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ORIGINAL PAPER

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Chloroplast SSR polymorphisms in the Compositae and the mode of organellar inheritance in *Helianthus annuus*

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Abstract Because organellar genomes are often uniparentally inherited, chloroplast (cp) and mitochondrial (mt) DNA polymorphisms have become the markers of choice for investigating evolutionary issues such as sex-biased dispersal and the directionality of introgression. To the extent that organellar inheritance is strictly maternal, it has also been suggested that the insertion of transgenes into either the chloroplast or mitochondrial genomes would reduce the likelihood of gene escape via pollen flow from crop fields into wild plant populations. In this paper we describe the adaptation of chloroplast simple sequence repeats (cpSSRs) for use in the Compositae. This work resulted in the identification of 12 loci that are variable across the family, seven of which were further shown to be highly polymorphic within sunflower (*Helianthus annuus*). We then used these markers, along with a novel mtDNA restriction fragment length polymorphism (RFLP), to investigate the mode of organellar inheritance in a series of experimental crosses designed to mimic the initial stages of crop-wild hybridization in sunflower. Although we cannot rule out the possibility of extremely rare paternal transmission, our results provide the best evidence to date of strict maternal organellar inheritance in sunflower, suggesting that organellar gene containment may be a viable strategy in sunflower. Moreover, the portability of these markers suggests that they will provide a ready source of cpDNA polymorphisms for use in evolutionary studies across the Compositae.

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Introduction

The use of chloroplast (cp) DNA and/or mitochondrial (mt) DNA polymorphisms to investigate evolutionary phenomena requires a knowledge of the mode of organellar inheritance. For example, the frequent occurrence of uniparental inheritance of organellar genomes results in a characteristic decrease in the expected level of standing organellar variation in a population (as compared to the biparental case; Birky et al. 1989). In addition, to the extent that they are uniparentally inherited, organellar polymorphisms can provide unique insights into phenomena such as sex-biased gene flow (McCauley 1994) and the directionality of introgression in populations of hybrid origin (Edwards-Burke et al. 1997). When investigating such phenomena, even infrequent biparental inheritance can be misleading. Thus, it is of critical importance that the mode of organellar inheritance in any particular taxon be established with a high level of certainty before proceeding.

Another area in which knowledge of the mode of organellar inheritance has become increasingly important is that of transgene containment. Assuming strict maternal inheritance, pollen cannot serve as a vector for the transmission of organellar genes. Based on this fact, Gressel (1999) argued that the insertion of an engineered gene into either the chloroplast or mitochondrial genome could dramatically reduce the likelihood of gene transfer from crop plants into their sexually compatible (and often weedy) wild relatives. Uniparental inheritance is, however, far from universal in plants (Smith 1989), making such strategies risky in the absence of clear evidence of strict maternal transmission (Stewart and Prakash 1998). Indeed, Haygood et al. (2004) argued that even very low 'leakage' rates might be sufficient for advantageous transgenes to become established in wild populations. Because transgenes which impart resistance to, or tolerance of, various biotic or abiotic stresses (e.g. drought, disease, or pests) might reasonably be favored in the wild, we need to ensure that the rate of escape of such genes is as low as possible.

Perhaps the most common approach used in the investigation of the mode of organellar inheritance is to analyze the progeny of a cross between individuals carrying distinguishable organellar genotypes. With regard to the statistical power associated with this sort of approach, however, Milligan (1992) argued that the sample sizes employed are often far too small to reliably detect rare events. Indeed, sample sizes of well over 100 progeny are necessary to detect infrequent leakage with any degree of certainty, yet to this day investigators often use far fewer than this number (e.g., Vaillancourt et al. 2004). Thus, even if such studies are consistent with strict uniparental inheritance, any such conclusions are often unwarranted.

Beyond the statistical difficulties associated with the detection of paternal leakage, genotypic approaches such as the one outlined above require a readily available source of polymorphic markers. Although the chloroplast genome is typically highly conserved at the nucleotide level, recent years have seen an increasing number of publications describing PCR primers that amplify regions of the chloroplast genome that harbor highly polymorphic simple sequence repeats (cpSSRs), which can be visualized as electrophoretic length variants (reviewed by Provan et al. 2001). Here we report on the utility of these primers for revealing polymorphism in the Compositae (synonym: the Asteraceae), which is one of the largest and most diverse of the flowering plant families (Heywood 1978). Comprising over one-tenth of all known angiosperm species, the Compositae contains over 40 economically important species that have been domesticated for food, medicinal, and ornamental purposes, as well as numerous noxious weeds (Kesseli and Michelmore 1997). We focused our efforts on what are perhaps the best-known representatives of the two major subfamilies of the Compositae—sunflower (*Helianthus* spp.) and lettuce (*Lactuca* spp.). We then used these markers, along with a novel mtDNA RFLP, to investigate the mode of organellar inheritance in *Helianthus annuus*.

Derived from the common sunflower (*H. annuus*), the cultivated sunflower (also *H. annuus*) is an important source of oil and confectionery seeds. Despite being morphologically distinct, cultivated and common sunflower are considered to be members of the same species, and are fully reproductively compatible. Results of previous research indicate that, whenever they come into contact and flower coincidentally, common and cultivated sunflower hybridize readily, sometimes over surprisingly long distances (Arias and Rieseberg 1994; Whitton et al. 1997; Linder et al. 1998). Moreover, a recent multi-year, range-wide survey revealed that the necessary geographic and phenological overlap exists throughout the range of sunflower cultivation in the United States, making crop-wild hybridization a virtual certainty (Burke et al. 2002a).

When combined with the fact that transgenic approaches have been successfully applied to disease and pest problems in cultivated sunflower (e.g., Burke and Rieseberg 2003; Snow et al. 2003), the high likelihood of crop-wild hybridization makes transgene containment strategies a topic of central importance for sunflower researchers. Given the high level of pollen flow from cultivated into common sunflower populations, one strategy might be to take the advice of Gressel (1999) and employ organellar gene containment to mitigate the risks of transgene escape. Unfortunately, although organellar inheritance in sunflower has been the subject of both cytological and genetic analyses (Corriveau and Coleman 1988; Razoriteleva et al. 1970; Rieseberg et al. 1991, 1994), all of which have been consistent with strict maternal inheritance, the sample sizes employed thus far have been too small to provide the necessary level of confidence in this conclusion. For example, in the largest genetic study to date, Rieseberg et al. (1994) surveyed only 52 progeny derived from an experimental cross of cultivated x wild *H. annuus*. In addition to the relatively limited sample size, the authors pollinated cultivated sunflower with wild pollen—exactly the opposite of what we should be concerned about when discussing transgene escape—and employed only a single cross.

In the present paper, we describe the screening and optimization of 36 previously published cpSSRs primer pairs for use in members of the Compositae, and report on their level of polymorphism. We then used seven of these loci, along with a novel mtDNA RFLP, to investigate the mode of organellar inheritance in a set of five crosses that were designed to mimic the initial stage of crop-wild hybridization in sunflower.

Materials and methods

Experimental crosses

Five wild *Helianthus annuus* individuals from a population originating near Keith County, Nebraska (ANN1238; Burke et al. 2002b) were hand-crossed in the greenhouse with pollen from unique individuals drawn from three landraces obtained from the North Central Plant Introduction Station (Ames, Iowa): two individuals each from the Seneca (PI 369360) and Hopi (PI 432504) landraces and one individual from the Havasupai landrace (PI 369358). Because wild *H. annuus* is self-incompatible and because heads were bagged to prevent cross-contamination, all progeny are known to have been sired by the cultivated pollen parent (see below). Seeds were collected from each plant at physiological maturity, sown in flats, and placed in the Vanderbilt University Department of Biological Sciences greenhouses where they were maintained under a 16/8-h (light/dark) photoregime with constant (bottom) watering. Following emergence, 200 mg leaf tissue was collected from seedlings from each of the five crosses. Total genomic DNA was extracted from each seedling using the Qiagen DNeasy Plant Mini kit (Qiagen, Valencia, Calif.), and 29–32 progeny from each cross were genotyped, as described below. In order to confirm that the offspring of each cross were of hybrid origin, and not the result of cryptic self-fertilization, approximately 20 individuals from each cross were grown to maturity. All

such individuals exhibited intermediate phenotypes (reduced branching and increased size of the primary inflorescence) that were consistent with F_1 crop × wild hybrids, a result which confirms that the progeny of our crosses were sired by the cultivated pollen parent.

cpSSR Screening

Based on a survey of the available literature, we selected 36 primer pairs that amplify cpSSRs from tobacco, potato, and soybean (Bryan et al. 1999; Powell and Morgante 1995; Weising and Gardner 1999). We then tested these primer pairs for amplification in two species each of sunflower (*H. annuus* and *H. petiolaris*) and lettuce (*Lactuca sativa*, and *L. serriola*). Reactions were performed in 10-µl total volumes containing 10 ng of template DNA, 30 m *M* Tricine pH 8.4-KOH, 50 m *M* KCl, 2 m *M* $MgCl_2$, 100 μM of each dNTP, 0.1 μM of each primer, and 2 U *Taq* polymerase. The cycling conditions consisted of a touchdown regime as follows: an initial denaturing step of 3 min at 95°C, followed by ten cycles of 30 s at 94°C, 30 s at 58°C (annealing temperature was reduced by 1°C per cycle), 45 s at 72°C, followed by 30 cycles of 30 s at 94°C, 30 s at 48°C, 45 s at 72°C, and a final extension time of 20 min at 72°C. The amplification products were then visualized on 2% agarose gels stained with ethidium bromide. Of the 36 primer pairs tested, only the 22 that yielded amplification products of 100 bp or larger in *H. annuus* were selected for fluorescent analysis on an automated DNA sequencer because PCR products smaller than 100 bp are often obscured by unincorporated, fluorescent primers and low-molecular-weight artifacts. Of these 22 primer pairs, two (ccmp 2 and NTCP 7) are known to amplify the same cpSSR-containing region, although they result in products of different lengths. All 22 of these primers were surveyed for polymorphism by genotyping a panel consisting of one individual of the Havasupai landrace, one wild sunflower individual each from Arizona (Ames 1440), Iowa (PI 597895), Kansas (PI 413027), North Dakota (PI 586810), Tennessee (PI 435552), Texas (Ames 7442), and Washington (PI 531016), two individuals of *H. petiolaris* and one individual each of *L. sativa* and *L. serriola*. The ten individuals that served as parents of our experimental crosses were then screened for differences at each of the loci that proved to be polymorphic within *H. annuus* in order to identify 'informative' loci that could be used to track organellar inheritance. All genotyping was performed as described below.

Our PCR protocol for the fluorescent analyses was a modified version of the method presented by Schuelke (2000), wherein we added an arbitrarily selected sequence [the M13 forward (F) (−29) sequencing primer, 5′-CAC GAC GTT GTA AAA CGA C-3′] to the 5′ end of the forward primer. Amplifications were performed as above, except that the forward primer concentration was reduced to 0.02 μM. In order to allow for the resolution of multiple loci per lane on an automated DNA sequencer, PCR products were fluorescently labeled by including 0.1 μM of the M13 F (−29) primer (labeled with either HEX, FAM, or TET) in the reaction mixture. Cycling conditions were as described above. The amplification products were visualized on an MJ Research BaseStation Automated DNA Sequencer (MJ Research, Waltham, Mass.) housed in the Vanderbilt University Department of Biological Sciences core sequencing/genotyping laboratory. Genescan 400 HD size standards (Applied Biosystems, Foster City, Calif.) were run in each lane to allow for the accurate determination of fragment size, and alleles were called with the software package CARTOGRAPHER (MJ Research).

mtDNA PCR-RFLP

The mitochondrial cytochrome oxidase and ATP synthase genes were surveyed for sequence variation using PCR primers from Bowe et al. (2000) and Barkman et al. (2000). Regions of both genes (*coxI* and *atpA*) were amplified from each of the ten individuals that served as the parents of our experimental crosses. Reactions were performed in 50-µl total volumes containing 50 ng of template DNA, 30 m *M* Tricine pH 8.4-KOH, 50 m *M* KCl, 2 m *M* $MgCl_2$, 100 μM of each dNTP, 0.2 μM of each primer, and 2 U *Taq* polymerase. Cycling conditions were as described above, except that the annealing temperature was 65°C at the beginning of the touchdown cycles, and 55°C for the cycles following the touchdown phase. The resulting PCR products were cleaned using the QiaQuick Cleanup kit (Qiagen, Valencia, Calif.) to remove unincorporated primers and to ready the templates for sequencing. The original PCR primers were then used to sequence the products in each direction using 1/8 reactions of the DYEnamic ET Terminator Cycle Sequencing kit (Amersham Pharm Biotech, Piscataway, N.J.). The sequences of both genes were then scanned for unique restriction sites that differentiate the mitochondrial haplotypes of the seed and pollen parents of each cross. Across the entire *atpA* sequence of 722 bp, we found a single variable site that, coincidentally, resulted in the presence of an *Hha*I restriction site in the maternal (wild) parent of three of our crosses. There were no polymorphisms across the entire 1,252-bp *coxI* sequence.

In order to score the mtDNA RFLP from the progeny of the three informative crosses, we amplified *atpA* (as described above) and digested 10 µl of the resulting PCR product from each individual using 20 U *Hha*I (New England Biolabs, Beverley, Mass.) and 1× NEBuffer no. 4 with 100 ng/µl bovine serum albumin, in a total reaction volume of 20 µl at 37°C for a minimum of 1 h. The digested PCR products were then separated on 2% agarose gels and visualized via staining with ethidium bromide.

Table 1 Primer sequences and results of our cpSSR screen in four species representing the two major subfamilies of the Compositae (*Helianthus annuus* and *H. petiolaris*; *Lactuca sativa* and *L. serriola*). Note that only those primer pairs that produced a clear, single-banded amplification product are included here and that all such primer pairs worked in all four taxa. Six primer pairs that either failed to produce an amplification product or which produced multi-banded, nonspecific priming are not listed here

Locus	Primer sequences (5′–3′)[a]	Result[b]
ccmp 1	Fwd:CAGGTAAACTTCTCAACGGA Rev: CCGAAGTCAAAAGAGCGATT	Poly
ccmp 2[c]	Fwd: GATCCCGGACGTAATCCTG Rev: ATCGTACCGAGGGTTCGAAT	Informative
ccmp 3	Fwd: CAGACCAAAAGCTGACATAG Rev: GTTTCATTCGGCTCCTTTAT	Poly
ccmp 4	Fwd: AATGCTGAATCGAYGACCTA Rev: CCAAAATATTBGGAGGACTCT	Poly
ccmp 5	Fwd: TGTTCCAATATCTTCTTGTCATTT Rev: AGGTTCCATCGGAACAATTAT	Poly
ccmp 6	Fwd: CGATGCATATGTAGAAAGCC Rev: CATTACGTGCGACTATCTCC	Mono
ccmp 7	Fwd: CAACATATACCACTGTCAAG Rev: ACATCATTATTGTATACTCTTTC	Informative
ccmp 10	Fwd: TTGGCTACTCTAACCTTCCC Rev: TTCTTTCTTATTTCGCAGDGAA	Poly
NTCP 2	Fwd: CTCGCCTACTTACATTCC Rev: AAGGAGAGGTTATTTTCTTG	Mono
NTCP 4	Fwd: TTGGATTAGATTTGTAGTTCCA Rev: ATCCACTTCATTTATCACAATG	< 100 bp
NTCP 5	Fwd: CGAATTGATAGATACGAAACC Rev: AATACACCAAACAACAAATCC	< 100 bp
NTCP 6	Fwd: GGTTCGAATCCTTCCGTC Rev: GATTCTTTCGCATCTCGATTC	No label
NTCP 7[c]	Fwd: TGATCCCGGACGTAATCC Rev: CGAATCCCTCTCTTTCCG	Informative
NTCP 9	Fwd: CTTCCAAGCTAACGATGC Rev: CTGTCCTATCCATTAAGACAATG	Informative
NTCP 13	Fwd: TTTCCTGTTCCTGGTGGTA Rev: TTGGGGTAGATACACAAATCAC	Mono
NTCP 16	Fwd: TCTAAACTAAAATAATCGAAAGA Rev: TGAAATTGTCAATATAATCGA	No label
NTCP 18	Fwd: CTGTTCTTTCCATGACCCCTC Rev: CCACCTAGCCAAGCCAGA	Informative
NTCP 19	Fwd: AATCGTTGTTTTAGACGATGC Rev: GAAACCCATTCTTACCACAAG	Mono
NTCP 20	Fwd: TCCTCGTAAGACTGAGAGAAAT Rev: TTACGAGTAATTCCGACAACTT	Mono
NTCP 24	Fwd: GACCGATGATTTGGACGAC Rev: GCTAGCGGACATTTATTTTGAA	No label
NTCP 26	Fwd: GCAATTGCAATGGCTTCTTTA Rev: TTTATGTTCGGTGGAAATCACA	< 100 bp
NTCP 27	Fwd: ATAAATACAGAACCCGTCGTAA Rev: TGCTTAGAGTTGGACACAGAAT	< 100 bp
NTCP 30	Fwd: GATGGCTCCGTTGCTTTAT Rev: TGCCGGAGAGTTCTTAACAATA	Informative
NTCP 32	Fwd: TGTTTCATCTTTTAGGTTTAT Rev: TCAAGCAAAGTTATCTCAAC	< 100 bp
NTCP 34	Fwd: GATCCATACAGCATTTCGTAT Rev: CTGTTTCTAGTGGGGTATTTGA	< 100 bp
NTCP 37	Fwd: TTCCGAGGTGTGAAGTGG Rev: CAGGATGATAAAAAGCTTAACAC	No label
NTCP 39	Fwd: GTCACAATTGGGGTTTTGAATA Rev: GACGATACTGTAGGGGAGGTC	Informative
NTCP 40	Fwd: TAATTTGATTCTTCGTCGC Rev: GATGTAGCCAAGTGGATCA	Informative
RP 19	Fwd: CTAAATATTACAAAATGGAATTCT Rev: ACCAATTCAAAAAATGGAATA	< 100 bp
SOYCP	Fwd: CATAGATAGGTACCATCCTTTTT Rev: CGCCGTATGAAAGCAATAC	< 100 bp

[a]Note that, for the purposes of fluorescent labeling, the M13 Forward (−29) primer sequence was prepended to the 5′ end of all forward primers. See Materials and methods for details

[b]Poly, locus was polymorphic across taxa; informative, locus was polymorphic within *H. annuus*; mono, locus was monomorphic across taxa; < 100 bp, amplification product was < 100 bp in length and locus was not pursued further; no label, amplification product failed to incorporate the fluorescently labeled M13 primer

[c]Both of these primer pairs amplify the same SSR locus, with the NTCP 7 amplification product being 16 bp shorter than that of ccmp 2

Results and discussion

Of the 36 cpSSR primer pairs that we screened, 30 produced a clear, single-banded amplification product of the expected size from all four tested taxa (Table 1). The remaining six primer pairs (ccmp 8, ccmp 9, NTCP 14, NTCP 22, NTCP 29 and NTCP 38) either failed to produce an amplification product or resulted in a multi-banded, non-specific amplification profile. Because the taxa included in our survey represent both of the major subfamilies within the Compositae, our results suggest that these primer pairs will be generally applicable across the family. The apparently high level of portability of these primer pairs, with a success rate of more than 80% in both *Helianthus* and *Lactuca*, is not necessarily surprising in view of the fact that ten of them (ccmp 1–10) were designed from a consensus sequence derived from the full sequence of the tobacco chloroplast genome (where the cpSSRs were initially identified) as well as from cpDNA sequences from 23 additional angiosperm taxa (Weising and Gardner 1999). It is interesting to note, however, that these primers fared no better than did the remaining 26 primer pairs, all of which were each designed on the basis of sequences derived from only a single taxon (tobacco for the NTCP primers and soybean for RP19 and SOYCP; Bryan et al. 1999; Powell et al. 1995).

In terms of our screen for polymorphism, four of the 22 primer pairs selected for fluorescent analysis on the automated DNA sequencer failed to incorporate the labeled primer. Thus, although these primer pairs might prove to be informative using an alternative labeling protocol, they could not be visualized using our methods. Of the remaining 18 primer pairs, 13 (corresponding to 12 unique loci) were polymorphic within the Compositae, with eight (corresponding to seven unique loci) revealing polymorphisms within *H. annuus* (Tables 1 and 2). Despite the relatively limited scope of our diversity survey within *H. annuus*, we detected two to five alleles at each locus, with estimates of gene diversity (calculated as $1\text{-}\Sigma\, p_I^2$, where p_I is the frequency of the ith allele at a given locus) ranging from 0.22–0.78. Moreover, these eight individuals correspond to eight unique haplotypes.

When compared to previous attempts at the identification of cpDNA polymorphisms in sunflower, it is clear that the cpSSRs described here reveal much more variability than the traditionally employed cpDNA RFLPs. For example, Rieseberg and Seiler (1990) used 17 restriction enzymes to digest entire chloroplast genomes isolated from 34 *H. annuus* accessions be were only able to identify four unique cpDNA haplotypes. Similarly, Welch and Rieseberg (2002) performed PCR-RFLP analysis on four regions of the chloroplast genome (spanning a total of approximately 14 kb) and identified only five unique cpDNA haplotypes in a sample of 87 individuals drawn from five wild *H. annuus* populations and one cultivated lineage.

The high level of polymorphism revealed by the cpSSRs allowed us to distinguish between the pollen and seed parents of all five of our experimental crosses using

Table 2 Results of our cpSSR polymorphism survey across one cultivated and seven wild *H. annuus* individuals. Allele sizes are reported in basepairs and reflect the inclusion of the 19-bp extension on the 5′ end of the forward primer (see Materials and methods for additional details) (*NA* not available)

Locus	Size range of alleles (bp)	Number of alleles	Gene diversity
ccmp2/NTCP 7[a]	228–230	3	0.41
ccmp 7	139–149	4	0.69
NTCP9	140–143	4	0.69
NTCP 18	207–216	3	0.41
NTCP30	179–181	3	0.59
NTCP 39	181–192	6	0.78
NTCP40	277–278	2	0.22
Mean	*NA*	3.6	0.54

[a] These two primer pairs each amplify the same locus, with the NTCP 7 product being 16 bp shorter than the ccmp 2 product, which is reported here

Table 3 Crosses employed to investigate the mode of organellar inheritance in sunflower (*H. annuus*). Each cross involved the pollination of a wild sunflower individual with pollen from a unique individual from one of three cultivated accessions. Note that the maternal and paternal cpSSR allele lengths are reported in basepairs and that the mitochondrial haplotype of the parents of crosses B and E were identical, such that mtDNA inheritance could not be investigated for these crosses (*NA* not available)

Cross ID	Parents	Maternal and paternal allele lengths at the diagnostic loci[a]						Frequency of maternal inheritance	
		Ccmp 7	NTCP 9	NTCP 18	NTCP 30	NTCP 39	NTCP 40	cpDNA	mtDNA
A	Wild 3 × Seneca 4	140/140	281/280	208/208	177/179	182/190	277/277	32 of 32	32 of 32
B	Wild 9 × Seneca 3	139/140	279/280	219/208	179/179	174/190	279/277	32 of 32	*NA*
C	Wild 10 × Havasupai 2	140/140	281/280	208/208	177/179	182/187	277/277	29 of 29	29 of 29
D	Wild 12 × Hopi 1	140/140	281/280	208/208	177/179	182/187	277/277	29 of 29	29 of 29
E	Wild 23 × Hopi 3	140/140	280/280	208/208	179/179	188/187	277/277	30 of 30	*NA*
Total								152	90

[a]Note that allele lengths reflect the inclusion of the 19-bp M13 Forward (−29) sequence (see Materials and methods for details). Thus, the actual amplicon lengths are 19 bp shorter than reported here

anywhere from one to five diagnostic loci. All of the progeny from our crosses carried the chloroplast haplotype of their seed parent, a result consistent with strict maternal inheritance (Table 3). Likewise, all progeny from crosses A, C and D (see Table 3) carried the mtDNA characteristic of their seed parent. This latter result is, once again, consistent with strict maternal inheritance. In total, we observed maternal cpDNA inheritance in 152 of 152 progeny from five crosses as well as maternal mtDNA inheritance in 90 of 90 progeny from three of these crosses (Table 3).

Based on the methods of Milligan (1992), we can be reasonably sure that we would have detected paternal chloroplast transmission if it were occurring at an appreciable rate. More specifically, we can be 95% certain that paternal cpDNA transmission is occurring at a rate less than approximately 2%, and 80% certain that the rate is less than approximately 1%. In terms of mtDNA inheritance, where we can be slightly less certain due to the reduction in sample size, the corresponding rates are approximately 3.5% and approximately 2.0%. Combining our data with data from the previous analysis of organellar inheritance in *H. annuus* performed by Rieseberg et al. (1994), and ignoring for the moment that their cross was performed in the 'wrong' direction with regard to assessing the risk of transgene escape, we now have 220 and 142 observations of maternal cpDNA and mtDNA inheritance, respectively, with no documented instances of paternal inheritance. This increase in sample size further increases our power to detect rare paternal leakage, meaning that we can be 95% (80%) certain that the rate of paternal cpDNA transmission is less than approximately 1.35% (approximately 0.75%). For mtDNA leakage, the corresponding upper limits are approximately 2.1% and approximately 1.1%.

The data presented here provide the best evidence to date in support of the strict maternal inheritance of both the chloroplast and mitochondrial genomes of *H. annuus*. When this fact is combined with the apparent portability of the cpSSRs surveyed in this study, as well as the high level of variation that they reveal, it seems likely that these markers will provide a ready source of maternally inherited polymorphisms for use in evolutionary studies across the Compositae. Perhaps more importantly, however, our data suggest that organellar gene containment may be a feasible method of mitigating the risks associated with transgene escape in sunflower. This latter point must, of course, be tempered by the realization that even low rates of paternal transmission might be sufficient for the escape of organellar transgenes (see Chamberlain and Stewart 1999; Haygood et al. 2004). Furthermore, the transfer of plastid DNA into the nuclear genome (Huang et al. 2003, 2004) could provide an alternate route for gene escape via pollen. Given these caveats, it seems that organellar transgene containment is perhaps best used in combination with more traditional risk assessment in which we work to identify those genes that pose the least environmental risk (Burke and Rieseberg 2003).

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