Transposable Element Proliferation and Genome Expansion Are Rare in Contemporary Sunflower Hybrid Populations Despite Widespread Transcriptional Activity of LTR Retrotransposons

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We sequenced a small number of RT-PCR bands (coding regions of LTR retrotransposons) for confirmation purposes. These sequences have been deposited in GenBank under accession numbers HQ665469–HQ665504.

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Abstract

Hybridization is a natural phenomenon that has been linked in several organismal groups to transposable element derepression and copy number amplification. A noteworthy example involves three diploid annual sunflower species from North America that have arisen via ancient hybridization between the same two parental taxa, Helianthus annuus and H. petiolaris. The genomes of the hybrid species have undergone large-scale increases in genome size attributable to long terminal repeat (LTR) retrotransposon proliferation. The parental species that gave rise to the hybrid taxa are widely distributed, often sympatric, and contemporary hybridization between them is common. Natural H. annuus × H. petiolaris hybrid populations likely served as source populations from which the hybrid species arose and, as such, represent excellent natural experiments for examining the potential role of hybridization in transposable element derepression and proliferation in this group. In the current report, we examine multiple H. annuus × H. petiolaris hybrid populations for evidence of genome expansion, LTR retrotransposon copy number increases, and LTR retrotransposon transcriptional activity. We demonstrate that genome expansion and LTR retrotransposon proliferation are rare in contemporary hybrid populations, despite independent proliferation events that took place in the genomes of the ancient hybrid species. Interestingly, LTR retrotransposon lineages that proliferated in the hybrid species genomes remain transcriptionally active in hybrid and nonhybrid genotypes across the entire sampling area. The finding of transcriptional activity but not copy number increases in hybrid genotypes suggests that proliferation and genome expansion in contemporary hybrid populations may be mitigated by posttranscriptional mechanisms of repression.

Key words: hybridization, transposable elements, derepression, genome evolution, repetitive DNA.

Introduction

Transposable elements are DNA sequences that can mobilize and replicate within the genome of their host. Two major classes of elements are recognized based on the nature of the transposition intermediate and mechanism of mobilization (Wicker et al. 2007). Class I elements transpose via an RNA intermediate transcribed from an existing element; intermediates are reverse transcribed prior to insertion at a new location. The class II elements transpose via a DNA intermediate that is either excised from its existing location via double-stranded cleavage followed by subsequent insertion elsewhere or by a phenomenon involving transposition in which only a single strand is cleaved (e.g., rolling circle replication, [Kapitonov and Jurka 2001]). The vast amounts of genome sequence data now available for a large and growing number of organisms coupled with rapid advances in analytical approaches for detecting repetitive elements...
have greatly facilitated studies of transposable element biology (Saha et al. 2008; Feschotte et al. 2009; Pritham 2009; Lerat 2010). Consequently, our understanding of these sequences and their potential roles in shaping genome evolution has grown significantly in recent years (Pritham 2009; Tenaillon et al. 2010).

Class I elements are particularly relevant in evolutionary analyses of genome size and structure because of their proliferative mode of replication. Autonomous long terminal repeat (LTR) retrotransposons are the most abundant Class I elements in plants and are subdivided into two superfamilies known as Ty3/Gypsy-like and Ty1/Copia-like (Kumar and Bennetzen 1999). These superfamilies display similar structural features (e.g., flanking LTRs in direct orientation, presence of GAG and POL genes) but represent separate ancient lineages diverged at the sequence level and that differ with regard to domain order within POL. Despite their proliferative capacity, transcriptional and/or transpositional activity of most Class I elements is repressed by the host genome through a combination of epigenetic mechanisms involving both transcriptional and posttranscriptional controls (Zilberman and Henikoff 2004; Slotkin and Martienssen 2007; Lisch 2009). Because of the highly mutagenic consequences of unchecked transposition, historical selection likely has been strong for the maintenance of these host repression mechanisms. Under certain genomic and environmental conditions, however, host repression may fail, resulting in large-scale and episodic activation and proliferation (Wessler 1996; Grandbastien 1998; Lisch 2009).

Hybridization is a naturally occurring phenomenon shown in some species to be associated with derepression of LTR retrotransposons (Waugh O’Neill et al. 1998; Labrador et al. 1999; Metcalfe et al. 2007; Lisch 2009; Michalak 2009). The molecular basis of hybridization-induced element derepression is not fully understood but likely involves disruption of one or more host repression systems as a consequence of merging differentiated genomes. A potential example of such derepression involves three annual sunflower species from North America. These species (Helianthus anomalus, H. deserticola, and H. paradoxus) arose via ancient hybridization events between the same two extant sunflower parental species, H. annuus and H. petiolaris. The hybrid species independently have undergone massive proliferation events of Ty3/gypsy-like elements and, to a lesser extent, Ty1/copia-like elements (Ungerer et al. 2006, 2009; Staton et al. 2009; Kawakami et al. 2010). These proliferation events occurred in recent evolutionary time, as evidenced both by the young ages of the hybrid species (Schwarzbach and Rieseberg 2002; Welch and Rieseberg 2002; Gross et al. 2003) and by estimates of proliferation events themselves (Ungerer et al. 2009).

Because of their broad geographic and often sympatric distributions, natural hybridization between H. annuus and H. petiolaris remains an ongoing and common phenomenon in wild populations. Contemporary H. annuus × H. petiolaris hybrid populations typically consist of a mixture of genotypes ranging from early generation hybrid to advanced backcross individuals and display a broad array of hybrid index scores (Rieseberg et al. 1998, 1999). Natural zones of hybridization likely served as sources for the establishment and evolution of the hybrid species and, as such, represent excellent natural laboratories to examine genome dynamics of hybridization, including the potential role of hybridization in transposable element derepression and proliferation.

In the current report, we examine whether natural hybridization between H. annuus × H. petiolaris may be a catalyst for transposable element derepression and genome expansion. We demonstrate that, despite massive proliferation events in the genomes of all three sunflower hybrid species, natural hybridization between the two parental species is not, in-and-of-itself, a major trigger of transposable element proliferation, and that genome size increases in hybrid genotypes are rare. Interestingly, however, we detect widespread transcriptional activity of Ty3/gypsy and Ty1/copia sequences in both hybrid and nonhybrid genotypes. These results suggest posttranscriptional mechanisms of repression may be preventing further copy number amplification of these sequences in wild sunflower populations.

Materials and Methods

Plant Materials

During the summer of 2008, seeds were collected from naturally occurring H. annuus × H. petiolaris hybrid populations and nearby (within ~1 km) stands of “pure” H. annuus and H. petiolaris from several locations in the central, western, and southwestern United States (table 1). At two locations (Keith Co., Nebraska and Quay Co., New Mexico), two or three nearby but distinct hybrid populations were identified and sampled. The two hybrid populations from Keith Co. were the focus of previous studies by Rieseberg et al. (1998, 1999). The remaining hybrid populations were newly identified for the current study.

At each hybrid and parental population, seeds were collected from mature capitula on 20–30 plants in the field. Seeds were transported to Kansas State University and germinated in the dark on moist filter paper in Petri dishes. Seedlings were transferred to 4-inch pots and allowed to grow in the Kansas State University greenhouses until suitable size for the harvesting and processing of leaf tissue for 1) genome size determination via flow cytometry and 2) DNA extraction for estimation of transposable element numerical abundance via quantitative polymerase chain reaction (PCR) and microsatellite analyses of hybridity. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer’s instructions. Sample sizes for the different experimental assays of hybrid and nonhybrid genotypes are indicated in table 1.
Microsatellite Assay

Natural *H. annuus × H. petiolaris* hybrid populations can be identified phenotypically as they consist of individuals displaying a range of phenotypes spanning the variability across the two species, with many individuals showing intermediate characteristics. However, to 1) confirm these hybrid populations as such and 2) examine aspect of genome size and LTR retrotransposon copy number variation in relation to hybrid index scores, we utilized an M13-tailed primer protocol (Schuelke 2000) with four possible fluorophores (NED, FAM, VIC, and PET). PCR reactions were performed in 15 µl volumes consisting of 1× PCR buffer (Promega), 2.5 mM MgCl2, 200 µM of each dNTP, 50 nM of a forward M13-tailed primer, 50 nM of a M13 dye-labeled primer (5’-AC-GACGTGGTAAAACGAC), 100 nM reverse primer, 1 unit of Taq polymerase, and 2–12 ng of template DNA.

PCR was conducted on an MJ Research PTC-100 thermal cycler using the following touchdown procedure: initial denaturing at 95 °C for 5 min; followed by 5 cycles of 95 °C for 45 s, 68 °C for 5 min (−2 °C/cycle), 72 °C for 1 min; followed by 5 cycles of 95 °C for 45 s, 58 °C for 2 min (−2 °C/cycle), 72 °C for 1 min; followed by 25 cycles of 95 °C for 45 s, 50 °C for 2 min, 72 °C for 1 min; and a final extension of 72 °C for 5 min. PCR was conducted separately for each locus but with amplification products of 3–4 loci (each utilizing a different dye) subsequently pooled for co-loading on an ABI 3730 DNA Analyzer. Raw data were scored using GeneMarker software Version 1.85 (SoftGenetics). Hybrid index scores were determined for each hybrid population using the program HINDEX V 1.42 (Buerkle 2005) with parental allele frequencies estimated from nearby stands of *H. annuus* and *H. petiolaris* populations.

Genome Size Determination

Nuclear DNA content (2C genome size) was estimated using a FACSCalibur flow cytometer (Becton Dickinson). A minimum of 10,000 nuclei was analyzed per sample. To prepare samples for assay, fresh leaves (50 mg) were chopped with razor blades in a buffer containing 15 mM N-2-hydroxyethylpiperoxazine-N’-2-ethanesulfonic acid, 1 mM ethylenediaminetetraacetic acid, 80 mM KCl, 20 mM NaCl, 300 mM sucrose, 0.2% Triton-X, 0.5 mM spermine, 0.1% β-mercaptoethanol (modified after Bino et al. 1993), filtered through 30-mm nylon mesh, and then centrifuged to collect nuclei. The samples were stained for 2 h with 700 ml of intercalating propidium iodide solution (BioSure) and 2 ml of an internal standard solution containing chicken erythrocyte nuclei (CEN; BioSure). Duplicate analyses were conducted for a subset of individuals from hybrid populations (5~6 individuals per population) to determine the repeatability and precision of the estimates.

Because considerable variation can exist in reported 2C values of CEN among studies (reviewed in Bennett and Leitch 1995 and references therein), we estimated 2C value of CEN by using *H. deserticola* (one of the sunflower hybrid species) as an external standard. Leaves from *H. deserticola*...
(n = 4 plants, Ames 26094; United States Department of Agriculture [USDA], National Plant Germplasm System [NPGS]) were analyzed with the method described above. The analysis was duplicated on two different days. Mean peak positions of CEN relative to H. deserticola nuclei (2C = 10.79 pg, [Baack et al. 2005]) gave an estimated 2C value of CEN = 2.689 pg (standard deviation [SD] = 0.152). To validate that this 2C value of CEN yields appropriate genome size estimates of samples, 2C genome size of H. anomalus (Ames 26095; USDA, NPGS) also was estimated. Our estimate of 2C genome size of H. anomalus (2C = 11.03−11.83, n = 2) was consistent with a previous report for this species (2C = 11.46 pg, [Baack et al. 2005]). The appropriateness of the 2C value of CEN in our experiment was thus confirmed. The same CEN batch was utilized for genome size estimates of all samples reported in this study.

**LTR Retrotransposon Copy Number Estimation**

Copy number estimates of specific lineages of Ty3/gypsy and Ty1/copia LTR retrotransposons in the genomes of hybrid and pure parental individuals were estimated via quantitative PCR using a standard curve method described in Ungerer et al. (2006) and Kawakami et al. (2010). Briefly, a 168 bp Ty3/gypsy integrase (Int) fragment and a 230 bp Ty1/copia RNase H fragment were generated by standard PCR using genomic DNA of H. annuus with the following primers: forward, 5′-TTATGCATTGCCACAAAG-3′, and reverse, 5′-TCGACTCACAAGTCGACAC-3′, for Ty3/gypsy Int domain, and forward, 5′-TCTCAAGACCCTCGGCAATCT-3′, and reverse, 5′-GGCGAGCCAAAGAGGAAAATG-3′, for Ty1/copia RNase H domain. These fragments were cloned using a StrataClone PCR Cloning Kit (Agilent Technologies). The concentration of isolated plasmids (and insert) was determined by a NanoDrop spectrophotometer (Thermo Scientific), and plasmid number per unit volume was estimated. A 5-fold dilution series of the cloned fragments was utilized (50, 10, 2, 0.4, and 0.08 unit volume was estimated. A 5-fold dilution series of the clones was used to generate standard curves that were used to estimate absolute abundances of elements in our samples.

Quantitative PCR assays were performed using 1 μl of cloned DNA for standard curves or sample DNA (standardized to 80 pg/μl) with an iCycler iQ™ quantitative PCR system (Bio-Rad). Each reaction was performed in duplicate using the iQ™ SYBR Green Supermix kit (Bio-Rad) following the manufacturer’s protocols, with the exception that reactions were conducted in 25 μl volumes. PCR efficiency for the standard curve exceeded 96%. Copy number estimates were converted to copy number per genome using mean C-values for H. annuus and H. petiolaris estimated from several populations ([Baack et al. 2005]). The average C-value for H. annuus and H. petiolaris (6.96 pg) was used for hybrid individuals. Assays of LTR retrotransposon copy number were conducted on the same set of individuals that were genotyped with microsatellite markers, with the exception of individuals from the Barton Co. Kansas sampling location.

**Statistical Analysis**

Estimated nuclear DNA content and copy number estimates of Ty3/gypsy-like and Ty1/copia-like sequences were compared by analysis of variance (ANOVA). Particular hybrid individuals exhibiting elevated nuclear DNA content or very high copy number estimates of Ty3/gypsy or Ty1/copia elements were subjected to the Dixon outlier detection test ([Verma and Quiroz-Ruiz 2006]). Correlation between nuclear DNA content and copy numbers of Ty3/gypsy-like or Ty1/copia-like sequences was examined by Pearson product-moment correlations. All statistical analyses were performed in the software JMP 7.0.1 (SAS institute).

**Transcriptional Assays**

Transcriptional assays of Ty3/gypsy and Ty1/copia elements were conducted using the AccessQuick reverse transcriptase (RT)-PCR System (Promega). The same primer pairs used to estimate genomic copy numbers of Ty3/gypsy and Ty1/copia elements (see above) were utilized in assays of transcriptional activity of these retrotransposons. Primers targeting the 18s rRNA subunit (forward, 5′-CTTCGGATCCGAGTAATGA-3′, and reverse, 5′-TAAGAGGCTGCGATTTGAG-3′) were used for positive control reactions. To test for genomic DNA contamination of RNA, negative control reactions were performed by withholding the AMV RT. RT-PCR amplification was carried out by incubating at 45 °C for 45 min for the reverse transcription step, followed by one cycle of 95 °C for 2 min, 22 cycles (94 °C for 30 s; 56 °C for 30 s; and 72 °C for 60 s), and a final incubation cycle at 72 °C for 5 min. The amplified products were size-separated via electrophoresis in 2% agarose gel and visualized with ethidium bromide. Total RNA was extracted using TRizol (Invitrogen) and purified with a RNeasy Plant Mini Kit (Qiagen) following the manufacturer’s instructions. RNA was treated with RNase-Free DNase (Qiagen) to eliminate genomic DNA contamination.

RT-PCR amplified cDNA fragments of Ty3/gypsy and Ty1/copia were cloned from one H. annuus, H. petiolaris, and hybrid individual from the Barton County, KS location using a StrataClone PCR Cloning Kit. From 7 to 10 clones, per element per individual were sequenced on an ABI 3730XL DNA Analyzer (Applied Biosystems). DNA polymorphisms and sequence diversity indices were estimated using DNA SP ver. 5 ([Librado and Rozas 2009]).

**Results**

**Genome Size Estimates in Hybrid and Nonhybrid Genotypes**

At each of the five regional sampling locations (table 1), individuals from H. annuus populations consistently exhibit
higher mean 2C values than those from *H. petiolaris* populations (7.31 ± 0.424 – 7.69 ± 0.247 for *H. annuus* vs. 6.66 ± 0.192 – 7.29 ± 0.234 for *H. petiolaris*) (fig. 1; table 2). At those same locations, individuals from hybrid populations display 2C values largely intermediate between those observed for *H. annuus* and *H. petiolaris* (fig. 1, table 2), indicating that large-scale genome expansion in individuals from hybrid populations is rare. Significant differences in 2C values among *H. annuus*, *H. petiolaris*, and the hybrid populations were found at all sampling locations (table 2). Variance in 2C values typically was greater in hybrid populations than in populations of parental species, suggesting that a diversity of hybrid genotypes likely exists within the hybrid populations sampled. Several individuals from the Quay County, NM, hybrid population and a single individual from the El Paso County, CO, hybrid population exhibit 2C values that exceed values observed for either parental species. These values, however, were only slightly higher than the maximum values observed for *H. annuus* individuals. The Dixon outlier detection test (Verma and Quir oz-Ruiz 2006) failed to identify these values as significant outliers (*P* > 0.05).
Table 2
Estimates of Nuclear DNA Content and Copy Numbers of Ty3/gypsy and Ty1/copia Sequences in Individuals from Helianthus annuus, H. petiolaris, and Natural Hybrid Populations

| County           | Population | Nuclear DNA Content (pg/2C Genome) | Ty3/gypsy Copy Number | Ty1/copia Copy Number |
|------------------|------------|-----------------------------------|-----------------------|-----------------------|
|                  |            | Mean  SD    F     P               | Mean  SD    F     P   | Mean  SD    F     P   |
| El Paso Co., Colorado | ElP-A      | H. annuus    | 7.32 0.274 30.141 <0.0001 | 53,369 3,609 34.541 <0.0001 | 57,134 3,915 26.517 <0.0001 |
|                  | ElP-HP1    | Hybrids      | 7.04 0.426             | 26,019 3,228 | 30,292 3,502 |
|                  | ElP-P      | H. petiolaris| 6.66 0.192             | 9,804 3,609  | 25,131 3,915 |
| Keith Co., Nebraska | Kei-A      | H. annuus    | 7.31 0.424 7.326 0.0002 | 62,376 3,403 31.213 <0.0001 | 53,261 3,691 11.029 <0.0001 |
|                  | Kei-HP1    | Hybrids      | 6.95 0.376             | 13,457 3,609  | 23,957 3,915 |
|                  | Kei-HP2    | Hybrids      | 7.03 0.275             | 35,495 4,168  | 23,422 4,521 |
|                  | Kei-P      | H. petiolaris| 6.85 0.276             | 12,187 3,228  | 29,565 3,502 |
| Sandoval Co., New Mexico | San-A    | H. annuus    | 7.38 0.253 27.182 <0.0001 | 37,664 3,228 27.163 <0.0001 | 21,590 3,691 22.646 <0.0001 |
|                  | San-HP1    | Hybrids      | 7.09 0.345             | 24,454 3,403  | 21,889 3,691 |
|                  | San-P      | H. petiolaris| 6.79 0.277             | 12,249 3,609  | 14,626 3,915 |
| Quay Co., New Mexico | Qu-A     | H. annuus    | 7.69 0.247 7.365 <0.0001 | 34,288 3,858 6.382 0.0005 | 36,446 3,502 3.354 0.0197 |
|                  | Qu-HP1    | Hybrids      | 7.67 0.345             | 22,658 3,609  | 23,914 3,691 |
|                  | Qu-HP2    | Hybrids      | 7.50 0.387             | 24,177 3,403  | 14,135 3,915 |
|                  | Qu-HP3    | Hybrids      | 7.39 0.435             | 16,784 3,403  | 35,302 4,186 |
|                  | Qu-P      | H. petiolaris| 7.29 0.234             | 7,243 3,609  | 21,356 3,915 |
| Barton Co., Kansas | Bar-A     | H. annuus    | 7.50 0.425 35.55 <0.0001 | 39,492 3,078 39.923 <0.0001 | 56,944 3,339 11.677 0.0001 |
|                  | Bar-HP1   | Hybrids      | 7.33 0.398             | 32,176 2,283  | 46,991 2,476 |
|                  | Bar-P     | H. petiolaris| 6.72 0.202             | 8,259 3,078  | 30,929 3,339 |

Within parental species, mean 2C values were significantly different among the five regional sampling locations (H. annuus: ANOVA, $F_{4,124} = 5.98$, $P = 0.0002$; H. petiolaris: ANOVA, $F_{4,125} = 31.39$, $P < 0.0001$). Mean 2C values also were significantly different among hybrid populations across the sampling area (ANOVA, $F_{7,214} = 13.44$, $P < 0.0001$). Populations of H. annuus and H. petiolaris from Quay County, NM, displayed the highest species-specific mean 2C values. Correspondingly, hybrid populations from this location also displayed higher 2C values than hybrid populations from other locations.

**LTR Retrotransposon Copy Number Estimates in Hybrid and Nonhybrid Genotypes**

Because flow cytometry may lack sensitivity to detect genome size changes associated with smaller-scale increases in LTR retrotransposon copy numbers, we performed quantitative PCR assays of Ty3/gypsy and Ty1/copia copy number abundance in a subset of hybrid individuals and nonhybrid individuals across the five regional collection locations. In previous reports (Ungerer et al. 2006; Kawakami et al. 2010), we identified specific sublineages of Ty3/gypsy and Ty1/copia that have undergone proliferation events in the hybrid sunflower species. Copy number estimates of these same lineages were determined for 6–20 individuals from each hybrid population and nearby stands of pure H. annuus and H. petiolaris. In accordance with observations of overall genome size (fig. 1), H. annuus individuals displayed a higher average abundance of Ty3/gypsy and Ty1/copia sequences compared with H. petiolaris (fig. 2, table 2), with estimates for hybrid populations again generally intermediate. A single hybrid individual from the Barton County, KS, population was identified with a copy number estimate of Ty3/gypsy that exceeded those observed for either parental species; however, this individual was not identified as an outlier (Dixon outlier test, $P > 0.05$). None of the hybrid individuals with slightly or moderately elevated 2C values (see fig. 1A and D) had correspondingly elevated copy number estimates of Ty3/gypsy and/or Ty1/copia sequences. Population-level differences in copy numbers of Ty3/gypsy and Ty1/copia sequences were found for H. annuus and H. petiolaris (ANOVA, $F_{4,40} = 8.02$, $P < 0.0001$; Ty1/copia: ANOVA, $F_{4,40} = 4.72$, $P = 0.0032$), and for the hybrid populations (Ty3/gypsy: ANOVA, $F_{7,71} = 3.74$, $P = 0.0017$; Ty1/copia: ANOVA, $F_{7,71} = 11.77$, $P < 0.0001$).

**LTR Retrotransposon Copy Number Estimates and Hybrid Index Scores**

Given differences in copy number abundance of retrotransposons between H. annuus and H. petiolaris (fig. 2) and given the lack of evidence of copy number amplification in individuals from hybrid populations (figs. 1 and 2), copy number estimates of Ty3/gypsy and Ty1/copia sequences in individuals from hybrid populations are expected to be correlated with hybrid index scores, which estimate the relative proportion of H. petiolaris versus H. annuus DNA in the genome. Figure 3 depicts the relationship between copy number abundance and hybrid index scores for Ty3/gypsy (fig. 3A) and Ty1/copia.
(fig. 3B) elements where hybrid index values of 0 and 1 represent *H. petiolaris*-like and *H. annuus*-like genotypes, respectively. Consistent with expectations, higher copy number estimates are associated with hybrid index scores closer to 1 (more *H. annuus*-like) for both superfamilies, although this relationship is stronger for *Ty3/gypsy* (fig. 3).

### LTR Retrotransposon Expression Assays

Transcriptional activity of *Ty3/gypsy* and *Ty1/copia* elements was assayed via RT-PCR for a total of 10 *H. annuus* individuals, 10 *H. petiolaris* individuals, and 19 individuals from hybrid populations across the five sampling locations. These assays were performed using the same primer pairs utilized to determine element copy number abundance via quantitative PCR (see Materials and Methods). Transcripts were detected for both types of element in all hybrid individuals as well as in all assayed individuals of *H. annuus* and *H. petiolaris* (fig. 4). DNA contamination was ruled out by negative control reactions in which the RT enzyme was withheld (fig. 4). Amplicons were confirmed as *Ty3/gypsy* and *Ty1/copia* elements where hybrid index values of 0 and 1 represent *H. petiolaris*-like and *H. annuus*-like genotypes, respectively. Consistent with expectations, higher copy number estimates are associated with hybrid index scores closer to 1 (more *H. annuus*-like) for both superfamilies, although this relationship is stronger for *Ty3/gypsy* (fig. 3).

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transcripts by cloning and sequencing PCR products for one H. annuus, one H. petiolaris, and one hybrid individual from the Barton Co, KS location. Between 7 and 10 clones were sequenced per amplicon per individual. Sequenced transcripts exhibited polymorphism within and between individuals (table 3), with higher levels of polymorphism observed for Ty3/gypsy. These patterns are consistent with a previous report documenting transcriptional activity of Ty3/gypsy and Ty1/copia elements in an inbred line of H. annuus (Vukich et al. 2009). All sequences have been deposited in GenBank under accession numbers HQ665469–HQ665504.

Discussion

Hybridization has been linked to activation of transposable elements in several organismal groups, with most examples documenting such activity in early generation hybrid genotypes (Waugh O’Neill et al. 1998; Labrador et al. 1999; Shan et al. 2005; Petit et al. 2010). In previous reports, we described the occurrence and dynamics of historical proliferation events of LTR retrotransposons in the genomes of three sunflower species that independently arose via hybridization between the same two parental taxa (Ungerer et al. 2006, 2009; Staton et al. 2009; Kawakami et al. 2010). Temporal estimates of the origins of the hybrid species are variable (Schwarzbach and Rieseberg 2002; Welch and Rieseberg 2002; Gross et al. 2003; Ungerer et al. 2009), but all estimates suggest their formation within the last 1 My. Although bouts of transposable element proliferation in these species genomes are clearly associated with hybridization followed by genome reorganization (i.e., hybrid speciation), the extent to which natural hybridization in-and-of-itself was, and may continue to be, a catalyst for large-scale changes in genome size is unclear. The parental species, H. annuus and H. petiolaris, which gave rise to the hybrid taxa, have broad and overlapping geographic distributions, and interspecific hybridization between them is common (Rieseberg et al. 1999). Natural H. annuus × H. petiolaris hybrid populations likely served as original source populations from which the three hybrid species lineages became established and, as such, represent excellent natural laboratories for investigating the direct role of hybridization on transposable element dynamics in this group.

In the current report, genome size was estimated for a total of 222 individuals from hybrid populations and 259 individuals from nearby populations of pure parental species (n = 129 for H. annuus; n = 130 for H. petiolaris) over multiple locations where these species occur in sympatry. Consistent with previous reports (Baack et al. 2005), genome size estimates for H. annuus were consistently higher than those for H. petiolaris. These interspecific differences in genome size were mirrored by differences in copy number abundance of both Ty3/gypsy and Ty1/copia sequences, suggesting that differences in genome size between these species are attributable, at least in part, to differential abundance of LTR retrotransposons. Genome size estimates of individuals from hybrid populations were generally intermediate between those observed for H. annuus and H. petiolaris, suggesting that hybridization is not a major trigger of transposable element derepression in natural populations.

A small number of individuals from hybrid populations displayed genome size values that exceeded those measured for either parental species (fig. 1). These increases were small, however, especially compared with those that took place historically in the sunflower hybrid species, H. anomalus, H. deserticola, and H. paradoxus, where 2C values are at least 50% greater than those estimated for either parental species (Baack et al. 2005). Hybrid individuals exhibiting slightly elevated genome size values typically displayed hybrid index scores closer to 1 (more annuus-like) and did not demonstrate elevated copy number estimates.

![Fig. 3.](image-url) —Genomic copy number estimates of Ty3/gypsy (A) and Ty1/copia (B) elements as a function of hybrid index score for 59 hybrid individuals from four different regional sampling locations. Hybrid index scores range from 0 (H. petiolaris-like) to 1 (Helianthus annuus-like). Individuals from the Barton Co. Kansas site were not included in this analysis (see Materials and Methods).
Fig. 4.—Transcriptional assays of Ty3/gypsy and Ty1/copia elements in Helianthus annuus, H. petiolaris, and hybrid individuals collected from five different regional sampling locations (subpanels A–E). Plus (+) and minus (−) signs indicate whether RT enzyme was provided or withheld from the RT-PCR reaction, respectively. Ann, H. annuus; Pet, H. petiolaris; Hyb, hybrid individual.
of LTR retrotransposons in direct assays of numerical abundance via quantitative PCR. Hybrid index score thus proved the best predictor of LTR retrotransposon abundance (especially for Ty3/gypsy elements) and genome size in hybrid genotypes. Taken together, these observations suggest that proliferation of transposable element sequences in natural sunflower hybrid populations is indeed rare. These findings are consistent with several recent studies demonstrating an absence of transposable element mobilization in both laboratory generated (Beaulieu et al. 2009) and naturally occurring (Kentner et al. 2003; Hazzouri et al. 2008; Parisod et al. 2009) interspecific plant hybrids.

The lack of evidence for genome size expansion and LTR retrotransposon copy number increases across these five regional locations of Helianthus annuus × Helianthus petiolaris hybridization is notable and contrasts sharply with the scale and repeatability of proliferation events that took place in the genomes of the ancient hybrid species. It should be noted, however, that assays of LTR retrotransposon abundance via quantitative PCR, whereas appropriate for detecting large-scale proliferation, are likely to miss smaller-scale mobilization events that could be documented by other methods, such as transposon display (Waugh et al. 1997; Van den Broeck et al. 1998; Mel-ayah et al. 2001). The quantitative PCR assay provides reasonable resolution, however, as determined by variation between the two technical replicates performed for each individual in the current study. For example, across the 169 samples assayed by this method (table 1), pairs of technical replicates differed on average by 8.4% (SD = 7.1%) and 10.3% (SD = 8.4%) for assays of Ty3/gypsy and Ty1/copia elements, respectively.

Although the goal of the current study was to evaluate natural hybridization as a possible catalyst for transposable element proliferation, several lines of evidence suggest that biotic and abiotic stressors also may facilitate activation of transposable element sequences (Wessler 1996; Grandbastien 1998; Lisch 2009). Interestingly, the three hybrid species whose genomes underwent proliferation are locally adapted to relatively harsh environmental conditions. Helianthus anomalus and Helianthus deserticola occupy desert regions, whereas Helianthus paradoxus inhabits saline marshes (Rieseberg et al. 2003; Gross et al. 2004; Donovan et al. 2010). It thus seems feasible that some combination of hybridization and abiotic stress may have been required for the proliferation events that took place in the hybrid taxa. Experiments testing these ideas have been initiated in the greenhouse where environmental stress can be more precisely controlled.

Although proliferation of LTR retrotransposons leading to genome size increases was not detected in this study, transcriptional assays indicate that lineages of Ty3/gypsy and Ty1/copia elements that proliferated in the hybrid species remain active transcriptionally in hybrid populations as well as in natural populations of Helianthus annuus and Helianthus petiolaris across the entire sampling area. Sequencing of a small number of transcripts from Helianthus annuus, Helianthus petiolaris, and hybrid individuals from one of the sampling locations indicates that multiple Ty3/gypsy and Ty1/copia variants are transcribed. Transcriptional activity of Ty3/gypsy and Ty1/copia lineages was recently documented in an inbred line of Helianthus annuus (Vukich et al. 2009) as well as in greenhouse-generated Helianthus annuus × Helianthus petiolaris hybrids and the parental lines used to construct them (B. Hartman-Bakken, Kawakami, Ungerer, unpublished data). The Ty3/gypsy and Ty1/copia transcripts isolated from Helianthus annuus, Helianthus petiolaris, and hybrid individuals bear strong resemblance to elements previously characterized in sunflower via sequence surveys (Ungerer et al. 2009; Kawakami et al. 2010) or shown previously to be transcriptionally active in Helianthus annuus (Vukich et al. 2009). In addition, Blast searches against a Helianthus expressed sequence tag database with these transcripts as queries identified highly similar sequences isolated from multiple Helianthus species, suggesting widespread transcriptional activity of these sublineages in sunflowers.

If, as our data indicate, transcriptional activity of these elements is commonplace and widespread, the lack of insertional activity and genome expansion in contemporary hybrids is intriguing and suggests mechanisms of transposable element repression acting posttranscriptionally, such as RNAi (Slotkin and Martienssen 2007; Lisch 2009). In plants with large genomes, LTR retrotransposons often exhibit nested insertional patterns with variation in element orientation (SanMiguel et al. 1996). Such conditions are likely to be conducive to the production of double-stranded RNAs that could trigger the RNAi pathway (Madlung and Comai 2004; Lisch 2009). Future work will be geared toward understanding how and at what point host repression mechanisms are functional in these sunflower populations.

**Supplementary Material**

Supplementary table S1 is available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).
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