Intraspecific Ploidy Variation in the Australian Endemic Shrub *Santalum acuminatum* (Santalaceae): Evidence for Polyploidy and B Chromosomes

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Santalum acuminatum (quandong) at Angels Rest Nature Conservation Reserve, Victoria.

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Abstract

Polyploidy is a pervasive force in plant evolution, facilitating speciation, the evolution of novel traits and adaptation into new environments. However, when ploidy levels vary within populations it can impact sexual reproduction and may cause fertility issues in progeny. Thus, intraspecific ploidy variation is an important consideration for revegetation efforts where seed from varying provenance may be mixed. *Santalum acuminatum* (Santalaceae) is an endemic Australian hemiparasitic shrub for which polyploidy has been suggested in some individuals. The establishment of seed production areas to produce high genetic quality seed for restoration, suggestions of low fecundity and recruitment from seed, and a possible reliance on clonal reproduction highlight the need to investigate polyploidy in this species. These studies aimed to determine the presence and prevalence of ploidy variation in *S. acuminatum*, and to identify techniques for assessing ploidy level in the field. To do this, microsatellite markers, chromosome counts, genome size estimation via flow cytometry, karyotyping, microsatellite marker mapping through fluorescence *in situ* hybridisation (FISH) and morphological comparisons were used. Microsatellite markers found some individuals that amplified more than two alleles across one or more loci, consistent with polyploidy. However, initial chromosome counts found no evidence for polyploidy in these individuals, prompting an investigation into possible gene/locus duplication, B chromosomes, and aneuploidy. Genome size estimation found one individual with a significantly larger genome. Karyotyping revealed chromosome numbers ranged from 2n = 20–22, with this variation attributed to B chromosomes. Two triploid plants were observed through direct chromosome counting and karyotyping, with one also amplifying additional microsatellite alleles and having a larger genome size. FISH revealed possible linkage in two microsatellite markers while additional evidence is required to determine whether

gene/locus duplication has occurred. Morphological comparison of leaf, stomate and seed sizes did not provide evidence for larger organ size in polyploid *S. acuminatum.* However, the rarity of polyploidy and the possibility of gene/locus duplication rather than polyploidy in individuals amplifying additional microsatellite alleles may have confounded results. Furthermore, correlations between morphological characters and environmental variables may mask variation in morphology caused by polyploidy. These studies have uncovered a complex system involving polyploidy, B chromosomes and possible gene/locus duplication, with evidence that polyploidy has occurred independently and repeatedly. Such chromosomal aberrations have potential implications for fertility and sexual reproduction across the distribution of *S. acuminatum*.

Statement of Authorship

Except for Chapter 2 or where reference is made in the text of the thesis, this thesis contains no material previously published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma.

Chapter 2 uses, in part, the data from Jeremy Benwell-Clarke's honours thesis submitted to La Trobe University, Bundoora, for the fulfillment of a Bachelor of Science, Honours (cited here as Hoebee et al. 2018; unpublished). I have reanalysed all his data and the work has not previously been published in any scientific journal. A breakdown of contributions has been included in the author contributions section of Chapter 2.

No other person's work has been used without due acknowledgement in the main text of the thesis. All figures have been constructed by me unless due acknowledgement made in the text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

Chapters 2–4 have been written for submission to scientific journals. Key words are included where appropriate for target journal. For ease of review, all tables and figures have been embedded in the text and references and Supplementary material grouped at the end of the thesis. All data and writing, unless otherwise stated, and including that already mentioned for Chapter 2, has been undertaken by me. The listed co-authors for each chapter have contributed through the provision of knowledge and/or facilities and equipment, writing edits and/or had input into the design, analysis, and conclusions behind each chapter. A breakdown of author contributions can be found at the end of each data chapter.

Simone Currie

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Chapter 1

General Introduction

Polyploidy and plant evolution

Polyploidy is a prominent force in plant evolution facilitating speciation (Soltis et al. 2009, Yu et al. 2010, Vamosi and McEwen 2013), the evolution of novel traits (Crow and Wagner 2006, Veron et al. 2007, Gavin and Kenneth 2008, Airoldi and Davies 2012, Guo-Qiang et al. 2017) and adaptation into new environments (Bierzychudek 1985, Sonnleitner et al. 2010, McGoey et al. 2014, Coughlan et al. 2017, Karunarathne et al. 2018). Polyploidy is a heritable state in which an individual has more than two sets of each chromosome (De Storme and Mason 2014). Occurring through whole genome duplication events, polyploidy is thought to be responsible for much of the variation in genome sizes (ranging 24,000-fold; Pellicer et al 2018) and chromosome numbers (i.e., ploidy) (2n = 2–1440; Stace 2000) seen across plant taxa (Otto and Whitton 2000, Bowers et al. 2003, Soltis et al. 2003). Estimates suggest that between 70 and 100% of all angiosperms (flowering plants) have undergone at least one polyploidisation event in their evolutionary history (Masterson 1994, Soltis 2005, Jiao et al. 2011), and polyploidy is known to occur independently and repeatedly in natural plant populations (Soltis and Soltis 1995). It is no surprise then that polyploidy is considered to be one of the most important forces shaping the evolution of angiosperms.

Polyploid incidence

Polyploidy results from errors during cell division and may occur in both reproductive and non-reproductive tissues. Meiotic non-reduction is the most common route to polyploidy in reproductive tissues, resulting from unreduced (2n) gamete formation (i.e., gametes with the full chromosome complement of the parent) (Brownfield and Kohler 2011, De Storme and Mason 2014, Loginova and Silkova 2017) (Figure 1.1 A and B). This route to polyploidisation can occur between individuals of the same species (autopolyploidy) or between individuals of different species (allopolyploidy) (Kihara and Ono 1926). While rates of unreduced gamete formation vary between taxa (Ramsey and Schemske 1998, Kreiner et al. 2017), environmental factors such as extreme temperatures (both hot and cold) can stimulate unreduced gamete production (Negri and Lemmi 1998, Zhang et al. 2003, Pecrix et al. 2011). In non-reproductive tissues, errors in mitosis can also cause genome doubling (Ramsey and Schemske 1998). However, this form of polyploidisation is only heritable if it occurs in generative tissues such as bulbs, rhizomes or shoot meristems that subsequently undergo meiosis, thereby producing 2n gametes (De Storme and Mason 2014) (Figure 1.1 C). Via these routes, polyploidy can occur at various levels, each of which is characterised based on the number of chromosome sets (i.e., triploids have three sets of each chromosome (3n), tetraploids have four sets (4n), and so on).

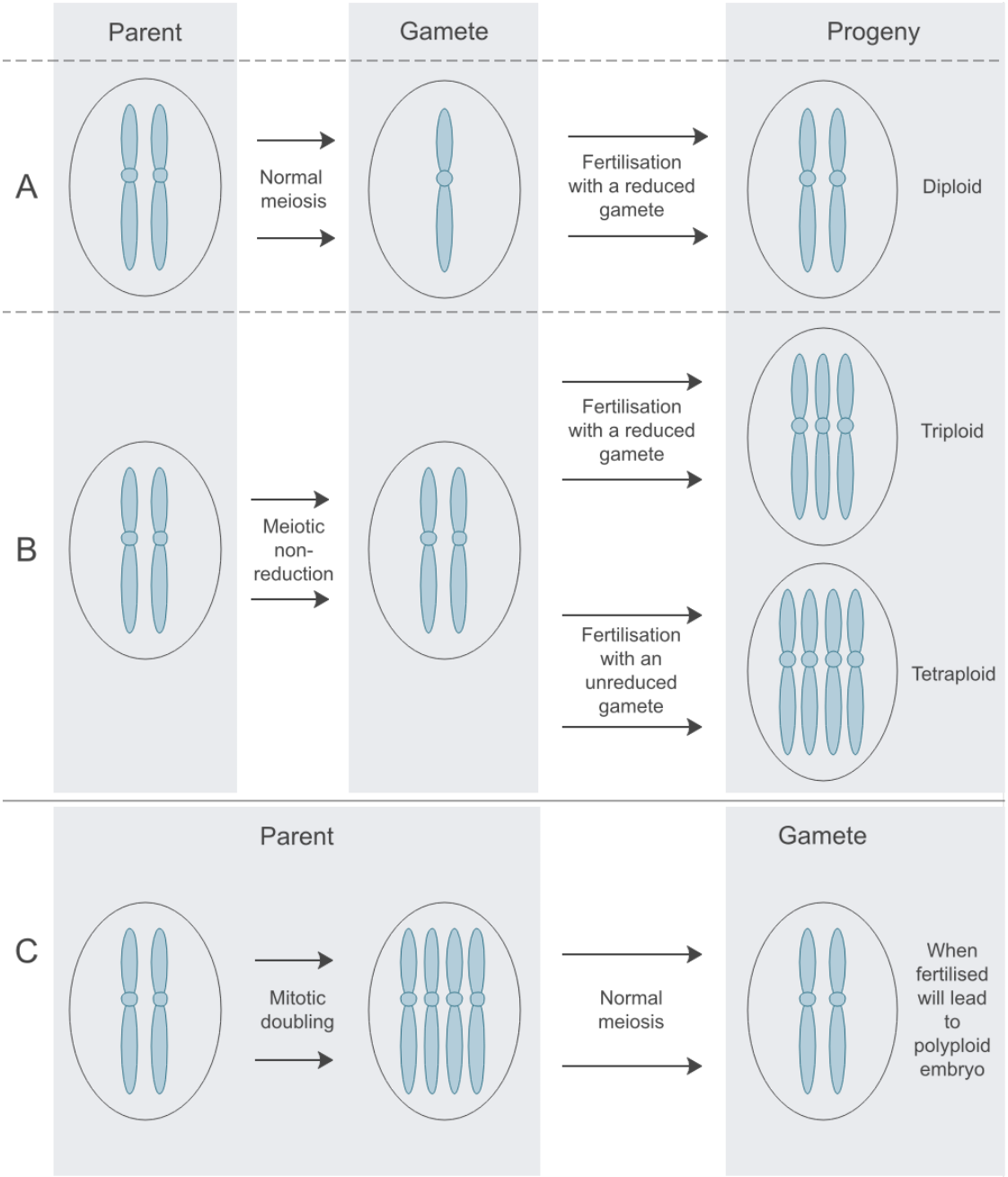

Figure 1.1: Meiotic and mitotic pathways to polyploidisation from diploid cells. For simplicity, only one chromosome is shown. The presence of one chromosome in a cell represents a haploid state, two chromosomes represent a diploid state, and three or more represent a polyploid state. A) Meiotic reduction during normal meiosis, resulting in a reduced gamete and diploid progeny. B) Meiotic non-reduction, resulting in an unreduced gamete and polyploid progeny. C) Somatic doubling in generative tissues (e.g., bulbs, rhizomes or shoot meristems) leading to unreduced gamete formation and polyploid progeny.

Consequences of polyploidy in plant populations

Polyploidisation introduces several pre- and postzygotic reproductive barriers between polyploids and their diploid progenitors (Husband and Sabara 2004). Shifts in phenology (flowering time) (Garbutt and Bazzaz 1983, Burton and Husband 2000, Yu et al. 2010), pollinator preference (Segraves and Thompson 1999, Husband and Schemske 2000), and reduced fertility or sterility in the progeny of inter-cytotype crosses (e.g. triploids) (Marks 1966) act to reproductively isolate polyploids from their progenitors. These barriers to gene flow can, over time, lead to speciation between ploidy races (Husband and Sabara 2004, Yu et al. 2010). However, in the short term they can interrupt sexual reproduction, reduce seed set, and offspring fitness (Ramsey and Schemske 1998). Sexual reproduction between individuals (outcrossing) is important for maintaining and generating genetic and genotypic diversity (Holsinger 2000), characteristics thought to be related to population fitness (Reed and Frankham 2003) and a species' ability to respond to environmental change (Frankham 2005, Jump et al. 2009). Through reproductive isolation, polyploidy within a population can reduce the number of sexually compatible individuals, thus limiting sexual reproduction (Aguilar et al. 2019, Chen et al. 2019). The effect of polyploidy on limiting sexual reproduction depends on polyploid frequency, spatial distribution, and the ploidy levels involved (Kramer et al. 2018). For example, high incidences of triploidy in a small or fragmented outcrossing population increases the frequency of failed fertilisation events owing to reduced fertility in triploids (Marks 1966, Burton and Husband 2000). Furthermore, when species have the capacity for clonal reproduction, sub-fertile or sterile individuals can persist in the landscape clonally for long periods of time (Lynch et al. 1998, Mock et al. 2012), occupying space and resources. Such decreases in mate availability can lead to assortative mating within ploidy races and, in small populations, this may result in increased reproduction between related individuals (i.e., inbreeding) (Newman and Pilson 1997) and self-fertilisation in self-compatible

species (Noel et al. 2016, Chybicki et al. 2019). Inbreeding and self-fertilisation reduce genetic diversity in populations and can have fitness consequences through the expression of deleterious alleles (Charlesworth and Charlesworth 1987, Wilcock and Neiland 2002, Aguilar et al. 2019). Though the purging of deleterious alleles through homozygote exposure may combat inbreeding depression in some species, its effects are inconsistent among taxa and as such, many self-fertilising populations show signs of inbreeding depression (Byers and Waller 1999). As a result, polyploidy is increasingly considered in species management (Chen et al. 2019) and revegetation efforts where the mixing of ploidy races is best avoided (Kramer et al. 2018).

Polyploid detection

Polyploidy can be detected in plants via several techniques. The most reliable and only direct method of assessing ploidy level is through the preparation and staining of chromosomes for counting (Sari et al. 1999). This technique, however, is laborious and difficult to undertake on some species (de Laat et al. 1987). Thus, other indirect methods for assessing ploidy level are commonly used. One method is to exploit the positive relationship between genome size and cell size (Beaulieu et al. 2008) which, for polyploids, tends to confer larger organ size (known as the ‘gigas effect’; Stebbins 1950). Studies have found polyploids to produce larger leaves (Sugiyama 2005), stomata (Vandenhout et al. 1995, McGoey et al. 2014, Snodgrass et al. 2016), flowers (Segraves and Thompson 1999), pollen (Lindstrom and Koos 1931, Marinho et al. 2014), and fruit (Wu et al. 2012) relative to their progenitors. However, these relationships are not present in all species (Segraves and Thompson 1999, Porturas et al. 2019) and are not consistent among plant growth forms (i.e., herbs, shrubs and trees; Beaulieu et al. 2008). Of the morphological characters commonly used to identify polyploids, stomata are among the most reliable, with studies finding no overlap in stomata sizes between ploidy levels

(Celarier and Mehra 1958, Marinho et al. 2014). For this reason, stomata size has been used to infer ploidy level in fossil and herbarium specimens (Masterson 1994). Other techniques are genetic in their approach involving amplification of genetic markers (i.e., microsatellites) and genome size estimation via flow cytometry (Besnard et al. 2008, Bourge et al. 2018). Amplification of co-dominant genetic markers may reveal polyploidy through the number of fragment lengths (amplicons; alleles) at any given locus. That is, diploids should amplify at most two alleles per locus, while polyploids may amplify additional alleles (Sosa et al. 2014). Due to the possibility of two or more alleles at any given locus being homozygous and thus amplifying the same fragment length, this method is used to infer rather than determine ploidy level (Robertson et al. 2010) and is often used in combination with other techniques (Besnard et al. 2008, Seeber et al. 2014, Marques et al. 2016). Finally, genome size estimation via flow cytometry exploits the increase in genetic material resulting from polyploidy (Kron et al. 2007). Compared to other methods of ploidy determination, flow cytometry is a simple and rapid method, garnering accurate results (Suda and Trávníček 2006). While fresh material is generally used (Galbraith et al. 1983), methods for the use of flow cytometry with dried leaf material have been developed, though fail to work for all species (Suda and Trávníček 2006). Together, these techniques allow for ploidy determination using a range of different material types, broadening the reach of research projects.

Study species

A genus that polyploidy has played a role in shaping is the sandalwood genus (*Santalum*) (Harbaugh 2008). *Santalum* comprises 15 extant species of hemiparasitic shrub endemic to the Indo-Pacific (Xin-Hua et al. 2010, Tamla et al. 2012). Some members of this genus are economically important sources of sandalwood oil with a history of heavy exploitation (Xin-Hua et al. 2010, Tamla et al. 2012). Polyploidy has been identified in Pacific Island species and is thought to have been important in the establishment of new species and the dispersal to and colonisation of islands (Harbaugh 2008). *Santalum acuminatum* (R. Br.) A. DC. (commonly known as quandong or native peach) is an Australian endemic species with cultural significance to Aboriginal Peoples (Clarke 1985, Smith 2007) and with a commercial bushfoods industry of mostly wild harvested fruit (RIRDC 2014). Evidence suggests that the species is capable of clonal reproduction and recruitment from seed is thought to be low (Tennakoon et al. 1997a, Fuentes-Cross 2015). Individuals are sparsely distributed across a broad distribution in Australia (Applegate and McKinnell 1991), with extensive habitat clearing and fragmentation in the south-east (Bradshaw 2012). Seed production areas to reliably produce high genetic quality seed have been established in this area to assist with revegetation (J. Begley, personal communication, 2018).

There is conflicting evidence as to the presence of polyploidy in *S. acuminatum*. Restriction fragment length polymorphism (RFLP) banding patterns in two Western Australian (WA) stands suggested polyploidy (Byrne et al. 2003). This was subsequently supported by evidence from microsatellite amplification in south-eastern Australian stands, where some individuals amplified three alleles across various loci (Hoebee et al. 2018; unpublished). Initial stomata size measurements found these putative polyploids to have larger stomata compared to putative diploids amplifying at most two alleles at each microsatellite locus (Hoebee et al. 2018; unpublished). Conversely, comparisons of leaf, stomate and pollen sizes in 50 South Australian herbarium specimens, found no evidence

of polyploids of larger size (Randall 2000). Similarly, chromosome counts on one WA individual and estimation of genome size by flow cytometry on two seed (WA and New South Wales), found both individuals were diploid with 2n = 20 chromosomes (Harbaugh 2000). These contradictory results observed using differing methodologies, small sample sizes and restricted geographies, mean that the existence of polyploidy in *S. acuminatum* remains unresolved.

Scope and Aims of Thesis

Intraspecific ploidy variation has the capacity to introduce reproductive barriers that constrain sexual reproduction in species with small population sizes, especially those occupying fragmented landscapes and/or have the capacity for clonal reproduction. As such, identifying the levels and distribution of polyploidy in populations is important in both species management and revegetation programs where seed of varying provenance may be mixed (Kramer et al. 2018). This study aimed to 1) resolve conflicting evidence as to the presence of polyploidy in *S. acuminatum*, 2) confirm ploidy levels in the species and 3) determine whether morphological characters (leaf, stomate and seed) correlate with ploidy level to support rapid field assessment of cytotypes. These aims were deconstructed into three data-based chapters, detailed below.

Chapter 2: New insights into genetic diversity, clonal extent, and ploidy in the hemiparasitic shrub *Santalum acuminatum* (Sanatalaceae), combines previous genetic diversity analyses (Hoebee et al. 2018; unpublished), with ploidy level determination using microsatellite marker amplification and chromosome counts.

Chapter 3: Genome size variation, B chromosomes, and polyploidy in the Australian endemic shrub *Santalum acuminatum* (Sanatalaceae) builds off questions raised in Chapter 2 regarding aneuploidy, B chromosomes, and gene/locus duplication. Methods employed in this chapter include flow cytometry, karyotyping and fluorescence *in situ* hybridisation to look for genome size variation, gain higher resolution of ploidy levels, and map the location of microsatellite markers on the genome.

Chapter 4: Cytotype and environmental influences on morphology in *Santalum acuminatum* (Santalaceae), seeks to identify techniques for rapid in-field identification of polyploids. Morphological characters (leaf, stomate and seed) were measured to look for evidence of the gigas effect in polyploids in a sample size representing the species' Australia-wide distribution. As the environment may also influence morphology, correlations between environmental variables (nutrient status, pH, bare soil cover, aridity, precipitation, and temperature) and each morphological character were undertaken. The aim of which was to gain insight into the potential for the environment to mask morphological variation resulting from ploidy variation.

Chapter 5 is a general conclusion synthesising the experimental results of Chapters 2 to 4 and makes recommendations for species management and future research.

Chapter 2

New insights into genetic diversity, clonal extent, and ploidy in the Australian hemiparasitic shrub quandong (*Santalum acuminatum*, Santalaceae)

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Abstract

Many plants are capable of both sexual and asexual reproduction with clonal reproduction the most common asexual strategy. In environments where sex is constrained, biases towards clonal reproduction can affect genotypic diversity and evolutionary capacity, potentially increasing extinction risk. The hemiparasitic shrub *Santalum acuminatum*, exhibits low genetic diversity, signs of sexual failure and clonal reproduction. Furthermore, suggestions of polyploidy may further limit sexual reproduction with inter-cytotype incompatibility reducing mate availability. Here, microsatellite markers were used to investigate genetic diversity, population structure and clonality in the fragmented south-eastern Australian populations of *S. acuminatum*. Ploidy level was investigated through direct chromosome counting and microsatellite amplification across the species' Australia-wide distribution. Low genotypic diversity, high heterozygosity and extensive clonality was observed, with over half of the examined populations consisting of single genotypes (genets) spanning up to 8660m^2. Six individuals amplified three microsatellite alleles at one or more loci, consistent with polyploidy. Nonetheless, ploidy levels, were largely within the diploid range of 2n = 18–22 across all individuals, regardless of their microsatellite profile. High heterozygosity, low genotypic diversity and a lack of population structure are attributed to clonal reproduction maintaining genetic diversity within genets while slowing processes that drive population differentiation. It is suggested that *S. acuminatum* is predominantly clonal in the study area which may serve as an adaptation to environmental stress.

Introduction

Plants reproduce via a range of strategies including sexual reproduction between individuals, self-fertilisation and asexual reproduction, with most species adopting a mixed strategy (Richards 1997). Of the plant species capable of asexual reproduction, the vast majority reproduce clonally, with investment in this strategy varying over time and space (Klimes et al. 1991). Clonal reproduction produces genetically identical structures (ramets) that may remain close to and/or physically connected to the parent (genet) (Caracco and Kelly 1991), but with the capacity to cover large areas (Mitton and Grant 1996, Lynch et al. 1998). Physiologically, integration between connected ramets can aid with nutrient, sunlight, and water capture for genets in heterogeneous environments, decreasing the risk of genet death by distributing risk across ramets (Caracco and Kelly 1991, Barrett 2015) and increasing genet longevity (de Witte and Stöcklin 2010). Furthermore, clonal reproduction provides reproductive assurance (Vallejo-Marín et al. 2010) while avoiding the energetic costs of sex (Richards 1997). As such, clonality can facilitate persistence in marginal and/or fragmented habitats where sexual reproduction may be constrained by mate and/or pollinator limitation or poor habitat quality (Rossetto et al. 2004, Hewitt 2020). However, increased clonal investment can lead to a trade-off with sexual reproduction, resulting in reduced floral displays and seed production (Barrett 2015). Similarly, competition between genets can increase population monoclonality if one genet outcompetes conspecifics (Hartnett and Bazzaz 1985), thereby reducing opportunities for outcrossing when sexual reproduction occurs. As such, biases towards clonal reproduction are often suggested to reflect sexual failure rather than population viability, delaying a species' path to extinction (i.e., extinction debt) (Eriksson 1994, Silvertown 2008).

Clonal reproduction has implications for genotypic diversity and population genetic structure. Unlike sexual reproduction, clonality produces genetically homogenous offspring that lack the genetic diversity gained through gamete union and recombination (Holsinger 2000). Though some diversity between clonal structures can be gained through somatic mutation (Wolf et al. 2000, Lamont and Wiens 2003), this is not a significant source of genetic diversity for all clonal species (Stoeckel et al. 2006, Hu et al. 2016). Clonal structures also have limited capacity for dispersal between populations (Vallejo-Marín et al. 2010), limiting gene flow and increasing population genetic structure for predominantly clonal populations (Heywood 1991, Roberts et al. 2016). In the short term, clonal reproduction can lead to the proliferation of locally adapted genotypes (Klekowski 2003), whilst avoiding genotype dilution through recombination (Otto and Lenormand 2002). Likewise, reproductive systems combining obligate outcrossing and clonal reproduction can lead to heterozygote excess as genetic diversity generated through sex is maintained clonally (Stoeckel et al. 2006). In the long term, however, clonal reproduction in the absence of sex can reduce genotypic diversity and ultimately, evolutionary capacity, owing to fewer genotypes upon which natural selection can act (Richards 1997, Holsinger 2000). Prolonged investment in clonal reproduction can also lead to a complete loss of sexual reproduction over time due to the accumulation of deleterious mutations (Eckert 2001, Barrett 2015). Genetic load tends to accumulate at a higher rate in clonal systems (Caetano-Anollés 1999) resulting from somatic mutations in clonal structures (Gross et al. 2012) which lower reproductive success over time (Lamont and Wiens 2003). These impacts on the generation and maintenance of genotypic diversity in clonal systems may render populations susceptible to novel pathogens (Hamilton 1980, Schmid 1994) and environmental change (Eckert et al. 2006, Silvertown 2008). Despite this, there are examples of clonal plants that have persisted for thousands of years (Lynch et al. 1998, Ally et al. 2008) and thus, this strategy may not always constitute a path to extinction.

In some plant species, intraspecific variation in chromosome number (ploidy) is another consideration that may limit sexual reproduction and trigger and/or maintain clonal reproduction. Ploidy variation is common in plants, generally involving whole genome duplication (polyploidy) (Soltis 2005, Jiao et al. 2011). Intraspecific ploidy variation impacts on sexual reproduction as fertilisation occurring between ploidy levels may be unsuccessful or produce sub-fertile or sterile progeny (Marks 1966, Ramsey and Schemske 1998, Burton and Husband 2000). In this way, intraspecific ploidy variation can lower both mate availability and effective population size, with the capacity to increase inbreeding and lower genetic diversity (Loveless and Hamrick 1984). Clonality is more prevalent among polyploid taxa with evidence suggesting polyploidy triggers clonality in angiosperms (Van Drunen and Husband 2019). Thus, newly formed polyploids may persist in landscapes via clonal propagation for long periods of time (Lynch et al. 1998, Mock et al. 2012, Roberts et al. 2016), occupying space, using resources, and extending reproductive barriers through time. Consequently, intraspecific ploidy variation is increasingly considered in species management, where revegetation and/or translocations may result in the unintentional mixing of ploidy races (Kramer et al. 2018, Chen et al. 2019).

The genus *Santalum* comprises 15 extant species of hemiparasitic shrub native to southern Asia, Indonesia, Australia, and the South Pacific. In Australia, many *Santalum* species have undergone range contraction and habitat fragmentation due to agricultural activities, exploitation for hardwood oils (Applegate and McKinnell 1991, Warburton et al. 2000) and increased grazing pressure from feral animals (Denham and Auld 2004, Box et al. 2016). *Santalum acuminatum* (R. Br) A. DC. is an Australian endemic plant with a broad distribution across the southern half of mainland Australia (Figure 2.1). The species has cultural significance to Aboriginal Peoples as a source of food and medicine (Clarke 1985,

Jones et al. 1995) and is part of a bushfoods industry of commercial orchards and wild harvested fruit (RIRDC 2014). While the horticultural potential of this species has seen its biology well studied (Sedgley 1982, Loveys and Jusaitis 1994, Jones et al. 1995, Tennakoon et al. 1997a, Loveys et al. 2002), the distribution of genetic diversity in natural populations is only beginning to be understood (Fuentes-Cross 2015). Suggestions of low seedling recruitment (Tennakoon et al. 1997a) and fertility issues (Randall 2000, Fuentes-Cross 2015), increased grazing pressure by feral animals (Denham and Auld 2004, Box et al. 2016), harvesting of wild fruit (Fuentes-Cross 2015), and the loss of individuals through land clearing, are predicted to reduce genetic diversity over time (Applegate and McKinnell 1991). Likewise, evidence of low genetic diversity in central and western populations (Fuentes-Cross 2015), clonal reproduction (Tennakoon et al. 1997a) and conflicting suggestions of polyploidy (Randall 2000, Byrne et al. 2003, Harbaugh and Baldwin 2007) highlight the need for additional research, especially in the understudied south-eastern distribution to determine the impact of contemporary stressors on population dynamics.

The aims of this study were twofold; 1) to investigate genotypic and genetic diversity in populations of the hemiparasitic plant *Santalum acuminatum* occupying the fragmented south-eastern edge of the species' distribution, and 2) to determine ploidy levels in a larger sample size representing the species' Australia-wide distribution. Population genetic diversity analyses were undertaken using microsatellite markers, while ploidy levels were determined using a combination of microsatellite amplification and chromosome counts. It was hypothesised that genetic diversity would be low, population genetic structure would be high and that genetic and geographic distances would be correlated. Based on prior studies, clonal reproduction was expected while suggestions of polyploidy prompted expectations that some variation in ploidy occurs in this species.

Materials and Methods

Study species

Santalum acuminatum, or quandong, is a small (< 5 m) shrub with a natural distribution across arid and semi-arid ecosystems in mainland Australia, extending into coastal regions of Western Australia and South Australia (Figure 2.1). Like other *Santalum* species, *S. acuminatum* is a root hemiparasite, obtaining part of its water and nutrient requirements from the roots of nearby plants (Tennakoon et al. 1997a). The species is a generalist in host choice, but studies have reported a preference for nitrogen fixing species, in particular *Acacia* species (Tennakoon et al. 1997a, Nge et al. 2019). Clonal reproduction occurs via root suckering (Tennakoon et al. 1997a), while pollination is thought to be by insects with some evidence for self-compatibility (Sedgley 1982). Seed dispersal is undertaken by emus (*Dromaius novaehollandiae*) and bettongs (*Bettongia* spp.). The loss of bettongs from much of their pre-European distribution (Lomolino and Channell 1995), however, suggests that these animals are no longer significant seed dispersal agents. Long distance seed dispersal is also suggested to have been facilitated by Aboriginal Peoples (Bonney 2013, Fuentes-Cross 2015).

Genetic diversity and population structure

Sampling

Fifteen sites along a northwest aridity gradient in south-eastern Australia were selected for genotypic and genetic diversity assessment (Table 2.1 A and B; Figure 2.1). Plants were sampled from August to November 2017. At each site up to 20 stems were sampled depending on population size. Stems at least 10 m apart were sampled across each population to reduce the likelihood of sampling clones (Lhuillier et al. 2006a), except for the smaller population at Devenish where all stems were sampled. Several leaves were collected from each stem, placed in paper envelopes, and stored in plastic bags with ~75 g of self-indicating silica gel beads (3–8 mesh; ChemSupply) for rapid drying. The latitude and longitude of each stem was taken using a hand-held Garmin GPS.

Table 2.1: *Santalum acuminatum* study sites for genetic diversity analysis (A, B) and ploidy assessment (B, C).

	Population ID	Location	State	Latitude	Longitude	N_D	$N_{P\ (material)}$
A)	An	Annuello Flora and Fauna Reserve	VIC	-34.888	142.709	1	-
	BR	Bronzewing Flora and Fauna Reserve	VIC	-35.241	142.463	11	-
	Ha	Hattah-Kulkyne National Park	VIC	-34.770	142.282	13	-
	HV	Happy Valley	VIC	-34.715	142.704	20	-
	IR	Inglewood Nature Conservation Reserve	VIC	-36.602	143.816	20	-
	Ma	Mallan	NSW	-35.162	143.833	5	-
	PBR	Pirlta Bushland Reserve	VIC	-34.364	141.897	13	-
	Th	Thule	NSW	-35.698	144.442	7	-
	TT	Tol Tol	VIC	-34.679	142.885	1	-
B)	ARR	Angels Rest Flora Reserve	VIC	-35.685	143.033	15	$1_{(leaf,\ leaf\ bud)}$
	Be	Berrigan	NSW	-35.741	145.796	5	$1_{(leaf,\ leaf\ bud)}$
	De	Devenish	VIC	-36.362	145.914	22	$1_{(seed)}$
	Ko	Koorlong	VIC	-34.342	142.088	4	$1_{(leaf,\ leaf\ bud)}$
	RC	Red Cliffs	VIC	-34.334	142.130	7	$1_{(leaf,\ leaf\ bud)}$
	Sa	Savernake	NSW	-35.749	146.030	6	$1_{(seed)}$
C)	CDR	Charles Darwin Reserve	WA	-29.570	116.870	-	$1_{leaf,\ seed)}$
	GL	Green Lake Reginal Park	VIC	-35.598	142.847	-	$1_{(leaf,\ leaf\ bud,\ seed)}$
	HR	Hamelin Reserve	WA	-26.500	114.350	-	$1_{(leaf,\ seed)}$
	INR	Ingalba Nature Reserve	NSW	-34.440	147.440	-	$3_{(leaf,\ leaf\ bud,\ seed)}$
	LE	Lyndavale Erldunda Station	NT	-25.194*	133.256*	-	$6_{(seed)}$
	Mb	Merbein	VIC	-34.188	142.058	-	$1_{(leaf,\ leaf\ bud,\ seed)}$
	MP	Mulga Park	NT	-25.906*	131.668*	-	$2_{(seed)}$
	Na	Nathalia	VIC	-36.064	145.201	-	$1_{(seed)}$
	Nm	Natimuk	VIC	-36.674	141.932	-	$1_{(leaf,\ leaf\ bud,\ seed)}$
	PNR	Pucawan Nature Reserve	NSW	-34.450	147.350	-	$2_{(leaf,\ leaf\ bud,\ seed)}$
	TFR	Torrita Flora and Fauna Reserve	VIC	-35.160	141.930	-	$2_{(leaf,\ leaf\ bud)}$
	UR	Umberumberka Reservoir	NSW	-31.830	141.210	-	$1_{(seed)}$
	WNR	Walpeup Nature Conservation Reserve	VIC	-35.142	142.031	-	$1_{(leaf,\ leaf\ bud)}$
	WN	Walpeup North	VIC	-35.122	142.016	-	$1_{(leaf,\ leaf\ bud,\ seed)}$
	Yu	Yulara	NT	-25.263	130.971	-	$1_{(seed)}$

VIC, Victoria; NSW, New South Wales; SA, South Australia; NT, Northern Territory; WA, Western Australia; N_D, number of stems sampled for genetic diversity analysis; N_P, number of stems sampled for ploidy assessment (material type collected). * denotes approximate (up to 100km error) coordinates.

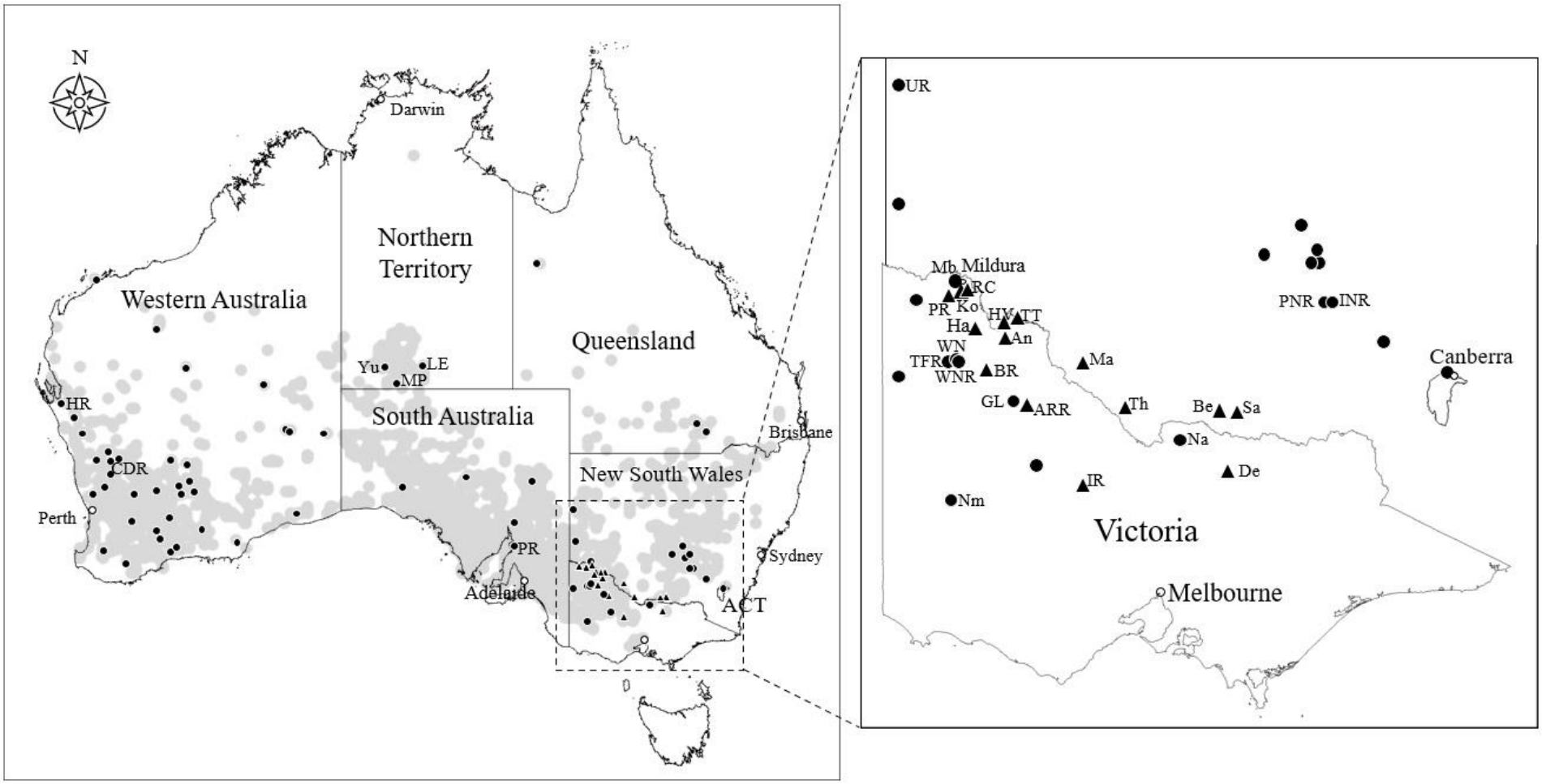


Figure 2.1: *Santalum acuminatum* sampling locations for this study. The distribution of *S. acuminatum* in Australia is represented in light grey (Atlas of Living Australia 2020). Triangles (▲) denote sites sampled for genotypic and genetic diversity analyses and black circles (●) denote sites sampled for ploidy assessment. Unnamed circles are herbarium specimens (Supplementary material, S2.1).

DNA extraction

DNA was extracted from 16 to 20 mg of dried leaf from each sampled stem. The material was pulverised in tubes containing two 3 mm tungsten carbide beads using a QIAGEN© TissueLyser II for 1 min at 25 Hz/s. DNA was then extracted following the QIAGEN© DNeasy Plant Mini-Kit and associated protocol, with the following modifications: (1) during cell lysis tubes were incubated at 65°C for 30 min; and (2) tubes were left for 5 min with lids off prior to elution to allow any residual ethanol to evaporate. Eluted DNA was stored at -20°C.

Microsatellite amplification and assessment

Twelve microsatellite markers developed for *S. insulare* (Lhuillier et al. 2006b), *S. austrocaledonicum* (Bottin et al. 2005a) and *S. lanceolatum* (Jones et al. 2010) were tested for use with *S. acuminatum* (Hoebee et al. 2018; unpublished). Of these, eight markers amplified within the expected size range and were selected for genotyping *S. acuminatum* samples. Primers were synthesised by Integrated DNA Technologies with modification of one of four universal primer sequence tails (Blacket et al. 2012) to forward primers to allow for fluorescent labelling and multiplex polymerase chain reaction (PCR) (Table 2.2).

Table 2.2: Microsatellite loci amplified in *Santalum acuminatum* for genetic and genotypic diversity analyses (A-H) and ploidy assessment (A, C, E, and H).

	Locus	Repeat motif	Primer sequence (5'–3')	Amplicon size range (bp)	N_a
A	Lanc07[1]	$(TC)_4(CT)_{10}$	F: [B]AAACCCCTTCTCCTCCCATT R: CCGATATTCCCCCATTTCTT	139–163	6
B	Lanc08[1]	$(GA)_8$	F: [A]AAGTGATCACAGCTCCGGTTA R: GGGGTCGTTGCATCTATTTC	-	-
C	Lanc09[1]	$(AG)_{15}$	F: [C]ATGAGAGCGAGAGGGAGACA R: GTCCACTCCTCACCAAAACC	190–196	4
D	mSaCIRE09[2]	$(CT)_{16}$	F: [C]GGAAAGGGTTGACAGGAAGAAAA R: TGCGAGTGAGTGGGAAAAGTAGA	172–200	12
E	mSaCIRF10[2]	$(GA)_{17}$	F: [A]TTAGGAAAACATAGCACACT R: GAGCACTTCACCACCATTAC	151–181	12
F	mSiCIR44[3]	$(TG)_8$	F: [B]CGCCTTTTTCACTTTTCGC R: ACACCTCACACAGTTCCCT	128	1
G	mSiCIR153[3]	$(CA)_2GA$	F: [D]ATGCTTTTGTGGTGATTC R: GCTTGGAGTATCTTGTTGG	-	-
H	mSiCIR185[3]	$(CA)_7$	F: [D]ACAACAACGCATAACCCT R: AAAACAATGGCACTGAGAA	285–289	3

[1],Jones et al. (2010); [2], Bottin et al. (2005a); [3], Lhuillier et al. (2006b); A, Tail A: 5'-GCCTCCCTCGCGCCA-3'; B, Tail B: 5'-GCCTTGCCAGCCCGC-3'; C, Tail C: 5'-CAGGACCAGGCTACCGTG-3'; D, Tail D: 5'-CGGAGAGCCGAGAGGTG-3'; N_a, number of alleles.

The loci were assessed for primer dimers using Multiple Primer Analyzer (Thermo Fisher) and split into three multiplex reactions: (1) mSiCIR44 and mSaCIRF10; (2) Lanc07, Lanc09 and mSiCIR185; and (3) Lanc08, mSaCIRE09 and mSiCIR153. Amplification via PCR was in a 15 µL admix of 7.5 µL 2x Type-it Multiplex PCR Master Mix (QIAGEN), 0.1 µM of each forward primer, 0.2 µM of each reverse primer, 0.2 µM of the relevant fluorophore (FAM, VIC, NED and PET; Blacket et al. 2012), 1.5 µL template DNA, and nuclease free water to total volume. Touchdown amplification was undertaken in a MyCycler™ Thermal Cycler System (BIORAD) under the following conditions: 5 min denaturation at 95°C, followed by 30 s at 94°C, 30 s at 65°C (decreasing 1.5°C per cycle) and 30 s at 72°C for 10 cycles, then 30 s at 94°C, 30 s at 50°C and 30 s at 72°C for 25 cycles, with a final extension of 5 min at 72°C. PCR products were visualised on a 1% agarose gel stained with Gel Red (Biotium). Amplicons were analysed using capillary

electrophoresis at the Australian Genomic Research Facility and scored using Geneious version 11.0.4 (Kearse et al. 2012).

Genetic diversity and population genetic structure

Two markers (Lanc08 and mSaCIR153) amplified inconsistently and mSiCIR44 was monomorphic. These loci were excluded, leaving five loci for genetic diversity analyses. Sequencing of these microsatellite loci confirmed that each primer amplified the target microsatellite loci in *S. acuminatum* (Supplementary material, S2.2). Unless otherwise indicated, all analyses were conducted in R version 3.4.3 (R Core Team 2019). The data were imported as a genclone object using the poppr package version 2.6.0 (Kamvar et al. 2014) and as a genambig object for analysis using the polysat package version 1.7-2 (Clark and Jasieniuk 2011). These packages were selected for their handling of polyploid (Clark and Jasieniuk 2011) and mixed ploidy datasets (Kamvar et al. 2014).

The discrimination power of the five loci was assessed using a genotype accumulation curve based on 1000 random samples of n loci using poppr. The number of alleles per locus, private alleles and multilocus genotypes (MLGs) were calculated using poppr. To test whether diversity in MLGs was an effect of sampling effort a Spearman's correlation coefficient was undertaken to look for a correlation between the number of ramets sampled and the number of MLGs observed using the R stats package (R Core Team 2019). Tests for linkage disequilibrium and departures from Hardy-Weinberg Equilibrium were not performed as populations consisted of too few genotypes (Douhovnikoff and Leventhal 2016) and the possibility of biased results for polyploids in the absence of allele dosage information (Meirmans et al. 2018).

The data were clone censored to remove duplicate MLGs originating from clonal propagation and MLGs assigned a unique number using poppr. Since we could not assume random mating and linkage disequilibria (Stenberg et al. 2003) for these populations, the probability of n repeated MLGs from sexual reproduction (P_{sex}) was not calculated. As long-lived clonal plants can accumulate somatic mutations over time (Halkett et al. 2005), slightly different MLGs may constitute the same multilocus lineage (MLL) (i.e., having originated from the same zygote) (Arnaud-Haond et al. 2007). Consequently, Bruvo's genetic distance (see below) threshold of 0.1 was used to assign MLGs to a MLL using polysat. Genotypic diversity for each site was estimated as G/N, where G = the number of genotypes detected (MLL) and N = number of ramets sampled (Ellstrand and Roose 1987), excluding sites for which one sample was collected. Observed heterozygosity (H_o) ± standard error (SE) and expected heterozygosity (H_e) ± SE were calculated using GenAIEx (Peakall and Smouse 2006, Peakall and Smouse 2012). Heterozygosity estimates excluded individuals amplifying more than two alleles at any given locus and were only reported for populations comprising more than one MLL.

To estimate genetic distance between MLGs, Bruvo's genetic distance was calculated using polysat as this measure allows comparisons between ploidy levels by including virtual alleles that simulate the origin of ploidy differences through a stepwise mutation model (Bruvo et al. 2004). However, examples of microsatellite mutation processes violating the stepwise mutation model have been reported (Selkoe and Toonen 2006), and thus Lynch's genetic distance was also calculated using polysat as this measures the proportion of shared alleles (Lynch 1990). Principal coordinates analysis (PCoA) was undertaken on genetic distance estimates and visualised in ordination space using the R 'stats' package (R Core Team 2019). An Isolation by Distance (IBD) model of genetic diversity was tested using separate Mantel tests performed on each matrix of Bruvo's and

Lynch's genetic distances and a matrix of geographic distances were performed using ade4 version 7.7-10 (Dray and Dufour 2007).

To better understand the spatial extent of clones the coordinates of all sampled stems were imported into Google Earth Pro version 7.1.8.3036. Clone size was estimated by joining each stem (ramet) belonging to the same MLG (genet) to form a polygon, within which area was calculated.

Ploidy assessment

Sampling

In 2019/20, six of the 15 sites sampled for the genetic diversity analysis were revisited to collect material for ploidy assessment (Table 2.1 B). To supplement this, plant material from 15 new sites was collected or donated (Table 2.1 C; Figure 2.1). To avoid re-sampling the same genet, clusters of stems separated from nearby clusters by at least 50 m were treated as separate genets. For each stand, juvenile leaves and growing leaf buds were collected from at least three separate branches of the largest stem. Leaves were sampled as previously described while leaf buds were wrapped in wet tissue and stored at ~4°C for up to six days prior to processing. Where possible, fresh fruit or seed were sampled from stems either directly from a branch or from the ground immediately beneath the canopy. Fruits were de-fleshed and stored in paper envelopes until use. Seed and leaf material from eight stands was donated and species identification confirmed based on endocarp texture, which differs among *Santalum* species (Harbaugh 2000). In addition, leaf material from 62 specimens representing the natural distribution of *S. acuminatum* were sampled from Australian herbaria for microsatellite amplification (Figure 2.1;

Supplementary material, S2.1). Only specimens collected within the last 15 years were selected to increase confidence in DNA yield.

Seed germination for chromosome counts

Where available, up to five seed from each site were germinated to obtain root tips for chromosome staining. Each seed was extracted from the woody endocarp using a hammer applied directly to the micropyle, and the seed coat removed before sterilisation in 10% bleach solution for 25–30 min. The seed were rinsed thoroughly in running distilled water and placed in a sterile glass petri dish containing autoclaved vermiculite. Distilled water (5–10 mL) was used to moisten vermiculite and the dishes sealed with parafilm. Germination took place in the dark in a Thermoline growth cabinet set to 20°C and seed monitored weekly. Once lateral root growth occurred, root tips were sampled for root tip squash.

Root tip preparations

Harvested root tips were pre-treated in a cold saturated solution of 1,4-dichlorobenzene for 18 h at 4°C followed by washing in distilled water and fixed in 1:3 glacial acetic acid: ethanol for 24 h at room temperature (18–22°C). Root tips were again washed in distilled water and stored in 70% ethanol at -20°C prior to preparation. Pre-treated root tips were washed thoroughly in distilled water and hydrolysed in 1M HCl at 60°C for 8–15 min depending on root thickness. Roots were again washed in distilled water before being placed on a microscope slide where the root caps were carefully removed and the root meristem (terminal ~2 mm of root) isolated. Excess distilled water was removed and a drop of aceto-orcein stain (1% orcein in 45% acetic acid) applied. Root tips were macerated for ~30 sec using the blunt end of a probe tool to separate cells and a cover slip

applied. Slides were heated at 60°C for ~30 sec before incubation at room temperature for up to 15 min to allow the stain to penetrate the cells. Filter paper was placed over the cover slip and pressure applied to flatten the cells. Clear nail varnish was then applied to the edges of the cover slip to seal contents. Mounted root tips were stored at -20°C.

Leaf bud preparations

Freshly growing leaf buds were processed according to the protocol outlined in Sinha et al. (2016) except that the leaf buds were stored in 70% ethanol at -20°C following fixation until preparation. Processed leaf buds were placed on microscope slides and the meristematic tissues isolated. Meristems were then macerated, sealed, and stored as described above for root tip preparations.

Chromosome visualisation and counting

Chromosomes from root tip and leaf bud preparations were viewed and counted using an Olympus CX22LED microscope at 1,000x magnification under oil immersion with Yang Wang YW500u3 USB3 industrial camera and associated S-EYE software version 1.2.5.147 (Hayear Electronics 2015). Counts were performed on two separate roots or leaf buds from each stand. This was repeated across several cells in the preparation to minimise errors from cell overlap or the contents of lysed cells. Ploidy levels are expressed as the total number of chromosomes in somatic cells (2n).

Microsatellite evidence of polyploidy

Four microsatellite loci that amplified more than two fragments (i.e., alleles) in samples analysed for genetic diversity were selected to screen for ploidy variation (Table 2.2 A, C,

E, and H). Loci were assessed for primer dimers as described above for genetic diversity and subsequently split into two multiplex reactions: (1) Lanc07 and Lanc09, and (2) mSiCIR185 and mSaCIRF10. Amplification, multiplex PCR and scoring of amplicons was undertaken as previously described, and samples that amplified more than two fragments were flagged as potential polyploids. A subset of these potential polyploids were re-analysed to confirm amplification. Due to the nature of microsatellite amplicons, polyploidy may be masked by the presence of homozygous alleles with different dosages (i.e., AA and AAA will both amplify one fragment) (Dufresne et al. 2014). As such, results from microsatellites were used to infer ploidy change rather than to determine the ploidy level.

Results

Genetic diversity and clonality

Thirty-seven alleles were amplified across the five loci with the number of alleles ranging from three for mSaCIR185 to twelve for both mSaCIRE09 and mSaCIRF10. The mean number of alleles per locus (N_a) was 7.4 (± 1.58 SE). Private alleles were found at Be (2), BR (2), IR (3), Ko (1) and PBR (1) (Table 2.3). Simulations indicated that the number of loci used was sufficient for differentiation (Figure 2.2). Of the 150 sampled stems, 27 unique multi-locus genotypes (MLGs) and 24 multi-locus lineages (MLL) were observed, with MLGs assigned to the same MLL at ARR, Ha and IR. The number of observed MLGs was not correlated with the number of stems sampled at each site. Single MLL were observed at Be, BR, De, HV, Ko, RC, Sa, and Th (genotypic diversity = 0), whereas

genotypic diversity was highest at IR (0.37). The mean genotypic diversity across all sites was 0.14 (Table 2.3). Overall, single MLGs were observed at 67% of sites, while 87% of sites comprised single MLLs. No MLGs were shared across sites. Observed heterozygosity (H_o) was lowest at IR (0.52 ± 0.14) and highest at PBR (0.71 ± 0.12), while the mean H_o was 0.61 ± 0.15 (Table 2.3). At two of the three sites with more than one MLL (Ma and PBR), H_o was greater than the expected heterozygosity. Within stands the estimated area occupied by single MLGs ranged from 11 m^2 (Ma, MLG 22) to 8660 m^2 (Ha) (Supplementary material, S2.3).

Table 2.3: Genetic diversity statistics for *Santalum acuminatum* sites spanning the species' south-eastern distribution in Australia. Heterozygosity estimates exclude individuals amplifying more than two microsatellite alleles at any given locus and are only reported for populations comprising multiple multilocus lineages.

Population ID	Ns	MLG	MLL	Genotypic diversity	Heterozygosity		Private alleles
					H_o	H_e	
An	1	1	1	-	-	-	0
ARR	15	2	1	0.07	-	-	0
Be	5	1	1	0.20	-	-	2
BR	11	1	1	0.09	-	-	2
De	22	1	1	0.05	-	-	0
Ha	13	2	1	0.08	-	-	0
HV	20	1	1	0.05	-	-	0
IR	20	8	7	0.37	0.52 ± 0.14	0.59 ± 0.08	3
Ko	4	1	1	0.25	-	-	1
Ma	5	2	2	0.2	0.60 ± 0.19	0.47 ± 0.12	0
PBR	13	3	3	0.17	0.71 ± 0.12	0.48 ± 0.06	1
RC	7	1	1	0.14	-	-	0
Sa	6	1	1	0.17	-	-	0
Th	7	1	1	0.14	-	-	0
TT	1	1	1	-	-	-	0
Total	150	27	24	-	-	-	9
Mean	-	-	-	0.14	0.61 ± 0.15	0.51 ± 0.09	-

Ns, number of stems sampled; MLG, number of multilocus genotypes; MLL, number of multilocus lineages at Bruvo's genetic distance threshold of 0.1; H_o, observed heterozygosity ± standard error (SE); H_e, expected heterozygosity ± SE.

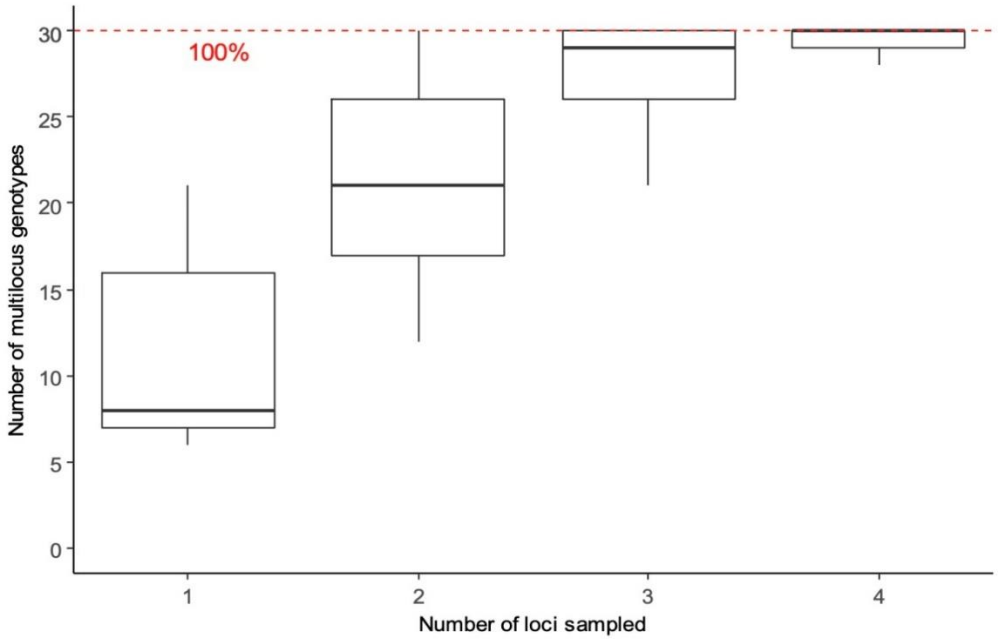
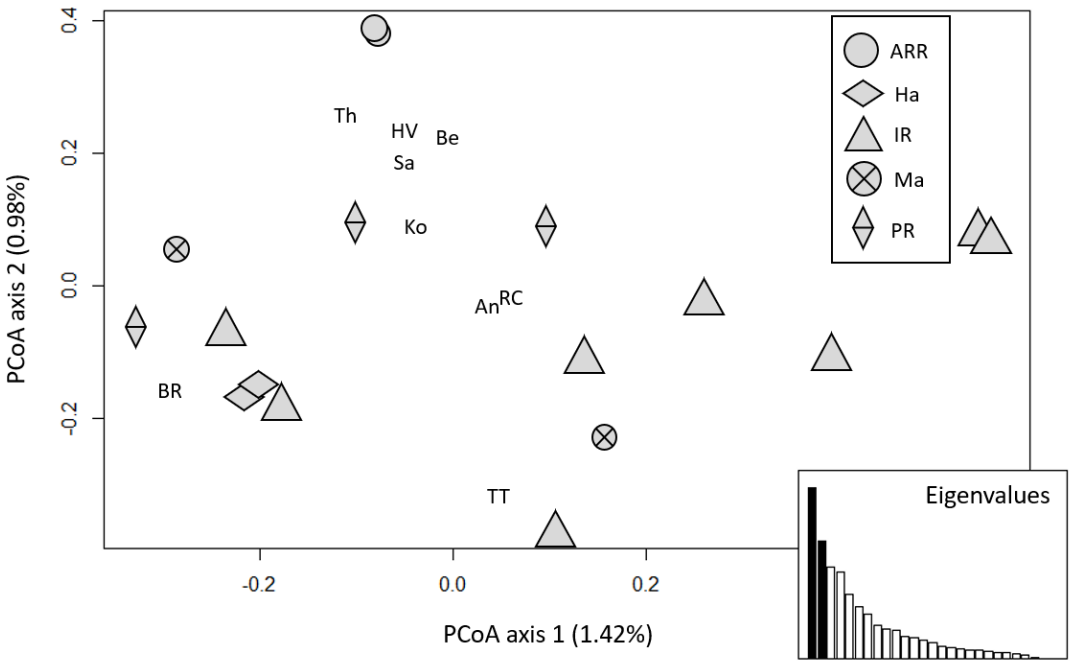


Figure 2.2: *Santalum acuminatum* microsatellite genotype accumulation curve of 1000 random samples of n loci.

Population structure

PCoAs based on both Bruvo's and Lynch's genetic distances did not indicate any population genetic structure with very little variation explained by each PCoA axis (Figure 2.3 A and B respectively). Mantel tests indicated that genetic and geographic distances were not correlated for both Bruvo's and Lynch's genetic distance measures.

A)



B)

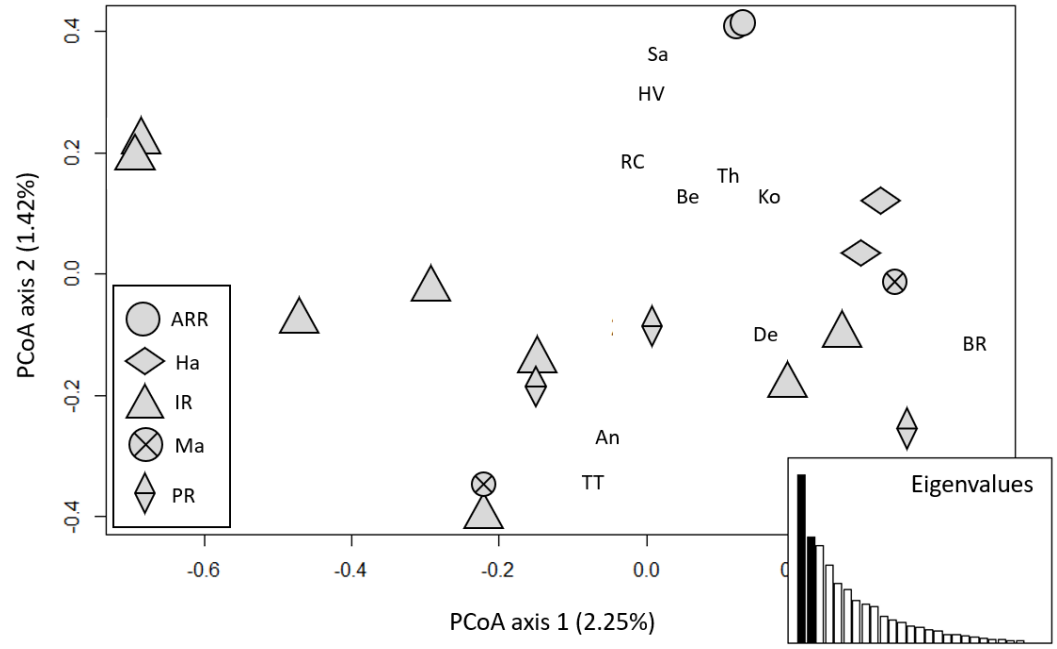


Figure 2.3: Plot of the first two Principal Coordinates Analysis (PCoA) axes for 27 *Santalum acuminatum* multilocus genotypes (MLG) based on A) Bruvo's genetic distance and B) Lynch's genetic distance. Eigenvalues represent the degree of variance displayed in PCoA axes (filled bars) relative to hidden axes (unfilled bars). Populations comprising more than one MLG are represented by symbols. Populations comprising single MLG's are denoted with population ID (see Table 2.1).

Chromosome counts

Chromosome numbers were obtained from the root tips of plants representing 14 stands and the leaf tips of plants representing ten stands. Due to variation in chromosome number between cells of the same individual – likely due to chromosome overlap and/or breakage – somatic chromosome numbers were expressed as a range. Chromosome numbers were within the range of 2n = 18–22 for all stands, except for one (ARR) that was triploid (Figure 2.4).

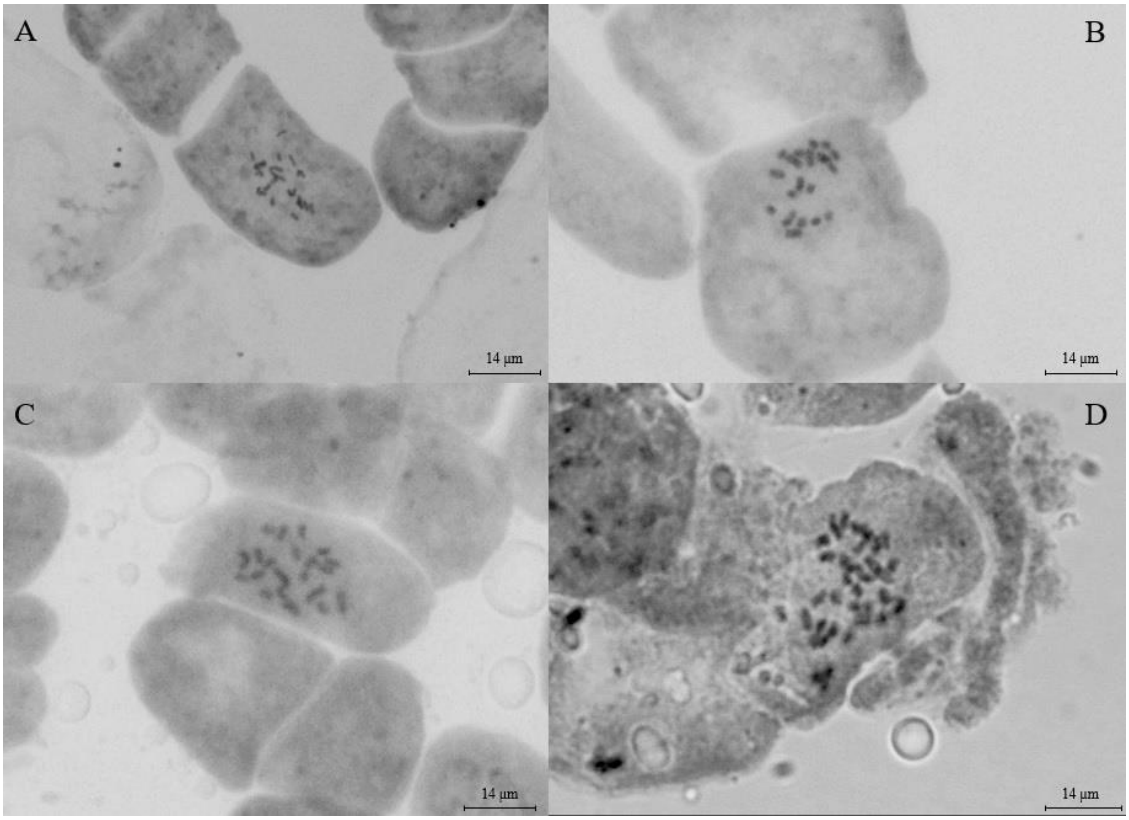


Figure 2.4: Chromosome images for *Santalum acuminatum* stands A) 2n = 18, B) 2n = 20, C) 2n = 21/22, D) 2n = 30.

Microsatellite evidence of polyploidy

From a total of 87 samples, six samples (four field collected, and two herbaria sourced) amplified three fragments within the expected size range and repeat motif (Table 2.4). Across all samples, amplicons were consistent in electropherogram size and appearance and repeated amplification undertaken on Be, Ko and PBR produced identical results, excluding PCR error. Private alleles for loci amplifying three fragments were seen for Lanc07 (PBR) and Lanc09 (Be). No samples amplified more than three fragments.

Table 2.4: Microsatellite fragment lengths, including primer, amplified in *Santalum acuminatum.* Only samples amplifying more than two lengths at any given locus are included. Lengths displayed as number of base pairs.

Population ID	State	Lanc07			Lanc09			mSaCIRF10			mSiCIR185		
		L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3
Be	NSW	145	149	-	190	194	196	163	167	171	285	289	-
Ko	VIC	145	145	-	194	196	-	163	173	177	285	289	-
PBR	VIC	139	145	147	192	194	196	173	175	-	287	289	-
RC	VIC	145	145	-	192	194	-	171	177	-	285	287	289
MEL 2329929	VIC	143	147	149	195	197	-	172	176	-	286	286	-
PERTH 7935315	WA	-	-	-	193	193	-	146	174	-	280	286	290

Discussion

This study aimed to identify the levels of genetic and genotypic diversity present in the south-eastern distribution of *Santalum acuminatum* and to determine whether population genetic structure and clonality were evident. Given mixed reports of polyploidy (see Randall 2000, Byrne et al. 2003, Harbaugh 2008), this study also aimed to establish if intraspecific ploidy variation occurs in *S. acuminatum* by assessing an increased number of samples from a wider distribution than had previously been considered. Heterozygosity estimates were relatively high for sites comprising more than one genotype. However, all but one population (IR) had low levels of genotypic diversity, with many consisting of single genotypes suggestive of monoclonal stands. No population structure nor evidence of increasing genetic isolation by distance was found across the study area. Six stands amplified three alleles across multiple microsatellite loci suggestive of either having additional chromosomes (Sosa et al. 2014, Roberts et al. 2017) or duplicated loci (Zhang and Rosenberg 2007). Chromosome counts revealed ploidy levels were largely within the same range for all stems assessed, but a single triploid stand was identified.

Clonality

Clonal reproduction has been reported in many *Santalum* species, including *S. album* (Dani et al. 2011), *S. insular* (Lhuillier et al. 2006a) and *S. lanceolatum* (Warburton et al. 2000, Brunton et al. 2021). Tennakoon et al. (1997a) reported clonal reproduction in *S. acuminatum* from root excavations and observations of genet death following fire in populations from south-western Australia. The results of this study provide supporting evidence of clonal reproduction in the species' south-eastern distribution as well as the first genetic evidence of clonality in the species. Furthermore, 87% of all sites contained just one MLL with genotypic diversity much lower than the average reported for other

clonal taxa (this study mean = 0.14 *cf.* mean of other taxa = 0.42; Vallejo-Marín et al. 2010). While low genotypic diversity could be in part due to the number of loci used, a genotype accumulation curve suggested that four loci were sufficient to differentiate between multi-locus genotypes in this study. Population monoclonality is thought to be rare for terrestrial clonal taxa (Silvertown 2008), however, it has been reported for other *Santalum* species. Allozyme and random amplified polymorphic DNA (RAPD) analysis found all five remnant *S. lanceolatum* populations from Victoria consisted of single genotypes resulting from clonal propagation, with genets comprising up to 116 ramets (Warburton et al. 2000). An analysis of clonality in French Polynesian populations of *S. insular* using microsatellite markers found that 81% of populations exhibited some degree of clonality with 42% being monoclonal (Lhuillier et al. 2006a). In both studies, the extent of clonal reproduction varied among populations, possibly due to environmental differences between sites (Warburton et al. 2000, Lhuillier et al. 2006a).

Investment in clonal reproduction may be an adaptation for species occupying stressful or patchy environments. For example, greater investment in clonal reproduction has been observed in plants occupying more arid Australian ecosystems (Kearney 2003, Roberts et al. 2016). Resource sharing between ramets is known to promote persistence and growth (Pennings and Callaway 2000), potentially offering a competitive advantage in these habitats. As a root hemiparasite, *S. acuminatum* may also profit from clonal reproduction as it allows access to a wider variety and number of host plants, facilitating persistence. On the other hand, stressors such as disturbance, grazing pressure and fire can also promote clonal reproduction in some species (Hewitt 2020). Many *S. acuminatum* populations occur along roadsides and at the fringes of reserves where disturbance from roadside grading and agricultural activities have been known to induce clonality in some tree species (Spooner 2005, Bognounou et al. 2009). Clonal investment may also be a

response to increased grazing pressure by introduced herbivores. The leaves of *S. acuminatum* are highly palatable (Applegate and McKinnell 1991), with grazing by rabbits threatening recruitment in New South Wales (Denham and Auld 2004) and camel grazing an issue in the Northern Territory (Box et al. 2016). The pressures of grazing are better tolerated by ramets than seedlings (Del Tredici 2001) which may explain the prevalence of clonal reproduction and lack of seedling establishment across the study area. Clonal reproduction is also a common adaptation for post-fire survival in many species (Clarke and Knox 2002) and increased clonality has been observed in *S. acuminatum* populations from south-western Australia following low intensity fire (Tennakoon et al. 1997a). Contemporary fires are unlikely to drive the observed clonality, with fires having been recorded at just two sites (An and Ha) post 1997 (Belbin 2011), but considering the potential longevity of these clones, historical fire may have played a role. On the other hand, seedling mortality in this species is thought to be high (Tennakoon et al. 1997a), potentially due to adverse and/or stochastic environmental conditions and/or this species' hemiparasitic nature. Studies have observed a growth benefit to forming host connections within the first year of establishment (Loveys et al. 2002). Thus, the extensive clonality observed in this study may be a by-product of seedling mortality rather than a bias towards clonal reproduction. It is possible, therefore, that *S. acuminatum* exhibits multiple reproductive strategies involving both sexual and clonal reproduction where clonality serves as an adaptation for survival in stressful environments with high seedling mortality limiting sexual output.

Genetic diversity

As most sites in this study were found to consist of single genotypes (MLL) with genotypic diversity low across the study area, population genetic diversity estimates were only possible for three sites. Across these sites, observed heterozygosity was generally high

(mean H_o = 0.61) and on average greater than expected heterozygosity (mean H_e = 0.51). Genetic diversity analyses using single nucleotide polymorphisms (SNPs) on *S. acuminatum* populations from Western Australia, South Australia and New South Wales found population heterozygosity to be low (0.062; Fuentes-Cross 2015). In comparison to other *Santalum* species where population heterozygosity was estimated using microsatellite markers, the heterozygosity estimate is comparatively high in *S. acuminatum*. For example, observed heterozygosity across all studied populations was 0.45 in *S. austrocaledonicum* (Bottin et al. 2005b), 0.39 for *S. insular* (Lhuillier et al. 2006a) and 0.41 for *S. lanceolatum* (Brunton et al. 2021). However, population sizes, the number of populations for which heterozygosity was estimated, and the number of microsatellite loci used were greater in these prior studies. Such differences may have allowed a greater portion of genetic diversity to be sampled. In this study, low genotypic diversity and small population sizes prevented the estimation of heterozygosity for most sites. On the other hand, such differences may be due to variation in reproductive strategy.

Compared to random mating, genetic diversity should, theoretically, be higher in obligately outcrossing populations (Holsinger 2000) and lower where there is inbreeding (Newman and Pilson 1997) and self-fertilisation (Loveless and Hamrick 1984). For clonal systems, however, overlapping generations can make inference of breeding system from genetic diversity estimates complex. Clonal plant populations may have elevated heterozygosity as, in the absence of recombination, heterozygosity is fixed in individuals (Balloux et al. 2003). Consequently, diversity gained through sexual reproduction (Kimpton et al. 2002, Rodger et al. 2021) or somatic mutation (Klekowski 1997, Caetano-Anollés 1999, Gross et al. 2012) is maintained clonally across generations, increasing heterozygosity over time (Judson and Normark 1996). Simulations provide theoretical evidence that heterozygosity increases in diploid populations with increasing clonality,

while genotypic diversity decreases (Balloux et al. 2003). Heterozygote excess was attributed to clonal reproduction in wild cherry (*Prunus avium*) (Stoeckel et al. 2006), while fixation of genetic diversity gained through historic sexual reproduction and maintained clonally has been suggested for *Grevillea infecunda* (Kimpton et al. 2002) and *Rutidosis leptorrhynchoides* (Rodger et al. 2021). For *Calystegia collina* a combination of somatic mutation and clonal reproduction was suggested to explain the high levels of genetic diversity despite small population sizes (Wolf et al. 2000). The relatively high population heterozygosity estimates, evidence of extensive clonality and low genotypic diversity observed in this study is consistent with such interpretations. Furthermore, the assignment of multiple MLGs to the same MLL provides evidence that somatic mutation may play a role in generating genetic diversity in this species.

Population structure

Breeding systems characterised by limited gene flow between populations are expected to have high population genetic differentiation (Loveless and Hamrick 1984). For clonal systems which can be characterised by low dispersal capacity of ramets and low investment in sexual reproduction (i.e., pollen and seed dispersal; Vallejo-Marín et al. 2010) strong population genetic structure is expected (Heywood 1991).

Unexpectedly, no population genetic structure was observed in this study and genetic differentiation was not explained by geographic distance. This suggested a high level of gene flow among these populations. Dispersal of ramets is an unlikely source of this gene flow and, indeed, no MLGs were shared among populations. Genetic analysis of central and western Australian populations of *S. acuminatum* found some population genetic structure in the region with evidence for long distance gene flow (Fuentes-Cross 2015). Pollen flow in *S. acuminatum* is facilitated by insects (Sedgley 1982) and seed dispersal is by emus (Dunstan et al. 2013) and bettongs (Murphy et al. 2015), with the burial of seed

by bettongs suggested as vital for seedling establishment (Leake 1962). Long distance seed dispersal by Aboriginal Peoples has also been suggested (Fuentes-Cross 2015), a practice which is thought to have facilitated the dispersal of other species such as the Australian baobab (Rangan et al. 2015). Given the activity of insects is generally at most a few kilometres (Pasquet et al. 2008) and the local extinction of bettongs in eastern Australia (Lomolino and Channell 1995), contemporary gene flow is unlikely to be responsible for the observed genetic continuity between these populations. Rather, a combination of historic gene flow and clonal reproduction may be responsible for the lack of genetic structure observed. Clonal reproduction slows genetic drift and selection which drive genetic differentiation between populations (Honnay et al. 2006). Such a phenomenon is thought to be responsible for the patterns of population genetic structure among clonal *Zingiber zerumbet* populations in comparison to related sexual and self-fertilising species (Huang et al. 2021). Thus, historic, or intermittent gene flow is likely to have driven population genetic similarity between *S. acuminatum* populations which has been maintained through clonal reproduction.

Ploidy variation

Prior amplification of restriction fragment length polymorphisms (RLFPs) in some Western Australian *S. acuminatum* stands found banding patterns consistent with polyploidy (Byrne et al. 2008). Here, amplification of microsatellite loci provided similar evidence in stands from Victoria, New South Wales, and Western Australia. However, through direct chromosome counts, three of these stands were found to have no more chromosomes than other stands. While the presence of additional microsatellite alleles is often interpreted as evidence of polyploidy (Sosa et al. 2014, Roberts et al. 2016, Roberts et al. 2017), gene/locus duplication may also lead to the amplification of additional alleles (Zhang and Rosenberg 2007). Gene duplication in the nuclear *waxy* gene (WX1) of

S. acuminatum has previously been reported (Harbaugh 2008) and may have included or occurred separately to the duplication of a microsatellite locus. Likewise, gene duplicates and microsatellite loci have been found on supernumerary (B) chromosomes in plants (Milani et al. 2014, Piscor and Parise-Maltempi 2016). Furthermore, the duplication of single chromosomes (aneuploidy) can also result in additional alleles without large changes to ploidy, though this is usually lethal in non-polyploids (De Storme and Mason 2014). While the likelihood of duplication events occurring independently across four microsatellite loci may be low, it may indicate that these loci are linked. Demonstrating linkage via recombination rates is, however, difficult in a mostly clonal species where genotypic diversity is low.

Using traditional root squashes and leaf preparations, the number of chromosomes within *S. acuminatum* was not found to vary significantly across the study area, suggesting that polyploidy is uncommon. While it was not possible to be specific in terms of ploidy due to small chromosome sizes, chromosome overlap and the possibility of chromosome breakage, the range observed was very similar to 2n = 20 as reported from two plants examined by Harbaugh (2008). This study is the first to report evidence of polyploidy in *S. acuminatum* and, while polyploidy occurs in other *Santalum* species, it has not yet been observed in mainland Australian populations (Harbaugh 2008). Interestingly, the single triploid stand (ARR) amplified at most two microsatellite alleles across all loci, a possibility if two alleles at each locus are homozygous (Sosa et al. 2014). Triploid formation largely occurs through two processes, sexually via unreduced gamete production or via hybridisation between a diploid and tetraploid (De Storme and Mason 2014). Due to the low frequency of triploids and lack of tetraploids it is likely this individual originated from unreduced gamete formation in a diploid parent. While unreduced gamete formation occurs randomly in nature, it may also result from

temperature stress (hot/cold shock) (Rezaei et al. 2010, De Storme and Geelen 2014). Plants with uneven chromosome numbers largely produce aneuploid gametes reducing fertility (Marks 1966, Ramsey and Schemske 1998, Burton and Husband 2000). While the reproductive status of the observed triploid is unknown, it is likely that fertility issues are present. Certainly, no seed was observed on or below this stand during field collections. Thus, the likelihood of this stand contributing to current or future sexual reproduction in the species is low.

Conclusion

This study has found evidence for extensive clonality, low population genotypic diversity, and a lack of population structure across the south-eastern distribution of *S. acuminatum*. Polyploidy in the form of triploidy was observed in a single population at the edge of the species' distribution in south-eastern Australia. These results suggest that the populations in the study area are predominantly clonal with, possibly very rare recruitment from seed. Such a predominance of clonal reproduction is likely due to high seedling mortality and environmental factors rather than variation in ploidy. Clonal investment may be an adaptation facilitating persistence in stressful or fragmented habitats where environmental disturbance is heightened and/or where mate availability is low. Given the longevity of clonal species such a life history trait may ensure long-term survival. However, the species' low genotypic diversity and seemingly low recruitment from sexual reproduction may limit adaptation to changing environments and/or novel pathogens. The high incidence of population monoclonality across the study area will limit outcrossing ability while prolonged investment in clonal reproduction may lower fertility due to somatic mutation. Thus, clonal reproduction in *S. acuminatum* may be a poor long-term survival strategy, possibly contributing to an extinction debt.

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Author Contributions

SC was responsible for the overall project design, data analysis and writing. SC was also responsible for all field and lab work in relation to ploidy level estimation by microsatellite amplification and all chromosome counts. Together SC contributed 60% to the study. JBC was responsible for project design, all lab work and field work in relation to the population genetic diversity and population genetic structure analyses, contributing 20% to the study. LB was responsible for project design, writing edits, data interpretation and conclusions, contributing 5% to the study. SH was responsible for project design, writing edits, data analysis and interpretation and conclusions, contributing 15% to the study.

Chapter 3

Genome size variation, B chromosomes, and polyploidy in the Australian endemic shrub *Santalum acuminatum* (Santalaceae)

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Abstract

Chromosomal evolution is underpinned by the processes of duplication, insertion, fission, and fusion, each with the capacity to facilitate speciation. Within plant populations, such processes can result in changes to genome size, ploidy and karyotype that may interrupt sexual reproduction through barriers to gene flow caused by inter-cytotype incompatibility. This study sought to understand the extent of genome size, ploidy, and karyotype variation in *Santalum acuminatum*, a species for which intraspecific ploidy variation has previously been observed. Flow cytometry, karyotype analysis and fluorescence *in situ* hybridisation (FISH) were used to estimate genome sizes (from leaf and seed material), determine ploidy levels, and understand whether gene/locus duplication has occurred – suspected from the prior amplification of additional microsatellite alleles in the absence of ploidy change. Genome sizes were consistent for all but one individual that had a larger than average genome size. This individual was also confirmed to be triploid (3n = 30) in a karyotype analysis while all other individuals were diploid (2n = 20) with some also showing evidence for 1–2 B chromosomes. The ratio of embryo to endosperm genome sizes in one individual differed from 2:3 as is expected for sexual seed, suggesting clonal seed production via autonomous apomixis or a triploid cytotype. FISH analysis revealed linkage between two microsatellite markers but lacked the resolution required to confirm gene/locus duplication. The correlation between amplification of additional microsatellite alleles, polyploidy and larger genome size raises questions of polyploidy in five additional individuals who previously amplified additional microsatellite alleles.

Key words: fluorescence *in situ* hybridisation, quandong, hemiparasite, linkage, triploidy, apomixis, clonality.

Introduction

In eukaryotic organisms the heritable unit of genetic transmission is the chromosome (Schubert 2007). While the general structure and function of chromosomes is highly conserved, taxa vary greatly in the size, shape, and quantity of chromosomes with implications for genome size and cellular processes (Heslop-Harrison and Schwarzacher 2011). Such variation is underpinned by processes of duplication, insertion, fission, and fusion leading to chromosomal evolution over time (Jones 1998). With a 24,000-fold difference in genome size (Pellicer et al. 2018) and chromosome numbers (i.e., ploidy) ranging from 2n = 2–1440 (Stace 2000), plant taxa possess great genomic diversity. Such genomic diversity has been correlated with species diversity, with chromosomal rearrangements and/or duplications marking the divergence of major clades (Kellogg and Bennetzen 2004). It is clear that genome size, structure and ploidy change are important processes in plant evolution and are useful tools for understanding species divergence and relationships (Vimala et al. 2021).

Of the processes involved in chromosomal evolution, polyploidy or whole genome duplication events are perhaps the most prolific (Soltis et al. 2009, Vamosi and McEwen 2013), leading to instantaneous changes in ploidy and genome size (Otto and Whitton 2000, Bowers et al. 2003). Polyploidy is widespread across plant taxa, having occurred in most plant lineages throughout evolutionary time (Masterson 1994, Soltis 2005, Jiao et al. 2011). Of the routes that lead to polyploidisation, unreduced gamete formation (2n) is the most common and may occur within a species (autopolyploidy) or between species (allopolyploidy) (Kihara and Ono 1926). As a result of incompatibilities between ploidy levels, newly formed polyploids (neopolyploids) are often reproductively isolated from their diploid progenitors (Ramsey and Schemske 1998). Lack of gene flow between ploidy races may therefore lead to speciation, a phenomenon thought to have contributed to 15%

(Vamosi and McEwen 2013) or more (Soltis et al. 2009) of plant speciation events. In addition to facilitating speciation, polyploidy is thought to have contributed to the evolution of novel traits (Airoldi and Davies 2012, Moriyama and Koshiba-Takeuchi 2018), and adaptation of species into new environments (Hahn et al. 2012). Polyploidy is thus considered to be an important force, having repeatedly shaped the evolution of plant linages.

In addition to polyploidy, changes involving single members of the chromosome complement and B chromosomes are additional sources of genomic variation commonly observed in plants. The full chromosome complement of an organism, (i.e., karyotype) evolves through chromosome addition or loss, chromosome rearrangements such as inversions and translocations and centric fission or fusion (Schubert and Lysak 2011). Any change to the karyotype may facilitate speciation by 1) reducing gene flow between cytotypes, underpinned by reduced fertility or sterility in inter-cytotype hybrids (Faria and Navarro 2010), and/or 2) suppressed recombination in genomic regions that over time accumulate sufficient incompatibilities (Navarro and Barton 2003). While rates of karyotype change vary across plant taxa due to genetic differences, environmental stress has also been suggested to increase karyotype change, specifically by increasing non-homologous recombination events (De Storme and Mason 2014). B chromosomes are supernumerary in nature, largely comprised of so called ‘junk’ DNA originally derived from the essential (A) set of chromosomes (Martis et al. 2012, Houben et al. 2014). They are present in many natural plant populations, although are often absent from cultivars and inbred populations (Jones et al. 2008). A diagnostic feature of B chromosomes is their presence in some but not all individuals of a species which in some cases may be a significant source of intraspecific genome size variation (Jones et al. 2008). In addition to increasing genome size, B chromosomes can have phenotypic consequences and can

reduce plant fertility and growth when they are present in high and/or odd numbers (Bougourd and Jones 1997, Jones and Houben 2003). However, it has also been observed, in both maize (Carlson 1969) and rye (Puertas and Carmona 1976), that pollen cells containing B chromosomes have a higher fertilisation success rate compared to pollen that lack B chromosomes. As such, B chromosomes are not necessarily disadvantageous. While B chromosomes are generally considered to be non-functional, genes also present on A chromosomes (Martis et al. 2012, Houben et al. 2014) and microsatellites (Milani et al. 2014, Piscor and Parise-Maltempi 2016) have been found on B chromosomes, with some evidence for gene activity (Leach et al. 2005). Unlike karyotypic change, B chromosomes are not thought to be involved in speciation events (Jones et al. 2008).

The same mechanisms that facilitate speciation following ploidy and/or karyotype change have the capacity to interrupt sexual reproduction within a population. Through inter-cytotype incompatibility, incidences of polyploidy and karyotype change can reduce the number of sexually compatible individuals within populations and thus, limit a population's capacity to reproduce sexually (Aguilar et al. 2019, Chen et al. 2019). Sexual reproduction between individuals (i.e., outcrossing) is important for many plant populations as a source of genetic diversity (Holsinger 2000) and to avoid the negative fitness effects that are often associated with inbreeding (Newman and Pilson 1997). Such genetic diversity may dictate a species' ability to cope with stress and disease (Frankham 2005) and respond to environmental change (Jump et al. 2009). Consequently, inter-cytotype incompatibility has gained recognition as a risk factor in several conservation and revegetation programs (Murray and Young 2001, Godfree et al. 2017, Kramer et al. 2018, Chen et al. 2019).

Santalum acuminatum is a hemiparasitic shrub native to mainland Australia. Polyploidy has been suggested in this species based on two independent molecular marker studies (Chapter 2, Byrne et al. 2003), where fragment amplification observed alleles in excess of diploidy. While the presence of additional alleles in such studies is often interpreted as evidence of polyploidy (Sosa et al. 2014, Roberts et al. 2016) it is possible for aneuploidy (De Storme and Mason 2014), gene/locus duplication (Wang et al. 2012) or B chromosomes (Milani et al. 2014, Piscor and Parise-Maltempi 2016) to produce similar results. On the other hand, following analysis of genome size based on flow cytometry and chromosome counts on two individuals (NSW and WA), Harbaugh (2008) suggested that *S. acuminatum* is diploid with a ploidy level of 2n = 20. However, more recent evidence from chromosome counts in a larger sample size than was examined by Harbaugh (2008) suggested there may be some intraspecific ploidy variation (2n = 18–22–(30); Chapter 2). A high incidence of polyembryony (twin seed) has also been observed in *S. acuminatum* (14% of germinated seed, Currie, 2021; personal observation) a trait often correlated with polyploidy (Muntzing 1938, Webber 1940). Consequently, multiple lines of evidence suggest ploidy change in *S. acuminatum* with the nature of genome variation in the species yet to be fully elucidated.

This study aimed to determine whether there is genome size variation in *S. acuminatum* and whether karyotype and/or ploidy change, or gene/locus duplications have occurred. Genome size variation was investigated via flow cytometry using fresh leaf, dried leaf, and seed material, allowing sampling from a broad geographic range. The ratio of embryo to endosperm genome sizes was also investigated to look for evidence of clonal seed production via apomixis. Apomixis is more common among polyploid lineages (Carman 1997) and may serve as a reproductive strategy to increase fertility among neopolyploids (Yamauchi et al. 2004). Karyotyping was undertaken to confirm chromosome number in

this species and to determine whether polyploidy, aneuploidy and/or B chromosomes are present. Finally, fluorescence *in situ* hybridisation (FISH) was performed using two microsatellite loci to determine their location in the genome and whether gene/locus duplication has occurred.

Materials and Methods

Study species

Santalum acuminatum is a root hemiparasite with non-specific host requirements, although there may be some preference for nitrogen fixing host species such as acacias (Tennakoon et al. 1997a, Nge et al. 2019). The species' distribution spans the southern half of mainland Australia from semi-arid ecosystems to coastal regions of south and western Australia. Clonal reproduction has been reported in the species (Tennakoon et al. 1997a, Harbaugh 2000), with evidence that this may be the species' main form of reproduction in its south-eastern distribution (Chapter 2).

Sampling

Leaf and seed material was sampled from seven stands in Victoria and New South Wales from November 2019 to February 2020. Leaf material was placed in paper envelopes and stored in plastic bags containing ~75 g of self-indicating silica gel beads (3–8 mesh; ChemSupply) for rapid drying. Dried leaf and seed material was donated from 13 stands across Australia. Several field collected and donated seed from multiple mother plants were germinated to generate fresh leaf and shoot meristems for flow cytometry,

karyotyping and FISH. Seed were extracted from the woody endocarp by applying force to the micropyle using a hammer. Seed were sterilised in 10% bleach for 25–30 min before being rinsed thoroughly in distilled water and placed in sterile glass petri dishes containing autoclaved vermiculite. Vermiculite was moistened with 5–10 mL of distilled water and petri dishes sealed using parafilm. Germination took place in the dark (petri dishes wrapped in aluminium foil) within a Thermoline growth cabinet set at 20°C. Seeds were monitored weekly and once shoot tips began to emerge from the endosperm, the seedlings were transferred to 30 x 40 cm pots containing juvenile *Acacia verticillata*, sourced from the La Trobe Wildlife Sanctuary, as the host species. These were grown in a 1:3 perlite: native soil media with a teaspoon of native slow-release fertiliser (Scotts Osmocote®) in a shade house at La Trobe University, Bundoora, Victoria and watered daily. Supplementing this, two sites (Berrigan and the Australian National Botanic Gardens; Table 3.1) were revisited during April 2021 to collect fresh leaf and shoot meristems, which were kept in water at ~4°C until processing. As each technique required a specific type of material, it was not possible to undertake each technique on all individuals (Table 3.1).

Table 3.1: *Santalum acuminatum* stands sampled for this study and the techniques undertaken on each.

Provenance	State	Latitude, longitude	Sample ID	Flow Cytometry			Karyotype	FISH
				Fresh leaf	Dried leaf	Seed		
Koorlong	VIC	-34.34, 142.09	Ko		x			
Nathalia	VIC	-36.06, 145.20	Na S2	x				
			Na S3	x				
			Na S4			x		
			Na S7			x		
			Na S10			x		
Prilta	VIC	-34.36, 141.90	PBR		x			
Red Cliffs	VIC	-34.33, 142.13	RC		x			
Berrigan	NSW	-35.74, 145.80	Be	x	x		x	
Broken Hill	NSW	-	BH SPA S1	x				
Ingalba	NSW	-34.44, 147.44	C2					
			B3 S1			x		
			B3 S4			x		
			B3 S6	x	x			
			B3 S7			x		
			C3 S1	x				
Pucawan	NSW	-34.45, 147.35	Pu A1 S4		x			x
Savernake	NSW	-35.75, 146.03	Sa S7			x		
			Sa S8			x		
			Sa S9			x		
Umberumberka Reservoir	NSW	-31.83, 141.21	UR S1	x	x		x	x
			UR S4		x			
			UR S5		x			
			UR T1		x			
			UR T2		x			
			UR T3		x			
Gladstone	SA	-	MK T20 S5				x	x
Whyalla/Ceduna	SA	-	MK T5 S1				x	
Gladstone	SA	-	MK T17 S1				x	
Gladstone	SA	-	MK T17 S2				x	
Unknown	-	-	MK T13 S4					
CDR	WA	-29.57, 116.97	CDR 2019		x			
			CDR S6		x		x	x
Hamelin	WA	-29.49, 114.11	HMLc					
			HMLc S1	x	x		x	x
Mulga Park	NT	-25.91, 131.67*	MP T1		x			
			MP T2		x			
			MP T2 S3	x				
			MP T2 S9			x		
Yulara	NT	-25.26, 130.97	Yu S20			x		
			Yu S23			x		
			Yu S23			x		
Australian National Botanic Gardens	-	-	CANB 879793.9	x			x	x
			CANB 879793.8	x				
			CANB 879793.3	x				

FISH = fluorescence *in situ* hybridisation; *, possible error of up to 100km.

Genome size estimation

Approximately 10 mg of fresh leaf, 20 mg of dried leaf or 20 mg of seed (endosperm and embryo) was macerated in a petri dish containing 500 µL CyStain® PI Absolute P Nuclei-Isolation Buffer (SYSMEX) with 20 mg of internal standard (*Glycine max*) using a razor blade for ~30 sec. Dried leaf and seed material was incubated in this buffer for 5–10 min prior to maceration to hydrate cells. The resultant material was then filtered using a nylon mesh (40 µm pore size) and transferred to 5 mL Falcon tubes (REF 352052) before addition of 1 mL staining mixture (20 ml SYSMEX CyStain® PI Absolute P Staining Buffer, 120 µL SYSMEX CyStain® PI Absolute P Propidium Iodide and 60 µL SYSMEX CyStain® PI Absolute P RNase A, for ~20 reactions). The samples were run on a BD Accuri™ C6 Plus Flow Cytometer using associated BD Sampler Plus software with a threshold of 80,000. Samples were run until at least 2,000 events were recorded under both target and standard peaks. Genome size was calculated using the equation:

$$\left(\frac{Mean\ PE\text{–}A\ target\ individual}{Mean\ PE\text{–}A\ standard}\right)\ x\ genome\ size\ of\ standard\ (2.5\ pg)$$

Each sample was repeated at least twice, and the mean genome size (± standard error) was recorded. For seed, both embryo and endosperm genomes sizes were recorded.

All statistical analyses, unless otherwise indicated, were undertaken using R version 3.4.3 and the R stats package (R Core Team 2019). All graphs were constructed using the package ggplot2 (Wickham 2016). To look for statistical differences in genome size among individuals an analysis of variance (ANOVA) was conducted and differences visualised using comparative box plots. Tukey pairwise comparisons were undertaken to look for statistical differences at an alpha of 0.05. The total mean genome size of seed embryos was used to calculate the expected genome size of the endosperm under a 2:3 and 1:2 embryo to endosperm ratio. The expected genome sizes were compared to the

observed endosperm genome sizes using ANOVA to test for deviations from a 2:3 ratio. Such a deviation may suggest asexual seed production (i.e., apomixis) is occurring in the species (Chen et al. 2019, Hojsgaard and Horandl 2019).

Karyotype

Fresh leaf buds from target individuals were sampled during the morning and pre-treated according to the protocol outlined in Anamthawat-Jonsson (2003) with the amendment that, leaf buds were hydrolysed in 1M HCl for 1 min at 60˚C before enzyme digestion for 3 hours where material was broken up using tweezers each hour. Approximately 8 µL of cell suspension was dropped from a height of ~20 cm onto a slide to lyse nuclei and spread chromosomes for better visualisation. Once dry, ~40 µL of antifade medium VECTASHEILD® (Vector Laboratories, Burlingame, CA, USA) containing 15 mg/mL DAPI was applied followed by a coverslip. Slides were incubated in the dark at room temperature (18–22°C) for 30 min before karyotyping using a ZEISS Axio Scope A1 microscope with ZEISS AxioCam MRm camera and MetaSystems ISIS Fluorescence Imaging Platform.

Karyotypes were obtained from 5 to 10 metaphase spreads per individual. For each cell chromosomes were arranged from longest to shortest homologous pair. Chromosome arms were measured from the centromere and averaged across three cells per individual to obtain the mean chromosome length and the ratio of long to short arm. Chromosomes were assessed for symmetry based on arm ratio and classified as metacentric (median), submetacentric (sub-median), subterminal or terminal based on the criteria established by Levan et al. (1964). Karyotype asymmetry was assessed based on the classifications described by Stebbins (1971). The karyotype formula for each cytology was reported as the number of metacentric (m) + submetacentric (sm) + subterminal (st) + terminal (t) + B chromosomes (b) (Stebbin's karyotype asymmetry classification).

Fluorescence in situ *hybridisation (FISH)*

Two microsatellite loci (Lanc09 and mSaCIRF10) that previously amplified more than two alleles in a genetic diversity analysis in *S. acuminatum* (Chapter 2; Table 2.2 C and E) were selected for FISH. Hybridising probes were synthesised by amplification of target loci via polymerase chain reaction (PCR) in four target individuals (Be, UR S1, UR S5 and CDR). PCR took place in an admix of 25 µL 2x MyTaq Red Mix, 2 µL forward and reverse primer, 10 µL template DNA, 0.6 µL of FITC-Fluorescein-12-dUTP (Roche) for Lanc09 and Texas Red-12-dUTP (Invitrogen) for mSaCIRF10 and 10.4 µL nuclease free water. PCR conditions were as follows; initial cycle of 5 min at 95˚C, followed by denaturation of 30 sec at 95˚C, annealing for 1.5 min at 62˚C for Lanc09 and 53.5˚C for mSaCIRF10 and extension of 30 sec at 72˚C for 30 cycles before a final cycle of 30 min at 60˚C. Following PCR, the products were pooled, and DNA precipitated in 1 µL of glycogen and 300 µL of absolute ethanol overnight at -20˚C. Probes were then centrifuged at 15 000 rpm for 30 min and the supernatant discarded. The tubes were then incubated at 37˚C for 5–10 min to completely remove any residual ethanol. Once dry, 40 µL of hybridisation buffer (50% formamide, 10% dextran sulfate, 2x saline sodium citrate (SSC), 40 mmol/L sodium phosphate pH 7.0 and 1x Denhardt's solution) pre-warmed to 37˚C was added and tubes incubated at 37˚C to resuspend pellet. Probes were stored at -20˚C until use. Cell suspensions prepared and dropped onto slides according to the protocol described for karyotyping were aged on a heat block set to 60˚C for 1 h before immersion in 70% ethanol for 2 min, 90% ethanol for 2 min and 100% ethanol for 2 min to dehydrate chromosomes. Once dry, 20 µL of probe mixture (Table 3.2) was added to slides followed a cover slip, sealed through application of rubber cement to cover slip edge. DNA was denatured on a 67˚C heat block for 5 min followed by 72 h hybridisation at 37˚C. Cover slips were removed, and slides washed in 0.4x SSC buffer and 0.3% Igepal at 37˚C for 2 min, followed by another wash in 2x SSC and 0.1% Igepal at room

temperature for 1 min. Slides were then immersed in 70% ethanol for 2 min, 90% ethanol for 2 min and 100% ethanol for 2 min. Once dry, ~40 µL of antifade medium VECTASHEILD® containing 15 mg/mL DAPI was applied to slides followed by a coverslip. Slides were viewed under a ZEISS Axio Scope A1 microscope with ZEIZZ AxioCam MRm camera and MetaSystems ISIS Fluorescence Imaging Platform.

Table 3.2: Fluorescence *in situ* hybridisation probes designed via PCR from and used on *Santalum acuminatum* samples.

PCR probe designed	Sample used on
UR S1	UR S1
CDR	CDR S6
UR S5	NBG 9
CDR	HMLc S1
Be	Pu A1 S4

Results

Genome size estimation

Genome size was estimated for 12 individuals using fresh leaf material and seven individuals using seed. While the genome size of the endosperm was estimated for all seed, six seed embryos could not be estimated, likely due to insufficient amounts of embryonic tissue. Genome size estimation from dried leaf material was inconsistent and subsequently excluded from analyses. Genome sizes ranges from 0.6 to 0.85 pg (± 0.01–0.03 pg respectively) for fresh leaf with the Be sample having a significantly larger genome size (0.85 ± 0.01 pg) than all other samples (0.6–0.69 pg ± 0.01–0.03 pg respectively) (Figure 3.1 A; $p < 0.01$ in all pairwise comparisons, see Supplementary material, S3.1). This increase in genome size was 1.35x the mean genome size of all other

samples. The mean genome size of seed embryos ranged from 0.5 to 0.63 pg (± 0.01–0.02 pg respectively), while seed endosperm ranged from 0.92 to 1.16 pg (± 0.01–0.05 pg respectively) (Figure 3.1 B). The mean genome sizes of all seed embryos did not differ statistically from one another, while the NaS7 endosperm was significantly larger than most other endosperm samples ($p < 0.02$ in all pairwise comparisons) except InB3S7 (see Supplementary material, S3.2). The mean genome size of seed embryos was 0.62 pg and the expected genome size of endosperm given a 2:3 and 1:2 ratio was 0.93 pg and 1.23 pg, respectively. In most pairwise comparisons the genome sizes of endosperm did not differ significantly from expected under a 2:3 ratio, except for NaS7 ($p < 0.001$ in all pairwise comparisons) which was consistent with a 1:2 ratio (see Supplementary material, S3.3).

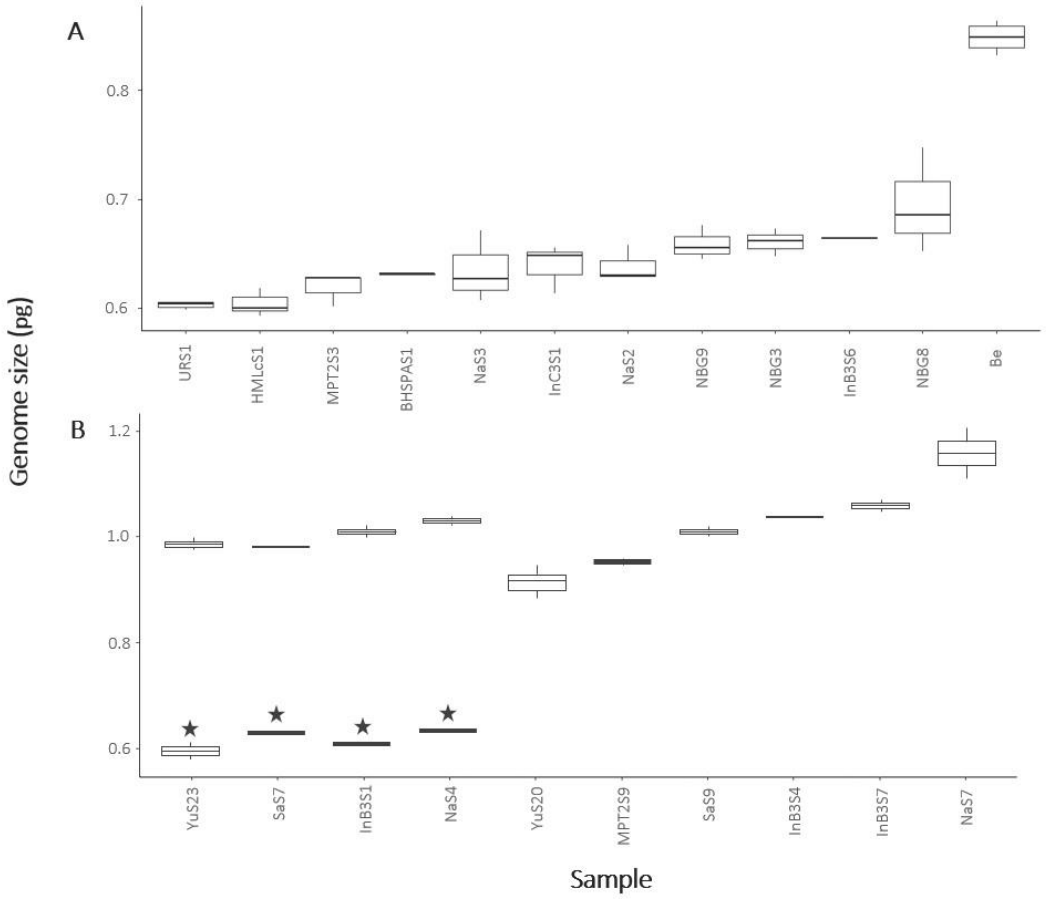

Figure 3.1: Comparative box plots of mean genome size in *Santalum acuminatum* stands measured from A) fresh leaf material, B) seed where genome sizes from embryo are denoted with a star and endosperm is unmarked. Boxes represent IQR with median represented by horizontal breaks, whiskers represent minimum and maximum.

Karyotype

Karyotypes were determined for nine individuals. Of these, eight were diploid with 2n = 2x = 20 chromosomes and one was triploid with 3n = 3x = 30 chromosomes (Figure 3.2). Of the diploid individuals, six had one or two B chromosomes. Overall, the chromosomes were small, with the mean length across all individuals ranging from 1.28 to 3.19 µm and the mean ratio of the longest to shortest chromosome 1.62. The karyotype formula for diploids was 2n = 20 = 16m + 4sm + 0–3b (2B) with a satellite on Chromosome 3. The karyotype formular for triploids was 3n = 30 = 24m + 6sm (2B) also with a satellite on Chromosome 3. Size dimorphism in Chromosome 1 was observed in five individuals.

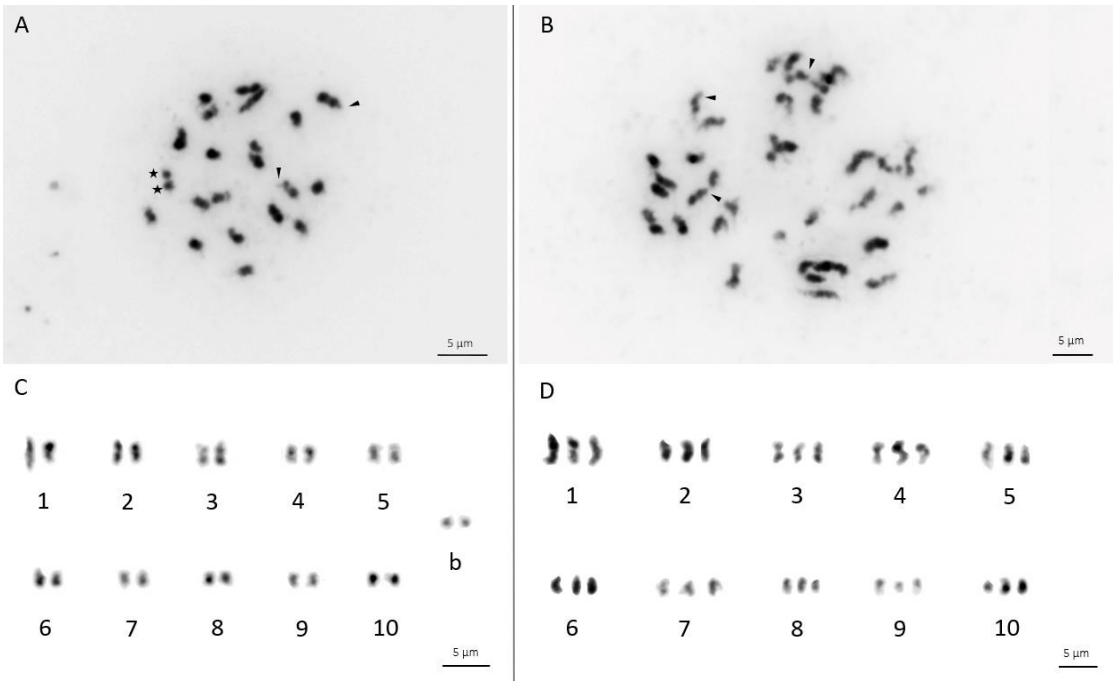

Figure 3.2: Karyotypes from diploid (A and C) and triploid (B and D) *Santalum acuminatum* individuals. The A chromosome set is numbered 1–10 with B chromosomes labelled b. Arrows indicate satellites on Chromosome 3. Stars indicate B chromosomes.

Fluorescence in situ *hybridisation (FISH)*

Hybridisation of both microsatellite probes was successful for four individuals. In each case, chromosome clarity and separation were poor. Consequently, discerning which chromosome the markers were located on was not possible. Similarly, hybridisation of each marker was inconsistent between cells of the same individual and thus, it was not possible to determine allele copy number. What was consistent between cells of an individual and between individuals was the location of the microsatellite markers in relation to each other. In every instance, both markers hybridised in the same location, indicating that these markers are located close to each other on the same chromosome (Figure 3.3).

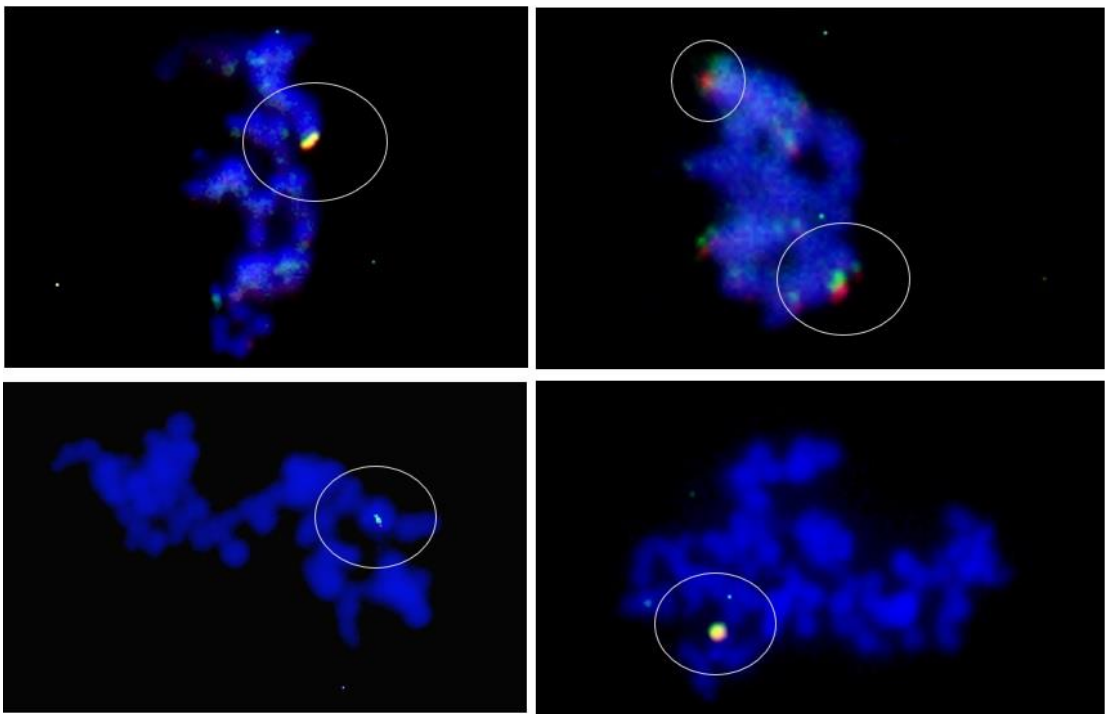

Figure 3.3: Fluorescence *in situ* hybridisation of two microsatellite markers; Lanc09, green and mSaCIRF10, red in *Santalum acuminatum.* Location of markers on the genome marked by circles.

Discussion

Genome size, karyotype, and ploidy variation can facilitate speciation events in plants by impeding gene flow between cytotypes. These same processes can interrupt sexual reproduction in species with the potential to reduce mate availability, increase inbreeding and reduce genetic diversity. This study sought to determine the presence and extent of genome size, ploidy and karyotype variation in the hemiparasitic plant *S. acuminatum.* Genome size estimation via flow cytometry found one individual to have a larger than average genome size, while the expected ratio of 2:3 (embryo to endosperm genome sizes) for sexually derived seed, differed for another individual. Karyotype analysis found most individuals to be diploid with a cytology of 2n = 20 A chromosomes with some individuals having 1–2 B chromosomes. Triploidy was observed in a single individual that also had a larger than average genome size, though the increase in genome size was not proportional to the increase in ploidy. Fluorescence *in situ* hybridisation revealed possible linkage in two microsatellite markers previously used in a genetic diversity analysis (see Chapter 2).

Genome size variation

A previously reported genome size for *S. acuminatum* was 0.67 ± 0.01 pg for two individuals originating from New South Wales and Western Australia (Harbaugh 2008). The genome sizes of individuals estimated from fresh leaf and seed embryo in this study were consistent with Harbaugh's results (mean = 0.63 ± 0.004 pg), except for Be whose genome size was significantly larger (0.85 ± 0.007 pg). The genome size of *S. acuminatum* is well below the average observed for angiosperms (*S. acuminatum* in this study = 0.62 pg *cf.* angiosperm mean = 5.9 pg; Heslop-Harrison and Schwarzacher 2011). Genome sizes may be influenced by several factors. For example, small genomes have been associated with faster growth and invasiveness (Bennett et al. 1998, Fridley and Craddock

2015). Contrastingly, large genomes are often absent from areas characterised by low precipitation and high temperatures, with evidence suggesting that smaller genomes are more adapted to extreme conditions (Knight and Ackerly 2002). Such correlations are thought to be underpinned by the positive relationships between genome size, cell size (Beaulieu et al. 2008), cell cycle duration (Bennett et al. 1977) and growth rates (Mukherjee and Sharma 1990). Smaller genome species are expected to benefit from smaller cell size – conferring smaller organ sizes such as stomata which mitigates water loss (Doheny-Adams et al. 2012, Durand et al. 2020), and a faster cell cycle that promotes rapid growth (Fridley and Craddock 2015). These relationships, however, are not consistent for all taxa (Mukherjee and Sharma 1990). As a species that occupies arid and semi-arid ecosystems, selection for small genome sizes or constraints on genome size increases in *S. acuminatum* may reflect the environment this species occupies (see Chapter 4). Likewise, *S. acuminatum* may benefit from small genome sizes should they confer a faster cell cycle and growth rates promoting rapid growth and/or establishment when conditions are favourable.

Based on chromosome counts of selected *Santalum* species, including *S. acuminatum*, Harbaugh (2008) estimated that n = 10 diploids (2n = 20) across the genus would have genome sizes within the range of 0.61 to 0.98 pg. The genomes sizes of all fresh leaf and seed embryo in this study are within this expected diploid range. Since genome sizes are largely consistent among individuals of the same species (Swift 1950) and the genome sizes of most individuals considered here (from leaf and seed embryo) were statistically similar, the significant increase in genome size observed for Be (1.35 x the mean of all other individuals) is suggestive of chromosomal change in this individual. Polyploidy is expected to confer an increase in genome size proportional to the increase in ploidy (Leitch and Bennett 2004, Kron et al. 2007) as has been observed in neotriploid (Puangpairote et

al. 2016) and neotetraploid lineages (Domínguez-Delgado et al. 2021). However, a 17% reduction in the genome size of synthetic neotetraploids has also been observed in *Phlox drummondii*, with further reductions in genome size of subsequent generations associated with increased fertility (Raina et al. 1994). Such immediate reduction may constitute adaptive diploidisation in the species (Parisod et al. 2010), whereby alterations to the polyploid genome over time return the genome to a functionally diploid state. Similar mechanisms may have led to an instantaneous reduction in genome size of triploid *S. acuminatum.* The adaptive potential of this reduction may be limited since no fruit production has been observed for this individual (Be) in the last 15 years (S. Logie, personal communication, 2018), suggesting that this individual is infertile.

Due to the presence of polyploidy and polyembryony in *S. acuminatum* – traits often associated with apomixis (Carman 1997) – the ratio between the genome sizes of the embryo and endosperm were assessed for deviations from a 2:3 ratio that may indicate apomictic seed (Chen et al. 2019, Hojsgaard and Horandl 2019). The ratio of embryo to endosperm genome sizes did not differ from 2:3 in all individuals except NaS7 for which the genome size of endosperm was consistent with an embryo to endosperm ratio of 1:2. This 1:2 ratio suggests that apomixis is occurring in the absence of fertilisation (autonomous endosperm), where the maternal contribution to endosperm is twice that of the embryo (i.e., 2n embryo to 4n endosperm) (Henderson et al. 2017, Hojsgaard and Horandl 2019). On the other hand, the endosperm size of NaS7 is consistent with a triploid embryo originating from an unreduced paternal gamete. The maternal contribution to endosperm in most angiosperms is twice that of the paternal contribution owing to the fertilisation of one sperm cell (paternally derived) with two polar nuclei (maternally derived) (Friedman 1995). Thus, an unreduced pollen cell would contribute equally to the endosperm following fertilisation with a reduced egg giving an embryo to endosperm

genome size ratio of 3:4. Based on a triploid embryo size of 0.85 pg, consistent with the Be sample, the endosperm size of NaS7 is in line with this ratio. As the genome size of the embryo was unknown for this individual, it was not possible to confirm whether this was apomictic or triploid seed.

Karyotype

The results of this study corroborate prior research describing *S. acuminatum* as a primarily diploid species with 2n = 20 chromosomes (Harbaugh 2000). Karyotype analysis found the centromeres of most chromosomes in a median position (metacentric) with B chromosomes present in most individuals. Prior karyotype analysis of *S. album* found a similar proportion of metacentric chromosomes (2n = 20 = 18m + 2sm) and evidence for B chromosomes while karyotype asymmetry between the species fell within the same category (2B) (Xin-Hua et al. 2010). Symmetrical karyotypes with a high proportion of metacentric and submetacentric chromosomes of similar size are thought to be more primitive than asymmetric karyotypes with centromeres in more terminal positions (Stebbins 1971). Despite being indexed within the same asymmetry classification, *S. acuminatum* was found to have a slightly higher proportion of submetacentric chromosomes compared to *S. album* (*S. acuminatum* this study = 4sm *cf. S. album* 2sm; Xin-Hua et al. 2010). This difference may be a source of differentiation between the species and emblematic of chromosome evolution in *S. acuminatum*. In addition to karyotype symmetry, shorter chromosomes are thought to be more derived than longer chromosomes (Vimala et al. 2021). Chromosome size in plants varies from 0.8 µm in *Chamaelirium luteum* (Tanaka 2020) to ~ 30 µm in *Paris japonica* (Vimala et al. 2021). The chromosome size range observed for *S. acuminatum* was at the shorter end of this range suggesting evolution towards or maintenance of shorter chromosomes in this

species. Since *S. album* has similar sized chromosomes (Xin-Hua et al. 2010), such processes are likely to have occurred in the genus prior to the divergence of these species.

In addition to the essential set of chromosomes, six of the nine individuals karyotyped in this study had 1–2 B chromosomes. B chromosomes can have a variety of consequences for individuals, such as reducing fitness in high and/or odd numbers (Jones and Houben 2003). As *S. acuminatum* has low numbers of B chromosomes any fitness consequences may be restricted to having an odd number - observed in three individuals in this study. On the other hand, B chromosomes can confer fitness benefits to pollen cells in some species (Carlson 1969, Puertas and Carmona 1976) raising the possibility of a selective advantage to B chromosomes in *S. acuminatum*. Another consequence of B chromosomes is their effect on genome size (Jones et al. 2008). The genome size of one individual confirmed to have one B chromosome was larger (0.65 pg) than two individuals confirmed to lack B chromosomes (mean = 0.60 pg). Additional sampling of individuals with and without B chromosomes is required to determine whether this trend is significant. The presence of B chromosomes is positively correlated with genome size, whereby B chromosomes are more common in taxa with larger genomes (Trivers et al. 2004). Consequently, the relatively small genome size of *S. acuminatum* may limit the number of B chromosomes. Despite occurring in low numbers, B chromosomes were widespread in this study, occurring in most individuals examined and across a wide geographic distribution. The presence of B chromosomes in *S. album* (Xin-Hua et al. 2010) may indicate that these chromosomes are a genus-wide phenomenon in *Santalum.*

Triploidy

Triploidy was observed in one individual (Be) where 3n = 3x = 30. It is suspected that this individual is an autotriploid based on similarity among homologous chromosomes.

Triploidy has previously been observed from leaf tip preparations of a stand in Victoria with microsatellite genotyping amplifying at most two alleles across all five loci (Chapter 2). In contrast, the Be individual amplified three alleles across two of five microsatellite loci in this prior analysis and was observed here to have a larger than average genome size (0.85 pg *cf.* mean = 0.63 pg). Amplification of additional microsatellite alleles, larger genome size and additional chromosomes are traits associated with polyploids (Otto and Whitton 2000, Bowers et al. 2003, Sosa et al. 2014) and raise the question of polyploidy in the other five individuals that amplified additional microsatellite alleles in prior analyses (Chapter 2). While polyploidy was not observed in these individuals using root tip and leaf bud squash protocols, a higher level of clarity obtained through karyotyping may help resolve the issue of polyploidy in these individuals.

Triploidy in *S. acuminatum* is likely a result of unreduced (2n) gamete formation in a diploid parent. This route to triploid formation is common in plants and is found to occur at higher rates under temperature and possibly other environmental stressors (Rezaei et al. 2010, De Storme and Geelen 2014). The two triploid individuals identified thus far in *S. acuminatum* occur at the south-eastern edge of the species' range where climatic conditions are more temperate with higher rainfall and lower temperatures (Stern et al. 2000). While geographical assessment of stress in *S. acuminatum* is outside the scope of this study, individuals occupying the fringes of a species' range often experience higher levels of stress (Castro et al. 2004, Hampe 2005, Viejo et al. 2011). Thus, unreduced gamete formation in these areas may be higher due to such stressors, leading to a higher incidence of triploid formation.

Linkage in microsatellite markers

An assumption of many analyses used to determine genetic diversity and population genetic structure is independence of genetic markers (Putman and Carbone 2014, Grünwald et al. 2017). Markers which are located on the same chromosome will be inherited together unless separated through recombination, thus violating the assumption of independent inheritance (Lawson et al. 2012). The results of fluorescence *in situ* hybridisation found that two microsatellite markers previously amplified in *S. acuminatum* for genetic diversity and population structure analyses (see Chapter 2) are potentially linked, occurring in the same location on the same chromosome. Each marker was developed for use on different *Santalum* species – *S. lanceolatum* (Lanc09; Jones et al. 2010) and *S. austrocaledonicum* (mSaCIRF10; Bottin et al. 2005a). Such linkage violates the assumptions of some genetic diversity analyses (Grünwald et al. 2017) and may distort analyses such as principal components analysis (PCA) which are commonly used to understand population genetic structure (Lawson et al. 2012, Baran et al. 2013). In these cases, linkage can be controlled for via the removal of one of the linked loci (Patterson et al. 2006), though other models exist (Lawson et al. 2012, Baran et al. 2013)[1].

[1]Both of these markers were used in the genetic diversity and population genetic structure analyses in Chapter 2 as the loss of one locus was unlikely to make a large difference to the results given 4–5 loci were sufficient for genotyping (see Figure 2.2).

Conclusion

The results of this study indicate that variation in chromosome number including the presence of B chromosomes is common in *S. acuminatum*, with B chromosomes occurring across a broad geographic range and triploidy localised to the south-eastern edge of the species' distribution. The potential correlation between larger genome size, polyploidy and microsatellite profile suggest that polyploidy may be more frequent in this species than has been confirmed thus far. If the six individuals amplifying additional microsatellite alleles (Chapter 2) are in fact polyploids, polyploidy in the species has likely arisen from independent events. However, the possibility of additional microsatellite alleles resulting from gene/locus duplication remains, as of yet, unresolved. The disproportionate increase in genome size compared to ploidy may suggest a diploidisation process in polyploid *S. acuminatum*. Such variation in ploidy is expected to constrain sexual reproduction if mate availability is low. Thus, consideration of ploidy is recommended in *S. acuminatum* seed production areas and where this species is used in revegetation programs to avoid mixing cytotypes.

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Author Contributions

SC was responsible for project design, field work, lab work, data analysis and writing, contributing 71% to the study. FS provided assistance and knowledge to karyotyping and FISH analyses, contributing 7% to the study. TE contributed to project design and data interpretation (karyotyping and FISH), contributing 5% to the study. AS-L provided knowledge and training for flow cytometry, contributing 2% to the study. LB and SH contributed to project design, data interpretation and writing edits, contributing 7 and 8% to the study respectively.

Chapter 4

Cytotype and environmental influences on morphology in *Santalum acuminatum* (Santalaceae)

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Abstract

Intraspecific ploidy variation is increasingly considered in species conservation and management as it can negatively affect sexual reproduction and lead to the production of infertile progeny. Direct techniques for assessing ploidy are laborious and unable to be undertaken in the field. An alternative method of ploidy level determination is comparative morphological assessment, as organ size tends to increase with increasing ploidy and genome size. However, morphology in plants is also influenced by the environment which may mask the effect of ploidy and/or genome size on morphology. *Santalum acuminatum* (R.Br.) A. DC. is an endemic Australian hemiparasitic shrub inhabiting a wide geographic range spanning diverse environmental conditions. Prior research has found evidence for variation in ploidy and genome size across the species range. This study used morphological comparisons (leaf, stomate and seed size measurements) to look for evidence of intraspecific ploidy variation across the range of *S. acuminatum* using both herbarium and field-collected material. Additionally, this study looked for possible interactions of environmental variables on morphology to gauge whether possible differences in morphology caused by cytotype may be masked by the environment. The distribution of mean leaf length and width, stomate length and seed size provided no evidence for bimodality that may have suggested underlying ploidy variation. Leaf length and seed length were not correlated with any environmental variable, whereas leaf width and stomate length were correlated with aridity, precipitation, and temperature variables. The presence of relationships with environmental variables may mask any effect of genome size on morphology when screening across large geographic areas.

Key words: quandong, polyploidy, stomate size, leaf size, seed size, hemiparasite, gigas effect.

Introduction

Sexual reproduction between individuals is important for maintaining and generating genetic diversity and preventing inbreeding in many plant populations (Holsinger 2000). These genetic characteristics are thought to increase population fitness (Reed and Frankham 2003) and allow populations to better respond to environmental change (Frankham 2005, Jump et al. 2009). Many environmental and genetic processes can influence sexual reproduction in plants. One such genetic process is variation in the number of chromosome sets (i.e., ploidy) between individuals of the same species. Ploidy variation is common in plants (Masterson 1994, Soltis 2005, Jiao et al. 2011), generally resulting from whole genome duplication events (i.e., polyploidy), although duplication or loss of single chromosomes (i.e., aneuploidy) and chromosomal rearrangements resulting in ploidy change (i.e., dysploidy) are also possible (De Storme and Mason 2014). Cytotype alterations can limit sexual reproduction between ploidy levels as inter-cytotype fertilisation may be unsuccessful or reduce fecundity in offspring (Marks 1966, Ramsey and Schemske 1998, Burton and Husband 2000). A reduction of sexually compatible individuals can increase inbreeding in populations (Newman and Pilson 1997), with the potential to reduce population genetic diversity (Reed and Frankham 2003). Such reductions in genetic diversity can impact population persistence and the ability of a population to respond to environmental change (Frankham 2005, Jump et al. 2009). Thus, intraspecific ploidy variation should be considered in species management and restoration efforts, especially where seed of differing ploidy may unintentionally be mixed (Kramer et al. 2018).

Ploidy variation in plant species may be detected directly through chromosome counts or indirectly via several techniques (see Chapters 2 and 3). While varying methodologies for chromosome counting exist, each is laborious and requires access to fresh meristematic or

gametophytic tissue (de Laat et al. 1987, Sari et al. 1999). Such material may be difficult to obtain, especially if individuals are sterile or occupy broad geographic areas. As a result, indirect methods such as genome size estimation via flow cytometry (Kron et al. 2007), molecular marker amplification (Besnard et al. 2008, Sosa et al. 2014) and morphological comparison (Sari et al. 1999) are commonly used *in lieu* of or in combination with chromosome counting.

Of the techniques used to assess ploidy, morphological comparison is a relatively simple method that can be used in the field and for herbarium specimens, allowing greater sampling through time and space. This method exploits the positive relationship between genome size and cell and organ size, termed the 'gigas effect' (Stebbins 1950). Numerous studies have found genome size to correlate positively with stomatal size (Beaulieu et al. 2008, Hodgson et al. 2010, Franks et al. 2012) and seed mass, though this relationship is relatively weak across taxa (Beaulieu et al. 2007). Ploidy level has also been found to positively correlate with leaf (Sugiyama 2005), stomate (Vandenhout et al. 1995, McGoey et al. 2014, Snodgrass et al. 2016), flower (Segraves and Thompson 1999), pollen (Lindstrom and Koos 1931, Marinho et al. 2014, Snodgrass et al. 2016), and fruit size (Wu et al. 2012). While the strength of these relationships varies among species, some studies have found clear separation in the distribution of stomata sizes between diploids and polyploids (Celarier and Mehra 1958, Speckmann et al. 1965, Marinho et al. 2014). Thus, determining the existence and consistency of the relationships between size morphology, genome size and ploidy level may greatly benefit species management, especially if ploidy estimation can be rapidly undertaken in the field.

Despite relative consistency in relationships with genome size and ploidy level, plant morphology is also influenced by environmental conditions. Plant phenotypes vary in response to a myriad of environmental variables to maximise growth and reproductive output (Grime 1977, Chapin et al. 1993). Such variation may be due to phenotypic plasticity (i.e., the range of phenotypes that can be expressed by the same genotype; Pigliucci et al. 2006), genetic variation (arguably through natural selection acting on populations; Hereford and Winn, 2008), or both (Sánchez-Gómez et al. 2013). As such, organ size can vary greatly among individuals of the same species that occupy different habitats (Petrík et al. 2020). Leaf traits such as size shape and density influence light capture (Poorter et al. 2009), while stomata size and density regulate CO_2 uptake and transpiration (Lawson and Matthews 2020). Together these traits affect photosynthesis and thus, the accumulation of plant biomass (Doheny-Adams et al. 2012). To maximise growth and survival, plants can respond to environmental conditions by manipulating organ size. For example, under water deficit, plants respond by reducing leaf and stomata size to minimise water loss through transpiration (Drake et al. 2013, George-Jaeggli et al. 2017, Caine et al. 2019). Similarly, light, temperature, and nutrient availability influence growth, with consequences for leaf (Cunningham et al. 1999, Poorter et al. 2009) and seed size (Murray et al. 2004, Liu et al. 2016). Thus, for species occupying broad geographic ranges and/or a variety of environmental conditions, morphological variation due to underlying cytological differences may be masked by the environment (Beaulieu et al. 2008).

Santalum acuminatum is an endemic Australian species with a broad distribution across the southern half of the mainland (Figure 4.1). Seed production areas have been established in the species' south-eastern distribution with the intention of using the seed produced for restoration projects (J. Begley, personal communication, 2018). Some

variation in ploidy level has been established across the species' distribution and evidence of genome size variation in south-eastern Australia has been observed (Chapter 3). Genetic evidence suggesting polyploidy has been observed in stands across Australia via microsatellites (Chapter 2) and restriction fragment length polymorphisms (RLFPs) (Byrne et al. 2003), with polyploidy having been confirmed in one individual amplifying three microsatellite alleles (Chapter 3) – amplification of more than two microsatellite alleles is suggestive of polyploidy (Sosa et al. 2014). Morphological traits (leaf, stomate and pollen) have previously been measured in 50 South Australian (SA) herbarium specimens with the aim of locating possible polyploids, though no evidence for multiple size classes that may have suggested polyploidy was found (Randall 2000). As ploidy and genome size variation is potentially rare in this species and was not observed in SA, sampling over a wider geography may reveal morphological variables in *S. acuminatum* correlated with ploidy increases.

By sampling more widely than previous studies, this study sought to assess whether the size of leaf, stomate and/or seed in *S. acuminatum* is correlated with ploidy level change. As this species occupies a broad distribution across Australia, environmental differences between populations may also affect morphological characters. Thus, this study also aimed to understand if environmental variables are correlated with morphological characters. The aim of which was to ascertain whether relationships with environmental variables may mask variation in morphology due to ploidy differences. It was hypothesised that leaf length and width, stomate length and seed length would increase in size with increasing ploidy. Depending on the strength of these relationships it was expected that potential polyploids would be evident through the presence of multiple modes in the distribution of each morphological character. Furthermore, it was expected that each morphological variable would be correlated with aridity, precipitation, and

temperature variables and that the relationships would be consistent with a water conservation strategy, whereby morphological characters decrease in size in more arid conditions.

Materials and Methods

Study species

Santalum acuminatum is a root hemiparasitic shrub that inhabits arid to semi-arid ecosystems throughout mainland Australia (Grant and Buttrose 1978) (Figure 4.1). The species is thought to be a generalist in host choice but favouring nitrogen-fixing species such as acacias (Tennakoon et al. 1997a, Nge et al. 2019). Studies have suggested the species is self-compatible (Sedgley 1982), however, natural populations may rely heavily on vegetative reproduction with seed recruitment being relatively rare (Chapter 2, Tennakoon et al. 1997a, Fuentes-Cross 2015). *Santalum acuminatum* produces large, edible, nutrient dense fruits that serve as an important source of food for emus (*Dromaius novaehollandiae*) (Dunstan et al. 2013), bettongs (*Bettongia* spp.) (Murphy et al. 2015) and historically Aboriginal Peoples (Bonney 2013). Despite its wide distribution, the existing populations are in decline and are becoming increasingly fragmented (Box et al. 2016).

Sampling

To maximise the geographic range of samples, material was obtained from several sources – herbaria, field collections and donations (Figure 4.1). In total 104 specimens were obtained from Australian herbaria representing the natural distribution of *S. acuminatum*

(Supplementary material, S4.1). These samples were supplemented with field collections (18 stands, 13 sites) in November 2019 during the fruiting season (Table 4.1). Owing to the species' propensity for clonality (Chapter 2, Tennakoon et al. 1997a), clusters of ramets separated from nearby clusters by at least 50 m were treated as separate genets (individuals) for the purpose of this study. For each stand, leaves were collected from at least three branches of the largest ramet and rapidly dried in paper envelopes in zip-lock bags containing ~75 g of silica gel self-indicating beads (3–8 mesh, ChemSupply). Where possible fresh fruit and seed were collected from ramets either directly from a branch or from the ground immediately underneath the canopy. All seed were de-fleshed and stored in paper envelopes for later use. Seed from 33 stands were donated by various seed collectors and growers (Table 4.1), including some with leaf material sourced from the mother tree.

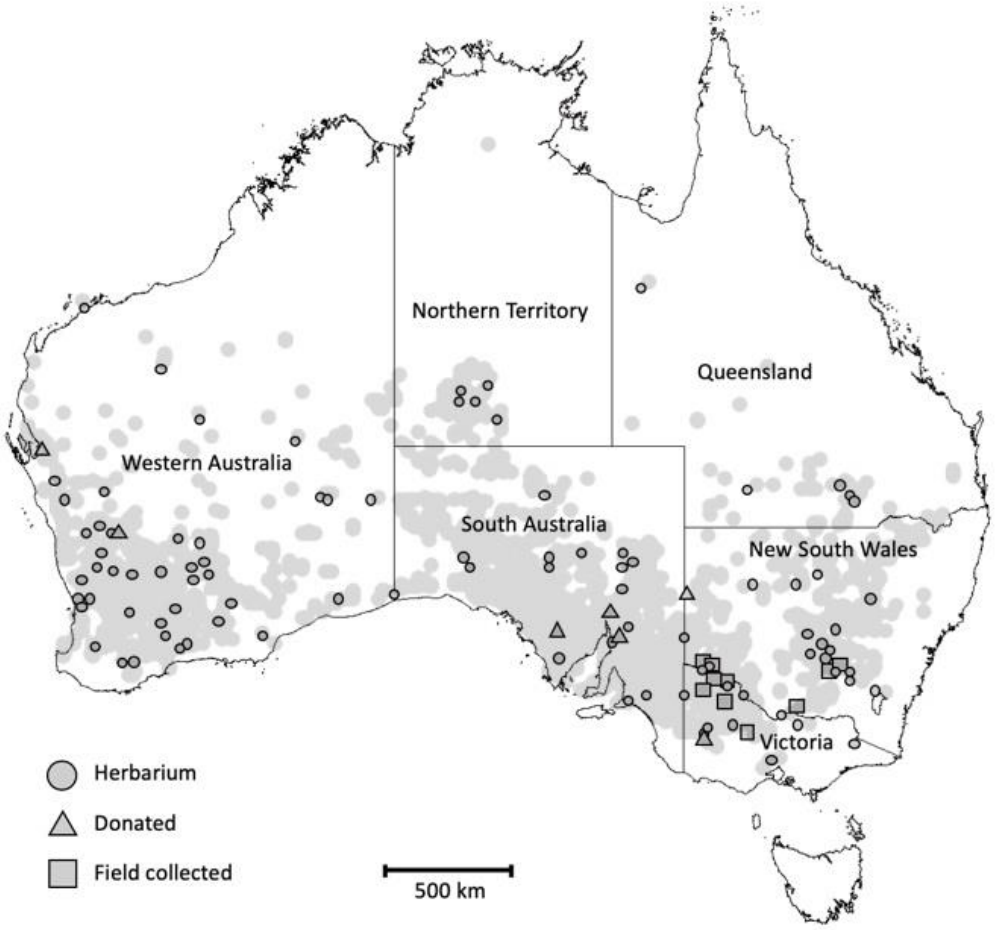

Figure 4.1: Map of *Santalum acuminatum* sampling sites across Australia categorised by source. Distribution of *S. acuminatum* in Australia is represented in light grey dots (Atlas of Living Australia 2020).

Table 4.1: Sites from which *Santalum acuminatum* stands were sampled during field collections (F) or donated (D) and the type of material received and subsequently measured for this study.

ID (individual)	Location	State	Latitude	Longitude	Source (F/D)	N	Material type	
							Leaf	Seed
An	Annuello Flora and Fauna Reserve	VIC	-34.87	142.71	F	2	x	
De	Devenish	VIC	-36.36	145.91	D	1		x
GL	Green Lake Reginal Park	VIC	-35.60	142.85	F	1	x	x
Ha	Hattah-Kulkyne National Park	VIC	-34.77	142.28	F	1	x	
INR	Inglewood Nature Conservation Reserve	VIC	-36.60	143.82	F	1	x	x
Ko	Koorlong	VIC	-34.34	142.09	F	1	x	
Mb	Merbein	VIC	-34.19	142.06	F	1	x	x
Nm	Natimuk	VIC	-36.67	141.93	D	1	x	x
Na	Nathalia	VIC	-	-	D	1		x
RC	Red Cliffs	VIC	-34.34	142.13	F	1	x	
TFR	Torrita Flora and Fauna Reserve	VIC	-35.16	141.93	F	2	x	
WP	Walpeup	VIC	-35.14	142.03	F	1	x	
WN	Walpeup North	VIC	-35.12	142.02	F	1	x	
BB	Barrabool	NSW	-	-	D	1		x
Be	Berrigan	NSW	-35.74	145.80	F	1	x	
INR (In, A, B, C)	Ingalba Nature Reserve	NSW	-34.44	147.44	F	3	x	x
PNR (A, B)	Pacawan Nature Reserve	NSW	-34.45	147.35	F	2	x	x
Sa	Savernake	NSW	-35.75	146.03	D	1		x
UR	Umberumberka Reservoir	NSW	-31.83	141.21	D	1		x
MKT2	CSIRO	NSW			D*	1	x	x
MKT5	Whyalla or Ceduna	SA	-	-	D*	1	x	x
MKT9	Whyalla or Ceduna	SA	-	-	D*	1	x	x
MKT10	Whyalla or Ceduna	SA	-	-	D*	1	x	x
MKT11	Whyalla or Ceduna	SA	-	-	D*	1	x	x
MKT13	Unknown	-	-	-	D*	1		x
MKT17	Gladstone	SA	-	-	D*	1	x	x
MKT19	Whyalla or Ceduna	SA	-	-	D*	1		x
MKT20	Gladstone	SA	-	-	D*	1	x	x
MKBOG	Gladstone	SA	-	-	D*	1	x	x
GNT (1–6)	Uluru or Kings Canyon or Streaky Bay (SA)	SA/NT	-	-	D*	6	x	x

LET (1–6)	Lyndavale Erldunda Station	NT	-25.19˜	133.26˜	D*	6	x	x
MPT (1, 2)	Mulga Park	NT	-25.91˜	101.67˜	D*	2	x	x
Yu	Yulara	NT	-25.26	130.97	D	1		x
CDR	Charles Darwin Reserve	WA	-29.57	116.97	D	1	x	x
HML	Hamelin Reserve	WA	-29.49	114.11	D	1	x	x

*Material donated from an orchard.; ˜, denotes approximate (up to 100km error) coordinates; N, Number of stands sampled per site.

Morphological characters

Leaf measurements

Leaf length and width (mm) were measured for 88 stands (46 from herbaria, 24 donated and 18 field collected). For each stand, 5–20 mature leaves were measured, and the mean (± standard error) calculated. Leaf width was measured at the widest point perpendicular to the leaf vein, whereas leaf length was measured along the leaf vein from tip to base, excluding the petiole. All measurements were taken with a ruler unless the leaf vein was curved, in which case string was used to follow the curvature and then measured on a ruler to obtain the total leaf length.

Stomate size measurements

Stomate lengths (μm) were measured for 146 stands (104 from herbarium specimens, 24 donated and 18 field collected) using a mature, dried leaf from each stand. Epidermal peels were performed by applying clear nail varnish to the upper $^{3}/_{4}$ section of the abaxial leaf surface which was then removed using clear adhesive tape (PostPack™) and mounted on a microscope slide. As stomate size tends to be more variable at the leaf tip and margins (Smith et al. 1989, Willmer 1996, Poole et al. 2000), 20 randomly selected stomata were measured from the centre of each leaf. Measurements were taken using an Olympus CX22LED microscope under 40x magnification with Yang Wang YW500u3 USB3 industrial camera and measured with the associated S-EYE software. The mean (± standard error) for each stand was calculated and used for analysis.

Seed measurements

Endocarp size was used as a proxy for seed size as *S. acuminatum* germinates within the endocarp and is therefore a more practical measurement for use in the field. Seed length and width (mm) were measured for all seed donated and obtained during field collections (n = 175 from 40 stands), and the mean (± standard error) for each mother plant was calculated. Seed length was measured using digital callipers from the micropyle to the opposite side of the seed, while width was taken at the widest point on the perpendicular axis.

Environmental data

Environmental data for each sample was obtained from the Atlas of Living Australia spatial portal (Belbin 2011; accessed 25 May 2021) (Table 4.2). Individuals from orchards were excluded from the analysis since environmental conditions are likely to differ greatly from original provenance and synthetic fertilisers and non-native hosts could also influence results.

Table 4.2: Environmental variables downloaded for each *Santalum acuminatum* stand in this study. Datasets obtained from the Atlas of Living Australia spatial portal (Belbin 2011). Resolution for each variable is ~1 km.

Environmental dataset	Abbreviation
Nutrient status index[1]	NS
Aridity index month minimum[1]	A_{min}
Aridity index month maximum[1]	A_{max}
Aridity index annual mean[1]	A_{mean}
Precipitation during the driest month (mm)[1]	P_{dry}
Precipitation during the wettest month (mm)[1]	P_{wet}
Precipitation annual mean (mm)[1]	P_{mean}
Precipitation total annual (mm)[1]	P_{annual}
Temperature annual minimum mean (˚C)[1]	T_{min}
Temperature annual maximum mean (˚C)[1]	T_{max}
Temperature annual mean (˚C)[1]	T_{mean}
Temperature annual range (˚C)[1]	T_{range}
Fractional bare soil cover (%)[2]	Bare soil
Soil acidity and alkalinity (pH)[3]	Soil pH

[1], Based on data up to November 2009 (Williams et al. 2012); [2], measured in 2012 (Guerschman 2019); [3], based on data up to 2001 (ABARES 2001).

Data analysis

All statistical analyses, unless otherwise stated, were undertaken using R version 3.4.3 and the R stats package (R Core Team 2019). All graphs were constructed using the package ggplot2 (Wickham 2016).

Variation in morphology

Mean leaf, stomate and seed sizes were visualised using frequency distributions. As clear separation in the distribution of stomata size between diploids and polyploids has been observed in some species (Speckmann et al. 1965, Marinho et al. 2014), each morphological variable was assessed for bimodality with a Hartigan's dip test (Hartigan and Hartigan 1985) using the R package diptest (Maechler 2016). Such bimodality may reflect an underlying genome size (Lomax et al. 2009) or ploidy difference between stands (Celarier and Mehra 1958).

Morphological variation between putative ploidy levels

Microsatellite amplicon profiles are an indirect method of assessing ploidy level whereby the presence of more than two alleles at a given locus may indicate polyploidy (Sosa et al. 2014). Using the results of Chapter 2, individuals were grouped based on their amplification of at most two alleles at each locus (denoted here as XX) and amplification of more than two alleles at one or more loci (denoted here as XXX). However, since gene/locus duplication (Wang et al. 2012) and/or aneuploidy (De Storme and Mason 2014) can garner similar amplicon profiles, the ploidy levels of these groups were putative only. To determine whether mean leaf length and width and stomate length differed in size between groups, Student's two sample t-tests were conducted (unless otherwise stated due to non-normally distributed variables or inequality of variance established using an F-test). Analyses based on genome size were unable to be undertaken due to small sample sizes (n = 1 for larger genome individuals). Similarly, differences in seed size between XX and XXX individuals were not possible as all seed, for which microsatellite profiles were available, were classed as XX.

Variation in seed size among mother plants

To look for differences in mean seed size among mother plants that may suggest differences in ploidy, an analysis of variance (ANOVA) was conducted. Differences were visualised using comparative boxplots and Tukey pairwise comparisons used to assess statistical differences at an alpha of 0.05.

Relationships between morphological characters and environmental variables

Each environmental and morphological variable was assessed for normality using a Shapiro-Wilk test. Relationships between morphometrics and environmental data were

undertaken using Pearson's correlation coefficient for normally distributed data and Spearman's correlation coefficient for non-normally distributed data. A Holm-Bonferroni adjustment was undertaken on *p*-values to control for the increased probability of type I error due to multiple comparisons.

Most environmental variables were significantly correlated with each other (Supplementary material, S4.2). Thus, to understand the relative importance of each environmental variable on each morphological character, multiple linear regression analyses were undertaken. For non-normally distributed variables, the package bestNormalize was used to select the best transformation using the orderNorm (OQR) technique, and the appropriate transformation performed (Peterson and Cavanaugh 2020). All significantly correlated environmental variables for each morphological character were included in the model, except where multiple aridity, temperature and precipitation variables were significantly correlated. In these cases, only the most significant variable (lowest *p*-value) for each measure was included.

Results

Variation in morphology

Santalum acuminatum leaf length ranged from 32.33 to 115.80 mm with a mean of 64.36 mm (± 1.97 mm) (Figure 4.2 A), while leaf width ranged from 3.4 to 17.3 mm with a mean of 7.46 mm (± 0.29 mm) (Figure 4.2 B). There was no statistical support for bimodality in the distribution of either variable. Stomate length ranged from 14.74 to 29.92 μm with a mean of 21.52 μm (± 0.24 μm) (Figure 4.2 C) and again no statistical support for bimodality was observed. Mean seed length for each mother plant ranged from 12.20 to 20.96 mm (± 0.12 and 0.37 mm respectively) (Figure 4.2 D), while width ranged from 12.82 to 22.17 mm (± 0.12 and 0.27 mm respectively). Seed length and width were strongly correlated (r^2 = 0.85) (Supplementary material, S4.3) and seed length was subsequently used in all further analyses. The distribution of seed length was not statistically bimodal.

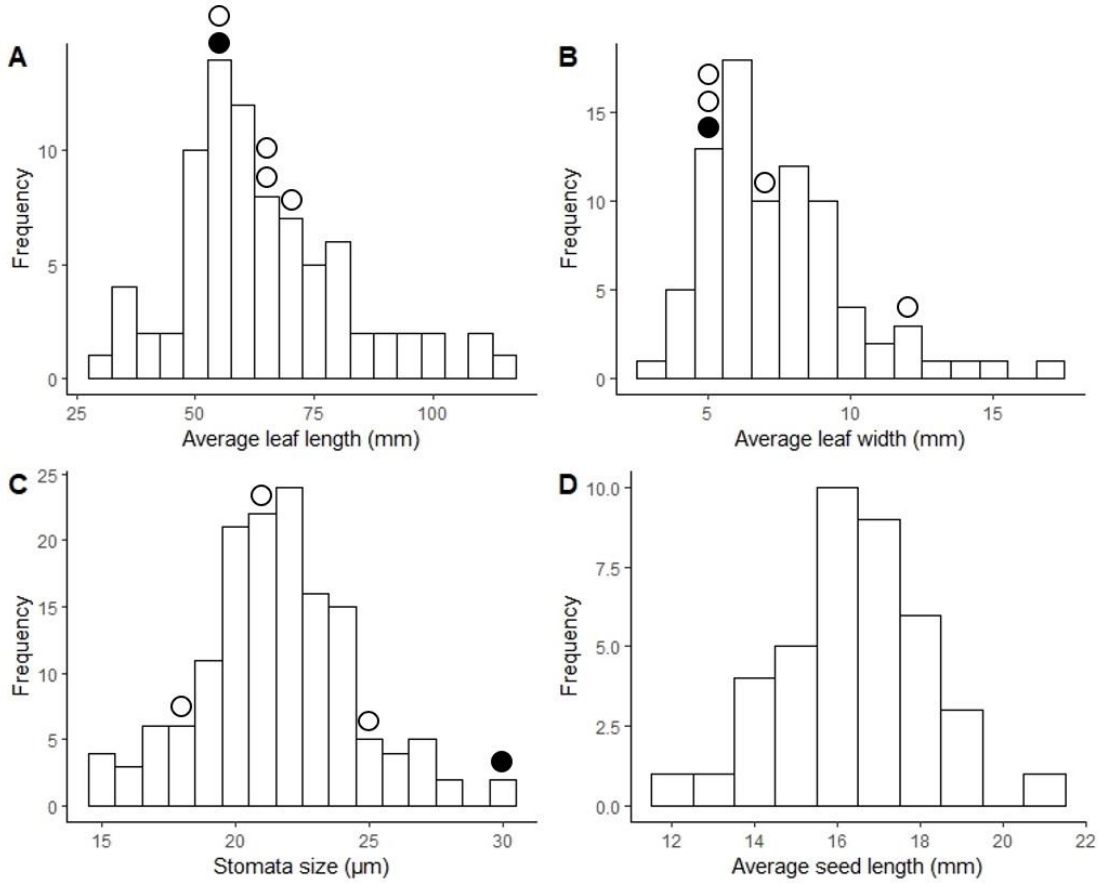

Figure 4.2: Frequency distributions of mean leaf, stomate and seed sizes in *Santalum acuminatum*. The y-axis represents the frequency of samples within each size interval. Closed circles indicate the position of the individual with a triploid cytology and open circles indicate the position of individuals amplifying three microsatellite alleles at any given locus (Chapters 2 and 3).

Morphological variation between putative ploidy levels

Mean leaf length and width were available for 78 XX individuals and five XXX individuals, while mean stomate length was available for 96 XX individuals and five XXX individuals. Wilcoxon signed rank tests were used for mean leaf length and width as each variable was non-normally distributed. No statistical differences were observed between the leaf length and width and stomata length of XX and XXX individuals.

Variation in seed size among mother plants

Mean seed length differed significantly among a few stands such as BB, which was significantly larger than most other stands in this study ($p < 0.05$ in all pairwise comparisons), except for CDR and Yu (Figure 4.3). Mean seed length for MKT9 was significantly smaller than most other stands ($p \leq 0.01$ in all pairwise comparisons), except for GL and INR. See Supplementary material, S4.4 for all pairwise comparisons.

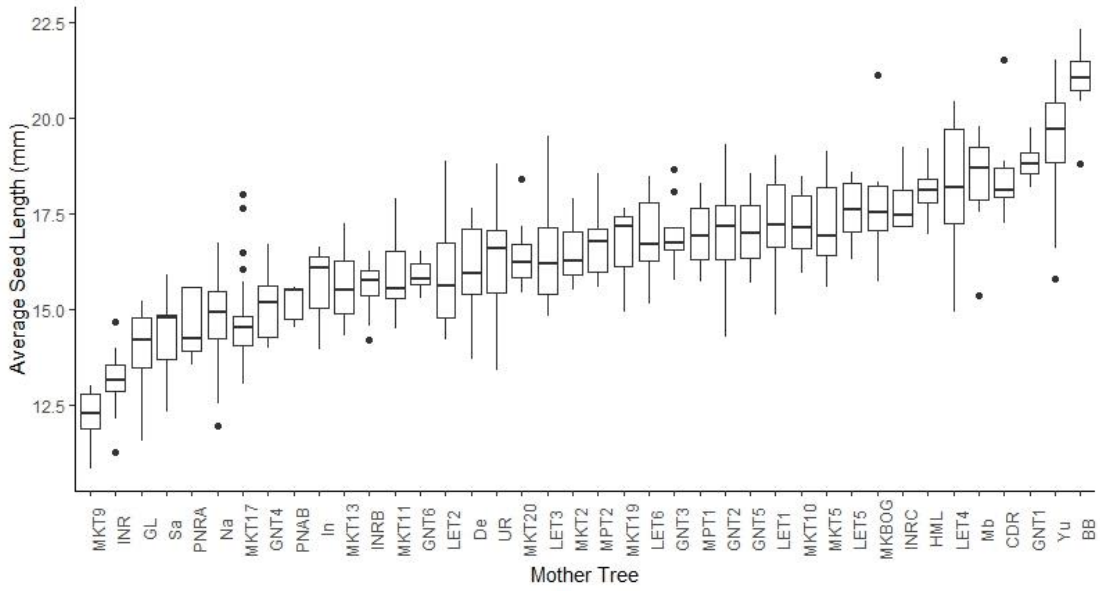

Figure 4.3: Box plots of *Santalum acuminatum* median seed length for each maternal stand measured in this study. Boxes represent inter quartile range (IQR), whiskers represent minimum and maximum (Q1 – and Q3 + 1.5*IQR) and dots are outliers. See Table 4.1 for sample prefix locations.

Relationships between morphological characters and environmental variables

Environmental variables were available for 127 individuals except soil pH, which was only available for 97 individuals. The range of each environmental variable in this study is displayed in Table 4.3.

Leaf width and stomate length were correlated with multiple environmental variables while leaf length and seed length were not significantly correlated with any environmental variable (Table 4.4; Figure 4.4; Supplementary material, S4.5). Mean leaf width was significantly and negatively correlated with A_{min} and P_{dry}, while significantly and positively correlated with T_{min}, T_{max} and T_{mean}. Mean stomate length was significantly and positively correlated with A_{mean}, while significantly and negatively correlated with each temperature variable.

Table 4.3: Range of values observed for environmental variables across the distribution of *Santalum acuminatum* stands in this study. See Table 4.2 for abbreviated variables.

Variable	Minimum	Maximum	Variable	Minimum	Maximum
NS	0.6	2.0	P_{annual}	144 mm	913 mm
A_{min}	0.003	0.23	T_{range}	19.7˚C	37.25˚C
A_{max}	0.11	3.48	T_{min}	6.7˚C	17.42˚C
A_{mean}	0.05	1.07	T_{max}	17.5˚C	31.33˚C
P_{dry}	1 mm	46 mm	T_{mean}	12.8˚C	25.1˚C
P_{wet}	19 mm	227.53 mm	Bare soil	0%	65%
P_{mean}	12.42 mm	94.11 mm	Soil pH	4	9

Table 4.4: Significant relationships from Spearman's correlation coefficient analyses on *Santalum acuminatum* morphometrics and environmental variables. A Holm-Bonferroni adjustment has been applied to *p*-values. See Table 4.2 for abbreviated environmental variables.

Morphometric	Environmental variable	n	S	*p*-value	rho
Mean leaf width	A_{min}	57	54110	0.0005	-0.50
	P_{dry}		54246	0.0004	-0.51
	T_{min}		19938	0.004	0.45
	T_{max}		20773	0.007	0.42
	T_{mean}		20420	0.006	0.43
Mean stomate length	A_{mean}	117	191418	0.02	0.28
	T_{range}		344382	0.02	-0.29
	T_{min}		353001	0.005	-0.32
	T_{max}		350408	0.007	-0.31
	T_{mean}		355179	0.004	-0.33

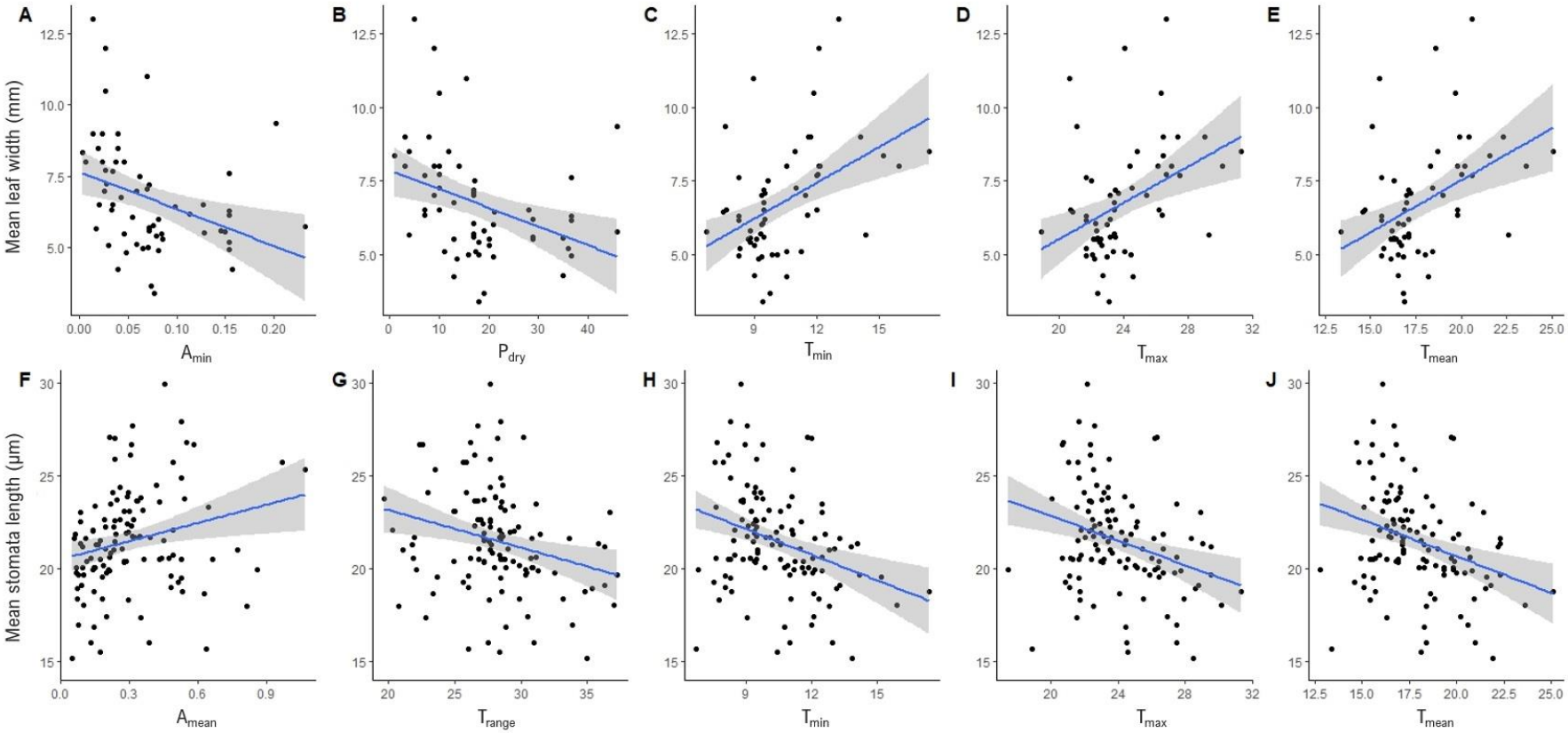

Figure 4.4: Statistically significant relationships between morphological characters (A–E, mean leaf width and F–J, mean stomate length) and environmental characters. Dots represent samples, blue line plots the general linear model and grey shading represents standard error. See Table 4.2 for abbreviated environmental variables and Table 4.4. for results of Spearman correlation coefficient analyses.

Of the environmental variables significantly correlated with one or more morphological character(s), all were non normally distributed and transformed to approximate a normal distribution (Supplementary material, S4.6). Mean leaf width was significantly correlated with three temperature variables (T_{min}, T_{max}, and T_{mean}), where the most significant relationship was observed with T_{min}. Subsequently, A_{min}, P_{dry} and T_{min} were included as explanatory variables in a multiple linear regression analysis. Of the three explanatory variables, the relationship with T_{min} was significant (Table 4.5 A). For mean stomate length, significant relationships were observed with A_{mean}, and each temperature variable. Of the temperature variables, the relationship between mean stomate length and T_{mean} was the most significant. Thus, the multiple linear regression analysis for mean stomate length used A_{mean} and T_{mean} as explanatory variables. Of these variables, the relationship with T_{mean} was significant (Table 4.5 B).

Table 4.5: Multiple linear regression analysis on *Santalum acuminatum* morphological variables and significantly correlated environmental variables. Significant *p*-values are bolded. See materials and methods for abbreviated explanatory variables.

	Response variable	Explanatory variables	Estimate	SE	*t* value	*p*-value	Intercept ± SE
A	Mean leaf width	A_{min}	1.83	27.88	0.07	0.95	2.93 ± 2.41
		P_{dry}	-0.01	0.14	-0.11	0.92	
		T_{min}	0.38	0.18	2.15	**0.04**	
B	Mean stomate length	A_{mean}	0.59	1.64	0.36	0.72	27.77 ± 2.95
		T_{mean}	-0.36	0.15	-2.49	**0.01**	

Discussion

Intraspecific ploidy variation can impact on sexual reproduction with the potential to cause reproductive failure and reduce fertility in offspring (Marks 1966, Ramsey and Schemske 1998). As such, techniques to determine ploidy level rapidly in the field can aid species management and revegetation efforts through both time and cost-effectiveness compared to more detailed genetic approaches (e.g., Chapter 3). This study sought to ascertain whether there was evidence for polyploids of larger size across the distribution of *S. acuminatum* evidenced through bimodality in the distribution of morphological characters. Additionally, size differences between individuals suspected to be polyploids, owing to the amplification of more than two alleles at microsatellite loci (i.e., XXX individuals), was investigated to determine whether the gigas effect was present in *S. acuminatum*. Furthermore, as the abiotic environment can influence morphology, this study sought to understand if morphological characters were related to environmental variables. Evidence from mean leaf, stomate and seed size measurements did not indicate bimodality in the distribution of any character, while size differences between XX and XXX individuals were not observed for any morphological character. Aridity, precipitation, and temperature variables were significantly correlated with mean leaf width and mean stomate length. Yet, multiple linear regression analyses indicated a stronger association between these morphological characters and temperature.

Variation in morphology due to ploidy

Prior research investigating possible polyploidy in *S. acuminatum* found no evidence for multiple size classes in mean leaf and stomatal traits (Randall 2000), although this study considered South Australian (SA) herbarium specimens only. Interestingly, mean leaf

length reported by Randall (2000) was significantly larger than that reported here using samples from all states (Randall mean = 66.13 ± 2.06 mm *cf.* this study mean = 59.30 ± 1.70 mm; Welch's two sample t-test, $t = 2.55$, df = 99, p-value = 0.01). While mean leaf width measured by Randall (2000) was also larger, the difference was not significant (Randall mean = 7.19 ± 0.27 mm *cf.* this study mean = 6.72 ± 0.24 mm). However, mean leaf length and width of SA stands measured in this study (57.54 ± 2.58 mm and 6.25 ± 0.48 mm respectively) were also smaller than those reported by Randall (2000) suggesting sampling or methodological differences. Mean stomate size reported here was also smaller than that reported by Randall (2000) (this study mean = 21.44 ± 0.26 μm *cf.* Randall mean = 34.18 ± 0.64 μm). Randall (2000) assessed stomate width, however, length may be the better measurement since stomate width changes throughout the course of a day in response to changes in the environment (Smith et al. 1989, Willmer 1996). These differences may be underpinned by ecology, ploidy, sampling, and/or methodology. Nevertheless, this study also found no evidence for multiple size classes for any of the measured traits across the study area, despite evidence for ploidy and genome size variation based on karyotyping and flow cytometry (Chapter 3).

The presence of additional microsatellite alleles is often interpreted as support for polyploidy (Sosa et al. 2014), with some evidence that this may hold true for *S. acuminatum* (Chapter 3). An *S. acuminatum* stand amplifying three fragment lengths across two microsatellite loci was found to have a triploid cytotype, suggesting that the presence of additional microsatellite alleles may be due to polyploidy. While this individual had larger than average stomata (mean = 29.92 ± 3.58 μm *cf.* 21.44 ± 0.26 μm respectively), leaf length and width were below average (mean = 53.19 ± 1.43 and 5.63 ± 0.25 mm *cf.* mean = 59.30 ± 1.70 mm and 6.72 ± 0.24 mm respectively). Furthermore, when comparing the mean of each morphological character for individuals amplifying at

most two (XX) or at least three (XXX) alleles at any locus, no differences were observed. Two possible scenarios may explain these results: 1) the presence of three microsatellite alleles does not constitute ploidy change in all individuals, or, 2) the *S. acuminatum* morphological characters measured here do not increase in size with increasing ploidy. Additional microsatellite alleles may result from gene/locus duplication events (Wang et al. 2012), duplication of single chromosomes (i.e. aneuploidy; De Storme and Mason 2014), or the presence of B chromosomes (Milani et al. 2014, Piscor and Parise-Maltempi 2016). Gene duplication of the nuclear waxy gene (WX1) in *S. acuminatum* has been reported (Harbaugh 2000) and possible linkage in microsatellite markers (Chapter 3) may mean gene or segmental duplication, aneuploidy or B chromosomes have caused the duplication of microsatellite alleles in the absence of polyploidy.

Many studies have found morphological characters, especially stomate size, to increase in size with increasing ploidy (Stebbins 1950, Vandenhout et al. 1995, Sugiyama 2005, Wu et al. 2012, McGoey et al. 2014, Snodgrass et al. 2016). However, evidence suggests that the strength of these relationships varies across plant growth forms with trees and shrubs failing to show consistent increases in stomate size with ploidy level (Beaulieu et al. 2008). As a shrub, *S. acuminatum* may be one such species that lacks a relationship between ploidy level and size morphology. Alternatively, as the increase in genome size of a triploid measured in Chapter 2 was less than proportional to the increase in ploidy, any associated increase in morphology may be small and/or undetectable. On the other hand, the lack of a relationship may be due to variation in morphology caused by environmental variation and/or small polyploid sample sizes across the species' distribution.

Relationships between morphological characters and environmental variables

Plants may adapt to environmental conditions in part through changes to their morphology (Wright et al. 2001, Wright et al. 2005, Sánchez-Gómez et al. 2013). The results presented here suggest that morphological characters in *S. acuminatum* are influenced largely by aridity, precipitation, and temperature. However, temperature may be a more important driver of these relationships.

Leaf size and shape respond to a variety of environmental variables including water deficit, irradiance, nutrient content, and atmospheric CO_2 (Wright et al. 2001, Poorter et al. 2009). For *S. acuminatum*, leaf width rather than length was correlated with environmental variables, suggesting that this species may respond to the environment by altering leaf width. The leaves of *S. acuminatum* in this study were wider at warmer temperatures and under more arid conditions. Though, the relationship with T_{min} appeared to be a more important driver of these relationships. Under a water saving strategy, leaf size has been seen to decrease with increasing aridity to reduce water loss through transpiration (Sánchez-Gómez et al. 2013, George-Jaeggli et al. 2017). For *S. acuminatum* the inverse relationship was observed, suggesting that this species is less affected by low water availability.

As a root hemiparasite *S. acuminatum* may not be as affected by environmental water as non-parasitic species. *Santalum acuminatum* maintains large and constant differences in water potential relative to neighbouring plants, much higher than other parasitic species (Loveys et al. 2001). This strong difference in water potential may ensure water and nutrient capture from a wide variety of host species (Loveys et al. 2001) while facilitating water and nutrient capture during periods of water deficit, when host water potential decreases. Thus, the response of leaf width to aridity may be due to other factors such as irradiance. While most parasitic species have some capacity for photosynthesis,

photosynthetic rates are usually lower than host species (Richter et al. 1995, Lechowski 1996). Photosynthetic rates in *S. acuminatum*, however, are comparable to host species and the ratio of CO_2 assimilation to transpiration is consistent with high water use efficiency (Loveys et al. 2001), also atypical of parasitic plants (Ulmann et al. 1985, Lechowski 1996). Thus, the leaves of *S. acuminatum* may widen under more arid conditions to maximise surface area available for light capture and CO_2 assimilation (Gifford et al. 1984). Though, increased photosynthetic capacity also relies on leaves possessing greater amounts of photosynthetic tissues (Poorter et al. 2009), which was not directly tested in this study. Furthermore, predictive models suggest that smaller leaves are favoured at high temperatures and irradiances to avoid overheating (Okajima et al. 2012). However, as CO_2 assimilation rates per unit leaf area of parasitic plants are generally lower than that of host species (Richter et al. 1995, Tennakoon et al. 1997b), *S. acuminatum* may avoid high leaf temperatures through lower photosynthetic potential of leaf tissue.

Stomate size in plants involves a trade-off between CO_2 uptake and water loss, whereby larger stomata are associated with increased CO_2 assimilation (Petrík et al. 2020) and higher rates of transpiration (Chaerle et al. 2005). Under arid conditions, stomate length has been observed to decrease to prevent water loss (Doheny-Adams et al. 2012, Durand et al. 2020). The results of this study were consistent with this expectation, with stomate length decreasing with increasing temperatures and in more arid conditions. Of these relationships T_{mean} appeared to be more strongly related to stomate length than A_{mean}. Aridity is measured as the ratio of precipitation to evaporation (Williams et al. 2012). Evaporation potential tends to increase with increasing temperature which is also associated with increased transpiration in plants (Downes 1970). Thus, stomata length in

S. acuminatum likely responds to increased transpiration at higher temperatures to reduce water loss via smaller stomata.

Experimental studies on the stomatal dynamics of five *Banksia* sp., found that smaller-sized stomata were associated with faster stomatal opening in response to light and higher CO_2 assimilation rates while maintaining water balance (Drake et al. 2013). This has also been observed in poplars (*Populus* species) where faster stomatal responses were associated with lower transpiration rates (Durand et al. 2020). On the other hand, larger stomata have been associated with higher photosynthetic potential (Bucher et al. 2016) and biomass accumulation (Doheny-Adams et al. 2012). Therefore, at higher temperatures, *S. acuminatum* may benefit from smaller sized stomata if this facilitates a more rapid response to light, allowing maximum exploitation of light resources with rapid stomatal closure under high temperatures. At lower temperatures, *S. acuminatum* may prioritise growth, increasing CO_2 assimilation rates and biomass accumulation via larger stomata. This interpretation is perhaps supported by evidence that assimilation rates in *S. acuminatum* shift from 11 am to 12 pm in winter to 9 am in summer (Loveys et al. 2001), suggesting exploitation of morning light during warmer times of the year.

Seed length was not correlated with any environmental variable tested in this study which may be a result *S. acuminatum's* hemiparasitic tendencies (Tennakoon et al. 1997a, Loveys et al. 2001), decreasing the species' dependence on environmental water and nutrients. *Santalum acuminatum* seed show adaptations to arid environments through the provision of large nutrient supplies (i.e., endosperm) and a tough endocarp to resist desiccation (Loveys and Jusaitis 1994). Increases in seed size have been observed at higher temperatures in some species (Liu et al. 2016), while seed mass in Australian

Glycine taxa has been observed to decrease with increasing temperatures in the arid interior and increase with solar radiation (Murray et al. 2004). Thus, it is surprising that relationships between seed size and aridity and temperature were not observed in this study. Although, relationships between seed length and environmental variables may have been constrained by small sample sizes.

Conclusions and future directions

While we found no evidence in *S. acuminatum* for polyploids of larger size across the Australian landscape, the potentially low incidence of polyploidy in this species and variation in morphology due to the environment, may have masked any effect of ploidy level on morphological characters. However, a relationship between ploidy level and morphology was not established in this study and may require a larger sample size of known polyploids to be fully elucidated. On the other hand, morphological characters in *S. acuminatum* may be unaffected by ploidy, potentially due to smaller than expected increase in genome size in known polyploids. Evidence suggests that morphological characters in *S. acuminatum* are less affected by environmental water than temperature with evidence that the species may increase leaf width and decrease stomate size under arid conditions to prioritise growth while mitigating water loss. The uptake of water from host species by *S. acuminatum* may therefore skew trade-offs between water conservation and biomass accumulation towards growth.

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Author Contributions

SC was responsible for project design, field collections, data collection, data analysis, interpretation, and writing, contributing 80% to the study. LB was responsible for data interpretation, and writing edits contributing 5% to this study. SH was responsible for project design, data interpretation and writing edits contributing to 15% of this study.

Chapter 5

General Discussion

Genome size evolution in plants is largely underpinned by processes of duplication, especially polyploidy (Soltis et al. 2003, Jiao et al. 2011), which plays a significant role in facilitating speciation (Otto and Whitton 2000, De Storme and Mason 2014), the creation of novel traits (Crow and Wagner 2006, Veron et al. 2007, Moriyama and Koshiba-Takeuchi 2018), and adaptation into new environments (Sonnleitner et al. 2010, Coughlan et al. 2017). Within populations, genomic change can skew reproduction by reducing investment in sexual reproduction (Levin 1975), instigating asexual modes of reproduction (clonal reproduction and apomixis; Van Drunen and Husband 2019) and/or increasing self-fertilisation (Barringer 2007). Such changes have implications for population genetic and genotypic diversity (Holsinger 2000), evolutionary capacity (Richards 1997, Agrawal 2006), and adaptation to environmental change and novel stressors (Frankham 2005, Jump et al. 2009). This study sought to understand the extent of genomic changes that have occurred in *Santalum acuminatum*, an endemic Australian shrub of cultural and economic importance. Through the application of several independent techniques, polyploidy (Chapters 2 and 3), genome size variation, B chromosomes and possible apomictic seed (Chapter 3) were observed. An examination of morphological characters known to vary in size with ploidy level found no evidence that such changes impact morphological characters (leaf, stomate and seed size) in this species (Chapter 4). These morphological characters were significantly correlated with environmental variables, potentially masking variation from cytology due to *S. acuminatum*'s occupation of a broad geography and range of climatic conditions. Together, the results of this study have uncovered a complex life history involving clonal

reproduction, possible apomixis and geographically widespread and recurrent chromosomal aberrations (polyploidy and B chromosomes).

Correlated evidence for polyploidy

Polyploidy can be determined via several techniques including direct chromosome counting from metaphase preparations (Sari et al. 1999), genetic marker amplification (Sosa et al. 2014), genome size estimation by flow cytometry (de Laat et al. 1987, Kron et al. 2007), and morphological size comparisons (Stebbins 1950). As chromosome counting techniques and flow cytometry require access to specific material types (Galbraith et al. 1983, de Laat et al. 1987), studies investigating ploidy variation over broad geographies can benefit from techniques that are not constrained by material type. As *S. acuminatum* is widely distributed across Australia, multiple approaches were used to broaden the scope of our analyses by allowing material of various types (i.e., fresh, dried and herbaria) to be used. Subsequently, it was not possible to undertake each technique on all individuals considered.

As polyploidy tends to have consistent and predictable effects on the genome (Comai 2005), it was expected that there would be a correlation among techniques used to infer ploidy level. For example, polyploidy results in the duplication of all genetic material and thus increases genome size (Kron et al. 2007) and allele copy number (Sosa et al. 2014), which often correlates with an increase in cell and organ size (Stebbins 1950, Beaulieu et al. 2008). Despite these relationships being reported for many plant taxa, a direct correlation between the techniques used here was not always apparent for *S. acuminatum*. Direct evidence for polyploidy was detected via chromosome counts in two individuals (Chapters 2 and 3), while only one of these individuals amplified additional microsatellite

loci (Chapter 2). This same individual also had a larger than average genome size (Chapter 3). However, the increase in genome size in this individual was not proportional to the increase in ploidy (Chapter 3), which may suggest an instantaneous diploidisation process (Chapter 3; Parisod et al. 2010). Furthermore, evidence of polyploidy in three other individuals amplifying additional microsatellite alleles was not observed (Chapter 2). This may be due to methodology since triploidy was observed by karyotyping in one individual thought to be diploid from leaf bud squash preparations (Chapters 2 and 3). However, gene/locus duplication in microsatellite loci (Zhang and Rosenberg 2007) or the presence of B chromosomes (Milani et al. 2014, Piscor and Parise-Maltempi 2016) may produce similar results.

In comparisons of leaf, stomate and seed sizes in a sample size representing the species' distribution, no evidence for individuals with morphological characters of larger size, suggestive of polyploidy, were found (Chapter 4). While one triploid individual with a larger than average genome size and additional microsatellite alleles was found to have the longest stomata (mean = 29.92 µm), the stomata lengths of all other individuals amplifying additional microsatellite alleles were consistent with the mean across the entire data set (mean = 21.67 µm *cf.* 21.52 µm respectively; Chapter 4). This suggests that either gene/locus duplication or B chromosomes rather than polyploidy are responsible for the amplification of additional microsatellite alleles, or that polyploidy does not increase stomata length in *S. acuminatum*. Indeed, leaf size was not related to ploidy or microsatellite profile (Chapter 4). Taken together, the lack of correlation between techniques suggests that the genome of *S. acuminatum* may be in flux, having undergone polyploidy, gene/locus duplication and formation of B chromosomes while avoiding gigas effects often associated with polyploidy.

Distribution of chromosomal aberrations

The distribution of chromosomal aberrations identified in *S. acuminatum* may be indicative of independent and repeated events. Polyploidy is known to arise multiple times in natural plant populations and polyploid lineages often have several origins (Soltis and Soltis 1995). If the presence of additional microsatellite alleles in *S. acuminatum* is indeed a result of polyploidy, these events have occurred over a wide geographic range consistent with independent origins (Figure 5.1). While some studies have suggested a higher incidence of polyploidy in the Australian arid zone (Kearney 2003, Kearney 2005, Roberts et al. 2016), the inverse relationship appears to be the case for *S. acuminatum*, with polyploidy observed in more temperate regions towards the boundaries of the species' range. Unreduced gamete formation, one of the most common routes to polyploidy, is affected by environmental stress (De Storme and Mason 2014). The most studied stressor leading to polyploidisation is temperature (hot/cold shock) (Rezaei et al. 2010, De Storme and Geelen 2014), although other stressors such as drought and salinity are known to affect male gamete production and may also induce polyploidisation (De Storme and Mason 2014). The temperature at which unreduced gametes are formed varies between species (see Negri and Lemmi 1998, Zhang et al. 2003, Pecrix et al. 2011). Thus, *S. acuminatum* may be more tolerant of hot temperatures experienced in more arid conditions and less tolerant of cold in more temperate regions. Contrastingly, the boundaries of a species' range are often associated with an increase in environmental stressors (Castro et al. 2004, Hampe 2005, Viejo et al. 2011), and an unknown stressor in these regions may have induced polyploidy. As polyploidy can facilitate adaptation into new environments, through increased phenotypic plasticity (Husband and Schemske 2000, Hijmans et al. 2007, Hahn et al. 2012), polyploidy may be observed at a higher rate due a selective advantage in these areas. This scenario has been suggested for the colonisation of formerly glaciated areas, where polyploids occur at a higher frequency than diploids (Brochmann

et al. 2004). Alternatively, polyploid incidence in these areas may reflect a greater sampling effort as 83% of sites in this study were within temperate or grassland climatic conditions with 17% in arid conditions (Köppen climate zones; Bureau of Meteorology 2005).

On the other hand, gene/locus duplication may be responsible for the amplification of additional microsatellite alleles in *S. acuminatum*. If this is the case, parsimony suggests that the duplication event occurred early in the species' evolution, prior to its dispersal across Australia. Among *Santalum* species, evidence for prior gene duplication, has only been observed in *S. acuminatum* (Harbaugh 2000). Whether this duplication event coincided with the duplication of the microsatellite loci used here is unknown, although it sets a precedent that such events may be a species-wide phenomenon. Conversely, gene/locus duplication may be lineage specific and long-distance seed dispersal of individuals from the east to west or visa-versa may explain the distribution of this trait. Indeed long distance seed dispersal by Aboriginal Peoples has been suggested for this species (Fuentes-Cross 2015).

The spatial distribution of B chromosomes in *S. acuminatum* may similarly be indicative of their formation prior to the species dispersal across Australia, or a result of independent long distance dispersal events (Figure 5.1). However, their presence in *S. album* (Xin-Hua et al. 2010) and evidence suggesting that *S. acuminatum* and *S. album* diverged early in the evolution of *Santalum* (Harbaugh and Baldwin 2007), suggests that B chromosomes may be a genus wide trait. If this is the case, it is expected that B chromosomes would be present in stands across Australia.

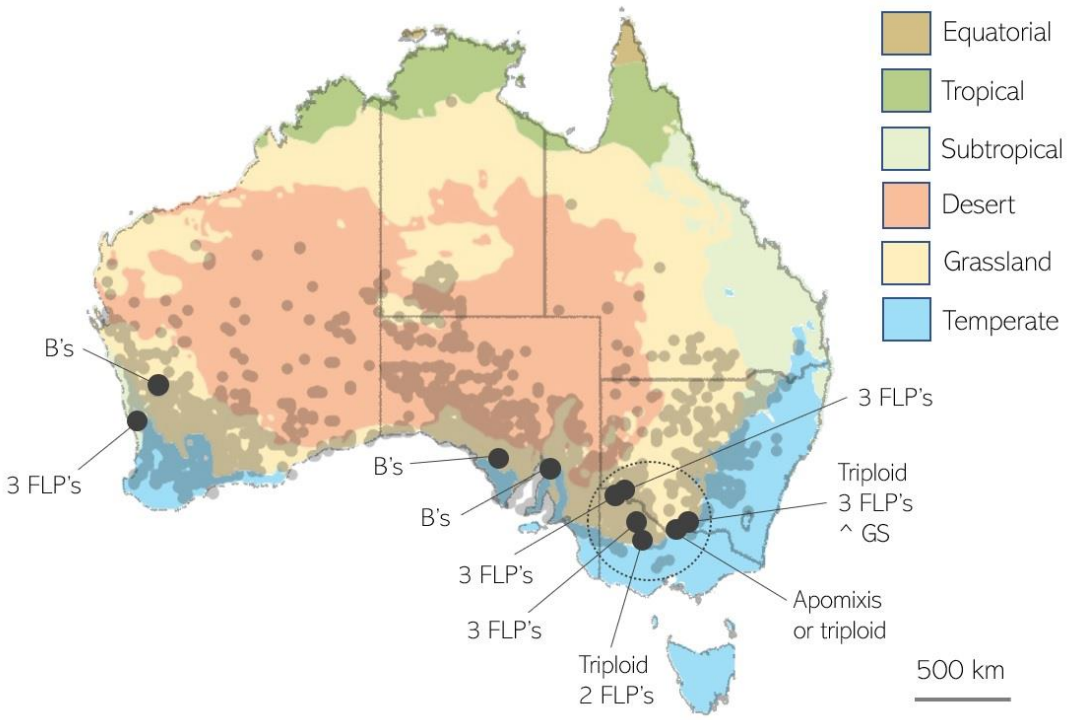

Figure 5.1: Map of *Santalum acuminatum* stands observed to have B chromosomes (B's), a triploid cytotype, amplification of three microsatellite alleles at one or more loci (3 FLP's), a larger than average genome size (^ GS) and possible apomixis. Climate zones (Köppen) categorised by colour (Bureau of Meteorology 2005). Distribution of *S. acuminatum* in grey dots (Atlas of Living Australia 2020). Dotted circle represents the area where a high degree of clonal reproduction was observed.

Polyploidy and reproduction

Whole genome duplication (i.e., polyploidy) is correlated with reproductive changes in many plant taxa including self-fertilisation (Barringer 2007, Husband et al. 2008), clonal reproduction (Stebbins 1971, Van Drunen and Husband 2019), and apomixis (Otto and Whitton 2000). Such changes are a possible adaptation to minority cytotype exclusion (MCE) (Levin 1975) whereby the fitness of neopolyploids is reduced by low mate availability and a disproportionate quantity of inter-cytotype fertilisation events (Otto and Whitton 2000). These events are often unsuccessful or result in sub- or infertile progeny (Marks 1966, Burton and Husband 2000). It should be noted however, that inter-cytotype

hybrids, characterised by uneven ploidy levels (i.e., triploids), can produce some viable haploid and diploid gametes, which may lead to the establishment of fertile tetraploid lineages (Ramsey and Schemske 2002).

Self-fertilisation is expected to be more common in polyploids due to: 1) the weakening or complete breakdown of genetic self-incompatibility systems caused by an increase in allele copy number (Karle et al. 2002, Stone 2002), 2) being a mechanism for, or a by-product of, reproductive assurance when cytotype incompatibility is high (Barringer 2007), and/or 3) a higher tolerance of inbreeding associated with self-fertilisation due to gene duplication in polyploids (Lande and Schemske 1985, Husband and Schemske 1997). Analysis of pollen tube growth following self-fertilisation (Sedgley 1982) and evidence of self-fertilised seed from microsatellite amplification of progeny arrays (Currie 2020; unpublished) suggest that *S. acuminatum* is self-compatible. However, genetic diversity analyses suggested that self-fertilisation is not prominent in this species' south-eastern distribution (Chapter 2). Furthermore, triploidy is associated with reduced or complete infertility, and as this was the only level of polyploidy observed in this study, self-fertilisation may not be a significant mechanism for reproductive assurance in polyploids.

Two pathways have been proposed to explain the association between polyploidy and clonality: 1) clonal reproduction in a diploid species promotes polyploid establishment, and 2) polyploidy instigates clonal reproduction in neopolyploids (Van Drunen and Husband 2019). Under each scenario, clonal reproduction is expected to benefit neopolyploids by facilitating persistence within landscapes until MCE is reduced by the establishment of other polyploids (Weiss-Schneeweiss et al. 2013, Van Drunen and Husband 2018). Both diploid and polyploid stands of *S. acuminatum* were found to exhibit

clonal reproduction, suggesting that this strategy pre-dates polyploid establishment in the species. Polyploidy may increase clonal reproduction for these stands which could be evident through stem quantity and/or stand area. The stand covering the largest area (Ha, 8660 m^2) however, was diploid and thus, investment may be better illustrated by stem quantity, or stand age may be a confounding factor. Consequently, clonal reproduction may serve as a mechanism that has facilitated the persistence of polyploids in *S. acuminatum* rather than a life history trait instigated by polyploidy.

Clonal investment in *S. acuminatum* may be a result of environmental factors that render other forms of reproduction disadvantageous. Sexual reproduction (including self-fertilisation) is energetically expensive (Richards 1997). For this reason, reduced investment in sexual reproduction is often observed in ecologically stressful environments such as fragmented habitats (Rossetto et al. 2004, Honnay and Bossuyt 2005), where disturbance is high (Del Tredici 2001, Kleijn and Steinger 2002, Ledo and Schnitzer 2014), and/or at the fringes of a species' distribution (Beatty et al. 2008). Such ecological stressors are characteristic of the area in which clonal reproduction was investigated in this study (Denham and Auld 2004, Bradshaw 2012). For *S. acuminatum*, clonal reproduction may also aid water and nutrient acquisition from host species by allowing associations with an increased number of and/or variety of host plants. This may be particularly useful in heterogenous or patchy environments or where host plants are sparsely distributed. On the other hand, high seedling mortality due to herbivory, environmental stress and/or lack of suitable host plants may lower output from sexual reproduction. Thus, the prevalence of clonal reproduction in *S. acuminatum* is plausibly a product of environmental stress and/or heterogeneity, rather than a response to polyploidy.

Asexual seed production (apomixis) has evolved repeatedly in angiosperms and is now recognised in at least 40 families (Asker 1992). Among apomictic species, the vast

majority are polyploids (Carman 1997, Otto and Whitton 2000), with some evidence that apomixis is instigated through dosage effects on apomictic genes conferred through unreduced gametes (Pessino et al. 1999, Whitton et al. 2008). In this study, a single seed with a larger than expected endosperm genome size was observed (Chapter 3). The genome size of this endosperm was consistent with a 1:2 embryo to endosperm genome size ratio and apomixis via autonomous endosperm (i.e., in the absence of fertilisation; Henderson et al. 2017, Hojsgaard and Horandl 2019) in a diploid. On the other hand, the genome size of this endosperm was also consistent with a triploid seed produced through fertilisation with an unreduced male gamete (i.e., 3:4 embryo to endosperm genome size ratio; see Chapter 3). Apomixis in diploids is more likely to occur via adventitious embryony compared to other forms of apomixis (Asker 1992). Adventitious embryony produces two or more embryos from a single seed (i.e., polyembryony) (Koltunow 1993), a phenomenon observed in 14% of *S. acuminatum* seed germinated for these studies (Currie 2021; personal observation; Figure 5.2). However, this form of apomixis does not produce seed with larger than expected endosperm genome sizes (Hojsgaard and Horandl 2019). Thus, it is more likely that this seed is triploid rather a diploid apomict. While polyembryony in *S. acuminatum* may be a result of apomixis via adventitious embryony, polyembryony may also arise sexually (Trapero et al. 2014). Either way, possible apomixis in *S. acuminatum* is likely unrelated to polyploidy and does not serve as a reproductive strategy improving the fertility of polyploid lineages.

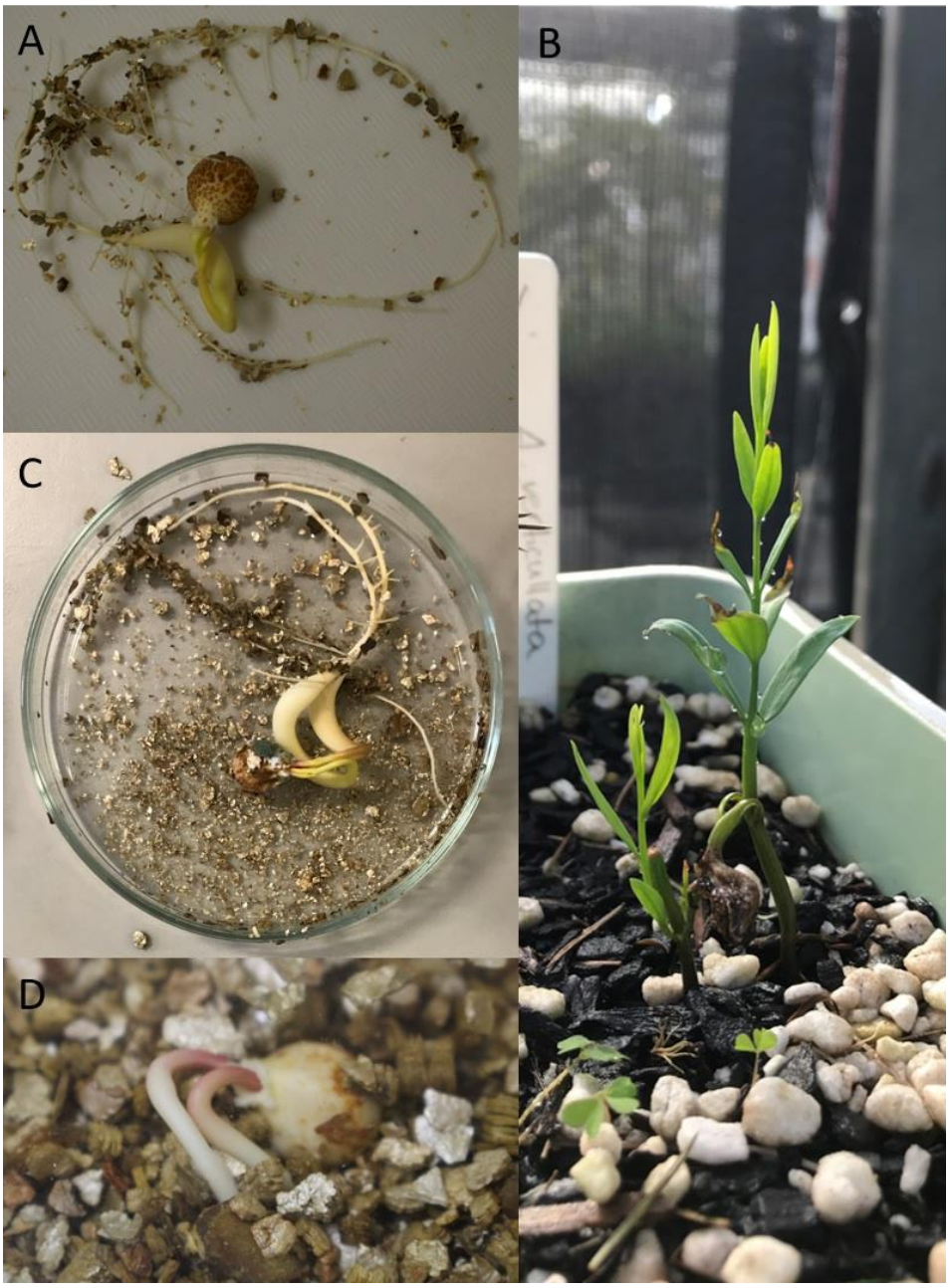

Figure 5.2: Polyembryony in *Santalum acuminatum*. A) a non polyembryonic germinant, B–D) polyembryonic germinants.

Consequences of clonal reproduction and polyploidy

Plant mating systems have direct and often predictable effects on population genetic diversity, genotypic diversity and population structure (Hamrick and Godt 1996, Charlesworth and Wright 2001), with implications for population fitness (Newman and Pilson 1997), adaptive potential (Frankham 2005, Jump et al. 2009) and evolutionary

capacity (Richards 1997). Predominantly clonal populations are expected to have lower genotypic diversity than sexual outcrossers (Holsinger 2000). This is due to clonal offspring being genetically identical to the parent, except for some diversity gained through somatic mutation (Wolf et al. 2000, Lamont and Wiens 2003). Furthermore, in the absence of sexual reproduction, genotypic diversity may be further reduced through inter-genet competition (Hartnett and Bazzaz 1985, Eriksson 1989) and genet death. This reproductive strategy is expected to be beneficial if genotypes are well adapted to environmental conditions and there is environmental stability (Holsinger 2000). If, however, there is a shift in environmental conditions or the introduction of a novel pathogen, clonal reproduction can be disadvantageous due to limited genotypic diversity for natural selection to act upon (Richards 1997). The limited capacity of ramets to geographically disperse (Vallejo-Marín et al. 2010) can also reduce a species' capacity to colonise new areas under shifting climatic niches (Parmesan and Yohe 2003). It is for such reasons that clonal reproduction in the absence of sexual reproduction is predicted to be a poor long term survival strategy and may lead to extinction (Honnay and Bossuyt 2005). Though, some clonal plants, such as *Lomatia tasmanica* (Lynch et al. 1998) and *Populus tremuloides* (Ally et al. 2010) have persisted in landscapes clonally for thousands of years. This may suggest that clonal reproduction does not constitute a path to extinction in all species, or an extinction debt is yet to be realised. On the other hand, polyploidy, through the duplication of loci, can increase heterozygosity and gene redundancy, with fitness benefits (Burton and Husband 2000), while protecting polyploid genomes from inbreeding depression (Comai 2005). Polyploidy can also increase phenotypic plasticity facilitating range shifts that may aid adaptation to environmental change (Parisod et al. 2010). For example, some polyploid lineages occur in disturbed habitats where diploids are unable to persist (Rivero-Guerra 2008) and traits associated with higher stress tolerance have been observed in some polyploid lineages (Stevens et al. 2019). It has also been suggested that polyploids may have better pathogen resistance than diploids due to increased

heterozygosity in polyploid populations (Oswald and Nuismer 2007). Thus, polyploidy may benefit species by facilitating persistence under changed environmental conditions or through the movement of species into new niches.

Santalum acuminatum exhibits extensive clonality and instances of triploidy in its south-eastern distribution (Chapters 2 and 3), and evidence of clonal reproduction (Tennakoon et al. 1997a, Fuentes-Cross 2015) and polyploidy (Byrne et al. 2003) in other areas. As such, the potential consequences for this species are diverse. While clonal reproduction has the capacity to limit a species' ability to adapt to environmental change (Richards 1997, Agrawal 2006) or a novel pathogen (Hamilton 1980, Schmid 1994), it does allow a species to persist across extensive time periods. Additionally, polyploidy may facilitate persistence under environmental stressors (te Beest et al. 2012, Ramsey and Ramsey 2014) and as such, polyploidy may be favoured in certain environments and/or geographies (Godfree et al. 2017). Although, polyploid fitness in comparison to diploids is yet to be explored in this species. Furthermore, polyploid advantage may depend on ploidy level and the traits considered. For example, triploids may benefit from increased growth (Wang et al. 2016, Li et al. 2019) while suffering from low fertility (Burton and Husband 2000, Farco and Dematteis 2014). Certainly, no fruit production has been observed in the last 15 years for the Be individual (S. Logie, personal communication, 2018) which was confirmed to be triploid in a karyotype analysis (Chapter 3), suggesting reduced fertility or sterility. On the other hand, polyploidy may limit this species' capacity to sexually outcross in the future due to inter-cytotype incompatibility. The fitness of cytotypes in a sexually outcrossing population is dependent upon the frequency of compatible cytotypes (Levin 1975). Thus, in populations where diploids occur in low frequency or are sparsely distributed across a large area, even low instances of polyploidy can lower mate availability. The majority of *S. acuminatum* sites in the species' south-

eastern distribution contained single genets and the distance between sites was on the scale of kilometres. Furthermore, with a potential incidence of five polyploids out of twenty-five genets identified in the species' south-eastern distribution, polyploid incidence may be high. Consequently, sexual outcrossing is predicted to be affected by polyploidy in this area.

Future research and management recommendations

The complexity of the results discussed here clearly indicate that further investigation of ploidy and genome change in *S. acuminatum* is warranted. Establishment of a correlation between microsatellite profiles and polyploidy will aid in understanding the extent of polyploidy across the species' range. Furthermore, such a correlation may identify microsatellite markers as a useful technique for ploidy level estimation when chromosome counts are impractical. Whole genome sequencing on the other hand would aid in understanding current and ancient gene and genome duplication events in this species. Future research into the absence of a relationship between polyploidy and morphological characters in *S. acuminatum* may aid in understanding the cellular mechanisms involved in the gigas effect, which are yet to be fully elucidated (Tsukaya 2013). If additional polyploid lineages are identified in the future, the addition of morphological data from these plants to the data gathered here would facilitate a reassessment of whether bimodality within traits is present. Comparison of fitness between diploid and triploid stands will reveal any potential advantages of triploidy in this species. Such advantages may aid survival of these stands and/or see the frequency of triploidy increase in some areas and/or over time. An understanding of the relative investment of clonal reproduction across the distribution of *S. acuminatum* in Australia will aid in determining the environmental, and/or genetic processes leading to extensive clonal reproduction with implications for species management. Management of seed production areas should

consider intraspecific ploidy variation prior to moving seed around the landscape. Currently, such screening is best undertaken using flow cytometry and/or karyotyping.

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Supplementary Material

S2.1: List of *Santalum acuminatum* specimens from herbaria used in the ploidy analysis in this study. Accessions are listed by herbaria – MEL, National Herbarium of Victoria; CANB, National Australian Herbarium; PERTH, Western Australian Herbarium. State abbreviations: VIC, Victoria; NSW, New South Wales; QLD, Queensland; SA, South Australia; NT, Northern Territory; WA, Western Australia.

Accession number	**Location**	**State**	**Latitude**	**Longitude**
MEL 2329929	Buloke	VIC	-36.37	143.15
MEL 2329930	Buloke	VIC	-36.37	143.15
MEL 2356002	Mildura	VIC	-34.37	141.9
MEL 2357300	Mildura	VIC	-34.43	141.44
MEL 2366014	Mildura	VIC	-35.33	141.19
MEL 2425594	Benalla	VIC	-36.36	145.91
MEL 2381975	Bland	NSW	-33.98	147.16
MEL 2344163	Balonne	QLD	-27.86	147.64
MEL 2414052	Balonne	QLD	-28.27	148.17
MEL 2365463	MacDonnell	NT	-25.22	133.2
MEL 2311629	Trayning	WA	-31.11	117.88
MEL 2361018	Yilgarn	WA	-30.96	119.1
MEL 2381845	Kondinin	WA	-32.23	119.75
CANB 760602.1	Gundagai	NSW	-34.91	148.22
CANB 746605	Temora	NSW	-34.44	147.44
CANB 746634	Lachlan	NSW	-33.53	146.99
CANB 867054	Bland	NSW	-33.84	147.21
CANB 866553.1	Bland	NSW	-33.86	146.46
CANB 728295	Bland	NSW	-33.98	147.15
CANB 805388.1	Unincorporated NSW	NSW	-33.27	141.18
CANB 879823.1	Australian National Botanic Gardens	ACT	-35.27	149.11
CANB 821416.1	Mount Isa	QLD	-20.22	139.23
CANB 825022.1	Bon Bon Homestead	SA	-30.42	135.47
CANB 886053.1	Port Pirie City and Dists	SA	-33.48	138.08
CANB 868449.1	Unincorporated SA	SA	-30.59	138.95
CANB 886509	Unincorporated SA	SA	-30.81	132.13
CANB 785042	Chapman Valley	WA	-28.33	115.07
CANB 785042	Chapman Valley	WA	-28.33	115.07
PERTH 8789614	Roebourne	WA	-20.98	115.87
PERTH 7888287	East Pilbara	WA	-23.35	119.06
PERTH 8819459	Wiluna	WA	-25.26	120.65
PERTH 8698473	Ngaanyatjarraku	WA	-26.12	124.74
PERTH 8067317	Laverton	WA	-28.27	126.06
PERTH 8112681	Laverton	WA	-28.23	125.90
PERTH 8741751	Laverton	WA	-28.40	127.98
PERTH 7269900	Dundas	WA	-32.05	126.53
PERTH 7517483	Esperance	WA	-33.31	123.38

PERTH 7897677	Esperance	WA	-32.75	121.44
PERTH 8412855	Ravensthorpe	WA	-33.74	119.86
PERTH 7971729	Ravensthorpe	WA	-33.54	120.12
PERTH 7346271	Cranbrook	WA	-34.25	117.45
PERTH 8507392	Boyup Brook	WA	-33.68	116.26
PERTH 7505485	Perenjori	WA	-29.20	116.52
PERTH 7405073	Carnamah	WA	-29.60	115.91
PERTH 8312087	Menzies	WA	-29.66	119.82
PERTH 8495734	Menzies	WA	-29.82	120.71
PERTH 8385092	Coolgardie	WA	-30.56	120.85
PERTH 8149712	Coolgardie	WA	-31.18	120.38
PERTH 8387567	Coolgardie	WA	-31.07	121.04
PERTH 9088156	Coolgardie	WA	-30.79	120.32
PERTH 7947100	Lake Grace	WA	-33.21	119.30
PERTH 8406634	Kondinin	WA	-32.23	119.75
PERTH 8978565	Lake Grace	WA	-32.81	119.14
PERTH 8339090	Northampton	WA	-27.58	114.67
PERTH 7391862	Dalwallinu	WA	-30.20	116.65
PERTH 7935315	Gingin	WA	-31.17	115.77
PERTH 7143710	Victoria Plains	WA	-30.84	116.38
PERTH 7380283	Perenjori	WA	-29.59	116.96
PERTH 8513392	Corrigin	WA	-32.41	117.76

S2.2: Direct amplicon sequencing of microsatellite markers in *Santalum acuminatum*.

Methods

Thirteen individuals were selected for direct amplicon sequencing to confirm that the primers were amplifying the correct microsatellite loci in *S. acuminatum.* Sampling included six individuals that had previously amplified three alleles at one or more loci and seven individuals that amplified at most two alleles across all loci. Repeated polymerase chain reaction (PCR) and sequencing was undertaken on three individuals to confirm consistent amplification and verify sequencing. Direct sequencing of the PCR product was undertaken for five loci (Table s2.2.1), four of which previously amplified three alleles in at least one individual.

DNA was extracted according to the protocol described in Chapter 2 for the diversity analysis. Primers were synthesised by Integrated DNA Technologies to include Illumina adapter sequences to their 5' end:

Forward adapter sequence: ACACTCTTTCCCTACACGACGCTCTTCCGATCT

Reverse adapter sequence: AGACGTGTGCTCTTCCGATCT

After testing for primer-dimer formation using Multiple Primer Analyser (Thermo Fisher), the loci were split into three multiplex (MP) reactions: (1) Lanc07 and Lanc09, (2) mSaCIRF10 and mSiCIR185, and (3) mSaCIRE09. PCR amplification was undertaken twice (PCR 1 for marker amplification and PCR 2 for hybridisation of Illumina indices). PCR1 was undertaken in a 15 µL mixture of 7.5 µL 2x Type-it Multiplex PCR Master Mix (QIAGEN), 0.1 µM each forward primer and 0.2 µM of each reverse primer, 1.5 µL template DNA and 4.5 µL nuclease-free water under the following conditions; initial cycle of 5 min at 95˚C, followed by denaturation of 30 sec at 95˚C, annealing for 1.5 min at

62˚C for MP1, 53.5˚C for MP2 and 65˚C for MP3 and extension of 30 sec at 72˚C for 20 cycles before a final cycle of 30 min at 60˚C. PCR2 was undertaken in a 15 µL mixture of 7.5 µL GoTaq® Green Master Mix x2, 0.75 µL Illumina index primer, 3 µL PCR 1 product and 4 µL nuclease-free water under the following conditions; initial cycle of 3 min at 95˚C, then denaturation of 20 sec at 98˚C, annealing of 15 sec at 60˚C and extension of 30 sec at 72˚C for ten cycles, and a final extension of 3 min at 72˚C. PCR2 product for MP1–3 were pooled for each sample. Primer dimer was removed in a mixture of 15 µL PCR2 product, 15 µL nuclease-free water and 45 µL magnetic bead solution prepared according to the protocol outlined in Jolivet and Foley (2015) in separate wells of a BIORAD Hard-Shell PCR 96 well plate and incubated at room temperature (18–22°C) for 10 min. Samples were then transferred to an Ambion® RNA by Life Technologies™ Magnetic stand-96 for 5 min and the supernatant removed from each sample. The DNA were cleaned in 190 µL of 80% ethanol for 30 sec. The supernatant removal and ethanol cleaning stages were repeated. The plate was then removed from the magnet and ethanol allowed to evaporate for 3 minutes at room temperature before 22 µL of nuclease-free water was added and each sample mixed thoroughly via pipetting. Samples were incubated at room temperature for 5 min before being placed on the magnetic stand (as described above) for a further 5 min, after which 20 µL of supernatant was removed for gel electrophoresis and sequencing. To ensure removal of primer dimer 10 µL of each sample was run on a 1% agarose gel for 60 min at 100 V. DNA concentration was measured using a NanoDrop Mini (ThermoFisher) and samples normalised for pooling, and a library of all samples prepared in accordance with Illumina MiSeq protocol. Sequencing was via MiSeq Reagent v2 (2x300bp) sequencing kit.

Illumina MiSeq amplicon sequences had their indices and forward and reverse primer sequences paired. Illumina adapters, low quality reads (minimum quality score 13) and short reads (< 30 bp) were trimmed using the BBDuk plugin (of BBMap package:

https://sourceforge.net/projects/bbmap/) in Geneious version 9.0.5 (Kearse et al. 2012). The sequences were then aligned and mapped using the Geneious mapper (medium sensitivity) to a reference list containing the microsatellite sequences from which the loci were developed. Reference sequences were downloaded from GenBank (Agarwala et al. 2016) (Table s2.2.1).

Results

PCR amplification was successful for all samples at four of the microsatellite loci (Lanc09, mSaCIR185, mSaCIRF10 and mSaCIRE09). A fifth locus (Lanc07) failed to amplify for seven samples and amplification was weak for the remaining individuals.

Sequencing of the amplified products from the four remaining loci showed that each contained the microsatellite motifs and flanking regions that aligned to the reference sequences. Some differences in microsatellite length were observed between amplified sequences and reference sequences. The largest change was seen for Lanc09 ($(AG)_{15}$ for reference and $(AG)_{8–10}$ here). Motif changes were observed for Lanc07 ($(TC)_4(CT)_{10}$ for reference and $(TC)_{10}$ here) and mSaCIR185 ($(CA)_7$ for reference and $(CT)_5(CA)_{1–3}$ here). There was no evidence to suggest that non-target sequences were amplified for any individual and at any locus.

Table s2.2.1: Microsatellite loci amplified in *Santalum acuminatum*. Reference sequences for each locus were downloaded from GenBank.

Locus	Repeat motif	Primer sequence	Amplicon size range (bp)	GenBank accession	Originating species and reference
Lanc07	$(TC)_4(CT)_{10}$	F: [B]AAACCCCTTCTCCTCCCATT R: CCGATATTCCCCCATTTCTT	139-163	HM448039	*S. la*[1]
Lanc09	$(AG)_{15}$	F: [C]ATGAGAGCGAGAGGGAGACA R: GTCCACTCCTCACCAAAACC	190-196	HM448041	*S. la*[1]
mSaCIRE09	$(CT)_{16}$	F: [C]GGAAAGGGTTGACAGGAAGAAAA R: TGCGAGTGAGTGGGAAAAGTAGA	172-200	AJ831397	*S. au*[2]
mSaCIRF10	$(GA)_{17}$	F: [A]TTAGGAAAACATAGCACACT R: GAGCACTTCACCACCATTAC	151-181	AJ831398	*S. au*[2]
mSiCIR185	$(CA)_7$	F: [D]ACAACAACGCATAACCCT R: AAAACAATGGCACTGAGAA	285-289	AM113985	*S. in*[3]

A, Tail A: 5'-GCCTCCCTCGCGCCA-3'; B, Tail B: 5'-GCCTTGCCAGCCCGC-3'; C, Tail C: 5'-CAGGACCAGGCTACCGTG-3'; D, Tail D: 5'-CGGAGAGCCGAGAGGTG-3'; *S. la*, *Santalum lanceolatum;* [1], Jones et al. (2010); *S. au*, *S. austrocaledonicum*; [2], Bottin et al. (2005a); *S. in*, *S. insulare*; [3], Lhuillier et al. (2006b).

S2.3*:* Area covered by *Santalum acuminatum* multilocus genotypes (MLG) in this study. N = number of stems sampled. See Table 2.1 for site information. Dashes indicate that too few samples were collected or that GPS information is missing.

Site ID	**MLG number**	**N**	**Area (m^2)**
An	1	1	-
ARR	2	9	455
ARR	3	6	79
Be	4	5	-
BR	5	11	6888
De	9	19	-
Ha	10	3	110
Ha	11	10	8660
HV	12	20	-
IR	13	2	-
IR	14	3	159
IR	15	1	-
IR	16	4	227
IR	17	1	-
IR	18	4	420
IR	19	4	78
IR	20	1	-
Ko	21	4	220
Ma	22	2	-
Ma	23	3	11
PR	24	4	971
PR	25	4	596
PR	26	5	2417
RC	27	7	566
Sa	28	6	-
Th	29	7	215
TT	30	1	-
An	1	1	-

S3.1: Tukey pair-wise comparison matrix of *p*-values from an ANOVA conducted on mean genome sizes of *Santalum acuminatum* fresh leaf material estimated by flow cytometry. Significant *p*-values are coded *****, <0.01; ****, 0.01; ***, 0.02; **, 0.03: *,0.04.

	Be	BHSPAS1	HMLcS1	InB3S6	InC3S1	MPT2S3	Na S2	NaS3	NBG3	NBG8
BHSPAS1	*****									
HMLcS1	*****	0.55								
InB3S6	*****	0.59	*****							
InC3S1	*****	1.00	0.22	0.87						
MPT2S3	*****	1.00	0.98	0.17	0.97					
NaS2	*****	1.00	0.22	0.87	1.00	0.97				
NaS3	*****	1.00	0.38	0.73	1.00	1.00	1.00			
NBG3	*****	0.74	*****	1.00	0.95	0.26	0.95	0.86		
NBG8	*****	****	*****	0.69	*	*****	*	***	0.53	
NBG9	*****	0.81	****	1.00	0.97	0.32	0.97	0.90	1.00	0.46

S3.2: Tukey pair-wise comparison matrix of *p*-values from an ANOVA conducted on mean genome sizes of *Santalum acuminatum* seed endosperm estimated by flow cytometry. Significant *p*-values are coded *****, <0.01; ****, 0.01; ***, 0.02; **, 0.03: *,0.04.

	InB3S1	InB3S4	InB3S7	MPT2S9	NaS4	NaS7	SaS7	SaS9	YuS2
InB3S4	0.99								
InB3S7	0.75	0.99							
MPT2S9	0.55	0.19	0.06						
NaS4	1.00	1.00	0.98	0.24					
NaS7	****	***	0.09	*****	***				
SaS7	0.96	0.59	0.22	0.99	0.68	*****			
SaS9	1.00	0.99	0.76	0.55	1.00	****	0.96		
YuS20	0.10	**	****	0.92	*	*****	0.46	0.10	
YuS23	0.99	0.72	0.30	0.96	0.81	*****	1.00	0.99	0.34

S3.3: Tukey pair-wise comparison matrix of *p*-values from an ANOVA conducted on mean genome sizes of *Santalum acuminatum* seed endosperm against expected endosperm sizes under a 2:3 and 1:2 embryo to endosperm genome size ratio. Expected endosperm sizes calculated based on the mean embryo size observed in this study (0.62 pg). Significant *p*-values are coded *****, <0.01; ****, 0.01; ***, 0.02; **, 0.03: *,0.04.

	2:3 ratio	1:2 ratio
2x	*****	-
InB3S1	0.36	*****
InB3S4	0.14	****
InB3S7	0.05	****
MPT2S9	1.00	*****
NaS4	0.17	*****
NaS7	*****	0.63
SaS7	0.83	*****
SaS9	0.35	*****
YuS20	1.00	*****

S4.1: List of *Santalum acuminatum* specimens from herbaria and the character(s) measured in this study. Specimens are listed by accession number and grouped by herbaria - National Herbarium of Victoria (MEL), National Australian Herbarium (CANB) and Western Australian Herbarium (PERTH). Victoria (VIC), New South Wales (NSW), Australian Capital Territory (ACT), Queensland (QLD), South Australia (SA), Northern Territory (NT), Western Australia (WA).

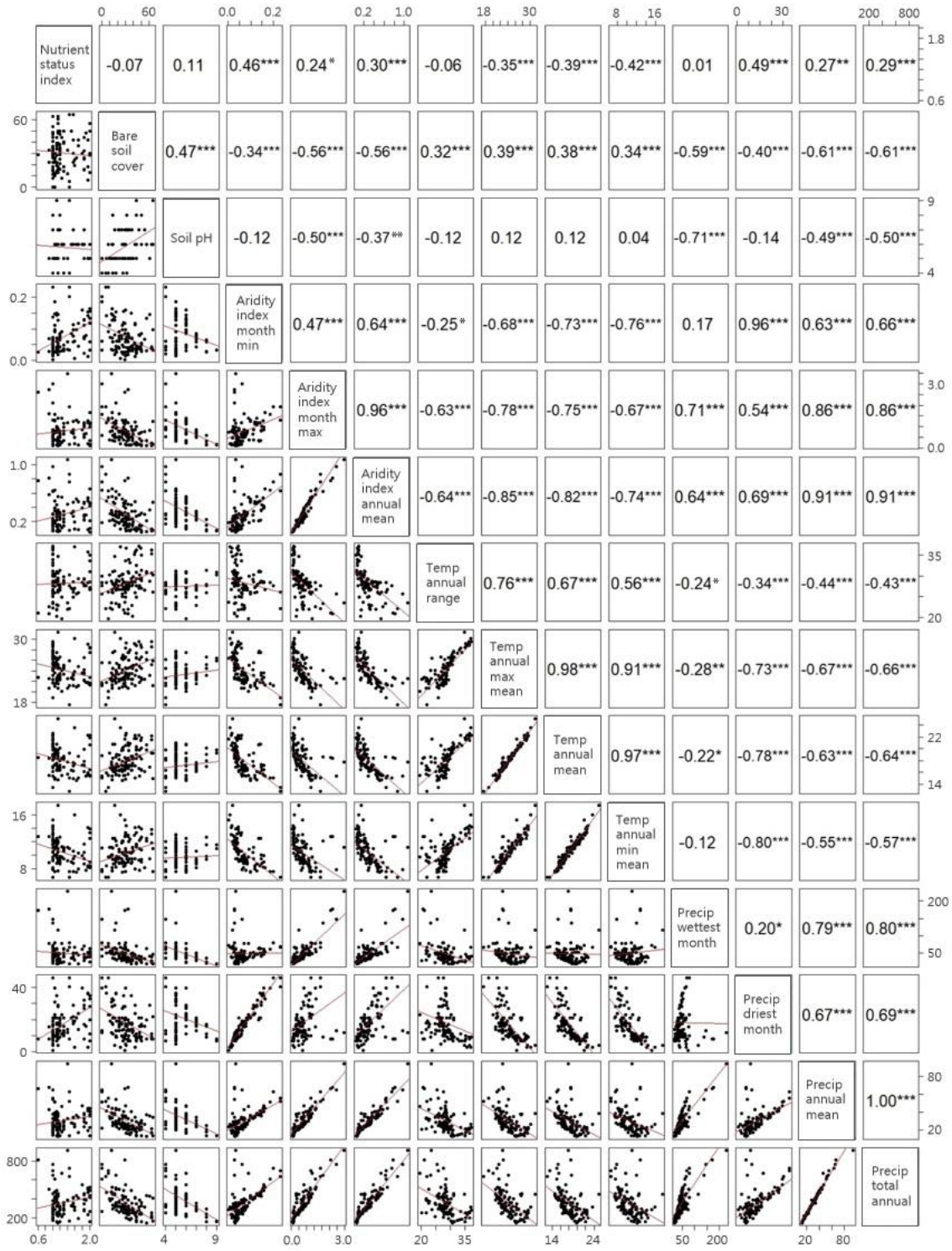
Accession	**Location**	**State**	**Latitude**	**Longitude**	**Character measured**
MEL 2329929	Buloke	VIC	-36.37	143.15	Stomate size
MEL 2329930	Buloke	VIC	-36.37	143.15	Stomate size
MEL 2356002	Mildura	VIC	-34.37	141.9	Stomate size
MEL 2357300	Mildura	VIC	-34.43	141.44	Stomate size
MEL 2366014	Mildura	VIC	-35.33	141.19	Stomate size
MEL 2425594	Benella	VIC	-36.36	145.91	Stomate size
MEL 1503834	Macedon Ranges	VIC	-37.48	144.77	Stomate size
MEL 0002500	Swan Hill	VIC	-35.05	142.87	Stomate size
MEL 0002550	Hindmarsh	VIC	-36.45	142.01	Stomate size
MEL 2227223	Mildura	VIC	-34.3	142.17	Stomate size
MEL 2046390	Horsham	VIC	-36.58	141.96	Stomate size
MEL 0002549	Moira	VIC	-36.01	145.25	Stomate size
MEL 0002686	Swan Hill	VIC	-35.31	143.55	Stomate size
MEL 2092274	Mildura	VIC	-34.3	142.23	Stomate size
MEL 2381975	Bland	NSW	-33.98	147.16	Stomate size
MEL 0002510	Lachlan	NSW	-33.0	147.5	Stomate size
MEL 0002520	-	NSW	-	-	Stomate size
MEL 0002524	Dubbo	NSW	-32.25	148.62	Stomate size
MEL 0002536	Bogan	NSW	-31.02	146.7	Stomate size
MEL 0002540	-	NSW	-	-	Stomate size
MEL 0002639	Cobar	NSW	-31.5	145.82	Stomate size
MEL 1513390	-	NSW	-	-	Stomate size
MEL 2067608	Lachlan	NSW	-33.22	146.37	Stomate size
MEL 2280541	Cootamunda	NSW	-34.52	148.03	Stomate size
MEL 2344163	Balonne	QLD	-27.86	147.64	Stomate size
MEL 2414052	Balonne	QLD	-28.27	148.17	Stomate size
MEL 0002615	Bulloo	QLD	-27.97	143.82	Stomate size
MEL 2230561	Balonne	QLD	-28.17	148.00	Stomate size
MEL 2230558	Peterborough	SA	-32.9	138.73	Stomate size
MEL 0002513	Maralinga Tjarutja	SA	-30.45	131.82	Stomate size
MEL 0002516	-	SA	-	-	Stomate size
MEL 0002523	Unincorporated SA	SA	-30.2	138.57	Stomate size
MEL 0002640	Unincorporated SA	SA	-31.5	138.5	Stomate size
MEL 0002641	Unincorporated SA	SA	-31.5	138.5	Stomate size
MEL 0002647	Unincorporated SA	SA	-30.82	138.4	Stomate size
MEL 0298251	Unincorporated SA	SA	-30.3	136.77	Stomate size
MEL 0535984	The Coorong	SA	-35.33	139.5	Stomate size

MEL 2106126	Unincorporated SA	SA	-30.78	135.35	Stomate size
MEL 2230554	Alexandrina	SA	-35.5	138.77	Stomate size
MEL 2230569	Unincorporated SA	SA	-28.17	135.3	Stomate size
MEL 2365463	MacDonnell	NT	-25.22	133.2	Stomate size
MEL 2230549	MacDonnell	NT	-24.62	132.27	Stomate size
MEL 0002683	MacDonnell	NT	-23.95	132.77	Stomate size
MEL 1558171	MacDonnell	NT	-24.23	131.63	Stomate size
MEL 2230555	MacDonnell	NT	-24.57	131.67	Stomate size
MEL 2311629	Trayning	WA	-31.11	117.88	Stomate size
MEL 2361018	Yilgarn	WA	-30.96	119.1	Stomate size
MEL 2381845	Kondinin	WA	-32.23	119.75	Stomate size
MEL 0002518	Freemantle	WA	-32.07	115.75	Stomate size
MEL 0002662	Dundas	WA	-31.72	128.87	Stomate size
MEL 0002711	Yilgarn	WA	-30.88	119.02	Stomate size
MEL 1536406	Kalamunda	WA	-31.98	116.07	Stomate size
MEL 1538740	Dowerin	WA	-30.87	117.08	Stomate size
MEL 1555306	Yalgoo	WA	-28.03	116.68	Stomate size
MEL 2106660	Dundas	WA	-32.11	122.02	Stomate size
MEL 2230544	Coolgardie	WA	-30.95	120.42	Stomate size
MEL 2230553	-	WA	-	-	Stomate size
MEL 0002501	City Beach	WA	-31.91	115.75	Stomate size
CANB 760602.1	Gundagai	NSW	-34.91	148.22	Leaf size, stomate size
CANB 746605	Temora	NSW	-34.44	147.44	Leaf size, stomate size
CANB 746634	Lachlan	NSW	-33.53	146.99	Leaf size, stomate size
CANB 867054	Bland	NSW	-33.84	147.21	Leaf size, stomate size
CANB 866553.1	Bland	NSW	-33.86	146.46	Leaf size, stomate size
CANB 728295	Bland	NSW	-33.98	147.15	Leaf size, stomate size
CANB 805388.1	Unincorporated NSW	NSW	-33.27	141.18	Leaf size, stomate size
CANB 879823.1	Australian National Botanic Gardens	ACT	-35.27	149.11	Leaf size, stomate size
CANB 821416.1	Mount Isa	QLD	-20.22	139.23	Leaf size, stomate size
CANB 825022.1	Bon Bon Homestead	SA	-30.42	135.47	Leaf size, stomate size
CANB 886053.1	Port Pirie City and Dists	SA	-33.48	138.08	Leaf size, stomate size
CANB 868449.1	Unincorporated SA	SA	-30.59	138.95	Leaf size, stomate size
CANB 886509	Unincorporated SA	SA	-30.81	132.13	Leaf size, stomate size
CANB 785042	Chapman Valley	WA	-28.33	115.07	Leaf size, stomate size
CANB 785042	Chapman Valley	WA	-28.33	115.07	Leaf size, stomate size
PERTH 8789614	Roebourne	WA	-20.98	115.87	Leaf size, stomate size
PERTH 7888287	East Pilbara	WA	-23.35	119.06	Leaf size, stomate size
PERTH 8819459	Wiluna	WA	-25.26	120.65	Leaf size, stomate size
PERTH 8698473	Ngaanyatjarraku	WA	-26.12	124.74	Leaf size, stomate size
PERTH 8067317	Laverton	WA	-28.27	126.06	Leaf size, stomate size
PERTH 8112681	Laverton	WA	-28.23	125.90	Leaf size, stomate size
PERTH 8741751	Laverton	WA	-28.40	127.98	Leaf size, stomate size
PERTH 7269900	Dundas	WA	-32.05	126.53	Leaf size, stomate size
PERTH 7517483	Esperance	WA	-33.30	123.38	Leaf size, stomate size
PERTH 7897677	Esperance	WA	-32.75	121.44	Leaf size, stomate size

PERTH 8412855	Ravensthorpe	WA	-33.74	119.86	Leaf size, stomate size
PERTH 7971729	Ravensthorpe	WA	-33.54	120.12	Leaf size, stomate size
PERTH 7346271	Cranbrook	WA	-34.25	117.45	Leaf size, stomate size
PERTH 8507392	Boyup Brook	WA	-33.68	116.26	Leaf size, stomate size
PERTH 7505485	Perenjori	WA	-29.20	116.51	Leaf size, stomate size
PERTH 7405073	Carnamah	WA	-29.60	115.91	Leaf size, stomate size
PERTH 8312087	Menzies	WA	-29.66	119.82	Leaf size, stomate size
PERTH 8495734	Menzies	WA	-29.82	120.71	Leaf size, stomate size
PERTH 8385092	Coolgardie	WA	-30.56	120.85	Leaf size, stomate size
PERTH 8149712	Coolgardie	WA	-31.18	120.38	Leaf size, stomate size
PERTH 8387567	Coolgardie	WA	-31.08	121.04	Leaf size, stomate size
PERTH 9088156	Coolgardie	WA	-30.79	120.32	Leaf size, stomate size
PERTH 7947100	Lake Grace	WA	-33.21	119.30	Leaf size, stomate size
PERTH 8406634	Kondinin	WA	-32.23	119.75	Leaf size, stomate size
PERTH 8978565	Lake Grace	WA	-32.81	119.14	Leaf size, stomate size
PERTH 8339090	Northampton	WA	-27.58	114.67	Leaf size, stomate size
PERTH 7391862	Dalwallinu	WA	-30.20	116.65	Leaf size, stomate size
PERTH 7935315	Gingin	WA	-31.17	115.77	Leaf size, stomate size
PERTH 7143710	Victoria Plains	WA	-30.84	116.38	Leaf size, stomate size
PERTH 7380283	Perenjori	WA	-29.59	116.96	Leaf size, stomate size
PERTH 8513392	Corrigin	WA	-32.41	117.76	Leaf size, stomate size

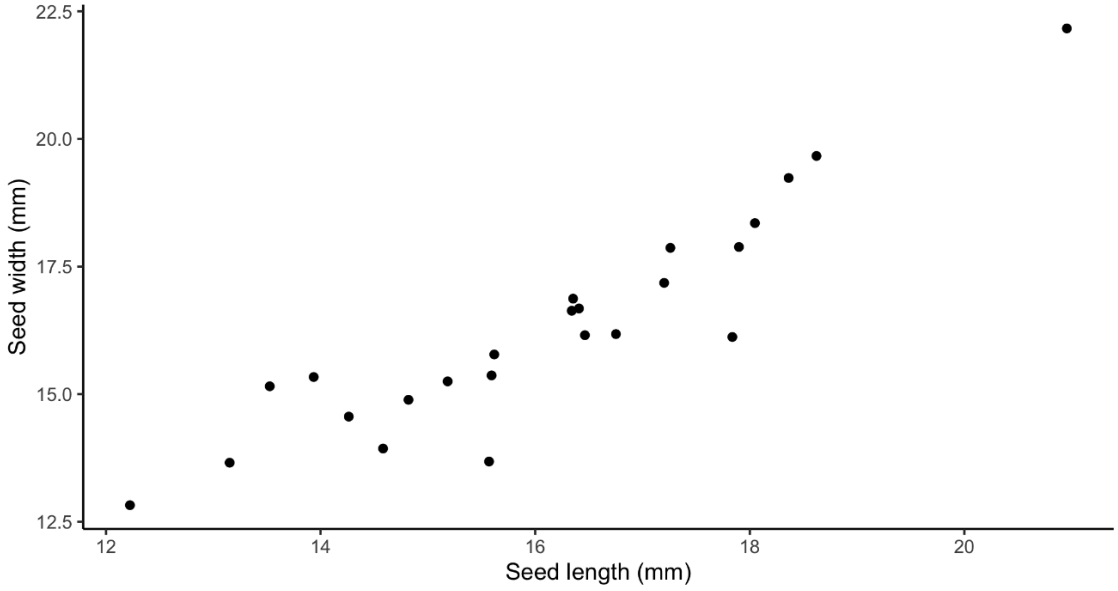
S4.2: Spearman's correlation coefficient matrix of environmental variables. Top right cells depict Spearman's rho and where correlations are significant the *p*-value has been coded * < 0.01, ** < 0.001, *** < 0.0001. Lower left cells display the scatterplot for each relationship where the red trend line plots the general linear model. Min = minimum, max = maximum, temp = temperature and precip = precipitation.

- Figure on next page -


Nutrient status index
Bare soil cover
Soil pH
Aridity index month min
Aridity index month max
Aridity index annual mean
Temp annual range
Temp annual max mean
Temp annual mean
Temp annual min mean
Precip wettest month
Precip driest month
Precip annual mean
Precip total annual
-0.07 0.11 0.46*** 0.24* 0.30*** -0.06 -0.35*** -0.39*** -0.42*** 0.01 0.49*** 0.27** 0.29***
0.47*** -0.34*** -0.56*** -0.56*** 0.32*** 0.39*** 0.38*** 0.34*** -0.59*** -0.40*** -0.61*** -0.61***
-0.12 -0.50*** -0.37*** -0.12 0.12 0.12 0.04 -0.71*** -0.14 -0.49*** -0.50***
0.47*** 0.64*** -0.25* -0.68*** -0.73*** -0.76*** 0.17 0.96*** 0.63*** 0.66***
0.96*** -0.63*** -0.78*** -0.75*** -0.67*** 0.71*** 0.54*** 0.86*** 0.86***
-0.64*** -0.85*** -0.82*** -0.74*** 0.64*** 0.69*** 0.91*** 0.91***
0.76*** 0.67*** 0.56*** -0.24* -0.34*** -0.44*** -0.43***
0.98*** 0.91*** -0.28** -0.73*** -0.67*** -0.66***
0.97*** -0.22* -0.78*** -0.63*** -0.64***
-0.12 -0.80*** -0.55*** -0.57***
0.20* 0.79*** 0.80***
0.67*** 0.69***
1.00***

S4.3: Relationship between seed length and width in *Santalum acuminatum*.

Scatterplot of seed length and width measurements, $r^2 = 0.85$.



S4.4: Tukey pair-wise comparison matrix of *p*-values from an ANOVA conducted on seed length between mother plants in *Santalum acuminatum*. Significant *p*-values are coded ***** < 0.01, **** = 0.01, *** = 0.02, ** = 0.03, * = 0.04.

- Table over next two pages -

	De	GL	GNT1	GNT2	GNT3	GNT4	GNT5	GNT6	HML	In	INR	INRB	INRC	LET1	LET2	LET3	LET4	LET5
GL	0.09																	
GNT1		*****																
GNT2	0.91	*****	0.05															
GNT3	0.99	*****	0.07	1.00														
GNT4	1.00	0.94	*****	****	0.09													
GNT5	0.91	*****	0.09	1.00	1.00	****												
GNT6	1.00	0.19	*****	0.92	0.99	1.00	0.91											
HML	****	*****	1.00	0.99	0.98	*****	1.00	****										
In	1.00	0.98	*****	0.99	1.00	1.00	0.99	1.00	0.23									
INR	*****	1.00	*****	*****	*****	*****	*****	*****	*****	0.21								
INRB	1.00	0.33	*****	0.32	0.71	1.00	0.33	1.00	*****	1.00	*****							
INRC	0.57	*****	1.00	1.00	1.00	***	1.00	0.56	1.00	0.78	*****	0.18						
LET1	0.79	*****	0.16	1.00	1.00	****	1.00	0.80	1.00	0.97	*****	0.19	1.00					
LET2	1.00	*	*****	0.76	0.97	1.00	0.76	1.00	*****	1.00	*****	1.00	0.44	0.57				
LET3	1.00	*****	*****	1.00	1.00	0.50	1.00	1.00	0.14	1.00	*****	1.00	0.97	1.00	1.00			
LET4	***	*****	1.00	0.98	0.97	*****	0.99	***	1.00	0.20	*****	*****	1.00	1.00	*****	0.17		
LET5	0.43	*****	0.84	1.00	1.00	*****	1.00	0.45	1.00	0.82	*****	0.06	1.00	1.00	0.24	0.96	1.00	
LET6	0.99	*****	***	1.00	1.00	0.07	1.00	0.99	0.93	1.00	*****	0.69	1.00	1.00	0.97	1.00	0.90	1.00
Mb	*****	*****	1.00	0.79	0.76	*****	0.86	*****	1.00	0.08	*****	*****	1.00	0.94	*****	***	1.00	1.00
MKBOC	0.09	*****	0.99	1.00	1.00	*****	1.00	0.10	1.00	0.50	*****	*****	1.00	1.00	**	0.57	1.00	1.00
MKT10	0.82	*****	0.21	1.00	1.00	****	1.00	0.83	1.00	0.97	*****	0.23	1.00	1.00	0.63	1.00	1.00	1.00
MKT11	1.00	0.27	*****	0.93	0.99	1.00	0.92	1.00	***	1.00	*****	1.00	0.58	0.82	1.00	1.00	**	0.48
MKT13	1.00	0.31	*****	0.22	0.60	1.00	0.22	1.00	*****	1.00	*****	1.00	0.13	0.12	1.00	0.99	*****	*
MKT17	0.76	1.00	*****	*****	*****	1.00	*****	0.93	*****	1.00	****	0.99	*****	*****	0.45	****	*****	*****
MKT19	1.00	*****	**	1.00	1.00	0.36	1.00	1.00	0.90	1.00	*****	0.96	1.00	1.00	1.00	1.00	0.87	1.00
MKT2	1.00	*****	*****	1.00	1.00	0.75	1.00	1.00	0.43	1.00	*****	1.00	0.99	1.00	1.00	1.00	0.42	1.00
MKT20	1.00	*****	*****	1.00	1.00	0.77	1.00	1.00	0.28	1.00	*****	1.00	0.98	1.00	1.00	1.00	0.30	0.98
MKT5	0.74	*****	0.28	1.00	1.00	****	1.00	0.75	1.00	0.95	*****	0.17	1.00	1.00	0.52	1.00	1.00	1.00
MKT9	*****	0.12	*****	*****	*****	*****	*****	*****	*****	*****	0.86	*****	*****	*****	*****	*****	*****	*****
MPT1	0.98	*****	0.06	1.00	1.00	*	1.00	0.97	0.99	1.00	*****	0.55	1.00	1.00	0.92	1.00	0.98	1.00
MPT2	1.00	*****	*****	1.00	1.00	0.33	1.00	1.00	0.70	1.00	*****	0.97	1.00	1.00	1.00	1.00	0.68	1.00
Na	0.70	1.00	*****	*****	*****	1.00	*****	0.91	*****	1.00	****	0.99	*****	*****	0.38	****	*****	*****
PNAB	1.00	1.00	*****	0.37	0.64	1.00	0.37	1.00	*****	1.00	0.17	1.00	0.15	0.25	1.00	0.98	*****	0.09
PNRA	0.97	1.00	*****	***	0.07	1.00	***	0.99	*****	1.00	0.90	1.00	****	****	0.93	0.34	*****	*****
Sa	0.24	1.00	*****	*****	*****	1.00	*****	0.44	*****	1.00	0.88	0.66	*****	*****	0.10	*****	*****	*****
UR	1.00	*****	*****	1.00	1.00	0.50	1.00	1.00	*	1.00	*****	1.00	0.90	0.99	1.00	1.00	0.06	0.83
Yu	*****	*****	1.00	*****	*****	*****	*****	*****	0.20	*****	*****	*****	0.72	*****	*****	*****	0.77	****

MET6	Mb	MKBOG	MKT10	MKT11	MKT13	MKT17	MKT19	MKT2	MKT20	MKT5	MKT9	MPT1	MPT2	Na	PNAB	PNRA	Sa	UR
0.55																		
1.00	1.00																	
1.00	0.96	1.00																
0.99	*****	0.13	0.84															
0.57	*****	*****	0.15	1.00														
*****	*****	*****	*****	0.97	0.99													
1.00	0.53	1.00	1.00	1.00	0.93	***												
1.00	0.12	0.85	1.00	1.00	1.00	0.09	1.00											
1.00	0.06	0.74	1.00	1.00	1.00	0.09	1.00	1.00										
1.00	0.98	1.00	1.00	0.77	0.10	*****	1.00	1.00	1.00									
*****	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****								
1.00	0.78	1.00	1.00	0.98	0.42	*****	1.00	1.00	1.00	1.00	*****							
1.00	0.27	0.97	1.00	1.00	0.93	****	1.00	1.00	1.00	1.00	*****	1.00						
*****	*****	*****	*****	0.96	0.98	1.00	****	0.07	0.07	*****	*****	*****	****					
0.65	*****	***	0.27	1.00	1.00	1.00	0.89	0.99	0.99	0.22	*****	0.52	0.91	1.00				
0.06	*****	*****	****	0.99	1.00	1.00	0.21	0.48	0.51	****	****	*	0.21	1.00	1.00			
*****	*****	*****	*****	0.57	0.64	1.00	*****	****	****	*****	*****	*****	*****	1.00	1.00	1.00		
1.00	*****	0.29	0.99	1.00	1.00	****	1.00	1.00	1.00	0.98	*****	1.00	1.00	*****	0.99	0.37	*****	
*****	0.83	0.05	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****

S4.5: Statistics for Spearman's correlation coefficient for *Santalum acuminatum* morphometrics and environmental variables. Holm adjustment has been applied to *p*-values. Statistically significant *p*-values are coded *, 0.04; ** 0.03; ***, 0.02; ****, 0.01 and *****, <0.01.

Morphometric	Environmental variable	n	S	*p*-value	rho
Mean leaf length (mm)	Soil pH	57	16902	1.0	0.08
	Nutrient status cover (index)		38890	1.0	-0.08
	Bare soil cover (%)		32301	1.0	0.10
	Aridity index month min		39227	1.0	-0.09
	Aridity index month max		38146	1.0	-0.06
	Aridity index annual mean		39721	1.0	-0.10
	Precipitation driest month (mm)		39467	1.0	-0.10
	Precipitation wettest month (mm)		33685	1.0	0.06
	Precipitation annual mean (mm)		38989	1.0	-0.08
	Precipitation annual total (mm)		38998	1.0	-0.08
	Temperature annual range (˚C)		32849	1.0	0.09
	Temperature annual min mean (˚C)		34000	1.0	0.06
	Temperature annual max mean (˚C)		31877	1.0	0.11
	Temperature annual mean (˚C)		32147	1.0	0.11
Mean leaf width (mm)	Soil pH	57	23027	0.52	-0.25
	Nutrient status cover (index)		44706	0.44	-0.24
	Bare soil cover (%)		31869	1.00	0.11
	Aridity index month min		54110	*****	-0.50
	Aridity index month max		37031	1.00	-0.03
	Aridity index annual mean		43836	0.52	-0.22
	Precipitation driest month (mm)		54246	*****	-0.51
	Precipitation wettest month (mm)		24150	0.09	0.33
	Precipitation annual mean (mm)		40083	1.00	-0.11
	Precipitation annual total (mm)		39416	1.00	-0.10
	Temperature annual range (˚C)		25366	0.18	0.30
	Temperature annual min mean (˚C)		19938	*****	0.45
	Temperature annual max mean (˚C)		20773	****	0.42
	Temperature annual mean (˚C)		20420	****	0.43

n = sample size, S = Spearman's statistic, rho = Spearman's rho.

- Table continues on next page -

Morphometric	Environmental variable	n	S	*p*-value	rho
Mean stomata size (µm)	Soil pH	90	112928	1.00	0.07
	Nutrient status cover (index)	117	244565	1.00	0.08
	Bare soil cover (%)		333015	0.05	-0.25
	Aridity index month min		208675	0.11	0.22
	Aridity index month max		200083	0.05	0.25
	Aridity index annual mean		191418	***	0.28
	Precipitation driest month (mm)		198513	0.05	0.26
	Precipitation wettest month (mm)		245006	1.00	0.08
	Precipitation annual mean (mm)		213783	0.13	0.20
	Precipitation annual total (mm)		204136	0.11	0.22
	Temperature annual range (˚C)		344382	***	-0.29
	Temperature annual min mean (˚C)		353001	****	-0.32
	Temperature annual max mean (˚C)		350408	****	-0.31
	Temperature annual mean (˚C)		355179	*****	-0.33
Mean seed length (mm)	Soil pH	13	408.87	1.00	-0.12
	Nutrient status cover (index)	16	929.25	0.77	-0.37
	Bare soil cover (%)		393.52	0.77	0.42
	Aridity index month min		977.09	0.77	-0.44
	Aridity index month max		1013.2	0.54	-0.49
	Aridity index annual mean		981.11	0.77	-0.44
	Precipitation driest month (mm)		1022.8	0.51	-0.50
	Precipitation wettest month (mm)		630.56	1.00	0.07
	Precipitation annual mean (mm)		973.08	0.77	-0.43
	Precipitation annual total (mm)		973.08	0.77	-0.43
	Temperature annual range (˚C)		427.07	0.77	0.37
	Temperature annual min mean (˚C)		302.61	0.31	0.55
	Temperature annual max mean (˚C)		296.59	0.30	0.56
	Temperature annual mean (˚C)		288.56	0.27	0.58

n = sample size, S = Spearman's statistic, rho = Spearman's rho.

S4.6: Results of a Shapiro-Wilk test for normality and transformation performed on each variable that was non-normally distributed. Only environmental variables that were significantly correlated with one or more morphological character(s) are included.

Variable	Shapiro-Wilk test for normality		Transformation
	W	*p*-value	
Aridity index – month min	0.90	< 0.001	Ordernorm
Aridity index – month mean	0.91	< 0.001	Standardised log_b(x + a)
Precipitation during driest month	0.92	< 0.001	Standardised log_b(x + a)
Temperature minimum month mean	0.96	< 0.001	Ordernorm
Temperature maximum month mean	0.96	< 0.001	Standardised asinh(x)
Temperature annual mean	0.96	< 0.001	Standardised Yeo-Johnson
Temperature range	0.95	< 0.001	Ordernorm
Leaf width (mm)	0.93	0.001	Standardised log_b(x + a)