

ORIGINAL ARTICLE

Detection of rare paternal chloroplast inheritance
in controlled crosses of the endangered sunflower
Helianthus verticillatus

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A variety of questions in population and evolutionary biology are studied using chloroplast DNA (cpDNA). The presumed maternal inheritance in angiosperms allows for certain assumptions and calculations to be made when studying plant hybridization, phylogeography, molecular systematics and seed dispersal. Further, the placement of transgenes in the chloroplast to lessen the probability of 'escape' to weedy relatives has been proposed since such genes would not move through pollen. In many studies, however, strict maternal inheritance is assumed but not tested directly, and some studies may have sample sizes too small to be able to detect rare paternal leakage. Here, we study the inheritance of cpDNA simple sequence repeats in 323

offspring derived from greenhouse crosses of the rare sunflower *Helianthus verticillatus* Small. We found evidence for rare chloroplast paternal leakage and heteroplasmy in 1.86% of the offspring. We address the question of whether one can extrapolate the mode of chloroplast transmission within a genus by comparing our results to the findings of another sunflower species study. The findings of occasional paternal transmission of the chloroplast genome are discussed in the framework of using these markers in studies of population and evolutionary biology both in *Helianthus* and other angiosperms.

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Introduction

Population and evolutionary biologists use chloroplast DNA (cpDNA) in a variety of applications, including studies of hybridization and phylogeography (Rieseberg and Ellstrand, 1993; Welch and Rieseberg, 2002; Dobeš *et al.*, 2004; Van Droogenbroeck *et al.*, 2006), plant molecular systematics (Olmstead and Palmer, 1994; Kelchner, 2000; Wolfe and Randle, 2004) and seed movement and dispersal in natural populations (McCauley, 1995; Ouborg *et al.*, 1999; Hamilton and Miller, 2002; Petit *et al.*, 2005). Since the chloroplast genome is largely maternally transmitted in angiosperms (Sears, 1980; Corriveau and Coleman, 1988; Zhang *et al.*, 2003), these types of applications assume maternal inheritance (Birky, 1995, 2001), though this assumption is only rarely tested. In fact, occasional paternal or biparental inheritance has been shown in some species (Sears, 1980; Corriveau and Coleman, 1988; Reboud and Zeyl, 1994; Röhr *et al.*, 1998; Hansen *et al.*, 2007; McCauley *et al.*, 2007). Thus, when it occurs, paternal or biparental inheritance of the chloroplast genome could lead to incorrect conclusions in studies involving seed dispersal, hybrid origins and

evolutionary relationships should maternal inheritance be assumed.

Further, researchers have posited that transgenes placed in the chloroplast genome of crops would reduce their probability of 'escape' as the genes would not move through pollen if maternally inherited (Gressel, 1999; Daniell *et al.*, 2005; Grevich and Daniell, 2005). In crop systems, non-maternal inheritance could lead to the escape of transgenes, for example that may confer herbicide resistance, thus leading to the possibility of creating 'superweeds' since many domesticated crops grow in close proximity to their weedy wild relatives (Smith, 1989; Haygood *et al.*, 2004; Chapman and Burke, 2006). In fact, Haygood *et al.* (2004) found that even with low levels of paternal transmission, the probability of transgene escape is considerable. Therefore, studying the transmission of the chloroplast genome can provide valuable information for the likelihood of transgene escape in domesticated crop species as well as accepting or rejecting the assumptions of maternal inheritance in population and evolutionary studies.

In many plant genera, consistency of inheritance has been observed among the several congeners that happen to have been studied (Sears, 1980; Corriveau and Coleman, 1988; Zhang *et al.*, 2003). These findings suggest that chloroplast inheritance may be conserved within a genus. If so, one could extend information on the mode of inheritance of one species to its congeners. However, there are a few exceptions to this observation in which different members of a genus have conflicting modes of inheritance (Sears, 1980; Zhang *et al.*, 2003)—

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thus raising the question: can one assume that the mode of chloroplast inheritance is identical among congeners? If not, then chloroplast inheritance needs to be assessed directly in each species in question.

Several factors influence whether paternal leakage can occur and what effect it has on the population biology of plant species: whether and how often pollen grains contain cpDNA, how frequently it is transmitted to the zygote, and how intraindividual drift affects copies within cells. Large-scale studies designed to infer organellar inheritance in angiosperms have screened pollen grains for evidence of plastids or plastid DNA in generative or sperm cells (Sears, 1980; Corriveau and Coleman, 1988; Zhang *et al.*, 2003). Using cytological evidence to determine the mode of plastid DNA inheritance, these studies have designated cpDNA transmission in hundreds of angiosperm species as either maternal or biparental. Due to their nature, these methods can only identify the potential mode of inheritance, that is, whether there is plastid DNA in the cell. Since these studies scan a great number of species, they often use a small number of individuals per species, which may miss rare paternal transmission. It is also not possible to determine the consequences for individual and population biology when the transmission mode is biparental since intraindividual drift of DNA copies during cell divisions will lead to individuals that sort almost completely to one type or the other (that is, a homoplasmic individual) or to individuals that carry a mixture of paternal and maternal copies (that is, a heteroplasmic individual) (Birky, 2001). Cell divisions represent founder effects or bottlenecks of each generation, which should enforce a highly homoplasmic state within the individual. However, occasional biparental inheritance would continue to introduce alternate alleles into the individual generating heteroplasmy. Chloroplast heteroplasmy has been documented in several angiosperms genera: *Passiflora* (Hansen *et al.*, 2006, 2007), *Senecio* (Frey *et al.*, 2005), *Medicago* (Johnson and Palmer, 1989) and *Turnera* (Shore *et al.*, 1994; Shore and Triassi, 1998).

We have investigated cpDNA inheritance and heteroplasmy in a rare sunflower species, *Helianthus verticillatus* Small, and compared it to the mode of inheritance found in a related economically important species, *H. annuus* (Rieseberg *et al.*, 1994; Wills *et al.*, 2005). These two prior studies found no evidence for paternal leakage of cpDNA in *H. annuus* crosses, and Wills *et al.* (2005) found no evidence for heteroplasmy (personal communication). Ellis *et al.* (2006) investigated population structure in *H. verticillatus* based on F_{ST} calculations for nuclear and cpDNA markers and found the latter to have a much greater F_{ST} . On the basis of the described cpDNA inheritance in *H. annuus*, the difference in magnitude between nuclear and chloroplast F_{ST} was explained in part by maternal inheritance of cpDNA. However, this assumed mode of inheritance has not been tested directly in *H. verticillatus*. Since we are focusing on just one species, we chose to look at chloroplast inheritance directly by examining progeny from controlled crosses in which the parents had different cpDNA haplotypes. This approach allows the direct observation of chloroplast transmission and includes much larger sample sizes, thus giving a greater chance of detecting rare paternal leakage and quantifying the rate of leakage accurately.

When designing an experiment to study rare events, one must take into account the power of detection. Milligan (1992) proposed a binomial model of organelle inheritance to determine the power of the analysis to detect paternal leakage at a given rate. He states that many studies that address organelle inheritance use insufficient sample sizes to detect leakage, and since some studies have found leakage rates of 0.01–2.5% (Simmonds, 1969; Tilney-Bassett, 1978; Medgyesy *et al.*, 1986), sample sizes in excess of 100 progeny are needed to detect leakage even at the 2.5% level. Further, since the probability of non-maternal chloroplast inheritance may vary among crosses (Birky, 1995; Mogensen, 1996), individuals will not be completely independent data points if they are from the same family. Consequently, when designing an experiment to evaluate organelle inheritance, one should choose as many families as possible recognizing the trade-off between the number of families and the number of offspring per family. Here we report on a study of cpDNA inheritance using cpSSRs (chloroplast simple sequence repeat; Provan *et al.*, 2001) in 323 *H. verticillatus* offspring comprising 53 families and provide evidence for occasional paternal leakage and heteroplasmy of cpDNA in controlled greenhouse crosses.

Materials and methods

H. verticillatus is an extremely rare, diploid ($n = 17$) self-incompatible, perennial sunflower restricted to only four locations in the southeast interior of the United States: two in western Tennessee (Madison Co. and McNairy Co.), one in northeastern Alabama (Cherokee Co.) and one in northwestern Georgia (Floyd Co.). It is a candidate for federal listing for the Endangered Species Act and is listed as endangered in each of the three states. First collected in western Tennessee in 1892, it was not found again in the field until 1994 in Georgia (Matthews *et al.*, 2002). In 1996 and 1998, the populations in Alabama and Madison Co., Tennessee, respectively, were discovered. In the fall of 2006, the fourth location in McNairy Co., Tennessee was discovered during an annual survey and search for the species. The Alabama and Georgia populations are about 3.5 km from each other, whereas the Tennessee populations are about 350 km from the others and about 40 km from one another. In a prior study, the Alabama and Tennessee populations were found to be fixed for different cpDNA haplotypes making it possible to detect paternal leakage easily in crosses between the two populations (Ellis *et al.*, 2006).

In order to detect rare paternal leakage, the appropriate sample size must be used. We used Milligan's (1992) equation for calculating the power of analysis for the number of individuals studied and the allowed percentage of non-maternal inheritance:

$$\beta = 1 - (1 - P)^N$$

where β is the power of the test to detect leakage, P is the probability of paternal transmission, and N is the number of progeny. We designed our experiment to be able to reject the strict maternal inheritance hypothesis 95% of the time at a rate of leakage equal to or $>1\%$. To have this statistical power, 300 individuals (that is, observations of inheritance) were needed according to this calculation.

Achenes from *H. verticillatus* were collected from the Alabama and Tennessee sites and grown in the Vanderbilt Biological Sciences greenhouse to serve as parents for the crosses. Since the parents need to differ at the markers studied in order to detect paternal leakage, we genotyped the parents for the cpSSRs (method described below) to choose individuals with differing cpDNA haplotypes. Alabama and Tennessee individuals carried different cpDNA haplotypes; therefore, any interpopulation crosses would have differing parental genotypes. The Tennessee population was polymorphic; thus, Tennessee by Tennessee intrapopulation crosses were also conducted with parents that differed in cpDNA haplotype. We used 272 offspring from 45 controlled greenhouse interpopulation crosses (24 Alabama \times Tennessee and 21 Tennessee \times Alabama) and 51 individuals from eight greenhouse intrapopulation (Tennessee) crosses of *H. verticillatus* for a total of 323 offspring.

The crosses were carried out as follows: inflorescences were bagged prior to anthesis to prevent any unwanted pollinations. Crosses were conducted by brushing pollen with a paintbrush from inflorescences at anthesis into aluminum foil and then brushing pollen onto the stigmas of another inflorescence in which the same pollen removal had been conducted. Pollinations were conducted within 1 h of collecting pollen and all pollinations were conducted at mid-morning (\sim 1000 h). Inflorescences were re-bagged and achenes were allowed to mature. Achenes (that is, the offspring) were nicked with a razor blade, germinated on moist filter paper and grown in the greenhouse. When the resulting plants were large enough, a leaf was collected for DNA extraction.

Total genomic DNA was isolated from \sim 200 mg of fresh leaf tissue using the Applied Biosystems 6100 Nucleic Acid PrepStation DNA extraction machine and associated protocols (Foster City, CA, USA). All parents and offspring were genotyped for three polymorphic cpSSRs, (N39 and N30 (Bryan *et al.*, 1999) and C7 (Weising and Gardner, 1999); also used in Wills *et al.*, 2005; Ellis *et al.*, 2006) using PCR and fragment length analysis. Any individuals that indicated paternal leakage were re-genotyped two additional times to verify the results. Briefly, SSR genotyping was performed using a modified version of the fluorescent labeling protocol of Schuelke (2000), as detailed in Wills *et al.* (2005). PCR was performed in a total volume of 20 μ l containing 2 ng of template DNA, 30 mM Tricine pH 8.4-KOH, 50 mM KCl, 2 mM MgCl₂, 125 μ M of each deoxyribonucleotide triphosphate, 0.2 μ M M13 Forward (–29) sequencing primer labeled with either HEX, 6FAM or TET, 0.2 μ M reverse primer, 0.02 μ M forward primer and two units of *Taq* polymerase. The PCR conditions were as follows: 3 min at 95 °C; ten cycles of 30 s at 94 °C, 30 s at 65 °C and 45 s at 72 °C, annealing temperature decreasing to 55 °C by 1 °C per cycle, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 45 s at 72 °C, followed by 20 m at 72 °C.

PCR products were visualized on an MJ Research BaseStation automated DNA sequencer (South San Francisco, CA, USA). MapMarker 1000 ROX size standards (BioVentures Inc., Murfreesboro, TN, USA) were run in each lane to allow for accurate determination of fragment size. Cartographer v 1.2.6 (MJ Research) was used to infer individual genotypes according to the fragment sizes of the PCR products. The parents used in

this study carried one of four haplotypes with Alabama and Tennessee containing different haplotypes. Haplotypes were denoted A1, T1, T2, T3 named by the three-locus SSR sizes (C7–N30–N39: 145–176–174 bp = A1; 148–177–181 bp = T1; 149–177–184 bp = T2; 149–177–185 bp = T3). Given that individuals showing paternal leakage would likely still carry some of the maternal haplotype (that is, heteroplasmy), we scored individuals first for their primary fragment peak and scored any secondary peak when it met two criteria: (1) it was the alternate allele that would be expected given the type of cross and (2) it was at least 10% of the primary peak fluorescence level. Any individual that indicated heteroplasmy was also genotyped two additional times (from the same DNA extraction). All parents were found to be of one primary haplotype.

Since paternal leakage is most likely a rare occurrence, it is critical that parentage be verified to ensure that mistakes were not made during the handling of individuals throughout cultivation, DNA extractions and genotyping. In order to detect any possible errors in mother-offspring assignment, parentage was verified in those offspring that did not indicate maternal cpDNA inheritance by genotyping the suspected leakage individuals as well as the maternal and paternal donors for nine previously described highly polymorphic nuclear EST-SSRs BL 1, 2, 3, 4, 5, 8, 10, 13 and 17 (for details see Pashley *et al.*, 2006; Ellis *et al.*, 2006).

Results

Out of 323 observations of inheritance, we found five cases of non-maternal inheritance equaling a leakage rate of at least 1.55%. Table 1 provides the cpDNA haplotypes for the offspring indicating paternal leakage. One offspring each of the AL2 \times TN2, TN4 \times AL3, TN6 \times AL3 families showed the paternal haplotype. Two individuals of the TN3 \times AL2 family had the paternal haplotype. Maternity and paternity was confirmed in each case that indicated paternal leakage using EST-SSRs. In each of the 323 offspring scored, a primary haplotype was observed; however, some offspring contained a secondary haplotype (at least 10% of the primary peak) that represented the alternate allele of the appropriate size for the cross type (see Figure 1 for a representative example). The highest ratio observed for primary to secondary haplotype was roughly 2:1. All five individuals that indicated primary non-maternal inheritance also carried at least 10% of the maternal haplotype, and one additional

Table 1 Mother and offspring haplotypes showing primary non-maternal inheritance

Family	Maternal/paternal haplotype	Offspring/haplotype
AL2 \times TN2	A1/T3	6A1/ 1T3
TN4 \times AL3	T1/A1	9T1/ 1A1
TN6 \times AL3	T2/A1	3T2/ 1A1
TN3 \times AL2	T2/A1	2T2/ 2A1

Mother and offspring haplotypes consisting of alleles at three cpSSRs from controlled greenhouse crosses within and among the Madison Co., Tennessee and Cherokee Co. and Alabama populations. Only families that showed evidence for primary nonmaternal inheritance are presented. Bold information indicates paternal leakage.

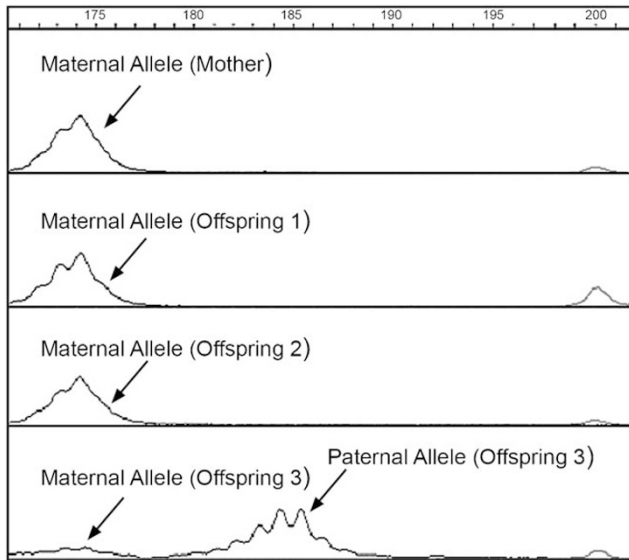


Figure 1 Electropherogram from cpSSR (chloroplast simple sequence repeat) locus N39 to show an example of chloroplast inheritance. A mother and three offspring from the AL2 × TN2 family are shown. Offspring one and two show maternal inheritance, while offspring three contains the paternal allele as well as evidence of the maternal allele, indicating paternal leakage and biparental inheritance. There is a size marker at the 200 bp position.

Table 2 Inheritance and heteroplasmy classification for all offspring

	Paternal inheritance	Maternal inheritance
Heteroplasmy	5	1
No heteroplasmy	0	317

Offspring from all crosses classified according to the type of inheritance and presence or absence of heteroplasmy (see text for details).

individual (from family AL2 × TN2) primarily contained the maternal haplotype but contained an observable percentage of the paternal haplotype according to our criteria (Table 2). Further, a Fisher's Exact Test indicated that the probability of detecting heteroplasmy is not independent of the probability of which parent provides the primary haplotype ($P < 0.001$).

Discussion

In this study we found paternal leakage of the chloroplast at a rate of 1.55% (5/323 observations) or 1.86% (6/323) accounting for the individual that showed secondary paternal heteroplasmy. This low level of paternal transmission was observable in our study since we designed the experiment to be able to detect leakage 95% of the time at a transmission rate of $\geq 1\%$ (Milligan, 1992). One caveat to discuss with regard to calculating the detection ability is that since there is sometimes a family association with leakage (Birky, 1995; Mogensen, 1996; McCauley *et al.*, 2007), the actual number of independent data points may not be the total number of observations, but rather probably lies somewhere between the number of families and the number of individuals. Thus when approaching the trade-off

between number of families and offspring, and given a constraint on the total number of samples in a study, it is better to sample as many families as possible. Studies that employ a small number of families to detect non-maternal inheritance may be more likely to miss rare events of paternal leakage. In this study we sampled 53 families—a number that is much larger than previous studies finding strict maternal chloroplast inheritance in other angiosperms (Vaillancourt *et al.*, 2004; Van Droogenbroeck *et al.*, 2005). We only found evidence for paternal leakage in interpopulation crosses. Often paternal leakage is reported in interspecific hybrids (Soliman *et al.*, 1987; Cruzan *et al.*, 1993; Hansen *et al.*, 2007) and thus it may be more likely for paternal leakage to occur in crosses between divergent populations than within population crosses. However, no formal statistical calculations were performed on this conclusion as we had so few within population crosses, thus limiting our statistical power.

We also found evidence for chloroplast heteroplasmy in six individuals. Documentation of chloroplast heteroplasmy is rare (but see Frey *et al.*, 2005) perhaps due in part to the dogma of strict maternal inheritance in angiosperms. Intraindividual variation has been observed and quantified for plant mitochondrial genes in several cases, (Hattori *et al.*, 2002; McCauley *et al.*, 2005; Welch *et al.*, 2006) but such observations remain rare. Perhaps not surprisingly heteroplasmy occurs if paternal leakage takes place given that the mother presumably always transfers organelles to her offspring. Chloroplast heteroplasmy has consequences for the population biology of *H. verticillatus*. Given that founder effects or bottlenecks of chloroplasts occur with each successive cell division, even a small amount of paternal leakage at fertilization could, by chance, lead to a mature offspring with the majority paternal haplotype just as it might often be lost. Genetic drift within the individual will also vary at different life stages of the plant. A young plant will likely have fewer cell divisions and may harbor a greater mixture of chloroplast genes, while an older plant has completed more cell divisions thus allowing genetic drift to create a more homoplasmic state (recall we sampled *H. verticillatus* offspring at the young plant stage).

The findings of this study indicate caution must be used when assuming strict maternal inheritance of the chloroplast genome in angiosperms and in unstudied species of *Helianthus* for several reasons. First, cpDNA is often employed in studies examining hybridization and introgression in plants (Rieseberg and Ellstrand, 1993; Edwards-Burke *et al.*, 1997; Welch and Rieseberg, 2002; Van Droogenbroeck *et al.*, 2006). When strict maternal inheritance is assumed but paternal leakage occurs, incorrect conclusions regarding parental contributions during hybridization and directionality of introgression may be drawn. The sunflower genus is noted for having significant amounts of hybridization and introgression (Rieseberg, 1991; Rieseberg *et al.*, 1995, 1996). Paternal transmission of the chloroplast found in *H. verticillatus* indicates the need to be cautious when studying aspects of hybridization in this genus.

Second, non-maternal inheritance may complicate cpDNA-based phylogenies since chloroplast transmission through pollen would increase the likelihood of its introgression from one species into another. This

'chloroplast capture' can result in incongruence between nuclear and organelle phylogenies (Soltis and Kuzoff, 1995; Tsitrone *et al.*, 2003). Schilling *et al.* (1998) found inconsistencies between nuclear and cpDNA phylogenies in *Helianthus*. Since paternal transmission of the chloroplast could lead to inconsistencies when comparing nuclear and chloroplast phylogenies, our findings suggest that the possibility of paternal leakage should be considered when examining evolutionary relationships in *Helianthus*.

Next, the difference between measures of population structure (F_{ST}) using cpDNA and nuclear DNA is often used to evaluate the relative contributions of seed and pollen movement to total gene flow. In theory, if there is strict maternal inheritance of organellar genes then seeds will carry copies of the nuclear and cytoplasmic genomes, while pollen will carry only nuclear genes (Birky *et al.*, 1983, 1989; Petit *et al.*, 1993). Paternal leakage will decrease values of F_{ST} based on organellar DNA, skewing estimates of the contributions of seeds and pollen to gene flow relative to that when maternal inheritance is assumed.

Finally, it has been proposed that transgenes be placed in the chloroplast genome of crop species to prevent their escape (Gressel, 1999; Daniell *et al.*, 2005; Grevich and Daniell, 2005). However, low rates of paternal transmission of the chloroplast have been shown in crops species including tobacco (Medgyesy *et al.*, 1986) and potato (Simmonds, 1969). Haygood *et al.* (2004) modeled transgene escape and found that even genes with leakage rates as low as that found in our study will have an appreciable probability of escape into the wild. The possibility of transgene escape at such low leakage rates further highlights the necessity for studies examining mode of organellar inheritance to have a high statistical power to detect rare leakage.

We were also interested in addressing the question of whether it is necessary to study chloroplast transmission in more than one species within the same genus (that is, is it possible to extrapolate within the genus if one member species' transmission has already been determined?). In order to address this, we considered the results of three surveys of potential chloroplast transmission in angiosperms (Sears, 1980; Corriveau and Coleman, 1988; Zhang *et al.*, 2003) and after accounting for overlap among them and assuming equal sampling intensities, we found that 6 out of 113 genera contained conflicting modes of inheritance. In three of the genera, there was predominantly one type of transmission with a low frequency of the other. In our study, we found *H. verticillatus* to have a low level of paternal leakage. This is in contrast to the observation of strict maternal inheritance found in another species, *H. annuus*, within the genus (Rieseberg *et al.*, 1994; Wills *et al.*, 2005). Combining their data set with that of Rieseberg *et al.* (1994), Wills *et al.* (2005) determined they were able to detect paternal leakage at a rate of $\geq 1.35\%$, 95% of the time, a study that is comparable in magnitude to ours. The findings of this literature survey and our results indicate that the mode of chloroplast inheritance cannot always be extrapolated within a genus. Furthermore, the methods used in these studies may only provide conservative estimates given the limited sample sizes per species and ability to detect only the potential mode of inheritance.

In conclusion, we found evidence for a modest amount paternal transmission of cpDNA and heteroplasmy in the perennial sunflower, *H. verticillatus*, in greenhouse crosses. We are also interested in the consequences of paternal leakage and heteroplasmy for the biology of natural populations. The next step is to address paternal leakage in the field. If we extrapolate our primary paternal leakage rate (1.55%) to natural populations and assume every leakage event is detectable (that is, the parents have different cpDNA haplotypes), at least 200 offspring from the field would be necessary to have a 95% chance of detecting leakage at the frequency we found in the crosses. However, every leakage event will not be detectable given the high level of chloroplast population structure, $F_{ST}=0.620$ (Ellis *et al.*, 2006). The probability of the parents being different, assuming random mating within populations, can be calculated for n number of haplotypes by

$$P = 1 - p^2 - q^2 - r^2 - s^2 \dots n^2$$

where p , q , r and s are cpDNA haplotype frequencies from a given natural population. For example, using the four haplotype frequencies from the Tennessee population of *H. verticillatus* (Ellis *et al.*, 2006), $P=0.48$ ($1-0.7^2-0.12^2-0.12^2-0.06^2$). Roughly, only half of the matings will be between parents that differ at these cpSSRs. Thus, multiplying 200, the number of offspring that must be inspected to observe leakage at a rate of 1.55 when parents differ at marker loci, by $1/P$ yields the expectation that 417 offspring must be examined to have a 95% chance of detecting leakage at a rate of 1.55% in natural populations given the population structure. These results indicate large sample sizes are necessary to ensure detection of rare paternal leakage in angiosperms especially in natural populations.

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