RELATIONSHIPS BETWEEN GENETIC DIVERSITY, CLONAL STRUCTURE AND SUDDEN ASPEN DECLINE IN KAIBAB NATIONAL FOREST, ARIZONA

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LIST OF ABBREVIATIONS

SAD: Sudden Aspen Decline (Worrall *et al*. 2008)

ha: hectare (10,000 m^2)

TPH: Trees per hectare (stems ha^{-1})

BA: Basal Area (m^2 ha^{-1})

UTMs: Universal Transverse Mercator

MLGs: Multilocus genotypes

PCR: Polymerase chain reaction

dNTP: Dideoxynucleotide triphosphate

$P_{(ID)}$: Probability of identity, a measurement of the probability that two individuals drawn at random from a population will have the same genotype at multiple loci.

H_{obs}: Observed Heterozygosity

H_{exp}: Expected Heterozygosity

F_{IS}: Inbreeding Coefficient (Equation 1).

P_{gen}: A metric of the probability of a multilocus genotype arising in a population from random sexual reproduction (Equation 2).

bp: Base pairs

MANOVA: Multivariate Analysis of Variance

AMOVA: Analysis of Molecular Variance

ABSTRACT

RELATIONSHIPS BETWEEN GENETIC DIVERSITY, CLONAL STRUCTURE AND SUDDEN ASPEN DECLINE IN KAIBAB NATIONAL FOREST, ARIZONA

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Rapid and extensive dieback of aspen stands in the western United States, termed 'Sudden Aspen Decline,' has been attributed to combinations of predisposing inciting and contributing factors. A recent study in the Kaibab National Forest near Flagstaff, AZ conducted by Zegler (2011) was intended in part to examine the relationships between aspen crown dieback and mortality with predisposing stand factors and contributing damaging agents. However, the genetic diversity and clonal structure of the sample sites used in this study had not been estimated. This provided a unique opportunity to combine a genetic dataset with preexisting measurements of stand degradation and environmental conditions to test for relationships between them. The objectives of this study were 1) to estimate the genetic diversity of aspen in the study area, 2) to assess clonal structure to make inferences of historical reproductive patterns, and 3) to test for relationships between genetic diversity, clonal structure, and signs of SAD. To accomplish this, microsatellite multilocus genotypes were generated from tissue samples taken from a subset of sample sites from Zegler (2011). Analysis of the genotypes from these sites revealed an association between genotypic diversity and northerly aspect, and levels of site degradation showed a positive relationship with mean heterozygosity. I speculate that the association between genotypic diversity and northerly aspect may be due to higher rates of aspen seedling recruitment among

northerly aspects, and that the relationship between heterozygosity and stand degradation results from ancient clonal lineages with both high levels of heterozygosity and poor fitness under current conditions. I conclude that conservation efforts encouraging the propagation of seedlings and younger clones would improve resistance of the greater aspen population in Kaibab National Forest to Sudden Aspen Decline.

CHAPTER 1: INTRODUCTION

In clonal plants, the spatial distribution of genetic diversity is related to growth habit (Namroud *et al*. 2005). Genetic diversity is also often connected with fitness, with greater diversity usually resulting in greater fitness (Mitton and Grant 1996; Hansson and Westerberg 2002; Reed and Frankham 2002), though unique circumstances may render the opposite relationship (Klekowski 2003; Ally *et al*. 2010). Therefore, among clonal plants, it is likely that historical growth patterns contribute to a population's fitness level under current conditions. Aspen are capable of extensive clonal growth, with offspring of a single seed capable of growing into expansive multi-stemmed individuals called genets or clones (Barnes 1966). Aspen have shade intolerant seedlings, and as a result usually grow as early successional species, dependent on canopy opening disturbances for successful seed germination and clonal expansion (Kay 1993). Disturbances that promote regeneration include fire, mudslides, windthrow, and insect outbreaks (Mitton and Grant 1980; Kay 1993; Romme *et al*. 2001; Kulakowski *et al.* 2006). Aspen seedlings are also intolerant to desiccation. Eckert (2002) proposed that the relative importance of sexual versus clonal recruitment among clonal plants will vary with habitat suitability to successful sexual reproduction. Consistent with this argument, in the semi-arid mountains of the western United States, seedling intolerance to desiccation makes successful sexual reproduction particularly rare (Kemperman and Barnes 1976; Jelinski and Cheliak 1992; Romme *et al*. 2001). Aspen in this region have been thought to reproduce almost exclusively by suckering, resulting in relatively small numbers of ancient, large, multiramet clones (Kemperman and Barnes 1976; Mock *et al*. 2008). However, more recent evidence has demonstrated that while clonal growth is indeed extensive, sexual reproduction likely plays a greater role than previously believed (Mock *et al*. 2008).

Particularly in the west, but also in the prairie provinces of Canada, eastern Canada, and the Great Lakes region, aspen has been in a state of decline (Shields and Bockheim 1981; Bartos and Campbell 1998; Rehfeldt *et al*. 2009). This decline was attributed largely to disruption of disturbance regimes (particularly fire suppression), causing succession in seral aspen communities toward more shade tolerant species and preventing establishment of new aspen communities. In the west, many believe that elk browsing exacerbates aspen decline by preventing successful sucker regeneration and seedling survival (Romme *et al*. 1995; Romme *et al*. 2001; Hessl and Graumlich 2002; Kaye *et al*. 2005; Halofsky *et al* 2008). In 2001, estimates of decline in aspen ranges were as high as 49% in Colorado and 95% in Arizona (Bartos 2001).

More recently, accelerated aspen dieback with distinct etiology has been observed (Frey *et al*. 2004; Fairweather *et al*. 2008; Hogg *et al*. 2008; Zegler 2011). Due to its rapid rate, Worrall *et al*. (2008) termed the phenomenon Sudden Aspen Decline (SAD), which they describe in terms of a decline disease (Manion 1991; Manion and LaChance 1992) brought about by the serial combination of predisposing, inciting, and contributing factors. Predisposing factors are relatively static, exist over long timescales, and cause general stress among populations. Examples include climate change, long term drought (Fairweather 1999), unfavorable site conditions related to slope, aspect and elevation, conifer encroachment, and stand demography (Worrall *et al*. 2008; 2010). Inciting factors are short term changes such as insect defoliation (Zegler 2011), frost (Fairweather 1999), or drought (Hogg *et al*. 2005; Fairweather *et al.* 2008) that cause acute stress in a population. Drought is considered the primary inciting factor (Rehfeldt *et al*. 2009). Contributing factors are often biological agents that would not normally cause extensive mortality, but take exaggerated tolls on populations weakened by predisposing and inciting factors, including canker fungi, wood-boring insects, and bark

beetles (Worrall *et al*. 2008; Worrall *et al*. 2010). Consistent with this role of contributing factors, a number of aspen's predators are known to feed particularly upon already weakened trees (Jones *et al*. 1985).

In Arizona, signs of SAD are prevalent. In 2003, aerial surveys detected ~29,500 hectares of aspen defoliation and decline throughout the state (USDA Forest Service 2004). Since then, aerial studies mapped progressively more aspen damage peaking at ~49,800 hectares in 2008, then decreasing to ~14,960 hectares in 2009, and ~8,461 hectares in 2010 (USDA Forest Service 2008 cited in Zegler 2011; USDA Forest Service 2009 and 2010). The damage is attributed primarily to drought and insect defoliation (USDA Forest Service 2009). The entire southwest region saw reductions in damage from ~71,225 hectares in 2008 to ~32,375 hectares in 2010, but reductions were greater in Arizona than New Mexico. This may have been due in part to a late season frost in Arizona that caused a crash in populations of the western tent caterpillar (*Malacosoma californicum* Packard; USDA Forest Service 2011). In a monitoring project on aspen in the Coconino National Forest in northern Arizona, Fairweather *et al*. (2008) observed increasing aspen mortality with decreasing elevation, with 16% mortality at high elevation sites (>2591 m), 61% mortality among mid-elevation sites (2286 –2591 m), and extensive mortality (95%) in low elevation sites (<2286 m). Cooler and moister conditions at higher elevations (Pearson 1920) suggests an important role of moisture levels in SAD in Arizona.

In the Kaibab National Forest in northern Arizona, Zegler (2011) surveyed 48 sample sites chosen by stratified random sampling with proportional allocation to characterize relationships between aspen and biotic and abiotic site conditions including elevation, slope, aspect, forest type, and damaging agents. His results were consistent with characteristics of SAD. Zegler (2011) found aspen health tended to increase with

elevation. He also found incidence of canker disease and conifer encroachment were associated with aspen mortality. Studies have indicated that clone identity (genotype) likely plays a role in susceptibility to factors contributing to SAD such as canker fungi (Copony and Barnes 1974; French and Hart 1978), climate (Ohms 2003), defoliation (Shields and Bockheim 1981; Hwang and Lindroth 1997; Donaldson and Lindroth 2007), and *Venturia* shoot blight (Holeski *et al.* 2009). Among aspen grown in a common garden, St. Clair *et al*. (2010) demonstrated clone based variation in physiology, growth, and survival. Given these observations, it seemed probable that there was a genetic component to susceptibility to SAD among aspen stands observed by Zegler (2011) in the Kaibab National Forest. To test this, I generated a dataset of microsatellite multilocus genotypes (MLGs) from aspen trees sampled from a subset of Zegler's (2011) 48 sample sites. My primary objectives were to 1) assess the genetic diversity of aspen in the study area, 2) assess clonal structure to make inferences of historical reproductive patterns, and 3) to test for relationships between genetic diversity, clonal structure, and signs of SAD.

CHAPTER 2: LITERATURE REVIEW

Aspen Importance and Ecology

Quaking aspen[1] is the most widely distributed tree species in North America (Holeski *et al.* 2009), and the most widespread deciduous tree species of the interior western United States (Bartos and Campbell 1998). As such, the species provides unique and disproportionally high ecological and societal benefits (Zegler 2011). *Populus* species (aspens and cottonwoods) support a wide variety of vertebrate and invertebrate herbivores, saprophytic invertebrates, fungi, lichens and coleopterans (Myking *et al*. 2011), and aspen communities have been associated with high levels of plant (Stohlgren *et al*. 1997), bird (Mills *et al*. 2000; Rumble *et al*. 2001), and butterfly biodiversity (Simonson *et al*. 2001). Many consider *P. tremuloides* and its close relative European aspen (*P. tremula* L.) to be keystone species (Jones *et al*. 2005; Myking *et al*. 2011). *Populus tremuloides'* wide distribution bears important implications for terrestrial carbon and nutrient cycles (King *et al*. 1999). It has also been shown that water yields decrease following replacement of aspen with conifers, which can negatively impact undergrowth vegetation, soil profiles, and stream flow (Bartos 2001).

Rapid growth and early reproduction make aspen a useful species for lumber and pulp harvest (Holeski *et al*. 2009). Kauter *et al*. (2003) propose *Populus* species as a potentially valuable source of solid biofuel, and in the Great Lakes region, aspen accounts for nearly half of total pulpwood production (Shields and Bockheim 1981). The species is also valuable for molecular genetic research. The *Populus* genus is a useful model tree for genetic studies because of its relatively small genomes (~520 Mbp) and

[1] 'Aspen' can refer to a collection of closely related species within the *Populus* genus, but is often used as short hand for quaking aspen (*Populus tremuloides*). In this document, I will use 'aspen' to refer to quaking aspen. When pluralized, 'aspen species' will indicate the group of similar species within the *Populus* genus. When other *Populus* are species are discussed specific names will be used.

availability of transformation systems (Tuskan *et al*. 2003), and aspen offers an ideal system for study of adaptive molecular evolution (Lexer *et al*. 2005; Mock *et al*. 2008). Aspen is also valuable for its esthetics, particularly during autumn leaf coloration (Romme *et al*. 1995).

To summarize, aspen provides forage for livestock, habitat for wildlife, watershed protection, water yield for downstream users, aesthetic value, recreational areas, wood fiber, biodiversity, and a useful study organism (Bartos and Campbell 1998; Bartos 2001). Substantial loss of aspen coverage threatens these benefits; therefore, it is important we improve our understanding of the causes of aspen decline and SAD. Better understanding will lead to better informed conservation efforts with increased probabilities of success in preserving aspen in the western United States.

In addition to the largest range of any tree species in North America, aspen is the second most widely distributed tree species on earth (Mitton and Grant 1996). This extensive natural range incorporates a wide variety of habitat types in which aspen shows great ecological flexibility (Romme *et al*. 2001). Spanning 40 degrees of latitude (Barnes 1975) and elevations from sea level to 3700 m (Mitton and Grant 1996), the greatest proportion of aspen's range is in the boreal and alpine forests of Canada and Alaska (Bartos 2001). However, the species is also found in the Rocky Mountains, throughout New England, and even at high elevations in Mexico (Little 1971; Mitton and Grant 1996). In the western United States, aspen is generally restricted to high elevation mountain sides and plateaus, and in xeric regions it is usually found only in riparian habitats (Mitton and Grant 1996; Bartos 2001). In an experiment on the viability of aspen seed sources from throughout its range, Pauley *et al.* (1963) demonstrated that seedlings from the western United States grew poorly under the environmental conditions in New England (Cited in Jones and DeByle 1985a). Root suckering

characteristics have also been shown to vary between eastern and western aspen, with western aspen generating root suckers from deeper roots than aspen from the great Lakes area (Schier *et al*. 1985). Comparing leaf and bud morphology, Barnes (1967) demonstrated phenotypic differences between eastern and western clones, with western clones showing greater pubescence on the basal scales of terminal buds and some western clones showing markedly decreased leaf serration. Among western aspen populations, Barnes (1975) found significant variation in leaf shape (blade width/blade length), size (blade length and width), and serration (number of teeth). Leaf shape correlated with latitude and elevation, with wider leaves in the more arid, lower elevations and southern areas, and longer leaves at higher elevations and further north. Of his sample populations, the northernmost population in Vancouver, BC had the largest leaves, which he argues likely result from the region's long moist growing seasons. MANOVA analyses reported by Barnes (1975) found only 17 of 406 possible pairwise comparisons of phenotypic variation between populations were not statistically significant. From these studies of intraspecific variation we see that *P. tremuloides* is a phenotypically diverse species capable of survival in wide range of habitat types throughout its impressive range.

Phenotypic diversity typically arises from recombination via sexual reproduction. However, *P. tremuloides* reproduces both sexually and clonally. The capability of both sexual and clonal reproduction is widespread in eukaryotes, occurring in nearly every major taxon except mammals and birds (Halkett *et al*. 2005). However few plant examples are as well recognized as aspen. Some have argued that 'Pando,' an aspen clone in southern Utah with a total area of 43.3 ha and 47,000 ramets, is the largest single organism on earth (Grant *et al*. 1992). In an assessment of multiple studies of clonal plants, Eriksson (1989) found greater dependence on clonal reproduction among

clonal plants that 1) inhabit woodlands rather than grasslands, 2) spread vegetatively underground rather than above ground, 3) possess mechanisms for long distance seed dispersal. Each of these characteristics applies to aspen. Clonal reproduction in *P. tremuloides* is carried out by root suckering, where vertical shoots grow from an expanding root system to form new stems (Romme *et al*. 2005). Suckers grow from meristems that develop from the cork cambium of parent roots (Schier *et al*. 1985). Meristems can develop any time during secondary growth, and can exist as suppressed shoot primordia until growth regulator signals from the parent stem signal developmental initiation (Schier *et al*. 1985). The result of this suckering capability is that a single genetically distinct individual called a clone, or *genet*, can consist of several, hundreds, or even thousands of individual stems (Grant *et al*. 1992). The clonally reproduced stems of a genet are referred to as *ramets*. The term *ortet* refers to an original seedling which is capable of clonal reproduction but may or may not do so in its lifetime. Although individual stems are relatively short lived and not particularly robust (Myking *et al*. 2011), clonal stem turnover allows clones to survive to well beyond the age of any single constituent stem (Kemperman and Barnes 1976).

Root suckering is considered aspen's primary method of reproduction (Mitton and Grant 1996; Myking *et al*. 2011), and many argue that in the western United States, successful reproduction by seed is exceptionally rare or even negligible (Kay 1993; Jelinski and Cheliak 1992; Manier and Laven 2001; Romme *et al*. 2005). Highly limited successful sexual reproduction is described in the "ancient clone hypothesis" (Mock *et al*. 2008) that is largely attributed to Kemperman and Barnes (1976). They describe aspen in the western United States as consisting of very few, large, ancient clones, persisting for thousands of years by generations of ramet turnover. Some estimate that a substantial portion of western aspen genets were established shortly after glacial

recession, ~10,000 years ago (Mitton and Grant 1996). This tendency toward clonal reproduction is not the result of low fertility. Adult aspen can generate millions of seeds annually, and viability tests have indicated that a large portion of the seed mass is capable of seedling establishment (Schopmeyer 1974; Mitton and Grant 1996). The low numbers of sexually reproduced individuals is attributed to the improbability of seedling survival (Kemperman and Barnes 1976; McDonough 1985; Jelinski and Cheliak 1992). Aspen seedlings are considered extremely intolerant to desiccation and light competition (Romme *et al*. 2001). High temperatures (> 40°C) also inhibit germination, decrease emergence, and retard seedling growth (McDonough 1985). To survive and develop, aspen seedlings require bare mineral soil, limited light competition, and moist topsoil during germination and the first growing season (Kay *et al.* 1993). Because of these limitations, in the west, successful sexual reproduction occurs only in rare 'windows of opportunity' when conditions are favorable for seedling establishment (Jelinski and Cheliak 1992). Romme *et al*. (2001) attribute high rates of seedling establishment during a moist season in Yellowstone National Park following the 1988 fire to such a window of opportunity. Recent evidence suggests reproduction by seed likely contributes more to overall reproduction than was previously believed (Suvanto and Latva-Karjanmaa 2005; Mock 2008), but it is still clear that root suckering plays an extensive part of the species' reproductive strategy.

The sensitive requirements of aspen regeneration shape its spatial and temportal distribution. Aspen communities are characterized as stable, seral (successional), or decadent (Bartos 2001; Manier and Laven 2001). Nevertheless, aspen is considered to grow most often as successional communities that are dependent upon disturbance for maintenance (Kulakowski *et al.* 2006; Myking *et al*. 2011). Aspen is dioecious and produces wind-born seeds or pollen from flowers in inflorescences (catkins) (Mitton and

Grant 1996). Catkins generally bear between 50 and 100 flowers, with each flower capable of producing 2-10 seeds each (McDonough 1985). Wind born pollen and seeds give aspen a fairly large reproductive range. Pollen has been found up to 320 km from its nearest possible source (Jones and DeByle 1985a), and the plumose seeds may be carried distances 500 m, or even several kilometers under high wind conditions (McDonough 1985). In this way, aspen can rapidly recruit to disturbed habitats by seed, and once established, single individuals can rapidly populate the areas through clonal reproduction (Myking *et al*. 2011). Root suckering can also prove faster than seeding for movement into adjacent open habitats (Heinze and Fussi 2008). These reproductive life history characteristics make stand-replacing disturbances highly beneficial to aspen seedling establishment and clonal expansion (Bartos and Campbell 1998). Fire is the principally important disturbance (Bartos and Campbell 1998, Worrall *et al*. 2008), but windthrow, fungal diseases, snow damage, tent caterpillars, other insect outbreaks, hail, lightening, rockslides, avalanches and sunscald are also recognized as contributing disturbances (Mitton and Grant 1980; Romme *et al*. 2001; Namroud *et al*. 2005; Kulakowski *et al.* 2006). Clear-cutting can also be an important human caused disturbance (Romme *et al.* 2001).

In addition to opening new areas for recruitment, disturbances benefit aspen by preventing succession to more shade tolerant tree species (Manier and Laven 2001). Fire has been shown to stimulate suckering from aspen root systems (Bartos and Mueggler 1981; Kay *et al*. 1993; Frey *et al*. 2003). Following destruction of the stem portion of the tree, suppressed shoot primordia and newly initiated meristems in the roots can develop into new suckers (Schier 1985). The timing of root suckering is controlled by the ratio of growth regulators called auxins and cytokinins (Bartos 2001). Auxins are transported polarly downward from the meristems of the aerial portion of the

clone and suppress root suckering in a phenomenon termed “apical dominance” (Schier 1985). If the stem is destroyed by disturbance, the downward flow of auxins is disrupted, causing a drop in concentration in the roots, after which suckering is initiated from new meristems and suppressed primordia (Schier 1985; Bartos 2001). From this underground reproductive source, established communities of aspen can easily repopulate even after severe stand-replacing fires which destroy all above ground seed sources (Romme *et al*. 2001). Thus, aspen has a unique and historically successful life history strategy that is heavily dependent upon disturbance. Though its life history strategy has shown great historical success, the future of aspen in the western United States is matter of concern.

Decline

Throughout North America, and particularly in the west, aspen populations are in a state of decline. Bartos and Campbell (1998) reported a 60% reduction in aspen dominated landscapes in Utah, and others estimate that aspen ecosystems in Arizona and New Mexico have decreased by nearly half from 197,000 to 106,000 hectares between 1962 and 1986 (Holmes and Jansen 1999). This long term phenomenon is often referred to as “aspen decline” (Romme *et al*. 1995; Worrall *et al*. 2008; Durham and Marlow 2010; Rogers *et al*. 2010; Zegler 2011). It has been largely attributed to successional patterns caused by altered disturbance regimes (Bartos and Campbell 1998). Succession in aspen communities is usually toward more shade tolerant species, including Engelmann spruce (*Picea engelmannii* Parry ex Engelm.), subalpine fir (*Abies lasiocarpa* (Hooker) Nuttall), white fir (*Abies concolor* (Gordon) Lindley ex Hildebrand), Douglas-fir (*Pseudotsuga menziesii* D. Don), and ponderosa pine (*Pinus ponderosa* Douglas ex C.Lawson) (Romme *et al*. 2001). Because aspen’s life history strategy benefits from disturbances that exclude competing species or open up new areas where rapid recruitment and growth are advantageous, fire exclusion is considered a primary cause of aspen decline (Durham and Marlow 2010). Fire regimes in the western US have been altered by human activities that began as early as the middle 1800s (Bartos and Campbell 1998). Livestock (cattle and sheep) and wild ungulate browsing are also considered an exacerbating factor of aspen decline (Romme *et al*. 1995; Bartos and Campbell 1998; Worrall *et al*. 2008). Increases in livestock and elk populations in the western US over the last 150 years concomitant with aspen decline support the possible role of over-browsing (Halofsky *et al*. 2008; Kimble *et al*. 2011). Romme *et al*. (1995) concluded that although there was significant stimulation of suckering following the fires

of 1988 in Yellowstone National Park, very few will grow to adult size because of chronic elk browsing.

In addition to the slower ecologic successional patterns of aspen decline, there is evidence of a rapid dieback of aspen with apparently distinct characteristics and etiology (Hogg *et al*. 2002; Frey *et al*. 2004; Fairweather *et al*. 2008; Rehfeldt *et al*. 2009; Michaelian *et al*. 2010; Zegler 2011). Worrall *et al*. (2008) term this rapid decline "sudden aspen decline" (SAD) but "aspen die-off" and "aspen dieback" also appear in the literature (Zegler 2011). Worrall *et al*. (2008; 2010) describe SAD in terms of a decline disease (Manion 1991; Manion and LaChance 1992) brought on by serial combinations of predisposing, inciting, and contributing factors that synergistically reduce the health of aspen stands and increase mortality. Predisposing factors are relatively constant over time, and cause general stress and weaken individuals. Predisposing factors associated with SAD include stand demography, climate change (Rehfeldt *et al*. 2009), and unfavorable site characteristics associated with elevation slope, aspect, conifer encroachment and soil type (Fairweather *et al*. 2008; Zegler 2011). Inciting factors increase stress from predisposing factors to acute levels (Worrall *et al*. 2008). Inciting factors include drought (Fairweather *et al.* 2008; Hogg *et al*. 2008; Michaelian *et al*. 2010), insect defoliation (USDA Forest Service 2011), late frost (Fairweather 1999), diseases, and air pollution (Worrall *et al*. 2008; 2010). Acute stress from the combination of predisposing and inciting factors opens individuals to damage and eventual mortality from contributing factors that would otherwise pose only minor health threats (Jones *et al*. 1985; Worrall *et al*. 2010). Known contributing factors are canker fungi, wood-boring insects, and bark beetles (Jones *et al*. 1985; Worrall *et al*. 2008; Zegler 2011). Thus, where 'aspen decline' describes a slow pattern of replacement of aspen through successional processes, SAD indicates a distinct threat involving rapid loss of aspen

forest area with high levels of crown dieback and stem mortality and a number of synergistic causal factors. Both aspen decline and SAD threaten *P. tremuloides* in the western United States.

Adding to the complexity of sudden aspen decline is the possibility that the causal agents described above may have different effects on particular clones, with some genets showing greater resilience or susceptibility than others (Copony and Barnes 1974; French and Hart 1978; Shields and Bockheim 1981; Hwang and Lindroth 1997; Mock *et al*. 2008; Worrall *et al*. 2008; Holeski *et al*. 2009; St. Clair *et al*. 2010). Sheilds and Bockheim (1981) reported incidences of deteriorating and well stocked clones within the same stand and under presumably similar site conditions. Hwang and Lindroth (1997) demonstrated significant variation in defoliation from tent caterpillars between different clones. Holeski *et al*. (2009) found that different clones varied in susceptibility to *Venturia* shoot blight, and both Copony and Barnes (1974) and French and Hart (1978) demonstrated varied resistance to hypoxylon canker fungi between clones from the same areas. Among different clones in a common garden, St. Claire *et al*. (2010) found significant clonal variation in survival, growth and physiological characteristics. Individual diversity has also been shown to affect aspen fitness. Jelinski and Cheliak (1992) and Mitton and Grant (1980; 1996) found isozyme heterozygosity to be positively linked with annual growth rates, and Cole *et al*. (2010) showed that microsatellite heterozygosity was linked with higher growth rates in response to elevated CO_2. Thus, differing genotypes can show varied phenotypic responses to similar conditions. From these studies, it is likely that genotype also plays a role in SAD.

If genotype influences SAD, clonal structure will also be an important factor. Because some clones may be better adapted to varied damaging factors of SAD, aspen stands consisting of genetic “mosaics” (Namroud *et al*. 2005; Mock *et al*. 2008) may

experience only partial dieback without threat of extirpation of the entire stand. It may also be that some arrays of predisposing, inciting and contributing factors amount to a threat that no members of the species can survive. If this is the case, then entire populations of *P. tremuloides* would be moribund without disruption of the threatening factors. Thus, the best course of action for conservation may depend on the natural selection (or lack thereof) imparted by SAD among specific clones. Comparing genetic diversity and clonal structure of aspen stands and their health will help determine the relative roles of genetics and environmental influences in SAD.

Clonal Structure

Given its prevalence, it comes as no surprise that clonal reproduction strongly influences *P. tremuloides* population structure. Since seedling survival is the limiting factor of sexual reproduction, it is not surprising that the relative instances of sexual and asexual reproduction vary with local and regional conditions that influence seedling survival rates. Suvanto and Latva-Karjanmaa (2005) used three microsatellite markers to study clonal structure in *P. tremula* in Finland. They found small clone sizes, with 70% single ramet clones and no clone larger than twelve ramets. They also found clones in old-growth forests were generally larger than those in managed forests where disturbance was more prevalent. In northwestern Quebec, Namroud *et al*. (2005) divided a sample of ramets into cohorts based on estimated age from core samples. Each cohort was associated with an age, and a historic disturbance (fire, tent caterpillar [*Malacosoma disstria* Hübner] outbreaks, and a spruce budworm [*Choristoneura fumiferana* Clem.] outbreak) that likely allowed for the cohort's establishment. They also found small clone sizes (mean = 3.26 ± 1.65 and 4.39 ± 4.15), a predominance (75%) of single ramet clones, and high genotypic diversities (0.63 - 0.92), where genotypic diversity is equal to the ratio of total genotypes to the number of samples (G/N). They posited their observations were due to dense post-fire seedling recruitment. They also indicated that small scale gap opening disturbances gradually degrade the spatial patterns of the post-fire cohorts. As adjacent clones produce new root suckers following gap opening disturbances, their ramets will intermingle. Over time, the intermingling will blur clonal boundaries. The ramets of a clone are predicted to be proximal to one another (spatial genetic autocorrelation). While Namroud *et al.* (2005) observed degradation of spatial genetic patterns, it was not sufficient to render spatial genetic autocorrelation undetectable. Comparing these studies, we see here how disturbance,

environmental conditions, and variable emphasis on sexual and clonal reproduction can shape aspen genetic structure.

These studies of clonal structure among northern populations of aspen contrast sharply with similar studies in the western United States where Mock *et al*. (2008) found genotypic diversities of only 0.31 and 0.15 in two study areas. Using a combination of statistical methods, Mock *et al.* (2008) were able to determine clone diversities (ratio of the total number of clones to N) of 0.24 and 0.11. These values were smaller than those for genotypic diversity because some of the unique genotypes they discovered resulted from somatic mutations within genets rather than independent sexual origin. According to the ancient clone hypothesis, one expects to find very few large genets in xeric regions of the western United States. While genotypic diversity was still lower than more mesic areas of aspen's range, Mock *et al*. (2008) attributed much of the genotypic diversity that they observed to sexual reproduction, and concluded that reproduction by seed plays a more significant role in shaping aspen genetic structure in the western United States than previously believed. Tuskan *et al*. (2008) found similar results in Rocky Mountain National Park, with only six of 86 sampled patches consisting of single clones. Results from these studies show that clonal dynamics among *P. tremuloides* vary within its range. It can be found growing as expansive multiramet genets or closely clustered genets with only a few or single ramets. This variation appears to be linked with seedling recruitment rates influenced by geography, climate, and disturbance regimes.

Genetics

P. tremuloides and the other members of the *Populus* genus have 19 chromosomes as haploid cells, and 38 as diploids (Jones and DeByle 1985a). Trees are most often diploid, but both triploids and tetraploids have been observed naturally and as the result of breeding programs (Lexer et al. 2005; Jones and DeByle 1985a). Recently, genetic studies have benefitted from the availability of the complete draft sequence of a black cottonwood (*Populus trichocarpa* Torr. & A. Gray ex Hook.)(Tuskan *et al*. 2003), and it is likely that with the increased use of massively parallel sequencing technologies (reviewed in Metzger 2010), plant genetics will continue to benefit as more and more species' genomes are published.

P. tremuloides can hybridize with a number of species within its genus (Jones and DeByle 1985a). Barnes (1961) described natural hybrids between *P. tremuloides* and big-tooth aspen (*P. grandidentata* Michx.) in the western United States, and artificial breeding programs have succeeded in hybridizing *P. tremuloides* with *P. tremula*, white poplar (*P. alba* L.), and *P. canescens* Ait. (Jones and DeByle *et al*. 1985). Studying a hybrid zone near Vienna, Austria, Lexer et al. (2005) used microsatellites and DNA restriction site polymorphisms to demonstrate preferential introgression of alleles from *P. tremula* to *P. alba*. They concluded natural hybrid zones may increase fitness in *P. tremuloides* by serving as 'evolutionary filters;' allowing introgression of beneficial alleles from one species to another, but halting introgression of deleterious alleles by natural selection in the hybrid population (Lexer *et al*. 2005). Mapping linkage groups using AFLP and microsatellite markers, Cervera *et al*. (2001) found 19 linkage groups consistent between *Populus deltoides*, *P. nigra*, and *P. trichocarpa*. The homology of these linkage groups suggests that reproductive barriers between species may be genic

(resulting from individual genes) rather than chromosomal (resulting from incompatibility of chromosomes (Lexer *et al*. 2005).

While sexual reproduction and hybridization can result in new combinations of alleles, clonal growth plays an important role in shaping the genetic diversity of aspen populations. Population genetic theory predicts that diploid populations undergoing extensive clonal reproduction with little or no sexual recruitment will decrease in genotypic diversity, and increase in heterozygosity (Balloux *et al*. 2003). Reduction in genotypic diversity will occur as a limited number of highly competitive genotypes clonally increase and exclude other genotypes (Eriksson 1989). Though genotypic diversity decreases, diversity at the scale of individual loci (heterozygosity) is expected to increase because of the accumulation of somatic mutations in the clonal lineages. Assuming selectively neutral markers, without introduction of new alleles from migration, a relatively small sexual population is expected to lose heterozygosity over time (Hartl and Clark 1989). This occurs because the allele pool is resampled with every generation through sexual recombination (Conner and Hartl 2004). However, as a clonal lineage perpetuates, recombination does not occur. Without recombination, each of the two alleles from each locus in a diploid lineage will accumulate somatic mutations independently and irreversibly (Birky 1996; Halkett *et al*. 2005). The result is that heterozygosity of neutral markers is expected to increase essentially indefinitely within purely clonal lineages (Balloux *et al*. 2003). This effect has been termed the “Meselson effect” (Balloux *et al*. 2003; Halkett *et al*. 2005). Evidence for this effect was demonstrated in the class Bdelloidea (phylum Rotifera) which is the largest known taxon of metazoans with no evidence of sexual reproduction (Welch and Meselson 2000). Because of this effect, heterozygosity is expected to be higher with increasing rates of asexual reproduction (Balloux *et al*. 2003; Halkett *et al*. 2005). Based on this reasoning,

Wyman *et al*. (2003) suggested that the high rates of heterozygosity found in western aspen populations reported in Cheliak and Dancik (1982) and Jelinski and Cheliak (1992) may be due to mutation accumulation associated with higher levels of clonal reproduction in the western populations. Similarly, Gross *et al*. (2011) found accumulation of somatic mutation contributed significantly to the genetic diversity of asexually reproducing populations of the shrub *Grevillea rhizomatosa* Olde & Marriott. Therefore, among *P. tremuloides,* which undergoes varying rates of sexual and clonal reproduction in different habitats throughout its range, theory predicts heterozygosity will be higher in areas of greater clonal reproduction.

Since genetic variation is the raw material for evolution by natural selection, genetic variation among aspen populations is considered important for coping with future environmental changes. Limited genetic variation may constrain adaptation, leaving populations unable to survive novel conditions (Tuskan *et al*. 2008). Climate change is recognized as a probable factor in aspen decline (Rehfeldt *et al*. 2009; Worrall *et al*. 2010) and will apply selective pressure on aspen populations in the future. In addition, the clonal growth habit of *P. tremuloides* renders unique evolutionary patterns (Eriksson 1989). Especially in the west, spanning clones reduce the number of sexually reproducing individuals (Kemperman and Barnes 1976), and persistence of ancient clones for hundreds of years may limit establishment of younger seedlings with allele combinations better suited for current or future conditions (Jones and DeByle 1985a; Jelinski and Cheliak 1992). Hence, evaluation of genetic diversity and clonal structure will provide important information to the potential viability of western aspen populations.

Clone Assignment

One of the primary challenges in assessing clonal structure is assigning clone identity based on genetic data. A number of methods can be used to distinguish unique genets. These methods vary depending on technical capabilities, types of data used, and specific needs of the investigator. For instance, the simplest method to ensure one is not sampling the same genet twice is to only sample ramets that are separated by a distance of aspen free landscape that sufficiently exceeds the longest known reach of roots, or are separated by some impassable barrier (Mitton and Grant 1980). Jones and DeByle (1985b) report a greatest distance between connected ramets to be 30 m. However, Suvanto and Latva-Karjanmaa (2005) reported members of the same genet of *P. tremula* growing 30-40 m apart, and a maximum of just over 60 m apart. Threshold distances vary in the literature from 30-50 m (Jelinski and Cheliak 1992; Wyman *et al*. 2003) to 400 m (Mitton and Grant 1980).

Addressing questions of aspen population structure and ecology often requires more than the assurance of sampling distinct genets. For example, attempts to characterize clonal structure, or compare fitness between clones require the determination of whether two sampled ramets are members of separate genets. In the 1970s and early 1980s this problem was addressed with observations of physiological characteristics such as leaf, bark and stem morphology, and branching patterns (Kemperman and Barnes 1976; Shields and Bockheim 1981). Descriptions of naturally variable characteristics of aspen and their use in delineating clones are found in Barnes (1969). Since the 1980s, newer methods in molecular ecology have demonstrated the limited discriminatory power of clone assignment based on physiological data (Wyman *et al*. 2003; Suvanto and Latva-Karjanmaa 2005). Wyman *et al*. (2003) identified clones both by morphology and genetically using microsatellites. They found that the number of

clones identified genetically was 1.62 to 2.2 times greater than the number of clones identified by morphology. Suvanto and Latva-Karjanmaa (2005) obtained similar results, identifying 113 clones genetically, where only 79 clones, which they called morphotypes, could be identified by morphology. In a few cases, multiple morphotypes were discovered within single genetically assigned clones. Thus, morphological clone assignment can underestimate clonal diversity by overlooking physiologically similar, but genetically distinct clones, and risks splitting single clones due to variation among their ramets. Molecular methods, including analyses of isozymes (Jelinski and Cheliak 1992), RAPDs (Randomly amplified polymorphic DNAs) (Stevens *et al*. 1999; Yeh *et al*. 1995), and microsatellites (Wyman *et al*. 2003; Mock *et al*. 2008; St. Clair *et al*. 2010) are not subject to the caveats associated with morphological clone assignment and can provide clearer delineation of the clonal structure of aspen populations (Myking *et al*. 2011). Of these molecular methods, microsatellites are currently favored for studies of population genetics and clonal structure (Rahman *et al*. 2000; Wyman *et al*. 2003; Suvanto and Latva-Karjanmaa 2005; Mock *et al*. 2008).

Microsatellites are stretches of tandemly repeated sequence motifs of 2 to 5 base pairs (Koreth *et al*. 1996). Spread throughout the genomes of organisms from all kingdoms, they tend to be highly polymorphic with variation in the number of repeats of the sequence motif (Li *et al*. 2002). These differences are known as simple sequence length polymorphisms, and due to their hypervariability and reproducibility, they make excellent genetic markers for constructing genetic maps (Rahman *et al*. 2000) and genetic population studies. Because the repeat regions are often flanked by conserved sequences, length polymorphisms for a microsatellite locus can be detected by PCR followed by electrophoretic size separation. The relatively high polymorphism of microsatellite markers is due to their instability compared with other genomic markers

(mutation rates estimated between 10^{-2} and 10^{-6} events per locus per generation) (Li *et al*. 2002). Microsatellites rarely appear in protein coding regions (Koreth *et al*. 1996), so they lack the selective pressure of maintaining functional polypeptide sequences. Indeed, though evidence exists of allele size constraints and functional significance (Garza *et al.* 1995; Kashi and Soller 1999), some investigators (Wyman *et al*. 2003; Suvanto and Latva-Karjanmaa 2005) assume microsatellite variation to have little or no effect on the fitness of the individual (Reviewed in Li *et al*. 2002).

Mechanistically, variation within microsatellites comes from replication slippage or uneven crossing over between homologous loci (Li *et al*. 2002). Both mechanisms occur with greater frequency among microsatellites because of their repetitive character, which explains the relatively high instability of microsatellites compared with other genomic loci (Eisen 1999). Uneven crossing over occurs when there is homologous recombination between alleles that have not aligned properly with one another. This causes the alleles in the homologous pair to gain or lose a number of repeat units to the other. Replication slippage, or slip-strand mispairing, occurs during DNA replication. Here, repeat units are added or removed to the newly replicated strand through misalignment. According to this model, DNA polymerases can slip on the template molecule from which they are replicating DNA. During a slippage event, the newly formed strand can disassociate with the template strand, and must realign before replication can continue. Incorrect realignment can render additions or deletions of repeat units from the newly formed template molecule (Eisen 1999). Replication slippage must also escape DNA repair mechanisms to become established in populations, so efficiencies of proofreading and mismatch repair should also be considered as effectors of microsatellite instability (Li *et al*. 2002). Thus, due to their repetitive character, microsatellite markers are prone to mutational events that alter the number of repeats.

The resulting instability renders highly polymorphic markers that are easily distinguished codominantly by size separation of tagged alleles. These characters make microsatellites excellent tools for population analyses and estimates of clonal structure (Halkett *et al*. 2005).

Molecular methods such as microsatellites, while able to provide diverse multi-locus genotypes from highly polymorphic loci, require further analysis for genet delineation. The methodology for supporting clonal assignments varies between studies. Namroud *et al.* (2005) address the problem by estimating the probabilities that two individuals drawn randomly from a population will have the same genotype at multiple loci. The statistic, called probability of identity $P_{(ID)}$ uses the frequencies of the alleles in the sample population for each locus used in the study to predict what can be considered the random-match-probability (Waits *et al*. 2001). By setting a maximum threshold for $P_{(ID)}$ (<0.01 in Namroud *et al*. 2005) one can reject the null hypothesis that two ramets share highly similar or identical genotypes simply by chance. In this way, one can infer from the similarity of the two ramets' genotypes that they are probably members of the same genet (Namroud *et al*. 2005). Jelinski and Cheliak (1992) assigned clones using data from 13 polymorphic enzyme systems and Simpson's Index of Diversity to measure the probability of two genotypes randomly selected from the population N would have identical genotypes. Suvanto and Latva-Karjanmaa (2005) use yet another statistic developed by Parks and Werth (1993) referred to as P_{gen}, which Suvanto and Latva-Karjanmaa describe as a measurement of the probability that consecutively sampled trees that actually belong to different clones would have the same genotype by chance (see Methods; Parks and Werth 1993). In each of the cases above, the strength of the statistic increases with the number of markers used. Dayanandan *et al*. (1998) demonstrated that 89% of sampled individuals could be

clonally assigned based on four microsatellite markers. Based on this estimate, Wyman *et al*. (2003) used four loci for clonal assignment in an assessment of genetic diversity of aspen in Quebec. St. Claire *et al*. (2010) also considered four microsatellites loci sufficient to ensure that ramets were from unique clones. A further challenge assessing clonal structure is that intraclonal somatic mutation causes a fundamental disconnect between genotypic diversity and clonal diversity (Mock *et al*. 2008). While genotypic diversity provides a good estimate of clonal diversity (Ellstrand and Roose 1987) the measurements are unlikely to be precisely equivalent among sample populations. New genotypes that differ as a result of somatic mutation could give an inflated estimate of the number of clones. The question becomes “how different must two genotypes be to infer that they represent two sexually produced individuals?” The most thorough methods of genet assignment are described by Mock *et al*. (2008), who used a combination of genet assignment statistics to resolve cases when any one statistic provided inconclusive results.

Clone Age

Clone age is considered a predisposing factor in susceptibility to SAD (Worrall *et al*. 2008). Unfortunately, estimation of clone age can be challenging. Coupling tree ring data with historical records of disturbances can allow researchers to infer when cohorts of ramets established (Namroud *et al*. 2005), but because genets can survive many generations of ramet turnover, tree rings can at best only provide a minimum age for an aspen genet (Ally *et al*. 2010). Individual ramets are generally shorter lived than other hardwood species (Myking *et al*. 2011). In the west, ramet age distribution appears to peak between 80 and 100 years, with ramets greater than 200 years in age occurring rarely (Jones and Schier 1985). Tree ring data is likely to be particularly uninformative in the case of genets from western North America, where clones are estimated to be thousands, and possibly up to one million years in age (Kemperman and Barnes 1976). Clone size is considered a possible analog for clone age with older clones larger than younger clones (Kemperman and Barnes 1976). However, other factors, such as original number of seedlings established in the area, light and moisture competition, disturbance frequency, as well as the inherent growth rates of genets may also influence clone size (Kemperman and Barnes 1976; Ally *et al*. 2008). The influence of these other variables on clone size limits its precision as an indicator of clone age.

Genetic diversity can be used to estimate clone age based on the accumulation of somatic mutations in vegetatively reproducing individuals (Klekowski 2003; Tuskan *et al*. 2008). This is related to the Meselson effect discussed previously (genetics section; page 27). Although accumulation of deleterious mutations within ramets likely occur in a genet's lifetime, Ally *et al*. (2010) argued that in regard to phenotypes affecting survivability and vegetative reproduction, natural selection should still restrict the frequency of deleterious alleles among the ramet population. However, natural selection

should have no effect on alleles that are neutral to fitness. Assuming microsatellite markers are neutral to fitness, they should slowly accumulate allelic diversity through somatic mutation as the genet produces more ramets (Ally *et al*. 2008). For this reason, diversity of neutral makers within clones can be related to clone age (Ally *et al*. 2008; Tuskan *et al*. 2008). Based on this expectation of allelic divergence within clonally reproducing populations, Ally *et al*. (2008) describes a method of estimating the age of genets from genetic divergence between its constituent ramets. Using 14 microsatellite loci and upper and lower age bounds based on glacial retreat and the age of the oldest constituent ramet, they calibrated an 'ontogenetic molecular clock' to age aspen genets. They used this method to demonstrate the limited accuracy of clone size as a proxy for clone age (Ally *et al*. 2008). Tuskan *et al*. (2008) used 39 microsatellite loci in a similar method to describe the age distribution of clones in Rocky Mountain National Park. Ally *et al*. (2010) explored an alternate method of estimating clone age through sexual senescence. They argued that in regard to phenotypes influencing survivability and clonal reproduction, natural selection will prevent accumulation of deleterious somatic mutations by selecting against asexually produced ramets that bear them. However, because sexual fitness will not fall under the constraints of natural selection in a clonally reproducing population of ramets, Ally *et al*. (2010) argued that decreased sexual fitness will be correlated with clone age. They supported this argument by estimating sexual fitness based on amounts of viable pollen in catkins of differently aged clones. Using accumulation of somatic mutations within clones as a proxy for clone age, they demonstrated that older clones showed significantly lower numbers of viable pollen than younger clones (Ally *et al*. 2010). The loss of sexual fitness among clonal lineages is not limited to aspen, and has been observed in a number of clonal taxa (Klekowski 1997; Eckert 2002).

To summarize, aspen clones in western North America are likely to be very old, surviving many turnovers in their ramets. Within asexually reproducing populations, it is expected that somatic mutations will accumulate over time, and render higher than expected levels of heterozygosity. This is especially likely in the case of markers that have little or no effect on fitness, such as microsatellites. Thus, high rates of heterozygosity in microsatellite markers are hypothesized to be associated with sustained asexual reproduction.

CHAPTER 3: MANUSCRIPT

Introduction

In clonal plants, the spatial distribution of genetic diversity is related to growth habit (Namroud *et al*. 2005). Genetic diversity is also often connected with fitness, with greater diversity usually resulting in greater fitness (Mitton and Grant 1996; Hansson and Westerberg 2002; Reed and Frankham 2002), though unique circumstances may render the opposite relationship (Klekowski 2003; Ally *et al*. 2010). Therefore, among clonal plants, it is likely that historical growth patterns contribute to a population's fitness level under current conditions. Aspen are capable of extensive clonal growth, with offspring of a single seed capable of growing into expansive multi-stemmed individuals called genets or clones (Barnes 1966). Aspen have shade intolerant seedlings, and as a result usually grow as early successional species, dependent on canopy opening disturbances for successful seed germination and clonal expansion (Kay 1993). Disturbances that promote regeneration include fire, mudslides, windthrow, and insect outbreaks (Mitton and Grant 1980; Kay 1993; Romme *et al*. 2001; Kulakowski *et al.* 2006). Aspen seedlings are also intolerant to desiccation. Eckert (2002) proposed that the relative importance of sexual versus clonal recruitment among clonal plants will vary with habitat suitability to successful sexual reproduction. Consistent with this argument, in the semi-arid mountains of the western United States, seedling intolerance to desiccation makes successful sexual reproduction particularly rare (Kemperman and Barnes 1976; Jelinski and Cheliak 1992; Romme *et al*. 2001). Aspen in this region have been thought to reproduce almost exclusively by suckering, resulting in relatively small numbers of ancient, large, multiramet clones (Kemperman and Barnes 1976; Mock *et al*. 2008). However, more recent evidence has demonstrated that while clonal growth is indeed

extensive, sexual reproduction likely plays a greater role than previously believed (Mock *et al*. 2008).

Particularly in the west, but also in the prairie provinces of Canada, eastern Canada, and the Great Lakes region, aspen has been in a state of decline (Shields and Bockheim 1981; Bartos and Campbell 1998; Rehfeldt *et al*. 2009). This decline was attributed largely to disruption of disturbance regimes (particularly fire suppression), causing succession in seral aspen communities toward more shade tolerant species and preventing establishment of new aspen communities. In the west, many believe that elk browsing exacerbates aspen decline by preventing successful sucker regeneration and seedling survival (Romme *et al*. 1995; Romme *et al*. 2001; Hessl and Graumlich 2002; Kaye *et al*. 2005; Halofsky *et al* 2008). In 2001, estimates of decline in aspen ranges were as high as 49% in Colorado and 95% in Arizona (Bartos 2001).

More recently, accelerated aspen dieback with distinct etiology has been observed (Frey *et al*. 2004; Fairweather *et al*. 2008; Hogg *et al*. 2008; Zegler 2011). Due to its rapid rate, Worrall *et al*. (2008) termed the phenomenon Sudden Aspen Decline (SAD), which they describe in terms of a decline disease (Manion 1991; Manion and LaChance 1992) brought about by the serial combination of predisposing, inciting, and contributing factors. Predisposing factors are relatively static, exist over long timescales, and cause general stress among populations. Examples include climate change, long term drought (Fairweather 1999), unfavorable site conditions related to slope, aspect and elevation, conifer encroachment, and stand demography (Worrall *et al*. 2008; 2010). Inciting factors are short term changes such as insect defoliation (Zegler 2011), frost (Fairweather 1999), or drought (Hogg *et al*. 2005; Fairweather *et al.* 2008) that cause acute stress in a population. Drought is considered the primary inciting factor (Rehfeldt *et al*. 2009). Contributing factors are often biological agents that would not normally

cause extensive mortality, but take exaggerated tolls on populations weakened by predisposing and inciting factors, including canker fungi, wood-boring insects, and bark beetles (Worrall *et al*. 2008; Worrall *et al*. 2010). Consistent with this role of contributing factors, number of aspen's predators are known to feed particularly upon already weakened trees (Jones *et al*. 1985).

In Arizona, signs of SAD are prevalent. In 2003, aerial surveys detected ~29,500 hectares of aspen defoliation and decline throughout the state (USDA Forest Service 2004). Since then, aerial studies mapped progressively more aspen damage peaking at ~49,800 hectares in 2008, then decreasing to ~14,960 hectares in 2009, and ~8,461 hectares in 2010 (USDA Forest Service 2008 cited in Zegler 2011; USDA Forest Service 2009 and 2010). The damage is attributed primarily to drought and insect defoliation (USDA Forest Service 2009). The entire southwest region saw reductions in damage from ~71,225 hectares in 2008 to ~32,375 hectares in 2010, but reductions were greater in Arizona than New Mexico. This may have been due in part to a late season frost in Arizona that caused a crash in populations of the western tent caterpillar (*Malacosoma californicum* Packard; USDA Forest Service 2011). In a monitoring project on aspen in the Coconino National Forest in northern Arizona, Fairweather *et al*. (2008) observed increasing aspen mortality with decreasing elevation, with 16% mortality at high elevation sites (>2591 m), 61% mortality among mid-elevation sites (2286 –2591 m), and extensive mortality (95%) in low elevation sites (<2286 m). Cooler and moister conditions at higher elevations (Pearson 1920) suggests an important role of moisture levels in SAD in Arizona.

In the Kaibab National Forest in northern Arizona, Zegler (2011) surveyed 48 sample sites chosen by stratified random sampling with proportional allocation to characterize relationships between aspen and biotic and abiotic site conditions including

elevation, slope, aspect, forest type, and damaging agents. His results were consistent with characteristics of SAD. Zegler (2011) found aspen health tended to increase with elevation. He also found incidence of canker disease and conifer encroachment were associated with aspen mortality. Studies have indicated that clone identity (genotype) likely plays a role in susceptibility to factors contributing to SAD such as canker fungi (Copony and Barnes 1974; French and Hart 1978), climate (Ohms 2003), defoliation (Shields and Bockheim 1981; Hwang and Lindroth 1997; Donaldson and Lindroth 2007), and *Venturia* shoot blight (Holeski *et al.* 2009). Among aspen grown in a common garden, St. Clair *et al*. (2010) demonstrated clone based variation in physiology, growth, and survival. Given these observations, it seemed probable that there was a genetic component to susceptibility to SAD among aspen stands observed by Zegler (2011) in the Kaibab National Forest. To test this, I generated a dataset of microsatellite multilocus genotypes (MLGs) from aspen trees sampled from a subset of Zegler's (2011) 48 sample sites. My primary objectives were to 1) assess the genetic diversity of aspen in the study area, 2) assess clonal structure to make inferences of historical reproductive patterns, and 3) to test for relationships between genetic diversity, clonal structure, and signs of SAD.

Methods

Field Methods

The study area was the Williams Ranger District of Kaibab National Forest, located Northern Arizona west of Flagstaff (Figure 1). The sampling strategy used for this study was developed by Zegler (2011) to test for relationships between SAD, environmental conditions and damaging agents. His 48 sample sites were established using stratified random sampling with proportional allocation, so that they represented variation in environmental conditions as similar as possible to the entire aspen population within the study area. The 25 sample sites used in this study were a subset of the 48 sites established by Zegler (2011). The subset was selected from the middle of the total range in elevation because these sites were likely to show the greatest variation in health (Zegler 2011). The range of elevation in Zegler (2011) was 2094 to 2888 m. The range for this study was 2271 to 2652 m. A list of these 25 sample sites, their elevations, slope, aspect, and geographic coordinates (UTMs) is shown in

Table **1**.

Each of Zegler's (2011) sample sites had a permanently marked center surrounded by four subplots in each of the four cardinal directions (Figure 2). The four subplots were 8 m in diameter centered 20 m from the site center. My tissue sampling strategy was devised to fit these pre-established sites. From each subplot, I collected tissue samples 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. I only sampled trees greater than 10.0 cm dbh because this was the size category for which I had percent mortality and crown dieback data from Zegler (2011; Table 1). Percent mortality was

described in terms of trees per hectare (TPH) which was the ratio of standing dead stems to total stems for a site, and on basal area (BA) (m^2 ha^{-1}), which was the

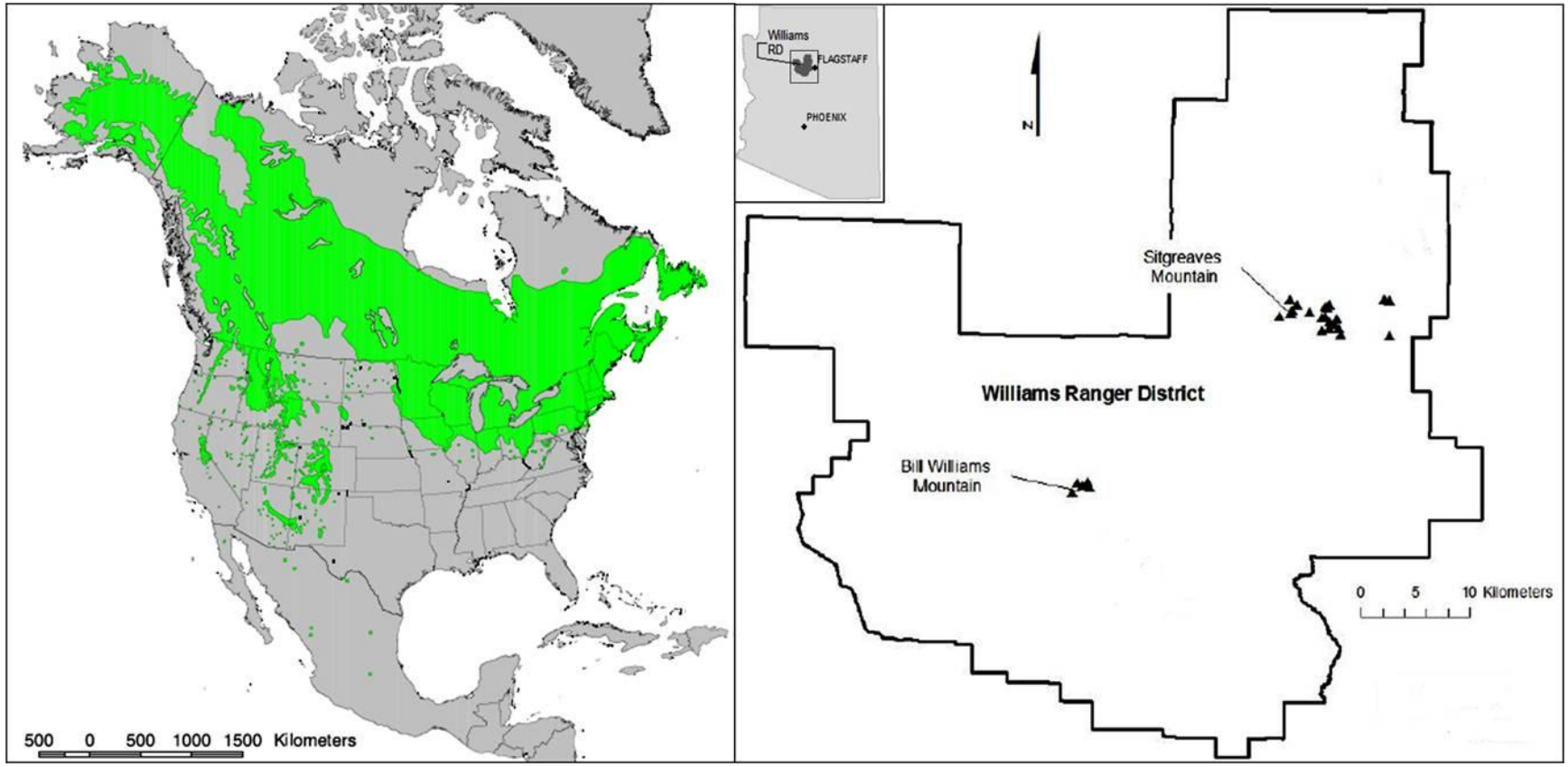

Figure 1: Left: Geographic range of Populus tremuloides (U.S. Geological Survey; Little 1971). Right: Location of sample sites in the Williams Ranger District of Kaibab National Forest west of Flagstaff, AZ. Figure adapted with permission from Zegler (2011).

Table 1: Sample sites selected for this study with abiotic conditions, health measurements and locations collected by Zegler (2011). [1]Percent mortality was calculated based on trees per hectare (TPH) and based on basal area per hectare (BA). [2]Percent crown dieback represents the proportions of trees from the sites with light, moderate, and heavy crown dieback, where light crown dieback indicates less than 33% crown dieback, moderate between 33% and 66% crown dieback, and heavy greater 66% crown dieback.

Site	Elevation	Slope	Aspect	% Mortality[1]		% Crown Dieback[2]			Location (UTMs)	
	(m)	(%)	(deg)	(TPH)	(BA)	Light	Moderate	Heavy	N	E
ANH12	2378	27	340	92	86	0	67	33	423446	3914240
BWM108	2652	24	340	24	17	19	30	51	390269	3896506
BWM159	2685	35	26	26	12	44	46	11	390524	3896210
BWM187	2483	38	354	44	33	74	20	6	388957	3895706
BWM190	2560	52	348	80	75	63	25	13	390394	3896714
BWM75	2547	32	290	55	46	26	26	48	389878	3896383
ER4	2374	20	350	93	94	0	0	100	410634	3911651
GH104	2479	7	322	72	66	40	30	30	413258	3910285
GH179	2439	23	344	70	67	33	33	33	412888	3910624
GH23	2386	44	300	61	76	86	14	0	412462	3910824
GH42	2320	14	350	53	52	43	39	17	413081	3911108
GH6	2387	43	2	67	52	67	17	17	413453	3909714
GH80	2529	37	274	36	16	63	25	13	412487	3910245
GM170	2424	59	322	10	8	69	12	19	417510	3912760
GM3	2369	52	24	33	23	60	10	30	417966	3912734
GP89	2309	34	2	50	35	6	41	53	417919	3909551
NT130	2446	20	207	37	35	44	41	15	420564	3917561
NT133	2418	8	278	24	16	73	20	7	420309	3917768
SGM196	2482	16	0	62	57	60	40	0	408818	3912762
SGM68	2595	25	240	58	51	57	43	0	407916	3911242
SVT100	2277	4	75	41	51	90	0	10	412302	3912040
SVT101	2271	3	80	15	19	62	24	14	412407	3912283
SVT102	2324	22	343	56	63	0	25	75	411810	3911195
SVT99	2281	3	100	50	35	33	67	0	412113	3912111
TT28	2317	22	84	36	25	32	50	18	417910	3923562

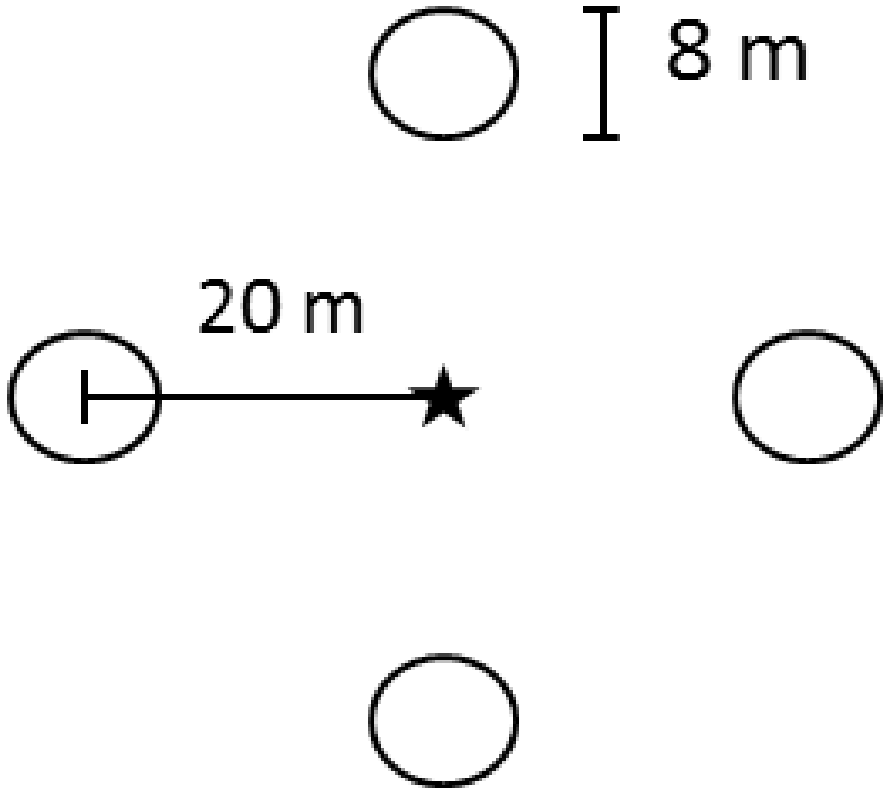

Figure 2: Diagram of a sample site. The site center is indicated with a star. Subplots are indicated by the four circles. Five trees were sampled from each of the four subplots of each site: the four trees farthest from the subplot center in each of the four cardinal directions, and the tree closest to the subplot center, allowing for a maximum of twenty trees sampled per site.

proportion of the basal area of dead aspen to total basal area of aspen in the site. Crown dieback was measured as the proportion of aspen stems that fell into three dieback categories: light (< 33% crown dieback), moderate (between 33% and 66% crown dieback), and heavy (> 66% crown dieback). Because the site centers were randomly selected, in many instances there were fewer than five trees in a subplot. In these cases I sampled as many trees as were there. The maximum number of trees that could be sampled for one site was 20 (5 trees from each of the 4 subplots), but most sites were represented by fewer trees with an average number of trees per site of 8.48 ± 4.75 standard deviation. Whenever possible, ~5 leaves were collected per tree. When leaves were out of reach, ~5 cm^2 pieces of cambial tissue were collected using a razor; cutting to a depth just beyond the green corticular layer beneath the outer bark. In a few unique instances, I sampled flower and bud tissue, taking two or three catkins or five or six buds. In the field, all tissue samples were temporarily stored in firm paper envelopes or brown paper lunch bags. Samples were dried by loosely packing silica gel or cat litter around the vertically stacked envelopes and bags for one to two weeks. Following desiccation, all samples were frozen at -20°C until DNA extraction. Of 212 genotyped samples, 107 were leaf samples, 99 were cambial samples, four were bud samples and two were flower samples.

DNA extraction, PCR, and Genotyping

I extracted genomic DNA from tissue samples using a modified protocol for a QIAGEN DNEasy® 96 Plant Kit. Proceeding cell lysis, the protocol calls for disruption of tissue using a QIAGEN tissue lyser. Lacking this instrument, I disrupted tissue samples using a Biospec Mini Bead Beater and 2.3 mm diameter steel beads. Tissue disruption was accomplished by loading 100 mg of tissue into a 2.0 ml disruption tube one third filled with 2.3 mm steel beads and 1.0 ml molecular biology grade water. Leaf, bud and flower tissue samples were disrupted with three 40 second intervals on the bead beater at maximum speed. Cambial tissue was disrupted with six 40 second intervals. The result was a homogenate of the tissue sample in water. I pipetted 100 µl of this homogenate into each well of the 96 well plate of a QIAGEN DNEasy® 96 Plant Kit. Assuming complete homogenization, an equivalent of 10 mg of tissue sample was used for each extraction.

Using these genomic extracts as template, PCR was used to amplify five of the microsatellite markers described in Mock *et al*. (2008): PMGC576, PMGC2571, WPMS14, WPMS15, and GCPM970-1 (Table 2). Alleles for each marker were amplified using 10.0 µl volume PCRs with the following reagent concentrations: 1.80 mM MgCl2, 0.20 mM each dNTP, 0.25 mM of each primer, 0.30 U polymerase and 1 X PCR buffer. The thermocyling program for my PCRs with adjustments of annealing temperature and cycle number (Table 2) was as follows: 5 minutes at 95°C, 32 or 36 cycles of 30 seconds at 94°C, 40 seconds at primer set specific annealing temperature, and 50 seconds at 72°C, cycles were followed by a 7 minute final elongation step at 72°C. For two primer sets, PMGC576 and WPMS14, 36 PCR cycles were necessary to consistently generate

Table 2: Microsatellite primer sets, thermocycling conditions, and marker characteristics

Locus	# of PCR cycles	Annealing Temp.(°C)	Dye label	Repeat Motif	Size Range (bp)
PMGC576[1]	36	52	Hex*	$[GA]_n$	150 - 168
PMGC2571[1]	32	56	Fam	$[GA]_n$	89 - 113
WPMS14[2]	36	55	Hex	$[CGT]_n$	200 - 230
WPMS15[2]	32	55	Hex	$[CCT]_n$	182 - 194
GCPM970-1[1]	32	57	Fam	$[TGC]_n$	117 - 126

[1](http://www.ornl.gov/sci/ipgc/ssr_resource.htm); [2](Smulders *et al*. 2001).

*Hex and Fam are fluorescently labeled dyes incorporated at the 5' end of one primer from each primer set to tag PCR products for genotyping.

sufficient peak signals. This may have been necessary because of limited DNA yields due to modification of the DNA extraction method. One of the two primers from each primer set included a fluorescent tag at its 5’ end. These were incorporated into the PCR product along with the primers, and allowed automated detection of the fragments during capillary electrophoresis.

PCR products were sized using a 3130xl genetic analyzer (Applied Biosystems). Genotyping reactions composed 9.5 µl of Hi-Di™ Formamide (Applied Biosystems), 2.0 µl of PCR product, and 0.5 µl of ROX size standard (Applied Biosystems). Electropherograms were genotyped and manually examined for peak morphology and artifacts using GeneMapper (Applied Biosystems).

Statistical Analyses

I used multilocus genotypes generated for each sample to assess the genetic diversity of the study area, examine clonal structure, and test for relationships between genetic diversity and signs of SAD. Health data collected by Zegler (2011) was used to indicate SAD (

Table **1**). Divergence from Hardy-Weinberg equilibrium, allele frequencies, genotypic distances, observed and expected heterozygosity, inbreeding coefficients ($F_{IS;}$ Equation 1) and AMOVA (Excoffier *et al*. 1992) were calculated using GenAlEx 6.4 (Peakall and Smouse 2006). Mean observed heterozygosity for each sample site was calculated as the sum of the sampled trees' percent heterozygosities divided by the number of trees sampled. Regression analyses, t-tests, Chi-Square tests for independence, and Fisher's exact tests were performed using R version 2.12.1 (The R Foundation for Statistical Computing 2010). Unless otherwise noted, uncertainties are given as standard deviations.

Equation 1: $F_{IS} = \frac{\bar{H}_{exp} - \bar{H}_{obs}}{\bar{H}_{exp}}$

The strength of my clone assignments was supported using the metric P_{gen} described in Parks and Werth (1993; Equation 2).

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

Where $p_i q_i$ is the product of the allele frequencies of the two alleles of each locus and h is number of heterozygous loci in the MLG. This metric estimates the probability of a given MLG arising randomly in a population by sexual reproduction (Parks and Werth 1993). It assumes random mate selection, independent loci, and that the experimentally observed allele frequencies are close estimates of the true allele frequencies. An MLG could be shared between trees from different clones if by chance they had the same alleles at each examined locus or because they are ramets of the same genet. As the number of examined loci is increased, the probability of two trees randomly sharing the same alleles at each becomes smaller and smaller. In this study, by default each tree was assumed to come from a unique genet. Low P_{gen} values (< 0.01; Parks and Werth 1993) for a MLG shared between two trees indicated that this default assumption was unlikely, in which case the alternative conclusion, that the trees were ramets of the same genet, was accepted. Here 'clonal assignment' refers only to the conclusion that two trees with identical MLGs were clonal propagates of the same genet. Studies have indicated that four microsatellite loci can provide confident clonal assignment (Dayanandan 1998; Wyman *et al*. 2003; Namroud *et al*. 2005). To test this, P_{gen} values were also calculated for multilocus genotypes with the least polymorphic marker (GCPM970-1) removed. It is also possible that the MLGs of two ramets from the same genet could differ slightly due to somatic mutation. This possibility was addressed by recalculating P_{gen} only for the loci that were identical between the two MLGs in question. If the P_{gen} value for only the shared loci was < 0.01, trees were thought to be ramets of the same clone and their slight genetic divergence was due to somatic mutation. This step expands the meaning of clonal assignment, allowing two different but highly similar genotypes to be assigned to the same clone.

MLGs from a large proportion of the samples showed three alleles for at least one locus (see Results). If the tri-allelic loci result from triploid or aneuploidy, it is possible that these samples had more undetected alleles (Mock *et al*. 2008). For this reason, samples that had three alleles for any locus were not included in calculations for Hardy-Weinberg equilibrium, allele frequencies, P_{gen} or AMOVA. Unless otherwise noted, for estimates of heterozygosity, genotypes with triple alleles were included and loci with triple alleles were considered heterozygous. Estimates of genotypic diversity included genotypes with triple alleles.

Results

Genetic Diversity

Multilocus genotypes were generated for 212 ramets from 25 sample sites. Among five microsatellite loci there were 31 unique alleles. The loci showed variable levels of polymorphism, with the number of alleles for each locus ranging from 3 to 10 with an average of 6.2 ± 2.9 (

Table **3**). Allele distributions for the loci are shown in Figure 3. Frequencies were similar whether they were calculated from all samples or all genets (data not shown). With each locus, one or two alleles were predominant, with no overlap of standard error bars with the lower frequency alleles. Thus, the majority of the allelic richness was represented by low frequency alleles. Four of the five loci (all except WPMS15) diverged from Hardy-Weinberg equilibrium (

Table **3**). For the pooled population, observed heterozygosity was higher when samples with triple alleles were included (mean = 0.662 ± 0.218), than when they were not included (mean = 0.556 ± 0.173;

Table **3**). Fixation indices (F_{IS}) ranged from -0.274 to 0.076 (mean = -0.046 ± 0.148) when triploids were included and were much higher (mean = 0.135 ± 0.133) when triploids were not included. Mean H_{obs} of the sample sites ranged from 0.20 to 1.00 with an average of 0.689 ± 0.211. AMOVA indicated a high degree of genetic structuring between sites, with 73% of the genetic variation occurring among the sample sites, while 27% occurred within the sites.

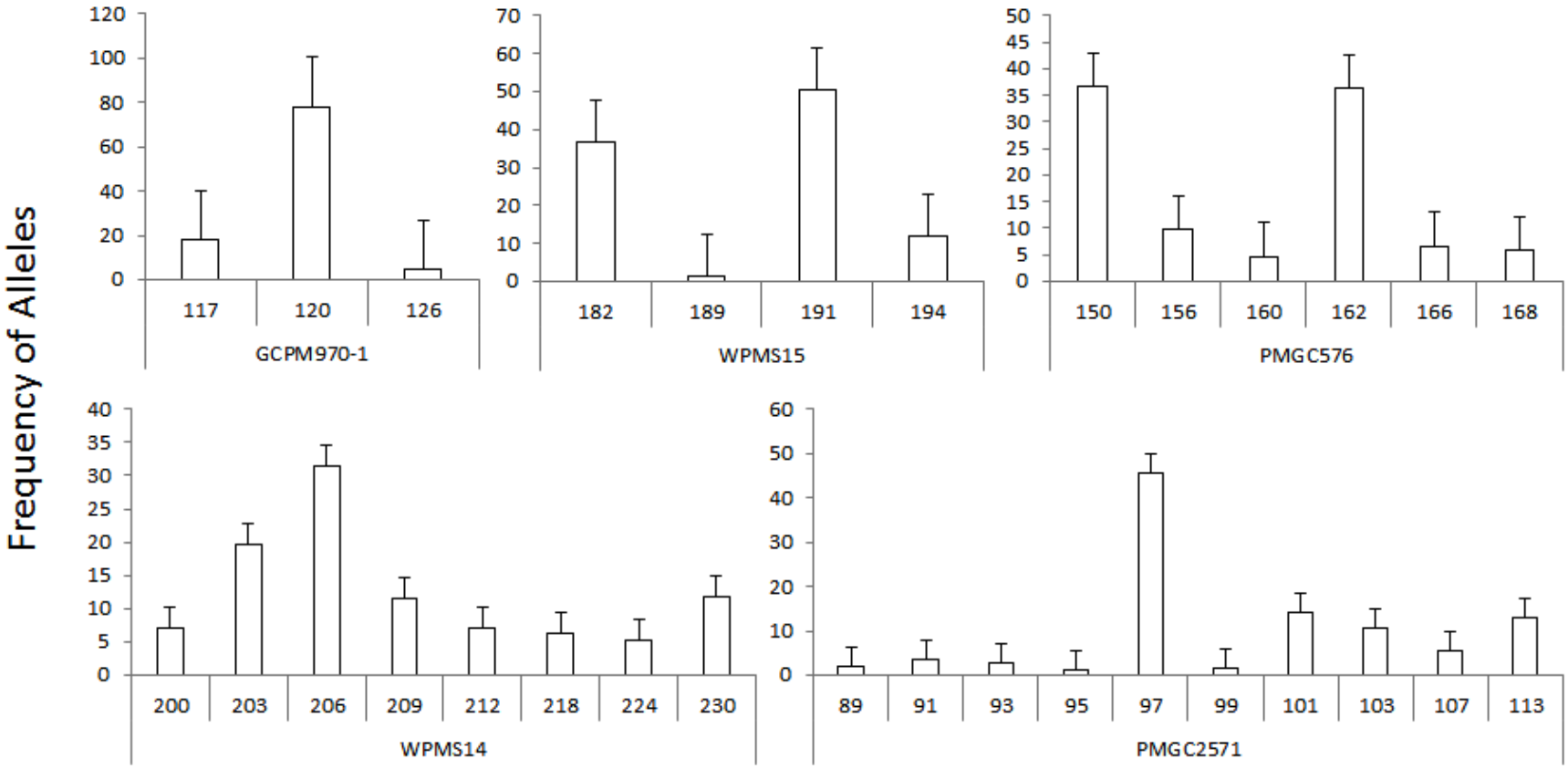

Figure 3: Allele distributions for five microsatellite loci for the entire sample population of aspen sampled from the Kaibab National Forest, AZ. Each bar represents the frequency of an allele. Numbers below bars indicate allele sizes in base pairs. Error bars indicate standard error. Samples showing triple alleles were not included.

Table 3: Genetic diversity of five loci in the entire population of aspen sampled in the Kaibab National Forest AZ, with and without samples showing triple alleles. Degrees of freedom vary for loci based on the number of possible genotypes that could be formed from their alleles. Samples showing triple alleles were not included for Hardy-Weinberg calculations.

Triploids Included					Triploids Excluded				Hardy-Weinberg	
Locus	# Alleles	H_{obs}	H_{exp}	F_{IS}	# Alleles	*H*obs	*H*exp	F_{IS}	df	X^2
GCPM970-1	4	0.297	0.322	0.076	3	0.315	0.359	0.123	3	104.56***
WPMS15	4	0.717	0.563	-0.274	4	0.606	0.598	-0.014	6	3.042
PMGC576	6	0.684	0.704	0.028	6	0.488	0.715	0.317	15	156.61***
WPMS14	10	0.882	0.790	-0.116	8	0.787	0.818	0.038	28	206.52***
PMGC2571	11	0.731	0.773	0.055	10	0.583	0.737	0.210	45	302.27***
Mean	7.00	0.662	0.630	-0.046	6.2	0.556	0.646	0.135	19.4	
Standard Deviation	3.317	0.218	0.194	0.148	2.864	0.173	0.179	0.133		

*** = significant at $p < 0.001$

Clonal Structure

P_{gen} values for multilocus genotypes ranged from 3.01×10^{-6} to 2.807×10^{-3} with a mean of $6.78 \times 10^{-4} \pm 8.30 \times 10^{-4}$ (Table 4). Removal of the least polymorphic marker (GCPM970-1) increased the P_{gen} values to a range of 5.584×10^{-5} to 6.088×10^{-3} with a mean of $1.757 \times 10^{-3} \pm 1.787 \times 10^{-3}$. No two trees from different sites had the same MLG. The pair-wise genetic distances between genotypes ranged from 2 to 17 with an average of 7.60 ± 2.74 (*n* = 379 comparisons; Appendix 1).The distribution of these distances is shown in Figure 4. The distribution was unimodal with only slight skewing toward greater genetic distance. The two most similar pairs of genotypes had pair-wise distances of two and three. In both cases the pair was from the same sample site, from BWM187 and GM3 respectively. Recalculation of P_{gen} values excluding non-identical loci for the MLGs resulted in a P_{gen} value of 0.017 for the pair with an original distance of two, and 0.032 for the pair with an original distance of three. Though these values were low, they were above 0.01, so the two pairs were considered to represent four unique clones.

The sample population showed 40 unique genotypes (triple alleles included), giving an overall genotypic diversity (G/N) of 0.184. Seventeen of the 25 samples sites were monoclonal (every tree sampled in the site had the same MLG). Of the eight sites showing multiple genotypes (multiclonal sites), five showed three or more genotypes (Figure 5). In five cases, trees with different genotypes were sampled from the same 8 m diameter subplot. In a single case, in one of the two most genotypically diverse sample sites, trees with three unique genotypes were sampled from a single subplot. The multiclonal sites tended to have northerly aspects. Only one multiclonal site's aspect diverged from due north by more than 26 degrees, and the mean divergence from due north (20.75 ± 21.43) was significantly lower for multiclonal sites than for monoclonal

Table 4: P_{gen} values for each genotype (triple allele genotypes not included) found in the sample population. Low values indicate low probabilities of the genotypes arising randomly in the population, and thus the strength of clone assignments. Allele frequencies were estimated according to their frequencies among the sample population. All values were ≤ 0.003, indicating the five microsatellite markers were sufficient for clonal assignment.

Genotype	Site	P_{gen}
1	BWM108	0.002715
2	BWM187	5.66E-05
3	BWM187	0.00014
4	BWM75	0.000865
5	BWM75	0.000712
6	GH179	0.001314
7	GH179	9.14E-05
8	GH42	7.06E-05
9	GH42	0.000329
10	GH42	0.002478
11	GH23	0.001726
12	GH80	4.18E-05
13	GH80	2.08E-05
14	GM170	5.81E-05
15	GM170	0.000958
16	GM3	0.000506
17	GM3	0.000395
18	GM3	0.000833
19	GM3	0.002807
20	NT133	0.001081
21	SGM196	0.000237
22	SVT100	0.000685
23	SVT101	4.31E-05
24	SVT102	2.57E-05
25	SVT99	0.000234
26	TT28	0.000144
27	TT28	0.000403
28	TT28	3.1E-06

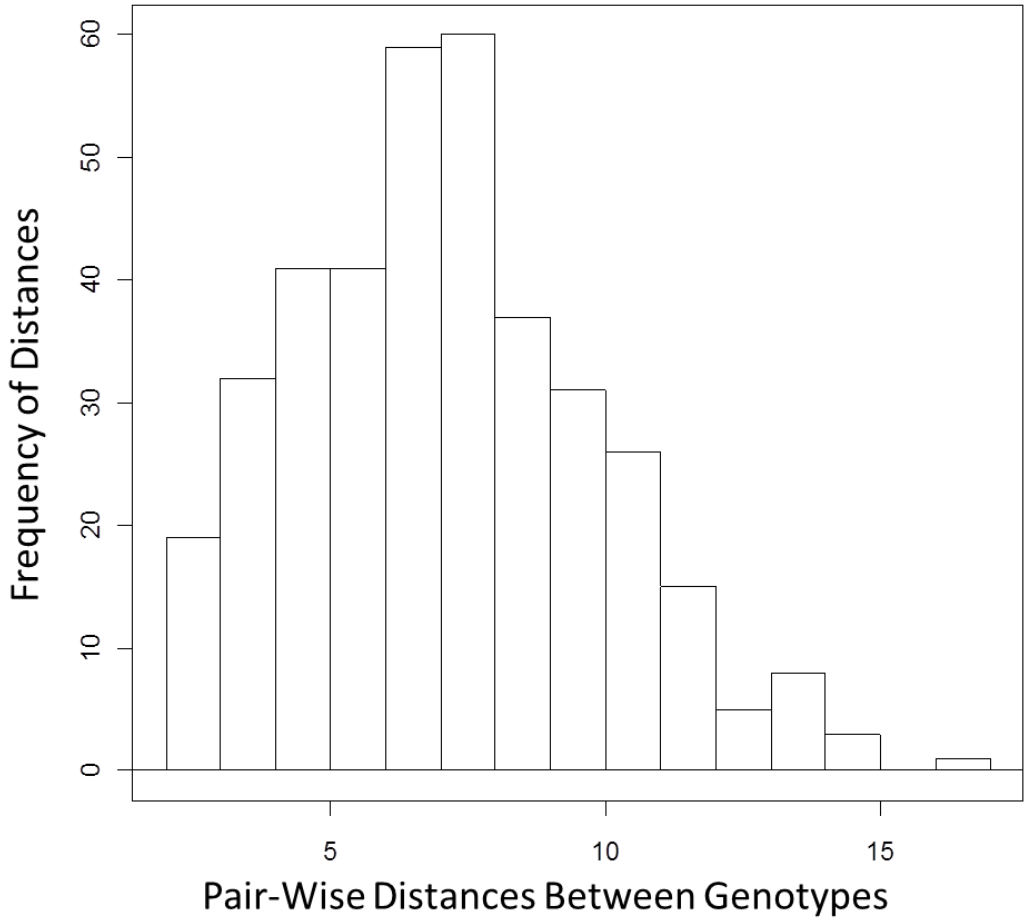

Figure 4: Distribution of pair-wise distances between genotypes calculated using GenAlEx (Peakall and Smouse 2006). Bins represent different genetic distances (based on allele mismatches) between the genotypes in my sample population.

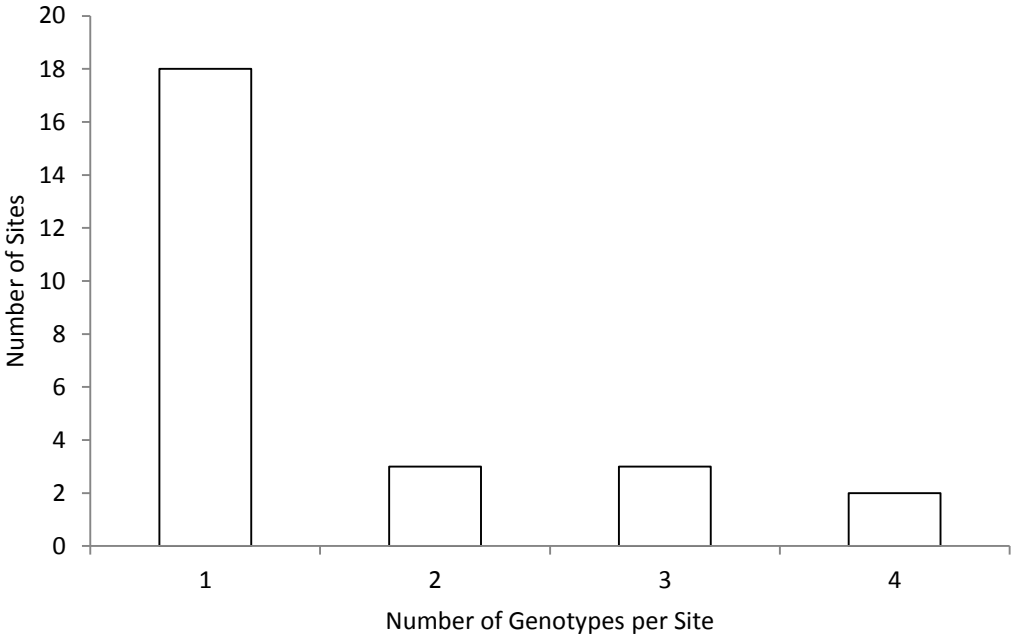

Figure 5: Total numbers of multilocus genotypes per sample site in the Kaibab National Forest, AZ (triple allele genotypes included).

sites (57.94 ± 44.48)(one tailed Welch two sample t-test: t = -2.8212; p = 0.005). Multiclonal sites also showed a tendency toward lower average heterozygosity, though the difference was not statistically significant (Two Sample t-test t = -1.593; p = 0.062). Comparisons of site characteristics and health conditions for multiclonal and monoclonal sites are shown in Figure 6. As would be expected, mean clone size was higher among monoclonal sites (mean = 7.647 ± 5.207) than among the multiclonal sites (mean = 3.522 ± 2.778; Welch's t-test: t = 2.969; p = 0.003).

To further test for trends between genetic diversity and aspect, the sample sites were divided into two sets: those with more northerly aspect (aspect divergence from north < 40 degrees; n = 15), and those with less northerly aspect (aspect divergence from north ≥ 50 degrees; n = 10; Table 5). This division was made based on the bimodal distribution of the sites' aspect divergence from north (Figure 7). For simplicity, these two sets are labeled as the 'northerly' and 'southerly' sites, though the 'southerly' sites may be thought of more properly as the non-northerly sites. While none of the comparisons between these sets were statistically significant, it should be noted that the northerly showed slight tendencies toward: 1) greater frequency of multiclonal sites (all but one multiclonal site), 2) higher genotypic diversity (G/N = 0.267 vs. 0.183), 3) fewer ramets per genet (mean = 4.74 ± 4.30 vs. 6.38 ± 4.70), 4) lower heterozygosity (mean = 6.444 ± 0.190 vs. 0.755 ± 0.234), and 5) greater proportion of single ramet clones, with 8 of the 11 (72.7%) single ramet clones found in northerly sites. Because I sampled a maximum of 20 trees per sample site it was unlikely that any genet would be represented by more than 20 ramets. The distribution of the number of ramets per genotype is shown in Figure 8. The largest genet had 18 sampled ramets, but many genets were represented by a single tree. The number of ramets per genet was variable, and averaged 5.27 ±

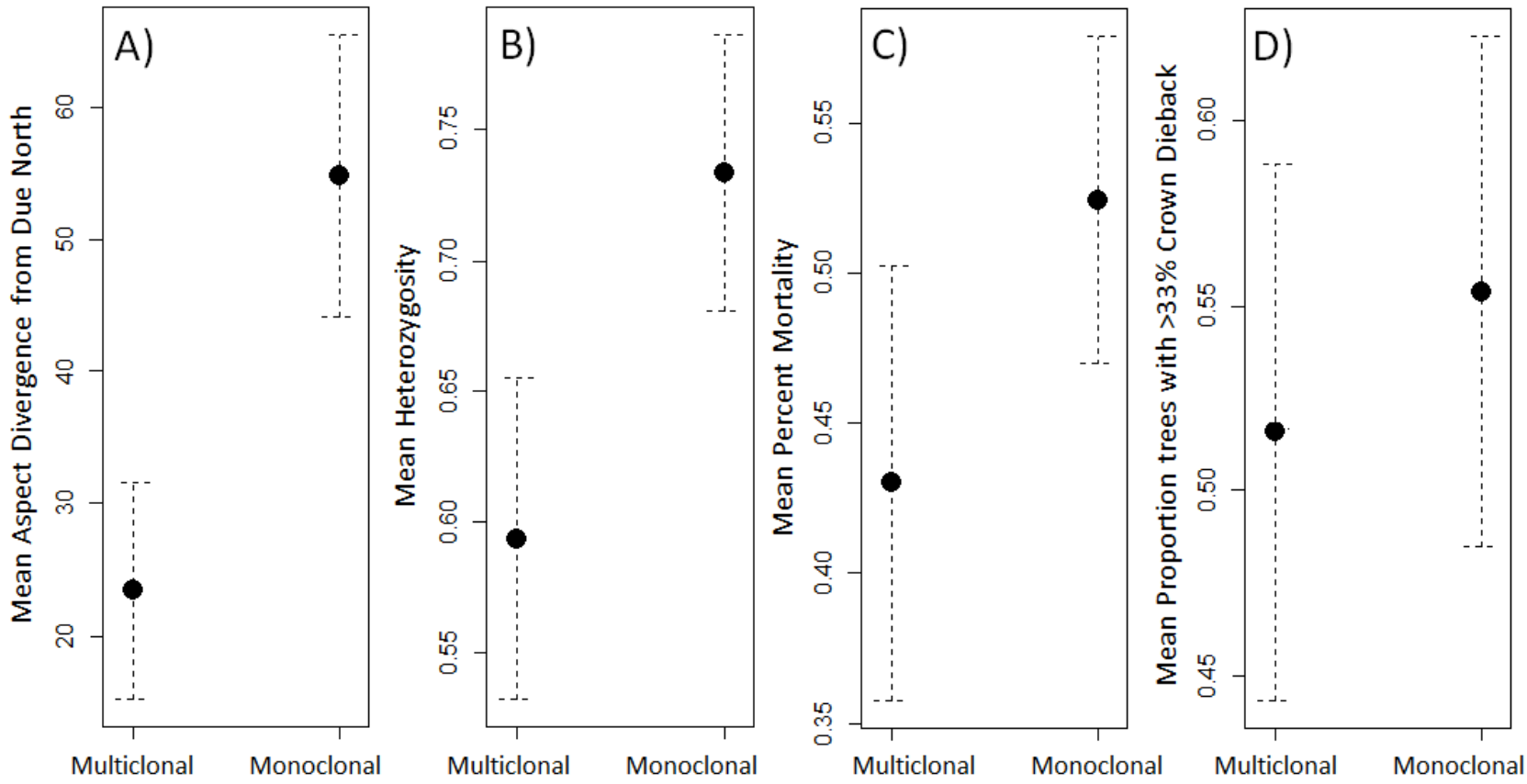

Figure 6: Comparisons of mean site conditions between multiclonal and monoclonal sites. A) Mean divergence from due north ($p < 0.005$). B) Mean heterozygosity (ns). C) Mean percent mortality (ns). D) Mean proportion of trees with > 33% crown dieback (ns).

Table 5: Distribution of genotypes among northerly and southerly aspects: ‘Northerly’ = Deviation from due north < 38 degrees and ‘Southerly’ = deviation from due north > 60 degrees. The label ‘Southerly’ is a misnomer used for the sake of simplicity. The sites do not show any tendency toward southern aspect, but are simply the less northerly portion of the sample sites.

Northerly			Southerly		
Site	# Genotypes	Deviation from due N	Site	# Genotypes	Deviation from due N
NT133	1	0	BWM159	1	60
ER4	1	2	GM3	4	70
GH80	2	2	SGM68	1	75
GH42	4	6	SVT100	1	80
BWM190	1	10	NT130	1	82
TT28	3	10	SVT99	1	84
GM170	2	12	GH104	1	86
BWM108	1	16	SVT102	1	100
SVT101	1	17	SGM196	1	120
BWM75	2	20	GP89	1	153
BWM187	3	20	Mean	1.3	91.00
GH6	1	24			
GH179	3	26			
ANH12	1	38			
GH23	1	38			
Mean	1.8	16.07			

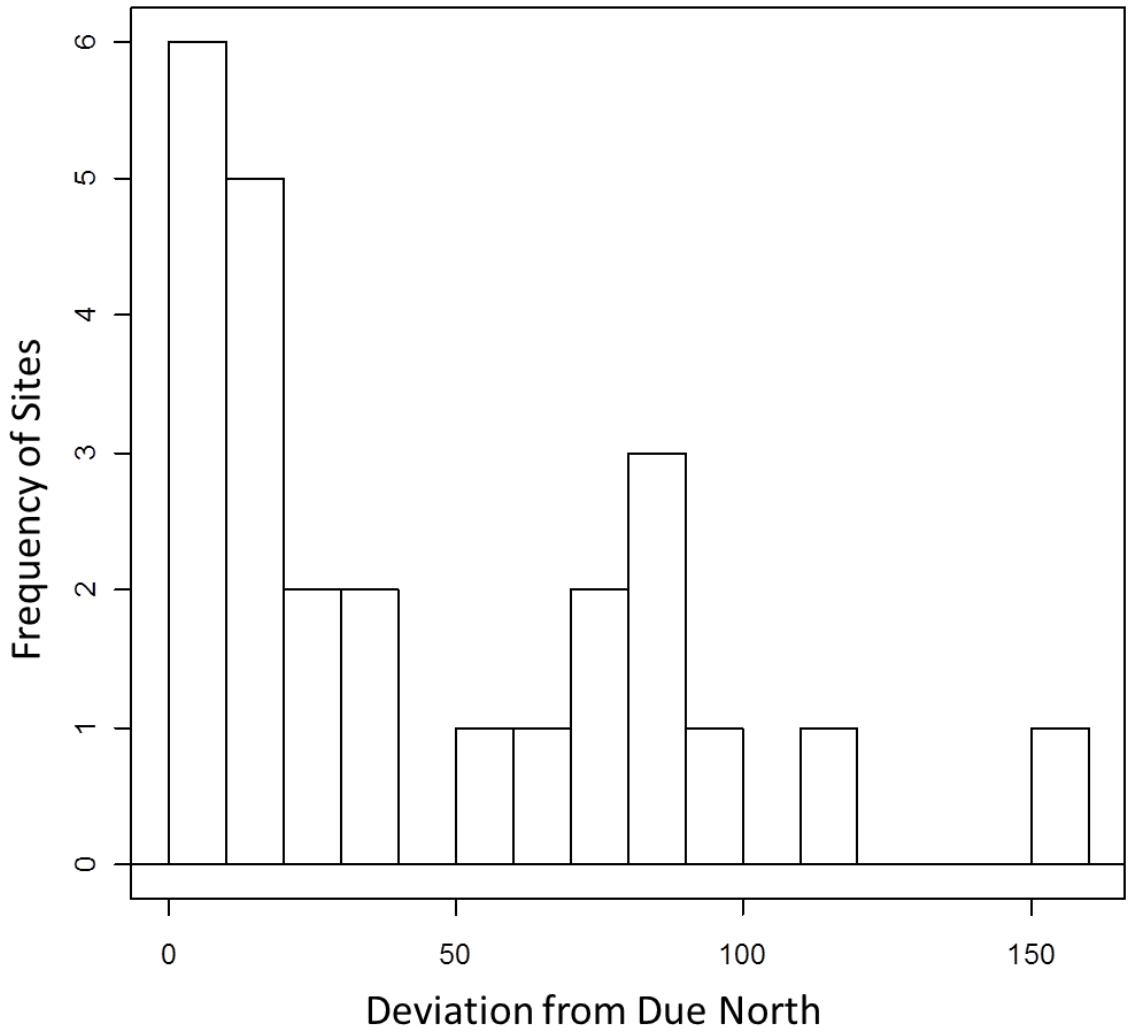

Figure 7: Distribution of site aspect deviation from north. Distribution was bimodal, which was used to separate the sites into ‘northerly’ and ‘southerly’ aspect categories (Table 4).

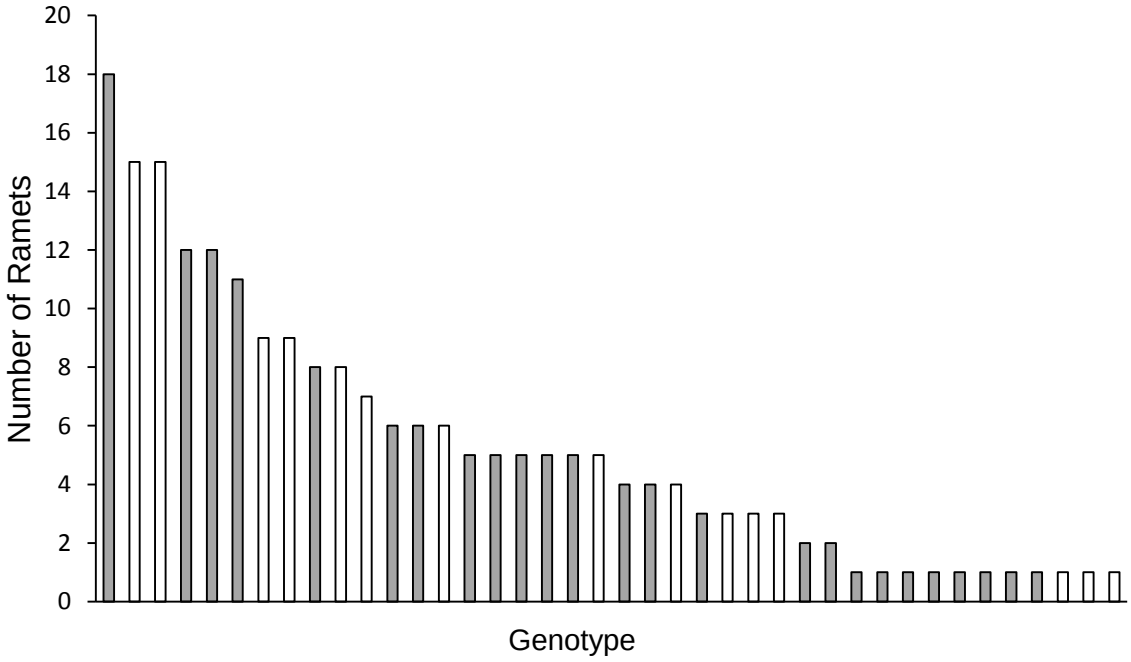

Figure 8: Distribution of the number of ramets sampled per genotype. Vertical bars represent the 40 genotypes from my sample population. Due to my sampling strategy, the total number of ramets sampled for a genotype was unlikely to surpass 20. Genotypes from northerly sites are shaded grey and genotypes from southerly sites are white**.**

4.44. The median was 4.50, indicating slight inflation of the mean by several genets with relatively large numbers of sampled ramets.

Sudden Aspen Decline

Health data from Zegler (2011) used to compare my estimates of genetic diversity with indicators of SAD is shown in

Table **1**. In my subset of sites elevation was not related to percent mortality (TPH) (R^2 = 0.002; p = 0.831), percent mortality (BA) (R^2 = 0.028; p = 0.421), nor the proportion of trees with greater than 33% crown dieback and (R^2 = 0.002; p = 0.830). Aspect deviation from due north also did not appear to be important to mortality (R^2 = 0.008; p = 0.669). While elevation did not appear to be an important explanatory variable, regression analyses revealed relationships between heterozygosity and site health (Figure 9).There were a positive relationships between mean heterozygosity of each sample site and percent mortality (TPH) (R^2 = 0.313; $p < 0.005$), and the proportion of trees from the site showing greater than 33% crown dieback (R^2 = 0.296; $p < 0.005$). This can be thought of as a negative relationship between mean percent heterozygosity and 'light' crown dieback (less than 33%). The relationship was weaker between heterozygosity and percent mortality (BA) was marginally significant (R^2 = 0.119; p = 0.0509) (Figure 9). The relationship between heterozygosity and percent mortality was similar whether sites showing triple alleles were included or excluded (R^2 = 0.327; p = 0.033), though it was less pronounced with the proportion of trees with >33% crown dieback when sites with triple alleles were removed (R^2 = 0.220; p = 0.091).

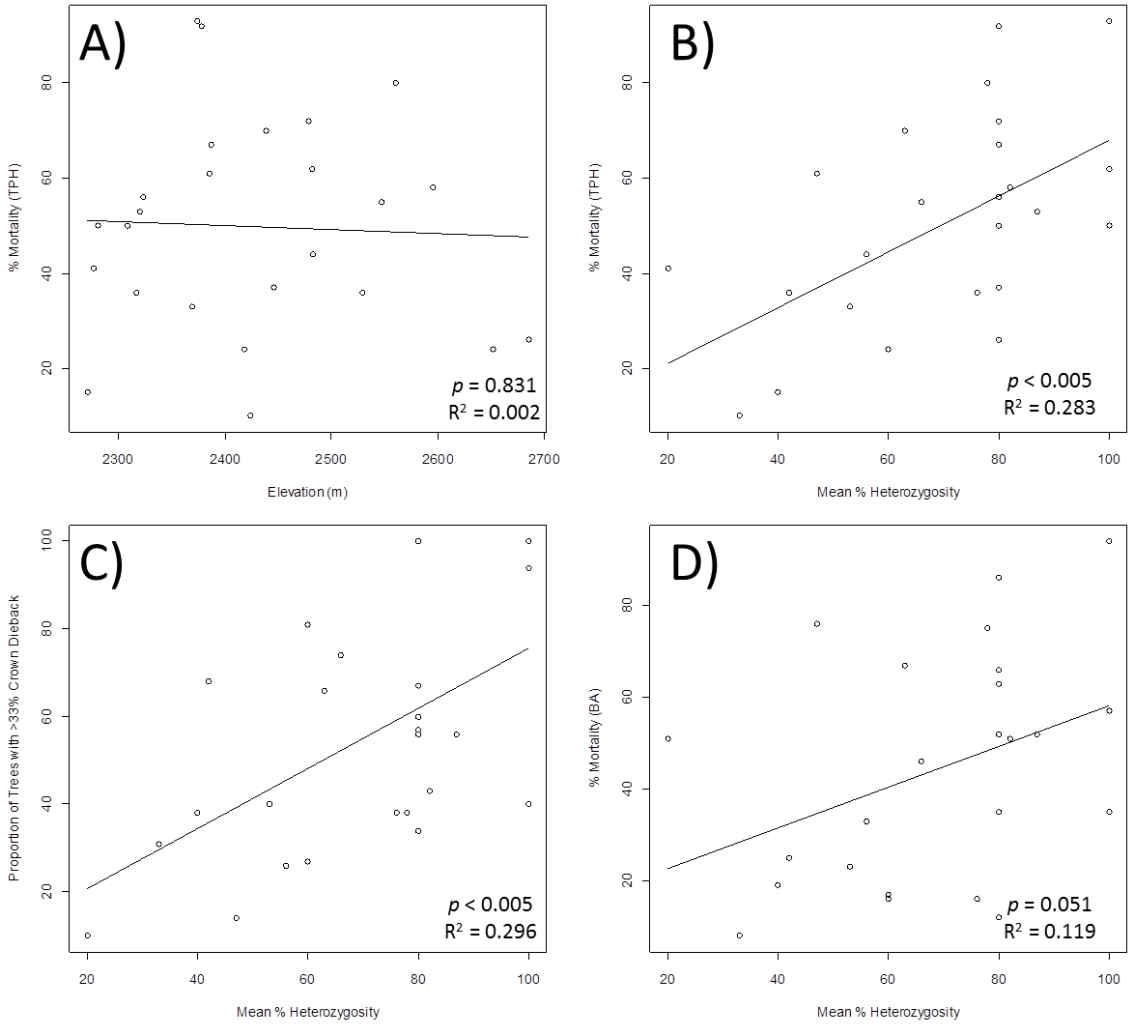

Figure 9: Relationships of health measurements for sample sites (n = 25) from my study region in Kaibab National Forest AZ. (A): Relationship between percent mortality (TPH) and sample site elevation (p = 0.831). (B): Relationship between percent mortality (TPH) and mean heterozygosity of sample sites (p < 0.005). (C): Relationship between the proportion of trees from each site with >33% crown dieback and mean heterozygosity of sample sites (p < 0.005). (D): Relationship between percent mortality (BA) and mean heterozygosity of sample (n = 25). Percent mortality (BA) values were arcsine transformed for normality (p = 0.051).

Triple Alleles

A large proportion of MLGs showed three allele peaks for one or more loci. Of the 40 genets found in the study, 11 had triple alleles at least one locus, and three had triple alleles at two loci. The 11 genets with triple alleles were represented by 85 out of 212 total samples, and the three genets with triple alleles at two loci were represented by 25 samples. Hence, about 40% of my sampled ramets and 28% of the genotypes showed triple alleles, with 11.8% of samples and 7.5% of genets showing triple alleles at two loci. Each marker except GCPM970-1 and PMGC576 showed three alleles in at least one sample, and the frequency of triple alleles for a locus showed a loose positive relationship to the number of alleles observed for the locus (R^2 = 0.476; ns). Instances of triple alleles for each locus was similarly related to mean heterozygosity for the locus (R^2 = 0.418; ns). For the entire population, the total number of alleles was lower when samples showing triple alleles were excluded (

Table **3**).

Comparisons between sites with and without triple allele genets demonstrated several differing tendencies. Sites with triple alleles (*n* = 11) had higher mean heterozygosity (mean = 0.799 ± 0.130) than sites without (*n* = 14) (0.602 ± 0.226) (Welch Two Sample t-test *t* = -2.731; *p* = 0.006). Sites with triple alleles also had higher percent mortality (mean = 0.626 ± 0.219) than sites that were without triple alleles (mean = 0.397 ± 0.171) (Two-Sample t-test: *t* = -2.947; *p* = 0.004). Similarly, sites with genets showing triple alleles tended to have higher proportions of trees with greater than 33% crown dieback than sites without, (means = 0.613 ± 0.265 and 0.489 ± 0.262, respectively) though the difference was not significant (Two Sample t-test: *t* = -1.170; *p* = 0.127).

Genets with triple alleles showed distributional patterns in regard to genotypic diversity. Genotypes showing triple alleles were more frequent in monoclonal sites than in multiclonal sites (Pearson's Chi Square test: $\chi^2 = 9.598$; $p = 0.002$). They also tended to be larger, with a higher average number of ramets (mean = 7.182 ± 5.36) than clones without triple alleles (mean = 4.552 ± 3.906; Two sample t-test: $t = -1.713$; $p = 0.047$). Genets with triple alleles made up a greater proportion of genets from the southerly sites (5 out of 13) than the northerly sites (6 out of 27), although the difference was not significant (Pearson's Chi Square test: $\chi^2 = 1.161$; $p = 0.2813$).

Discussion

Genetic Diversity and Clonal Structure

Low P_{gen} values for multilocus genotypes (Table 4) demonstrated that the five microsatellite markers used in this study provided sufficient strength to conclude that trees from the same site sharing the same multilocus genotype were ramets of the same genet. (Parks and Werth 1993; Suvanto and Latva-Karjanmaa 2005). Removal of the least polymorphic marker (GCPM970-1) from the dataset increased the mean P_{gen} value to 0.0018, however, all values were still below 0.01. So while five markers improved the statistical strength of the clonal assignments, my four most polymorphic markers would have been sufficient with an acceptable threshold for error of 0.01. These results are consistent with results from Dayanandan *et al*. (1998) that four microsatellite loci were sufficient for identification of most aspen clones. This method of genet assignment can result in over splitting of genets that have undergone somatic mutations (Mock *et al*. 2008). This may have occurred for at least two pairs of MLGs from the sites BWM187 and GM3 (APPENDIX A), which had relatively low pair-wise distances and came from the same sample site. P_{gen} values recalculated from only the matching loci of the two pairs were 0.0167 and 0.032. Thus is it possible that these two MLGs differed only by somatic mutation, and did not represent unique clones. Likely, the inclusion of more loci would clarify these uncertain clone assignments. So while four or five loci provide confident assignment for identical MLGs, more markers are likely needed to confidently address highly similar but unique genotypes. It should be noted that the two sites where the similar genotype pairs were found each had at least one additional, more genetically distant genotype, so that assignment of the sites as multiclonal sites would not change if the pairs were considered a single genet.

Studies have shown that extensive clonal growth can lead to reduced genotypic diversity and elevated heterozygosity. Eriksson (1989) proposed that in the absence of seedling recruitment, a clonal population will lose genotypic diversity due to selective elimination of genotypes as highly competitive clones exclude less competitive ones. Competition with persistent clones may then further reduce seedling establishment (Jelinski and Cheliak 1992). These factors will reduce genotypic diversity. Consistent with expectations of limited seedling recruitment and high rates of clonal reproduction of aspen in the relatively xeric western United States (Kemperman and Barnes 1976; Jelinski and Cheliak 1992), genotypic diversity was low among my sample sites (mean = 0.184). This result was comparable to Mock *et al*. (2008) who reported G/N values of 0.107 and 0.233 from two study regions in Southern Utah. From the east and west sides of the Rocky Mountain National Park, Tuskan *et al*. (2008) reported genotypic diversities of 0.291 and 0.383. These values from the western United States contrast with studies of populations from higher latitude eastern regions. In northwestern Quebec, Canada, Namroud *et al*. (2005) reported G/N values of 0.63 to 0.64, and proposed their sample populations had historically undergone high rates of seedling recruitment. Also from northwestern Quebec, Wyman *et al*. (2003) reported even higher genotypic diversities ranging from 0.733 to 0.917. Thus, estimates of genotypic diversity from this study, along with other studies are consistent with predictions of lower genotypic diversity from regions less suited to aspen seedling establishment.

Further evidence of the role of seedling recruitment in shaping clonal dynamics lies in comparison of proportions of single ramet clones. Namroud *et al*. (2005) reported 74.7 and 82.4% of genets had only a single ramet. From their sample populations of *P. tremula* in northeastern Finland, Suvanto and Latva-Karjanmaa (2005) reported 63% and 69% incidence of single ramet clones in old growth forests, and 78% in a managed

forest, with no clone exceeding 12 ramets in size. From these small clone sizes they concluded that most clones were young, and sexual reproduction was common. Studies of western populations reported much lower instances of single ramet clones, with 38.1 and 46.7% from Mock *et al.* (2008), and 27.5% from this study (Figure 8). AMOVA between my sites revealed 73% variation between sample sites. High genetic variation between sample sites was not surprising as the majority (17 out of 25; 68%) of my sample sites were monoclonal, and further indicates a historically high degree of clonal reproduction in my study area.

According to the "somatic mutation theory of clonality" described in Klekowski (1997) and addressed in Eckert *et al.* (1999) and Ally *et al*. (2010), extensive clonal growth will result in low sexual reproductivity, as genets accumulate somatic mutations in the absence of selection for sexual fitness. Similarly, in a diploid population reproducing exclusively clonally, it can be expected that without recombination, alleles of neutral markers will diverge independently and irreversibly through somatic mutation, resulting in a general increase in heterozygosity (Binky 1996; Halkett *et al*. 2005). This is sometimes referred to as the "Meselson effect," and predicts greater heterozygosity with increasing duration of clonal lineages (Balloux *et al*. 2003; Halkett *et al*. 2005). The result is that high rates of perpetuated clonal reproduction will positively affect heterozygosity (Balloux *et al*. 2003; Bengtsson 2003). Gross *et al*. (2011) found accumulation of somatic mutation contributed significantly to the genetic diversity of asexually reproduced populations of the shrub *Grevillea rhizomatosa* Olde and Marriott. High rates of heterozygosity found by Cheliak and Dancik (1982), and Jelinski and Cheliak (1992) have been attributed to mutation accumulation associated with very limited seedling recruitment and high average clone age (Wyman *et al*. 2003). Higher rates of seedling recruitment and succession among clonal plants are expected to decrease mutation

accumulation and thus lower heterozygosity (Jelinski and Cheliak 1992; Bengtsson 2003). Heterozygosity in this study was high (mean H_{obs} = 0.662 ±0.218 and mean H_{exp} = 0.630 ± 0.194), and contrasted with values from studies of aspen populations from more eastern higher latitude regions: Namroud *et al*. (2005) reported mean H_{obs} values ranging from 0.45 to 0.48, and Wyman *et al*. (2003) found a mean H_{obs} of 0.556, considerably lower than mean H_{exp} of 0.718. Populations of *P. tremula* in Finland showed even lower levels of microsatellite heterozygosity, with mean H_{obs} and H_{exp} of 0.348 and 0.407, respectively (Suvanto and Latva-Karjanmaa 2005).

Thus, among my sample sites from the Kaibab National Forest, AZ I found low genotypic diversity, low incidence of single ramet clones, and high heterozygosity. Each of these results is consistent with expected associations of low G/N and high heterozygosity with greater degrees of clonal reproduction, and the understanding that successful aspen seedling recruitment in the west is infrequent compared with other regions within its range. These comparisons between eastern and western aspen populations demonstrate how influences of geography and climate on seedling recruitment can shape the genetic diversity and clonal structure of aspen populations.

If climate and resulting suitability for aspen seedling establishment influence clonal structure on a regional scale, it seems reasonable that similar effects may be observed at smaller scales. The direction a slope faces can result in different temperature and moisture conditions, which can cause variation in biological composition and structure in montane communities (Birdsell and Hamrick 1978; Robichaud 2000; Bennie *et al*. 2006). In the northern hemisphere, north facing slopes tend to have lower temperature and higher moisture content (Pearson 1920). Moisture is a principal factor limiting successful recruitment of desiccation-intolerant aspen seedlings (Romme et al. 2001), so it is probable that aspect and moisture affect clonal

structure by controlling rates of seedling recruitment. Jelinski and Cheliak (1992) propose that moister environments more suitable to seedling establishment are likely to possess younger aspen clones than drier habitats less suitable for recruitment. Trends between aspect and the genotypic diversity of my sample sites supported this proposition. The mean aspect divergence from due north of my eight multiclonal sites was significantly lower than the monoclonal sites (Figure 6). Thus, more genotypically diverse sites tended to have northerly aspects. This suggests that the cooler, moister, northerly slopes support greater rates of seedling recruitment. The higher proportion of single ramet clones coming from the northerly sample sites (33.33% of the northerly clones) compared with 15.4% of clones from southerly sites further supports this suggestion. Sites with multiple genotypes also tended to have lower mean heterozygosity, though the difference was not significant (Figure 6). Returning to the "Meselson effect," lower heterozygosity among the multiclonal sites is consistent with expected relationships between genotypic diversity, heterozygosity, clone age, and rates of seedling recruitment. The genotypically diverse (multiclonal) sites tended to be northerly, where higher moisture would benefit sexual recruitment, and monoclonal sites tended to be more heterozygous, as would be expected from accumulation of somatic mutations during exclusive clonal growth (Balloux *et al*. 2003; Bengtsson 2003; Halkett *et al*. 2005). This study and others have suggested that on a geographic scale, the relative importance of sexual and clonal reproduction may vary based on rates of successful sexual reproduction in different environments (Eckert 2002). These comparisons between the monoclonal and multiclonal sites in my study area suggest a similar pattern is occurring at a more local scale.

Because sites with signs of higher seedling recruitment (the multiclonal sites) tended to have northerly aspect, I tested the converse relationship that northerly aspect

sites tended to have signs of higher seedling recruitment. Though not statistically significant, the comparisons did not contradict this expectation, with higher mean genotypic diversity and lower mean heterozygosity among the northerly sites (Table 5; Figure 7). However, these trends may have simply been due to a large majority (all but one) of multiclonal sites sorting into the northerly aspect category. This suggests that while northerly aspect increases the probability of seedling recruitment it does not alone seem sufficient to significantly increase genotypic diversity. As five of the eight multiclonal sites showed three or more genotypes, it seems likely that these sites had characteristics in addition to northerly aspect that made them particularly well suited for genet establishment. Historical canopy opening disturbance is a probable candidate, because aspen seedlings also require minimal light competition for successful development (McDonough 1985). This is consistent with required “windows of opportunity” for seedling establishment described in Jelinski and Cheliak (1992) in which a combination of factors including high moisture levels and noncompetitive conditions must coincide to enable seedling survival and development.

Heterozygosity, Demography and SAD

Drought is considered both a predisposing and inciting factor in SAD (Worrall 2010). In western areas such as the Rocky Mountains and Arizona, higher elevations generally have lower temperatures and higher moisture levels (Pearson 1920). Zegler (2011) found that elevation was a significant explanatory variable for both aspen crown dieback and percent mortality, with dieback and mortality decreasing with increasing elevation. The sites sampled in this study were only a subset of those from Zegler (2011); representing the middle elevational range of aspen in the study area. For my subset of sites, univariate regressions indicated elevation was not a significant factor in the variation of mortality or crown dieback, likely because the range of elevation was too small to detect the elevational trends in the health measurements reported by Zegler (2011). There was, however, a significant relationship between observed heterozygosity and both percent mortality and the proportion of trees with greater than 33% crown dieback, with mortality and dieback increasing with increasing heterozygosity (Figure 9). So while elevation was a key factor in explaining variation in site health throughout the entire elevational range, within the middle elevational range observed heterozygosity was a stronger explanatory variable. Jelinski and Cheliak (1992), Mitton and Grant (1980; 1996), and Cole *et al*. (2010) all found positive relationships between heterozygosity and growth rate. As growth rate is often used as an analog for fitness (Mitton and Grant 1980), I expected to find a negative relationship between heterozygosity and signs of SAD. However, my results indicated the reverse relationship, with both crown dieback and percent mortality increasing with mean heterozygosity ($p < 0.005$). This relationship can be explained in the context of clonal growth theory.

I have discussed how my results of increasing heterozygosity and decreasing genotypic diversity suggest higher degrees of clonal reproduction, which is supported by other studies of aspen genetic diversity. Among aspen clones, extensive clonal growth may also lead to poor fitness. With low genotypic diversity expected from extensive clonal reproduction (Eriksson 1989; Balloux *et al*. 2003), fewer unique combinations of alleles will be available for selection, resulting in diminished adaptation rates. Also, because of the high potential age of genets, and competitive advantages of already established clones, ancient clones may persist and exclude seedling recruitment despite being poorly adapted to current conditions (Jones and DeByle 1985a; Jelinski and Cheliak 1992). Because it occurs without recombination of genes, exclusive clonal reproduction renders the population dependent on fixation of beneficial mutations for adaptation (Honnay and Bossuyt 2005), which likely reduces adaptability to long term climate changes (McDonough 1985). Each of these effects of extensive clonal growth could result in poor adaptation among older aspen clones to current environmental conditions. As older clonal lineages are also likely to show high levels of heterozygosity (Birky 1996; Balloux *et al*. 2003; Bengtsson 2003; Halkett *et al*. 2005), poor fitness of older aspen clones in regard to SAD offers an explanation for the positive relationship between heterozygosity and stand degradation (Figure 9). Klekowski (2003) argues that even with selection among ramets, mutation accumulation within a clone may slowly degrade fitness, leading to decreases in clone size in which genetic drift would further outstrip selection leading slowly to extinction of the ramets. Lynch *et al*. (1993) describes a similar process among asexual populations as “mutational meltdown,” which occurs because of the population’s inability to reconstitute highly fit genotypes through sexual recombination. These views impose a sort of senescence among clones due to mutation accumulation. Mutational meltdown would result in a positive relationship between

heterozygosity and lower fitness similar to those reported in this study. Thus, among aspen stands that have undergone extensive clonal reproduction, such as appears to be the case in my study area particularly among the southerly sites, we may expect to see not only low genotypic diversity and high heterozygosity, but possibly lower fitness. Lower fitness could result from mutation accumulation, suboptimal adaptation to current environmental conditions or both. Therefore, the positive relationship between mean heterozygosity and both percent mortality and crown dieback ($p < 0.005$;) found in this study may result from the combination of increased heterozygosity and reduced fitness of stands that have undergone many generations of ramet turnover in the absence of seedling recruitment. I have suggested that the multiclonal sites from this study have experienced higher levels of genet establishment because of more suitable conditions for seedling recruitment. If this suggesting is true, and older clonal lineages do indeed show higher heterozygosity and lower fitness, we would expect the multiclonal sites in my study to have lower heterozygosity and greater fitness. Though neither difference was statistically significant, comparisons between the multiclonal sites and monoclonal sites in my study did not contradict this expectation, with lower mean heterozygosity and percent mortality among the multiclonal sites (Figure 6).

While observed heterozygosity was significantly related to measurements of site health, it only explained a limited proportion of the variation (R^2 ranged 30 – 31%). Zegler (2011) was able to explain 78% of percent mortality by basal area and 34% of overstory crown dieback using multivariate models with explanatory variables not included in my study. Zegler's (2011) explanatory factors for percent mortality were forest type, percent of overstory basal area that was coniferous, and percent incidence of grouped canker diseases. Explanatory factors for crown dieback were elevation and percent incidence of grouped canker diseases. It is likely that some of these variables

covary with genotype, indicating genetically based susceptibilities to damaging agents, differential selection among forest types, or environmentally influenced demography and clonal structure. For example, it has been shown that in montane environments in the west, fire suppression may lead to encroachment by shade tolerant conifers (Cocke *et al.* 2005). Fire suppression is also considered a principal factor in aspen decline (Bartos and Campbell 1998). Disturbance can be highly beneficial to aspen seedling recruitment and can encourage genotypic diversity (Kay *et al*. 1993; Stevens *et al*. 1999). Therefore, fire suppression could lead to both increased heterozygosity through extensive clonal growth such as supported by my results and conifer encroachment through succession. In this way, conifer encroachment, increased heterozygosity, and poor health could be linked. “Mutational meltdown”’ (Lynch *et al.* 1993; Klekowski 2003) causes reduced fitness through accumulation of deleterious somatic mutations, and would cause an association between poor health and heterozygosity. Reduced fitness, which was positively related to heterozygosity in my study, could be manifest in susceptibility to disease (Lynch *et al*. 1993) or possibly damaging agents, such as the canker diseases measured by Zegler (2011). Copony and Barnes (1974) found that clones from the same area varied markedly in their susceptibility to hypoxylon canker, and French and Hart (1978) confirmed their observation by inoculating clones in the field. Therefore, it is reasonable that genetic diversity described in my study and Zegler’s (2011) explanatory variables could be interconnected in their associations with signs of SAD.

Triple Alleles

Many (27.5%) of the MLGs in this study showed triple alleles, which is consistent with results from Mock *et al*. (2008), where 15% and 28% of observed genets from two study sites showed triple alleles. Mock *et al*. (2008) concluded that consistent amplification of triple alleles from ramets suggests the triple alleles are biological and not the result of laboratory error, and recommend chromosome counts to resolve whether triple alleles are due to polyploidy or aneuploidy resulting from somatic mutation. While I cannot reject the explanation of full triploidy, several pieces of evidence support somatic aneuploidy as the mechanism behind the triple alleles. Here somatic aneuploidy refers to somatic mutations occurring within clonal lineages that result in duplications of examined markers.

With robust methods of genet assignment, Mock *et al*. (2008) were also able to identify four instances of somatic mutation resulting in triple alleles, indicating that somatic aneuploidy does occur within aspen genets. If the triple alleles result from accumulation of somatic aneuploidy, they should appear more often in older clones that have undergone extensive clonal reproduction. Mock *et al*. (2008) found genets with triple alleles tended to be larger, with a higher average number of ramets. This was also true in my dataset ($p = 0.047$), suggesting that genets with triple alleles tend to have undergone greater clonal growth and may be of greater age (Kemperman and Barnes 1976). Mock *et al*. (2008) also observed that genets with triple alleles were common among spatially fragmented clones. Fragmented clones are likely to be older because older clones have a greater probability of being fragmented by historical small scale disturbances. Hence, preference of triple alleles among fragmented clones also suggests they tend to occur in older clones which would also be more likely to have undergone somatic mutations resulting in aneuploidy. I have proposed the multiclonal

sites in this study had characteristics that made them particularly well suited for seedling establishment, and that the genets found among them are likely younger than those from monoclonal sites. Therefore, if triple alleles result from somatic mutation, triple alleles should be more frequent among the older clones of the monoclonal sites than the multiclonal sites. My results supported this, with significantly fewer (only 2 of the 11) genets showing triple alleles came from multiclonal sites. I also attributed the relationship between heterozygosity and poor health to lower fitness among older genets that have undergone extensive clonal reproduction. I found significantly higher percent mortality among genets with triple alleles. This is consistent with the positions that older clones tend to have triple alleles because of accumulation of somatic aneuploidy, and that older clones have reduced fitness in response to SAD.

Summary and Conclusions

I have shown that in the Williams Ranger District of Kaibab National Forest multiclonal sites tended to have northerly aspect, stand degradation was positively related to microsatellite heterozygosity, and that genets with triple alleles tended to be found among monoclonal sites and show poor health. All three results were explained largely in the context of clonal dynamics based on varying degrees of sexual and asexual reproduction (Table 6). I attributed the tendency of multiclonal sites to have northerly aspect to higher rates of seedling establishment on moister northerly slopes. From this I concluded that small scale variation in environmental conditions within my study area shaped clonal structure. The positive relationship between heterozygosity and site degradation was attributed to excessive clonal reproduction among some genets, which could lead to both increased heterozygosity and poor fitness. Thus, my data suggest that at middle elevations, clone age may increase susceptibility to SAD. Evidence from my study and Mock *et al*. (2008) suggests accumulation of somatic aneuploidy as the mechanism behind consistent observation of triple alleles. From this it seems that like heterozygosity, increased incidence of triple alleles may be indicative of persistent clonal growth and old age among aspen clones in the west. If, as this study suggests, excessive clonal reproduction and ancient clones are linked with poor fitness, increasing the prevalence of younger, better adapted genotypes should improve aspen health in the Kaibab National Forest. This could be accomplished by encouraging seedling recruitment with planned canopy opening disturbances. In addition to encouraging sexual reproduction, transplanting ramets from healthier clones to other sites where they could clonally propagate would increase local genotypic diversity while bypassing the challenges of successful seedling recruitment in drier sites in the study areas. Each of these strategies could encourage the spread of younger clonal lineages

Table 6: Summary of relationships expected with increasing prevalence of clonal and sexual reproduction, relevant citations, and supporting results. Superscripts indicate references supporting expectations of characteristics. [1](Eckert 2002), [2](Balloux 2003), [3](Eriksson 1989), [4](Namroud *et al*. 2005), [5](Jelinski and Cheliak 1992), [6](Kemperman and Barnes 1976), [7](Mock *et al*. 2008), [8](Birky 1996), [9](Halkett *et al*. 2005), [10](Jones and DeByle 1985a), [11](Klekowski 2003), [12](Lynch *et al*. 1993).

Characteristic	**Sexual Reproduction**	**Clonal Reproduction**	**Supporting Results**
Seedling Recruitment[1]	higher	lower	Genets with triple alleles more frequent among monoclonal sites (p = 0.002)
G/N[2,3]	higher	lower	Multiclonal Sites tended to have northerly aspect (p = 0.005)
Aspect	‘northerly’	'southerly'	Genets with triple alleles more frequent among monoclonal sites (p = 0.002)
Single Ramet Clones[4]	more common	less common	Single Ramet Clones more frequent among Northerly sites (ns)
Clone Age[5]	younger	older	
Clone size[6]	smaller	larger	Genets from monoclonal sites were larger ($p = 0.003$) Genets with triple alleles were larger (p = 0.047)
Clone Fragmentation[7]	less common	more common	Triple alleles were common among fragmented clones (Mock *et al*. 2008)
Heterozygosity[2,8,9]	lower	higher	Lower heterozygosity among multiclonal sites (p = 0.062)
Fitness[5,10,11,12]	higher	lower	Relationship between heterozygosity and site degradation (p < 0.005)
Triple alleles[7]	less common	more common	Higher mortality among genets with triple alleles (p = 0.004)

in the study area, and, given the conclusions from this study, would likely improve the resilience of the overall population to SAD.

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APPENDIX A: Pair-wise Genetic Distance Matrix Between All Genotypes (triple alleles excluded)

BWM 108	BWM187a	BWM187b	BWM75a	BWM75b	GH 179a	GH 179b	GH 42a	GH 42b	GH 42c	GH23	GH80a	GH80b	GM 170a	GM 170b	GM 3a	GM 3b	GM 3c	GM 3d	NT133	SGM196	SVT 100	SVT 101	SVT 102	SVT 99	TT 28a	TT 28b	TT 28c	
0																												BWM 108
7	0																											BWM187a
6	2	0																										BWM187b
7	11	10	0																									BWM75a
3	8	8	5	0																								BWM75b
4	9	8	9	3	0																							GH 179a
6	10	9	9	7	7	0																						GH 179b
7	15	11	7	6	4	7	0																					GH 42a
7	11	7	7	7	5	8	4	0																				GH 42b
5	11	9	9	5	5	8	6	4	0																			GH 42c
4	6	5	4	5	5	4	6	5	7	0																		GH23
11	12	11	12	11	11	5	10	12	12	7	0																	GH80a
9	10	9	8	6	8	9	7	8	7	8	6	0																GH80b
4	7	10	10	4	5	8	11	10	7	7	14	11	0															GM 170a
7	10	10	3	7	9	6	8	7	8	3	9	8	9	0														GM 170b
7	14	11	7	5	4	8	2	4	5	6	8	4	10	7	0													GM 3a
8	11	8	7	6	3	9	4	2	6	5	12	7	10	7	3	0												GM 3b
2	10	7	7	4	5	6	5	6	6	5	10	8	7	8	5	7	0											GM 3c
8	7	9	8	7	5	7	7	8	8	6	9	7	6	7	8	7	10	0										GM 3d
10	12	9	6	8	7	9	3	5	8	6	9	6	14	7	4	5	8	7	0									NT133
4	4	3	10	5	3	8	9	6	7	4	11	9	6	10	8	5	5	7	10	0								SGM196
9	17	13	8	9	8	10	4	6	7	8	13	11	14	9	6	8	8	10	5	12	0							SVT 100
10	14	14	11	9	8	8	8	10	8	8	10	11	11	9	9	11	12	7	8	12	8	0						SVT 101
3	10	9	6	4	4	7	7	7	5	4	11	9	6	6	6	6	4	8	9	5	9	10	0					SVT 102
8	8	7	7	7	5	8	5	5	7	4	10	8	9	7	6	5	9	4	5	6	7	4	8	0				SVT 99
9	11	9	13	8	8	7	11	11	12	8	12	11	13	12	10	9	9	14	11	8	15	15	10	12	0			TT 28a
6	12	11	7	5	4	5	5	4	5	4	8	8	6	5	3	4	6	7	8	7	8	8	5	6	11	0		TT 28b
6	14	10	8	6	3	8	3	3	5	6	13	10	8	9	4	3	5	8	7	6	6	10	5	6	12	4	0	TT 28c

Distance scoring method for each locus where *i* and *j* represent different alleles of a single locus. Total pairwise distance represents the sum of the distances for each of the five loci.

- (*ii, ii*) = 0
- (*ij, ij*) = 0
- (*ii, ij*) = 1
- (*ij, ik*) = 1
- (*ij, kl*) = 2
- (*ii, jk*) = 3
- (*ii, jj*) = 4

APPENDIX B: Table of Multilocus Genotypes for All Genotyped Samples

The three numbered columns below each locus represent alleles. Alleles are given as their estimated size in base-pairs.

			WPMS15			GCPM970-1			PMGC576			WPMS14			PMGC2571		
Sample	**Site**	**Subplot**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
9g	ANH12	N	182	191		117	120		150	150		203	212	230	101	113	
1c	BWM108	E	182	191		120	120		162	162		203	206		97	101	
1d	BWM108	E	182	191		120	120		162	162		203	206		97	101	
1l	BWM108	E	182	191		120	120		162	162		203	206		97	101	
1m	BWM108	E	182	191		120	120		162	162		203	206		97	101	
1n	BWM108	E	182	191		120	120		162	162		203	206		97	101	
1a	BWM108	N	182	191		120	120		162	162		203	206		97	101	
1b	BWM108	N	182	191		120	120		162	162		203	206		97	101	
1e	BWM108	N	182	191		120	120		162	162		203	206		97	101	
1f	BWM108	N	182	191		120	120		162	162		203	206		97	101	
1g	BWM108	N	182	191		120	120		162	162		203	206		97	101	
1h	BWM108	S	182	191		120	120		162	162		203	206		97	101	
1i	BWM108	S	182	191		120	120		162	162		203	206		97	101	
1j	BWM108	S	182	191		120	120		162	162		203	206		97	101	
1k	BWM108	S	182	191		120	120		162	162		203	206		97	101	
1o	BWM108	W	182	191		120	120		162	162		203	206		97	101	
1p	BWM108	W	182	191		120	120		162	162		203	206		97	101	
1q	BWM108	W	182	191		120	120		162	162		203	206		97	101	
1r	BWM108	W	182	191		120	120		162	162		203	206		97	101	
6v	BWM159	N	182	191		120	120		150	168		206	230		85	95	103
6x	BWM159	N	182	191		120	120		150	168		206	230		85	95	103

6y	BWM159	N	182	191	120	120	150	168	206	230		85	95	103
6z	BWM159	N	182	191	120	120	150	168	206	230		85	95	103
7a	BWM159	N	182	191	120	120	150	168	206	230		85	95	103
6r	BWM159	S	182	191	120	120	150	168	206	230		85	95	103
6s	BWM159	S	182	191	120	120	150	168	206	230		85	95	103
6t	BWM159	S	182	191	120	120	150	168	206	230		85	95	103
6u	BWM159	S	182	191	120	120	150	168	206	230		85	95	103
6w	BWM159	S	182	191	120	120	150	168	206	230		85	95	103
6o	BWM159	W	182	191	120	120	150	168	206	230		85	95	103
6p	BWM159	W	182	191	120	120	150	168	206	230		85	95	103
6q	BWM159	W	182	191	120	120	150	168	206	230		85	95	103
7b	BWM159	W	182	191	120	120	150	168	206	230		85	95	103
7c	BWM159	W	182	191	120	120	150	168	206	230		85	95	103
7p	BWM187	E	191	191	120	120	150	156	206	212	230	97	113	
7q	BWM187	E	191	191	120	120	150	156	206	212	230	97	113	
7r	BWM187	E	191	191	120	120	150	156	206	212	230	97	113	
7s	BWM187	E	191	191	120	120	150	156	206	212	230	97	113	
7t	BWM187	E	191	191	120	120	150	156	206	212	230	97	113	
7n	BWM187	N	191	191	120	120	150	156	206	212	230	97	113	
7m	BWM187	S	182	182	117	117	162	162	200	206		97	99	
7o	BWM187	S	182	182	117	117	162	162	200	206		97	99	
7u	BWM187	S	182	191	117	117	162	162	200	203		97	99	
8k	BWM190	E	182	191	120	120	150	162	203	209		97	99	113
8n	BWM190	E	182	191	120	120	150	162	203	209		97	99	113
8o	BWM190	E	182	191	120	120	150	162	203	209		97	99	113
8d	BWM190	N	182	191	120	120	150	162	203	209		97	99	113

8e	BWM190	N	182	191		120	120	150	162	203	209		97	99	113
8f	BWM190	N	182	191		120	120	150	162	203	209		97	99	113
8l	BWM190	N	182	191		120	120	150	162	203	209		97	99	113
8p	BWM190	S	182	191		120	120	150	150	203	209		97	99	113
8g	BWM190	W	182	191		120	120	150	162	203	209		97	99	113
8h	BWM190	W	182	191		120	120	150	162	203	209		97	99	113
8i	BWM190	W	182	191		120	120	150	162	203	209		97	99	113
8j	BWM190	W	182	191		120	120	150	162	203	209		97	99	113
8a	BWM75	S	191	191		117	120	150	168	206	206		93	101	
8b	BWM75	S	191	191		117	120	150	168	206	206		93	101	
7w	BWM75	W	191	191		117	120	150	168	260	206		93	101	
7x	BWM75	W	191	191		117	120	150	168	206	206		93	101	
7y	BWM75	W	191	191		117	120	150	168	206	206		93	101	
7z	BWM75	W	182	191		120	120	162	168	206	230		93	97	
8c	BWM75	W	191	191		117	120	150	168	206	206		93	101	
9h	ER4	N	191	194		120	132	150	162	203	206		85	99	113
5x	GH104	E	182	191	194	120	120	150	160	206	212		89	97	113
5w	GH104	N	182	191	194	120	120	150	160	206	212		89	97	113
5y	GH104	N	182	191	194	120	120	150	160	206	212		89	97	113
5z	GH104	N	182	191	194	120	120	150	160	206	212		89	97	113
6a	GH104	N	182	191	194	120	120	150	160	206	212		89	97	113
6b	GH104	N	182	191	194	120	120	150	160	206	212		89	97	113
6c	GH104	W	182	191	194	120	120	150	160	206	212		89	97	113
6d	GH104	W	182	191	194	120	120	150	160	206	212		89	97	113
6e	GH104	W	182	191	194	120	120	150	160	206	212		89	97	113
5u	GH179	E	191	191		120	132	150	160	206	209	224	97	97	

5v	GH179	E	191	191	120	132	150	160	206	209	224	97	97
5t	GH179	N	191	191	120	132	150	160	206	209	224	97	97
9i	GH179	N	191	191	120	132	150	160	206	209	224	97	97
5q	GH179	S	182	191	120	120	150	162	212	230		97	97
5s	GH179	S	182	191	120	120	150	162	212	230		97	97
5r	GH179	W	194	194	120	126	150	162	203	206		97	113
3f	GH42	E	191	191	120	120	150	150	203	230		97	113
3g	GH42	E	191	191	120	120	150	150	203	230		97	113
3j	GH42	E	191	191	120	120	150	150	203	230		97	113
3k	GH42	E	191	191	120	120	150	150	203	230		97	113
3l	GH42	E	191	191	120	120	150	150	203	230		97	113
3n	GH42	N	182	191	120	120	156	160	203	209		91	97
3o	GH42	N	182	191	120	120	156	160	203	209		91	97
3p	GH42	N	182	191	120	120	156	160	203	209		91	97
3q	GH42	N	182	191	120	120	156	160	203	209		91	97
3m	GH42	S	191	191	117	120	150	156	203	209		97	97
3r	GH42	S	182	191	120	120	156	160	203	209		91	97
3d	GH42	W	182	191	120	120	156	160	203	209		91	97
3e	GH42	W	182	191	120	120	156	160	203	209		91	97
3h	GH42	W	182	191	120	120	156	160	203	209		91	97
3i	GH42	W	182	191	120	120	156	160	203	209		91	97
8r	GH23	E	191	194	117	120	150	162	200	206		97	101
8s	GH23	E	191	194	117	120	150	162	200	206		97	101
8t	GH23	E	191	194	117	120	150	162	200	206		97	101
8u	GH23	E	191	194	117	120	150	162	200	206		97	101
8v	GH23	E	191	194	117	120	150	162	200	206		97	101

8w	GH23	W	191	194	117	120	150	162	200	206		97	101	
9b	GH6	E	182	191	120	120	150	162	203	206	230	85	99	107
9c	GH6	E	182	191	120	120	150	162	203	206	230	85	99	107
9d	GH6	E	182	191	120	120	150	162	203	206	230	85	99	107
9e	GH6	E	182	191	120	120	150	162	203	206	230	85	99	107
9f	GH6	E	182	191	120	120	150	162	203	206	230	85	99	107
7g	GH80	E	182	191	117	120	156	168	224	230		113	113	
7h	GH80	E	182	191	117	120	156	168	224	230		113	113	
7i	GH80	E	182	191	117	120	156	168	224	230		113	113	
7k	GH80	E	182	191	117	120	156	168	224	230		113	113	
7l	GH80	E	182	191	117	120	156	168	224	230		113	113	
7j	GH80	N	194	194	117	120	150	162	224	224		113	113	
7d	GH80	S	182	191	117	120	156	168	224	230		113	113	
7e	GH80	S	182	191	117	120	156	168	224	230		113	113	
7f	GH80	S	182	191	117	120	156	168	224	230		113	113	
3x	GM170	E	182	182	120	120	162	166	206	206		97	97	
3z	GM170	E	182	182	120	120	162	166	206	206		97	97	
4b	GM170	E	191	194	117	120	150	156	206	206		95	101	
3w	GM170	N	191	194	117	120	150	156	188	206		95	101	
4a	GM170	N	191	194	117	120	150	156	206	206		95	101	
3s	GM170	S	182	182	120	120	162	166	206	206		97	97	
3u	GM170	S	182	182	120	120	162	166	206	206		97	97	
3v	GM170	S	182	182	120	120	162	166	206	206		97	97	
4d	GM170	S	182	182	120	120	162	166	206	206		97	97	
4e	GM170	S	182	182	120	120	162	166	206	206		97	97	
3t	GM170	W	182	182	120	120	162	166	206	206		97	97	

3y	GM170	W	182	182		120	120	162	166	206	206	97	97
4c	GM170	W	182	182		120	120	162	166	206	206	97	97
4f	GM170	W	182	182		120	120	162	166	206	206	97	97
4g	GM170	W	182	182		120	120	162	166	206	206	97	97
6i	GM3	E	191	191		120	120	150	156	224	230	97	113
6k	GM3	E	191	191		117	120	150	156	212	230	97	97
6l	GM3	E	191	191		120	120	162	162	203	206	97	113
6m	GM3	E	191	191		120	120	162	162	203	206	97	113
6n	GM3	E	191	191		120	120	162	162	203	206	97	113
6g	GM3	N	191	191		120	120	150	156	224	230	97	113
6f	GM3	W	182	182		117	120	150	150	206	212	97	113
6h	GM3	W	182	182		117	120	150	150	206	212	97	113
6j	GM3	W	182	182		117	120	150	150	206	212	97	113
2j	GP89	S	182	191	194	120	126	150	162	206	212	97	113
2n	GP89	S	182	191	194	120	126	150	162	206	212	97	113
2o	GP89	S	182	191	194	120	126	150	162	206	212	97	113
2i	GP89	W	182	191	194	120	126	150	162	206	212	97	113
2k	GP89	W	182	191	194	120	126	150	162	206	212	97	113
2l	GP89	W	182	191	194	120	126	150	162	206	212	97	113
2m	GP89	W	182	191	194	120	126	150	162	206	212	97	113
2p	GP89	W	182	191	194	120	126	150	162	206	212	97	113
2d	NT130	E	182	191	194	120	120	150	162	203	206	97	99
2e	NT130	E	182	191	194	120	120	150	162	203	206	97	99
2f	NT130	E	182	191	194	120	120	150	162	203	206	97	99
1s	NT130	N	182	191	194	120	120	150	162	203	206	97	99
1w	NT130	N	182	191	194	120	120	150	162	203	206	97	99

1y	NT130	N	182	191	194	120	120	150	162	203	206	97	99		
2b	NT130	N	182	191	194	120	120	150	162	203	206	97	99		
2c	NT130	N	182	191	194	120	120	150	162	203	206	97	99		
1z	NT130	S	182	191	194	120	120	150	162	203	206	97	99		
2a	NT130	S	182	191	194	120	120	150	162	203	206	97	99		
2g	NT130	S	182	191	194	120	120	150	162	203	206	97	99		
2h	NT130	S	182	191	194	120	120	150	162	203	206	97	99		
1t	NT130	W	182	191	194	120	120	150	162	203	206	97	99		
1u	NT130	W	182	191	194	120	120	150	162	203	206	97	99		
1v	NT130	W	182	191	194	120	120	150	162	203	206	97	99		
9j	NT133	W	191	191		117	120	150	150	209	230	99	113		
2q	SGM68	E	182	191	194	120	126	150	156	203	206	93	97	113	
2z	SGM68	E	182	191	194	120	126	150	156	203	206	93	97	113	
2t	SGM68	N	182	191	194	120	126	150	156	203	206	93	97	113	
2w	SGM68	N	182	191	194	120	126	150	156	203	206	93	97	113	
2r	SGM68	W	182	191	194	120	126	150	156	203	206	93	97	113	
2s	SGM68	W	182	191	194	120	126	150	156	203	206	93	97	113	
2u	SGM68	W	182	191	194	120	126	150	156	203	206	93	97	113	
2x	SGM68	W	182	191	194	120	126	150	156	203	206	93	97	113	
2y	SGM68	W	182	191	194	120	126	150	156	203	206	93	97	113	
8y	SGM196	E	182	191		117	120	162	162	200	212	97	97		
9a	SGM196	E	182	191		117	120	162	162	200	212	97	97		
8x	SGM196	S	182	191		117	120	162	162	200	212	97	97		
8z	SGM196	S	182	191		117	120	162	162	200	212	97	97		
5c	SVT100	E	191	191		120	120	150	150	203	209	107	107		
5h	SVT100	N	191	191		120	120	150	150	203	209	107	107		

5i	SVT100	N	191	191	120	120	150	150	203	209	107	107
5j	SVT100	N	191	191	120	120	150	150	203	209	107	107
5k	SVT100	N	191	191	120	120	150	150	203	209	107	107
5a	SVT100	W	191	191	120	120	150	150	203	209	107	107
5b	SVT100	W	191	191	120	120	150	150	203	209	107	107
4t	SVT101	E	182	194	120	120	150	150	209	218	103	103
4p	SVT101	N	182	194	120	120	150	150	209	218	103	103
4q	SVT101	N	182	194	120	120	150	150	209	218	103	103
4r	SVT101	N	182	194	120	120	150	150	209	218	103	103
4s	SVT101	N	182	194	120	120	150	150	209	218	103	103
4v	SVT101	N	182	194	120	120	150	150	209	218	103	103
4u	SVT101	W	182	194	120	120	150	150	209	218	103	103
4w	SVT101	W	182	194	120	120	150	150	209	218	103	103
4x	SVT101	W	182	194	120	120	150	150	209	218	103	103
4y	SVT101	W	182	194	120	120	150	150	209	218	103	103
4z	SVT101	W	182	194	120	120	150	150	209	218	103	103
5n	SVT102	S	189	191	120	120	160	162	206	212	97	101
5o	SVT102	S	189	191	120	120	160	162	206	212	97	101
5p	SVT102	S	189	191	120	120	160	162	206	212	97	101
5d	SVT99	S	182	191	117	120	150	150	200	218	97	103
5e	SVT99	S	182	191	117	120	150	150	200	218	97	103
5m	SVT99	S	182	191	117	120	150	150	200	218	97	103
5f	SVT99	S	182	191	117	120	150	150	200	218	97	103
5g	SVT99	S	182	191	117	120	150	150	200	218	97	103
3a	TT28	E	191	194	126	126	162	162	230	230	89	97
3b	TT28	E	191	194	126	126	162	162	230	230	89	97

4m	TT28	E	191	194	126	126	162	162	230	230	89	97
4n	TT28	E	191	194	126	126	162	162	230	230	89	97
4o	TT28	E	191	194	126	126	162	162	230	230	89	97
3c	TT28	N	191	194	120	120	150	156	206	224	97	97
4h	TT28	S	191	191	120	120	150	166	203	212	97	97
4i	TT28	S	191	191	120	120	150	166	203	212	97	97
4j	TT28	S	191	191	120	120	150	166	203	212	97	97
4k	TT28	S	191	191	120	120	150	166	203	212	97	97
4l	TT28	S	191	191	120	120	150	166	203	212	97	97