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Exact Microsatellite Density Differences among *Capsicum* Tissues and Development Stages

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ABSTRACT

Density and position differences of microsatellites in genomes may indicate important roles of microsatellites in genetic development and regulation of gene expression. However, there is no or limited study on microsatellite density differences among tissues of development stages. In the present study, exact microsatellite densities and motifs among 7 different tissues and development stages were determined using *Capsicum annuum* L. expressed sequence tags (ESTs), which were reassembled into *in silico* libraries. Results indicated that densities of exact microsatellites (1 to 6 bp repeats) in housekeeping and tissue specific ESTs of anther, flower bud, and placenta specific ESTs were statistically different, being low in comparison to that of leaf, fruit, early and hairy root. Further analyses also indicated that exact microsatellite density of anther and placenta was significantly low while exact microsatellite density of flower bud, early and hairy root was significantly higher. There were density differences among mono-, di-, tri- and hexa-nucleotides between housekeeping and tissue specific ESTs. Density of tri- and penta-nucleotides was not statistically significant. Overall results of the present study indicated that since the microsatellite densities differed between housekeeping and tissue specific genes, genes containing microsatellites may differ among tissues and development stages.

Keywords: Microsatellite densities, Repeat types; *In silico* analysis

Capsicum Doku ve Gelişme Dönemlerinde Düzenli Mikrosatellit Yoğunluk Farklılıkları

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ÖZET

Genomda mikrosatellitlerin yoğunluk ve konum farklılıkları mikrosatellitlerin genetik gelişim ve gen ekspresyonunun düzenlenmesi konularında önemli roller oynadıklarını göstermektedir. Ancak, doku ve gelişme dönemleri arasında mikrosatellit yoğunluk farklılıkları üzerine yapılan çalışmalar ya yoktur ya da oldukça sınırlıdır.

Bu çalışmada, 7 farklı doku ve gelişme dönemi arasında düzenli mikrosatellit yoğunluğu ve motifleri biber (*Capsicum annuum* L.) bitkisine ait ifade edilmiş gen parçası (EST) sekanslarından oluşturulan *in silico* kütüphaneleri kullanılarak belirlenmiştir. Sonuçlar anter, çiçek tomurcuğu ve plasenta dokularına özgü EST'lerde yaprak meyve, genç ve kılcal kök dokularına özgü EST'lere göre fiili işlevsel (housekeeping) ve doku spesifik EST'lerde düzenli mikrosatellit yoğunluğunun istatistiksel olarak önemli düzeyde düşük olduğunu göstermiştir. Buna ilaveten yapılan analizlerde de anter ve plasenta dokularında düzenli mikrosatellit yoğunluğu istatistiksel olarak önemli seviyede düşük, çiçek tomurcuğu, genç ve kılcal kök dokularında ise önemli düzeyde yüksek olduğu bulunmuştur. Housekeeping ve doku spesifik EST'lerde mono-, di-, tri- ve hekza-nükleotit mikrosatellit motiflerinin yoğunlukları arasında önemli farklılıklar olduğu saptanmıştır. Tri- ve penta-nükleotit yoğunluklarının ise önemli düzeyde olmadığı görülmüştür. Genel olarak housekeeping ve doku spesifik genlerde mikrosatellit yoğunluklarının farklı olduğu belirlenmiş ve bu sonuçlara göre de mikrosatellitlerin doku ve gelişme dönemine göre farklı ekspresyona neden olabilecekleri gösterilmiştir.

Anahtar sözcükler: Mikrosatellit yoğunluğu; Tekrar tipleri; *İn silico* analizler

1. Introduction

Microsatellites and minisatellites found in plant and animal genomes have been traditionally thought of as functionally unimportant but they have been commonly used as genetic markers. Microsatellite DNA motifs can consist of a single base to six bases, which are repeated several times. The repeats can be either exact (perfect) tandem repeats or interrupted by several non-repeat nucleotides (inexact or imperfect) or compound repeats (Bilgen et al 2004). Microsatellite repeat variations in plant species have been extensively used as markers of choice in genetic research since they exhibit high level of polymorphism within species; inherit as co-dominant fashion discriminating the homozygous from heterozygous individuals (Karaca et al 2002; Karaca et al 2004; Tyrka et al 2008; Ince et al 2009a; Ince et al 2010a).

Recent studies have shown that densities of microsatellites were considerably higher than they would be predicted purely on the grounds of base composition in many organisms (Bilgen et al 2004; Ince et al 2009b; Polat et al 2010). Including or excluding mononucleotide repeats in a genome greatly affect densities of microsatellites. For instance, the human genome contains approximately one million mononucleotide repeats which are longer than 9 bp (Cohen et al 2004). However, there still exist controversies in the microsatellite density differences among literatures in which some studies exclude mononucleotides and in some other studies the upper limit of repeat number is decreased as low as 5 bp or as great as 10 bp (Chambers & MacAvoy 2000; Ellegren 2004; Karaca & Ince 2011). Regardless of the definition of microsatellites, studies in animal genome have shown that microsatellites play a more active role in terms of gene regulation, development and evolution (Li et al 2004; Kashi & King 2006). However, there is limited information about the microsatellite density differences among plant tissues and development stages as well as between genes specific to a tissue or housekeeping.

This study was undertaken to identify exact microsatellite density differences in *Capsicum annuum* L. tissues and development stages as well as the genes specific to a tissue or housekeeping functions. In order to investigate microsatellite density differences *in silico* databases were constructed and these data were used in this study.

2. Materials and methods

2.1. ESTs

A total of 116,535 *Capsicum annuum* L. ESTs from National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/ containing 129,149,486 base pair nucleotide information were initially used. Keyword Finder and Organism Miner (Ince et al 2008) were implemented to obtain ESTs specific to each of anther, hairy root, early root, leaf, young fruit, placenta and flower bud library. A total of 20,738 ESTs containing 9.93 mega base nucleotides were selected from the database based on the library

identification number (Lib ID) and assembled into contiguous sequences (contigs) using Sequencher software (Gene Codes, Ann Arbour, MI). Contig assembly parameters were set to minimum overlap of 50 bases and 95% identity match.

2.2. Microsatellite analyses

Microsatellites in each dataset were identified using the Tandem Repeats Analyzer 1.5 (TRA 1.5) program (Bilgen et al 2004). Microsatellites in the present study were considered sequences containing a minimum of 18, 9, 7, 5, 5 and 4 nucleotide perfect (exact) repeats for mono- di-, tri-, tetra-, penta- and hexa-nucleotides, respectively. These repeat numbers were chosen since they are commonly used in other plant species (Karaca et al 2005; Li et al 2004; Lawson & Zhang 2008).

2.3. Statistical analysis

Chi-square (χ^2) goodness-of-fit tests with 1 degree of freedom were applied to test whether microsatellite densities were significantly different within and between datasets mentioned above.

$$E_i = \frac{N}{L} \times L_i \quad (1)$$

where E_i is the expected number of microsatellites in a dataset; N is the total number of microsatellites in the two different datasets; L is the total length in base pairs of the two datasets; and L_i is the length in base pairs of the dataset under investigation (Lawson & Zhang 2008).

3. Results and Discussion

3.1. ESTs analyses

A total of 20,738 ESTs containing 9.93 mega base nucleotide information as shown in Table 1 were studied. The highest number of ESTs were present in leaf tissues while anther tissues contained the lowest number of ESTs. There were lengths differences among the ESTs of seven tissues and development stages. The average number of base pairs in anther ESTs was 602.37 bp while the average number of base pairs in early root ESTs was 398.6 bp. The average number of the seven different tissues was 478.73 bp (Table 1).

3.2. Construction of in silico databases

All the EST sequences given in Table 1 were assembled into 22 *in silico* libraries (Figure 1). These *in silico* libraries consisted of singletons (S), consensus mutual (CM) and consensus specific (CS) for each of the seven cDNA sets to investigate microsatellite density differences among the genes specific to tissues and development stages (Table 2). Classification of sequences in Table 2 was obtained from the analyses summarized in Figure 1.

Tissue specific singletons (S) and contigs (CS) were considered those ESTs that had no homolog to other ESTs. On the other hand, those singletons and contigs with homology to other ESTs were considered non-tissue specific (CM). For instance a total of 510 anther ESTs were divided into CS, CM and S. CS of anther consisted of 20 Type I AO consensuses and 355 Type I A0 S sequences. CS and S of anther were only present in anther tissues while anther CM consisted of Type II A consensus sequences (135) that were also present in some other tissues and development stages.

In Figure 1 tissue and development specific *in silico* libraries are shown. This shema represented 22 (numbers 0 to 15) *in silico* libraries. For instance, anther *in silico* libraries have ESTs specifically expressed in anther (indicated as A0) and numbers 2, 3, 5, 6, 7, 8, 9, 14 and 15 represent ESTs which are also expressed in other tissues or development stages. A0 *in silico* anther library contained a total of 375 ESTs, which are the combination of 20 anther CS and 355 anther S shown in Table 2. On the other hands, 135 ESTs represented ESTs which collected from the all possible combination of seven tissues or development stages.

3.3. Microsatellite densities among tissues and development stages

As shown in Table 3, exact microsatellite density of tissue specific sequences consisting of singletons and consensus (TS + CS) for a tissue or development stage was compared to total number of sequences, which were 13,261 (Total). As shown in Table 3 exact microsatellite densities

Table 1-A summary of expressed sequence tags (ESTs) used in the study
Çizelge 1-Araştırmada kullanılan ifade edilmiş gen parçalarının (EST'ler) özeti

Source	*Total base (mega bases)*	*Number of ESTs*	*Average length (bp)*
Leaf	2.81	5,145	545.19
Flower Bud	1.67	3,524	474.12
Anther	0.40	666	602.37
Young Fruit (0.5-2 cm)	1.71	3,681	464.61
Hairy Root	0.84	1,926	435.92
Early Root	0.75	1,893	398.60
Placenta	1.77	3,903	447.47
Total	9.93	20,738	478.73

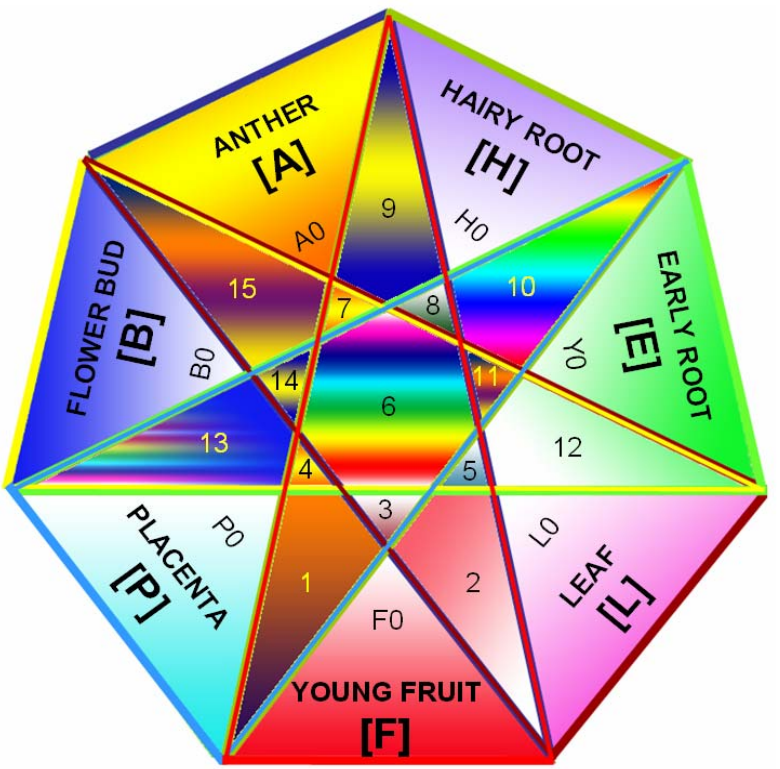

Figure 1-Figural representation of tissue and development specific in silico libraries. This shema represented 22 (numbers 0 to 15) in silico libraries. 0 represents tissues specific gene or gene segments. Other numeric numbers represent gene expressed in more than one tissue type
Şekil 1-In silico kütüphanelerinin doku gelişme dönemlerinin şekilsel temsili. Bu şema 22 (0 ila 15) in silico kütüphanesini temsil etmektedir.0 doku spesifik gen veya gen segmentlerini temsil etmekte, diğer sayısal numaralar ise birden fazla doku tipinde ifade edilen genleri temsil etmektedir

Table 2-Data of in silico libraries constructed in this study
Çizelge 2-Araştırmada oluşturulan in silico kütüphaneler

Databases	*Length (bp)*	*# Sequences*	*Types*
Anther (CS)	15,617	20	Type I A0
Anther (CM)	129,201	135	Type II A
Anther (S)	207,552	355	Type I A0
Flower bud (CS)	117,187	189	Type I B0
Flower bud (CM)	679,964	795	Type II B
Flower bud (S)	442,203	935	Type I B0
Early root (CS)	16,771	31	Type I E0
Early root (CM)	614,350	750	Type II E
Early root (S)	265,423	679	Type I E0
Young fruit (CS)	80,528	133	Type I F0
Young fruit (CM)	736,799	917	Type II F
Young fruit (S)	429,446	948	Type I F0
Hairy root (CS)	21,132	34	Type I H0
Hairy root (CM)	596,692	713	Type II H
Hairy root (S)	312,459	726	Type I H0
Leaf (CS)	237,795	353	Type I L0
Leaf (CM)	893,738	1,019	Type II L
Leaf (S)	992,164	1,775	Type I L0
Placenta (CS)	82,828	136	Type I P0
Placenta (CM)	823,926	1,027	Type II P
Placenta (S)	707,647	1,591	Type I P0

S: singletons, CM: consensus mutual, CS: consensus specific

among the 7 tissues and development stages were different with the exception of leaf and fruit. Results indicated that ESTs expressed in early root, hairy root, flower bud contained more microsatellites. On the other hand, placenta and anther contained lower amount of microsatellite densities.

Exact microsatellite densities between leaf and other tissues and between fruit and other tissues were not statistically different. These findings indicated that microsatellite containing ESTs in leaf and fruit also expressed in other tissues. Flower bud, hairy root and early root ESTs contained more microsatellite densities whereas anther and placenta ESTs contained fewer amounts of microsatellites (Table 3).

Mononucleotide repeat differences were statistically different with the exception of fruit and leaf tissues. Di-nucleotide repeat densities were significantly low in flower bud whereas it was significantly higher in leaf. There were no significant tri- and hexa-nucleotide repeat densities between the tissues. Placenta contained higher tetra-nucleotide density, while it contained less amount of mono-nucleotide density. Penta-nucleotide density of early root was significantly higher than the others. Based on the *in silico* studies we observed that mono-nucleotide densities are higher or lower in many tissues, whereas tri- and hexa-nucleotide repeat densities randomly distributed among the all tissues and development stages. Among the all 6 microsatellite motif densities mono-nucleotides were the most different repeat types, followed by the di-, tetra and penta-nucleotide repeats. On the other hand tri- and hexa-nucleotide repeats randomly distributed among the tissues and developmental stages (Table 3).

3.4. Microsatellite densities between tissue specific and housekeeping ESTs

Table 3-Exact microsatellite densities among tissues and development stages of *C. annuum* L.
Çizelge 3-C. annuum'da doku ve gelişme dönemleri arasında düzenli mikrosatellit yoğunlukları

Tissue types	*# Bases*	*#EST*	*#EST-SSR*		*Mono-*		*Di-*		*Tri-*		*Tetra-*		*Penta-*		*Hexa-*	
			O	E	O	E	O	E	O	E	O	E	O	E	O	E
Anther (TS+CS)	352,370	510	41	101.2	35	95.7	3	6.3	3	2.9	0	0.5	0	0.1	0	1.4
Total	8,051,052	12,751	2,509	2,312.8	2,248	2,187	148	144.7	68	68.1	12	11.5	1	0.9	32	30.6
χ^2			42.420**		40.210**		1.830		0.0002		0.525		0.044		1.401	
Early Root	896,544	1,460	328	257.6	300	244	17	16.1	7	7.6	1	1.3	1	0.1	2	3.4
Total	7,506,878	11,801	2,222	2,156.4	1,983	2,039	134	134.9	64	63.4	11	10.7	0	0.9	30	28.6
χ^2			12.880**		14.640**		0.055		0.049		0.069		8.373**		0.656	
Flower Bud	1,239,354	1,919	472	356.0	449	337	7	22.3	8	10.5	2	1.8	0	0.2	6	4.7
Total	7,164,068	11,342	2,078	2,058.0	1,834	1,946	144	128.7	63	60.5	10	10.2	1	0.9	26	27.3
χ^2			28.690**		43.930**		12.281**		0.684		0.035		0.173		0.408	
Fruit	1,246,773	1,998	395	358.2	355	339	19	22.4	13	10.5	1	1.8	0	0.2	7	4.8
Total	7,156,649	11,263	2,155	2,055.9	1,928	1,944	132	128.6	58	60.5	11	10.2	1	0.9	25	27.2
χ^2			0.862		0.919		0.607		0.678		0.402		0.174		1.255	
Hairy Root	930,283	1,473	318	267	293	252.7	14	16.7	6	7.9	2	1.3	0	0.1	3	3.5
Total	7,473,139	11,788	2,232	2,147	1,990	2,030	137	134.3	65	63.1	10	10.7	1	0.9	29	28.5
χ^2			5.079*		7.214**		0.496		0.495		0.382		0.125		0.094	
Placenta	1,614,401	2,754	314	464	260	438.6	30	29.1	14	13.6	5	2.3	0	0.2	5	6.2
Total	6,789,021	10,507	2,236	1,950	2,023	1,844.4	121	121.9	57	57.4	7	9.7	1	0.8	27	25.8
χ^2			78.166**		90.010**		0.419		0.012		3.899*		0.238		0.265	
Leaf	2,123,697	3,147	682	610	591	577	61	38.2	20	17.9	1	3.1	0	0.3	9	8.1
Total	6,279,725	10,114	1,868	1,804	1,692	1,706	90	112.8	51	53.1	11	8.9	1	0.7	23	23.9
χ^2			2.931		0.457		18.293***		0.316		1.823		0.338		0.138	

O: observed, E: expected, *: $P \leq 0.0031$, **: $P \leq 0.0005$

In order to investigate whether there existed exact microsatellite density differences between tissue specific (TS) and housekeeping (HS) gene or gene segments (ESTs), comparison analyses were performed and shown in Table 4. Results indicated that genes specifically expressed in anther, flower bud and placenta contained less density of microsatellites than expected while other tissues contained expected number of microsatellite densities.

Among the microsatellite motifs, densities of mononucleotides between tissue specific and housekeeping genes were significantly different in anther and placenta ESTs (Table 4). Dinucleotide microsatellite density was significantly low in early root tissue specific ESTs and trinucleotide microsatellite density was also low in anther tissue specific ESTs. Leaf specific ESTs contained more amount of hexanucleotide microsatellites than housekeeping ESTs. Flower bud housekeeping ESTs contained more hexanucleotides than flower bud specific ESTs (Table 4). There were no statistically significant differences between tissue specific and housekeeping ESTs for tetra-nucleotides and penta-nucleotides.

Up to date, limited research on variations in microsatellite density has been studied among tissues, populations, and species in plants. In a previous study, using a total of 16 cDNA samples obtained from different pepper tissues and at different developmental stages, it was observed that some types of microsatellite-containing genes showed differential expression patterns (Ince et al 2010b). In this study the use of *in silico* databases clearly showed that some types of microsatellite differently expressed among different tissues and there were microsatellite density differences between tissues specific and housekeeping genes.

Table 4- Exact microsatellite densities between tissue specific and housekeeping genes in *C. annuum* L.
Çizelge 4-C. annuum'da doku spesifik ve housekeeping genler arasında düzenli mikrosatellit yoğunlukları

Tissue types	*# Bases*	*#Ent*	*#Ent-SSR*		*Mono-*		*Di-*		*Tri-*		*Tetra-*		*Penta-*		*Hexa-*	
			O	E	O	E	O	E	O	E	O	E	O	E	O	E
Anther (TS+CS)	223,169	375	13	25	10	22.2	3	1.9	0	1.9	0	0	0	0	0	0
Total	129,201	135	28	15	25	12.8	0	1.1	3	1.1	0	0	0	0	0	0
χ^2			17.660**		18.210**		1.740		5.180*							
Early Root	282,194	710	109	103.2	105	94.4	1	5.4	1	2.2	1	0.3	1	0.3	0	0.6
Total	614,350	750	219	224.8	195	205.6	16	11.6	6	4.8	0	0.7	0	0.7	2	1.4
χ^2			0.469		1.728		5.160*		0.959		2.177		2.177		0.919	
Flower Bud	559,390	1,124	189	213.0	183	202.7	1	3.2	4	3.6	1	0.9	0	0	0	2.7
Total	679,964	795	283	259.0	266	246.3	6	3.8	4	4.4	1	1.1	0	0	6	3.3
χ^2			4.944*		3.476		2.690		0.076		0.019				4.936*	
Fruit	509,974	1,081	152	161.6	135	145.2	8	7.8	5	5.3	1	0.4	0	0	3	2.9
Total	736,799	917	243	233.4	220	209.8	11	11.2	8	7.7	0	0.6	0	0	4	4.1
χ^2			0.959		1.214		0.0114		0.032		1.445					
Hairy Root	333,591	760	105	114.0	98	105.1	5	5.0	1	2.2	0	0.7	0	0	1	1.1
Total	596,692	713	213	204.0	195	187.9	9	9.0	5	3.8	2	1.3	0	0	2	1.9
χ^2			0.721		0.481		0.112		1.054		2.612				0.557	
Placenta	790,475	1,727	89	153.7	65	127.3	13	14.7	5	6.9	4	2.4	0	0	2	2.4
Total	823,926	1,027	225	160.3	195	132.7	17	15.3	9	7.1	1	2.6	0	0	3	2.6
χ^2			53.430**		59.750**		0.381		0.984		1.927				0.161	
Leaf	1,229,959	2,128	394	395.0	335	342.3	41	35.3	8	11.6	1	0.6	0	0	9	5.2
Total	893,738	1,019	288	287.0	256	248.7	20	25.7	12	8.4	0	0.4	0	0	0	3.8
χ^2			0.006		0.368		2.163		2.634		1.000				6.540*	

Ent: Entry, TS: tissue specific, HS: housekeeping, O: observed, E: expected, *: $P \leq 0.0105$, **: $P \leq 0.0001$

Lawson & Zhang (2008), based on *in silico* analyses in mouse and human, indicated that microsatellite densities of housekeeping genes were about 1.7 times higher than those in tissue-specific genes and also showed that microsatellite domain contents were different between housekeeping and tissue-specific genes. In this study we observed that microsatellite density differences between housekeeping and tissue specific genes were also present in plant species. Furthermore this study also clearly showed the existence of microsatellite density differences among tissue and development stages.

Among the microsatellite motif, tri- and hexa-nucleotide motifs in plants (Bilgen et al 2004) and in human (Karaca et al 2005) have been shown to occur more than other repeats types excluding the mononucleotides. The occurrences of more tri-nucleotides indicate that genes with trinucleotide repeats may play significant roles in the maintenance of cellular physiology. For example, Huntington's disease and spinocerebellar ataxia (SCA) disease in human that alteration in CAG trinucleotide repetitive sequences was found to be associated with expansion in length. In another example, changing in GCG repeat numbers causes oculopharyngeal muscular dystrophy disease in human (Yamada et al 2002; Krol et al 2007; Pizzi et al 2007). These examples indicate that some disease may occur as a result of trinucleotide repeat variations. In the present study it was observed that tri-nucleotide repeat differences were not statistically different among the tissues and development stages.

In this study using publicly available cDNA libraries a total of 22 *in silico* libraries were constructed. Using these libraries genic microsatellite and single nucleotide markers can be identified. The use of ESTs for microsatellite

primer pairs has been intensively utilized in *Capsicum* species and other species (Ince et al 2010a; Polat et al 2010). However EST-based microsatellites show low level of polymorphisms than genomic microsatellites (Blair et al 2011). The level of polymorphism in EST-based microsatellites could be improved by the use of CAPS-microsatellite technique (Ince et al 2010c).

4. Conclusion

In this study it was observed that microsatellite densities among tissues and development stages as well as between tissue-specific and housekeeping ESTs were different. Although the number of ESTs studied in the present study is relatively low to represent the whole *Capsicum* genome, it is the first study to investigate densities of microsatellite motifs distributed among 22 *in silico* libraries. In spite of the fact that some EST numbers were low in some microsatellite motifs these findings may indicate that densities of microsatellites were higher than they would be predicted purely on the grounds of base composition. Tissue specific microsatellites with known function can be effectively used in genetic studies in plants. In the present study we also demonstrated that cDNA libraries could be reassembled to construct tissue and development stage specific *in silico* libraries which could be used in gene identification and annotation studies as well as identification of single nucleotide polymorphism.

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