 doi:10.20944/preprints202104.0521.v1

Article

Microsatellites as agents of adaptive change: An RNA-seq based comparative study of transcriptomes from five *Helianthus* species

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Abstract: Mutations that provide environment dependent selective advantages drive adaptive divergence among species. Many phenotypic differences among related species are more likely to result from gene expression divergence rather than from non-synonymous mutations. In this regard, cis-regulatory mutations play an important part in generating functionally significant variation. Some proposed mechanisms that explore the role of cis-regulatory mutations in gene expression divergence involve microsatellites. Microsatellites exhibit high mutation rates and are abundant in both coding and non-coding regions and could influence gene function and products. Here we tested the hypothesis that microsatellites contribute to gene expression divergence among species with 50 individuals from nine closely related Helianthus species using an RNA-seq approach. Differential expression analyses of the transcriptomes revealed that genes containing microsatellites in non-coding regions (UTRs and introns) are more likely to be differentially expressed among species when compared to genes with microsatellites in the coding regions and transcripts lacking microsatellites. We detected a greater proportion of shared microsatellites in 5'UTRs and coding regions compared to 3'UTRs and non-coding transcripts among *Helianthus* spp. Further, allele frequency differences measured by pairwise F_{ST} at single nucleotide polymorphisms (SNPs), indicate greater genetic divergence in transcripts containing microsatellites compared to those lacking microsatellites. A gene ontology (GO) analysis revealed that microsatellite-containing differentially expressed genes are significantly enriched for GO terms associated with regulation of transcription and transcription factor activity. Collectively, our study provides compelling evidence to support the role of microsatellites in gene expression divergence.

Keywords: Gene expression, *Helianthus*, microsatellites, transcriptomics

1. Introduction

Understanding the genomic basis of adaptive divergence among species remains a central theme in evolutionary biology. A major contributor to species divergence is

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variation in gene expression regulation [1]. Mutations within the cis-regulatory regions appear to be especially important in generating evolutionarily significant variations among species [2]. Indeed, studies analyzing the relative contribution of cis-regulatory mechanisms and protein coding changes in species divergence suggest that ontogenetic change among species is frequently cued by cis regulatory elements [3-5]. Some of the most frequently found cis-regulatory elements are highly mutable microsatellites or short tandem repeats [6]. A growing body of research now suggests that microsatellites in cis-regulatory regions can play a major role in gene expression variation [7, reviewed in 8].

Microsatellites consist of short tandem repeats, typically with 2 to 6 base pair long motifs, repeated several to dozens of times [9, 10]. Long considered non-functional and evolutionarily neutral, microsatellites are frequently used in genetic studies due to their high polymorphism [11]. Despite the high mutation rates associated with microsatellites, they are an integral part of all eukaryotic genomes and transcriptomes [12]. Initially, microsatellites were viewed as sequences that could be detrimental if present in the transcribed regions of the genome. For example, uncontrolled expansion of microsatellites in genic regions is known to cause nearly 30 human neurodegenerative diseases [13]. Yet, over the years, several studies have shown that microsatellites could be functionally beneficial and could facilitate rapid adaptation [14,15].

Microsatellites have recently been implicated in morphogenesis and reproductive phenology in plants [16-18], neuronal and craniofacial development in primates [19,20], limb and skull morphology in domesticated dogs [21], circadian clock cycles in fungi [22], and courtship behaviors in mammals [23] among other traits. With the advent of high-throughput sequencing, microsatellites and their functional role in gene regulation are now being studied at the genome level. Several such large-scale genome-wide and transcriptome-wide studies indicate that microsatellite-linked variations in gene expression are neither isolated nor sporadic, but widespread across genomes [24-27].

In this study, we investigate the role of microsatellites in gene expression divergence among species by comparing transcriptomes of several individuals belonging to the genus of North American annual wild sunflowers (*Helianthus*). *Helianthus* has a well characterized ecological and evolutionary history [28], and the genomic basis of local adaptation, ecotype formation, and speciation is well-documented in several species [29-31]. This makes them a good system for studying adaptive processes such as those that can result from microsatellite polymorphisms in genes.

Recent transcriptome-based studies of the common sunflower (*Helianthus annuus L.*) revealed that a substantial number of transcribed microsatellites in sunflower can be functional. Over 400 transcribed microsatellites have been linked to gene expression variation in common sunflower populations across a latitudinal gradient in North America [27]. Further, microsatellites of A and AG motif types have been linked to gene expression divergence among populations of common sunflower [32]. These previous studies on functional microsatellites in common sunflower provide impetus to explore the potential evolutionary role of transcribed microsatellites in gene expression divergence among closely related *Helianthus* species.

We designed the current study to answer the following questions:

1) Are microsatellite-containing genes more likely to show evidence of expression divergence among species, as compared to genes lacking microsatellites?

2) Do microsatellites-containing genes exhibit greater levels of genetic divergence compared to genes lacking microsatellites?

2. Materials and Methods

2.1 Plant sampling and sequencing

Seeds were collected from plants belonging to five *Helianthus* species – *H. annuus*, *H. bolanderi*, *H. debilis*, *H. exilis*, and *H. petiolaris* – growing in their natural ranges. These seeds were grown in greenhouses at University of British Columbia. Further protocols for plant sample preparation, RNA extraction, and Illumina GAII 2x100 paired end sequencing for

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50 individuals belonging to five major *Helianthus* species are described in detail elsewhere [33, 34].

Post sequencing data collection

A reference transcriptome was constructed from one *H. annuus* individual ("Canal2") using the de novo transcriptome assembly software Trinity [35]. The reference assembly consists of 51,468 transcripts as is available here: https://doi.org/10.5061/dryad.9q1n4 [36]. Each fastq file containing the raw reads for an individual was aligned to the reference transcriptome using BWA [37]. The resulting aligned files in BAM format were indexed and reads mapped to each transcript were measured as counts using SAMTools v.0.1.17 [38].

2.2 Functional annotation

A standalone BLASTX [39] search of the reference transcriptome was performed against a publicly available *H. annuus* protein sequence database. First, we built a local protein sequence database with common sunflower protein sequences available at https://www.heliagene.org/ (version HanXRQr2.0-SUNRISE). Using the reference transcriptome as the query, we performed a BLASTX search against the sunflower protein sequence database with an E-value cutoff set at 0.0001, gap open penalty score set at 11, gap extension penalty score of one, and minimum word size of three. We used BLOSUM62 as the matrix of choice for the search. To minimize the number of hits for each query sequence, we set the best hit overhang at 0.25 and the maximum target sequence value at one. The output of best hits produced by the BLASTX search was further filtered based on E-value and bit score to retain the hit with the lowest E-value and the highest bit score for each query sequence.

2.3 Mining and genotyping SNPs and microsatellites:

To identify and genotype SNPs present in all individuals, we used the "bcftools" and "mpileup" option in SAMTools v.0.1.17 [38]. SNP genotypes with Phred scaled genotype likelihoods below 30 were excluded from analyses and hence, only high-quality SNPs were used in further analyses. The methodology has been previously detailed in [36].

Microsatellites were identified using SciRoKo v 3.4 [40]. The parameters in SciRoKo were set to mine microsatellites of repeat sizes 1 to 6 bp, a minimum total length of 15 bp, and to find impure microsatellites, i.e., microsatellites that are interrupted by few substitutions or indels, using the "mismatch variable penalty" option, which allows for selection of impure microsatellites adjusted with respect to the total length of the tract.

Microsatellite alleles at a locus can be genotyped using RepeatSeq v 0.8.2 [41]. RepeatSeq works in conjunction with Tandem Repeats Finder (TRF) v. 4.07b [42]. Hence, microsatellites were mined from the reference transcriptome using TRF. This list of microsatellites generated by TRF is not as exhaustive as the list provided by SciRoKo, due to the more stringent criterion used by TRF that prevents mining shorter microsatellites. TRF was run using default settings, except for the parameter, raw score adjusted from the default 50 to 35, and the maximum repeat size adjusted to 6. All individuals were genotyped at the TRF generated list of microsatellites using RepeatSeq. RepeatSeq was run with default settings along with parameters set to output "calls" and "repeatseq" files, which contain a list of all microsatellite genotype assignments and alignments at each microsatellite region, respectively.

2.4 Differential expression

We performed differential gene expression analysis in OmicsBox version 1.4 (BioBam Bioinformatics S.L., Valencia, Spain) with the edgeR package [43]. False discovery rate (FDR) was set to 0.05 with a log fold change of ≤ 2 or ≥ 2, and read counts were normalized for relative expression and effective library size with the TMM (Trimmed Mean of M-

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values) method implemented in edgeR. The minimum number of samples reaching more than zero counts per million reads (CPM filter) was set to three to match the number of samples in the smallest group (*H. bolanderi)* in the data set. We performed differential expression analyses for each pair of species (10 comparisons), and genes with log fold change of ≤ 2 or ≥ 2 at FDR <0.05 for each comparison were identified as significantly differentially expressed (DE) genes for each pairwise species comparison and used in downstream analyses.

2.5 Gene Ontology (GO) Enrichment Analysis

A complete list of gene ontology (GO) terms associated with all annotated *H. annuus* genes available as part of the reference genome [44], was downloaded from https://www.heliagene.org/HanXRQr2.0-SUNRISE/downloads/2.1/HanXRQr2.0-SUNRISE-2.1.Blast2GO-20181213.zip. We extracted GO terms associated with the transcripts in the reference transcriptome from the downloaded complete list of GO terms and created a background list for GO enrichment analysis with OmicsBox version 1.4 (BioBam Bioinformatics S.L., Valencia, Spain).

Microsatellite-containing differentially expressed genes for each pairwise species comparison were extracted from the list of microsatellite-containing genes identified by SciRoKo that were successfully mapped to the reference genome. *Helianthus annuus* gene IDs (HanXRQr2.0 IDs) associated with microsatellite-containing differentially expressed genes were used as the "Test-set", and the background list of all *H. annuus* gene IDs associated with the reference transcriptome was used as the "Reference-set" in the GO enrichment analysis for each pairwise species comparison. Additionally, we conducted a GO enrichment analysis of all microsatellite-containing genes that were differentially expressed in at least one pairwise species comparison against the background list of all expressed genes in the reference transcriptome. All GO enrichment analyses were conducted in OmicsBox version 1.4 by performing Fisher's Exact Test with FDR set to 0.05.

2.6 Relative importance of microsatellites in species divergence

We are interested in understanding the role of microsatellites in species divergence via gene expression changes. To test this hypothesis, Chi-squared tests were performed to detect if microsatellite-containing genes were more likely to be differentially expressed than genes lacking microsatellites. We performed Chi-squared tests for each pairwise species comparison, and across all comparisons with microsatellites that were identified in DE genes in at least one pairwise species comparison. If microsatellites are important for bringing about species level divergence, then an elevated proportion of microsatellite-containing genes would show differential expression as opposed to genes lacking microsatellites.

Further, using the alignment start and end sites from the BLASTX output of the reference transcriptome and the microsatellite start and end sites from the SciRoKo output of the microsatellite search, we identified the location of the microsatellites (non-coding versus coding) within the transcripts. Previous large-scale studies on humans and plants have shown that in transcribed regions, more microsatellites are located within non-coding regions than in coding regions [45-47]. We observed similar patterns of microsatellite distribution in the reference transcriptome used in this study. Given the abundance of microsatellites within non-coding regions, which includes UTRs, and their implicated role in gene expression regulation [48-50], we tested with Chi-squared tests whether genes containing microsatellites within non-coding regions were more likely to be differentially expressed compared to genes that contained microsatellites in coding regions and genes lacking microsatellites.

 doi:10.20944/preprints202104.0521.v1

2.7 Population genetic analyses

Individual genotypes for SNPs for the five species were used to conduct population genetic analyses. PGDSpider v. 2.0.4 [51] was used to convert the SNP genotypes files into formats suitable for further population genetic analyses. To assess the level of genetic divergence among species, F_{ST} values [52] were estimated in R v.2.15.3 [53] using package Hierfstat v. 0.04-10 [54] for SNPs in both microsatellite-containing transcripts and those lacking microsatellites.

Microsatellites on average tend to have higher mutation rates when compared to the rest of the genome [55], hence, to obtain a neutral estimate of genetic divergence between genes harboring microsatellites and gene lacking microsatellites, F_{ST} values at SNPs were estimated separately for each category. With respect to microsatellites' role in species divergence, we assume that microsatellite alleles of different lengths could aid in differential response to the environment, hence, different microsatellite alleles are likely positively selected in different species. As such, if variation in microsatellite lengths is contributing to differences among species, then allele frequency differences at microsatellite- containing genes are likely to be elevated among species when compared to the background rate of divergence. Hence, to ascertain if genetic divergence observed at microsatellite-containing genes is likely to be higher or lower than background rate of divergence, we compared F_{ST} values from SNPs at microsatellite-containing genes versus those lacking microsatellites. Wilcoxon Rank Sum tests were carried out in R v.15.2.0 [53] to evaluate significance.

2.8 Shared microsatellites

To estimate the proportion of microsatellite tracts identified in *H. annuus* that are shared across other *Helianthus* species, a subset of 1,129 microsatellite loci with sufficient genotype data across 48 out of the 50 individuals was extracted from microsatellites detected by TRF. Coverage was determined based on SAMtools idxstats gene-by-gene expression data, with > 0 reads found at a gene considered to indicate coverage. This set of 1,129 was then analyzed by RepeatSeq, with successful genotyping of a locus in an individual considered as evidence for that locus being shared with that individual. Motif size and gene region information obtained via ESTscan [56, 57] data were then used to test whether the location of the microsatellite within a gene determined the likelihood of that microsatellite being shared among the five *Helianthus* species. Values were normalized relative to the average detection rate of loci in *H. annuus*.

3. Results

3.1 SNPs and microsatellites mined

Approximately 200,000 high quality SNPs corresponding to 99.9% genotyping accuracy were mined from the data using parameters previously described in [36]. All individuals were genotyped at these high-quality SNPs.

A total of 11,166 putative microsatellites were identified in the reference transcriptome by SciRoKo and of those, 9,479 microsatellites located in 7,247 transcripts were successfully mapped to genes in the reference genome (Supplementary Table S1). A fraction (3,786/11,166) was identified using TRF and used for further genotyping with RepeatSeq to assess the proportion of microsatellites shared among species. High quality microsatellite loci were genotyped at 1,129 of the original list of 3,786 microsatellite loci in 48 individuals with RepeatSeq and that information was used to identify the proportion of microsatellites that each species shared with *H. annuus.* This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3.2 Differential expression analysis

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Significantly differentially expressed (DE) genes were identified for each pairwise species comparison with edgeR. The highest number of DE transcripts were identified between *H. exilis* and *H. petiolaris* (20,851), of which 14,756 were mapped to annotated genes in the *H. annuus* genome (Supplementary Table S2). This estimate was closely followed by 20,476 (13,789 mapped to the *H. annuus* genome) DE transcripts identified between *H. annuus* and *H. exilis* (Supplementary Table S2). The lowest number of DE transcripts was observed between *H. annuus* and *H. debilis* (601), and 433 of those transcripts were successfully mapped to annotated genes in the *H. annuus* genome (Supplementary Table S2). No transcript was identified as significantly differentially expressed across all 10 pairwise species comparisons.

3.3 Microsatellite-containing differentially expressed genes

Using the list of 7,247 microsatellite-containing genes in the reference transcriptome, we identified microsatellite-containing DE genes in all pairwise species comparisons (Supplementary Table S3). The highest percentage of microsatellite-containing DE genes were identified between *H. bolanderi* and *H. debilis* (26.4% of the 7,671 DE genes) (Figure 1a). It was followed by 1,952 (25.9% of the 7,539 DE genes) microsatellite-containing DE genes identified in the *H. annuus* and *H. bolanderi* comparison (Figure 1a). The lowest percentage of microsatellite-containing DE genes was observed between *H. annuus* and *H. petiolaris* at 22% (1,208 of 4,282 DE genes) (Figure 1a). Across all 10 pairwise species comparisons, we identified 4,978 microsatellite-containing DE genes (23.8% of 20,886 DE genes). In these microsatellite-containing genes that were identified as differentially expressed in at least one pairwise species comparison, the highest percentage of microsatellites were located in non-coding regions (68.4% of 5,451 microsatellites). We observed similar patterns of microsatellite distribution in DE genes identified in pairwise species comparisons, and the greatest percentage of non-coding microsatellites were identified in the DE genes between *H. annuus* and *H. debilis* (74.5% of 106 microsatellites) (Table 1).

Table 1. Distribution of microsatellites in differentially expressed genes between different *Helianthus* spp.

Pairwise species comparison	Number of differentially expressed (DE) genes	Microsatellites in DE genes	
		In non-coding regions	In coding regions
H. annuus v H. bolanderi	7,539	1,490	669
H. annuus v H. debilis	433	79	27
H. annuus v H. exilis	13,789	2,416	1,120
H. annuus v H. petiolaris	5,490	910	401
H. bolanderi v H. debilis	7,671	1,526	701
H. bolanderi v H. exilis	470	76	37
H. bolanderi v H. petiolaris	9,593	1,865	865
H. exilis v H. debilis	12,567	2,350	1,132
H. exilis v H. petiolaris	14,576	2,653	1,233
H. petiolaris v H. debilis	1,492	266	122

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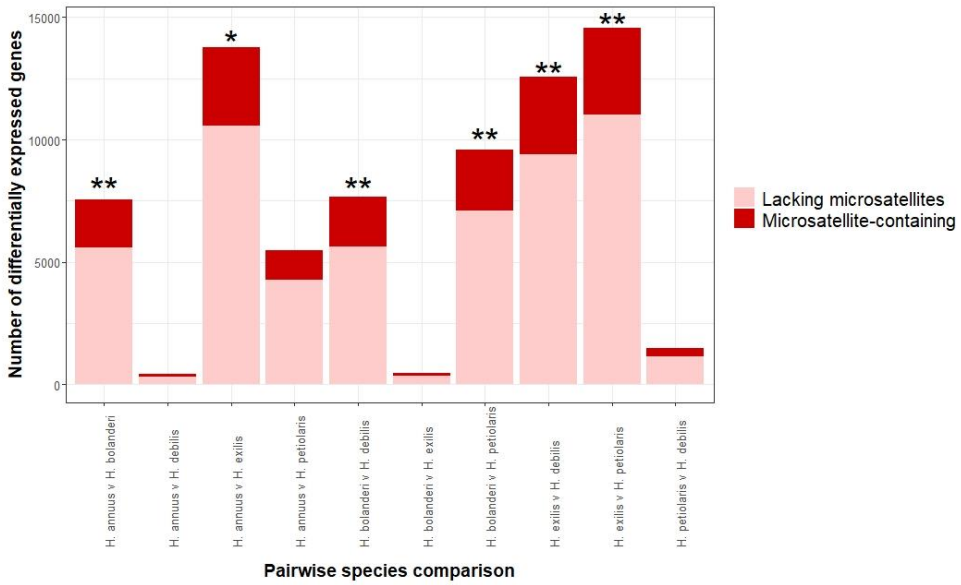
(**a**)



(**b**)

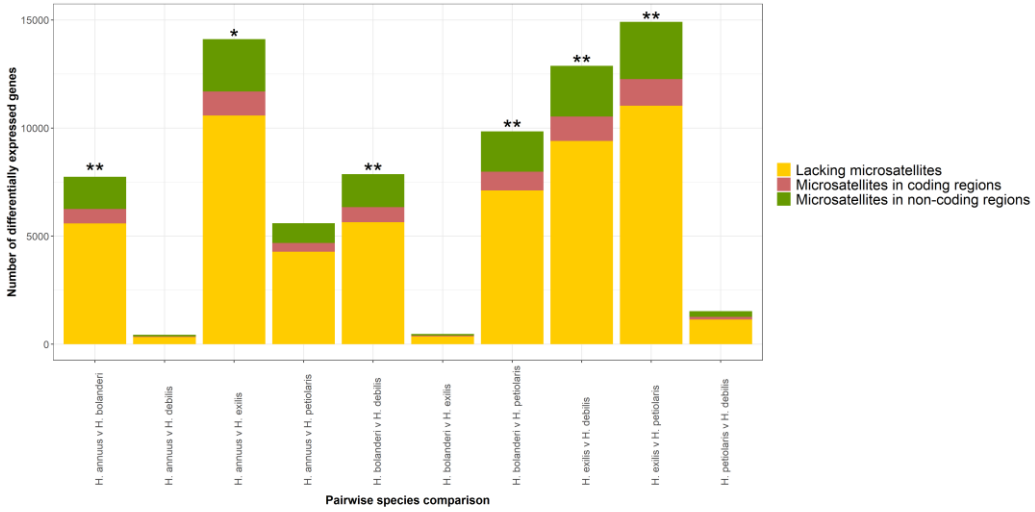


Figure 1. (a) Distribution of microsatellite-containing differentially expressed genes in pairwise *Helianthus spp.* comparisons. Chi-squared tests were performed to test whether microsatellite-containing genes were more likely to be differentially expressed between species pairs (* p-value < 0.0001, **p-value < 0.00001). (b) Distribution of microsatellites in different transcript regions within differentially expressed genes in pairwise *Helianthus spp.* comparisons. Chi-squared tests were performed to test whether genes with microsatellites in non-coding regions were more likely to be differentially expressed compared to genes with microsatellites in coding regions and genes lacking microsatellites (*p-value < 0.01, **p-value < 0.00001).

In general, when genes that were identified as differentially expressed in at least one pairwise species comparison (20,886) were considered, microsatellite-containing genes were more likely to be differentially expressed than genes lacking microsatellites (Chi-squared test, p-value < 0.00001, Figure 1a). Similarly, in six of the 10 pairwise species comparisons, we found that microsatellite-containing genes were more likely to show expression divergence compared to genes lacking microsatellites (Figure 1a). In four pairwise species comparisons (*H. petiolaris v H. debilis*, *H. bolanderi v H. exilis*, *H. annuus v H. petiolaris*, and *H. annuus v H. debilis*), presence of microsatellites did not affect the likelihood of differential expression in genes (Chi-squared test, p-value >0.05) (Figure 1a).

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We further tested whether genes containing microsatellites within non-coding regions (UTRs and introns) are more likely to be differentially expressed compared to genes with microsatellites in the coding regions and genes lacking microsatellites. Generally, across genes that were identified as differentially expressed in at least one pairwise species comparison (20,886), a gene with a microsatellite in non-coding regions was more likely to be differentially expressed compared to genes with microsatellites in the coding regions and genes lacking microsatellites (Chi-squared test, p-value < 0.0001) (Figure 1b). We observed similar associations of non-coding microsatellites and differential expression in six of the 10 pairwise species comparisons, except in *H. petiolaris v H. debilis*, *H. bolanderi v H. exilis*, *H. annuus v H. petiolaris*, and *H. annuus v H. debilis* (Chi-squared test, p-value >0.05) (Figure 1b). However, trends were consistent with an elevated rate of differential expression between most species comparisons, and the relatively low numbers of DE genes identified in some of these pairwise comparisons may have likely reduced the power to detect significant associations.

3.4 Functional annotation and gene ontology enrichment analysis

The BLASTX search of the reference transcriptome against the annotated *H. annuus* genome produced best hits for 32,425 out of 51,468 transcripts (Supplementary Table S4). Using the output of SciRoKo we identified 7,247 microsatellite-containing genes from the list of best hits (Supplementary Table S1).

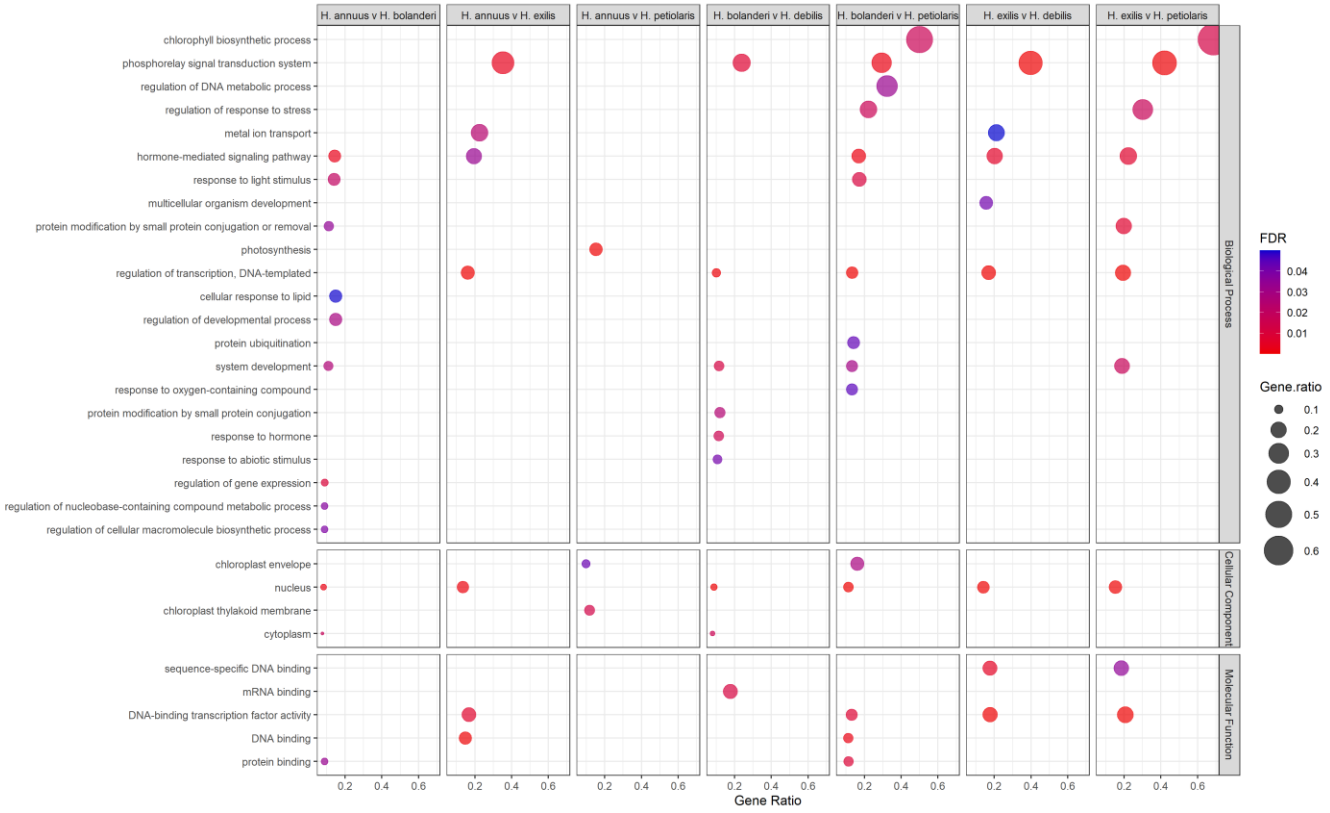

Figure 2. Gene Ontology (GO) terms enriched within microsatellite-containing differentially expressed genes in each pairwise *Helianthus* spp. comparison. The identified GO terms represent the three GO categories, biological process, molecular function, and cellular component. False Discovery Rate (FDR) associated with each comparison is represented by the color of dots for each GO term, and the size of each dot represents Gene Ratio (Number of genes associated with the GO term in the test set/ Number of genes associated with the GO term in the reference transcriptome).

GO enrichment analysis of microsatellite-containing DE genes compared to genes in the reference transcriptome identified enriched GO terms in seven out of the 10 pairwise

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species comparisons (Supplementary Table S5). The number of GO terms enriched in pairwise species comparisons ranged from 12 to 73 with most GO terms identified in the *H. bolanderi v H. petiolaris* comparison (Supplementary Table S5). These enriched GO terms were further reduced to most specific GO terms with OmicsBox (v 1.4) (Supplementary Table S6, Figure 2). The enriched GO terms represented the three GO categories, namely, biological process, molecular function, and cellular component. Some noteworthy, enriched GO terms in microsatellite-containing DE genes across multiple pairwise species comparisons included "regulation of transcription, DNA-templated" (GO:0006355, in five comparisons), "DNA-binding transcription factor activity" (GO:0003700 in four comparisons), and "hormone-mediated signaling pathway" (GO:0009755 in five comparisons) (Figure 2).

When microsatellite-containing genes that were identified as DE in at least one pairwise species comparison was used as the 'test-set', we identified 48 enriched GO terms across all three GO categories Supplementary Table S7. These 48 GO terms were further reduced to 10 specific GO terms that included some of the GO terms associated with transcription regulation identified in pairwise species comparisons Supplementary Table S8.

3.5 Population genetic estimates

To infer the relative divergence of microsatellite-containing genes to those lacking microsatellites, F_{ST} values at SNPs in these two sets of genes were compared. Mean pairwise F_{ST} at microsatellite-containing genes were significantly greater than those of genes lacking microsatellites (Table 2).

Table 2. Pairwise species differences as measured by F_{ST} at SNPs in microsatellite-containing and microsatellite lacking genes are shown in this table.

Pairwise comparison	Mean F_{ST} for genes lacking microsatellites	Mean F_{ST} for microsatellite-containing genes	Wilcoxon rank sum test p-value
H. annuus v. H. argophyllus	0.413	0.449	2.95E-10
H. annuus v. H. debilis	0.385	0.426	8.04E-13
H. annuus v. H. petiolaris	0.326	0.357	2.17E-10
H. debilis v. H. argophyllus	0.545	0.586	4.16E-10
H. petiolaris v. H. argophyllus	0.506	0.538	4.50E-09
H. petiolaris v. H. debilis	0.281	0.309	2.82E-12

3.6 Shared microsatellites

Across species, we found that *H. bolanderi* shared the largest portion of *H. annuus* microsatellite loci (94.2%); *H. debilis*, *H. exilis*, and *H. petiolaris* showed considerably lower percentages of shared microsatellites (79.1%, 72.7%, and 70.7%, respectively). Of repeat motifs, mono- and dinucleotide repeat tracts were less shared across all species (Figure 3). Trinucleotide tracts were much more likely to be shared than other repeat sizes in *H. debilis* (0.971; average 0.791) and *H. petiolaris* (0.858; average 0.707) (Figure 3). *H. annuus* hexanucleotides were preferentially shared with *H. exilis* (0.893; average 0.727) (Figure 3). The position of microsatellite tracts within a gene was also seen to influence the proportion of shared microsatellites. In all species, the percentage of shared microsatellites found in the 3'UTR and in transcripts that did not detectably code for proteins was lower than the percentage of shared microsatellites found in the 5'UTR and in the coding region (Figure 4).

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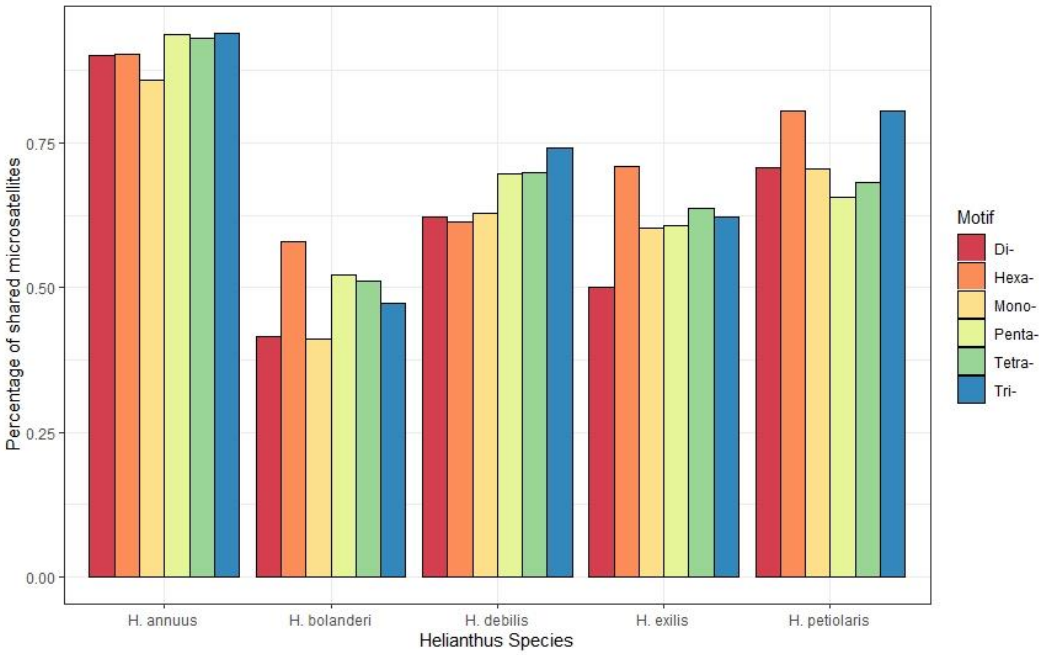

Figure 3. Estimated shared proportion of microsatellite loci of different motif sizes across five *Helianthus* species. Values shown are averages for all individuals of a species, relative to overall shared proportion in *H. annuus*.

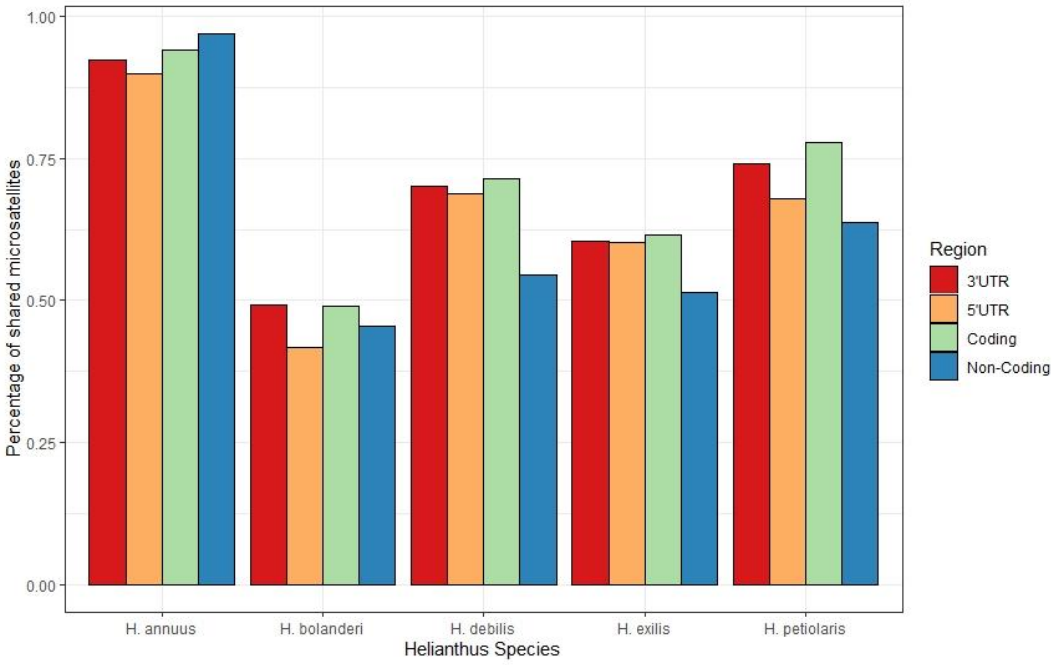

Figure 4. Estimated shared proportion of microsatellite loci in different transcript regions across five *Helianthus* species. "Non-coding" indicates microsatellites found in transcripts which were not predicted to encode protein sequence. Values shown are averages for all individuals of a species, relative to overall shared proportion in *H. annuus*.

4. Discussion

Microsatellites are an integral part of eukaryotic genomes and are abundant in both functional and non-functional regions. Frequently found in cis-regulatory regions of genes, microsatellites have been implicated in gene function and products linked to several traits. Here we tested the prediction that microsatellites in transcribed regions of the genome are involved in gene expression divergence among species with 50

 doi:10.20944/preprints202104.0521.v1

individuals from nine closely related *Helianthus* species using an RNA-seq approach. Our results show that microsatellite-containing genes in general are more likely to be differentially expressed among species. Further, compared to genes with microsatellites in coding regions and genes lacking microsatellites, genes that harbor microsatellites in non-coding regions are more likely to be differentially expressed among species. We found that microsatellite-containing differentially expressed genes were significantly enriched for GO terms such as "regulation of transcription, DNA-templated" (GO:0006355) and "DNA-binding transcription factor activity" (GO:0003700) among others. Population genetic analyses indicate greater levels of divergence in genes that contained microsatellites compared to those lacking microsatellites.

In most pairwise species comparisons and across all, we observe that microsatellite-containing genes are more likely to show gene expression divergence compared to those lacking microsatellites (Figure 1a) which highlights the potential contribution of microsatellites to *Helianthus* species adaptation through gene expression regulation. Similar patterns linking microsatellites to gene expression divergence have been reported in studies on primates. With a large panel of microsatellites from humans and other primates, [58] reported that genes harboring microsatellites in promoters, introns, UTRs, and coding regions showed greater levels of gene expression divergence among species when compared to genes lacking microsatellites. This study further revealed that genes with microsatellites in transcribed regions consistently show elevated inter-species gene expression levels across different tissue types [58]. At the intra-species level, microsatellites of short motif types, specifically A and AG repeats have been implicated in gene expression divergence among common sunflower populations, which suggests that specific microsatellite motif types may be more likely to influence gene expression divergence [32]. In addition, a recent study on common sunflower populations across a narrow latitudinal range detected hundreds of transcribed microsatellites linked to gene expression variation among and within populations, which provides further evidence of the regulatory role of microsatellites at an even finer scale [27].

The location of microsatellites within transcripts could be crucial in determining their regulatory role. Our results indicate that a gene containing a microsatellite in non-coding regions that may include UTRs are more likely to show gene expression divergence among sunflower species. Previous reports on UTR microsatellites have linked them to gene expression variation in several species [50, 59]. Some of the proposed mechanisms by which UTR microsatellites can regulate gene expression involve altering transcription start and end sites [59,60] and influencing mRNA stability [61]. Certainly, our results from the GO enrichment analysis of microsatellite-containing differentially expressed genes appear to suggest that microsatellite-mediated gene expression regulation could be linked to transcription factor binding.

Population genetic analysis indicates that microsatellite-containing genes could be under stronger selective pressures than those lacking microsatellites. These patterns are significant and consistent across all pairwise species comparisons. Collectively, these estimates provide compelling evidence to support the potential contribution of microsatellites to species divergence in *Helianthus.* However, we acknowledge that our use of a single *Helianthus annuus* individual transcriptome as the reference could have introduced some bias when mapping and mining SNPs. Further, short-read technology and RNA-seq data come with their own set of limitations and challenges. Short reads could have limited access to some longer microsatellite alleles in some species, which could have affected RepeatSeq based estimates of shared microsatellites. RNA-seq only provides access to the transcribed regions of the genomes, therefore microsatellites upstream of 5'UTRs that could potentially influence gene expression divergence are beyond our reach. Given these limitations, we believe that the evidence that this study provides supporting microsatellites' role in gene expression divergence is consistent across multiple species comparisons and substantial. Collectively, our study shows that transcribed microsatellites could significantly contribute to species divergence; therefore, we expect that integrating analysis of these highly mutable repetitive elements in gene

expression studies could greatly enhance our understanding of the genomic basis of species divergence.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: List of microsatellite-containing transcripts in the reference transcriptome mapped to the *Helianthus annuus* reference genome, Table S2: Differentially expressed transcripts identified in the ten pairwise species comparisons, Table S3: List of microsatellite-containing transcripts differentially expressed in each pairwise *Helianthus* species comparison, Table S4: BLASTX results from mapping the reference transcriptome to the annotated *Helianthus annuus* reference genome, Table S5: Table S5. Gene Ontology (GO) terms enriched within microsatellite-containing differentially expressed genes identified in pairwise *Helianthus* species comparisons compared to the reference transcriptome, Table S6: Gene Ontology (GO) terms enriched (reduced to most specific) within microsatellite-containing differentially expressed genes identified in pairwise *Helianthus* species comparisons compared to the, Table S7: Gene Ontology (GO) terms enriched within microsatellite-containing genes differentially expressed in at least one pairwise species comparison compared to the reference transcriptome, Table S8: Gene Ontology (GO) terms enriched (reduced to most specific) within microsatellite-containing genes differentially expressed in at least one pairwise species comparison compared to the reference.

Author Contributions: Conceptualization, M.E.W, L.H.R, S.R, and SP; data analysis, C.R, S.P, S.R, and G.W.; writing—review and editing, C.R, S.P, S.R, A.D.P, G.W, L.H.R, and M.E.W; supervision, M.E.W, L.H.R, and A.D.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Science Foundation grant MCB- 1158521 to M.E.W and the Natural Sciences and Engineering Research Council of Canada grant number 327475 to L.H.R.

Data Availability Statement: Reference transcriptome used in this study has been deposited in the Dryad Digital Repository (http://datadryad.org/resource/doi:10.5061/dryad.9q1n4). All sequence data have been deposited at the National Center for Biotechnology Information (NCBI) under project PRJNA193050.

Conflicts of Interest: The authors declare no conflict of interest.

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