54.8	AAGAAGTGGACGTGATGATAA	208
psSSR-30 -R	54.93	GTCAAACATCTGAGTGATGGT	
psSSR-31 -F	55.13	TCATGTTAAGAGTCGCTTGAT	200
psSSR-31 -R	54.54	TGAAAGATACGTTAGGGAATG	
psSSR-32 -F	55.15	AGGAGTTCGGAGATGATAAAG	205
psSSR-32 -R	56.49	GCAAGTTCATCTTCCAAAAAC	
psSSR-33 -F	54.19	GACCGATTATTCCTTAACCTT	211
psSSR-33 -R	55.25	TGGGAAAAGATAGAATTAGCC	
psSSR-34 -F	53.64	GCACCAACTTCTATGAAGAAT	191
psSSR-34 -R	55.28	CATCATTTTCATGGACATACC	
psSSR-35 -F	55.04	TGAAAGCTAAAATTTCAGTGC	213
psSSR-35 -R	54.9	CTAACTTGTCTTGGGTTGATG	
psSSR-36 -F	55	CGATGGTTGTCTTTCTTAATG	202
psSSR-36 -R	54.97	CCTGATTCTAGCACAATTCAC	
psSSR-37 -F	55.14	TCTGAACTGAGATCAACAGAGA	200
psSSR-37 -R	54.88	GATTAGTACGAAGGGGATTGT	
psSSR-38 -F	54.38	TGTGATTGTCAACAATTGAAC	194
psSSR-38 -R	55.66	ATGGGTAACCGTACCTAAATG	
psSSR-39 -F	54.96	CCTAAGGTTAAAGGAGAATCG	198
psSSR-39 -R	54.81	TCGTTTCTGAAATTATTCTGC	

(cont. on next page)

Table A.1. (cont.)

SSR primers	Tm (°C)	Sequence	Product size (nt)
psSSR-40 -F	54.73	TTGTGACATGTGTTGATGTCT	171
psSSR-40 -R	55.88	AAACAAAGGGTTGCAATAATC	
psSSR-41 -F	55.03	ACCATGGTTTTCTCATTCAG	179
psSSR-41 -R	55.02	TTAACCCTTGTCGAAAATACA	
psSSR-42 -F	55.42	ATGAAGGTTTTACGGTGTTTT	225
psSSR-42 -R	54.97	GTAATGAGCCTCTTGATGTTG	
psSSR-43 -F	55.81	GGCACTCTTCCCAATTTTAT	215
psSSR-43 -R	54.52	CGACTTTAGGGAAACTCGT	
psSSR-44 -F	54.97	ATCATCCACATCCCATTTT	202
psSSR-44 -R	55.34	CACTATCATCCAGATCACCAC	
psSSR-45 -F	54.8	TCATCAATAAAAGGTATGTGGA	197
psSSR-45 -R	55.08	GATGAACGTAGCTAAAGCAAA	
psSSR-46 -F	55.12	TGTACAATTACGAACCAATCC	229
psSSR-46 -R	54.61	CGTGTATATTGACTTGGCTCT	
psSSR-47 -F	55.17	GACGAGATTTCCTTGAGAACT	206
psSSR-47 -R	55.22	ATGTTCTTCAGCTTAGGGTTC	
psSSR-48 -F	55.5	AGAAGAGAGGAAGATGCAGAG	204
psSSR-48 -R	55.02	AAAATGAACGACACAGAAGAA	
psSSR-49 -F	54.41	CACACGTATTCTTTCTTCCAT	203
psSSR-49 -R	55.35	AGTTGCAACAATGGTAATTTG	
psSSR-50 -F	54.93	GTTGAGCTTTAGACTTCACGA	198
psSSR-50 -R	54.79	CGCAACATCAAATGTGTATAA	
psSSR-51 -F	55.05	TAAACACTTCGTCTCATCGTT	199
psSSR-51 -R	55.23	CAATCTGTCTCCCACAGATAA	
psSSR-52 -F	54.94	TTCCTTTGTTGTTTCTGTTGT	148
psSSR-52 -R	54.6	TCTCTGGAATTTCAATCAAAG	
psSSR-53 -F	55.63	TTGAGACAAGAAGTCGTATGC	126
psSSR-53 -R	54.8	CGAAGTGAAAAGACTTACCAA	
psSSR-54 -F	54.83	TACGGATTTAGAGAGCATGAG	218
psSSR-54 -R	54.17	GACTCAAGTCTATTTTGTTGTTTC	
psSSR-55 -F	53.23	TGCTCTAAACTTTCTCTCTCC	200
psSSR-55 -R	54.91	ATCCTGCGAAAATTAGAGAGT	
psSSR-56 -F	55.23	AAGAGGTTCCATTCGTAGAAG	204
psSSR-56 -R	54.7	GCTTGTTTAGGTCTTCATTCA	
psSSR-57 -F	55.33	GGCATAGAGGCTTCATCTACT	201
psSSR-57 -R	54.59	GAAGGGGTGTTGTATGTGTAG	
psSSR-58 -F	55.04	CTTTGTAACGGATTATGCAAC	182
psSSR-58 -R	55.03	TATCCGTGTATGAAATTCACC	
psSSR-59 -F	54.23	GGTGAAAGTGTATTCAACGAT	195
psSSR-59 -R	55.21	TATAAAACTGGCGTGGAACTA	
psSSR-60 -F	54.86	GAACACATCATTCATCAGGTT	214
psSSR-60 -R	54.74	TGCTTAGTGTCCGACTTATTC	
psSSR-61 -F	54.82	TATGAAATGCTCAAAAGAAGG	198

(cont. on next page)

Table A.1. (cont.)

SSR primers	Tm (°C)	Sequence	Product size (nt)
psSSR-61 -R	54.29	TCCCTGATATTTGGATAGTG	
psSSR-62 -F	54.7	TACGTTTCGAAATATGAGAGG	200
psSSR-62 -R	54.67	TTGAACATTGGTCTTTGGTAT	
psSSR-63 -F	55.31	GGTGCAAAGAAAACTTTAGGT	178
psSSR-63 -R	54.53	AATTGTGGACCCTCTTAAACT	
psSSR-64 -F	55.16	TAGGATTTCCTGCAACTACAA	202
psSSR-64 -R	55.18	TTACAAATTCAAGCACCCTAA	
psSSR-65 -F	55.25	AAGCTTCTGACTTGTGTTTCA	199
psSSR-65 -R	55.08	CATGAGAGCATAAGTGCTTCTA	
psSSR-66 -F	55.05	CATGGTACTTCCATAAATCCA	186
psSSR-66 -R	54.76	GTGCAAAAATACCATGAAATC	
psSSR-67 -F	55.6	TTCTTGCAACAATTCTCAAAC	210
psSSR-67 -R	55.21	ATCCCCATAATTAGTTCCTCA	
psSSR-68 -F	54.84	TGGGTTTTCTTCTATACCACA	203
psSSR-68 -R	55.2	CCCATTACTCCTCAAGCTAAT	
psSSR-69 -F	54.61	ATAGATTTATTTTGGCCACCT	156
psSSR-69 -R	55.16	CACCTATTGATTGAGGATGAA	
psSSR-70 -F	55.36	CTAATAACCCGCTCTCATTTT	211
psSSR-70 -R	55.05	CCCACAATAAAATACTGCAAG	
psSSR-71 -F	55.05	TCTGGCAAGTTACAAGACATT	139
psSSR-71 -R	54.19	CTGCTCCAAAATATCAAAATC	
psSSR-72 -F	55.23	TGAGAATCAACATTTCGTACC	205
psSSR-72 -R	54.78	AAAACAATAGAGCCAAGGAAT	
psSSR-73 -F	54.69	CAAAAAGGTGCAAATACAAGT	188
psSSR-73 -R	55.69	GTATCAGTACCTCCACGGTTT	
psSSR-74 -F	55.19	TTGGAAGAATAACTAGCGTTTT	197
psSSR-74 -R	54.85	CACAGACGCAAACAATACATA	
psSSR-75 -F	54.77	CATTCTTCTTCCGCTATATCA	194
psSSR-75 -R	55.36	TCCTATTAAGCCGAGTAAACC	
psSSR-76 -F	55.15	TGGATACCTCTCAAGTAAGCA	204
psSSR-76 -R	55.6	CATGCAGATCATACAAAATCC	
psSSR-77 -F	55.06	ACACAAATCATCATTTTCCAG	196
psSSR-77 -R	55.05	TACCGGTACGTTCTTTATTGA	
psSSR-78 -F	54.75	AGAAGTTAGCTTGGAGTAGGC	198
psSSR-78 -R	55.23	TTGATGATCTAACGAACAACC	
psSSR-79 -F	54.93	TCGAGACGTGTTATGGTTACT	215
psSSR-79 -R	54.99	GAAATTACAAGGCACAAGATG	
psSSR-80 -F	54.89	ACAGAGACAGTTCACTTCCAA	190
psSSR-80 -R	54.8	ATGAGTCGTTTTTGTTGTTGT	
psSSR-81 -F	57.2	TTGGTGGTGAGGAAAAATCTA	190
psSSR-81 -R	58.91	CTTGCCTCCCACGACTCT	
psSSR-82 -F	54.76	TTGTAAGATTCACTCCCCATA	203
psSSR-82 -R	55.12	ACATGGAAGTTCCCAAATTAT	
psSSR-83 -F	54.35	AGGGTTTCATTTAATCCTAGC	203

(cont. on next page)

Table A.1. (cont.)

SSR primers	Tm (°C)	Sequence	Product size (nt)
psSSR-83 -R	55.05	TTCGGGTTCTTTCAAAATTA	
psSSR-84 -F	54.63	ATGACACTTTGCAAGAGGTAG	165
psSSR-84 -R	55	TGTTGGATTAGGAAGTTACGA	
psSSR-85 -F	55.25	TTACAAACTTTGGGATCATTG	211
psSSR-85 -R	55.43	AACATGATTGAGATAGCAACG	
psSSR-86 -F	54.47	TCGTAAATTGGATTATTCGTC	188
psSSR-86 -R	55.04	ATGTCAATTGGGGTGAGAT	
psSSR-87 -F	55.15	CCATTTCCTGAATGTTCCTA	191
psSSR-87 -R	55.13	TGTTAAAAGAATCCAGAACGA	
psSSR-88 -F	55.21	GTTCTGGCTCCACTAGAGAAT	183
psSSR-88 -R	55.16	TACCTATGTAATGGGGTTCCT	
psSSR-89 -F	54.89	CGTTACTTCAATCTGTGGTTC	205
psSSR-89 -R	55.21	CCTTCCTATTTTGCATAGGTT	
psSSR-90 -F	55.04	TGTAAACCACCAAACCAATAC	194
psSSR-90 -R	54.97	AATAATCAATGGCTACCTTCC	
psSSR-91 -F	54.94	AAGAATCTTGCAGAGAAAGGT	197
psSSR-91 -R	54.78	TGAGCGAGAATTTCATAGAAC	
psSSR-92 -F	55.46	ACAAGCAGCTTTACAAACAGA	198
psSSR-92 -R	54.65	ATGAAGAAGAAGGGGATTCTA	
psSSR-93 -F	55.26	TTTCTTGATTTTGAGCGTTT	194
psSSR-93 -R	55.92	GCTATTATTCTTGCATGTATTCG	
psSSR-94 -F	55.33	AGTGAGGAATATCTCGGTGTT	202
psSSR-94 -R	55.37	AAGCATAAAACTCTCGACTCC	
psSSR-95 -F	55.32	TCCTTATTAGCGTTTGAGACA	204
psSSR-95 -R	55.28	GAGAAATTCGGACTAAGGAAA	
psSSR-96 -F	54.65	TGAATGTTCTTACAAGACAAGG	190
psSSR-96 -R	55	AACCATATCCGAAAATAAAGG	
psSSR-97 -F	54.9	AACCTAAAGTCAAGGATGGAC	210
psSSR-97 -R	55.22	TCAACAGTACCATCGATCTTC	
psSSR-98 -F	55	CCTTTATTTTCGGATATGGTT	204
psSSR-98 -R	55.66	CAGAATCTCGCAGCAATAAT	
psSSR-99 -F	55.23	CTCTAAGTCGGGTGAAAGATT	197
psSSR-99 -R	55.17	AACACACACCACTTAGTTTGG	
psSSR-100 -F	54.67	GGAATGTTAGTTGAATGTTGG	200
psSSR-100 -R	55.74	TGTCCCTAATTTTGACACTCA	