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ARTICLE

Microsatellite survey reveals possible link between triploidy and mortality of quaking aspen in Kaibab National Forest, Arizona

Groves B. Dixon and Laura E. DeWald

Abstract: In the southwestern United States, populations of quaking aspen (*Populus tremuloides* Michx.) are experiencing widespread mortality. Although environmental factors contributing to mortality have been well characterized, less is known about how genotype and particularly ploidy level affect susceptibility. We used five microsatellite markers to infer the ploidy level of 212 aspen stems in Kaibab National Forest, Arizona. Many multilocus genotypes showed three alleles at one or more loci, suggestive of frequent triploidy among our samples. Sites populated with putative triploids had higher mortality. In addition, heterozygosity was positively associated with mortality and crown dieback. Our results suggest that triploidy is a predisposing factor for aspen mortality in Kaibab National Forest.

Key words: Populus tremuloides, quaking aspen, clonal, mortality, decline, triploid.

Résumé : La mortalité est très répandue dans les populations de peuplier faux-tremble (*Populus tremuloides* Michx.) du sud-ouest des États-Unis. Alors que les facteurs environnementaux qui contribuent à la mortalité ont été bien caractérisés, la façon dont le génotype et plus particulièrement le degré de ploïdie influencent la susceptibilité est moins connue. Nous avons employé cinq marqueurs de type microsatellite afin de déterminer le degré de ploïdie de 212 tiges de peuplier faux-tremble à la Forêt nationale de Kaibab en Arizona. Plusieurs génotypes multilocus avaient trois allèles à un ou plusieurs loci, indiquant que la triploïdie est fréquente parmi les échantillons. La mortalité était plus élevée dans les sites occupés par des triploïdes présumés. De plus, l'hétérozygotie était associée positivement à la mortalité et au dépérissement de la cime. Nos résultats indiquent que la triploïdie est un facteur prédisposant qui contribue à la mortalité chez le peuplier faux-tremble de la Forêt nationale de Kaibab. [Traduit par la Rédaction]

Mots-clés : Populus tremuloides, peuplier faux-tremble, clonal, mortalité, déclin, triploïde.

Introduction

Quaking aspen (*Populus tremuloides* Michx.) is the most widespread tree species in North America (Little 1971). Root suckering results in extensive genets (clones) through expansive interconnected root systems with numerous stems (Barnes 1966). Some credit aspen with the largest single genetic individual on earth: the "Pando" clone in central Utah, USA (DeWoody et al. 2008). As an early successional species, quaking aspen (hereafter "aspen") demonstrates rapid growth and widespread dispersal via windborne pollen and seeds (Mitton and Grant 1996). Seedlings are shade-intolerant and moisture-sensitive, which restricts conditions required for successful seedling recruitment (Jelinski and Cheliak 1992). It has been proposed that among partially clonal plant species, the relative importance of sexual, rather than clonal, reproduction will vary with habitat suitability for sexual reproduction (Eckert 2002). For example, aspen in the relatively xeric southwestern regions of its range relies more on vegetative reproduction, resulting in relatively small numbers of old, multistemmed clones (Kemperman and Barnes 1976). More recent evidence has demonstrated that although clonal growth is indeed extensive, sexual reproduction in aspen likely plays a greater role than previously believed (Long and Mock 2012).

Particularly in western North America, but also in the Great Lakes region (Shields and Bockheim 1981), aspen has been in a state of decline (Rehfeldt et al. 2009). Decline is attributed largely to disruption of disturbance regimes, particularly fire suppression, resulting in slow shifts toward more shade-tolerant communities (Bartos and Campbell 1998). Browsing by livestock (Kay and Bartos 2000) and elk (*Cervus elaphus* L.) can exacerbate decline by suppressing sucker regeneration and seedling survival (Halofsky et al. 2008). Populations in southwestern North America are considered to be especially vulnerable from increasing drought risk associated with climate change (Rehfeldt et al. 2009).

A major contributor to decline of aspen populations is a process called dieback in which mature aspen stands undergo extensive aboveground mortality, often followed by breakup of the stand (Frey et al. 2004). Particularly in western North America, an accelerated form of dieback has been recognized and termed "sudden aspen decline" (SAD) (Worrall et al. 2008). Both dieback and SAD are described in terms of a decline disease (Manion and Lachance 1992) brought about by the serial combination of predisposing, inciting, and contributing factors. Predisposing factors are relatively static, exist over long time scales, and cause general stress among aspen populations (Worrall et al. 2008). Examples include climate change, long-term drought, elevation, conifer encroachment, and stand demography (Worrall et al. 2010). Inciting factors are short-term changes such as insect defoliation (Zegler 2011), frost, and drought (Hogg et al. 2008) that cause acute stress in aspen populations. Contributing factors are usually biological

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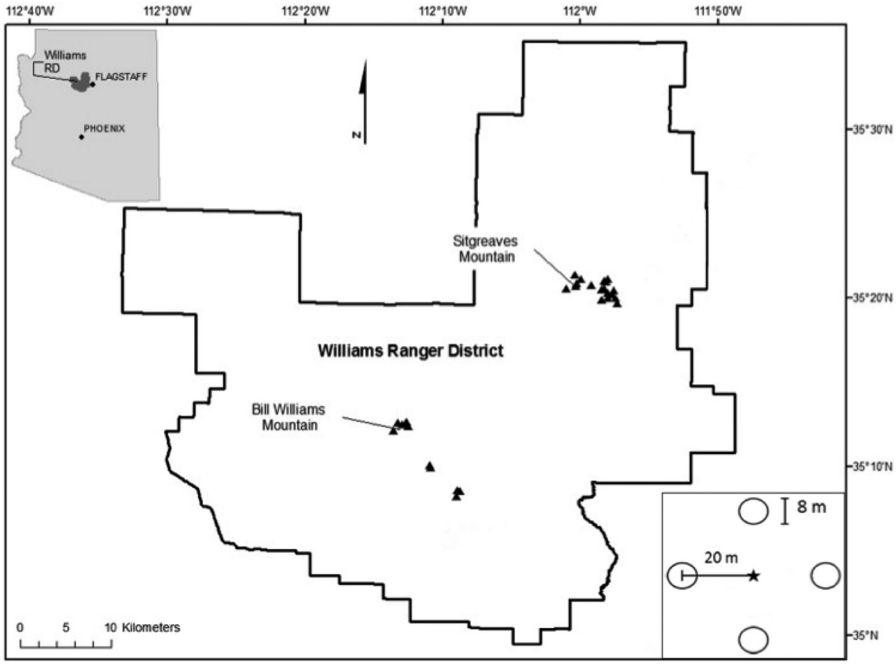
Fig. 1. Overview of the study area with inset showing schematic of a sample site. Sample sites are marked on map with triangles. Each site comprised a permanently marked center (indicated by star) surrounded by four subplots, each 8 m in diameter and centered 20 m from the site center in each of the four cardinal directions. Up to five trees were sampled from each subplot, one from the furthest point from the subplot center in each of the four cardinal directions and one from the subplot center. A maximum of 20 samples were taken per site. Figure reproduced with permission from Zegler et al. (2012).



agents that would not normally cause extensive mortality but take exaggerated tolls on aspen populations weakened by predisposing and inciting factors (Worrall et al. 2008). Examples of contributing factors include canker fungi, wood-boring insects, and bark beetles (Zegler et al. 2012).

While environmental conditions associated with aspen dieback are fairly well established (Frey et al. 2004), less is known about the role of genetics. Studies have indicated that aspen clone identity (genotype) influences susceptibility to various stressors such as canker fungi (French and Hart 1978), defoliation (Hwang and Lindroth 1997), and shoot blight (Holeski et al. 2009). Among aspen grown in a common garden experiment, St. Clair et al. (2010) showed that different genotypes varied significantly in physiology, growth, and survival. In another common garden experiment, vegetative propagules from wet and dry sites differed in total height, leaf number, water use, and stem structure, indicating heritable variation between wet and dry habitats (Kanaga et al. 2008). Hence, variation in aspen mortality occurring in western North America (Zegler et al. 2012) is likely to have a strong genetic component. Aspen are known to exhibit natural variation in ploidy level, with concomitant differences in growth and physiology (Jones and Debyle 1985). In the western United States, triploidy is particularly common (Mock et al. 2012). It has been proposed that ploidy level, along with clone size and age, could be major components of variation in mortality among western aspen populations (Mock et al. 2012). In this study, we hypothesized that triploid clones are at higher risk of mortality than diploid clones. To test this, we used microsatellite genotyping to infer the ploidy level of 212 aspen stems from Kaibab National Forest. We drew on previous reports of stem mortality and crown dieback to test for associations between inferred ploidy level and measures of stand health, as well as several factors associated with aspen diebck, including incidence of canker fungi, incidence of wood-boring insects, conifer encroachment, and site slope. Specifically, we tested the null hypothesis that rates of mortality and crown dieback were independent of inferred ploidy levels.

Materials and methods

Field methods

Our study area was the Williams Ranger District of Kaibab National Forest, located in northern Arizona west of Flagstaff (Fig. 1). The sampling strategy was developed by Zegler et al. (2012) to test for environmental conditions and damaging agents associated with aspen mortality in the Kaibab National Forest. Briefly, 48 sample sites were established using stratified random sampling with proportional allocation across two elevation (≤2400 m, >2400 m), two slope (≤28%, >28%), and five aspect (flat, north, east, south, and west) classes (Zegler et al. 2012). Proportional allocation was used so that variation in environmental conditions among the sample sites would be similar to that of total aspen coverage within the study area. As moisture often covaries with these conditions (Pearson 1920), the sampling strategy likely captured proportionally similar variation in moisture as well. Sites were selected from total area of aspen coverage within the study region (<1% of the ~382 400 ha study area). For further information regarding the study area and the methods of site selection, we direct the reader to the original publication (Zegler et al. 2012). A subset of 25 sample sites was selected for our study. Previously, it was shown that elevation was a major factor for mortality. Aspen in the middle range of elevation showed variation in mortality, whereas those at high elevations were predominantly healthy and those at lowest elevations were all in severe decline (Zegler et al. 2012). To capture variation in mortality that was not due to elevation, we selected our sample sites from the middle range of elevation. The range of elevation for all sample sites included in Zegler et al. (2012) was 2094 to 2888 m, whereas the range for our subset of 25 sites was 2271 to 2652 m. Sample sites were comprised of a permanently marked center surrounded by four subplots in each of the four cardinal directions (Fig. 1, inset). Subplots were 8 m in diameter and centered 20 m from the site center. From each subplot, tissue samples were collected from up to five trees: four trees furthest from the subplot center in the four cardinal directions,

and the tree closest to the subplot's center. Only stems with a diameter greater than 10.0 cm were sampled. Because site centers were randomly selected, there were often fewer than five aspen trees in a subplot, and some subplots had no stems. The number of stems sampled per plot ranged from 1 to 18 (Supplementary Fig. S1[1]). Whenever possible, we collected leaf tissue. When leaves were too high to be obtained, 5 cm^2 pieces of cambial tissue were collected using a clean razor, cutting to a depth just beyond the green corticular layer beneath the outer bark. Of our resulting 212 genotyped samples, 113 were leaf samples and 99 were cambial samples. Following collection, all tissue samples were stored in paper envelopes surrounded by desiccant (mixture of silica gel and silica cat litter) for two weeks. Following desiccation, samples were frozen at –20 °C until DNA extraction.

DNA extraction, PCR, and genotyping

Leaf and cambial tissue samples were mechanically disrupted using a Biospec Mini Bead Beater and 2.3 mm diameter steel beads. For disruption, tissue (100 mg) was loaded into a 2.0 mL disruption tube filled with 0.66 mL steel beads and 1.0 mL molecular biology grade water. Leaf samples were disrupted using three 40 s intervals at maximum speed. Cambial tissue was disrupted with six 40 s intervals. The result was a homogenate of tissue in water. For each sample, we added 100 μL of homogenate to a single well of a DNEasy 96 well plate (QIAGEN). From this point, extraction was continued according to the DNEasy 96 protocol.

Primer sets described in Mock et al. (2008) were used to amplify five microsatellite markers: PMGC576, PMGC2571, WPMS14, WPMS15, and GCPM970-1. Wyman et al. (2003) reported that as few as four microsatellite loci could provide confident clonal assignment in aspen. PCR components were mixed to final volume of 10.0 μL with 1.80 mmol·L^{-1} $MgCl_2$, 0.20 mmol·L^{-1} of each dNTP, 0.25 mmol·L^{-1} of each primer, 0.30 U Taq polymerase, and 1× PCR buffer. Thermocyling programs with primer-specific adjustments of annealing temperature and cycle number (Supplementary Table S1[1]) were as follows: 5 min at 95 °C, 32 or 36 cycles of 30 s at 94 °C, 40 s at primer set specific annealing temperature, and 50 s at 72 °C, followed by a 7 min final elongation step at 72 °C. For each primer set, the reverse primer included a fluorescent tag at its 5′ end for automated detection of fragments during capillary electrophoresis. PCR products were sized using a 3130xl genetic analyzer (Applied Biosystems). Genotyping reactions were comprised of 9.5 μL of Hi-Di Formamide (Applied Biosystems), 2.0 μL of PCR product, and 0.5 μL of ROX 500 size standard (Applied Biosystems). Electropherograms were genotyped and manually examined for peak morphology and artifacts using GeneMapper (Applied Biosystems).

Clonal assignment

Stems from different sample sites were assumed to be unique clones. When stems from the same site have different multilocus genotypes (MLGs), there are two biological explanations. The first is that the stems come from different clones. Alternatively, the differences between the MLGs could result from somatic mutation within a single clone. To test between these two alternatives, we calculated P_{gen} values (Parks and Werth 1993; eq. 1) based on the shared alleles between the two MLGs:

$$(1) \qquad P_{gen} = \left(\prod_{i=1}^{N} p_i q_i\right) 2^h$$

Here, N is the number of loci, and p_i and q_i are the estimated frequencies for the shared alleles between the pair of MLGs. When only one allele was shared, q_i was assigned a value of 1. The value for h is the number of heterozygous loci. The P_{gen} values provided an estimate of the probability that the similarity between the two MLGs was due to chance. If P_{gen} was less than 0.01, the null hypothesis that the two MLGs represented separate clones was rejected and the two MLGs were scored as a single clone.

Eleven of the 39 MLGs detected in this study had three allele peaks for one or more loci. Three different allele peaks at a locus can be an indication of triploidy (Mock and Long 2012). In two cases in which P_{gen} was calculated for triallelic MLGs, any of the three alleles could count as "shared" with the other MLG. For instance, a locus with triallelic genotype *a*/*b*/*c* would share two alleles with *a*/*b*, *a*/*c*, or *b*/*c*. This measure was intended to prevent oversplitting of MLGs with three alleles. However, both MLGs in question were still sufficiently distinct to be scored as separate clones. As allele frequencies could not be confidently resolved from putative triploids, these clones were not included when estimating allele frequencies. When two MLGs were collapsed into a single clone, a consensus MLG was generated by replacing mismatched alleles with the one with higher frequency. Allele frequencies, observed and expected heterozygosity, and fixation indices were calculated using GenAlEx 6.4 (Peakall and Smouse 2006). The frequency of null alleles for each locus was estimated using Brookfield's (1996) estimate. Mean site heterozygosity was measured as the observed heterozygosity averaged across all stems. When estimating heterozygosity, loci with three alleles were scored as heterozygous. Final values for allelic richness, observed heterozygosity, expected heterozygosity, and fixation index were calculated using only putative diploids after collapsing of similar genotypes.

Inference of triploidy

Putative triploids were inferred based on the presence of three allele peaks in the clonal genotype. Third peaks were called if they had comparable height to the two other allele peaks and were within the range of repeat units described for the marker in Mock et al. (2008). When the number of markers is small and especially when allelic richness is low, this method can fail to identify triploids because they do not have three different alleles at any of the genotyped loci. We estimated the probability of this occurring with our dataset using eq. 2:

$$(2) \qquad \text{Probability of a false-negative triploid} = \prod_{i=1}^{N}(1 - P_i)$$

where N is the total number of microsatellite markers and P_i is the probability that a triploid individual would have three unique alleles for marker i. To calculate P_i, we used the R package gtools (R Core Team 2013; Warnes et al. 2014) to take permutations of length = 3 (representing three chromosomes in a triploid individual) without replacement from the set of alleles for each marker. The set of permutations represented all of the possible ways that an individual could inherit three different alleles for that marker. The alleles for each permutation were then replaced with their respective frequency estimates, so that the product was equal to the probability of a triploid inheriting that permutation of alleles. The products were summed across all of the permutations to give P_i, the total expected frequency of genotypes with three different alleles for the marker. By multiplying $(1 - P_i)$ across all five markers, we could estimate the probability that a true triploid would not have three different alleles for any of the five markers. We used this as an estimate for the frequency of false-negative triploids in our dataset. The estimate assumes random mating, independent assortment, and that the allele frequency estimates are close to actual values. Allele frequency estimates used for this analysis were based on clones without triple allele peaks. This was

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjfr-2014-0566.

done because P_{gen} assumes a diploid population and because of the uncertainty in deriving allele counts from microsatellite traces of putative triploids.

Data analysis

We used linear regressions to test for associations between putative triploids and site health. The proportion of putatively triploid stems at each site was correlated with mortality and crown dieback. Mortality was measured as the ratio of standing dead stems to total aspen stems found within the subplots of each site. Crown dieback was measured as the proportion of aspen stems that had >33% crown dieback. Measurements of mortality and crown dieback were collected by Zegler (2011). As these data were collected before any genotyping was performed, they describe the sites themselves with no regard to clonal identity. We also tested for correlations between site health and mean percent heterozygosity. Mean percent heterozygosity was calculated for each site as the observed heterozygosity averaged across all genotyped stems.

We also analyzed mortality using multiple regression. To select an optimal set of variables for this analysis, we used stepwise selection based on the Akaike information criterion (AIC), implemented in the R package MASS (Venables and Ripley 2002). The initial set of variables included environmental factors measured by Zegler et al. (2012): canker disease, wood-boring insects, relative conifer density, elevation and slope, as well as variables based on microsatellite data: mean percent heterozygosity, clonal mixture (either monoclonal or polyclonal), presence or absence of putative triploids, and the proportion of putatively triploid stems. Factors that did not decrease second-order information criterion in both directions were excluded from the final model. Descriptions of the variables included in the final model are shown in Table 1. To further test for associations between site health and putative triploids, monoclonal sample sites (those populated by single clones; n = 19) were divided into two groups, those with putative triploids and those without, and mean mortality and crown dieback were compared between them. The comparison was also made using all sites with any putative triploids compared with all other sites.

Results

Genotyping

Multilocus genotypes (MLGs) were generated for 212 samples from 25 sample sites (Supplementary Table S2[1]). The number of alleles per locus ranged from 3 to 10, with an average of 6.2 ± 1.5. The allelic richness, observed heterozygosity, expected heterozygosity, fixation indices, and estimates of frequency of null alleles are shown in the supplemental materials (Supplementary Table S3[1]). Estimates of null allele frequency ranged from 0 to 0.07. Hence heterozygosity for one or more loci may be underestimated due to null alleles.

Clonal assignment

No samples from different sites had the same MLG. In five cases, two MLGs from the same site were found to represent a single clone (P_{gen} < 0.01), thus 39 unique MLGs were considered to represent 34 separate clones. The number of mismatches between collapsed MLGs ranged from 2 to 5. The distributions of mismatches between pairs of MLGs within sites and across all sites were normally distributed and had similar means (Supplementary Fig. S2[1]). Of 25 sample sites, 19 were monoclonal (every sample from the site was from the same clone), and six were multiclonal (at least two clones were found at the site) (Supplementary Fig. S3[1]).

Identification of putative triploids

Of 34 clones, 11 had three allele peaks for one or more loci. We refer to these clones these as putative triploids. Of 11 putative triploids, eight had three alleles at one locus and three had three alleles at two loci. It should be noted that a triploid individual may not display three alleles at any marker. This would occur if the triploid had two or more copies of the same allele for all of the five genotyped loci. We estimated the frequency of such false negatives (see eq. 2) to be 17.6%, or between two and three falsely identified diploids. As would be expected, we found that heterozygosity (having at least two unique alleles at a locus) was significantly higher for putative triploids than diploids (mean = 0.82 compared with 0.59; p < 0.001) (Supplementary Fig. S4[1]).

Putative triploids appeared more frequently among the monoclonal sites than the multiclonal sites (Fisher's exact test: p < 0.05). Indeed, roughly half of the clones from monoclonal sites were putative triploids (nine of 19) compared with only two of the 15 clones from the multiclonal sites. Putative triploids also showed higher stem counts (mean = 7.73 ± 1.48 compared with 5.52 ± 0.87), although the difference was not significant (p = 0.11).

Table 1. Factors included in the final multiple regression model for percent mortality.

Factor	Estimate	P
Canker disease (%)[a]	0.48	<0.001
Wood-boring insects (%)[b]	0.33	<0.001
Relative conifer density (%)[c]	0.16	0.031
Slope (%)[d]	0.26	0.016
Mean heterozygosity (%)[e]	0.29	0.001

Note: Data for all variables except mean heterozygosity were collected by Zegler (2011) and Zegler et al. (2012).

[a]Proportion of stems showing evidence of canker disease.
[b]Percentage of stems showing evidence of wood-boring insects.
[c]Relative density reported in basal area ($m^2 \cdot ha^{-1}$).
[d]Percentage of 45°.
[e]The mean observed heterozygosity across all samples from each sight.

Association between putative triploids and mortality

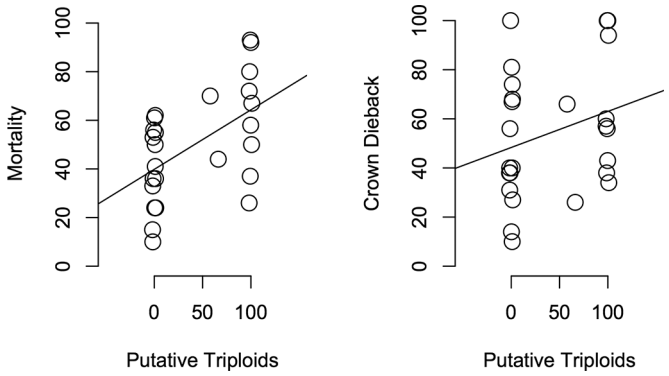
The proportion of putatively triploid stems at a sample site was positively correlated with mortality (R^2 = 0.271; p < 0.008; Fig. 2) but not with crown dieback (R^2 = 0.067; p = 0.21). Compared with all other sites (n = 14), sites with putative triploids (n = 11) had higher average mortality (62.64 ± 6.59 compared with 39.71 ± 4.57; p < 0.01) and showed a tendency toward elevated crown dieback (61.27 ± 7.98 compared with 48.86 ± 7.01; p = 0.13). Similar results were found comparing monoclonal sites with and without putative triploids (mortality: p < 0.05; crown dieback: p = 0.12). Because some of our sites were represented by very few samples, we repeated the regression analysis for mortality using only sites with more than four genotyped ramets. The trend was similar but not significant (R^2 = 0.127; p = 0.063). For some sites, low sample counts were due to extensive mortality, so it is not surprising that removal of poorly represented sites weakened the trend. Based on AIC, the proportion of triploid stems did not improve the multiple regression model of mortality. Surprisingly, another genetic factor, mean site heterozygosity, did improve the model (AIC = 102.43 compared with 114.85). Mean site heterozygosity was also significantly associated with crown dieback (R^2 = 0.266; p < 0.005). The best model for mortality included five variables (incidence of canker fungi, incidence of wood-boring insects, conifer encroachment, slope, and mean site heterozygosity) and accounted for 90% (adjusted R^2) of the variation in mortality between sites (p < 0.0001; Table 1).

Discussion

Link between putative triploids and mortality

We found that putative triploids were positively associated with mortality. When comparing monoclonal sites, mortality was on average 20% higher for sites with putative triploids. These results suggest that triploid aspen are at greater risk of mortality than diploids in Kaibab National Forest. The proportion of triploid stems shown in Fig. 2 is predominantly 0% or 100%. This reflects

Fig. 2. Linear regressions showing relationships between the percentage of putatively triploid stems and measures of aspen decline from each sample site. Relationship with mortality was significant ($R^2 = 0.271$; $p < 0.008$), but relationship with crown dieback was not ($R^2 = 0.067$; $p = 0.21$). Points were slightly offset to prevent overlap.



that the putative triploids generally occupied the entirely of the site that they came from. In only two instances were putative triploids found intermingled with other clones. This indicates that triploids in our study area are not only more prone to mortality, but also may tend toward larger clone size. Our interpretation, however, is subject to the uncertainty of using microsatellites to infer cytotype. Assuming that the presence of three alleles for a single locus indicates triploidy, there is a still chance for false-negative triploids. This would occur if a triploid clone did not have three unique alleles for any of our five markers. We estimated the probability of this to be 17.6%. As we detected 11 putative triploids, we expect to have missed between two and three true triploids. A likely candidate is the clone from site SGM196, which had an observed heterozygosity of 100% but did not show three alleles at any locus. In general, triploids are less likely to be homozygous (having three identical alleles) than diploids (having two identical alleles). Consistent with this expectation, observed heterozygosity was higher among putative triploid clones (Supplementary Fig. S4[1]). Hence, false-negative triploids are likely to display higher heterozygosity levels. To examine how the presence of false-negative triploids could alter our results, we recalculated the proportion of putatively triploid stems after removing diploid clones that had relatively high heterozygosity. Removing putative diploids with heterozygosity greater than 0.9 ($n = 1$) strengthened the linear relationship with mortality, shown in Fig. 2 ($R^2 = 0.31$; $p < 0.005$). A cutoff of 0.7 ($n = 8$ clones removed) gave similar results ($R^2 = 0.32$; $p < 0.009$). Therefore, the trend was robust to removal of uncertain cytotype calls.

It should be emphasized that identifying triploids based on three allele peaks can also lead to false positives, especially when only a single locus is required. Three allele peaks can occur for reasons other than triploidy such as aneuploidy (abnormal copy number for a single chromosome, rather than all chromosomes) or PCR artifacts such as contamination or nonspecific binding. Hence our results do not conclusively identify a link between triploidy and mortality but do provide evidence that such a link may exist. Further studies using more direct measures of cytotype such as flow cytometry or karyotyping are needed to confirm this trend and to further elucidate the role of cytotype in aspen mortality in western North America.

Drought sensitivity

In western regions of Canada (Hogg et al. 2008) and the United States (Hanna and Kulakowski 2012), drought is considered a primary inciting factor for aspen dieback. This appears to be the case in our study area. Zegler et al. (2012) found strong covariation between mortality and elevation. Aspen at the highest areas were predominantly healthy, whereas those in the lowest areas were in extensive decline. As drought is predicted to be less severe at higher elevations (Allen and Breshears 1998), it is plausible that moisture availability is a critical factor for aspen dieback in our study area. It is possible that sites in the middle range of elevation chosen for this study are near a threshold moisture level at which some genotypes are resilient but others are not. Known physiological differences between cytotypes could potentially cause such variation in drought sensitivity. A long-recognized difference between diploid and triploid aspen is growth rate (Benson and Einspahr 1967). In Utah, tree-ring comparisons between diploid and triploid clones revealed that the triploids tended to grow faster (DeRose et al. 2015). In a study of the closely related European aspen (*Populus tremula* L.), micropropagated triploids had higher growth rates, higher chlorophyll content, and higher net assimilation rates (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$) compared with diploid counterparts (Pärnik et al. 2014). We suggest that triploids in our study area suffer greater drought sensitivity as a trade-off for elevated photosynthesis. Under moisture stress, aspen reduce stomatal conductance, likely as an adaptive mechanism to prevent cavitation (Hogg et al. 2000). It is unclear whether diploids and triploids would vary in their capacity to regulate stomatal conductance, but if triploids always maintain higher photosynthetic rates, they would experience greater xylem tension under moisture stress, potentially leading to greater drought sensitivity and mortality risk. Such trade-offs between growth rate and tolerance have long been theorized (Orians and Solbrig 1977) and have been described in woody legumes (Fabaceae) (Polley et al. 2002). To use the concepts of predisposing, inciting, and contributing factors (Frey et al. 2004), triploids may be predisposed to the inciting factor of drought, which in turn opens them to contributing factors such as wood-boring insects and canker fungi. Testable predictions for this hypothesis are (1) stomatal conductance and net assimilation rate will be higher in triploids than in diploids under both normal conditions and moisture deficits and (2) cavitation will be more severe among triploids than diploids under moisture-deficient conditions.

Relationships between heterozygosity and decline

In this study, we have focused on the correlation between putative triploids and mortality. However, we also detected a trend linking mean site heterozygosity with both mortality and crown dieback. This was a surprising result because heterozygosity is often associated with robust populations. As triploid clones should have higher heterozygosity levels, this result is consistent with our main result that putative triploids were associated with mortality. However, heterozygosity was a significant factor in the multiple regression analysis, whereas the proportion of triploid stem was not. Moreover, mean site heterozygosity was the only factor significantly associated with crown dieback. So, heterozygosity showed associations with decline beyond that expected from its covariation with triploidy. The trend could be due to underdominance (reduced fitness compared with homozygotes). Evidence of underdominance has been reported in natural hybrids between *Populus* species in Colorado (Hersch-Green et al. 2014) and across Europe (Caseys et al. 2015). However, both of these studies found evidence of both underdominance and overdominance at different markers, indicating that the effects were locus-specific and not general across the genome. To test if the correlation between heterozygosity and mortality was dependent on any particular marker in our study, we removed each marker in series and recalculated the regression, finding that the correlation always remained significant ($p < 0.012$). Moreover, the relationship between mortality and heterozygosity for each individual locus was always positive (Supplementary Fig. S5[1]). From this, we conclude that the trend linking heterozygosity and mortality was not driven by a single causal underdominant locus. Similarly, al-

though null alleles could bias heterozygosity estimates for particular loci, they seem unlikely to have produced the consistent relationship observed here. It is interesting that, like triploidy, heterozygosity in aspen has been associated with higher growth rates. This has been demonstrated in wild aspen using both allozymes (Mitton and Grant 1996 and citations therein) and microsatellites (Cole et al. 2010). It is possible that more genetically diverse individuals tend toward higher growth rates, at a cost of greater susceptibility to drought. This could explain the relationship between heterozygosity and crown dieback, which is often a first sign of dieback incited by drought (Frey et al. 2004). It will be interesting to see if future studies of genetic diversity and aspen mortality reveal this as a general trend or peculiar to our study region.

Conclusion

Our results suggest that genetic factors such as heterozygosity and cytotype influence severity of aspen dieback. This is potentially due to variation in susceptibility to inciting factors such as drought. As drought becomes more prevalent in the coming years, cytotype may prove a critical factor for aspen health in western North American forests.

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References

Allen, C.D., and Breshears, D.D. 1998. Drought-induced shift of a forest–woodland ecotone: rapid landscape response to climate variation. Proc. Natl. Acad. Sci. U.S.A. **95**: 14839–14842. doi:10.1073/pnas.95.25.14839.

Barnes, B.V. 1966. The clonal growth habit of American aspens. Ecology, **47**: 439–447. doi:10.2307/1932983.

Bartos, D.L., and Campbell, R.B. 1998. Decline of quaking aspen in the Interior West — examples from Utah. Rangelands, **20**: 17–24.

Benson, M.K., and Einspahr, D.W. 1967. Early growth of diploid, triploid and triploid hybrid aspen. For. Sci. **13**: 150–156.

Brookfield, J.F.Y. 1996. A simple new method for estimating null allele frequency from heterozygote deficiency. Mol. Ecol. **5**(3): 453–455. doi:10.1046/j.1365-294X.1996.00098.

Caseys, C., Stritt, C., Glauser, G., Blanchard, T., and Lexer, C. 2015. Effects of hybridization and evolutionary constrains on secondary metabolites: the genetic architecture of phenylpropanoids in Europoean *Populus* species. PLoS One, **10**: e0128200. doi:10.1371/journal.pone.0128200.

Cole, C.T., Anderson, J.E., Lindroth, R.L., and Waller, D.M. 2010. Rising concentrations of atmospheric CO_2 have increased growth in natural stands of quaking aspen (*Populus tremuloides*). Glob. Chang. Biol. **16**: 2186–2197. doi:10.1111/j.1365-2486.2009.02103.x.

DeRose, R.J., Mock, K.E., and Long, J.N. 2015. Cytotype differences in radial increment provide novel insight into aspen reproductive ecology and stand dynamics. Can. J. For. Res. **45**: 1–8. doi:10.1139/cjfr-2014-0382.

DeWoody, J., Rowe, C., Hipkins, V., and Mock, K. 2008. "Pando" lives: molecular genetic evidence of a giant aspen clone in central Utah. West. North Am. Nat. **68**: 493–497. doi:10.3398/1527-0904-68.4.493.

Eckert, C.G. 2002. The loss of sex in clonal plants. Evol. Ecol. **15**: 501–520. doi:10.1007/978-94-017-1345-0_15.

French, J., and Hart, J. 1978. Variation in resistance of trembling aspen to *Hypoxylon mammatum* identified by inoculating naturally occurring clones. Phytopathology, **68**: 485–489. doi:10.1094/Phyto-68-485.

Frey, B.R., Lieffers, V.J, Hogg, E.H., and Landhäusser, S.M. 2004. Predicting landscape patterns of aspen dieback: mechanisms and knowledge gaps. Can. J. For. Res. **34**: 1379–1390. doi:10.1139/x04-062.

Halofsky, J.S., Ripple, W.J., and Beschta, R.L. 2008. Recoupling fire and aspen recruitment after wolf reintroduction in Yellowstone National Park, U.S.A. For. Ecol. Manage. **256**: 1004–1008. doi:10.1016/j.foreco.2008.06.002.

Hanna, P., and Kulakowski, D. 2012. The influence of climate on apsen dieback. For. Ecol. Manage. **274**: 91–98. doi:10.1016/j.foreco.2012.02.009.

Hersch-Green, E.I., Allan, G.J., and Whitham, T.G. 2014. Genetic analysis of admixture and patterns of introgression in foundation cottonwood trees (Salicaceae) in southwestern Colorado, U.S.A. Tree Genet. Genomes, **10**: 527–539. doi:10.1007/s11295-014-0701-9.

Hogg, E.H., Saugier, B., Pontailler, J.Y., Black, T.A., Chen, W., Hurdle, P.A., and Wu, A. 2000. Responses of trembling aspen and hazelnut to vapor pressure deficit in a boreal deciduous forest. Tree Physiol. **20**: 725–734. doi:10.1093/treephys/20.11.725.

Hogg, E.H., Brandt, J.P., and Michaelian, M. 2008. Impacts of a regional drought on the productivity, dieback, and biomass of western Canadian aspen forests. Can. J. For. Res. **38**: 1373–1384. doi:10.1139/X08-001.

Holeski, L.M., Vogelzang, A., Stanosz, G., and Lindroth, R.L. 2009. Incidence of Venturia shoot blight in aspen (*Populus tremuloides* Michx.) varies with tree chemistry and genotype. Biochem. Syst. Ecol. **37**: 139–145. doi:10.1016/j.bse.2009.02.003.

Hwang, S.Y., and Lindroth, R.L. 1997. Clonal variation in foliar chemistry of aspen: effects on gypsy moths and forest tent caterpillars. Oecologia, **111**: 99–108. doi:10.1007/s004420050213.

Jelinski, D.E., and Cheliak, W.M. 1992. Genetic diversity and spatial subdivision of *Populus tremuloides* (Salicaceae) in a heterogeneous landscape. Am. J. Bot. **79**: 728–736. doi:10.2307/2444937.

Jones, J.R., and Debyle, N.V. 1985. Genetics and variation *In* Aspen: ecology and management in the western United States. *Edited by* N.V. Debyle and R.P. Winokur. USDA Forest Service, Gen. Tech. Rep. RM-119. pp. 35–39.

Kanaga, M.K., Ryel, R.J., Mock, K.E., and Pfrender, M.E. 2008. Quantitative-genetic variation in morphological and physiological traits within a quaking aspen (*Populus tremuloides*) population. Can. J. For. Res. **38**: 1690–1694. doi:10.1139/X08-012.

Kay, C.E., and Bartos, D.L. 2000. Ungulate herbivory on Utah aspen: assessment of long-term exclosures. J. Range Manage. **53**: 145–153. doi:10.2307/4003274.

Kemperman, J.A., and Barnes, B.V. 1976. Clone size in American aspens. Can. J. Bot. **54**: 2603–2607. doi:10.1139/b76-280.

Little, E.L. 1971. Atlas of United States trees. Vol. 1. Conifers and important hardwoods. 1st ed. U.S. Department of Agriculture, Washington, D.C. pp. 202.

Long, J.N., and Mock, K. 2012. Changing perspectives on regeneration ecology and genetic diversity in western quaking aspen: implications for silviculture. Can. J. For. Res. **42**: 2011–2021. doi:10.1139/x2012-143.

Manion, P., and Lachance, D. 1992. Forest decline concepts. APS Press, St. Paul, Minnesota.

Mitton, J.B., and Grant, M.C. 1996. Genetic variation and the natural history of quaking aspen. Bioscience, **46**: 25–31. doi:10.2307/1312652.

Mock, K.E., and Long, J.N. 2012. Changing persepctives on regeneration ecology and genetic diversity in western quaking aspen: implications for silviculture. Can. J. For. Res. **42**: 2011–2021. doi:10.1139/x2012-143.

Mock, K.E., Rowe, C.A., Hooten, M.B., Dewoody, J., and Hipkins, V.D. 2008. Clonal dynamics in western North American aspen (*Populus tremuloides*). Mol. Ecol. **17**: 4827–4844. doi:10.1111/j.1365-294X.2008.03963.x.

Mock, K.E., Callahan, C.M., Islam-Faridi, M.N., Shaw, J.D., Rai, H.S., Sanderson, S.C., Rowe, C.A., Ryel, R.J., Madritch, M.D., Gardner, R.S., and Wolf, P.G. 2012. Widespread triploidy in western North American aspen (*Populus tremuloides*). PLoS One, **7**: e48406. doi:10.1371/journal.pone.0048406.

Orians, G.H., and Solbrig, O.T. 1977. A cost–income model of leaves and roots with special reference to arid and semiarid areas. Am. Nat. **111**: 677–690. doi:10.1086/283199.

Parks, J.C., and Werth, C.R. 1993. A study of spatial features of clones in a population of bracken fern, *Pteridium aquilinum* (Dennstaedtiaceae). Am. J. Bot. **80**: 537–544. doi:10.2307/2445369.

Pärnik, T., Ivanova, H., Keerberg, O., Vardja, R., and Niinemets, U. 2014. Tree age-dependent changes in photosynthetic and respiratory CO_2 exchange in leaves of micropropagated diploid, triploid, and hybrid aspen. Tree Physiol. **6**: 585–594.

Peakall, R., and Smouse, P.E. 2006. Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes, **6**: 288–295. doi:10.1111/j.1471-8286.2005.01155.x.

Pearson, G.A. 1920. Factors controlling the distribution of forest types, part II. Ecology, **1**: 289–308. doi:10.2307/1929564.

Polley, H.W., Tischler, C.R., Johnson, H.B., and Justin, D. 2002. Growth rate and survivorship of drought: CO_2 effects on the presumed tradeoff in seedlings of five woody legumes. Tree Physiol. **22**: 383–391. doi:10.1093/treephys/22.6.383.

R Core Team. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from http://www.R-project.org/.

Rehfeldt, G.E., Ferguson, D.E., and Crookston, N.L. 2009. Aspen, climate, and sudden decline in western U.S.A. For. Ecol. Manage. **258**: 2353–2364. doi:10.1016/j.foreco.2009.06.005.

Shields, W.J., and Bockheim, J. 1981. Deterioration of trembling aspen clones in the Great Lakes region. Can. J. For. Res. **11**: 530–537. doi:10.1139/x81-073.

St. Clair, S.B., Mock, K.E., Lamalfa, E.M., Campbell, R.B., and Ryel, R.J. 2010. Genetic contributions to phenotypic variation in physiology, growth, and vigor of western aspen (*Populus tremuloides*) clones. For. Sci. **56**: 222–230.

Venables, W.N., and Ripley, B.D. 2002. Modern applied statistics with S. 4th ed. Springer, New York.

Warnes, G.R., Bolker, B., and Lumley, T. 2014. Gtools: various R programming tools. R package version 3.4.2. Available from http://CRAN.R-project.org/package=gtools.

Worrall, J.J., Egeland, L., Eager, T., Mask, R.A., Johnson, E.W., Kemp, P.A., and Shepperd, W.D. 2008. Rapid mortality of *Populus tremuloides* in southwestern Colorado, U.S.A. For. Ecol. Manage. **255**: 686–696. doi:10.1016/j.foreco.2007.09.071.

Worrall, J.J., Marchetti, S.B., Egeland, L., Mask, R.A., Eager, T., and Howell, B. 2010. Effects and etiology of sudden aspen decline in southwestern Colorado, U.S.A. For. Ecol. Manage. **260**: 638–648. doi:10.1016/j.foreco.2010.05.020.

Wyman, J., Bruneau, A., and Tremblay, M.F. 2003. Microsatellite analysis of genetic diversity in four populations of *Populus tremuloides* in Quebec. Can. J. Bot. **81**: 360–367. doi:10.1139/b03-021.

Zegler, T.J. 2011. Site factors related to decline of aspen *P. tremuloides* Michx. in the Kaibab National Forest Arizona. M.Sc. thesis, School of Forestry, Northern Arizona University, Flagstaff, Arizona.

Zegler, T.J., Moore, M.M., Fairweather, M.L., Ireland, K.B., and Fulé, P.Z. 2012. *Populus tremuloides* mortality near the southwestern edge of its range. For. Ecol. Manage. **282**: 196–207. doi:10.1016/j.foreco.2012.07.004.