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Biparental chloroplast inheritance leads to rescue from cytonuclear incompatibility

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Summary

- Although organelle inheritance is predominantly maternal across animals and plants, biparental chloroplast inheritance has arisen multiple times in the angiosperms. Biparental inheritance has the potential to impact the evolutionary dynamics of cytonuclear incompatibility, interactions between nuclear and organelle genomes that are proposed to be among the earliest types of genetic incompatibility to arise in speciation.
- We examine the interplay between biparental inheritance and cytonuclear incompatibility in *Campanulastrum americanum*, a plant species exhibiting both traits. We first determine patterns of chloroplast inheritance in genetically similar and divergent crosses, and then associate inheritance with hybrid survival across multiple generations.
- There is substantial biparental inheritance in *C. americanum*. The frequency of biparental inheritance is greater in divergent crosses and in the presence of cytonuclear incompatibility. Biparental inheritance helps to mitigate cytonuclear incompatibility, leading to increased fitness of F_1 hybrids and recovery in the F_2 generation.
- This study demonstrates the potential for biparental chloroplast inheritance to rescue cytonuclear compatibility, reducing cytonuclear incompatibility's contribution to reproductive isolation and potentially slowing speciation. The efficacy of rescue depended upon the strength of incompatibility, with a greater persistence of weak incompatibilities in later generations. These findings suggest that incompatible plastids may lead to selection for biparental inheritance.

Introduction

Organelle inheritance is predominantly uniparental across plants and animals and is achieved by numerous mechanisms, leading to the hypothesis that uniparental inheritance is under positive selection (Birky, 1995, 2001; Mogensen, 1996). However, biparental inheritance of the chloroplast genome has arisen multiple times within the angiosperms (Hu *et al.*, 2008). Large-scale screens using DAPI DNA staining have determined that *c.* 20% of angiosperm species have the potential for biparental inheritance, defined as the presence of plastid DNA in pollen generative cells (Corriveau & Coleman, 1988; Zhang *et al.*, 2003). While the presence/absence of potential biparental inheritance generally matches patterns of inheritance determined via genetic studies (e.g. *Oenothera* and *Pelargonium*, Corriveau & Coleman, 1988; Chiu & Sears, 1993; Weihe *et al.*, 2009), it is not universally indicative of realized biparental inheritance (e.g. *Pisum satvia*; Corriveau & Coleman, 1988; Polans *et al.*, 1990). This discrepancy can be explained by the fact that multiple mechanisms exist to achieve uniparental inheritance beyond exclusion of plastids from pollen generative cells (Mogensen, 1996). Therefore, the actual frequency of biparental plastid inheritance in angiosperms remains uncertain.

Several reasons have been proposed to explain why biparental inheritance may have evolved. Biparental plastid inheritance may have been favored as a mechanism to overcome incompatible plastids in species that have cytonuclear incompatibility (Hu *et al.*, 2008; Zhang & Sodmergen, 2010; Jansen & Ruhlman, 2012). Biparental plastid inheritance may also have been selected for to allow for low levels of organelle recombination to counteract the accumulation of deleterious mutations (Muller's ratchet; Greiner *et al.*, 2015). Alternatively, biparental plastid inheritance could be a signature of conflict between the plastid and nuclear genomes regarding patterns of plastid inheritance (Reboud & Zeyl, 1994), similar to the dynamics between the nuclear and mitochondrial genomes thought to underlie cytoplasmic male sterility (CMS; Gouyon *et al.*, 1991; Chase, 2007).

Once biparental plastid inheritance has evolved, it has the potential to impact evolution. Cytonuclear incompatibility, negative interactions between the nuclear and organelle genomes, is proposed to be among the earliest types of genetic incompatibility to arise in speciation (Levin, 2003; Fishman & Willis, 2006; Greiner *et al.*, 2011; Burton *et al.*, 2013). However, biparental inheritance has the potential to mitigate this incompatibility. First, the occurrence of biparental inheritance in crosses that result in cytonuclear incompatibility increases the likelihood that hybrids will inherit a chloroplast that is compatible with the nuclear genome. Second, biparental inheritance introduces genetic variability among the organelles, allowing selection to occur that may lead to the loss of an incompatible chloroplast genome (Birky, 2001). In plants, loss of an incompatible genome can be facilitated by within-individual genetic drift ('vegetative sorting' or 'sorting out'), which typically results in the loss of one genome as heteroplasmic individuals grow. Losing the incompatible plastid genome may lead to a recovery in fitness in the second-generation (F_2) hybrids. These mechanisms could enable biparental chloroplast inheritance to rescue hybrids suffering a loss of fitness as a result of cytonuclear incompatibilities, reducing the role of cytonuclear incompatibility in reproductive isolation and speciation. These dynamics are likely to be important in many plant taxa, as biparental inheritance frequently occurs in taxa that exhibit cytonuclear incompatibility (reviewed in Jansen & Ruhlman, 2012). While biparental inheritance has been shown to increase the survival of first-generation (F_1) hybrids in the presence of cytonuclear incompatibility (Kirk & Tilney-Bassett, 1978; Ureshino *et al.*, 1999; Bogdanova, 2007), these studies have been limited to crosses between species or cultivars, and have not examined the effects of biparental inheritance on fitness across multiple generations.

Here we examine how biparental inheritance influences the dynamics of cytonuclear incompatibility using the plant species *Campanulastrum americanum*. Previous studies found a strong cytonuclear incompatibility between genetically divergent populations of *C. americanum*, leading to chlorosis and greatly reduced survival in F_1 hybrids (Galloway & Etterson, 2005; Etterson *et al.*, 2007; Barnard-Kubow, 2015). Green and white variegation was also frequently observed in F_1 hybrids; variegation is thought to be a result of biparental inheritance when one of the chloroplasts is incompatible on the hybrid nuclear background (Kirk & Tilney-Bassett, 1978; Ureshino *et al.*, 1999; Bogdanova, 2007; Weihe *et al.*, 2009; Greiner *et al.*, 2011). In addition, studies have found plastid DNA present in the pollen generative cells in the Campanulaceae (Sears, 1980; Corriveau & Coleman, 1988; Zhang *et al.*, 2003), suggesting the potential for biparental plastid inheritance in *C. americanum*.

We characterized the patterns of biparental chloroplast inheritance and its evolutionary consequences for cytonuclear incompatibility in *C. americanum* by determining patterns of chloroplast inheritance and survival across multiple hybrid generations. Specifically, we addressed the following questions. What is the typical pattern of chloroplast inheritance in *C. americanum*? Does hybridization between genetically divergent lineages trigger or increase the frequency of biparental inheritance? Does biparental inheritance lead to increased fitness of F_1 hybrids in genetically divergent crosses that show cytonuclear incompatibility? Does biparental inheritance and vegetative sorting allow for selection against and ultimately the loss of the incompatible plastid, leading to recovery in future hybrid generations?

Materials and Methods

Study system

Campanulastrum americanum (L.) Small (= *Campanula americana* L.) is an autotetraploid, monocarpic herb found in the eastern half of the United States. Individuals are annual or biennial, insect-pollinated, and highly outcrossing (Galloway *et al.*, 2003). Plants grow as a vegetative rosette, and, after vernalization, bolt and flower. *C. americanum* typically grows in disturbed habitats and seeds are passively dispersed, characteristics that probably contribute to its patchy population structure. Chloroplast markers resolve three genetic clades: Western, Appalachian, and Eastern (Barnard-Kubow *et al.*, 2015). These clades differ in their distribution, with the Appalachian and Eastern clades restricted primarily to the Appalachians, and the Western clade spread throughout the range. Populations are generally fixed for chloroplast haplotype (Barnard-Kubow *et al.*, 2015). *C. americanum* is a member of the Campanulaceae, which have been shown to have highly rearranged chloroplast genomes with increased rates of nucleotide substitution (Cosner *et al.*, 2004; Barnard-Kubow *et al.*, 2014; Knox, 2014), in addition to the potential for biparental chloroplast inheritance.

Chloroplast inheritance and fitness

F_1 samples and survival To examine patterns of chloroplast inheritance and survival in *C. americanum*, F_1 hybrid seed was used from crosses carried out in earlier studies (K. B. Barnard-Kubow *et al.*, unpublished; Barnard-Kubow, 2015). Three crosses within clades were chosen to determine the typical mode of inheritance (W1 × W2: 69 × 59, 44 × 20, and 34 × 7; Fig. 1; Supporting Information Table S1). All six parental populations contained chloroplast haplotypes from the previously characterized Western haplotype clade (Barnard-Kubow *et al.*, 2015), henceforth referred to as W haplotypes. Crosses within clades of *C. americanum* do not show reproductive isolation, and always produce green, viable F_1 hybrids (Barnard-Kubow, 2015). Six crosses between clades were chosen to determine the mode of chloroplast inheritance in genetically divergent crosses (W × A: 68 × 73, 52 × 72, 10 × 72, 29 × 5, 86 × 92, and 88 × 91; Fig. 1; Table S1). All six crosses were between the W and Appalachian (A haplotypes) chloroplast clades and have been found to exhibit cytonuclear incompatibility (K. B. Barnard-Kubow *et al.*, unpublished; Barnard-Kubow, 2015). Altogether this resulted in nine crosses (three within- and six between-clade) each with two crossing directions (defined by which population was maternal) for a total of 18 cross-types. All within-clade crosses and four of the between-clade crosses were grown from F_1 seed generated at the same time and under the same environmental conditions. Two of the between-clade crosses (86 × 92 and 88 × 91) were from a separate experiment in a different year, though with plants grown and crossed under similar environmental conditions as the first experiment.

Seed from the 18 cross-types were grown under controlled conditions to determine chloroplast inheritance and survival of F_1 hybrids. For each cross-type, multiple replicates of two seeds each were surface-sown onto potting medium (3 : 1, Promix : Turface) in 2.54 × 2.54 cm cells in 9 × 18 germination flats. Number of replicates ranged from 40 to 85 based on expected germination rate (affected by age of seed and reproductive isolation for germination; Barnard-Kubow, 2015). Ten replicates of two seeds each were also planted for each parental population. Replicates were spread evenly among *c.* 10 maternal families for each cross-type, with a total of 1150 replicates planted. Seed location was fully randomized and germination flats were placed in a growth chamber with a 12 : 12 h, 24 : 14°C, day : night temperature regime, and were watered daily. Seedlings were scored for germination and nongreen phenotypes were recorded (e.g. variegated, white, chlorotic with yellowing as a result of insufficient production of Chl; Fig. 2). Owing to lower than expected germination in some cross-types, additional F_1 seed was planted for a subset of the between-clade crosses to obtain larger sample sizes. Seed was planted and grown as described earlier, except that replicates contained 15 seeds each.

To determine chloroplast haplotype, seedlings from within-clade crosses and parental populations were harvested whole once they had at least two true leaves. A total of 480 within-clade cross and 313 parental individuals were harvested. For the between-clade crosses, seedlings were not harvested unless they were completely white, as previous work found that white seedlings do not live beyond a week (K. B. Barnard-Kubow, unpublished). Any seedlings starting to die were also harvested. Once germination had slowed, plants were moved to 5°C for vernalization to stimulate flowering. The first batch of plants was vernalized for 10 wk, while the second batch was vernalized for 7 wk.

To estimate F_1 survival to reproduction, after vernalization a subset of plants was transplanted into conetainers and moved to a glasshouse, where supplemental light increased day length to 16 h. For most of the between-clade crosses, 25 randomly selected plants were transplanted per crossing direction, distributed across maternal families. However, for crosses where low survival was expected as a result of the high frequency of non-green phenotypes, all surviving plants (up to 70 individuals) were transplanted. Plants not transplanted or that were starting to die were harvested. Smaller plants were harvested whole, while a leaf was harvested that was representative of the overall phenotype for larger plants. Transplanted plants had rosette leaf samples taken before or within a few days of starting to bolt. For variegated individuals, both green and white leaf tissue samples were taken to test if phenotype (green vs white) was correlated with chloroplast haplotype. Combining pre- and post-transplant, a total of 978 tissue samples were taken from between-clade hybrid individuals (summing the first and second plantings). Plants were monitored for survival and flowering.

Plants initially heteroplasmic for chloroplast genomes may transmit only a single haplotype to the next generation as a result of vegetative sorting as individuals grow. To examine whether vegetative sorting contributes to changes in chloroplast

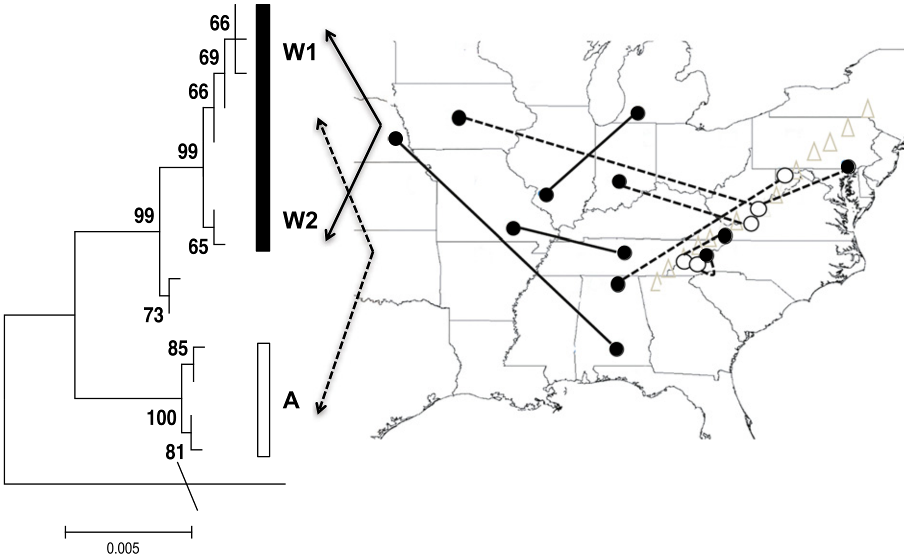

Fig. 1 Phylogenetic tree and map depicting crosses used to estimate degree and consequences of nonmaternal chloroplast inheritance in *Campanulastrum americanum*. Maximum likelihood phylogenetic tree with 1000 bootstrap replicates based on chloroplast DNA sequence from five loci (Barnard-Kubow *et al.*, 2015). Location of populations used are marked on the map, and shaded black or white according to chloroplast clade. The three solid lines represent genetically similar within-clade crosses, while the six dashed lines represent genetically divergent between-clade crosses. W1 and W2 are the two branches of the Western clade, while A is the Appalachian clade.

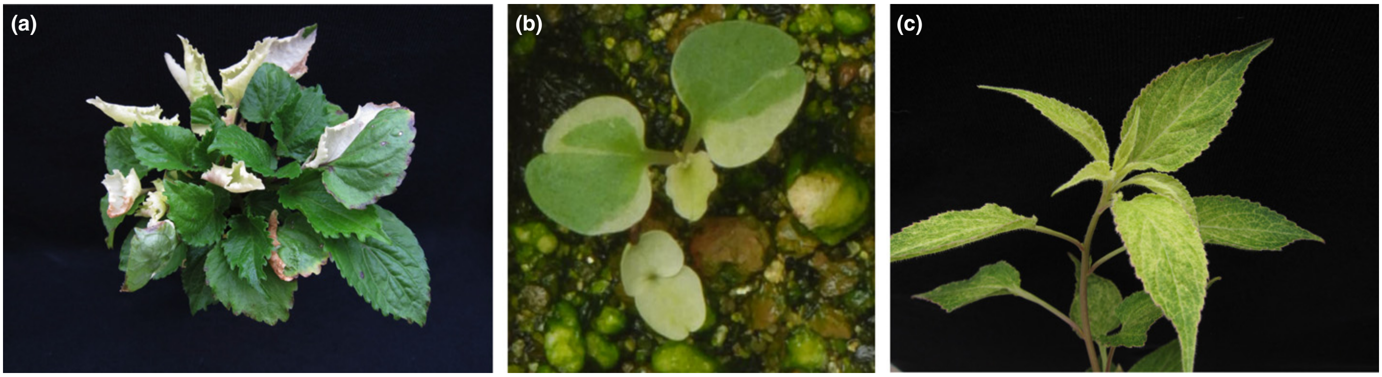

Fig. 2 Examples of variegated (a, b, upper plant) and white (b, lower plant) seedlings as a result of a strong incompatibility, and a seedling with a weaker chlorotic incompatibility (c), observed in genetically divergent *Campanulastrum americanum* F_1 hybrids.

haplotype, a leaf subtending a flower was collected for 24 plants that were genotyped as containing both A and W haplotypes when juvenile (see genotyping methods later). For comparison, comparable apical leaf tissue samples were also taken from individuals that were genotyped as containing only A or W haplotypes ($n = 37$ and 13, respectively).

F_2 samples and survival To determine chloroplast haplotype and survival of F_2 hybrids, F_2 seed was made by carrying out two pollinations on each between-clade F_1 plant. Individuals were randomly crossed to four other plants from the same cross-type, with each individual serving twice as a pollen recipient and twice as a pollen donor. The number of individuals pollinated ranged from 12 to 28 per cross-type, with pollinations within families avoided to minimize inbreeding.

Chloroplast haplotype and survival in the F_2 generation were evaluated using the methods for the F_1 generation detailed earlier. Forty to 56 replicates of two seeds each, evenly distributed across 10–14 families, were planted for each cross-type (476 replicates total). F_2 seed was chosen from maternal plants representing each haplotype category (A, W, AW) to get an accurate estimate of performance of offspring from F_1 mothers with differing chloroplast haplotypes. As a result, the haplotype distribution of maternal plants contributing to the F_2 generation was not always representative of the total haplotype distribution for that cross. For example, cross-type 88 × 91 had two A, 13 W, and five AW individuals flower. However, seeds from two A, five W, and three AW individuals were planted. As in the F_1, germination and nongreen seedling phenotypes were recorded, and any seedlings that were completely white or dying were harvested. After germination slowed, seedlings were randomly thinned to one per cell, with a subset of thinned seedlings harvested. The germination flats were moved to 5 °C for 7 wk to stimulate flowering, and then transplanted to conetainers and moved to the glasshouse. Plants were monitored for survival and flowering. Plants were scored as surviving if they were still alive after the majority of plants had flowered, even if they never flowered. Again, any plants that started to die were harvested and all surviving plants had rosette leaf samples taken before or within a few days of bolting. A total of 823 leaf tissue samples were collected from between-clade F_2 hybrids.

Genotyping samples for chloroplast haplotype

A subset of leaf samples was selected for each cross-type to determine chloroplast haplotypes in F_1 and F_2 hybrid individuals. Up to 62 samples per cross-type for both the F_1 and F_2 generation were chosen. Samples were distributed among maternal families, with most cross-types having eight to 10 families. Eight samples were also chosen from each parental population as a control. For the F_1 generation, 15 of 18 cross-types had sample sizes > 45 (Table 1), with an average of six individuals per family. The remaining three had smaller sample sizes as a result of poor germination and survival. For the F_2 generation, 11 of 12 cross-types had samples sizes > 40; the exception was 73 × 68, which had a sample size of 15.

Table 1 Chloroplast inheritance for F_1 hybrids from within- and between-clade crosses of *Campanulastrum americanum*

		n	Nonmaternal	*n*	Nonmaternal
Type	Cross	W1 × W2		W2 × W1	
Within clade	69 × 59	53	0.36	47	0.06
	44 × 20	49	0.31	60	0.10
	34 × 7	60	0.22	60	0.33
		W × A		A × W	
Between clade	68 × 73	60	0.53	6	0.17
	52 × 72	62	0.51	53	0.34
	10 × 72	62	0.42	23	0.30
	29 × 5	60	0.42	37	0.19
	86 × 92	61	0.26	59	0.19
	88 × 91	59	0.19	59	0.15

Number of individuals genotyped and proportion of individuals with nonmaternal inheritance are given for each cross and crossing direction separately. The maternal population is listed first for a given cross. W, Western clade; A, Appalachian clade; see Fig. 1.

DNA extractions were carried out using a modified cetyltrimethylammonium bromide procedure either in single-tube or 96-well plate format. Chloroplast haplotype was determined for each sample using Custom Taqman SNP Genotype Assays (Thermo Fisher Scientific, Waltham, MA, USA), similar to methods used for tracking mitochondrial inheritance (Bentley *et al.*, 2010). Three different assays were used for genotyping based on SNPs in the chloroplast genome. For each assay, a standard curve was first constructed by precisely mixing together various ratios (e.g. 2 : 98, 5 : 95, 10 : 90, 20 : 80, etc.) of the two parental haplotypes using parental population DNA extractions. These mixtures were run in triplicate on an ABI 7500 fast real-time PCR (RT-PCR) system (Thermo Fisher Scientific). The average difference in cycle threshold value between the two probes was calculated and graphed against the log of the artificial mixture ratios. This comparison resulted in a straight line whose equation could then be used to back-calculate ratios obtained from DNA samples to determine the ratio of the two chloroplast haplotypes present in a given sample (Fig. S1). Each DNA extraction was run at least once using the RT-PCR assay. A subset of individuals was run three times to examine inherent variation in the RT-PCR assays for estimating error in the calculated haplotype ratios. The chloroplast haplotype data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.pn25d.

As the SNP assays were based on chloroplast SNPs, only chloroplast inheritance patterns were documented. Inheritance of the chloroplast and mitochondria are independently controlled in plants (Nagata, 2010), with the mitochondria more strictly maternally inherited than the chloroplast (Greiner *et al.*, 2015). Therefore, whether or not the mitochondria are biparentally inherited cannot be determined from patterns of chloroplast inheritance. Attempts to identify mitochondrial SNPs in *C. americanum* have thus far proven unsuccessful, and therefore patterns of mitochondrial inheritance remain unknown.

Statistical analysis

Genotyping results were used to examine if the degree of biparental inheritance was impacted by genetic divergence and maternal plastid haplotype. The frequency of nonmaternal inheritance for each cross-type was determined from the RT-PCR results. For statistical analysis, inheritance was treated as a binomial trait, with individuals having either maternal or nonmaternal inheritance. Individuals were conservatively scored as having nonmaternal inheritance only when the RT-PCR assay estimated a 10% or greater contribution of the paternal chloroplast. This was based on threshold detection values of 5% when calculating the standard curve, and an estimated variance of 5% when running the same sample multiple times. The one exception were 47 between-clade individuals (spread across all six crosses) that were phenotyped as variegated shortly after germination, indicating biparental inheritance, but that, when genotyped, were scored as having maternal inheritance. The majority of these individuals were harvested when dying, after having been transplanted to the glasshouse. The difference in phenotype and genotype was probably a result of vegetative sorting, with these initially heteroplasmic individuals having drifted towards fixation of the incompatible plastid genome as they grew. This means the RT-PCR assay underestimates biparental inheritance, particularly when harvesting individuals later in development. Accordingly, we decided to score these individuals as having biparental inheritance. Scoring these individuals as having maternal inheritance led to qualitatively similar results.

A log-linear analysis assuming a binomial distribution and a logit link was conducted to test the effect of genetic divergence (within-clade vs between-clade crosses) as well as cross (nested within genetic divergence) on degree of nonmaternal inheritance (PROC GENMOD, SAS 9.3; SAS Institute Inc., 2011). To test whether inheritance patterns differed if populations with A or W haplotypes were maternal, a similar log-linear analysis was conducted using only between-clade crosses, with cross, crossing direction, and the interaction of these two factors in the model.

To examine the impact of chloroplast haplotype on the fitness of between-clade F_1 hybrids, the effect of cross, chloroplast haplotype, and their interaction on F_1 hybrid survival was tested using a log-linear analysis assuming a binomial distribution and a logit link (PROC GENMOD, SAS 9.3; SAS Institute Inc., 2011). The data indicated that W chloroplast haplotypes were predominantly incompatible on an AW hybrid nuclear background, but that the strength of this incompatibility varied among crosses. To test this observation, a log-linear analysis determined the effect of cross on F_1 hybrid survival for individuals containing only a W haplotype. Finally, a correlation analysis was run to determine the relationship between the probability of individuals in a given cross surviving when having only a W haplotype and degree of nonmaternal inheritance for that cross (PROC CORR, SAS 9.3; SAS Institute Inc., 2011). Only data from the crossing direction where populations with the W haplotype were maternal were used for this correlation analysis.

To examine the impact of nonmaternal inheritance on the fitness of F_1 hybrids, we first determined the proportion of surviving F_1 hybrid offspring for each cross that exhibited maternal vs nonmaternal inheritance. Then, the proportion of surviving F_2 offspring resulting from nonmaternal inheritance was estimated by first determining the mean survival of F_2s from maternal plants that had been genotyped as A, W, or AW. Finally, using the number of A, W, or AW F_1 hybrids that flowered for each cross, the proportion of F_2 offspring that would have come from F_1 maternal plants with nonmaternal inheritance had all F_2 families been planted was calculated.

We then examined whether biparental inheritance led to an increased survival of F_1 hybrids over maternal inheritance when combining both cross directions. We compared the survival of F_1 hybrids in each cross with the expected survival if inheritance had been strictly maternal. To estimate survival under maternal inheritance, we multiplied the number of F_1 individuals for each crossing direction by the survival of individuals that inherited only the maternal haplotype. For crosses where the cytonuclear incompatibility is complete (i.e. no survival with the incompatible W haplotype), maternal inheritance would result in no surviving offspring when populations with the W haplotype were maternal. By contrast, in crosses where the cytonuclear incompatibility was weaker, maternal inheritance would still lead to some survival when populations with the W haplotype were maternal. For all crosses, survival would be high under maternal inheritance when populations with the A haplotype were maternal. We then tested for a difference between the observed survival data and the estimated maternal inheritance survival data using a log-linear analysis assuming a binomial distribution and a logit link (PROC GENMOD, SAS 9.3; SAS Institute Inc., 2011). We tested for an effect of inheritance (biparental or maternal), cross, and their interaction. When presenting the results, back-transformed least-square means are used. Because of very low amounts of germination for 73 × 68, only five of the six between-clade crosses were included in this analysis.

We examined whether there was a reduction in the frequency of the incompatible W haplotypes in the F_2 relative to the F_1 hybrid generation in the crossing direction where W populations were maternal. First the frequency of the W haplotype for each cross in the F_1 and F_2 generation was calculated ((no. of W offspring + no. of AW offspring/2)/total no. of offspring). The proportionate reduction of the W haplotypes in the F_2 relative to the F_1 was then calculated $((f(W_{F1}) - f(W_{F2}))/f(W_{F1}))$. Positive values indicate a reduction in frequency of the W haplotypes in the F_2, while negative values indicate an increase in frequency. A correlation analysis was run to determine if there was a relationship between the proportionate reduction of the W haplotypes and the probability of F_1 hybrids surviving when only inheriting a W haplotype (PROC CORR, SAS 9.3; SAS Institute Inc., 2011).

To examine whether a reduction in the frequency of the W haplotypes leads to a concomitant recovery in fitness, we tested whether there was significant recovery in the F_2 generation relative to the F_1 when W populations were maternal. Recovery was examined by testing for an effect of cross, generation (F_1 vs F_2), and their interaction on hybrid survival using a log-linear analysis assuming a binomial distribution and a logit link. As the interaction was significant, similar analyses were run for each cross individually, testing for an effect of generation.

Results

Patterns of chloroplast inheritance

Substantial biparental and paternal chloroplast inheritance was found in all crosses (Table 1; Figs S2, S3), although inheritance was predominantly maternal. The existence of some paternal inheritance, despite the dominance of maternal inheritance, suggests that *C. americanum* exhibits a *Pelargonium*-type pattern of biparental inheritance (Hagemann, 2004). The frequency of nonmaternal inheritance ranged from 6% to 53% among combinations of cross and crossing direction with a mean and SD of 28 ± 13% (Table 1). Crossing between clades led to an increase in the frequency of nonmaternal inheritance relative to when crossing within a clade (31% vs 23%; $\chi_1^2 = 10.41$, $P = 0.001$). There was also an overall effect of crossing direction in between-clade crosses ($\chi_1^2 = 11.27$, $P < 0.001$), with a greater degree of nonmaternal inheritance when W populations were maternal (39% vs 22%; Table 1).

Chloroplast inheritance and fitness

Survival of F_1 between-clade hybrids varied among chloroplast haplotypes (A, W, or AW; $\chi_2^2 = 291.18$, $P < 0.001$) and among crosses ($\chi_5^2 = 20.49$, $P = 0.001$), but the effect of haplotype was consistent across crosses (haplotype × cross; $\chi_{10}^2 = 14.63$, $P = 0.146$). The likelihood of survival was consistently lowest for individuals with W haplotypes (14% survival), highest for those with A haplotypes (96% survival), and intermediate for those that inherited both A and W haplotypes. These results indicate that W haplotypes are incompatible on the AW hybrid nuclear background, while the A haplotypes are not. The incompatibility of W haplotypes was further confirmed in variegated seedlings where genotyping white and green leaf tissue punches from 36 individuals found that white leaf tissue always contained W haplotypes and green tissue A haplotypes (Fig. S4). The likelihood of surviving with a W haplotype varied among between-clade crosses from 0% to 32% ($\chi_5^2 = 26.41$, $P < 0.001$, Fig. 3). In the crossing direction where W populations were maternal, the likelihood of surviving was positively correlated with degree of maternal inheritance ($r = 0.86$, $P = 0.026$; Fig. 3a).

These findings strongly suggest that the W chloroplasts are incompatible with the AW hybrid nuclear background. While the possibility that the mitochondrial genome is also playing a role in the incompatibly cannot be eliminated, it is extremely unlikely, as it would require not only the same pattern of mitochondrial inheritance as chloroplast inheritance, but also similar patterns of vegetative sorting. These processes occur independently for chloroplasts and mitochondria.

Nonmaternal inheritance contributed substantially to the fitness of F_1 hybrids in the crossing direction where W populations were maternal. In the three crosses where the W haplotypes were almost entirely inviable (68 × 73, 52 × 72, and 10 × 72; *x*-axis Fig. 3), 92–100% of surviving F_1 hybrids were the result of nonmaternal inheritance (Fig. 4a). While in crosses where *c.* 15% of individuals with a W haplotype survived (29 × 5 and 86 × 92), 58–68% of surviving F_1 hybrids were the result of nonmaternal inheritance. The final cross, 88 × 91, had 30% survival of hybrid individuals with a W haplotype and only 32% of surviving F_1 hybrids were the result of nonmaternal inheritance. These differential effects of nonmaternal inheritance on hybrid fitness were also found when examining performance of the F_2 hybrid offspring (Fig. 4c). For 68 × 73, 52 × 72, and 10 × 72, 96–100% of surviving F_2 offspring were estimated to have come from F_1 individuals with nonmaternal inheritance. This number was also high at 83% for 29 × 5 and 86 × 92. However, for 88 × 91, only 32% of surviving F_2 offspring were estimated to come from F_1 individuals with nonmaternal inheritance.

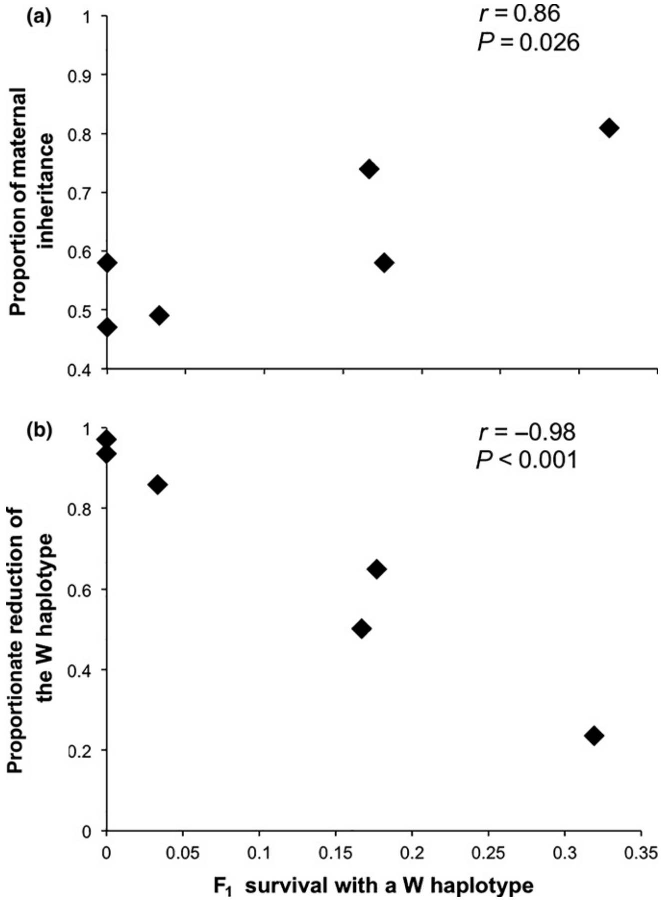

Fig. 3 Correlation between the likelihood of between-clade *Campanulastrum americanum* F_1 hybrids surviving when only inheriting a Western (W) haplotype and either: (a) the proportion of maternal inheritance in F_1 hybrids; or (b) the proportionate reduction of the W haplotype in the F_2 relative to the F_1. Each point represents the mean of a genetically divergent cross. Only data from the crossing direction where W populations were maternal is included.

By contrast, fitness of the F_1 hybrids in the alternate crossing direction was primarily as a result of maternal inheritance, as individuals with maternal inheritance contained compatible A

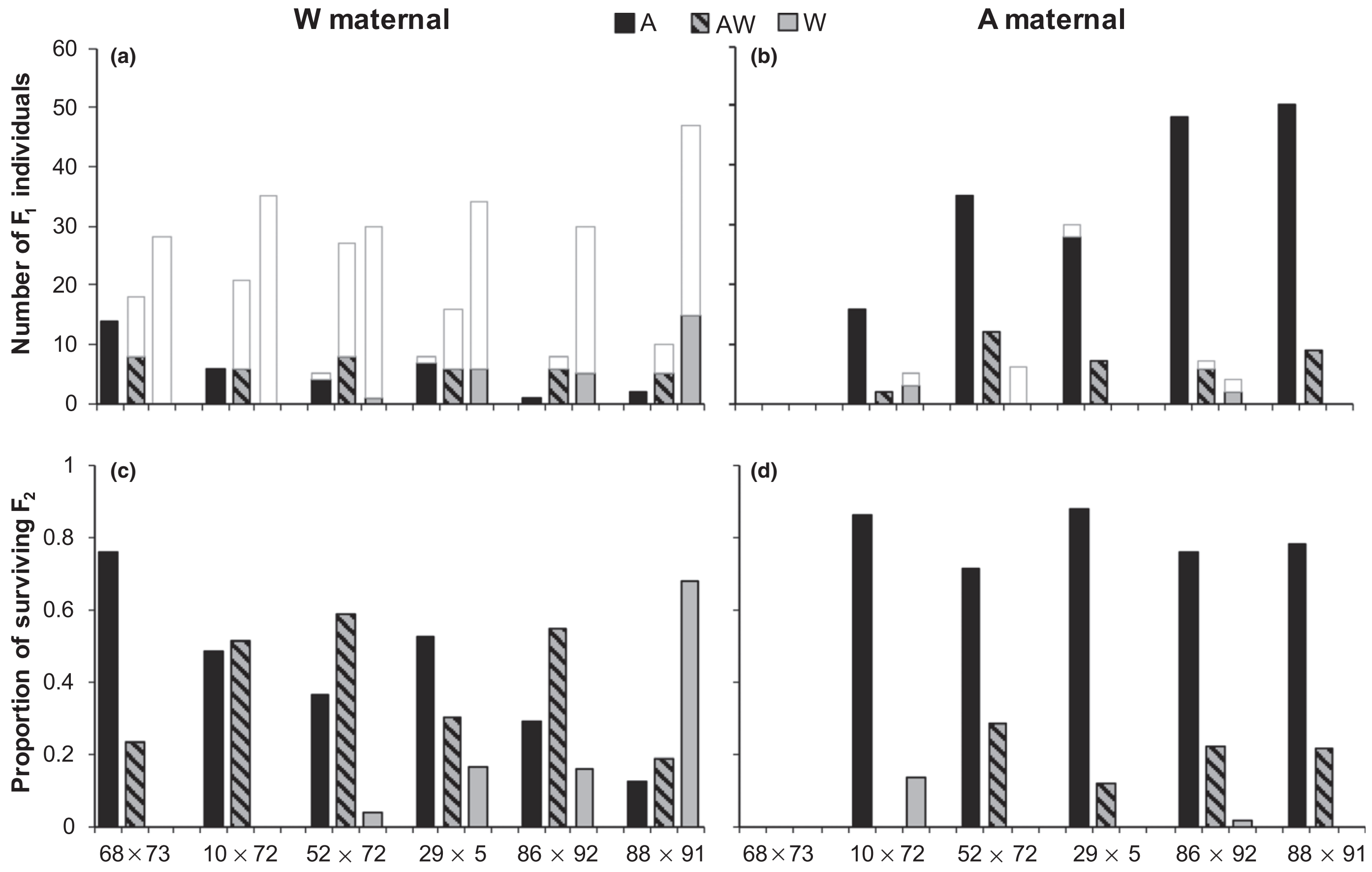

Fig. 4 Survival and fitness of each chloroplast haplotype through F_2 survival for *Campanulastrum americanum* F_1 hybrids from between-clade crosses. Cross IDs are listed across the bottom of the graphs, with the order of crosses matching that along the *x*-axis in Fig. 5. (a, b) Number of F_1 hybrid individuals that survived or died for each genetically divergent cross, according to chloroplast haplotype. White bars outlined in gray represent the number of individuals that died for each category. (c, d) The estimated proportion of surviving F_2 offspring for each genetically divergent cross that originated from F_1 progeny containing the alternate chloroplast haplotypes. Crossing direction where Western (W) populations were maternal (a, c) and Appalachian (A) populations were maternal (b, d).

haplotypes. Only 0–26% of surviving F_1 hybrids were the result of nonmaternal inheritance (Fig. 4b), with 0–28% of surviving F_2 offspring estimated to have come from F_1 individuals with nonmaternal inheritance (Fig. 4d).

When combining both crossing directions, biparental inheritance was found to lead to higher F_1 hybrid survival (66.7%) relative to strict maternal inheritance (51.9%) across all crosses ($\chi_1{}^2 = 4.19$, $P = 0.041$). The effect of cross was also significant, indicating rates of survival varied among crosses ($\chi_4{}^2 = 65.30$, $P < 0.001$). However, the interaction was not significant, indicating that biparental inheritance consistently led to higher fitness than maternal inheritance.

Evidence for vegetative sorting was found when comparing rosette and apical leaf tissue samples from between-clade hybrids. Of the 24 variegated individuals genotyped AW as rosettes, two-thirds of them were genotyped as containing only a single haplotype when flowering (haplotype A in all but two cases), indicating that vegetative sorting had occurred (Fig. S5). However, the remaining eight individuals retained both haplotypes, indicating that this process was not always complete. In comparison, of the 50 individuals genotyped as either A or W when rosettes, all but one retained those genotypes when flowering. The one remaining individual was genotyped as AW when flowering, suggesting that genotyping rosettes by a representative leaf, rather than sacrificing entire seedlings, occasionally results in missing occurrences of biparental inheritance.

All six between-clade crosses experienced a reduction in the frequency of the W haplotypes in the F_2 generation relative to the F_1 when focusing on the crossing direction where W populations were maternal. The degree of loss was negatively correlated with the likelihood of F_1 hybrid survival when inheriting only a W haplotype ($r = -0.98$, $P < 0.001$; Fig. 3b). Crosses in which some F_1 hybrids with a W haplotype survived (29 × 5, 86 × 92, and 88 × 91) experienced an 11–53% loss of the W haplotypes, while crosses where F_1 hybrids with a W haplotype were always inviable experienced a larger reduction (81–97%). Although reductions in the W haplotypes were substantial, none of the crosses showed a complete loss of this haplotype, probably as a result of occasional incomplete vegetative sorting in AW plants.

All six between-clade crosses had improved survival in the F_2 generation relative to the F_1 when the W populations were maternal (Fig. 5). Across all between-clade crosses there was a

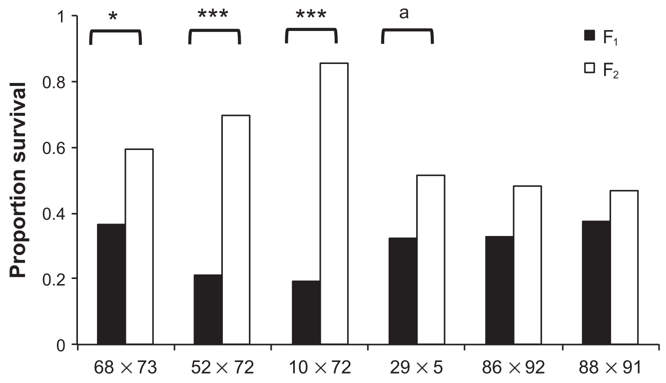

Fig. 5 Proportion survival of *Campanulastrum americanum* F_1 and F_2 generation hybrids for each between-clade cross. Data shown for the crossing direction where Western (W) populations were maternal. a, $P < 0.07$; *, $P < 0.5$; ***, $P < 0.001$.

significant effect of generation on survival ($\chi_1^2 = 44.73$, $P < 0.001$), indicating an overall recovery in survival of the F_2 generation relative to the F_1. However, the degree of recovery varied among crosses, as indicated by the significant interaction between cross and generation ($\chi_5^2 = 19.48$, $P = 0.002$). The three crosses that had the greatest loss of the W haplotype exhibited significant recovery in the F2 relative to the F_1 (Fig. 5; 10 × 72: $\chi_1^2 = 30.46$, $P < 0.001$; 52 × 72: $\chi_1^2 = 17.16$, $P < 0.001$; 68 × 73: $\chi_1^2 = 3.86$, $P = 0.05$). Recovery in 29 × 5 was near significant ($\chi_1^2 = 3.40$, $P = 0.065$), while there was only modest recovery for 86 × 92 ($\chi_1^2 = 2.15$, $P = 0.142$) and 88 × 91 ($\chi_1^2 = 0.79$, $P = 0.375$).

Discussion

Substantial biparental plastid inheritance (up to 53%) was found in *C. americanum* regardless of genetic divergence. However, the frequency of biparental inheritance was greater in genetically divergent crosses and in the presence of stronger cytonuclear incompatibility. Biparental inheritance allowed for increased survival of F_1 hybrid offspring, and also enabled selection against and loss of the incompatible plastid genome, leading to greater survival in the F_2 hybrid generation. As such, the occurrence of biparental plastid inheritance in genetically divergent crosses enabled rescue from cytonuclear incompatibility.

Patterns of inheritance

Biparental plastid inheritance occurred in both genetically similar and genetically divergent crosses of *C. americanum*, ranging from 6% to 53%. Finding biparental inheritance in genetically similar crosses suggests that the occurrence of biparental inheritance in C. *americanum* is not triggered by hybridization between genetically divergent lineages, but is a phenomenon common to the species as a whole. This result fits with the observation that all Campanulaceae species screened have plastid DNA in their pollen generative cells (Sears, 1980; Corriveau & Coleman, 1988; Zhang *et al.*, 2003), raising the potential for biparental inheritance. Other taxa with biparental inheritance, such as *Oenothera*, *Pelargonium*, and *Zanteschedia*, also show a wide range in inheritance patterns among different crosses (Kirk & Tilney-Bassett, 1978; Chiu & Sears, 1993; Snijder *et al.*, 2007). However, these studies use variegation as a marker for inheritance, and therefore only estimate biparental inheritance in crosses that exhibit cytonuclear incompatibility. Here we use genetic markers to demonstrate wide variation in the frequency of biparental inheritance not only in divergent crosses that exhibit cytonuclear incompatibility, but also in genetically similar crosses that produce only green offspring. Our finding of biparental inheritance in fully green offspring suggests that using variegation as an indicator of biparental inheritance probably underestimates its prevalence.

The frequency of biparental inheritance in *C. americanum* was associated with genetic divergence and cytonuclear incompatibility. Increased biparental inheritance was found in more genetically divergent between-clade crosses relative to within-clade crosses (see also Chiu & Sears, 1993; Xu, 2005; Bogdanova, 2007; Hansen *et al.*, 2007). In between-clade crosses of *C. americanum*, greater biparental inheritance was also found when populations with the incompatible W haplotypes were maternal, and the degree of biparental inheritance in this crossing direction was strongly correlated with strength of cytonuclear incompatibility. These results indicate an interaction between strength of cytonuclear incompatibility and degree of biparental inheritance in *C. americanum*. However, it is not clear whether cytonuclear incompatibility directly influences biparental inheritance in *C. americanum* or whether selection has driven an association between these traits.

Variance in germination also has the potential to influence apparent patterns of biparental inheritance. If the incompatible W haplotype led to reduced germination in addition to survival, in crosses with lower rates of germination maternal inheritance would be underestimated when populations with the W haplotype are maternal, but overestimated when they are paternal. However, we found no consistent relationship between germination success and degree of biparental inheritance (Fig. S6). In addition, other work in *C. americanum* demonstrated reductions in germination in between-clade F_1s are not caused by cytonuclear incompatibility (K. B. Barnard-Kubow *et al.*, unpublished). Therefore, the relationship between biparental inheritance and cytonuclear incompatibility is not likely to be driven by germination, reinforcing the idea that selection may have led to the covariation of these traits.

Biparental plastid inheritance leads to rescue from cytonuclear incompatibility

Biparental plastid inheritance in *C. americanum* allowed for increased survival of between-clade F_1 hybrids when populations with the incompatible W haplotypes were maternal. In particular, in crosses where the W haplotypes were lethal on the hybrid AW nuclear background, survival was only possible when biparental inheritance led to inheritance of a compatible A haplotype. The substantial fitness contributions of biparental inheritance in *C. americanum* also cascaded down to the next generation, as F_1

individuals with biparental inheritance contributed the majority of surviving F_2 offspring in five of the six between-clade crosses. Our results demonstrate the ability of biparental chloroplast inheritance to increase the fitness of F_1 hybrids in species with cytonuclear incompatibility (Kirk & Tilney-Bassett, 1978; Ureshino *et al.*, 1999; Bogdanova, 2007), and extends this fitness increase to the F_2 generation.

Biparental inheritance also led to a reduced frequency of the incompatible W haplotypes in the F_2 hybrid generation and a corresponding increase in survival. Therefore, biparental inheritance, along with vegetative sorting, allows for selection against, and a reduction in, the incompatible plastid, leading to a recovery in fitness in future hybrid generations. However, there was variance in the degree of recovery among crosses. Crosses in which the W haplotypes were completely inviable had substantial increases in survival in the F_2, while crosses where the cytonuclear incompatibility was weaker (individuals with a W haplotype sometimes survived) had less of a survival increase as a result of biparental inheritance and did not exhibit significant recovery in the F_2. The cross with the weakest cytonuclear incompatibility, 88 × 91, was particularly striking in this regard. This cross experienced only a 14% reduction in the W haplotype between the F_1 and F_2 generations, and it had the lowest F_2 survival with almost no recovery relative to the F_1 generation. One potential explanation for this variance in strength of cytonuclear incompatibility and ability of biparental inheritance to lead to recovery may be secondary contact and gene flow. Two of the crosses that exhibited weaker cytonuclear incompatibility and no significant recovery (86 × 92 and 88 × 91) are between populations that are found in the part of *C. americanum*'s range where genetically divergent lineages now co-occur (Barnard-Kubow *et al.*, 2015). Populations 88 and 91, in particular, are separated by only 68 km.

While biparental inheritance clearly led to increased fitness in divergent crosses when populations with the incompatible W haplotypes were maternal, biparental inheritance can only truly rescue cytonuclear incompatibility if it does better than strict maternal inheritance when combining both crossing directions. Biparental inheritance had a negative impact on the alternate crossing direction as a result of the introduction of the incompatible W haplotype. However, as biparental inheritance was less frequent when populations with the A haplotype were maternal, it resulted in only moderate reductions in fitness, with survival of F_1 hybrids ranging from 89% to 100%. Accordingly, when combining both crossing directions, the benefits of biparental inheritance outweighed the negative consequences, with biparental inheritance leading to a 15% increase in survival relative to strict maternal inheritance.

Overall our results demonstrate that biparental inheritance enables rescue from cytonuclear incompatibility. This finding is in agreement with the hypothesis that biparental inheritance has been selected as a mechanism for overcoming incompatible plastids in species with cytonuclear incompatibility. However, the occurrence of biparental plastid inheritance in the absence of cytonuclear incompatibility in *C. americanum*, suggests that while cytonuclear incompatibility may selectively favor this mode of inheritance, it is not necessary for its presence. Accordingly, biparental inheritance may have arisen as a result of other forces, including genomic conflict or genetic drift, but once present could be selected upon to mitigate the effects of cytonuclear incompatibility.

Biparental inheritance and the potential for speciation

Several taxa that have biparental plastid inheritance, such as *C. americanum*, also exhibit accelerated plastid evolution, having highly rearranged plastid genomes, with increased repetitive DNA, and accelerated rates of nucleotide substitution (Jansen & Ruhlman, 2012; Barnard-Kubow *et al.*, 2014). Accelerated plastid evolution may increase the propensity for cytonuclear incompatibility as it can lead to more rapid coevolution within populations. In a similar manner, rapid rates of mitochondrial evolution have been proposed to lead to compensatory nuclear evolution and mitochondrial-nuclear coadaptation (Burton & Barreto, 2012; Osada & Akashi, 2012; Sloan *et al.*, 2014), potentially increasing the propensity for mitochondrial-nuclear incompatibility in hybrids (Burton & Barreto, 2012). Variation in evolutionary rates of the organelle genomes relative to the nuclear genome has also been proposed to lead to a systematic directional asymmetry in terms of cytonuclear incompatibility. In this case, the incompatibility is expected to be greater when populations with a higher relative rate of organelle evolution are maternal (Turelli & Moyle, 2007; Bolnick *et al.*, 2008), although the effect is thought to be small and support from empirical studies has been mixed (Turelli & Moyle, 2007; Bolnick *et al.*, 2008; Brandvain *et al.*, 2014). However, it could be interesting to examine if the consistent directional asymmetry for the cytonuclear incompatibility observed in this study may be attributed to a difference in the relative rates of accelerated plastid evolution between the Appalachian and Western lineages.

As accelerated plastid evolution may increase the propensity for cytonuclear incompatibility, an association between biparental inheritance and cytonuclear incompatibility could be explained by greater selection for biparental plastid inheritance in taxa with accelerated plastid evolution. Alternatively, in these taxa, biparental inheritance and accelerated plastid evolution may both be manifestations of a general destabilization of the recombination and repair machinery in the plastid genome (Jansen & Ruhlman, 2012), where accelerated plastid evolution also independently increases the propensity for cytonuclear incompatibility. However, taxa with biparental inheritance do not always exhibit accelerated plastid evolution, indicating that the evolution of biparental inheritance is not dependent upon a general destabilization of the plastid genome. Finally, accelerated plastid evolution could also increase the potential for conflict between the nuclear and organelle genomes to lead to biparental inheritance (Reboud & Zeyl, 1994), independent of cytonuclear incompatibility, as it might enable the organelle genome to more rapidly evolve biparental inheritance and escape nuclear restorers.

The ability of biparental inheritance to rescue cytonuclear incompatibility has the potential to impact the evolutionary dynamics of cytonuclear incompatibility and its role in the speciation process, particularly as these traits often co-occur (Jansen & Ruhlman, 2012). Previous studies in *C. americanum* demonstrated the presence of a strong cytonuclear incompatibility leading to reduced survival in intraspecific hybrids (Barnard-Kubow, 2015), supporting the idea that cytonuclear incompatibilities may be among the first genetic incompatibilities to evolve (Levin, 2003; Greiner *et al.*, 2011; Burton & Barreto, 2012). However, results from the current study demonstrate that biparental inheritance enables rescue from this cytonuclear incompatibility, reducing its contribution to reproductive isolation and potentially slowing the speciation process.

The exact outcome of this interplay, though, depends on the both the asymmetry and strength of the cytonuclear incompatibility (i.e. strength of selection), as well as the pattern of biparental inheritance. The ability of biparental inheritance to enable rescue from cytonuclear incompatibility will be most effective when one chloroplast is fully compatible, and the other is highly incompatible. Rescue will also depend on the extent to which biparental inheritance is paternally biased (cf. Hagemann, 2004) and whether or not an asymmetry in biparental inheritance between crossing directions consistently favors the compatible haplotype. Finally, rescue will be more effective when the incompatibility is strong, as selection then leads to a loss of the incompatible haplotype and recovery in the F_2. When the incompatibility is weaker, selection is also weaker, and the incompatible chloroplast continues to be maintained in the F_2, negatively impacting fitness. Almost paradoxically then, weaker cytonuclear incompatibility is maintained to a greater extent in future hybrid generations, resulting in an overall greater contribution to reproductive isolation. Along the same lines, if divergent lineages were to experience secondary contact, populations with greater cytonuclear incompatibility would be more likely than those with weaker incompatibility to experience a collapse of reproductive isolation and subsequent introgression.

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Author contributions

K.B.B-K. designed the study, collected and analyzed the data, and drafted the manuscript. M.A.M. assisted with data collection. L.F.G. assisted with study design, data analysis, and drafting the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Graphs showing standard curves used for calculating chloroplast ratios in *Campanulastrum americanum* hybrids according to CT values from RT-PCR Taqman SNP Assays.

Fig. S2 Distribution of chloroplast inheritance in F_1 hybrids from within-clade crosses of *Campanulastrum americanum.*

Fig. S3 Distribution of chloroplast inheritance in F_1 hybrids from between-clade crosses of *Campanulastrum americanum.*

Fig. S4 Genotyping results of white and green leaf tissue punches taken from 36 variegated F_1 hybrids from between-clade crosses of *Campanulastrum americanum.*

Fig. S5 Comparing chloroplast haplotypes of basal and apical leaf tissue samples from F_1 hybrids from between-clade crosses of *Campanulastrum americanum.*

Fig. S6 Relationship between proportion germination and proportion maternal inheritance for between-clade crosses of *Campanulastrum americanum.*

Table S1 Geographic location and chloroplast haplotype for the *Campanulastrum americanum* populations used in within-clade (W1 and W2 haplotypes) and between-clade (W and A haplotypes) crosses to examine patterns and consequences of nonmaternal chloroplast inheritance