COMPUTATIONAL MINING AND SURVEY OF SIMPLE SEQUENCE REPEATS (SSRs) IN EXPRESSED SEQUENCE TAGS (ESTs) OF DICOTYLEDONOUS PLANTS

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DEDICATION

This thesis is dedicated to the memory of my father,

Venkateswara Rao Kumpatla,

My role model for hard work and perseverance

Acknowledgments

“Imagination is more important than knowledge…”

- Albert Einstein (1879 - 1955)

What started out to be an idea for a small bioinformatics project, later developed into a full blown project culminating in the current thesis. I am extremely grateful to the guidance and help extended to me by the faculty of School of Informatics and a number of colleagues and friends during this journey of converting my imagination and desire of mining SSRs in a large number of dicotyledonous species into reality.

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Abstract

DNA markers have revolutionized the field of genetics by increasing the pace of genetic analysis. Simple sequence repeats (SSRs) are repetitions of nucleotide motifs of 1 to 5 bases and are currently the markers of choice in many plant and animal genomes due to their abundant distribution in the genomes, hypervariable nature and suitability for high-throughput analysis. While SSRs, once developed, are extremely valuable, their development is time consuming, laborious and expensive. Sequences from many genomes are continuously made freely available in the public databases and mining of these sources using computational approaches permits rapid and economical marker development. Expressed sequence tags (ESTs) are ideal candidates for mining SSRs not only because of their availability in large numbers but also due to the fact that they represent expressed genes. Large scale SSR mining efforts in plants to date focused on monocotyledonous plants. In this project, an efficient SSR identification tool was developed and used to mine SSRs from more than 53 dicotyledonous species. A total of 92,648 non-redundant ESTs or 6.0% of the 1.54 million dicotyledonous ESTs investigated in this study were found to contain SSRs. The frequency of non-redundant-ESTs containing SSRs among the species investigated ranged from 2.65% to 16.82%. More than 80% of the non-redundant ESTs having SSRs contained a single SSR repeat while others contained 2 or more SSRs. An extensive analysis of the occurrence and frequencies of various SSR types revealed that the A/T mononucleotide, AG/GA/CT/TC dinucleotide, AAG/AGA/GAA/CTT/TTC/TCT trinucleotide and TTTA and TTAA tetranucleotide repeats are the most abundant in dicotyledonous species. In addition, an analysis of the number of repeats across species revealed that majority of the mononucleotide SSRs contained 15-25 repeats while majority of the di- and tri-nucleotide SSRs contained 5-10 repeats. By providing valuable information on the abundance of SSRs in ESTs of a large number of dicotyledonous species, this study demonstrates the potential of computational mining approach for rapid discovery of SSRs towards the development of markers for genetic analysis and related applications.

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I. Introduction

The advent of DNA marker technology has revolutionized the field of genetics (Cullis, 2002; Dodgson *et al.*, 1997; Rafalski and Tingey, 1993). Although phenotypic or morphological markers and later isozyme (protein) markers have dominated the field of classical genetics for more than a century, the introduction of DNA-based markers during the second half of 20th century changed the pace and precision of genetic analysis (Dodgson *et al.*, 1997). During the last two decades DNA-based markers have led to the construction of whole genome linkage maps in many plant and animal genomes, a crucial step for several downstream applications such as gene cloning, genome analysis and marker-assisted selection of agricultural crops (Cullis, 2002; Dodgson *et al.*, 1997; Paterson, 1996a). DNA markers are also being increasingly used in genetic diagnostics, population studies, comparative genomics, pharmacogenomics, drug discovery and molecular evolution studies (Bennetzen, 2000; McCarthy and Hilfiker, 2000; Pfost *et al.*, 2000; Rafalski and Tingey, 1993; Terauchi and Konuma, 1994).

A. Molecular marker technology and simple sequence repeats (SSRs)

While the earliest DNA markers, restriction fragment length polymorphism (RFLP) markers, have proved to be very useful, their development and utilization is laborious, time-consuming, expensive and not suitable for high-throughput automation (Paterson, 1996b; Rafalski and Tingey, 1993). For these reasons, PCR-based markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs) etc., have become popular for molecular genetic studies (Paterson, 1996b). Out of the PCR-based markers, SSR markers quickly became the markers of choice for plant and animal genomes during the last decade because of the small sample size (genomic DNA) requirement for their analysis and their suitability for automation and high-throughput (Hearne *et al.,* 1992). SSRs, also called microsatellites or short tandem repeats (STRs), are in general repetitions of nucleotide motifs of 1 to 5 bases (Powell *et al.,* 1996). Two major features that made SSRs very popular are their abundant distribution in the genomes examined to date and their hypervariable nature (Powell *et al.,* 1996; Tautz and Renz, 1984; Toth *et al.,* 2000). For example, Cardle *et al.,* (2000) have reported that SSRs occur as abundantly as once in approximately every 6 kb in plant genomes. The term 'hypervariable' refers to the property where the number of repeats in an SSR stretch differs from individual to individual or genotype to genotype thus making them extremely useful as genetic markers. While di-, tri- or tetra-nucleotide SSRs are most commonly used for the construction of linkage maps of nuclear genomes, single nucleotide repeats have been used in the population genetic analyses of chloroplast genomes (Powell *et al.,* 1995). Since SSRs can be assayed using PCR technology, they can be screened using high-throughput platforms for molecular genetic linkage (Morgante and Olivieri, 1993) and population (Powell *et al.,* 1995) studies. In humans, triplet repeat SSRs are known to be associated with more than fourteen inherited neurodegenerative diseases and SSR typing is being used as a diagnostic measure in these cases (Bryant-Greenwood, 2002; Sinden *et al.,* 2002).

Another area where SSR markers are extremely valuable and are increasingly becoming popular is comparative genomics where SSR markers developed from one species could be utilized in a related or heterologous species towards genetic mapping, characterization, gene cloning, diversity and evolutionary studies (Cordeiro *et al.,* 2001; Eujayl *et al.,* 2001; Killian *et al.,* 1997; Moore *et al.,* 1991; Peakall *et al.,* 1998; Rallo *et* *al.,* 2003; Westman and Kresovich, 1998). This approach gained momentum in plant genomics during the recent years based on the observation that despite a wide range in genome sizes, plants were found to exhibit extensive conservation of both gene content and gene order (Bennetzen and Freeling, 1993). Comparative genetic analyses have begun to show that different plant species often use homologous genes for very similar functions (Ahn *et al.,* 1993; Bennetzen and Freeling, 1997).

B. Importance of the subject

Bioinformatics approaches are increasingly being used for molecular marker development since the sequences from many genomes are made freely available in the public databases (Gu *et al.,* 1998; Kantety *et al.,* 2002; Varshney *et al.,* 2002). These sources could be mined for SSRs using computational tools thereby eliminating the need for costly library construction and screening required for obtaining sequence information. This not only reduces marker development costs but also permits development of a large number of markers in a short span of time. Additionally, bioinformatics tools also supplement existing approaches by automating the task of SSR identification from available DNA sequences. One source of sequences for marker development are expressed sequence tags (ESTs) that are obtained by sequencing 5' and/or 3' ends of a complementary DNA (cDNA) molecule synthesized from a messenger RNA (mRNA). ESTs are particularly attractive for marker development since they represent coding regions of the genome and are also being developed at an extremely faster pace for many genomes (Gu *et al.,* 1998; Kantety *et al.,* 2002; Picoult-Newberg *et al.,* 1999). Moreover, recent studies have observed that the frequency of microsatellites was significantly higher in ESTs than in genomic DNA in several plant species investigated (Morgante *et al.*, 2002; Toth *et al.*, 2000).

Most of the efforts to date for finding SSRs in EST sequences of plants focused on monocotyledonous crops (Kantety *et al.*, 2002; Thiel *et al.*, 2003; Varshney *et al.*, 2002). Also, efforts in the identification of transferable SSRs for comparative genetic studies in dicotyledonous species (dicots) were limited to a few studies where portability of SSRs was assessed between closely related species or genera (Decroocq *et al.*, 2003; Peakall *et al.*, 1998; Whitton *et al.*, 1997; Yamamoto *et al.*, 2001). Dicots comprise many economically important plants such as soybean, cotton, sunflower, tomato, potato, brassicas, beans etc., as well as the model plant for plant genomics, *Arabidopsis thaliana*. The discovery of SSRs from the ESTs of a large number of dicot species and information on the occurrence and frequency of different SSR categories is extremely valuable not only for the development of SSR markers in different species but also for understanding the abundance SSRs in dicots. Moreover, SSRs derived from ESTs essentially represent expressed genic sequences and hence are potential candidates for markers for comparative genomic studies.

C. Knowledge gap and contribution of the project

The most important requirement for the computational mining of SSRs is an efficient tool to accomplish the task. Although several public domain tools are available, as described in section III, they have one or more drawbacks because of which not all SSRs could be mined efficiently. For example, several existing programs do not identify sequences containing single nucleotide SSRs that are very useful for chloroplast genome analyses as well as for some crucial regions of nuclear genomes that do not contain di-, tri- or tetra-nucleotide repeats. Moreover, users of several of these tools have to spend a considerable amount of time in interpreting and organizing results. For some relatively efficient tools, the web interfaces are not available until a long time following their development and/or publication. Thus, there is a need for a simple, efficient and high-throughput SSR identification tool. Such a tool can quickly discover SSRs in previously unfathomed EST sequences in several dicot species or improve the SSR identification efficiency in other dicot species for which some work exists.

As mentioned in the previous section, although dicotyledons comprise many important crop species, large scale studies on SSR identification and survey in EST sequences are currently not available. SSR markers derived from ESTs could be used for standard genetic analysis and applications while some of them could even be used for cross-taxa or cross-specific comparative studies. However, efforts to date for identification of markers for comparative studies were limited to cereal or monocot species (Kantey *et al.,* 2002; Varshney *et al.,* 2002). This is due to the fact that a number of cereals were known to have remarkable micro-colinearities i.e., homology of DNA segments across different genomes (Bennetzen and Freeling, 1997; Bennetzen, 2000). Although a few reports of such studies exist in dicots, they are limited to comparisons of closely related species but not across different dicot plants (Decroocq *et al.,* 2003; Yamamoto *et al.,* 2001). Thus, there is a need for mining and survey of SSRs in a large number of dicotyledonous species.

The major contribution of this project is threefold: (i) developing an efficient tool for mining SSRs from large sequence datasets; (ii) mining of SSRs from ESTs of a large number of dicotyledonous genomes using the developed tool and surveying the occurrence and frequencies of various SSRs and (iii) identifying non-redundant EST sequences containing SSRs for marker development and other molecular genetic analyses.

II. Sequence data sources and Methods

A. Sequence data sources

EST sequences for all of the plants investigated in this project have been downloaded from GenBank at NCBI website (http://www.ncbi.nlm.nih.gov). Initially, a total of 53 dicotyledonous species were selected where the number of ESTs available per species ranged from 49 to 342,407 as of August 2003. In addition, two groups of sequences representing (i) additional *Rosa* species and hybrids and (ii) additional *Vicia* species were also selected to have more ESTs from these genera. As shown in Table 1 (Section IV), sequences of these selected species and groups have been grouped into a total of 49 dicotyledonous species sequence datasets for the purpose of the project. Five of these 49 sequence sets (*Citrus* spp., *Cucumis* spp., *Rosa* spp., *Vicia* spp., and *Vigna* spp.) represent groups that were generated by combining ESTs from two or more species belonging to the respective genera. Batch files of EST sequences for these species were downloaded from GenBank in FASTA format. It must be noted that for some of the genes, full-length cDNA sequences are available as opposed to ESTs. In those cases, entire cDNAs are utilized for mining purpose. Thus, for a given species, all entries in the GenBank that belong to EST or cDNA categories are included in the datasets.

B. SSR mining with RepeatFetcher

RepeatFetcher tool, the development of which is described in section III, has been used for the identification SSRs in EST sequences. This is a command line program and has been run on Phoenix server (phoenix.cs.iupui.edu) of IUPUI computer science department. Four classes of SSRs were targeted for identification using this program: mono-, di-, tri-, and tetra-nucleotide repeats. The default settings used in the program for minimum number of repeats is 15 for mononucleotides and 5 for di-, tri-, or tetra-nucleotides. In addition, the program also identifies interrupted repeats with a maximum interruption (spacer) of 7 base pairs. Batch files of the target species were exported to Phoenix server using an FTP protocol and were run through RepeatFetcher by passing the sequence file as input to the program at the command prompt. The output files from a given session were transferred to desktop using FTP protocol and were opened using Notepad or Wordpad programs for visualizing the results.

C. Trimming of poly A and poly T ends of ESTs

TRIMEST program of EMBOSS (http://ngfnblast.gbf.de/uk/emboss.html) has been used for removing the poly A / poly T ends of the EST sequences prior to running the sequence sets through RepeatFetcher so that they are not retrieved by the program as mononucleotide repeats. This is a publicly available free software tool and the interface available at http://ngfnblast.gbf.de/cgi-bin/emboss.pl?_action=input&_app=trimest has been used for this purpose. Batch sequences were loaded to the program using the ‘Browse’ option of the interface and the program was run using the following settings that were found to give optimal performance based on trial runs on several sequences:

Minimum length of a poly-A tail (integer): 4

Number of contiguous mismatches allowed in a tail (integer): 4

Write the reverse complement when poly-T is removed? Yes

Remove poly-T tails at the 5’ end of the sequence? Yes

D. EST clustering and analysis

SSR-containing EST sequences identified after using trimmed sequences were clustered by using Sequencher (Gene Codes Corporation, Ann Arbor, Michigan) or BAG program (at computing facility of IU, Bloomington) to eliminate redundancies. Contigs for 45 sequence sets were assembled using Sequencher, whereas the 4 large sequence datasets (*Arabidopsis thaliana*, *Glycine max*, *Lycopersicon esculentum* and *Medicago truncatula*) were clustered on a computer cluster at IU, Bloomington using BAG software developed by Dr. Sun Kim (Kim, 2003) using cut-off scores of 200, 300 and 400. For Sequencher contig assembly, the rigorous data algorithm option was chosen with the settings of minimum match percentage of 85 and a minimum overlap of 20 bases. In order to further correlate the BAG results with those of Sequencher that has been used for majority of the datasets (45 out of 49), the ESTs that were assembled into clusters by BAG program were run again on Sequencher. BAG results obtained with 400 cut-off score are in close agreement with Sequencher as judged by the minimum number of sequences left over as singletons by Sequencher. Clusters from all of the species were manually inspected, evaluated and redundancies were removed using the following criteria:

(i) If a contig has 2 or more identical sequences, longest EST sequence was retained

(ii) If the overlaps are just due to stretches of homopolymers i.e., poly A/T/C/G or SSRs, those contigs were not considered valid and all members were retained as unique

(iii) If the members of a contig were similar and of varying lengths, one representative member of each of the variant group has been retained such that the total possible contig length is retained through the selected members

(iv) Contigs generated due to overlap of ambiguous bases were not considered and the sequences with large stretches of ambiguous bases were discarded

(v) ESTs that contained short homopolymer stretches or SSRs as the only sequences were discarded

E. Compositional analysis of SSR mining results

The analysis of occurrence and frequency of SSRs among the 49 species was carried out by exporting the RepeatFetcher results to Microsoft Excel spreadsheets. Results on repeat types, number of repeats and frequency were first collected for each of the species by using a combination of sorting and counting functions and the results across all species were tabulated and significant results and observations were charted.

III. Development of a tool for SSR identification

A. Background

While SSRs, once developed, are very useful markers, their development is expensive since it requires prior knowledge of sequence information. The initial step in the development of SSR markers is the identification of sequences containing such repeats from the genomic sequences. This will be followed by the design of PCR primers flanking the SSR repeat stretch. There are two approaches for the identification of SSR containing sequences: 1) Molecular and 2) Computational. The molecular approach for the development of SSRs is to construct genomic libraries (with or without enrichment for SSRs), screen the libraries, sequence candidate clones and identify SSR motifs either manually or using computer programs. The computational or bioinformatics approaches take advantage of the available sequences such as those in the public databases and by scanning through them, they identify the ones that contain SSRs. They also supplement the molecular approaches by identifying SSR repeats in candidate sequences derived from the libraries.

Based on the nature of the algorithms used, the computational approaches for the identification of SSRs could be broadly divided into 1) Model-based approaches and 2) Dictionary approaches. In model-based approaches, a model is defined for a repeat and then used on the sequences to locate regions satisfying the definition. This approach provides a comprehensive list of repeats, either exact or degenerate, and does not require prior knowledge of the type of repeat motifs for its implementation. The programs such as Tandem Repeat Finder (Benson, 1999) and Sputnik (Abajian, 1994) belong to model-based approach. The dictionary approach provides a faster and more scalable solution to the problem of repeat identification when the repeat sequence motifs are known *a priori*. In this method, the program uses a dictionary of known motifs and scans the input sequence for the entries in the dictionary.

B. Review of existing SSR-finding tools

A high-throughput tool that can efficiently identify all SSR types and provides a simple and user-friendly output is a crucial prerequisite for this study. Before initiating the development of a program for SSR identification for this project, a survey and evaluation of existing SSR-finding tools has been conducted to see if any one of them serves the purpose of high throughput identification of SSR-containing sequences. The following criteria have been set as the ideal features for a desired SSR-identification program:

- Ability to identify mono-, di-, tri- and tetra-nucleotide repeats
- Ability to identify a compound repeat, i.e., a combination of two or more types of repeat motifs
- Ability to identify a large number of SSR types and locations in the target sequence
- Ability to identify repeats that are interrupted by a spacer sequence
- Ability to handle and process large batch files of thousands of sequences for SSR identification i.e., high throughput
- Ability to identify repeats in large input sequences that are of the order of several thousand or hundreds of thousands of base pairs (not for ESTs but for genomic sequences and large clones)
- Output should contain sequence ID, type, number and location of the repeats

Following is the assessment of dedicated or general-purpose SSR-finding tools for which web interfaces were available at the time of the survey.

Sputnik: This is a simple program written in C programming language that searches DNA sequence files in FASTA format for microsatellite repeats (Abajian, 1994). A sequence file is provided as an input to the program and the resulting hits are written to standard output along with their position in the sequence, length, and a score determined by the length of the repeat and the number of errors. Sputnik is intended to search for repeated patterns of nucleotides of length between 2 and 5. Insertions, mismatches and deletions are tolerated but affect the overall performance score. Sputnik is suited for low throughput applications and it cannot identify mononucleotide repeats. Also, it is not currently supported by a web interface.

FindPatterns: This is one of the programs available in the Genetics Computer Group (GCG), now Accelrys, package (www.accelrys.com). It looks through large data sets and identifies short nucleotide or amino acid patterns specified by the user. Although FindPatterns can recognize patterns with some symbols mismatched, it can not identify patterns that are interrupted. FindPatterns writes its results to an output file that can be directly used for other programs in the GCG package. This is convenient if the downstream applications are also part of GCG. However, FindPatterns is not very efficient in identifying compound repeats due to its inability to identify interrupted patterns. Moreover, the output does not quite match with the criteria set for efficient integration into other applications.

RepeatFinder: RepeatFinder is a web-based program specifically developed for the identification of SSRs (http://www.genet.sickkids.on.ca/~ali/ repeatfinder.html). This program was originally developed for identifying repeats in a single input sequence, however, later upgraded to handle batch files containing multiple sequences. Although

RepeatFinder is a good program for identifying SSRs from small to medium throughput datasets, currently it has the following limitations: (i) cannot identify single nucleotide repeats; (ii) slower performance with large batch files (iii) speed of the program reduces significantly for sequences larger than 3 kb; (iv) output is a single long concatenated sequence that makes the identification of individual sequences time consuming.

SSRIT (Simple Sequence Repeats Identification Tool): SSRIT is a simple program available through Gramene / Genome databases portal at Cornell University (http://brie2.cshl.org:8082/gramene/searches/ssrtool). The program as available is good for the identification of “perfect” simple sequence repeats and can handle moderate-sized datasets. Although the output does contain sequence ID, motif (repeat) type, no. of repeats, SSR start and end, it does have the following limitations against criteria: (i) the program currently is not capable of detecting mononucleotide repeats; (ii) the output is not perfected currently due to which it requires some additional work by the user which is especially cumbersome when dealing with medium-sized (hundreds of sequences) datasets.

After the development of the SSR identification tool for this project, interfaces for two new SSR identification tools were made available on the web: Tandem repeats finder or TRF (Benson, 1999) and Tandem repeat occurrence locator or TROLL (Castelo *et al.*, 2002). Out of these two programs, TROLL meets several of the criteria mentioned before. However, it still does not meet other criteria such as handling very large sequences and processing of very large batches of sequences. Moreover, it occasionally mis-identifies the motif due to a phenomenon called "motif shifting" wherein repeats are counted starting from second base of the 'repeat stretch' rather than the first base. This results in the non-identification of anticipated repeat motif type and recording of less than expected number of repeats.

C. Development of RepeatFetcher program

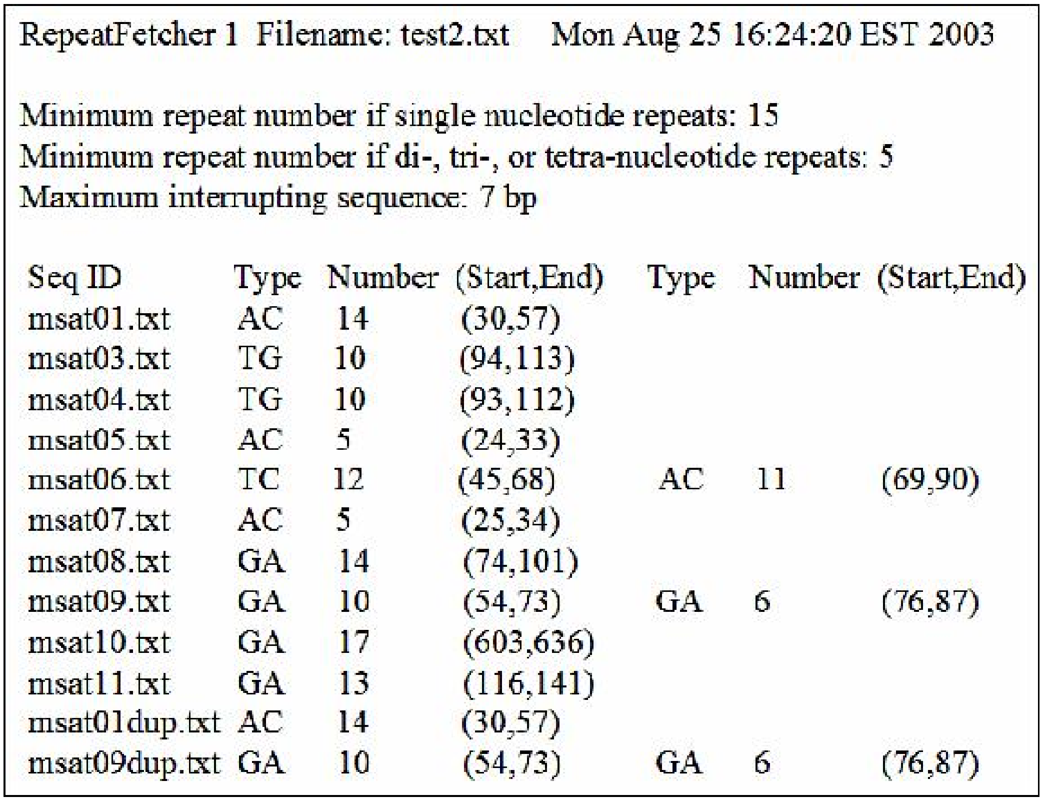
As discussed above, existing SSR-identification tools surveyed have one or more limitations with respect to the desirable features sought for an efficient and high-throughput program for this project as well as for routine use. Hence, an attempt has been made to develop a new program that meets the preset criteria. The result is a program, named RepeatFetcher that has been developed using Perl scripting language. This is a command-line program and has been run on Phoenix (Unix) platform for mining ESTs for this study. A brief outline of the algorithm behind RepeatFetcher is provided below:

<u>Algorithm</u>:

- The program has been created using the pattern recognition (regular expression) concept of Perl language
- Set the minimum number of repeats (mono-, di-, tri- and tetra-nucleotide) and maximum spacer length allowed in the program code
- Scan the sequence file (in FASTA format) from the beginning to the end and look for '>' sign and sequence ID headers, eliminate white spaces and specified characters; after finishing each line eliminate \n feature using 'chomp' function
- Find repeats with (2-4)n or (1-4)n pattern: Find the sequence length, divide it by the repeat size and move through the whole sequence by the repeat size; repeat the process for all frames and all repeat patterns; after completing the sequence, i.e, when the next '>' sign is encountered, repeat the process until the end of the file is reached

- Save the sequence ID, repeat type, number of repeats and the start and end of the repeats for all the 'hits' in an array and the ‘hits’ information in a file

In order to assess the functionality of the tool, several test files that interrogate different criteria were created and run through RepeatFetcher. Figure 1 shows the output from RepeatFetcher using a test file that has sequences containing one or more SSRs. As can be seen, RepeatFetcher has identified SSRs from all of the sequences and displayed the sequence IDs, repeat type, number and locations of each of the SSRs. RepeatFetcher has been shown to perform well on all datasets tested to date including large sequences such as the whole chromosome 1 sequence of yeast (230,207 bp).



```
RepeatFetcher 1  Filename: test2.txt     Mon Aug 25 16:24:20 EST 2003

Minimum repeat number if single nucleotide repeats: 15
Minimum repeat number if di-, tri-, or tetra-nucleotide repeats: 5
Maximum interrupting sequence: 7 bp

Seq ID          Type  Number  (Start,End)   Type  Number  (Start,End)
msat01.txt      AC     14      (30,57)
msat03.txt      TG     10      (94,113)
msat04.txt      TG     10      (93,112)
msat05.txt      AC     5       (24,33)
msat06.txt      TC     12      (45,68)       AC     11      (69,90)
msat07.txt      AC     5       (25,34)
msat08.txt      GA     14      (74,101)
msat09.txt      GA     10      (54,73)       GA     6       (76,87)
msat10.txt      GA     17      (603,636)
msat11.txt      GA     13      (116,141)
msat01dup.txt   AC     14      (30,57)
msat09dup.txt   GA     10      (54,73)       GA     6       (76,87)
```

Figure 1. Output from RepeatFetcher showing the results obtained using a test sequence file in FASTA format

IV. Mining of SSRs from EST sequences of dicotyledonous plants

The standard molecular biology method for developing SSR markers is the construction of small insert libraries followed by nucleic acid hybridization-based identification of candidate clones and sequencing (Liu *et al.*, 1996). While improved SSR enrichment methods reduce marker development costs, they still require some time-consuming steps for the development (Kumpatla *et al.*, 2004). Computational methods to mine SSRs from sequences freely available in public databases provide a novel alternative for rapid development of SSR markers with minimal development costs.

A. ESTs and target dicotyledonous species for the project

ESTs provide an attractive source for mining SSRs since they are fast accumulating in public databases due to current emphasis on functional genomics in several organisms. ESTs are sequences that are typically a few hundred base pairs in length that are obtained by the single-pass sequencing of the 5’ or 3’ ends of cDNA clones generated from gene transcripts. Currently there are more than 2 million ESTs available for major monocotyledonous species and more than 1.5 million ESTs for dicots. However, studies on multi-species computational SSR mining in plants to date were conducted on monocot species only. As mentioned in the introduction, dicots comprise of several economically important crop species and SSR mining from ESTs of these species could greatly enhance development of markers for genetic mapping, marker-assisted selection as well as for cross-species comparative studies. This study represents the first attempt to mine SSRs from EST sequences of a large number of dicotyledonous species using a computational tool. A total of 53 dicotyledonous species and two groups (genera) containing varying numbers of species have been chosen for this study (Table 1).

Dataset No.	**Species**	**Common name**	**No. of ESTs**
1	*Allium cepa*	Onion	1,269
2	*Arabidopsis thaliana*	Thale cress	252,033
3	*Arachis hypogaea*	Peanut	1,397
4	*Beta vulgaris*	Sugar beet	19,774
5	*Brassica napus*	Canola, Oilseed rape	37,548
6	*Brassica oleracea*	Cabbage, Cauliflower, Kale, Broccoli etc.,	269
7	*Brassica rapa*	Chinese cabbage, Turnip	5,570
8	*Capsicum annuum*	Chili pepper	22,601
9	*Cicer arietinum*	Chickpea	285
10	*Citrullus lanatus*	Watermelon	709
11	*Citrus* spp. *Citrus sinensis* *Citrus unshiu*	 Sweet orange Satsuma mandarin	10,990 (8,391) (2,599)
12	*Coffea arabica*	Coffee	496
13	*Crocus sativus*	Saffron crocus	553
14	*Cucumis* spp. *Cucumis melo* *Cucumis sativus*	 Muskmelon Cucumber	587 (77) (510)
15	*Daucus carota*	Carrot	209
16	*Glycine max*	Soybean	342,407
17	*Gossypium arboreum*	Tree cotton	38,932
18	*Gossypium barbadense*	Sea Island cotton	152
19	*Gossypium herbaceum*	Levant cotton, Indian cotton	200
20	*Gossypium hirsutum*	Upland cotton	13,847
21	*Helianthus annuus*	Sunflower	60,007
22	*Hevea brasiliensis*	Para rubber tree	1,005
23	*Ipomoea batatas*	Sweet potato	4,301
24	*Juglans regia*	Walnut	951
25	*Lactuca sativa*	Lettuce	68,242

Table 1. Dicotyledonous species selected for SSR mining and the ESTs available in the GenBank as of August, 2003

Dataset No.	Species	Common name	No. of ESTs
26	*Linum usitatissimum*	Flax	1,328
27	*Lotus corniculatus*	Bird’s-foot trefoil	36,379
28	*Lycopersicon esculentum*	Tomato	151,312
29	*Lycopersicon hirsutum*	Wild species of tomato	2,518
30	*Lycopersicon pennellii*	Wild species of tomato	8,361
31	*Manihot esculenta*	Cassava	889
32	*Medicago truncatula*	Barrel medic	187,933
33	*Mentha piperita*	Peppermint	1,329
34	*Nicotiana tabacum*	Tobacco	11,197
35	*Phaseolus coccineus*	Scarlet runner bean	20,129
36	*Phaseolus vulgaris*	French bean, Kidney bean	804
37	*Pisum sativum*	Pea	3,713
38	*Prunus armeniaca*	Apricot	4,685
39	*Prunus dulcis*	Almond	3,904
40	*Prunus persica*	Peach	10,286
41	*Pyrus communis*	Pear	263
42	*Raphanus sativus*	Radish	169
43	*Ricinus communis*	Castor bean	821
44	*Rosa* spp.		4,709
	Rosa chinensis	China rose	(1,800)
	Other Rosa spp.		(2,909)
45	*Solanum tuberosum*	Potato	95,611
46	*Spinacea oleracea*	Spinach	249
47	*Vicia* spp.		179
	Vicia faba	Broad bean	(148)
	Other Vicia spp.		(31)
48	*Vigna* spp.		307
	Vigna unguiculata	Black-eyed pea, Cowpea	(155)
	Vigna radiata	Mung bean	(103)
	Vigna angularis	Adjuki bean	(49)
49	*Vitis vinifera*	Grape	111,948
		TOTAL	**1,543,357**

Table 1 (contd). Dicotyledonous species selected for SSR mining and the ESTs available in the GenBank as of August, 2003

As indicated in Sequence data sources and Methods section (section II), these were grouped into 49 sequence datasets representing individual species as well as groups of closely related species under the same genera (Table 1, column 1). The 49 species datasets together contain a total of more than 1.5 million EST sequences.

B. Mining of SSRs using RepeatFetcher

For the initial SSR mining, sequence batch files of 49 species in FASTA format, downloaded from GenBank at NCBI website (www.ncbi.nlm.nih.gov/Entrez/html), were run through RepeatFetcher program. Table 2 (column 3) shows the number of SSR-containing ESTs for all the 49 species. A total of 250,641 ESTs or 16.24% of total available ESTs for the 49 species contained SSRs. In general, di-, tri- and tetra-nucleotides are widely used for molecular genetics work and hence a majority of the existing reports address the discovery of these SSRs only (Morgante and Olivieri, 1993; Varshney *et al.,* 2002). However, one of the goals of this study is to mine mono- (single) nucleotide SSRs in addition to the di-, tri- and tetra-nucleotide SSRs. Since ESTs are the target sequences for SSR mining, it must be noted that several of them still contain poly A or poly T sequence stretches at their ends unless they are trimmed prior to their submission to GenBank. For this reason, it is important to trim the poly A/T sequences prior to SSR mining otherwise the As and Ts at the ends of ESTs would be identified by the RepeatFetcher as mononucleotide repeats. The trimming step ensures that the A or T at the ends of ESTs are not identified by RepeatFetcher as SSRs.

All of the 49 sequence batch files were subjected to trimming using TRIMEST program of EMBOSS (www.hgmp.mrc.ac.uk/software/EMBOSS/Apps/trimest.html) using the settings mentioned in Methods section (section II). The trimmed sequences of

Species	Total ESTs	No. of SSR-containing ESTs		Percent of Trimmed ESTs out of total
		Before trimming	After trimming	
Allium cepa	1,269	181	116	9.14%
Arabidopsis thaliana	252,033	24,911	20,230	8.03%
Arachis hypogaea	1,397	175	158	11.31%
Beta vulgaris	19,774	3,287	2,222	11.24%
Brassica napus	37,548	5,620	5,471	14.57%
Brassica oleracea	269	88	40	14.87%
Brassica rapa	5,570	719	648	11.63%
Capsicum annuum	22,601	3,844	2,186	9.67%
Cicer arietinum	285	189	28	9.82%
Citrullus lanatus	709	73	63	8.89%
Citrus spp.	10,990	3,586	1,205	10.96%
Coffea arabica	496	175	29	5.85%
Crocus sativus	553	288	38	6.87%
Cucumis spp.	587	117	61	10.39%
Daucus carota	209	72	24	11.48%
Glycine max	342,407	49,091	28,959	8.48%
Gossypium arboreum	38,932	17,222	4,991	12.82%
Gossypium barbadense	152	41	17	11.18%
Gossypium herbaceum	200	34	33	16.5%
Gossypium hirsutum	13,847	3,257	1,682	12.15%
Helianthus annuus	60,007	5,176	3,708	6.18%
Hevea brasiliensis	1,005	248	168	16.72%
Ipomoea batatas	4,301	1,443	496	11.53%
Juglans regia	951	812	89	9.36%
Lactuca sativa	68,242	7,248	7,194	10.54%
Linum usitatissimum	1,328	192	186	14.01%

Table 2. Microsatellite-containing ESTs identified using RepeatFetcher

Species	**Total ESTs**	**No. of SSR-containing ESTs**		**Percent of Trimmed ESTs out of total**
		Before trimming	**After trimming**	
Lotus corniculatus	36,379	3,746	3,370	9.26%
Lycopersicon esculentum	151,312	14,540	11,189	7.39%
Lycopersicon hirsutum	2,518	158	153	6.07%
Lycopersicon pennellii	8,361	682	661	7.91%
Manihot esculenta	889	84	36	4.05%
Medicago truncatula	187,933	35,630	25,589	13.62%
Mentha piperita	1,329	307	222	16.70%
Nicotiana tabacum	11,197	857	481	4.30%
Phaseolus coccineus	20,129	1,943	1,257	6.24%
Phaseolus vulgaris	804	318	93	11.57%
Pisum sativum	3,713	426	230	6.19%
Prunus armeniaca	4,685	4,212	245	5.23%
Prunus dulcis	3,904	1,877	1,010	25.87%
Prunus persica	10,286	1,378	1,323	12.86%
Pyrus communis	263	86	17	6.46%
Raphanus sativus	169	43	14	8.28%
Ricinus communis	821	61	42	5.12%
Rosa spp.	4,709	912	726	15.42%
Solanum tuberosum	95,611	5,807	5,373	5.62%
Spinacea oleracea	249	93	43	17.27%
Vicia spp.	179	55	16	8.94%
Vigna spp.	307	105	30	9.77%
Vitis vinifera	111,948	49,232	10,753	9.61%
TOTAL	**1,543,357**	**250,641**	**142,915**	**9.26%**

Table 2 (contd). Microsatellite-containing ESTs identified using RepeatFetcher all 49 species were again subjected to SSR mining using RepeatFetcher. Table 2 (column 4) shows the SSR-containing ESTs obtained using the trimmed EST datasets. There is a reduction in the number of SSR-containing ESTs in all of the 49 species when trimmed sequences were subject to SSR identification (compare columns 3 and 4 of Table 2). As a result the total number of SSR-containing ESTs for the 49 species came down from 250,641 to 142,915, a 40% reduction. This reduction is much more pronounced in species such as *Glycine max*, *Gossypium arboreum*, *Prunus armeniaca* and *Vitis vinifera* suggesting that a majority of SSR-containing ESTs observed in these species when non-trimmed sequences were used for mining have poly A/T as the only available SSR repeat (Table 2, column 3, before trimming results). This observation strongly recommends the trimming of ESTs prior to SSR mining if the goal is to identify mononucleotide repeats. It must also be noted that TRIMEST is not infallible since it also trims non-poly A/T stretches of A/T that just happen to be at the ends of ESTs. Also, depending on the interruption of poly A/T stretches at the ends of ESTs, some of these stretches are not trimmed by the program. It is possible that some of the A/T mononucleotide SSRs observed in this study may have resulted from poly A/T that were not trimmed by TRIMEST. An analysis of trimming efficiency of several of the 49 species indicated that TRIMEST, in general trimmed majority, if not all, of the poly A/T.

In order to assess the frequency of SSRs in EST sequences of dicotyledonous species, percentages of SSR-containing ESTs (after trimming poly A/T) were calculated for all the species (Table 2, column 5). A total of 142,915 ESTs contained SSRs (9.26% of the total 1,543,357 ESTs). Among the 49 species, the frequency of SSR-containing ESTs ranged from 4.05% to 25.87% (Table 2, column 5) with 23 out of 49 species containing more than 10%. Studies on the abundance of SSRs in monocots revealed that SSRs were present in about 7% to 10% of the total ESTs (Varshney *et al.*, 2002).

Species	**ESTs subjected to clustering**	**No. of contigs (sequences fell into contigs)**	**Sequences / contig**	**Singletons**
Allium cepa	116	8(17)	2-3	99
*Arabidopsis thaliana**	20,230	2,032(10,122)	2-262	10,108
Arachis hypogaea	158	29(75)	2-10	83
Beta vulgaris	2,222	438(1,172)	2-13	1,050
Brassica napus	5,471	933(4,024)	2-102	1,447
Brassica oleracea	40	7(15)	2-3	25
Brassica rapa	648	86(233)	2-11	415
Capsicum annuum	2,186	326(1,120)	2-34	1,066
Cicer arietinum	28	1(2)	2	26
Citrullus lanatus	63	10(26)	2-5	37
Citrus spp.	1,205	152(773)	2-93	432
Coffea arabica	29	2(4)	2	25
Crocus sativus	38	7(14)	2	24
Cucumis spp.	61	5(14)	2-4	47
Daucus carota	24	2(4)	2	20
*Glycine max**	28,959	1,638(8,388)	2-85	20,571
Gossypium arboreum	4,991	630(2,657)	2-27	2,334
Gossypium barbadense	17	-	-	17
Gossypium herbaceum	33	2(5)	2-3	28
Gossypium hirsutum	1,682	211(755)	2-12	927
Helianthus annuus	3,708	442(2,431)	2-99	1,277
Hevea brasiliensis	168	18(81)	2-19	87
Ipomoea batatas	496	57(367)	2-68	129
Juglans regia	89	11(30)	2-5	59
Lactuca sativa	7,194	1,046(5,475)	2-264	1,719

Linum usitatissimum	186	29(63)	2-4	123

Table 3. Clustering results of microsatellite-containing ESTs

Species	**ESTs subjected to clustering**	**No. of contigs (sequences fell into contigs)**	**Sequences / contig**	**Singletons**
Lotus corniculatus	3,370	517(2,662)	2-115	708
*Lycopersicon esculentum**	11,189	712(4,147)	2-143	7,042
Lycopersicon hirsutum	153	14(45)	2-9	108
Lycopersicon pennellii	661	77(484)	2-46	177
Manihot esculenta	36	1(2)	2	34
*Medicago truncatula**	25,589	1,517(7,005)	2-96	18,584
Mentha piperita	222	34(110)	2-18	112
Nicotiana tabacum	481	40(81)	2-3	400
Phaseolus coccineus	1,257	184(870)	2-36	387
Phaseolus vulgaris	93	4(8)	2	85
Pisum sativum	230	24(126)	2-40	104
Prunus armeniaca	245	30(119)	2-33	126
Prunus dulcis	1,010	58(868)	2-158	142
Prunus persica	1,323	160(1,034)	2-322	289
Pyrus communis	17	1(2)	2	15
Raphanus sativus	14	2(4)	2	10
Ricinus communis	42	8(25)	2-11	17
Rosa spp.	726	70(438)	2-213	288
Solanum tuberosum	5,373	1,247(4,472)	2-61	901
Spinacea oleracea	43	6(13)	2-3	30
Vicia spp.	16	-	-	16
Vigna spp.	30	-	-	30
Vitis vinifera	10,753	1,418(8,931)	2-420	1,822

*Contigs obtained from the clustering of ESTs resulted from BAG program

Table 3 (contd). Clustering results of microsatellite-containing ESTs

Compared to these numbers, the observed frequencies in several of the dicotyledonous species are much higher. Two most likely reasons for these observations are: (i) the frequency estimates in some species may not represent the actual values due to the availability of smaller number of ESTs and (ii) several of the ESTs in species with high frequency of SSR-ESTs may be redundant.

C. Clustering and identification of non-redundant ESTs

In order to address the possibility of redundancy among ESTs, all of the SSR-containing ESTs (after trimming) were subjected to clustering using Sequencher software (www.genecodes.com) as per the settings mentioned in Methods. Since Sequencer can not handle larger datasets (>5,000 sequences) efficiently, they were first run through BAG clustering program (Kim, 2003) followed by a second clustering analysis using Sequencher. Table 3 provides EST clustering results for the 49 species. As can be seen from number of contigs obtained (column 3), contigs were obtained in all but three species (*Gossypium barbadense*, *Vicia* spp. and *Vigna* spp.). Whereas about half of the ESTs in species such as *Arabidopsis thaliana*, *Beta vulgaris* and *Capsicum annuum* fell into contigs (Table 3, column 3, contigs and the number of sequences per contig), majority of the ESTs remained as singletons (unique) in case of *Glycine max*, *Medicago truncatula* etc. One likely reason for the presence of a large proportion of singletons in these species is the elimination of redundancies prior to the loading of sequences in GenBank.

The number of sequences per cluster (contig) varied widely. Species such as *Arabidopsis thaliana*, *Lactuca sativa*, *Prunus persica*, *Vitis vinifera* recorded some large clusters containing as many as 260 sequences per cluster. As described in Methods, all of the clusters were manually analyzed as per preset criteria and only unique/representative members from each cluster were retained which, together with the singletons, formed the

Species	**Total ESTs**	**EST-SSRs after trimming**	**NR ESTs containing SSRs**
Allium cepa	1,269	116 (9.14%)	109 (8.59%)
Arabidopsis thaliana	252,033	20,230 (8.03%)	12,693 (5.04%)
Arachis hypogaea	1,397	158 (11.31%)	127 (9.10%)
Beta vulgaris	19,774	2,222 (11.24%)	1,575 (7.97%)
Brassica napus	37,548	5,471 (14.57%)	2,602 (6.93%)
Brassica oleracea	269	40 (14.87%)	37 (13.75%)
Brassica rapa	5,570	648 (11.63%)	515 (9.07%)
Capsicum annuum	22,601	2,186 (9.67%)	1,508 (6.67%)
Cicer arietinum	285	28 (9.82%)	27 (9.47%)
Citrullus lanatus	709	63 (8.89%)	51 (7.19%)
Citrus spp.	10,990	1,205 (10.96%)	669 (6.09%)
Coffea arabica	496	29 (5.85%)	28 (5.64%)
Crocus sativus	553	38 (6.87%)	32 (5.79%)
Cucumis spp.	587	61 (10.39%)	53 (9.03%)
Daucus carota	209	24 (11.48%)	23 (11.00%)
Glycine max	342,407	28,959 (8.48%)	23,367 (6.82%)
Gossypium arboreum	38,932	4,991 (12.82%)	3,616 (9.29%)
Gossypium barbadense	152	17 (11.18%)	17 (11.18%)
Gossypium herbaceum	200	33 (16.5%)	30 (15.00%)

Gossypium hirsutum	13,847	1,682 (12.15%)	1,324 (9.56%)

Table 4. Non-redundant (NR) ESTs containing SSRs

Species	**Total ESTs**	**EST-SSRs after trimming**	**NR ESTs containing SSRs**
Helianthus annuus	60,007	3,708 (6.18%)	2,117 (3.53%)
Hevea brasiliensis	1,005	168 (16.72%)	108 (10.75%)
Ipomoea batatas	4,301	496 (11.53%)	213 (4.95%)
Juglans regia	951	89 (9.36%)	160 (16.82%)
Lactuca sativa	68,242	7,194 (10.54%)	3,346 (4.90%)
Linum usitatissimum	1,328	186 (14.01%)	181 (13.63%)
Lotus corniculatus	36,379	3,370 (9.26%)	1,356 (3.73%)
Lycopersicon esculentum	151,312	11,189 (7.39%)	7,143 (4.72%)
Lycopersicon hirsutum	2,518	153 (6.07)	123 (4.88%)
Lycopersicon pennellii	8,361	661 (7.91%)	440 (5.26%)
Manihot esculenta	889	36 (4.05%)	35 (3.94%)
Medicago truncatula	187,933	25,589 (13.62%)	19,962 (10.62%)
Mentha piperita	1,329	222 (16.70%)	155 (11.66%)
Nicotiana tabacum	11,197	481 (4.30%)	449 (4.01%)
Phaseolus coccineus	20,129	1,257 (6.24%)	614 (3.05%)
Phaseolus vulgaris	804	93 (11.57%)	90 (11.19%)
Pisum sativum	3,713	230 (6.19%)	139 (3.74%)
Prunus armeniaca	4,685	245 (5.23%)	178 (3.80%)
Prunus dulcis	3,904	1,010	281

		(25.87%)	(7.20%)
Prunus persica	10,286	1,323 (12.86%)	513 (4.99%)
Pyrus communis	263	17 (6.46%)	16 (6.08%)

Table 4 (contd). Non-redundant (NR) ESTs containing SSRs

Species	**Total ESTs**	**EST-SSRs after trimming**	**NR ESTs containing SSRs**
Raphanus sativus	169	14 (8.28%)	12 (7.10%)
Ricinus communis	821	42 (5.12%)	26 (3.17%)
Rosa spp.	4,709	726 (15.42%)	379 (8.05%)
Solanum tuberosum	95,611	5,373 (5.62%)	2,532 (2.65%)
Spinacea oleracea	249	43 (17.27%)	36 (14.46%)
Vicia spp.	179	16 (8.94%)	16 (8.94%)
Vigna spp.	307	30 (9.77%)	31 (10.10%)
Vitis vinifera	111,948	10,753 (9.61%)	3,594 (3.21%)
TOTAL	**1,543,357**	**142,915 (9.26%)**	**92,648 (6.00%)**

Table 4 (contd). Non-redundant (NR) ESTs containing SSRs

non-redundant (NR) ESTs containing SSRs. Table 4 shows the number of SSR-containing non-redundant ESTs obtained for each of the 49 species (column 4) in comparison to the SSR-ESTs obtained after trimming (column 3). It is clear from the comparison that there is a marked decrease in the number of ESTs due to the elimination of redundancies. In particular, few hundred to few thousand sequences were eliminated from the datasets containing >2,000 sequences. For example, *Arabidopsis thaliana*, *Glycine max* and *Medicago truncatula* lost 37.3%, 19.3% and 22.0% sequences, respectively, due to elimination of redundancies (Table 4, compare columns 3 and 4).

Examples on the higher side include species such as *Lactuca sativa* and *Vitis vinifera* that lost 53.4% and 66.6% of the sequences, respectively, compared to SSR-ESTs obtained after trimming (compare values from Table 4, columns 3 and 4).

The total number of non-redundant ESTs mined from all 49 species thus came to 92,648 which is 6.0% of the 1.54 million dicotyledonous ESTs investigated in this study. The frequency of non-redundant-ESTs containing SSRs among 49 species ranged from 2.65% to 16.82%. Thirty three of the 49 species had more than 5% SSR containing NR ESTs out of total ESTs and further breakdown of the frequency among these 33 revealed that 12, 9 and 12 species had frequencies in the ranges of 5%-8%, 8%-10% and >10%, respectively. It must be noted that the frequencies in species with smaller number of available ESTs may not represent the actual scenarios.

Based on the frequencies observed in 18 species containing large number of sequences (>10,000) (Table 4, column 4), 13 of them had frequencies in the range of 4.72% to 10.75% whereas the remaining 5 had frequencies in the range of 2.65% to 3.73%. Based on the data from these 18 species it can be concluded that the frequency of SSRs in dicotyledonous plants ranges from 2.65% to 10.75% and that half of these species contain SSR-containing ESTs in the frequency range of 6.09% to 10.75%. Thus, about half of the 18 dicotyledonous species with large datasets have SSR-containing ESTs frequencies comparable to the 7% to 10% range observed for monocots (Varshney *et al.*, 2002), whereas the remaining have relatively lesser frequencies. It is possible that more ESTs would have been identified in these species with lesser frequencies if lower stringencies had been implemented in eliminating redundancies.

V. Analysis of occurrence and frequency of different SSR motifs

Simple sequence repeats have proven to be highly abundant and uniformly distributed in human and other mammalian genomes (Weber and May, 1989). Several studies have demonstrated the occurrence, distribution, informativeness and Mendelian inheritance of SSRs in plant genomes also (Wang *et al.,* 1994 and references therein). It has also been reported that SSRs occur as frequently as once in about 6 kb in case of plant genomes (Cardle *et al.,* 2000). Recent studies on several plant genomes have also demonstrated that the frequencies of SSRs were significantly higher in ESTs than in genomic DNA (Morgante *et al.,* 2002). The knowledge of the occurrence and frequency of different types of SSRs in different genomes is valuable not only for an understanding of their distribution but also in developing SSR markers for genetic analysis and diagnostics. To this end the frequencies of different classes and types of SSRs has been carried out for the dicotyledonous species investigated in this study.

A. Single vs. Multiple SSR stretches

ESTs, in general, are a few hundred base pairs in length and could harbor one or more stretches of SSRs. The utility of a given EST containing multiple SSRs could be higher since one or more of the available SSRs could be variable thereby improving the usefulness of that EST in developing a marker. Figure 2 (a, b) shows the frequency of ESTs containing a single or multiple (2 or more) SSRs among the non-redundant SSR-containing ESTs. It is clear that in all the 49 species investigated, ESTs containing a single SSR stretch are predominant. While the frequency of single SSR-containing ESTs range from 71.3% (*Hevea brasiliensis*) to 100% (*Vicia* spp.) majority of the species had 80% or more ESTs containing single SSRs. In species that have large number of non-redundant SSR-containing ESTs (>10,000) such as *Arabidopsis thaliana*, *Glycine max* and *Medicago truncatula*, the percent of single SSR-containing ESTs is 84.4%, 83.1% and 87.2%, respectively. The average frequency of ESTs containing multiple SSRs across all 49 species is about 14%. It is interesting to note that although the total number of ESTs containing SSRs is lower in *Hevea brasiliensis* and *Linum* usitatissimum, the

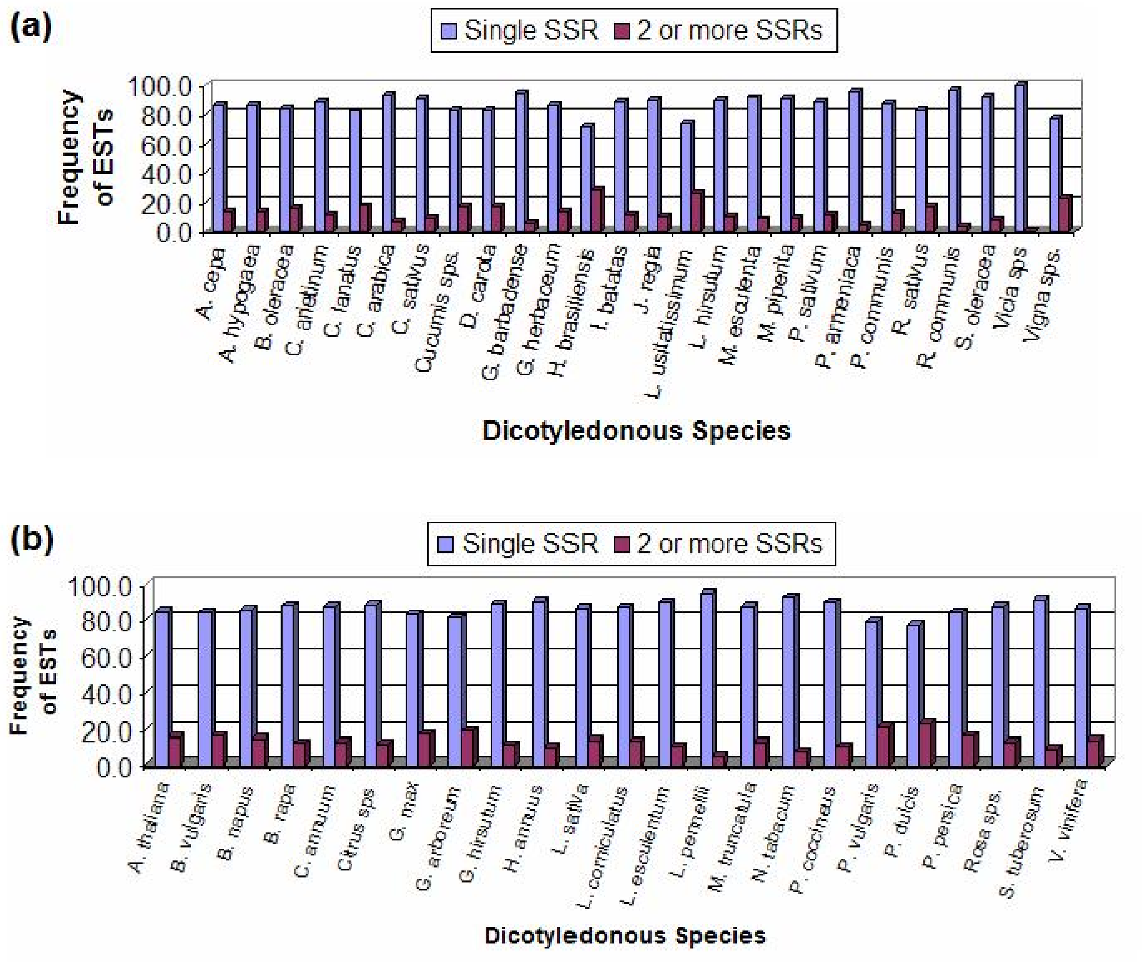


Figure 2. ESTs containing single or multiple stretches of SSR repeats

number of ESTs with multiple SSRs in these species is as high as 28.7% and 26.0%, respectively.

B. Distribution of repeat length classes in ESTs

The relative abundance of mono-, di-, tri- and tetra-nucleotide repeats in all of the 49 dicotyledonous species were determined by calculating their frequencies in NR ESTs containing single SSR stretches. Figure 3 (a, b, c) shows these different classes of SSRs. While the length of the bars shows the frequency of classes, the numbers inside them indicate the actual number of sequences of that particular category. Theoretically, the probability of finding mononucleotide repeats in a genome is higher followed by dinucleotide repeats and then by trinucleotide repeats followed by tetranucleotide repeats. While the results observed for ESTs in Figure 3 show this trend for some species (*A. cepa*, *H. brasiliensis*, *L. usitatissimum*, *P. armeniaca*, *C. annuum*, *G. arboreum*, *G. hirsutum* and *M. truncatula*), in majority of the remaining species (38 species), dinucleotide SSRs are the most abundant followed by tri- or mononucleotide repeats. On the other hand, trinucleotide repeats are the most abundant class of repeats in *C. arabica* and *L. sativa*. In *M. piperita*, di- and tri-nucleotide repeats are observed in equal proportions while the mononucleotide repeats are predominant class. Computational mining and analysis of SSRs in ESTs of some cereal species revealed that trimeric repeats are the most abundant class followed by dinucleotide repeats (Varshney *et al.*, 2002). Although the number of ESTs available in several of the dicotyledonous species investigated here is small, nevertheless the general trend shows that dinucleotides are the most abundant repeats in 38 out of the 49 species followed by mono- or tri-nucleotide repeats. Excluding mononucleotide repeats, di- and trinucleotides are the most abundant in ESTs in all of the species investigated.

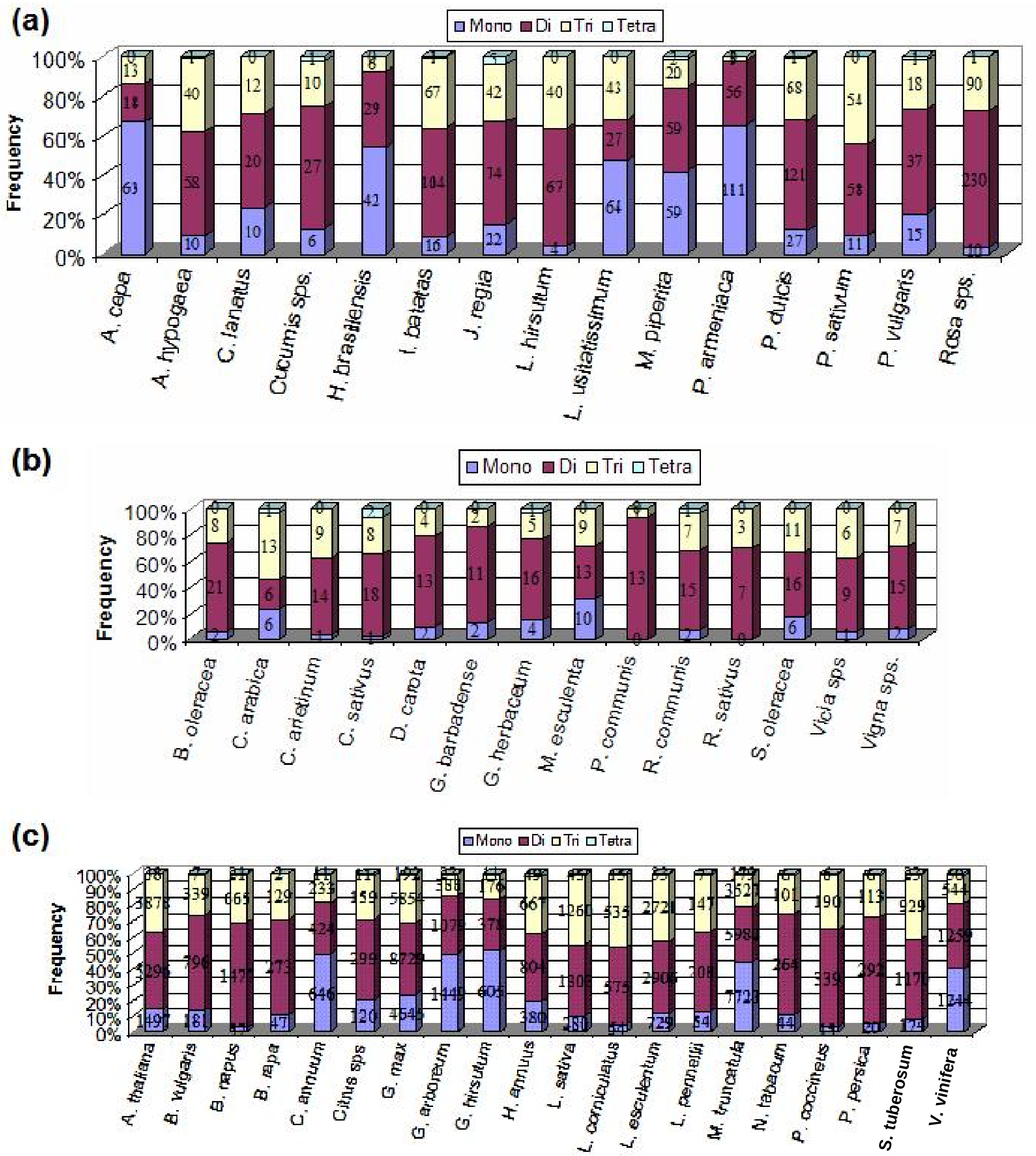


Figure 3. Frequency of mono-, di-, tri- and tetra-nucleotide SSRs in ESTs containing a single SSR.

C. Relative frequencies of different SSR repeat types

The available SSR motif combinations could be grouped into unique classes based on the property of DNA base complementarity. For mononucleotides, although A, T, C and G are possible, A and T could be grouped into one since an A repeat on one strand is same as a T repeat on the opposite strand and a polyC on one strand is the same as a polyG on the opposite strand, resulting in two unique classes of mononucleotides, A/T and C/G (Katti *et al.,* 2001). Similarly, all dinucleotides can be grouped into four unique classes: (i) AT/TA; (ii) AG/GA/CT/TC; (iii) AC/CA/TG/GT and (iv) GC/CG. Thus, the number of unique classes possible for mono-, di-, tri- and tetra-nucleotide repeats is 2, 4, 10 and 33, respectively (Katti *et al.,* 2001; Jurka and Pethiyagoda, 1995).

The relative frequencies of repeats were calculated for 20 species (Figures 4, 5, 6 and 7) that have >1,000 non-redundant SSR-containing ESTs in order to obtain relevant estimates. Moreover, the frequency estimates shown are based on the total number of SSRs observed in all NR ESTs that have either single or multiple SSRs. Figure 4 shows the frequencies of A/T and C/G repeats. It is clear that A/T repeats are the predominant mononucleotides in all of the 20 species. It can also be seen that A/T SSRs represent more than 40% of the total SSRs in *C. annuum*, *G. arboreum* and *G. hirsutum* species. It is also interesting to note that although not abundant as A/T repeats, C/G repeats make up as much as 15% of the total SSRs observed in *G. arboreum*. Relative frequencies of four unique classes of dinucleotide repeats are shown in Figure 5. Out of the dinucleotide repeats, AG/GA/CT/TC group is the predominant class of dinucleotide repeats in all of the species investigated (14.6% to 54.5% of the total SSRs observed in a species) except *L. pennelli* (Figure 5, b) where AT/TA repeats are present at a slightly higher frequency

(23.6%) than AG/GA/CT/TC class (21.2%). AT/TA is the second most abundant dinucleotide repeat type in all of the species investigated excluding *N. tabacum* where

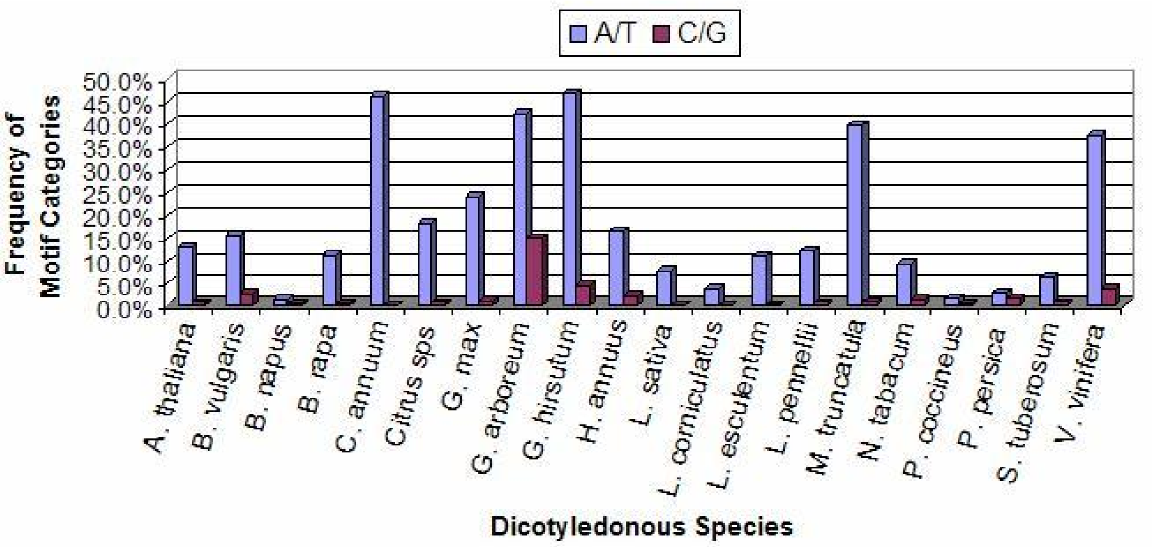
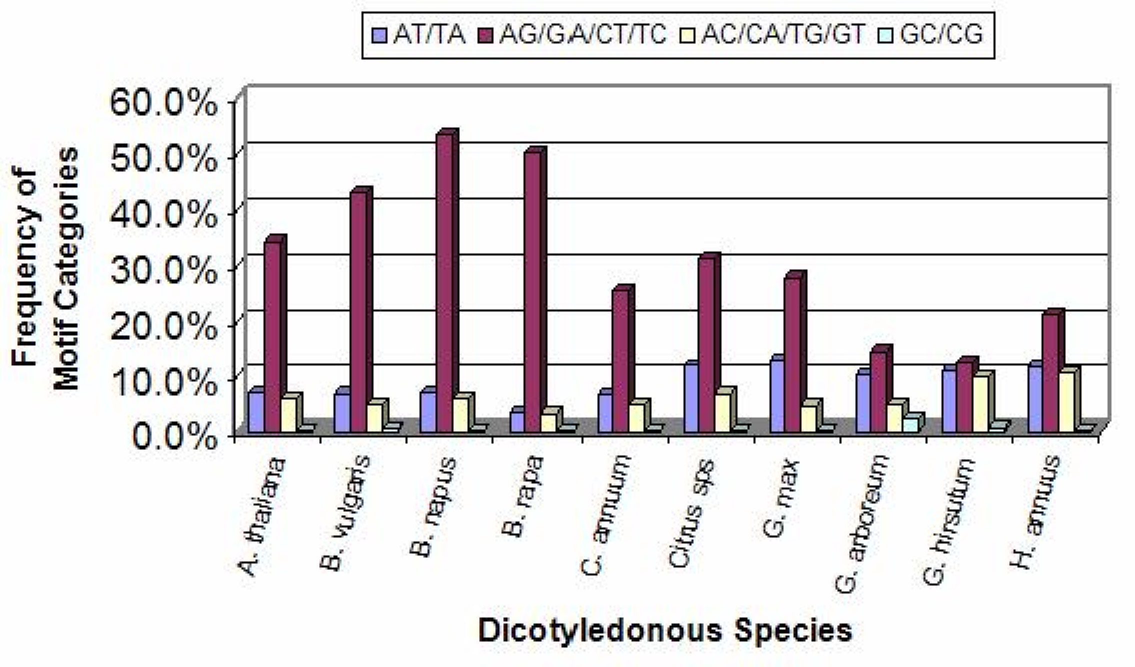


Figure 4. Frequency of mononucleotide SSRs in ESTs of dicotyledonous plants

AC/CA/TG/GT is the abundant class next to AG/GA/CT/TC (Figure 5, b). That AG/GA/CT/TC is the predominant class of repeats is in concurrence with the results observed by Varshney *et al.,* (2002) in some cereal species. However, the second most abundant repeat observed by Varshney *et al.,* (2002) was AC repeat (same as AC/CA/TG/GT group in the present study), whereas AT/TA is the second most frequent repeat in the present study following AG/GA/CT/TC.

An analysis of the frequencies of trinucleotide repeats out of total SSRs observed indicate the predominance of AAG/AGA/GAA/CTT/TTC/TCT repeat class in 16 out of 20 species (2.7% to 15.5% of all the SSRs available in those species) (Figures 6 and 7). Out of the remaining 4 species, the class ACC/CCA/CAC/GGT/GTG/TGG is predominant in two species (*H. annuus* and *L. sativa*), ATG/TGA/GAT/CAT/ATC/TCA is the predominant class in one species (*B. vulgaris*) and AGC/GCA/CAG/GCT/CTG/TGC is the most frequent class in other (*Citrus*) species.



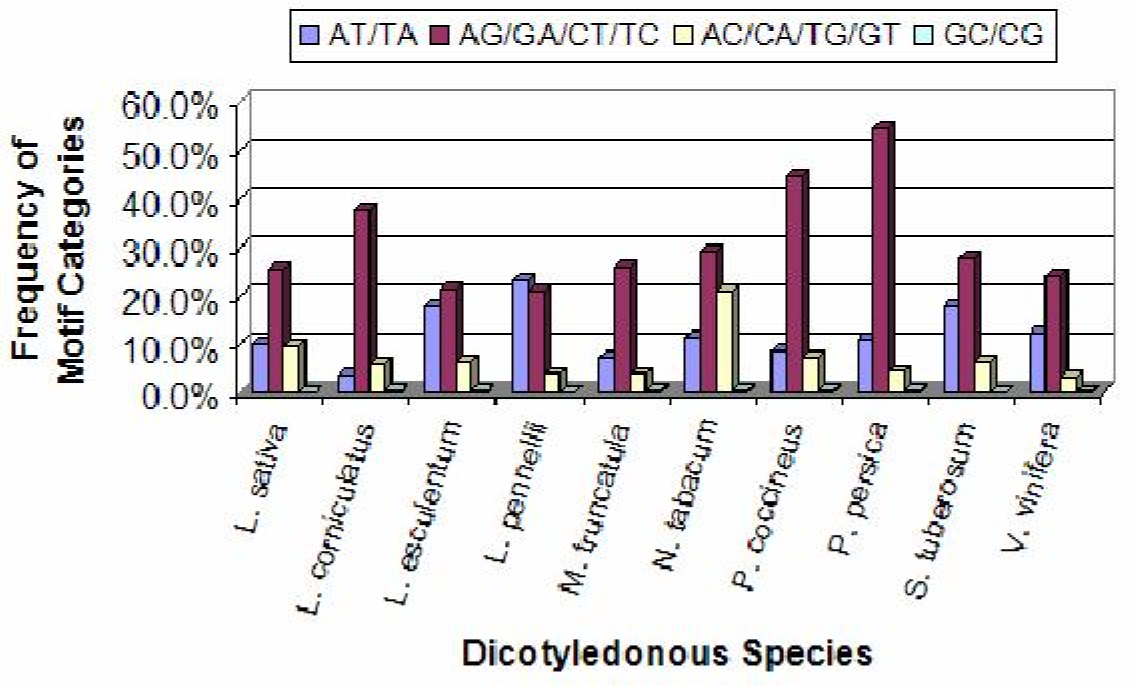


Figure 5. Frequencies of dinucleotide SSRs in ESTs of dicotyledonous plants

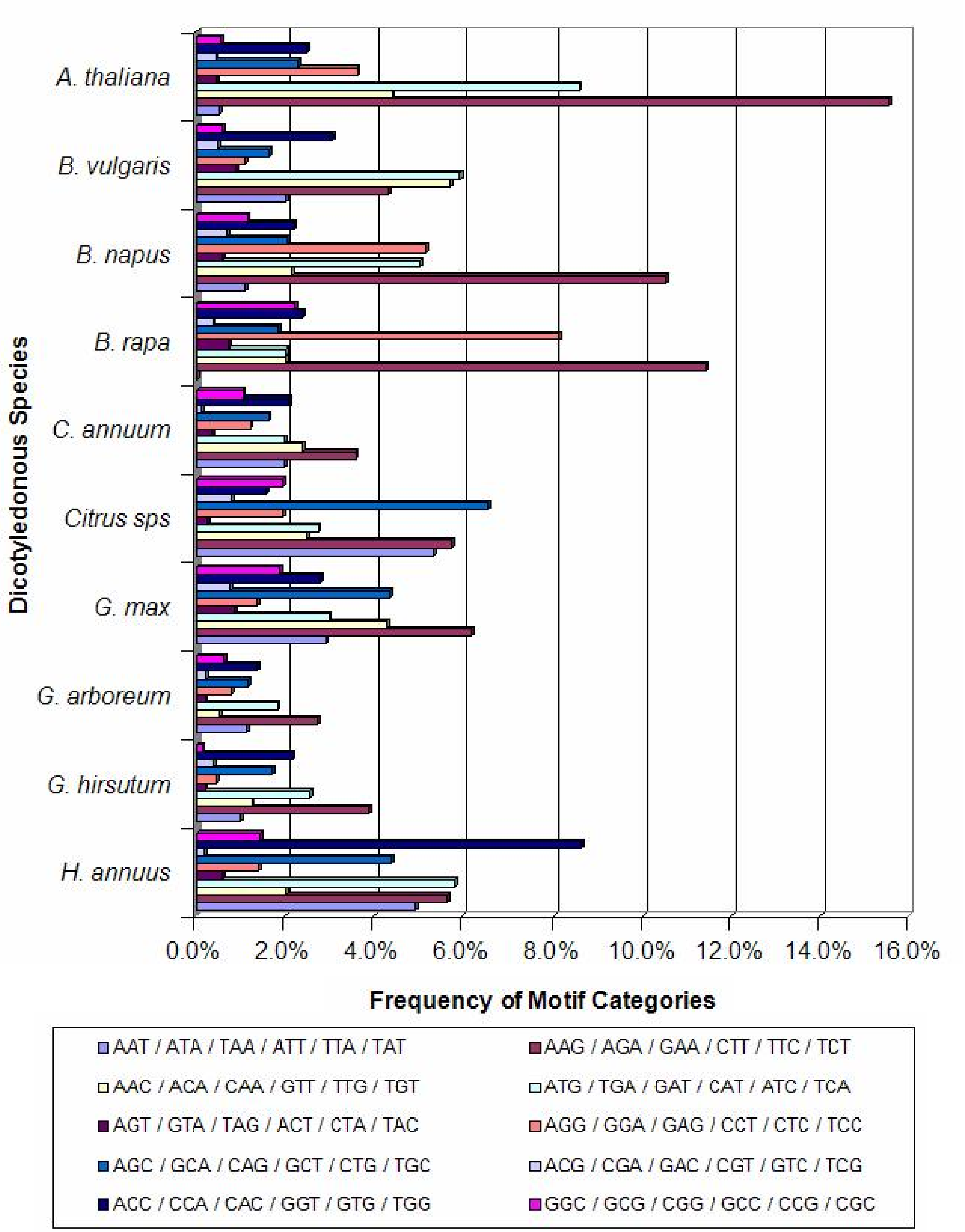


Figure 6. Frequencies of trinucleotide SSRs in ESTs of dicotyledonous plants

(Set 1)

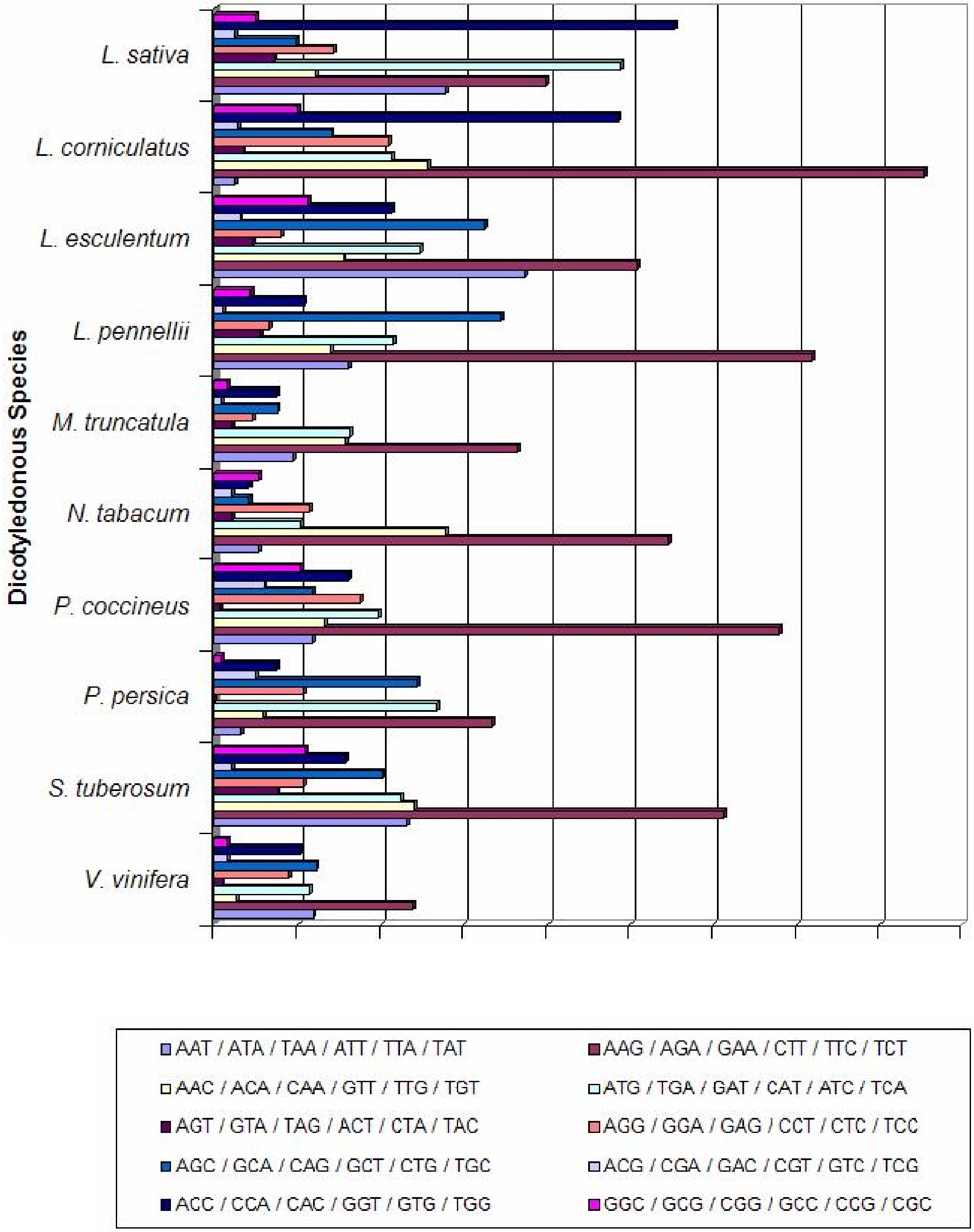


Figure 7. Frequencies of trinucleotide SSRs in ESTs of dicotyledonous plants

(Set 2)

The second most frequent repeat class is different across the 20 species. While ATG/TGA/GAT/GAT/ATC/TCA is the second frequent repeat class in 8 species, AAC/ACA/CAA/GTT/TTG/TGT is the second predominant in 5 species. Varshney *et al.,* (2002) observed that the CCG trinucleotide repeat (belongs to the GGC/GCG/CGC/GCC/CCG/CGC class) is the most predominant SSR in cereal species. However, this repeat is not the predominant class in any of the 20 species investigated here for which large numbers of ESTs are available. This probably reflects the higher G+C content of monocot species compared to dicots (Morgante *et al.,* 2002).

As mentioned above, 33 unique classes of tetranucleotide repeats are possible. However, only a small number of tetranucleotides were observed among the species investigated here. The top 5 species containing the highest number of tetranucleotides are *G. max*, *M. truncatula*, *V. vinifera*, *L. sativa* and *H. annuus* with 256, 233, 67, 61 and 56 repeats, respectively.

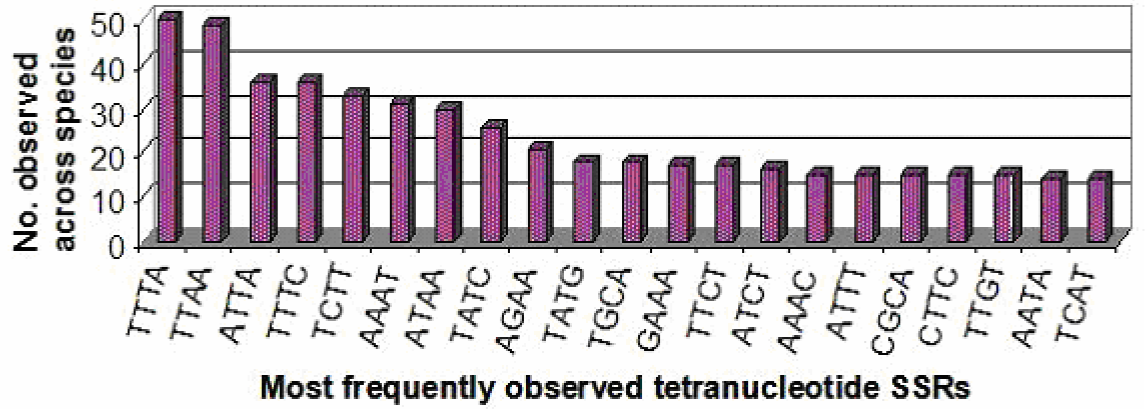


Figure 8. Predominant tetranucleotide SSRs in ESTs of dicotyledonous plants

Since the numbers are too low for frequency estimates in individual species, all of the observed tetranucleotide repeats for 20 species were collated in order to identify the most frequent tetranucleotide SSRs across these dicotyledons. Figure 8 shows the top 21 tetranucleotide repeats observed in these species. The TTTA and TTAA repeats seem to be the most abundant SSRs followed by ATTA and TTTC.

Thus, the analysis of mono-, di- and tri-nucleotide repeats across 20 dicotyledonous species shows that A/T mononucleotide repeats, AG/GA/CT/TC dinucleotides and AAG/AGA/GAA/CTT/TTC/TCT trinucleotides are the predominant repeat types in majority of the species. Although the number of tetranucleotide repeats observed are low, trends show that TTTA and TTAA are found most frequently in these dicotyledonous species compared to other tetranucleotides.

D. Relative frequencies of different SSR repeat length classes

One of the important features of SSRs that make them ideal candidates for genetic analysis is their highly polymorphic nature, i.e., a large number of allelic variants are possible across different genotypes (Akkaya *et al.*, 1992; Powell *et al.*, 1996). A knowledge of the distribution of SSRs into different repeat length classes is useful in assessing the abundance of potentially informative markers. It is a general experience in molecular genetics community that the utility or informativeness of SSRs increases with increased number of repeats in a given SSR stretch. For example, di- and tri-nucleotide repeats with 5 or more repeats are very likely to be informative compared to 2-4 repeats. This is the reason behind choosing 5 repeats as the minimum criteria for di- and tri-nucleotide repeats mining using RepeatFetcher program. In order to assess the frequency of SSRs belonging to different repeat length classes (number of repeats) in 20 species containing large number of NR SSR-ESTs, three classes were arbitrarily chosen for mono-, di- and tri-nucleotide repeats (see Figures 9, 10 and 11).

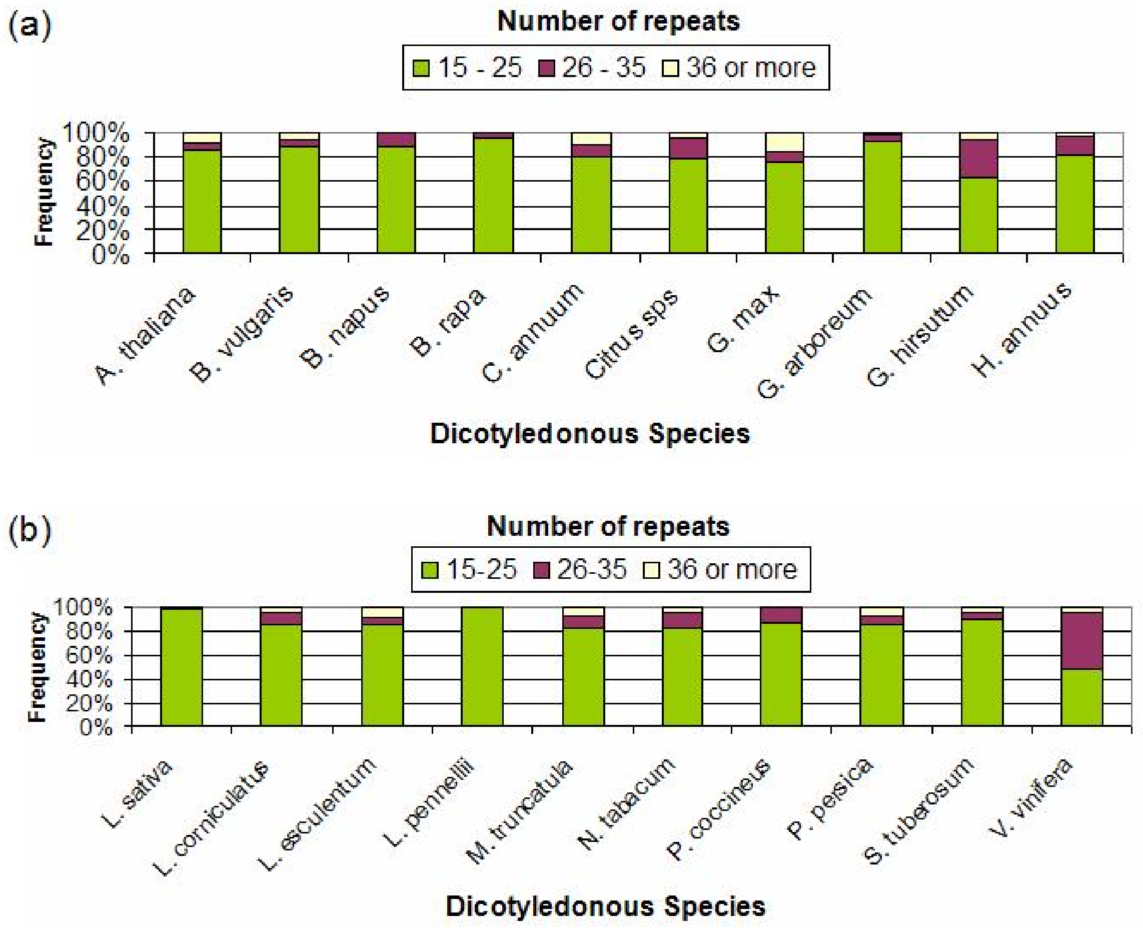


Figure 9. Distribution of mononucleotide SSRs into different repeat length classes

While the three classes chosen for mononucleotide SSRs are 15-25 repeats, 26-35 repeats and 36 or more repeats, the classes chosen for di- and tri-nucleotide repeats are 5-10 repeats, 11-16 repeats and 17 or more repeats. As can be seen from Figure 9, in 19 out of 20 species, majority of mononucleotide SSRs fall in 15-25 repeat class followed by 26-35 repeat class. In case of *Vitis vinifera*, the 15-25 and 26-35 classes share nearly equal proportions of the SSRs. Although SSRs with 36 or more repeats are less frequent, in species like *Glycine max*, they make up as much as 15.3% of the total mononucleotide SSRs and are even more frequent than 26-35 repeat class (9.3%) in this species.

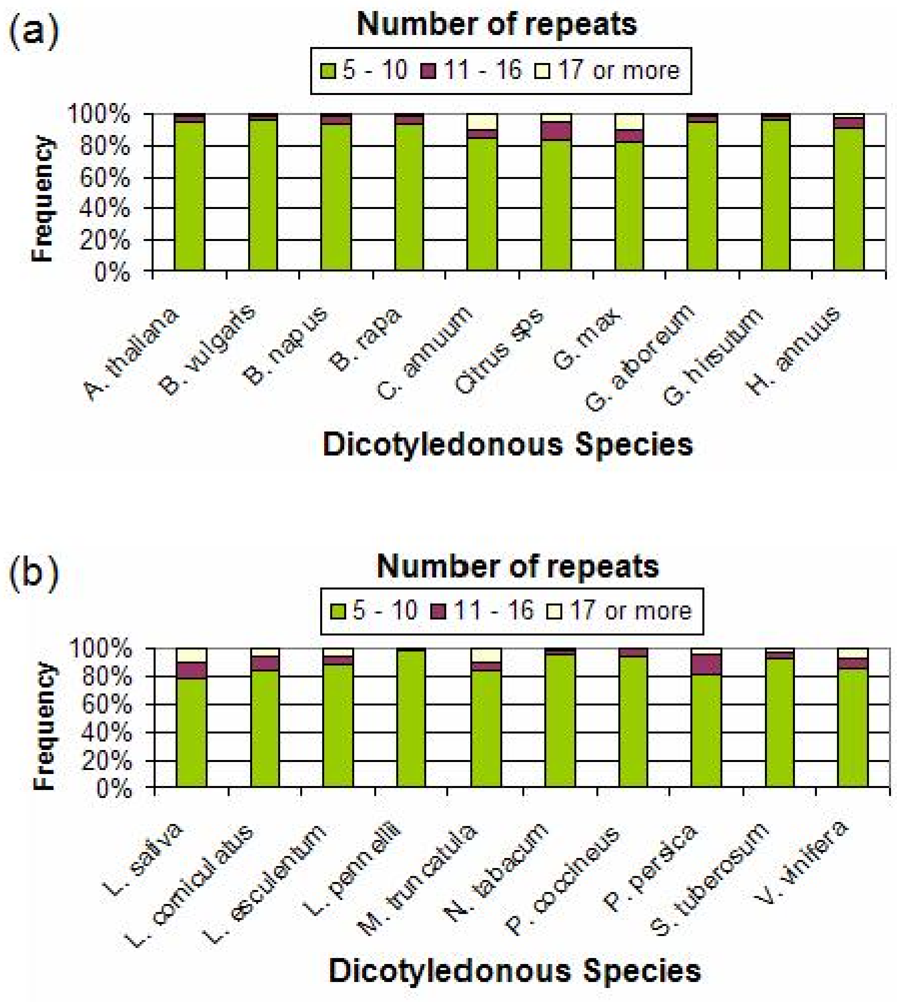


Figure 10. Distribution of dinucleotide SSRs into different repeat length classes

Distribution of dinucleotide SSRs (Figure 10) shows that in majority of the species, they fall in the class of 5-10 repeats followed by 11-16 repeat class. Exceptions to this generalization include *C. annuum, G. max* and *M. truncatula* species where more SSRs are observed in 17 or more repeat class compared to 11-16 class.

With respect to the distribution of trinucleotide SSR distribution into repeat length classes, as shown in Figure 11, the 5-10 repeat class is predominant in all of the species investigated containing more than 99% of the SSRs in this class.

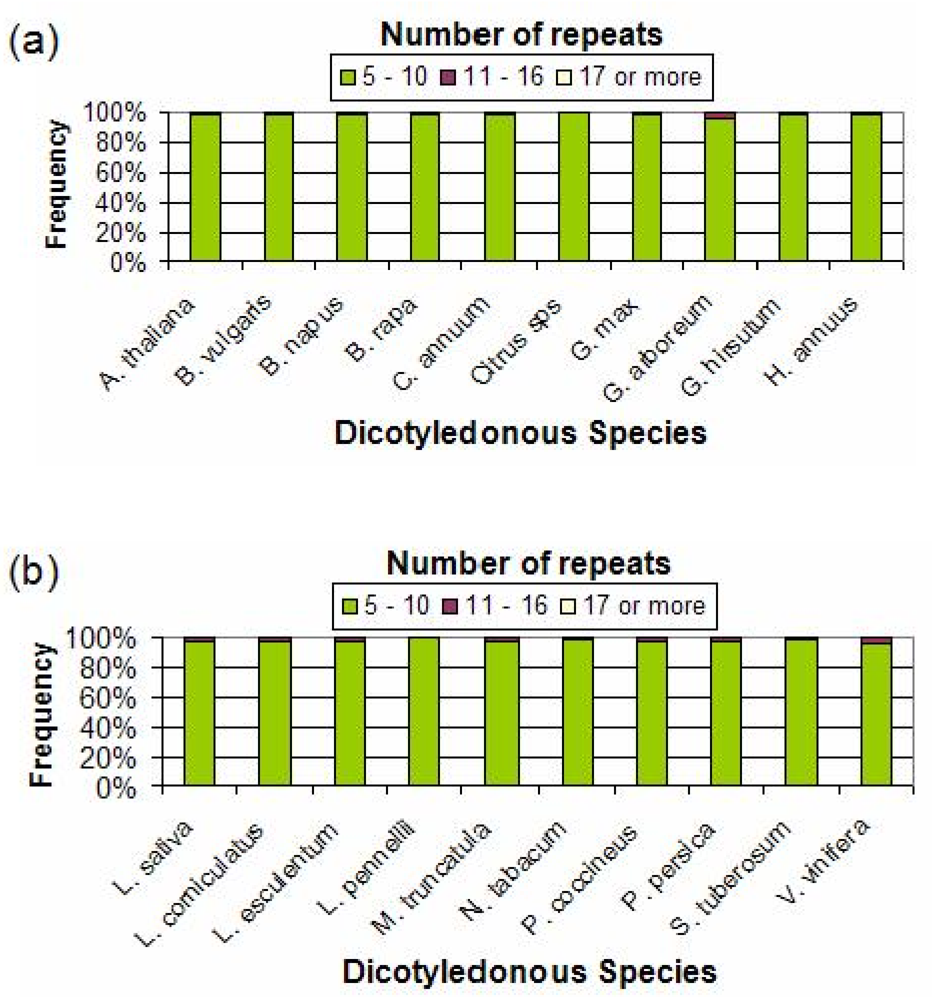


Figure 11. Distribution of trinucleotide SSRs into different repeat number classes

Thus, the distribution analysis of SSRs in non-redundant ESTs in 20 dicotyledonous species provided here clearly indicates the abundance of mononucleotide SSRs containing 15-25 repeats and di- and tri-nucleotide SSRs containing 5-10 repeats. This information coupled with the frequencies of different types of mono-, di- and trinucleotide motifs detailed in previous section demonstrates that ESTs are a rich source of SSRs towards marker development for genetic analysis in dicotyledonous species.

VI. Conclusion and biological implications of the findings

The development of DNA-based genetic markers has been the driving force behind the current revolution in animal and plant genetics (Dodgson *et al.*, 1997). The abundance and hypervariability associated with SSRs make them ideal candidates for the development of markers for genetic mapping, fingerprinting, gene tagging, marker-assisted selection and evolutionary studies (Kantety *et al.*, 2002; Powell *et al.*, 1996; Rafalski and Tingey, 1993; Tautz, 1989). Computational approaches provide an attractive alternative to conventional laboratory methods for rapid and economical development of SSR markers by utilizing freely available sequences in public databases. A knowledge of the occurrence and composition of SSRs across a large number of species also helps a great deal in targeting specific SSRs for marker development.

A. Overview of significant results

The contributions of this project could be divided into three areas: (i) development of a high-throughput tool for efficient and rapid identification of SSRs; (ii) mining of SSRs from a large number of dicotyledonous species and demonstrating the potential of ESTs as a source of SSRs and (iii) compositional analysis of mined SSRs to understand the abundance and distribution of different types of SSRs in ESTs.

Since several of the existing SSR identification tools did not meet all of the requirements of an ideal tool for this project, a program, named RepeatFetcher, was developed. This tool has been found to be very efficient in handling large datasets (hundreds of thousands of sequences) as well as large input sequences. A user can directly utilize the results obtained from RepeatFetcher for downstream processes such as designing PCR primers for target sequences towards marker development.

One of the noteworthy observations of this project in terms of SSR mining from ESTs is that several of the ESTs in GenBank still contain polyA / polyT stretches at their ends due to their non-processing prior to deposition in GenBank. If the goal of SSR mining includes mononucleotide repeats also, it is essential to trim the ESTs prior to mining; otherwise these poly A / T stretches would be retrieved as mononucleotide repeats although in reality they are post-transcriptional additions. As detailed in section IV, as many as 107,726 out of 250,641 ESTs contained mononucleotides A or T at ends and thus have been eliminated from SSR-EST set by introducing a trimming step prior to SSR mining by RepeatFetcher. Another important aspect is to remove the redundancies in the ESTs. This step can be undertaken before subjecting the ESTs for mining or after obtaining results from trimmed ESTs. The second approach has been taken in this project since it considerably reduced the size of the datasets for easier handling and analysis for downstream steps such as in-depth contig/cluster analysis and elimination of redundancies. Obtaining a non-redundant set of SSR-containing ESTs also saves marker development costs due to elimination of duplicates. The frequency of NR-ESTs containing SSRs among 49 species investigated ranged from 2.65% to 16.82% of the total ESTs available. Thirty three of the 49 species recorded frequencies of more than 5% while 21 out of these 33 species contained 8% or more NR-ESTs containing SSRs.

An analysis of abundance of SSRs in 18 species containing large number of sequences (>10,000) led to the conclusion that the frequency of SSRs in these dicotyledonous plants ranges from 2.65% to 10.75% and that half of these species contain SSR-containing ESTs in the range of 6.09% to 10.75% of total ESTs. In all of the 49 species investigated, ESTs containing a single SSR stretch are predominant (frequencies of single SSR-containing ESTs ranged from 71.3% to 100%) with majority of the species containing 80% or more ESTs with a single SSR stretch. Although the average frequency of multiple SSR-containing ESTs across 49 species is about 14%, *Hevea brasiliensis* and *Linum usitatissimum* are exceptions where the averages are 28.7% and 26.0%, respectively.

The information on the occurrence and composition of different SSRs in a large number of dicotyledonous species is important for understanding their abundance in ESTs towards using them as a source for marker development and other applications. The frequency analysis of mono-, di-, tri- and tetra-nucleotide repeats for all of the 49 species revealed interesting and useful trends in this regard. In 38 out of 49 species, dinucleotide SSRs are the most abundant followed by tri- or mono-nucleotide repeats. There are two species; *C. arabica* and *L. sativa,* where trinucleotides are the most frequent class of repeats.

The mononucleotide A/T has been found to be most abundant SSR in this category based on the analysis of 20 species with large number of NR-ESTs. They represent as high as 40% of total SSRs in *C. annuum*, *G. arboreum* and *G. hirsutum* species. An interesting observation is that C/G repeats make up as much as 15% of the total SSRs in *G. arboreum* although they are lower in abundance than A/T in that species. Relative frequencies of dinucleotide repeats revealed that AG/GA/CT/TC group is the predominant class of dinucleotide repeats in almost all of the species investigated (make up 14.6% to 54.5% of the total SSRs). AT/TA is the second most abundant dinucleotide repeat class in all species investigated with the exception of *N. tabacum* where AC/CA/TG/GT is the abundant class next to AG/GA/CT/TC.

The trinucleotide repeat AAG/AGA/GAA/CTT/TTC/TCT is the most frequently observed (16 out of 20 species investigated) comprising of 2.7% to 15.5% of the total SSRs while ACC/CCA/CAC/GGT/GTG/TGG is predominant in two species (*H. annuus* and *L. sativa*), ATG/TGA/GAT/GAT/ATC/TCA is the predominant class in *B. vulgaris* and AGC/GCA/CAG/GCT/CTG/TGC is the most frequent class in *Citrus* spp. Although the second most frequent repeat class is different across the 20 species, ATG/TGA/GAT/GAT/ATC/TCA and AAC/ACA/CAA/GTT/TTG/TGT are second most frequent repeat classes in 8 and 5 species, respectively. Although not many tetranucleotides were observed relative to other repeat types, based on the analysis of frequently observed tetranucleotide motif patterns, TTTA and TTAA repeats are the most abundant across the species followed by ATTA and TTTC.

B. Findings in context of current knowledge

The frequency of SSR-containing NR ESTs identified among 49 dicotyledonous species ranged from 2.65% to 16.82% with 33 species recording more than 5%. These frequencies are higher than those observed in previous studies and strongly support recent observations in selected plant species that SSRs are abundant in ESTs compared to genomic sequences (Morgante *et al.,* 2002). For example, an extensive comparison of abundance of SSRs in genomic DNA and ESTs of three monocotyledonous species (rice, maize and wheat) and two dicotyledonous species (*Arabidopsis thaliana* and soybean) revealed that the frequency of SSRs was significantly higher in ESTs (Morgante *et al.*, 2002). It is possible that many more SSR-containing ESTs would have been mined and retained in the species investigated here if lower stringencies had been implemented with respect to RepeatFetcher criteria (minimum number of repeats) and elimination of redundancies. The frequencies observed for 9 out of 18 species with large number of ESTs are in the range of 6.09% to 10.75% and are comparable to the 7% to 10% range observed for monocots where SSRs from ESTs were mined using computational approaches (Varshney *et al.,* 2002).

Dinucleotide repeats are found to be the most abundant SSRs (in 38 out of 49 species), followed by tri- or mono-nucleotide repeats. While two species, *C. arabica* and *L. sativa*, have trinucleotide repeats as the most frequent class of repeats, mononucleotide repeats are the predominant in other species. *In silico* mining and analysis of SSRs in ESTs of some cereal species revealed that trinucleotide repeats are the most abundant class followed by dinucleotide repeats (Varshney *et al.,* 2002) and this prevalence of trinucleotides has been attributed to the non-interruption of codons, which are triplets.

The repeat pattern AG/GA/CT/TC is found to be the most abundant of dinucleotide repeats in the dicotyledonous species (14.6% to 54.5% of the total SSRs in a given species) followed by AT/TA repeat. Morgante *et al.*, (2002) and Varshney *et al.,* (2002) have demonstrated that AG/CT are the most frequently observed dinucleotide SSRs in ESTs of plants and the results observed here with respect to this repeat are in strong agreement with their findings. However, unlike the observations by Morgante *et al.*, (2002) that AT repeats occur at a lower frequency, it was found that they are the second most abundant in the dicotyledonous species investigated here. The second most frequently observed repeat in ESTs of some cereals as reported by Varshney *et al.*, (2002) was AC repeat (same as AC/CA/TG/GT group in the present study), whereas AT/TA is the second most frequent repeat in the present study. With respect to AC/CA/TG/GT repeats, the findings of this study are in general agreement with the observations of Morgante and Olivieri (1993) and Lagercrantz *et al.*, (1993) who reported the scarcity of AC/GT repeats in plants compared to mammalian genomes. However, the dicotyledonous species *N. tabacum* is an exception where AC/CA/TG/GT is the second most abundant repeat next to AG/GA/CT/TC.

The most abundant trinucleotide repeat observed in the present study is AAG/AGA/GAA/CTT/TTC/TCT (16 out of 20 species) making up between 2.7% and 15.5% of the total SSRs mined in the species investigated. Varshney *et al.*, (2002) observed that the CCG trinucleotide repeat (belonging to the GGC/GCG/CGC/GCC/CCG/CGC group) is the most predominant SSR in cereal species. However, this repeat is not the predominant class in any of the 20 species investigated here for which large numbers of ESTs are available. Previous studies have indicated that trinucleotide repeats are significantly more abundant in ESTs, especially in rice (Morgante *et al.*, 2002). For example, the CCG/CGG repeats accounted for half of the trinucleotide repeats in rice, whereas they were rare in the dicotyledonous plants (*Arabidopsis* and soybean) and moderately abundant in monocots other than rice (maize and wheat). The results observed here strongly support the notion of rarity of CCG/CGG repeats in dicotyledonous plants. As hypothesized by Morgante *et al.*, (2002), the higher

G+C content and consequent codon usage bias in monocot ESTs may largely account for the differential occurrence of CCG/CGG in monocots and dicots.

C. Biological implications and practical applications of the findings

ESTs are ideal candidates for mining SSRs not only because of their availability in large numbers but due to the fact that they represent expressed genes. SSR markers developed from ESTs could be of great value in filling the existing linkage maps and in identifying markers for cross species studies. For example, Monforte (2003) reported a higher transferability of SSRs developed from melon ESTs between melon and cucumber species compared to SSRs developed from genomic libraries. The use of SSRs for comparative genomics and intra and inter-specific studies is gaining momentum in both monocots and dicots (Eujayl *et al.*, 2002; Rallo *et al.*, 2003 and Westman *et al.*, 1998) and ESTs are ideal candidates since the expressed sequences have a higher degree of conservation across species.

Simple sequence repeats are hypothesized to originate due to unequal crossing-over or replication errors leading to the formation of unusual DNA secondary structures such as hairpins or slipped strands (Pearson and Sinden, 1998; Tautz and Schlotterer, 1994). For a long time, it has been assumed that SSRs are abundant in repetitive sequences or non-coding fraction of the genome and that SSR densities increase with increasing genome size in animals (The Arabidopsis Genome Initiative; Hancock, 1996). However, a growing body of knowledge in plants is indicating that SSRs are preferentially associated with transcribed regions of the genomes (Morgante *et al.*, 2002). It has been demonstrated that 3’ untranslated regions (UTRs) contain a higher SSR frequency than the whole genome, with trinucleotide and tetranucleotide repeats contributing most to this increase (Morgante *et al.,* 2002). Similarly, 5' UTRs were also shown to have about threefold higher frequency of SSRs than any other genomic fraction due to the abundance of dinucleotide (in particular AG/CT) and trinucleotide repeats (in particular AAG/CTT) (Morgante *et al.*, 2002). Based on these and other results, it has been hypothesized that (i) coding sequences are under negative selection for all SSR types except trinucleotides (since they do not interrupt codon usage); (ii) 5'UTRs are under very strong positive selection; (iii) 3' UTRs are under moderate positive selection; (iv) repetitive DNA is under mutational (slippage) pressure that increases SSR frequency (Tautz and Schlotterer, 1994) and (v) single-copy nontranscribed DNA sequences are at equilibrium (Morgante *et al.,* 2002). Since the SSRs other than trinucleotides, if expanded could be detrimental to the coding region by interrupting codons and reading frames, it is likely that majority of the mono-, di- and tetra-nucleotides observed in the present study are actually located in 5' and 3'UTRs. Even then these are useful since markers developed from these SSRs are adjacent (linked) to functional genes.

Studies on EST-derived SSRs are also extremely important in dissecting their origin and distribution. While previous reports emphasized the repetitive DNA-derived origin of SSRs in animals (Nadir *et al.*, 1996) and plants (Ramsay *et al.*, 1999), the comprehensive analysis of Morgante *et al.*, (2002) in 5 species (including 2 dicots) demonstrated that low-copy fraction of plant genomes (transcribed regions, especially UTRs) are the sources of SSRs. If this is the case, we should observe more SSRs in non-repetitive fraction of genomes. In a study that addressed this aspect, highest frequency of SSRs were observed in *Arabidopsis thaliana* with increasingly lower frequencies in genomes with increasing genome size (Morgante *et al.,* 2002). This clearly indicates that SSR frequencies are higher in more streamlined genomes compared to large genomes where repetitive DNA makes up the bulk of a genome. With respect to the composition of SSRs, it has been reported for *Arabidopsis thaliana* that AT repeats are typical of nontranscribed regions and AG/CT are predominant in transcribed regions. Moreover, the frequency of these SSR motifs in 5'UTRs is found to be more than an order of magnitude higher compared to whole genome. The results observed here for dicotyledons show that AG/CT are the most abundant dinucleotides in ESTs that represent the transcribed regions of the genome. However, it is interesting to note that AT/TA repeats are the second most frequent repeats in several of the dicotyledonous ESTs investigated here. While their association with specific components of transcribed regions has not been addressed here, it is likely that they are coming from 5' and/or 3' UTRs.

Practical applications: One major and direct utility of SSR mining is the development of markers for genetic mapping and downstream applications. In order to experimentally validate the EST-SSR approach, SSR-containing ESTs identified in cotton (*Gossypium* species) were subjected to marker development. Since di-, tri- and tetra-nucleotide SSRs are most commonly used, initial efforts were focused on these categories. In the preliminary analysis, PCR primers could be designed for a total of 500 di-, tri- and tetra-nucleotide SSRs (65% primer design success rate). Out of these 500, successful PCR amplifications were achieved for 315 markers (63% success rate). These markers were screened on a panel of 5 cotton varieties and 79 (25%) of them were found to be polymorphic or informative and could be used for genetic mapping and other studies. Another application is the transferability of EST-SSRs to other related species or crops. As discussed above, SSRs are increasingly being used for cross-species applications and the SSRs identified in this study could be used for screening on species of interest for the identification of potential candidates for such comparative genomic studies.

VII. Discussion and Future Work

A. Overview of the study

This study demonstrates the utility of a computational approach for mining SSRs from ever increasing sequence information in biological sciences by focusing on dicotyledonous plants. While such approaches have been used for mammalian and several monocotyledonous plant species, large-scale mining studies corroborating the abundance of SSRs in ESTs of a wide range of dicotyledonous plants are lacking and this study attempts to fill this void. There are some aspects of the study that could have been further enhanced to improve the knowledge regarding SSRs in dicots. For example, a comparison of SSR information derived from genomic sequences with that of EST-SSRs and the distribution of EST-SSRs across different parts of the transcribed regions could be very valuable. However, such an analysis is currently possible only for species such as *Arabidopsis thaliana* for which complete genomic sequence information is available and a few others for which at least some genomic coverage is achieved. Another aspect that would extend the value of the findings of this study for comparative genomics is the cross-species clustering of ESTs to understand the extent of homology and the frequency of ESTs that fall into these clusters could potentially be useful for comparative mapping studies.

B. Recommendations for future work

By mining and evaluating the composition of SSRs from a large number of dicotyledonous species, this project has demonstrated that ESTs could be used as potential sources for marker development. In order to effectively utilize this information and to extend the utility of SSRs to comparative genomics and other studies, future work should focus on both computational and molecular biology fronts. In case of computational enhancements, it is very valuable to have a pipeline for the entire process of SSR mining. For example, automated sequence handling, trimming and SSR mining improves the speed of the process and when coupled with PCR primer design and SSR information archiving, it also enhances the pace of marker development. Another bioinformatics project that would extend the value of current project is the comparison of genomic SSR frequencies with those of EST-SSRs in dicotyledonous plants and distribution analysis of EST-SSRs among different transcribed regions (5' and 3' UTRs and open reading frame). On the molecular biology front, one project that could be of great value is the study of portability of EST-SSR markers for cross-species or cross-genera amplification and their utilization in comparative mapping and related applications.

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