Reproductive biology, flowering and genetics of Fontainea picrosperma (Euphorbiaceae)

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FLOWERING, REPRODUCTION, POLLINATION BIOLOGY, AND GENETICS OF *FONTAINEA PICROSPERMA* (EUPHORBIACEAE)

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Abstract

This thesis investigated the ecology of a plant species that has commercial application, *Fontainea picrosperma* (Euphorbiaceae). *Fontainea picrosperma* is of interest following the discovery of a small molecule, natural product with anti-cancer activity from its fruit. The novel epoxy-tigliane called tigilanol tiglate has been approved for use as a therapy for canine mast cell tumours and is being developed for the treatment of human head and neck squamous cell carcinoma. Tigilanol tiglate cannot be synthesised on a commercial scale and it is manufactured by extraction and purification from the seed of *F. picrosperma*. As such, an understanding of the reproductive characteristics that determine fruit-set of this species is critical.

Fontainea picrosperma is a subcanopy, tropical rainforest tree endemic to the Australian Wet Tropics (AWT), northern Australia. Rainforests in this region are notable for their high level of endemism and distinctive Gondwanan taxa, especially in the uplands where populations of *F. picrosperma* are geographically confined. *Fontainea picrosperma* is dioecious but little else was known of the ecology of this tropical rainforest plant. This thesis contributes to an understanding of the floral morphology, phenology, pollination and genetics of *Fontainea picrosperma*. Knowledge of the ecological aspects of the species helps to secure sustainable seed production for commercial manufacture of tigilanol tiglate. Moreover, this thesis improves our understanding of fine-scale ecological interactions within a poorly studied tropical rainforest community, the AWT, and helps to highlight the challenges and conservation strategies for this dioecious, subcanopy species. Research into plant ecology (modes of reproduction and gene flow) in tropical rainforests systems is dominated by canopy species, while subcanopy plant-pollinator interactions remain underrepresented.

This research provides the first description of the floral and reproductive biology of this species and reveals that *F. picrospmera* is pollen limited. *Fontainea picrosperma* inflorescences bear small, white, actinomorphic flowers with a shallow receptacle. These floral traits are often associated with a generalist, entomophilous pollination syndrome and are common to dioecious tropical rainforest flowers. Male panicles contained significantly more flowers than female inflorescences, and male

flowers opened sequentially on a panicle. This is most likely to ensure that pollen is available across the entire female flowering period and to encourage pollinator movement between inflorescences within the population. Conversely, female flowers opened almost simultaneously within an inflorescence to create a greater visual display. Individual female flowers persisted on the tree and remained receptive for long periods post-anthesis suggesting an adaptation to low pollinator activity. Indeed, *F. picrosperma* is pollen limited, as hand-pollinated female flowers produced almost double the final fruit set of open pollinated flowers. This conclusion is supported by a dearth of insects that were observed visiting the flowers. Therefore, optimised production of tigilanol tiglate may rely on improving pollen flow from male to female trees, by hand pollination or by managing natural or introduced pollinators.

We used several methods to elucidate the mode of pollen delivery from male to female flowers. Wind pollination in *F. picrosperma* is incidental, if it occurs at all and the flowers do not produce nectar. Female flowers, that offer no obvious reward, mimic the smell of reward offering male flowers and are pollinated by deceit, though floral parts themselves could attract certain pollinators. Many of the scent compounds present in the floral bouquet of *F. picrosperma* are ubiquitous in nature and known to attract a wide variety of insects. Both day time and night time pollinators contribute to successful reproduction and we observed several Orders of insects visiting the flowers such as beetles, predatory wasps, flies and thrips. Thrips were the most frequently observed flower visitor, otherwise, observations were characterised by low numbers of visitors to both male and female flowers. The unspecialised structure of *F. picrosperma* flowers together with the low frequency with which the wide variety of small insect taxa were observed visiting both sexes indicates that pollination occurs from the pool of low-energy, generalist insects found beneath the canopy in the tropical rainforest.

This thesis provides a direct analysis of pollen-mediated gene flow and estimates of genetic parameters and genetic structure between subpopulations of adult and juvenile groups in natural populations of *F. picrosperma.* Pollen movement affects genetic variation in plant populations and is an important consideration in conservation and plant domestication. Our results show pollination events occur over much shorter distances than reported for tropical canopy species. Pollinators preferentially travel short distances between conspecific trees, most likely due to the opportunistic feeding

patterns of small generalist insects. Many of the local male population contributed to successful reproduction of *F. picrosperma* with most fathers siring a single seed. However, the contributions to reproduction were uneven with larger male trees bearing more flowers having greater reproductive success than those with less flowers.

There were comparatively low levels of genetic variation across the species, owing in part to this predominant near neighbour mating. We found no loss of genetic diversity between adult and juvenile trees despite proximate plants being significantly related to each other than expected from random mating. Short distance pollen flow, skewed reproductive success of males and low genetic diversity is theoretically a prelude to genetic impoverishment. However, the species has persisted through multiple significant climatic oscillations during the Plio-Pleistocene era. Together, this suggests that this species is highly adapted to its environment, short distance pollen flow does not affect the species capacity to persist in the environment, and there is sufficient long-distance (> 30 m) gene flow to keep the level of genetic diversity stable across the species distribution. Despite the species' ongoing resilience, low overall genetic diversity may compromise the ability of *F. picrosperma* to adapt to changing environmental conditions and extreme stochastic events. Moreover, the remaining low genetic diversity is of potential concern for domestication programs, which require maximal genetic diversity to facilitate efficient selective breeding and genetic improvement of this commercially significant species.

The findings of this thesis extend established literature on the genus *Fontainea* by showing results that are novel for the species *F. picrosperma*. These results highlight the importance of the need for carefully designed plantations to optimise gene flow between male and female trees. Further, these results emphasise the importance of conserving the remaining species habitat and maintaining genetic connectivity between natural populations of *F. picrosperma*.

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: Elektra Grant

Date: 31 January 2020

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Chapter 1: General Introduction

Flowering, Reproduction, Pollination Biology and Gene flow of Vascular Plants in the Tropical Rainforest

1.1 INTRODUCTION TO TROPICAL RAINFORESTS

The tropical rainforest biome spreads along the equatorial region and naturally covers approximately 7 % of the Earth's land surface (Wilson, 1988). Tropical rainforests are unique due to their biological richness across both local and global spatial scales (Eiserhardt et al., 2017). These biologically diverse, geographically concentrated areas are estimated to harbour half of the world's biodiversity, including around half of the world's vascular plant species (Lewis, 2009; Eiserhardt et al., 2017).

Tropical rainforests occur in five main biogeographical regions along the equatorial belt: Africa; Madagascar; southeast Asia; New Guinea (including Australia and some Pacific Islands) and tropical America (south and central America) (Primack and Corlett, 2005). These regions contain a range of climates that have variations in annual rainfall, in the magnitude and frequency of extreme events and in the degree of seasonality (Walsh and Blake, 2015). Most rainforest regions experience frequent, short dry periods, though a few locations can experience droughts of up to 6 months during ENSO events and a small fraction are aseasonal (Walsh and Blake, 2009). For example, rainforests in the Australian Wet Tropics (AWT) are properly characterised as seasonally dry (van Schaik et al., 1993; Stork et al., 2008), whereas the dry and rainy seasons in southeast Asian rainforests are not clearly demarcated (Nishida, 2001). Further deviations in climatic correlates occur at local scales, which further obscures a clear definition of a tropical rainforest biome (Moncrieff et al., 2015). Moncrieff et al. (2016) suggests that biome definitions should be based on functional similarities or plant traits. This assumes that disjunct assemblages in similar physical environments have similar structural and functional similarities than assemblages from other environments because they are subject to the same evolutionary pressures (Lusk et al., 2016). The world's tropical rainforests have similar growth forms and the same

characteristic general appearance despite harbouring many independent lineages of plants (Eiserhardt et al., 2017).

The floristic links that occur at the plant family level exist between tropical rainforest regions due to distant geological history, when many of the Earth's land surfaces were linked on the ancient southern supercontinent of Gondwana (Raven and Axelrod, 1974). However, different lineages of plants have evolved in different rainforest biomes around the world due to long-term dispersal constraints since the break-up of this land mass (Primack and Corlett, 2005; Eiserhardt et al., 2017). Differences between global rainforest biomes at the sub-family level, have evolved such that they can be classified separately, into different realms (Raven and Axelrod, 1974; Singh and Sharma, 2009). Tropical rainforests of the south and central America are referred to as the Neotropical realm (new world tropics). The more recent linkage in evolutionary history between the modern day African and Indomalayan rainforest regions (India, mainland Asia, southeast Asia) is why they are often classified together as the Palaeotropical realm (old world tropics). The rainforests of Africa now tend to be drier and less diverse than both the Neotropical and southeast Asian rainforests (Singh and Sharma, 2009). Southeast Asian rainforests are dominated by trees of the family Dipterocarpaceae, which occur in the canopy and emergent layers and regenerate through unique, irregular episodes (~2-7 year intervals) of general or mass flowering (Ashton et al., 1988; Corlett, 2004). Lastly, the Australasian realm (including Australia, New Guinea, New Caledonia and other small fragments) separated from Gondwana when moist tropical environments were not present in the region (Metcalfe and Ford, 2009). These environments arose when the land mass moved northward towards the equator. Thus, the long isolation of the tropical rainforests in this region from developing floras in other parts of the tropics resulted in a distinctive flora, with East-Gondwanan origins (Webb and Tracey, 1981; Metcalfe and Ford, 2009).

Tropical rainforests provide local and globally important ecosystem services including storing biomass carbon, serving as habitats for endangered flora and fauna and preserving major elements of the global hydrological cycle (Singh and Sharma, 2009; Gibbs et al., 2010). Moreover, the species diversity concentrated in tropical rainforests provide a reservoir of natural products that could be developed into new pharmaceuticals (Kingston, 2011; Naman et al., 2017). For example, natural products

(small molecule chemical compounds produced by a living organism), or their synthetic analogues have attributed to approximately 50 % of approved cancer drugs since the 1940s and are significantly relied upon in other areas such as anti-infection (Newman and Cragg, 2016). The discovery of natural products relies implicitly on the preservation of biodiversity as the source material for new pharmaceuticals (Kingston, 2011). Thus, drug discovery represents an economic value latent within tropical rainforests that can be leveraged to conserve tropical rainforest biodiversity (Naman et al., 2017). Despite their significance, tropical rainforests are severely threatened by deforestation and fragmentation (Asner et al., 2009). Land conversion from intact tropical rainforests to crop and pasture land were the primary source of agricultural expansion in recent decades (1980-2000) (Gibbs et al., 2010). In addition, a rapidly changing climate means that plant traits may be less adaptive to the unprecedented rates of change (Metcalfe and Ford, 2009).

There are a few well studied tropical rainforest communities globally. Much of the research has emerged from permanent forest-dynamic plots established by the Smithsonian Tropical Research Institute (STRI) and its Centre for Tropical Forest Science (CTFS) (Carson and Schnitzer 2008). In the Neotropics for example, extensive research has been conducted in the 50 ha Forest Dynamics Plot, in the Barro Colorado Island in Gatun Lake in central Panama, which was the first CTFS plot to be established (STRI, 2020). Fewer studies exist of plant species in the Paleotropic realm (Vizentin-Bugoni et al., 2018), though the Canopy Biology Plot Lambir Hills National Park, Sarawak, Malaysia is probably the most heavily researched tropical rainforest in this region (Momose et al., 1998b; Sakai et al., 1999; Yumoto, 2000). Tropical rainforests in the Australasian realm remain very poorly studied (Gross 2005; Vizentin-Bugoni et al., 2018).

This literature review focuses on reproductive biology, pollination and gene flow in rainforests globally, with an emphasis on dioecious species. Plant species ecology (modes of reproduction and gene flow) is a key aspect in the structuring of rainforest systems by maintaining species diversity and driving speciation. Thus, increasing our knowledge in this field is an important consideration in management of species and conservation of these diverse, tropical rainforest communities.

1.2 REPRODUCTIVE ECOLOGY OF DIOECIOUS, VASCULAR PLANTS IN THE TROPICAL RAINFOREST

High species richness and concomitant low population densities led to predictions of restricted gene flow and high levels of self-fertilisation in tropical rainforest taxa by early authors (e.g. Corner 1954; Baker, 1959; Fedorov, 1966). These initial predictions have been superseded and current estimates suggest that most tropical rainforest plants are facultative or obligate outcrossers that rely almost exclusively on animal pollinators for seed production (Bawa et al., 1985b; Bawa, 1992; Carpenter et al., 2003; Ollerton et al., 2011). Dioecy, where male and female flowers occur on different plants, is the most extreme mechanism of outcrossing. Dioecious species represent only 5-6 % of all angiosperms globally (Renner, 2014), whereas about 20 % of tropical rainforest floras globally are dioecious (Bawa and Opler, 1975; Bullock, 1985; Russell-Smith and Lee, 1992; Hansman, 2001; Vary et al., 2011). Thus, this reproductive strategy is overrepresented in tropical rainforest biomes. It is unclear why a high incidence of dioecy occurs at similar levels in rainforest communities that have different evolutionary histories and different contemporaneous environmental conditions (Richards, 1997).

1.2.1 Reproductive characteristics of dioecious species

Similar selection pressures have produced similar flowering strategies in dioecious rainforest plants globally (Bawa and Opler, 1975; Renner and Ricklefs, 1995; Vamosi et al., 2003; Queenborough et al., 2007; Gao et al., 2012). Dioecious species typically produce large fleshy fruits and seed (Vamosi et al., 2003). Females do not have to allocate resources to male functions and are therefore able to invest more into higher quality offspring that encourages dispersal by animal vectors (Murcia, 1996; Matallana et al., 2005). Dioecy also allows plants to allocate different resources to male and female flowers and these investments have led to notable differences in the reproductive strategy between male and female trees (Gao et al., 2012).

Specialisation of male and female flowers have generally led to different inflorescence structures and flowering patterns within a species (Ainsworth, 2000). Specifically, male trees produce flowers at smaller sizes, flower more frequently, flower for a longer period of time and produce twice as many flowers as female trees

(Nicotra, 1998; Osunkoya, 1999; Gao et al., 2012; Queenborough et al., 2013). This ensures that pollen is available across the entire female flowering period, provided that male and female trees flower synchronously (House, 1993; Barrett and Harder, 1996; Williams and Adam, 2010). Flowers on female inflorescences are also known to open simultaneously to create a greater visual attraction for pollinators, whereas male inflorescences open sequentially, which encourages the movement of pollinators between trees (Yamasaki and Sakai, 2013). The flowering phenology of dioecious species therefore influences the foraging behaviour of pollinators and affects patterns of gene flow within plant populations (Dick et al., 2008).

Floral displays as well as spatial considerations between conspecific trees are important factors in determining reproductive success (Barrett and Harder, 1996; Degen and Roubik, 2004; Duminil et al., 2016). For dioecious species, sex ratios can determine the distance between individuals of the opposite sex, which influences pollen transfer between trees. For example, aggregation of males and females aids pollen transfer by reducing the distance between individuals of opposite sex (House 1992). Moreover, male-biased sex ratios commonly occur in species in rainforest ecosystems (Bawa and Opler, 1975; Queenborough et al., 2007; Gao et al., 2012; Field et al., 2013). Since male trees commonly produce more flowers than female trees, and are therefore more attractive to pollen consuming insects, the size of the local male neighbourhood can influence reproductive success in female individuals (House, 1993).

Male trees can also grow faster, survive longer and occupy different microhabitats compared with females (Nicotra, 1998; Wheelwright and Logan, 2004). More generally, there is a clear correlation between dioecy and species longevity (the proxy being woody growth) (Renner and Ricklefs, 1995). Species longevity, combined with intense competition with other plant species, predators, pathogens and abiotic agents in tropical rainforest biomes could promote outcrossing in favour of inbreeding (Janzen, 1970; Levin, 1975). Furthermore, high quality offspring produced by female only trees, owing to sex-specific resource allocation and genetic recombination, may represent a long-term evolutionary advantage despite lower fecundity over self-compatible hermaphrodites (Aguilar et al., 2006; Davila et al., 2012). Dioecious species are dependent on pollen vectors for reproduction, which

could make them particularly vulnerable to external pressures including habitat fragmentation and climate change (Bond et al., 1994; Aguilar et al., 2006).

The modification of habitat through destruction and forest fragmentation can disrupt natural patterns of gene flow by creating environments that are stressful for pollinator survival and activity (Bradshaw et al., 2009; Eckert et al., 2010). Altering the number of reproductive individuals in the population can negatively impact genetic diversity in progeny due to disruptions in pollen diversity and pollinator mobility (Breed et al., 2012b). However, the impacts to mating patterns vary between species and context (Hamrick, 2004; Lowe et al., 2005). Furthermore, successful reproduction in dioecious species measured in terms of both male success (pollen flow) and female success (fruit set) depends on many, often interacting factors. These include flowering patterns (House, 1992), pollinator composition, abundance and flight behaviours (Aguilar et al., 2006), adult sex ratios (Queenborough et al., 2007), and spatial distribution of the species population (Gao et al., 2012).

1.2.2 Floral attractants of dioecious species

Dioecious species are not able to produce seed by self-pollination and must invest resources to attract pollinators for successful reproduction (Williams and Adam, 2010). Individual fecundity relies on attracting foragers to both male and female flowers. The plant traits likely to be involved in pollinator attraction include flower colour, shape, size, scent and food reward (Barrett and Harder, 1996; Momose et al., 1998b; Boulter et al., 2006a). Floral biology, which includes both floral traits and flowering time, are recognised as adaptations to attract and exploit certain types of pollinators and exclude low-efficiency visitors (Faegri and van der Pijl, 1979). These relationships between floral biology and pollination systems are interpreted as ‘pollination syndromes’ (Faegri and van der Pijl, 1979). Thus, floral biology can predict the most likely and effective pollinators regardless of breeding system or region (Ollerton et al., 2011; Rosas-Guerrero et al., 2014). For example, the few tropical or subtropical dioecious rainforest species known to have obligate mutualisms with thrip (Thysanoptera) species possess floral morphological adaptations that act as protective brood and residential sites (Williams and Adam, 1994; Adam and Williams, 2001; Moog et al., 2002). Alternatively, dioecious species in tropical rainforests typically possess floral structures consistent with a general entomophilous pollinator syndrome

(Bawa and Opler, 1975; House, 1989; Renner and Feil, 1993). That is, small actinomorphic and pale-coloured flowers that have an open access receptacle inferring little specialisation towards pollinators of any kind (Bawa and Opler, 1975; Machado and Lopes, 2004; Boulter et al., 2006a).

Nectar is generally the most common floral reward for pollinators in plant species (Kevan and Baker, 1983). However, nectar is absent in flowers that are wind pollinated or flowers that attract pollinators by deception (Williams and Adam, 2010). Pollination by deceit, where female flowers offer no obvious reward (nectar or pollen), is common in tropical, dioecious species that are insect pollinated (Renner and Feil, 1993). Although, it must be noted that for some nectarless species the flowers themselves are an indirect food resource for pollinating taxa with adult chewing herbivores such as Coleoptera and Hemiptera (Sakai et al., 1999; Williams and Adam, 2010). Nectar production exerts a drain on plant resources and cost-saving by 'cheater flowers' is suggested to be the most predominant selective force in the evolution of nectarless flowers (Thakar et al., 2003). The trade-off for cost-saving in nectarless flowers might be an unreliable pollination system since nectariferous flowers are less likely to be pollen-limited than nectarless flowers in self in-compatible species (Larson and Barrett, 2000; Wilcock and Neiland, 2002). Female flowers that offer no obvious reward are likely to deceive pollinators due to their perceptual similarity to pollen-offering male flowers (Renner, 2006). Nectarless species may also depend on other species in the community to provide floral rewards, particularly in areas where co-occurring species are highly attractive to pollinators (Ghazoul, 2005). Therefore, pollination is the consequence of foraging errors made by pollinators as they move between trees (Endress, 1996; Schaefer and Ruxton, 2009).

When female flowers offer no nectar reward, Bakerian mimicry (where female flowers mimic male flowers and cheat pollinators out of a reward) often occurs (Voeks, 2002; Borchsenius et al., 2016). In these circumstances, floral volatiles can provide a cost-efficient way to attract pollinators as well serve as defensive cues against herbivores and pathogens (Dudareva et al., 2006; Junker and Blüthgen, 2010). Many insect pollinators have acute olfactory senses that can act over long distances before any visual cues are apparent at close range. Thus, chemical signals can aid in finding food over greater areas (Kite, 1998). Scent rather than flower colour may be a stronger attraction signal below the rainforest canopy where visual cues are less obvious in a

relatively dark environment with dense foliage (Knudsen et al., 1999). Floral scent chemistry has been largely overlooked in pollination biology (Dötterl and Vereecken, 2010). Identifying the main scent constituents present among plant species could increase our knowledge of the potential pollinators of a plant species (Irvine and Armstrong, 1991). Currently, the lack of literature in this field means few generalisations can be made about the main scent constituents and their relative composition and plants pollinated by different functional groups of insects (Raguso, 2008; Cordeiro et al., 2019).

1.2.3 Pollen limitation of dioecious species

Failure to attract pollinators can result in reduced fruit set due to pollen limitation (Larson and Barrett, 2000; Knight et al., 2005; Aguilar et al., 2006; Davila et al., 2012). Pollen limitation occurs when pollen augmentation increases seed production relative to open pollinated controls (Knight et al., 2005). Environmental conditions affect the degree of pollen limitation more strongly than common ancestry (Larson and Barrett, 2000) and species within tropical rainforests are more prone to pollen limitation than temperate species (Larson and Barrett, 2000; Knight et al., 2005; Vamosi et al., 2013). Tropical rainforests have a high percentage of obligate outcrossing species that typically occur in low densities (Bawa, 1990). Dioecious and self-incompatible species generally exhibit lower fecundity compared with related self-compatible hermaphrodites (Larson and Barrett, 2000; Ashman et al., 2004; Knight et al., 2005). This is likely to be exacerbated by the temporal variability in pollination services that is often encountered in tropical rainforests where pollinator abundance and competition from co-flowering species is highly unpredictable (Williams and Adam, 2010; Vamosi et al., 2013).

Species that exhibit pollen limitation are vulnerable to habitat fragmentation and loss when individual population sizes are reduced and plant-pollinator mutualistic interactions are disrupted (Kearns et al., 1998; Knight et al., 2005; Memmott et al., 2007). Small plant populations that have fewer mates available offer less reward and thus attract fewer insect pollinators (House 1993). With fewer pollinators, flower visits decline, limiting pollen transfer between trees within the population and ultimately leads to reduced seed production (Eckert et al., 2010). Subtle impacts on population genetic diversity due to altered gene flows are also likely to occur (Lamont

et al., 2016). Despite this concern, pollination, fruit-set and seed production have not been studied empirically for many dioecious rainforest trees.

1.3 TROPICAL RAINFOREST POLLINATION

Pollination by wild animal species is one of the most important ecological services provided to agriculture and natural ecosystems (Kearns et al., 1998; Garibaldi et al., 2011). Globally, around 85 % of angiosperms are reliant on animal pollination, with species in temperate regions relying proportionally less (78 %) than in tropical regions (94 %) on pollination by animals (Ollerton et al., 2011). Thus, plant-pollinator interactions are critically important in tropical rainforest systems where many plant species exhibit dioecy, self-incompatibility, and low plant densities in the canopy (Bawa, 1990). Facultative or obligate outcrossing bears costs both directly through the energy necessary to produce attractants and rewards and indirectly through fluctuations in the abundance of pollinators (Williams and Adam, 2010). In return, pollinating animals are equally reliant on flowering plants and would decline without floral rewards (Kearns et al., 1998; Memmott et al., 2007; Ollerton et al., 2011). Fragmented and disturbed environments are particularly vulnerable to failure of plant-pollinator mutualisms and fragmentation may accelerate the erosion of biodiversity (Bawa, 1990; Bond et al., 1994; Renner, 1998; Quesada et al., 2011). Despite the significance of plant-pollinator interactions, these systems remain poorly studied in most tropical rainforest regions (Winfree, 2010; Winfree et al., 2011; Vizentin-Bugoni et al., 2018). Therefore, research on plant-pollinator interactions is essential to understand plant reproduction in tropical environments and improve our ability to predict and manage these interactions.

1.3.1 Pollinator assemblies in tropical rainforest regions

Bees are thought to be the most important class of pollinators in tropical rainforests around the world (Dick et al., 2008), especially in the Neotropics, where bee species have been extraordinarily successful (Bawa, 1990; Machado and Lopes, 2004). The genus *Apis* (honeybees) is absent in south and central America but the region is otherwise rich in bee fauna (Nishida, 2001). Large solitary bees, Lepidoptera and vertebrates are relatively more important pollinators in the better-studied

Neotropics than in other rainforest regions (Corlett, 2004). Euglossine bees (long-tongued bees) and Hummingbirds (long-billed nectar-feeding birds) are moderately specialised, important long-distance pollinators confined to this region (Bawa, 1990; Kress and Beach, 1994; Dick et al., 2008). Bat and bird pollination overall are infrequent in both the Indomalayan region and in the Australian tropics, though few studies have been published in the latter region (Hansman, 2001; Corlett, 2004; Gross, 2005).

Southeast Asian rainforests appear to have fewer species pollinated by nectarivorous vertebrates, Lepidoptera and large solitary bees than the Neotropics (Kress and Beach, 1994; Sakai et al., 1999; Corlett, 2004). Most probably because these potential pollinators are the most vulnerable to long term fluctuations in resource availability (Momose et al., 1998a). They do not store food or switch diets thus they are not equipped to survive outside the general flowering episodes that are unique to the tropical lowland Dipterocarp rainforests in southeast Asia (Sakai et al., 1999). Pollination in these forests is dominated by highly social bees (mainly *Trigona* and *Apis* species), followed by beetles and then other bees and flies (Corlett, 2004). These pollinating taxa are able to survive through the fluctuating floral resources, for example, *Trigona* bees can store resources for 2-5 years without resupply and some *Apis* spp., e.g. *Apis dorsata* can migrate over 100 km at the onset of a general flowering period (Momose et al., 1998a). Herbivorous beetles are probably the second most important pollinators in the canopy of Dipterocarp forests (Momose et al., 1998b; Sakai et al., 1999; Corlett, 2004). Beetles feed on leaves during the non-flowering period and can proliferate rapidly during times of flowering (Sakai et al., 1999). Generalist floral visitors are also common in plant species across the Indomalayan region, though coverage of studies in the region is patchy (Corlett, 2004; Vizentin-Bugoni et al., 2018).

The initial perception of bee-dominated pollination in the Australian tropical rainforests has been superseded (Boulter et al., 2005; Kitching et al., 2007). Long-tongued bees and large social bees such as *Apis dorsata* or *A. cerrana* are entirely absent in the Australian tropics (Gross, 2005; Williams and Adam, 2010). The New Caledonian fauna appears to be similarly depauperate in medium to large bee species (Carpenter et al., 2003). It appears that the predominant floral visitors in Australian tropical forests are small generalist insects, commonly from the Orders, Coleoptera,

Diptera and Hymenoptera (Irvine and Armstrong, 1991; Boulter et al., 2005; Gross, 2005; Worboys and Jackes, 2005; Boulter et al., 2009). However, there are few published descriptions of the pollination of plants from Australian tropical rainforests and only tentative generalisations can be made at this stage (Boulter et al., 2006b). Beetles are thought to be important pollinators in the Australian tropics, with estimates suggesting that up to one quarter of Australian rainforest species might be pollinated by beetles (Irvine and Armstrong, 1991; Hansman, 2001; Webber et al., 2008; Wardhaugh et al., 2015). Studies indicate beetle pollination in Australian rainforests is more common than in Costa Rica, Venezuela and in the dry tropical rainforests of Brazil where only one beetle pollinated species is known (Bawa et al., 1985a; Irvine and Armstrong, 1991; Machado and Lopes, 2004).

1.3.2 Plant-pollinator interactions

Generalisation and specialisation in plant-pollinator interactions refer to the range of different resource items used that can be quantified by the number of pollinators visiting a plant species, or alternatively, the number of plant species a pollinator visits (Renner, 2006). Some authors suggest that tropical plants have relatively specialised and more reliable pollen vectors than temperate ones (Renner and Feil, 1993; Olesen and Jordano, 2002). However, others propose that specialisation is rare and generalised or inefficient pollinators including small insects such as small bees, butterflies, beetles, flies and wasps may be more common, especially in the canopy (Bawa, 1990; Ollerton and Cranmer, 2002; Corlett, 2004; Boulter et al., 2009). Differences between regions and breeding systems as well as different methods for measuring specificity contribute to this lack of consensus (Johnson and Steiner, 2000; Vazquez and Aizen, 2006; Waser and Ollerton, 2006). Plant-pollinator interactions present a continuum that ranges from complete mutual dependence (highly specialised) to nectar robbing by animals and pollination by deceit by plants (Waser and Ollerton, 2006). It is these complex interactions that are thought to play a role in governing and maintaining the rich biodiversity in tropical rainforests (Nishida, 2001).

The most commonly documented example of tropical rainforest trees with highly specialised pollinators are Figs (*Ficus* spp.) (Hill 1967; Dick et al., 2008). Fig species

have a pantropical distribution, and all are pollinated by tiny (2-3 mm) parasitic wasps belonging to the Agaonidae family (Machado Carlos et al., 2001). There are few other known instances of plant species visited by a single pollinator, and even sympatric fig taxa are known to share pollinators (Wang et al., 2016). A single species of Thysanoptera (thrip), *Neoheegeria* sp., dominates pollination of *Macaranga hulletti* (Moog et al., 2002), though it is unknown whether this thrip species pollinates other *Macaranga* species. Moderately specialised interactions move away from highly specific mutualisms with single plant species (Bawa, 1990). Euglossine bees (long tongued bees), commonly known as orchid bees, are a more well-known semi-specialised, plant-pollinator relationship (Costa and Francoy, 2017). This is an asymmetrical interaction where long tube flowers are dependent on a few species of Euglossine bees, but Euglossine bees forage on a greater number of nectar hosts (Listabarth, 1993; Borrell, 2005). Plant species with specialised or semi-specialised pollinators exhibit floral traits that attract certain pollinators and exclude low-efficiency visitors (Momose et al., 1998b). Euglossine bees with longer tongues are known to visit a wider variety of plant species than those with shorter tongues and flowers with longer corollas have fewer species visit than flowers with shorter corollas (Borrell, 2005). Orchids and hawkmoths also exhibit semi-specialised interactions, for example, several species of orchids are pollinated by a single species of hawkmoth, *Panogena lingen*, in Madagasca (Nilsson et al., 1987). Semi-specialised bee species (other than Euglossine bees), bats, lepidopterans, and beetles such as scarab beetles, are further examples of these interactions in tropical rainforest systems (Listabarth, 1993; Momose et al., 1998b; Stangler et al., 2016). Hummingbirds were originally thought to have moderately specialised relationships with plant species (Stiles, 1975) but these interactions are not always tightly prescribed (Cotton, 1998; Dziedzioch et al., 2003).

Generalist pollination occurs when pollinators use a wide range of floral resources. This type of plant-pollinator interaction is usually associated with an array of beetles, flies, non-specialised bees (e.g. eusocial *Apis* spp. and *Trigona* spp.), moths, bats and thrips (Waser and Ollerton, 2006). Generalised plant-pollinator interactions are a common pollination strategy across rainforest communities (Bawa et al., 1985a; Corlett, 2004; Boulter et al., 2009). In the lowland forests of central America, many tree species are pollinated by a diverse array of insects, second only to medium-large

sized bees (Bawa et al., 1985a). Most plant-pollinator relationships in the Indomalayan region appear to be relatively generalised (Corlett, 2004) and most woody plants in Australian tropical rainforest communities are also putatively pollinated by small, generalist insects (Irvine and Armstrong, 1991; Williams and Adam, 1994; Boulter et al., 2005; Worboys and Jackes, 2005; Webber et al., 2008). Plant species that possess a floral morphology consistent with the general entomophilous pollination syndrome dominates Australian tropical flora, New Caledonian rainforest trees and may account for up to 31 % of all species in tropical lowland rainforests of central America (Bawa, 1990; Hansman, 2001; Carpenter et al., 2003; Gross, 2005).

Unspecialised pollen vectors may only be opportunistic, faithful for short distances, potentially ineffective as long-distance pollen vectors and/ or inefficient due to the long periods they spend on individual flowers consuming pollen (e.g., beetle pollinators) when compared to pollinator specialists (House, 1989; House, 1993; Mack, 1997; Momose et al., 1998b; Gross, 2005). Despite these shortcomings, functional generalists are commonly less pollen limited than more specialised species in tropical rainforests (Wolowski et al., 2014). The close association between dioecy and generalised pollination systems in tropical rainforest communities is potentially beneficial with plant species that have no reproductive assurance (Bawa et al., 1985b). Flexibility in the pollination strategy of rainforest plants allows species to continue to successfully reproduce in the absence of one or more insect groups that visit the flowers making this system more resilient to fluctuations in pollinator services than specialised species (Ghazoul, 2005). However, in the event of pollinator loss or decline due to habitat degradation or fragmentation, species with a generalised pollination system may incur consequences because not all pollinators are equal (Murcia, 1996). Individual fecundity and or subtle alterations to gene flow can occur if pollinator species are not replaced by species with similar pollination efficiency (Corlett, 2004; Wolowski et al., 2014).

1.3.3 Differences in pollination among vertical strata and challenges of pollination beneath the canopy

Pollination services vary across tropical rainforest communities, in terms of specificity and among vertical strata within rainforest communities. Vertical strata can

be delineated as follows: herbs shrubs and small trees less than 5 m make up the understorey; trees greater than 5 m tall but less than canopy stature at reproductive maturity make up the subcanopy; and trees flowering at the top of the forest, including emergent trees, represent canopy species (Kress and Beach, 1994). Most of the floristic diversity is found beneath the canopy of tropical rainforests (Bawa et al., 1985a; Hubbell, 2005; Ashley, 2010). The differences in plant species composition as well as temporal availability of floral resources among the vertical strata result in a complex functional relationship with pollinator diversity (Kitching et al., 2007). In addition, the microclimate beneath the rainforest canopy likely contributes to differences in pollinator assemblages between strata (Appanah, 1991; New, 2018). The understorey and subcanopy is a markedly different environment to the forest canopy where highly productive flowers, in terms of floral rewards, are derived from the high light energy input (Bawa et al., 1985a; Appanah, 1991; Mabberley, 1992). The total radiation reaching the understory in tropical rainforests is 2-3 % of the incident light energy (Mabberley, 1992).

The rainforest understorey and subcanopy has dense evergreen foliage that creates physical barriers for pollinator movement, stable diurnal air temperatures, high relative humidity and low wind speeds and/or turbulent wind patterns compared to the canopy (Kato, 1996; Corlett 2004; New, 2018). These conditions accordingly pose challenges for pollen vectors and can reduce the effectiveness of aerial pollen transport (Kato, 1996; Corlett, 2004). Wind pollination is less important in rainforests systems generally and in subcanopy and understorey environments particularly, where the conditions and heavy rain precludes efficient pollination (Whitehead 1969; Linskens 1996; Corlett, 2004; Machado and Lopes, 2004). The high species diversity and typically low conspecific density of canopy species also favours pollen transport by animal vectors compared to wind transported pollen (Corlett, 2004).

Only a few studies have examined community wide differences in pollinator or flower visitor assemblages among vertical strata within tropical rainforest communities (Bawa et al., 1985a; Kress and Beach, 1994; Momose et al., 1998b). Flowers on canopy trees in Costa Rica receive more visits by pollinators such as medium-large sized bees than subcanopy trees that overall, receive a greater diversity of pollinators including hummingbirds, beetles, and sphingid moths (Bawa et al., 1985a; Kress and Beach, 1994). Beetle pollinators are confined to strata below the

canopy, possibly due to the proximity to adult floral rewards (pollen) and oviposition sites (Bawa et al., 1985a). Beetles, however, are prominent flower visitors of both canopy and subcanopy species in the AWT, where beetle species composition is known to vary between strata (Kitching et al., 2007; Stork et al., 2016).

Particular taxa, notably bees, generally have higher species diversity and dominate pollination systems in the canopy compared to the subcanopy or understorey (Bawa et al., 1985a; Ramalho, 2004; Stangler et al., 2016). Proposed reasons for the differences in bee abundance and species diversity among vertical strata include: a greater floral resource availability in the canopy due to the high light energy input; a lower risk of being parasitised in the canopy due to fewer antagonistic species; a higher risk of predation in the more exposed canopy leading species to prefer the lower strata; and nesting resource availability where natural cavities for nesting bees are more abundant in the canopy due to the light availability and drier conditions (Roubik, 1993; Morato and Martins, 2006; Stangler et al., 2016). The subcanopy and associated relative high humidity compared to the canopy encourages fungal growth that could also reduce the availability of appropriate nesting sites in these environments (Kato, 1996; Morato and Martins, 2006). Semi-specialised bees such as Euglossine bees are typically more abundant in the understorey or subcanopy (Janzen et al., 1971; Stangler et al., 2016). A low-light environment affects resource availability for plants and species may be unable to support the energy requirements of the entire pollinator population of bees due to the energetic costs associated with increasing nectar production (Appanah, 1991; Borrell, 2005). Floral morphological traits therefore can serve to exclude inefficient pollinators. For example, plant species with long tubed corollas exclude short-tongued bees as nectar consumers, but can still provide adequate nectar resources to specific, effective pollinators, i.e. Euglossine bee species, that learn to include the species in its foraging route (Kay and Schemske, 2003; Borrell, 2005). In the low energy environment beneath the canopy where light energy resources are limited, many species do not produce any nectar and only offer pollen as a food reward (Bawa et al., 1985a; Appanah, 1991).

Taxa including birds (Chmel et al., 2016) and insects such as Lepidoptera spp. (Schulze et al., 2001), thrips (Sakai, 2001), flies (House, 1989; Kato, 1996; Sakai et al., 1999), and beetles (Irvine and Armstrong, 1991; Sakai et al., 1999; Corlett, 2004), have all been shown to be important pollinators within the rainforest canopy. Flower

visitors or pollinators of rainforest subcanopy or understorey species are relatively understudied compared to canopy species. Two community studies focussing on understorey or subcanopy species have been conducted in lowland dipterocarp forests in Sarawak where pollination syndromes included mammal, bird, social and solitary bees, chrysomelid beetles and generalised insects (moth, wasp, butterfly, fly and beetles) (Kato, 1996; Momose et al., 1998b). Social bees, flies and diverse insects were the most common pollination modes discovered among 22 species of understorey shrubs in a wet evergreen forest in the southern Western Ghats of India (Devy and Davidar, 2006). Flies and diverse insects contributed significantly more to outcrossed pollination than did bees and other solitary pollinators (Devy and Davidar, 2006). Of the few studies on single species of subcanopy or understorey species available, a similarly wide variety of animal taxa have been documented as pollinators or flower visitors (Kato, 1996; Momose et al., 1998b; Zerega et al., 2004; Devy and Davidar, 2006; Aguirre et al., 2011; Berecha et al., 2015; Borchsenius et al., 2016; Sanfiorenzo et al., 2018).

Studies of understorey or subcanopy species in the Neotropics have been dominated by palm species (Listabarth, 1993; Knudsen et al., 1999; Otero-Arnaiz and Oyama, 2001; Tschapka, 2003; Luna et al., 2005; Aguirre et al., 2011; Borchsenius et al., 2016). A variety of beetles, bees, and flies, and other visitors from the Order, Orthoptera were attracted to the flowers of many palm species (Listabarth, 1993; Knudsen et al., 1999; Luna et al., 2005; Aguirre et al., 2011; Borchsenius et al., 2016). To date, there are very few published records on the potential pollinators of subcanopy or understorey species in the tropical region of the AWT. These studies include plant species pollinated by an undescribed species of beetle, *Monolepta* sp. (Webber et al., 2008), small unspecialised insects belonging to the Orders Diptera, Coleoptera and Hymenoptera (2 species, House, 1989) and a wide range of vertebrate and invertebrate species including bats, birds and insects visited the flowers of a final species studied (Crome and Irvine, 1986). The paucity of research on the floral visitors and pollinators of individual subcanopy species makes generalisations between differences among strata difficult.

1.4 POLLEN-MEDIATED GENE FLOW IN TROPICAL RAINFORESTS

Mating patterns, mediated by the movement of pollen are one of the main factors determining the genetic structure of plant populations at different spatial scales (Connell and Slatyer, 1977; Hamrick and Godt, 1996; Burczyk et al., 2004; Matsuki et al., 2008). Gene flow influences the reproductive success and fitness of individuals because it can counteract the potentially detrimental effects of genetic drift and may be a source of new alleles within populations (Burczyk et al., 2004; Dick et al., 2008). Alternatively, when gene flow is restricted, inbreeding or biparental inbreeding (mating with close relatives) can occur and ultimately lead to a loss of genetic diversity, directional selection and genetic drift (Ellstrand and Elam, 1993; Akihiro et al., 2000). Restricted gene flow can occur in species with limited dispersal capabilities or due to habitat fragmentation and degradation that leads to a reduced number of local and immigrant pollen sources in populations (Ellstrand and Elam, 1993; Collevatti et al., 2001; Sork and Smouse, 2006; Castilla et al., 2017).

Gene flow is important in conservation biology, primarily because of its influence on the effective population size of a species (Lande, 1988; Schemske et al., 1994). Accurate estimation of levels of mating distance and number of mating partners is fundamental to understanding whether populations are functionally connected (Adams and Burczyk, 2000). Knowledge of realised gene flow therefore permits the appropriate design of management and conservation strategies that maximise interconnectedness of populations while minimising unwanted gene flow (Adams and Burczyk, 2000; Wang et al., 2007; Bittencourt and Sebbenn, 2008). This will aid management of species in the face of anthropogenic habitat modification and exploitation of forest resources.

1.4.1 Pollen vectors

In tropical rainforest plants, most woody species are strongly outcrossed and rely on insects for pollinations (Bawa, 1992; Ollerton et al., 2011). Therefore, successful reproduction, measured in terms of gene flow, is determined by the composition and abundance of pollinators and their flight behaviour (House, 1992; House, 1993; Stacy et al., 1996; Aguilar et al., 2008).

There is much evidence to suggest that despite the low population densities and animal-mediated pollination of tropical forest trees, long-distance pollen dispersal is common, at least among canopy species. Studies conducted using genetic marker-based analyses of mating systems in canopy species in the Neotropical region have tended to reveal long-distance pollen dispersal for a range of pollination syndromes (e.g. (Chase, 1996; Ward et al., 2005; Hardesty et al., 2006; de Lacerda and A.E.M., 2008; Carneiro et al., 2009; Ottewell et al., 2012). For example, pollen dispersal in undisturbed habitats ranged from a mean of 200 m to over 19 km for species pollinated by small insects or bats (reviewed in, Ward et al., 2005) and trees pollinated by large bees have demonstrated pollen flow distances exceeding 500 m (Hamrick and Murawski, 1990; Loveless et al., 1998; Latouche-Hallé et al., 2004). Long-distance-specific pollinators are thought to be less common in the Paleotropics compared with the Neotropics (Momose et al., 1998b). The general flowering events unique to the Diptereocarp dominated rainforests in southeast Asia likely preclude many pollinating taxa capable of long-distance dispersal where irregular floral resources are inadequate for their survival (Momose et al., 1998b). Long-distance pollinators in this region are largely represented by highly eusocial bees that can store resources as well as large bees such as *Apis dorsata* that are capable of long-distance migrations and have also shown to disperse pollen over 600 m (Koeniger and Koeniger, 1980; Akihiro et al., 2000; Kenta et al., 2004). There are no large social bees such as *Apis dorsata* or *A. cerrana* (that occur in nearby Asia) for long-distance pollen dispersal in the AWT (Gross, 2005). There are limited, if any studies on marker-assisted pollen dispersal distances in species located in Australasian tropical rainforests.

Plants that have highly specialised pollinators are known to exhibit long distance gene flow, even for very small species traditionally thought of as weak flyers. For example, species-specific fig wasp pollinators, 1-2 mm in size, routinely disperse pollen 5.8-14.2 km between low density host trees in Barro Colorado Island, Panama (Nason et al., 1998). This finding is supported by a fig wasp species from the Southeast Asian mainland that can transfer pollen over large areas (Kobmoo et al., 2010). Long distance pollen dispersal has also been demonstrated in species pollinated by thrips that are passively dispersed long distances by wind once they are above the canopy (Fiala et al., 2011; Guicking et al., 2013). Hummingbirds, and Euglossine bees are also important long distance semi-specialised pollinators that occur in the understorey

and subcanopy in the Neotropics (Webb and Bawa, 1983; Bawa et al., 1985a; Kress and Beach, 1994). In contrast, small generalist pollinators that are known to visit unspecialised flowers have low energy requirements and often move shorter distances compared to specialised insects, larger insects, or vertebrates (Appanah, 1991; Dick et al., 2008).

Heterogeneity in pollinator composition over space and time can produce different patterns of gene flow (Dick et al., 2003; Kenta et al., 2004). Habitat disturbance can also cause changes in pollinator assemblages that leads to pollen limitation and fitness of the progeny produced through changes in the composition of pollen loads (Kearns et al., 1998). The impact of fragmentation on genetic diversity may be mitigated by pollinators that can cross gaps and thereby maintain connectivity between habitat fragments (Aguiar and Gaglianone, 2012). For example, this may occur when feral honeybees with long distance dispersal capabilities replace native pollinators in disturbed habitats (Dick, 2001; Dick et al., 2003). How pollen dispersal shapes local genetic structure is not well studied in animal pollinated plants in tropical rainforests (Ottewell et al., 2012), particularly outside Neotropical rainforest communities or in the strata beneath the canopy. To make more precise links between pollination vector and gene flow distances and population structure for tropical trees, studies should incorporate pollinator observations of their study populations to be able to test for associations between pollen vector and genetic diversity within and between subpopulations (Dick et al., 2008).

1.4.2 Plant species spatial distribution

Pollen-mediated gene flow, measured in terms of successful reproduction, is affected by density measures including population density, spatial distribution, species phenology and its synchronicity, as well as the size of the nearest pollen source (House, 1993; Stacy et al., 1996; Ghazoul, 2005; O'Connell et al., 2018). Gene flow is also influenced by the sex ratio in dioecious species (House, 1992; Mack, 1997; Silva et al., 2008; Gaino et al., 2010). Variation in these density factors influence pollinator foraging behaviour, pollination distances, pollen dispersal patterns and levels of genetic isolation (Castilla et al., 2017).

The long dispersal distances reported for many canopy species are partly because these taxa generally occur at low population densities (Akihiro et al., 2000; Kenta et al., 2004; Ward et al., 2005; Hardesty et al., 2006; Born et al., 2008; Monthe et al., 2017). Pollen dispersal patterns of many insect-pollinated tropical trees are influenced by preferential visitations to the closest neighbouring trees (Silva et al., 2010; Theim et al., 2014; Noreen et al., 2016). If only distant trees are flowering, pollinators must travel long distances to locate resources. Commonly, pollinator flight distances tend to increase with lower plant densities and decrease when flowering plants exhibit a clumped distribution or occur at high densities (House, 1992; Stacy et al., 1996; Hardy et al., 2006; Born et al., 2008; Naoki et al., 2009; Ashley, 2010; Silva et al., 2010; Duminil et al., 2016). This is because near-neighbour mating increases the foraging economy of the pollinating insect by maximizing the net energy gained by the pollen or nectar source (Levin and Kerster, 1974; Degen and Sebbenn 2016). There are relatively few studies describing gene flow within subcanopy or understorey tropical rainforest species compared to canopy species (see, Stacy et al., 1996; Lasso et al., 2011; Castilla et al., 2016; Hahn et al., 2017). Nevertheless, a high proportion of short distance pollination events have been found in this environment, particularly in species with high local density, aggregated populations (Stacy et al., 1996; Lasso et al., 2011; Castilla et al., 2016).

Seed set has been negatively correlated with distance to nearest pollen donor in self-incompatible or dioecious species, especially if pollinators cannot effectively move large distances (House, 1993; Mack, 1997). Moreover, for self-compatible species, self-fertilisation rates are known to vary inversely with population density in undisturbed habitats in the Neotropics (Ward et al., 2005; Naoki et al., 2009). If pollination services are low due to long distances between male and female individuals, cascading effects means the resulting fruit crop might attract few frugivores and depress reproductive success further (Mack, 1997). Species spatial distribution can also influence the diversity of pollen received on an individual. The theory of density-dependent animal pollination follows that tree species occurring at low population densities receive pollen from fewer individuals than trees in denser populations that receive pollen from a greater pool of pollen donors. However, dispersal distances tend to be lower in higher density populations (Murawski and Hamrick, 1991; Bianchi and Cunningham, 2012; Castilla et al., 2017).

The attractiveness of floral displays of individual trees is an important factor in determining pollinator-assisted gene flow (Barrett and Harder, 1996; Degen and Roubik, 2004; Duminil et al., 2016). Pollen movement by generalist insect fauna between trees appears to be a function of the response to the density of flowers (House, 1992). The size of nearest pollen source becomes important because a large but more distant male may contribute more pollen than a smaller, closer one. Thus, large male trees, with large floral displays can contribute disproportionately to seed production (Latouche-Hallé et al., 2004; Naoki et al., 2012; Tambarussi et al., 2015; Monthe et al., 2017; Younginger et al., 2017). Determining variations in male fecundity within populations is important because mating partners can influence genetic diversity and population fitness (Breed et al., 2012a). Unfit combinations of pollen and ovules are more likely to occur in offspring when fewer males contribute to reproduction. With more mating partners, there is a smaller probability that recessive deleterious alleles will be involved in reproduction (Breed et al., 2012b). It follows that species with small or fragmented populations, with altered population densities, may experience a loss of fecundity because of the genetic consequences of inbreeding (Young et al., 1996; Lowe et al., 2005).

1.5 RESEARCH GAPS AND SIGNIFICANCE

An understanding of a species' floral traits and phenology are necessary to elucidate potential pollinators and understand pollinator behaviour (Barrett and Harder, 1996; Rosas-Guerrero et al., 2014). Failure to attract effective pollinators can result in pollen limitation and reduced seed production (Larson and Barrett, 2000; Ashman et al., 2004) which, in some cases, can reduce whole-plant reproductive output (Trueman, 2013). Dioecious and self-incompatible species generally exhibit lower fecundity than related self-compatible species (Larson and Barrett, 2000; Knight et al., 2005; Aguilar et al., 2006; Davila et al., 2012). Nevertheless, pollination, fruit-set and seed production have not been studied empirically for many dioecious rainforest trees.

Pollination services are a critical ecosystem service with great ecological and economic importance (Winfree, 2010). The significance of animal mediated pollination increases dramatically in tropical rainforest systems where an estimated 94 % of plant species rely on pollination by animals for successful reproduction

(Ollerton et al., 2011). Despite this, most tropical pollination systems remain poorly studied (Winfree, 2010; Winfree et al., 2011; Garibaldi et al., 2013). Our understanding of the pollination system of species in the AWT is generally sparse (Boulter et al., 2009) and relatively few studies globally have focused on plant-animal interactions in subcanopy rainforest species, an environment that is markedly different to the rainforest canopy where much of the literature is focused. It is thus imperative to include subcanopy species in research to gain a more complete understanding of pollination in tropical rainforest systems. Moreover, knowledge of pollinator behaviour becomes economically important when plants reliant on pollination for reproduction are introduced into cultivation. Plantation designs and conditions may not favour the behaviour or life cycle of the pollinator, which can negatively affect pollination and fruit set (Irvine and Armstrong, 1991; Blanche and Cunningham, 2005). Therefore, it is critical to establish whether pollination is achieved via generalist or specialist animal vectors or through abiotic means.

Pollen dispersal and its impact on local genetic structure is not well studied in animal pollinated plants in tropical rainforests (Ottewell et al., 2012), particularly outside Neotropical rainforest communities. Most studies have examined gene flow and population genetic structure in rainforest canopy species and gene flow in tropical subcanopy species remains an area that is poorly addressed in the literature. To make more precise links between pollination vector and gene flow distances and population structure for tropical trees, studies should incorporate pollinator observations of their study populations to be able to test for associations between pollen vector and genetic diversity within and between subpopulations (Dick et al., 2008).

This thesis contributes to an understanding of the floral, reproductive and pollination biology and genetics of *Fontainea picrosperma*, a dioecious, subcanopy tropical species that is of considerable commercial interest because its fruit contains the small molecule natural product, tigilanol tiglate (Boyle et al., 2014; Linkliter et al., 2015; Panizza et al., 2019). This research will extend established literature on the genus *Fontainea* by showing results that are novel for the species *F. picrosperma*.

1.6 RESEARCH SCOPE

1.6.1 Background

Fontainea picrosperma (Euphorbiaceae) is a model species to investigate plant reproduction in a dioecious, subcanopy, tropical rainforest species. This species is of considerable commercial interest following the discovery of a novel epoxytigliane with anti-cancer activity from its fruit. The small molecule, natural product, tigilanol tiglate (Figure 1.1) is an effective treatment for cancer having been approved for use as a therapy for canine mast cell tumours by the European Medicines Agency (Miller et al., 2019; QBiotics, 2020) and being developed for the treatment of human head and neck squamous cell carcinoma (Boyle et al., 2014; Panizza et al., 2019). Tigilanol tiglate cannot be synthesised on a commercial scale and so it is manufactured by extraction and purification from the seed of *F. picrosperma*. A reliable and economical supply of the Active Pharmaceutical Ingredient is a key element in the development and commercialisation of a therapeutic agent. As such, domestication of *F. picrosperma* to supply raw materials for the manufacture of Tigilanol tiglate is crucial. Consequently, an understanding of the floral, reproductive and pollination biology of *F. picrosperma* is essential for sustainable seed production and commercial production of tigilanol tiglate. *Fontainea picrosperma* is dioecious but little else is known of the ecology of this tropical rainforest plant.

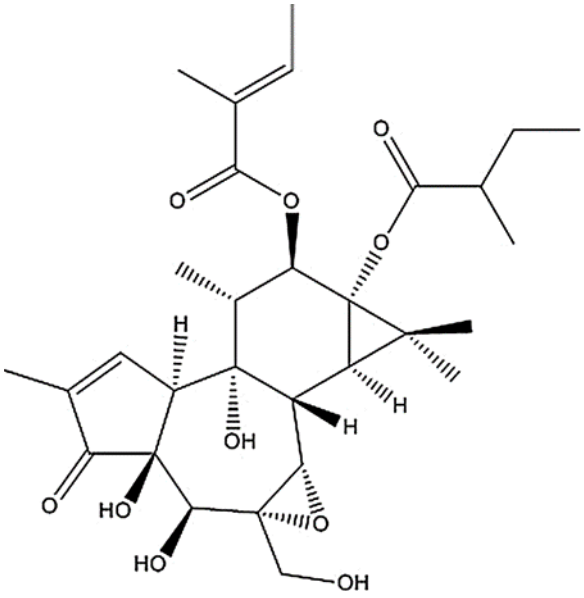

Figure 1.1 Chemical structure of the putative cancer therapeutic, tigilanol tiglate, isolated from *Fontainea picrosperma.*

1.6.2 Study species and study site

The distribution of the genus *Fontainea* (Euphorbiaceae) extends from northern New South Wales, through Queensland in Australia and into the South Pacific (New Guinea, New Caledonia and Vanuatu) and consists of nine species (Jessup and Guymer, 1985; Forster, 1997). Six species occur in Australia, four of which are considered Vulnerable (*F. australis*; *F. rostrata*; *F. venosa*) or Critically Endangered (*F. oraria*) under the EPBC Act 1999. A fifth species (*F. fugax*) is considered Vulnerable under the Queensland Nature Conservation Act 1992. *Fontainea picrosperma* is the only species in the genus that is not classified as a threatened species in Australia.

Fontainea picrosperma C.T. White (1933) is a small, dioecious, subcanopy tree endemic to upland tropical rainforests on the Atherton Tablelands, in northern Queensland, Australia. The natural population is likely to comprise of several thousand individuals covering a geographically restricted natural range of approximately 1000 km^2 (Agostini et al., 2013). Euphorbiaceae is a family that exemplifies the post-Cretaceous diversification of the Australian rainforest flora and *Fontainea* first appears in the fossil record in the early Tertiary period (Williams and Adam, 2010). During the climatic oscillations of the Pleistocene over the last 230,000 years, expansion and contraction of *F. picrosperma* populations led to the species retreating to moist refugia when climate conditions cooled and recolonized surrounding areas once climate conditions improved (Kershaw et al., 2007). Thus, the region that *F. picrosperma* inhabits has been subject to natural habitat fragmentation as a result of these climatic fluctuations. *Fontainea picrosperma's* current discontinuous distribution is due to anthropogenic habitat fragmentation primarily as a result of agricultural expansion, but also due to urban settlements (Lamont et al., 2016).

The species is found in both complex mesophyll and notophyll vine forests growing on basalt soils at restricted altitudes between 700 and 1200 m (Cooper, 2004). These rainforests are part of the AWT that form part of the Australasian rainforest realm. The AWT is a mega-diverse region covering less than 0.2 % of the continents land surface (Stork et al., 2008). The total Australian rainforest area is small in global terms, but the forests are unique due to the range of climates, and the considerable biodiversity and endemism that exists within plant families (Stork et al., 2008).

Moreover, the forests are notable for their distinctive Gondwanan taxa (Webb and Tracey, 1994) especially in the uplands (Boulter et al., 2009). The large number of identifiable species that represent angiosperm families with primitive origins include 16 of the 28 near-basal (or 'primitive') lineages of flowering plants (Metcalfe and Ford, 2009). Conserving this evolutionary history and the ecosystems in which these species survive should be given particular attention, especially as climate change is occurring at an unprecedented rate, and within a more challenging, fragmented landscape, rainforest taxa have less potential for adaptive change in the face of environmental change and may not be able to retreat to climate refugia and persist as they have done so in the past (Metcalfe and Ford, 2009).

1.6.3 Research aims

This thesis aims to improve our understanding of the ecology (modes of reproduction and gene flow) of *Fontainea picrosperma.* Subcanopy tropical rainforest species are poorly represented in the literature and this research will highlight the challenges and conservation strategies for dioecious rainforest species more generally. This research is also important for securing the commercial manufacture of tigilanol tiglate by enhancing *F. picrosperma* fruit supply; the raw material for tigilanol tiglate production.

This research aims to:

1. Increase knowledge of the flowering and reproductive biology of *F. picrosperma* (**Chapter 2**)
2. Identify the floral attractants and the potential pollinators of *F. picrosperma* (**Chapter 3**)
3. Elucidate the contemporary and historical patterns of gene flow due to pollination between individual trees of *F. picrosperma* (**Chapter 4**)

Specifically, I examine (1) the floral and pollen morphology, (2) the flowering phenology and (3) the breeding system of *F. picrosperma* and determine if the species is pollen limited (Chapter 2). To determine the species pollination biology, I investigate if *F. picrosperma* (1) is wind pollinated, (2) is pollinated by diurnal and/or nocturnal pollinators, (3) produces floral nectar and (4) produces floral scent

compounds. I further investigate (5) what animals visit the flowers or inhabit the inflorescences and (6) what types of insects are attracted to the main scent compounds present in *F. picrosperma*'s floral bouquet (Chapter 3). To determine contemporary patterns of gene flow I specifically determine (1) what proportion of seeds are sired by the local male population, (2) the number of males that contribute to progeny to mother trees and (3) how successful reproduction relates to specific paternal tree characteristics including flowering effort and location (direction and distance) relative to the mother tree (Chapter 4). I further examine historical patterns of gene flow by investigating (1) the levels of genetic diversity in adult and juvenile trees in the population and differences in genetic variation between generations and, (2) whether individuals growing near to each other are more related than expected from mating between random individuals (Chapter 4).

1.7 THESIS OUTLINE

Chapter 2: Floral and reproductive biology of the medicinally significant rainforest tree, *Fontainea picrosperma* (Euphorbiaceae)

This chapter describes the floral characteristics and reproductive biology of the medicinally significant Australian rainforest plant species, *Fontainea picrosperma*. This research provides the first description of the floral and reproductive biology and pollen limitation in this dioecious species.

Chapter 3: Flora attraction and flower visitors of *Fontainea picrosperma* (Euphorbiaceae)

This chapter aims to identify the main floral attractants in a subcanopy rainforest species, *Fontainea picrosperma* and what is attracted to the flowers in a subcanopy environment. No other studies on pollination of species in the *Fontainea* genus have been examined.

Chapter 4: Short distance pollen dispersal and low genetic diversity in a subcanopy tropical rainforest tree, *Fontainea picrosperma* (Euphorbiaceae)

This chapter describes pollen dispersal patterns and genetic diversity of the subcanopy rainforest species, *Fontainea picrosperma*. I identify localised patterns of gene flow and estimate the population genetic structure between subpopulations of adults and juveniles in natural populations of *F. picrosperma* using microsatellite

markers. This research provides the first direct analysis of pollen flow in *F. picrosperma*.

Chapter 5: Synopsis

This chapter summarises the findings of the studies included in this thesis and provides recommendations concluded from these findings and suggestions for further research.

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Chapter 2: Floral and Reproductive Biology of the Medicinally Significant Rainforest Tree, *Fontainea picrosperma* (Euphorbiaceae)

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2.1 ABSTRACT

Fontainea picrosperma (Euphorbiaceae) is a dioecious rainforest tree from northern Australia that is of commercial interest following the recent discovery of the putative anti-cancer agent, tigilanol tiglate, in its seed. Production of tigilanol tiglate will rely on purification from harvested fruit and therefore an understanding of the reproductive characteristics that determine fruit set of this species is critical. Most rainforest plant species rely exclusively on animal vectors to transport pollen between plants for successful reproduction. Flower traits and phenology can facilitate sexual reproduction by attracting pollinators whereas failure to attract pollinators can result in low fruit set due to pollen limitation. Here, we describe the floral morphology, flowering phenology and reproductive biology of *F. picrosperma*. This species bears small, white, actinomorphic flowers with a shallow receptacle. These floral traits are often associated with generalist insect pollination and are common to other dioecious tropical rainforest flowers. Individual female flowers persisted on the tree for several days longer than individual male flowers. Male panicles contained significantly more flowers than female inflorescences, and male flowers opened sequentially on a panicle whereas female flowers opened almost simultaneously within an inflorescence. *F. picrosperma* was pollen limited, as hand pollinated female flowers produced almost double the final fruit set (39.6 ± 4.4 %) of open pollinated flowers (21.3 ± 3.4 %). Optimised production of tigilanol tiglate may therefore rely on improving pollen flow from male to female trees.

Keywords: Cancer, EBC-46, Dioecy, Pollination, Pollen limitation, Tigilanol tiglate

2.2 INTRODUCTION

Tigilanol tiglate (EBC-46) is a novel compound from the tropical rainforest tree, *Fontainea picrosperma* (Euphorbiaceae) that is being developed as a cancer therapeutic for veterinary and human markets (Boyle et al., 2014; Linkliter et al., 2015). Tigilanol tiglate (Figure 1.1) cannot be synthesised easily and so it is manufactured for research and development solely by extraction and purification from the seed of *F. picrosperma*. A reliable and economical supply of the Active Pharmaceutical Ingredient is a key element in the development of a therapeutic agent. Consequently, an understanding of the floral and reproductive biology of *F. picrosperma* is essential for sustainable seed production and commercial production of tigilanol tiglate. *Fontainea picrosperma* is dioecious (i.e. male and female flowers are located on separate trees) but little else is known of the floral and reproductive biology of this tropical rainforest plant.

Most tropical rainforest plants are facultative or obligate outcrossers that rely almost exclusively on animal pollinators for seed production (Ollerton et al., 2011). Dioecious species in particular are not able to produce seed by self-pollination and those that rely on animal pollination must invest resources to attract pollinators (Williams and Adam, 2010). Plant traits likely to be involved in pollinator attraction include flower colour, shape, size, scent and food reward (Barrett and Harder, 1996; Boulter et al., 2006). Dioecious species in tropical rainforests globally display similar flowering strategies to attract animal pollinators (Bawa and Opler, 1975; Renner and Feil, 1993; Adam and Williams, 2001; Queenborough et al., 2007; Gao et al., 2012; Field et al., 2013a, b). These include producing small, actinomorphic, pale-coloured flowers that attract generalist insect pollinators (Machado and Lopes, 2004; Boulter et al., 2006). Male and female flowers have become specialized in dioecious species and generally have different inflorescence structures and flowering patterns within a species (Ainsworth, 2000). Male trees generally flower earlier and for a longer period and produce twice as many flowers as female trees (Gao et al., 2012). An understanding of a species' floral traits and phenology are necessary to elucidate potential pollinators and understand pollinator behaviour (Barrett and Harder, 1996; Rosas-Guerrero et al., 2014). Failure to attract effective pollinators can result in pollen limitation and reduced seed production (Larson and Barrett, 2000; Ashman et al., 2004) which, in some cases, can reduce whole-plant reproductive output (Trueman,

2013). Dioecious and self-incompatible species generally exhibit lower fecundity than related self-compatible species (Larson and Barrett, 2000; Knight et al., 2005; Aguilar et al., 2006; Davila et al., 2012). Nevertheless, pollination, fruit set and seed production have not been studied empirically for many dioecious rainforest trees.

In this study, we assessed the floral and reproductive biology of *Fontainea picrosperma*, which is endemic to the wet tropical rainforests of north Queensland, Australia (Agostini et al., 2013; Lamont et al., 2016). We provide the first description of flowering phenology and pollen limitation in this dioecious species.

2.3 MATERIALS AND METHODS

2.3.1 Species and study sites

Fontainea picrosperma is a small dioecious subcanopy tree found in complex mesophyll and notophyll vine forests (Tracey, 1982) on the Atherton Tablelands of north Queensland, Australia. The species grows on basalt soils between 700 m and 1200 m altitude (Cooper, 2004). Flowering occurs from September to November, occurring later at the higher altitudes. The red drupaceous fruit (up to 3 cm diameter) ripen in December and January and are eaten by cassowaries, musky rat kangaroos and giant white-tailed rats (Cooper, 2004).

Data in this study was collected from trees in the natural population (Boonjie, Evelyn Highlands and Malanda; see Lamont et al., 2016), potted nursery trees (grown in 20 L pots under 50 % shade in Yungaburra, Queensland) derived from Malanda, and trees in a commercial plantation (grown under 50 % shade near Yungaburra, Queensland) also derived from Malanda. Natural stands of *F. picrosperma* often form small but relatively dense (2–10 m inter-tree spacing) and clumped populations with ~50:50 male:female ratios. The potted nursery trees were between 1 m and 2 m in height and placed 1 m apart with a random ~50:50 distribution of male and female trees. Plantation trees were planted with 1.5 m intra-row and 2.5 m inter-row spacing with a random ~50:50 distribution of male and female trees. Investigations were performed during four reproductive seasons in 2011/2012, 2012/ 2013, 2013/2014 and 2014/2015. We examined floral morphology (inflorescence structure, flower structure,

anther morphology and pistil morphology) and flowering phenology (male flower longevity, stigma receptivity, and patterns of flower opening on male and female inflorescences). We also performed controlled pollinations to determine final fruit set and assess whether fruit set was pollen limited. Pollinations were performed using male parents from the same natural population.

2.3.2 Floral morphology

We examined inflorescence structure in the natural population in 2011. Flowers were counted on a total of 24 male inflorescences across four male trees and 46 female inflorescences across eight female trees. Flowers were counted on 2–8 inflorescences per tree depending on availability. Flower diameter was measured in the commercial plantation in 2014 on 30 flowers from each of three male and three female trees (180 flowers in total). Diameter was measured at the widest part of the corolla of open flowers.

Pollen morphology, anther dehiscence, and stigma morphology were examined by scanning electron microscopy. Open male and female flowers were collected and fixed in 3 % glutaraldehyde at room temperature and then stored overnight. The samples were then dehydrated in an aqueous ethanol series of increasing concentration (10, 30, 50, 70, 80, 90 and 100 % ethanol), critical point dried (Quorum K850, Quorum Technologies, East Sussex, UK), sputter coated with gold (Quorum Q150T S, Quorum Technologies, East Sussex, UK), and examined using a JSM-6010LA scanning electron microscope (JEOL, Tokyo, Japan).

2.3.3 Flowering phenology

Individual male flower longevity was observed in the natural population in 2011 on eighteen inflorescences across six male trees (78 flowers in total). Flowers were observed daily to determine the number of days that each individual flower remained open. The number of days from anthesis to abscission was calculated for each flower.

Female flowers were monitored for timing of stigma receptivity in the potted trees in 2012 and in the natural population in 2014. Inflorescences were enclosed in fine mesh bags (0.5 mm × 1.0 mm pore size) to exclude pollen. Individual unopened

buds were tagged and monitored daily for anthesis. To test stigma receptivity by peroxide activity, two to four flowers were collected at each age (-1, 0, 1, 3, 5, 7 and 11 d post anthesis) from each of five trees in the natural population, where 0 d post-anthesis represented flowers that had opened within the past 12 h. Stigma receptivity was determined by testing for the presence of peroxide on the stigma immediately after the flower was collected from the tree. Peroxide activity was examined using a Peroxtesmo Ko paper indicator test (Macherey-Nagel, Dueren, Germany) as previously described (Kearns and Inouye 1993), with one drop of deionised water placed on the test paper to increase the effectiveness of the test (Dafni and Maués 1998). To test receptivity by observing fruit set, between seven and 12 panicles were hand pollinated at each age (0, 1-2, 3-4, 6- 8, and 9-11 d post anthesis) across six to nine replicate potted trees, with 0 d post-anthesis representing flowers that had opened within the past 12 hours. One panicle on each of five trees was included as a bagged control. Fruit set was observed after 7-9 weeks, when the fruit were ~2 cm in diameter.

We assessed the patterns of flower opening on male and female inflorescences in the natural population and on potted nursery trees in 2011 and 2012. Nine inflorescences across three male trees and two inflorescences from one female tree were observed in the natural population in 2011. In addition, one inflorescence from each of 20 male trees and 20 female trees was observed on potted nursery plants in 2012. The number of open flowers on each inflorescence was counted at the same time each day until all flowers on the inflorescence had opened and the petals had browned and shrivelled.

2.3.4 Fruit set and pollen limitation

Controlled pollination experiments were conducted over two reproductive seasons in the natural population using 11 female trees in 2012/2013 and ten female trees in 2013/2014. Three to nine inflorescences per tree were selected and assigned randomly to one of three treatments: ‘bagged’ to exclude flower visitors (n = 126 flowers); ‘open’ to flower visitors (n = 143 flowers); or ‘hand-pollinated’ (n = 127 flowers). Any flowers that had already opened were removed from the inflorescence and the remaining unopened flowers were counted. Inflorescences assigned to the ‘bagged’ treatment were enclosed in fine mesh bags (0.5 mm × 1.0 mm) to exclude pollen. The bags were fastened to the stem with a wooden peg and left in place until

all flowers had withered. Inflorescences left 'open' were not enclosed in mesh bags, allowing flower visitors access to flowers. Inflorescences assigned to the 'hand pollinated' treatment were also enclosed in fine mesh bags. Their flowers were hand pollinated within 12 h of anthesis (i.e. 0 d post-anthesis) by brushing the stigma with anthers that had been dried over silica gel for 12 h to assist with pollen release. A hand lens was used to confirm that pollen had been deposited on the stigma. Hand pollination continued until all petals of all flowers in the treatment had browned and shrivelled. Initial fruit set was determined by counting the number of flowers with swelling of the ovary; i.e. at 5 mm diameter (~2 weeks). Final fruit set was counted when fruit were considered ripe; i.e. when they reached 20 mm diameter (~10 weeks). Fruit set was calculated as the percentage of the total number of flowers per inflorescence that formed fruit.

We also assessed the success of pollination treatments by observing pollen grains on the stigma and counting pollen tubes in the style. Three replicate inflorescences per treatment (bagged; open; and hand pollinated) on each of three female trees were selected in either the natural population, commercial plantation or potted trees in each of 2012 and 2013. The flowers in the bagged and hand pollinated treatment groups were either bagged or hand pollinated, as described above. The flowers of all treatments (23 open pollinated, 26 hand pollinated, and 9 bagged flowers) were removed 3 d post-anthesis and placed in fixative (25 % glacial acetic acid, 75 % ethanol; v,v). Flowers were examined for the presence of pollen grains and pollen tubes using a fluorescence microscope (Leica DM5500B, Leica Microsystems, Wetzlar, Germany) and a confocal microscope (Nikon Eclipse Ti/Nikon C2S, Nikon Inc, New York, USA) after staining with decolourised aniline blue (Shepherd, 2000).

2.3.5 Statistical analysis

Data comparing the number of flowers on male and female inflorescences were analysed using a two-way ANOVA with tree and sex as fixed factors. Data for flower diameter were log transformed to meet assumptions for normality and analysed using a nested ANOVA with sex and tree (nested within sex) as factors. Tree differences in male flower longevity were tested using a one-way ANOVA with tree as a fixed factor. Data for inflorescence opening duration were initially analysed by two-way ANOVA with site and sex as factors. However, due to significant site × sex interactions, data

were then analysed with a one-way ANOVA with sex × site combined as a single factor (i.e. male inflorescences in natural populations; female inflorescences in natural populations; male inflorescences on potted trees; and female inflorescences on potted trees). Stigma receptivity data were analysed by a one-way ANOVA. Controlled pollination and fruit set data were analysed with a two-way ANOVA (treatment × year). Almost no bagged-treatment flowers produced initial fruit (5 out of 126) or final fruit (2 out of 126) and so the bagged treatment was excluded from the analysis. No significant year effects or interactions between treatment and year were detected in the analyses of initial and final fruit set. Tukey's HSD tests were used to assess differences between the means when significant differences among the means were detected by ANOVA.

2.4 RESULTS

2.4.1 Floral morphology

Fontainea picrosperma bears mostly terminal inflorescences (Jessup and Guymer, 1985). The inflorescences that bore male flowers were paniculate whereas female flowers were arranged in a flat umbel pattern (Figure 2.1A and 2.1B). Male inflorescences contained many more flowers (25.8 ± 1.5) than female inflorescences (4.9 ± 0.2) (***$P < 0.001$). There were no significant differences in flower number per inflorescence between trees within a sex ($P = 0.366$). *Fontainea picrosperma* had actinomorphic planar flowers with a shallow receptacle and white coloured petals. Male flowers had five petals with well exposed anthers and an orange staminal disk (Figure 2.1C). Female flowers had five petals and the pistil had five deeply bi-lobed stigmas (Figure 2.1D). Stigmas were greenish-yellow upon opening but turned yellow and then brown as the flower matured and senesced or was successfully pollinated. The syncarpous gynoecium contained five locules but generally produced a single-seeded fruit following successful pollination. Flower size was significantly different between male and female trees (***$P < 0.001$) and between trees within each sex (***$P < 0.001$) (Table 2.1).

Anthers began to dehisce 1 to 2 d after anthesis. Dehiscence occurred via an external stomium; i.e. extrorse dehiscence (Figure 2.2A). Pollen grains were

spheroidal, relatively small with diameter of approximately 40 μm and released as a monad. The pollen had an exine ornamentation consisting of clavate elements with triangular sculpturing arranged in a croton-type pattern (Figure 2.2B) (after the classification of Punt et al., 2007). This surface pattern is a unique synapomorphy within the subfamily Crotonoideae to which *F. picrosperma* belongs. The stigmatic surface was wet with an exudate, and pollen grains were observed germinating on the wet stigma (Figure 2.2B).

Figure 2.1 Flower arrangement on inflorescences and flower morphology of *Fontainea picrosperma*. (A) Male inflorescence. (B) Female inflorescence. (C) Male flowers. The open flowers on the left and right have undehisced and dehisced anthers, respectively. (D) Female flower showing the stigma with five double lobes.

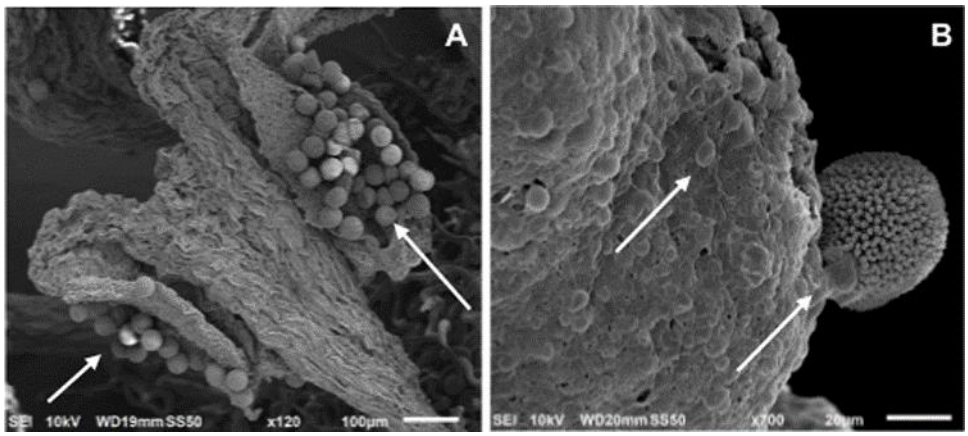

Figure 2.2 Scanning electron micrographs of *Fontainea picrosperma* flower parts. (A) Anther. Arrows point to anther dehiscence via longitudinal slits, showing the release of numerous spherical pollen grains. (B) Stigma. Arrows point to stigma exudate and a germinating pollen grain. Note the exine ornamentation consisting of clavate elements with triangular sculpturing arranged in a croton-type pattern, unique to the Euphorbiaceae subfamily Crotonoideae.

Table 2.1 Diameter of male and female *Fontainea picrosperma* flowers.

Sex	Tree number	Mean ± s.e. diameter (mm)
Male[a]	1	19.8 ± 0.20[a]
	2	19.6 ± 0.19[a]
	3	14.3 ± 0.21[b]
Female[b]	1	18.0 ± 0.18[a]
	2	21.4 ± 0.19[b]
	3	20.2 ± 0.15[c]

Significant differences (ANOVA and Tukey's test, $p < 0.05$, n=30) are represented by different letters.

2.4.2 Flowering phenology

Individual male flowers opened for 1 to 2 d (1.6 ± 0.1 d) and no significant within-tree variation in male flower longevity was observed. All stigmas gave a positive result for peroxidase activity, indicating that stigmas were receptive at least 1 d before anthesis until at least 11 d after anthesis (Table 2.2). However, only 3.7 ± 3.7 % of flowers hand pollinated at 0 d post-anthesis and none of the flowers pollinated

at 9-11 d post-anthesis set fruit, compared with 76.1 ± 7.6 %, 84.9 ± 6.9 % and 54.8 ± 8.7 % of flowers pollinated at 1-2, 3-4 and 6-8 d post-anthesis, respectively (Table 2.2). No set fruit was observed from the bagged control flowers in this experiment.

Table 2.2 Timing of receptivity of female *Fontainea picrosperma* flowers as determined by stigma peroxide activity and fruit set after hand pollination.

Technique	Time post-anthesis (d)					
	Flower harvest date					
	-1	0	1	3	5	11
Stigma peroxide activity	+	+	+	+	+	+
	Hand pollination date					
	0	1-2	3-4	6-8	9-11	
Mean ± s.e. fruit set (%)	3.7 ± 3.7	76.1 ± 7.6^{ab}	84.9 ± 6.9^{a}	54.8 ± 8.7^{b}	0 ± 0	

'+' indicates a positive result for peroxide activity. Significant differences among three means (ANOVA and Tukey's test, $p < 0.05$, $n = 7$ trees) are represented by different letters.

There were no significant differences in inflorescence opening duration in the natural population; however, male inflorescences on potted plants had flowers open for significantly longer than in natural populations (***$P < 0.001$) and the number of days to peak flowering was also longer (***$P < 0.001$) (Table 2.3; Figure 2.3). Male inflorescences in the natural population were open for a maximum of 14 d and the mean peak of flower opening was at 6.1 ± 0.9 d, while female inflorescences were open for 11 d and the mean peak of flower opening was at 6.0 ± 0.0 d (Figure 2.3A). Male inflorescences in the potted plants were open for a maximum of 49 d and the mean peak of flower opening was at 14.9 ± 0.7 d, while female inflorescences were open for 12 d and the mean peak of flower opening was at 3.0 ± 0.4 d (Figure 2.3B).

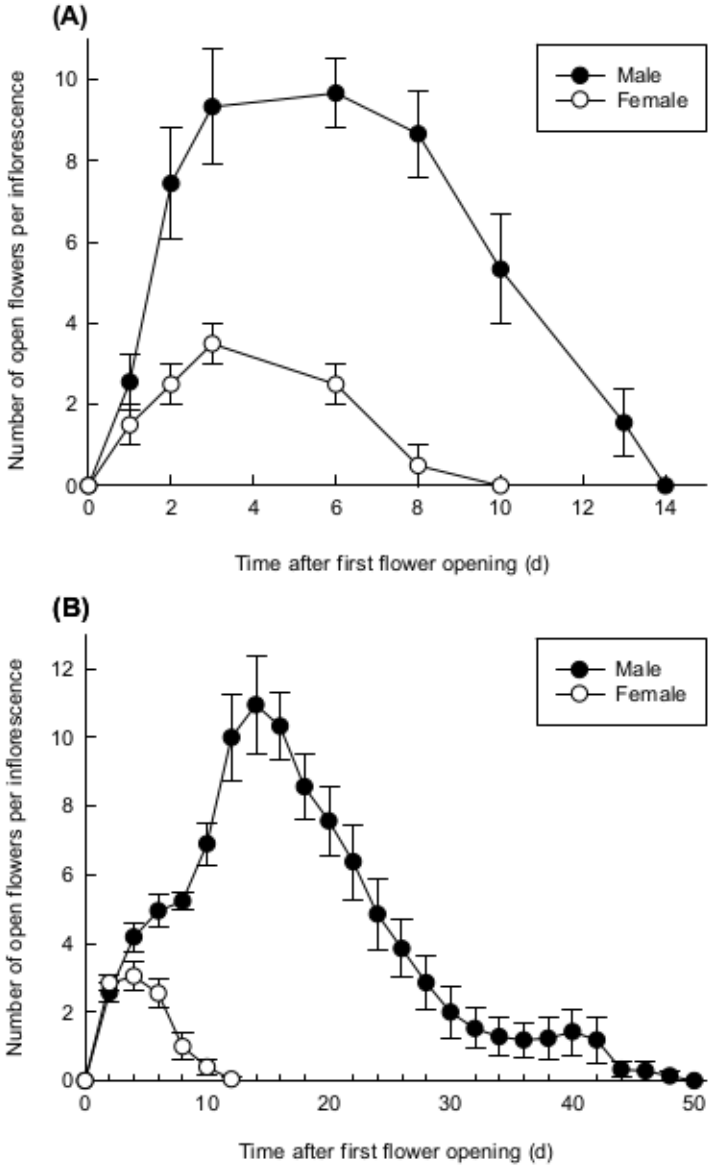

Figure 2.3 Patterns of flower opening within male and female *Fontainea picrosperma* inflorescences. Mean (± s.e.) number of open flowers on individual inflorescences over the lifespan of the inflorescence in (A) the natural population (female inflorescences: n = 2; male inflorescences: n = 9) and (B) potted nursery plants (female inflorescences: n = 20; male inflorescences: n = 20).

Table 2.3 Duration that individual male and female *Fontainea picrosperma* inflorescences possessed open flowers in the natural population and on potted plants.

Site location and sex	Mean ± s.e. duration (d)
Males from natural population	12.5 ± 0.6^{a} ($n = 9$)
Females from natural population	10.0 ± 1.0^{a} ($n = 2$)
Male potted plants	30.9 ± 1.9^{b} ($n = 20$)
Female potted plants	7.3 ± 0.6^{a} ($n = 20$)

Significant differences (ANOVA and Tukey's test, $p < 0.05$) are represented by different letters

2.4.3 Fruit set and pollen limitation

Hand pollination resulted in higher initial fruit set (82.6 ± 3.9 %) than did open pollination (45.6 ± 4.7 %) (***P < 0.001, Figure 2.4A). Initial fruit set from bagged, unpollinated flowers was very low (3.8 ± 1.5 %) (Figure 2.4A). This may be the result of rare insect movement through the mesh by particularly small insects such as thrips, although a very low level of apomictic reproduction in this species cannot be ruled out. Hand pollination also resulted in higher final fruit set (39.6 ± 4.4 %) than did open pollination (21.3 ± 3.4 %) (***P < 0.001, Figure 2.4B). Final fruit set from bagged, unpollinated flowers was negligible (1.7 ± 1.2 %) (Figure 2.4B).

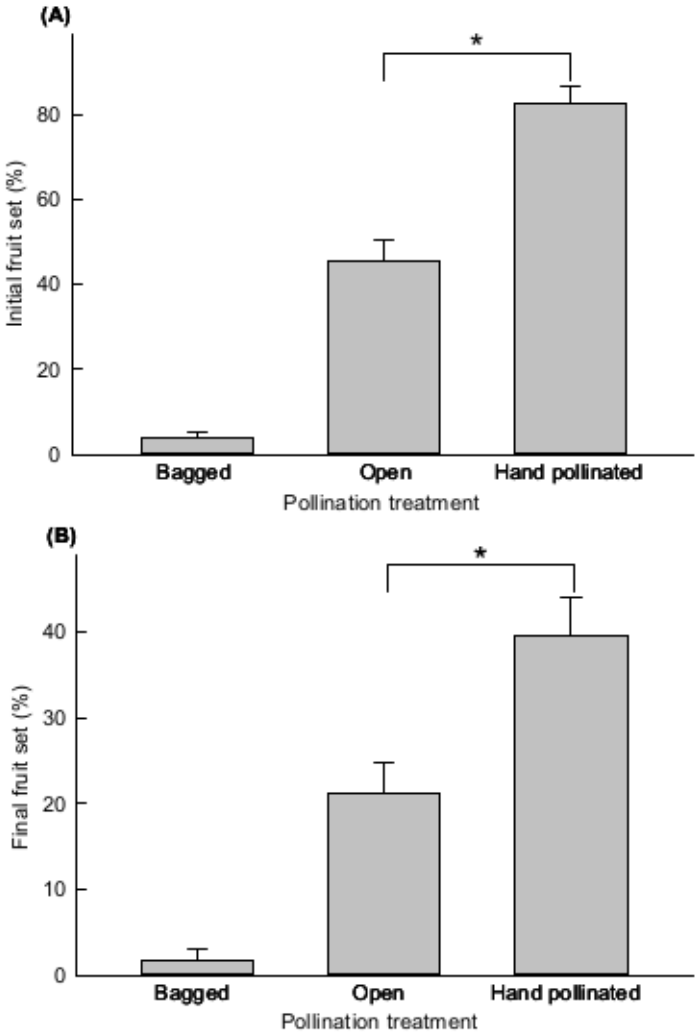

Figure 2.4 Fruit set of *Fontainea picrosperma* inflorescences. (A) Initial fruit set and (B) final fruit set (mean ± s.e.) following three different pollination treatments: Bagged flowers were isolated from insect visitors; Open flowers were left open to insect visitors; Hand pollinated flowers were bagged but manually pollinated. * Indicates differences between open and hand pollinated inflorescences were significant (two-way ANOVA, ***$P < 0.001$, n = 10–11 trees).

No pollen grains or pollen tubes were observed on or within any flowers from the open or bagged pollination treatments at 3 d after anthesis. Pollen grains and pollen tubes were observed on the stigma and within the style of every hand pollinated flower that was sampled (Figure 2.5). We observed 66 ± 9 (n = 12) and 36 ± 9 (n = 14) pollen tubes in the upper style of hand pollinated flowers in 2012 and 2013, respectively. However, the pollen tubes that were observed within the style had not reached the ovules by 3 d after anthesis.

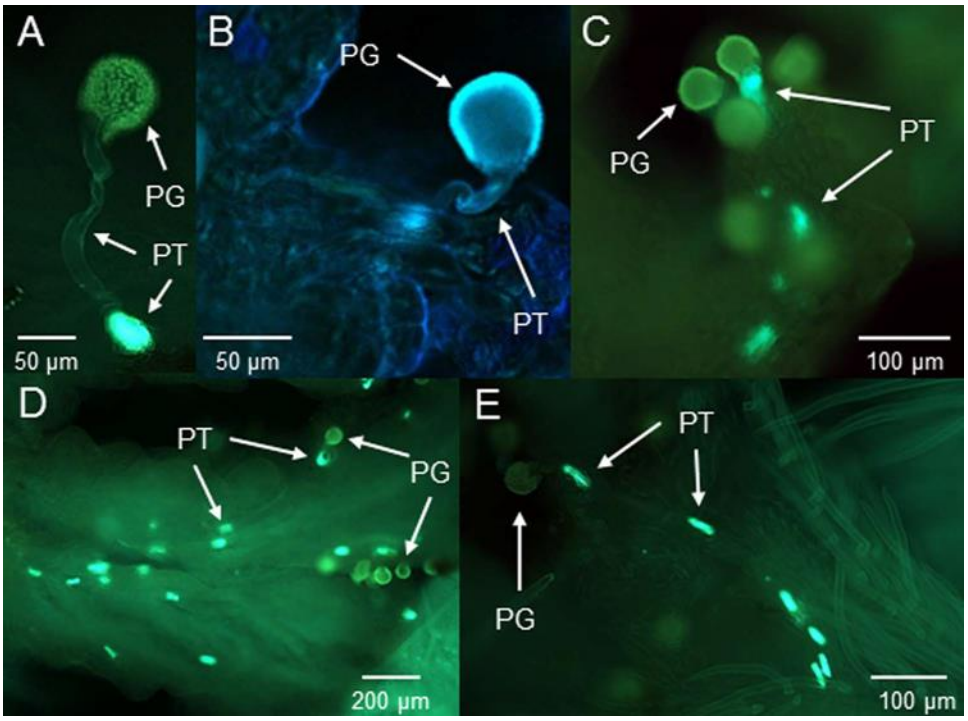

Figure 2.5 Pollen grains and pollen tubes of *Fontainea picrosperma*. (A–C) Germinating pollen grains on the stigma and (D–E) pollen grains on the stigma and pollen tubes within the style of hand pollinated flowers. Arrows point to pollen grains (PG) and pollen tubes (PT).

2.5 DISCUSSION

The floral morphology and flowering phenology of *F. picrosperma* were typical of many dioecious plants found in rainforest communities. Many woody Australian rainforest species possess small flowers that are either white or green with an unspecialised flower structure and a shallow open-access receptacle (Bawa, 1990; Williams and Adam, 1999; Boulter et al., 2010). These common floral traits are often associated with generalist entomophilous pollination (Armstrong and Irvine, 1989; Hansman, 2001; Carpenter et al., 2003; Corlett, 2004; Machado and Lopes, 2004; Gross, 2005; Rosas-Guerrero et al., 2014). These features were all evident in *F. picrosperma* and it is therefore likely to be pollinated by insects. Male *F. picrosperma* flowers were clustered together in paniculate inflorescences that contained significantly more flowers than female inflorescences. This may ensure that pollen is available across the entire female flowering period (House, 1993; Barrett and Harder, 1996; Williams and Adam, 2010), provided that male and female trees flower more or less synchronously. Individual flowers within the male inflorescence opened

sequentially and individual flowers senesced 1 to 2 days after anthesis, possibly encouraging pollinators to visit more inflorescences within the population (House, 1993; Osunkoya, 1999; Moog et al., 2002; Yamasaki and Sakai, 2013).

Male and female flower diameters varied by approximately 33 % and 17 %, respectively, in plantation *F. picrosperma* trees. Flower size is a potentially significant commercial trait as it has been correlated with pollinator visitation (Klinkhamer and van der Lugt, 2004), fruit set (Scorza et al., 1991; Johnson et al., 2011; Wetzstein et al., 2013) and fruit size (Andersson, 1993; Scorza et al., 1991; Rosati et al., 2009; Johnson et al., 2011, 2011; Wetzstein et al., 2013). Relationships between flower diameter and fruit set or fruit size in *F. picrosperma* warrant further research to ensure that fruit yield is maximised through plantation management practices or genotypic selections that optimise flower quality.

No pollen grains or pollen tubes were observed on or within open-pollinated female flowers of *F. picrosperma* after 3 days of flower opening. This was despite stigmas being receptive within the first two days after anthesis and despite fruit set in open-pollinated flowers being observed in approximately 20 % of flowers. This implies very slow or limited pollen movement in the plantation and suggests that this species requires an extended period of stigma receptivity to produce a high fruit load. Female *F. picrosperma* flowers remained open for long periods and remained receptive for up to eight days post-anthesis, suggesting an adaptation to low pollinator activity. While strong phylogenetic constraints operate at the family level to limit the evolution of flower longevity (Kochmer and Handel, 1986; Stratton, 1989), flower longevity can vary at the species level because of low pollination activity (Stratton, 1989; Bawa, 1990; Devaux and Lande, 2010). Final fruit set of *F. picrosperma* was 21.3 ± 3.4 % when the flowers were open to insect visitors whereas almost no fruit were produced when insects were excluded from the flowers. This demonstrates that *F. picrosperma* did not reproduce apomictically, at least not at levels that are likely to be reproductively significant, and that individual fecundity relied on attracting foragers to both male and female flowers. Pollen limitation occurs when pollen augmentation increases seed production relative to open pollinated controls (Trueman and Wallace, 1999; Knight et al., 2005). We found that hand pollinating flowers almost doubled fruit set relative to open pollinated flowers (39.6 ± 4.4 % v. 21.3 ± 3.4 %), indicating that fruit set of *F. picrosperma* was limited by pollen transfer under natural pollination

conditions. The floral traits of *F. picrosperma* suggested that it was adapted for generalist insect pollination, but we have not yet identified the pollinators and it remains possible that this species has a specialist pollinator. Pollen limitation is common in tropical rainforests (Vamosi et al., 2013) and environmental conditions affect the degree of pollen limitation more strongly than common ancestry (Larson and Barrett, 2000). Although seed production and, therefore, tigilanol tiglate production will depend on insect pollinators or hand pollination to produce fruit, further research is required to determine the potential implications of pollen limitation on the whole-tree yield of seed and tigilanol tiglate in this species. The availability of maternal resources to support seed development may also affect the retention, size and tigilanol tiglate content of fruit. Higher frequencies of fruit set associated with hand pollination may result in compensatory effects that reduce average seed size or seed quality including tigilanol tiglate content.

Reproductive strategies strongly influence the ability of plant species to bear viable seed (Adam and Williams, 2001). Dioecy can be highly beneficial for species by allowing sex-specific resource allocation and promoting out-crossing, but it can also lead to pollen limitation and reduced fruit set (Larson and Barrett, 2000; Knight et al., 2005; Aguilar et al., 2006; Davila et al., 2012). Our results from two reproductive seasons clearly showed that *F. picrosperma* was pollen limited. Pollen limitation may be the result of temporal variability in pollination services, often encountered in rainforests where pollinator abundance and competition from co-flowering species is highly unpredictable (Freeman et al., 1980; Williams and Adam, 2010; Vamosi et al., 2013). However, species that exhibit pollen limitation are vulnerable to habitat fragmentation and loss when individual population sizes are reduced (Kearns et al., 1998; Warburton et al., 2000; Knight et al., 2005; Memmott et al., 2007; Williams and Adam, 2010). Although *F. picrosperma* has persisted through rainforest contraction and expansion in response to glacial-interglacial cycles, it has been affected by anthropogenic habitat fragmentation and so subtle impacts on population genetic diversity are possible due to altered gene flows (Lamont et al., 2016).

In conclusion, *F. picrosperma* displayed floral characteristics, reproductive strategies and pollen limitation that are common amongst woody, dioecious, tropical rainforest species. Seed production for the extraction of tigilanol tiglate could be

enhanced by improving pollen transfer from flowers on male trees to flowers on female trees. This could be achieved by increasing the ratio of male to female trees in plantations. Optimising seed production of *F. picrosperma* could also depend upon improving pollen transfer between male and female trees by managing natural or introduced pollinators and optimising the placement of male trees around female trees. This will rely on a better understanding of gene flow in *F. picrosperma* populations and the identification of flower visitors that contribute most significantly to pollination and fruit set.

2.6 ACKNOWLEDGEMENTS

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Chapter 3: Floral Attraction and Flower Visitors of *Fontainea picrosperma* (Euphorbiaceae)

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3.1 ABSTRACT

Flowering plants in tropical rainforests rely heavily on pollen vectors for successful reproduction. Pollinator assemblages vary across tropical rainforest communities geographically and among vertical forest strata within rainforests. Research into the pollination systems is dominated by canopy species, while subcanopy plant-pollinator interactions remain underrepresented. The rainforest subcanopy is a markedly different microclimate from the high-light energy environment in the canopy. Using a range of methods, we studied the floral attractants and floral visitors of a dioecious, subcanopy tree, *Fontainea picrosperma* (Euphorbiaceae) in the Wet Tropics bioregion of northern Queensland, Australia. We found that wind pollination is incidental, if it occurs at all, and the flowers do not produce nectar. Female flowers that offer no obvious reward are pollinated by deceit, though floral parts themselves could attract incidental pollinators. Floral scent was the primary floral attractant in the shaded understorey where visual cues are less important. Female flowers had the same scent profile as male flowers that offer pollen as a reward and floral scent compounds present in the floral bouquet of *F. picrosperma* were generally ubiquitous in nature and known to attract a wide variety of insects. Both day time and night time pollinators contribute to successful reproduction and we observed several Orders of insects visiting the flowers such as beetles, predatory wasps and thrips. Thrips were the most frequently observed flower visitor, otherwise, observations were characterised by an overall dearth in insect visitors to both male and female flowers. *Fontainea picrosperma* is likely to be pollinated by a diverse array of small generalist insects that are relatively ineffective pollinators in a challenging environment beneath the rainforest canopy.

Key words: Australian Wet Tropics; understorey; dioecious; pollination; generalist pollination syndrome; floral scent; cancer; drug.

3.2 INTRODUCTION

Many angiosperms in tropical rainforests exhibit breeding mechanisms that promote outcrossing and rely almost exclusively on animals for pollination (Ollerton et al., 2011). Thus, pollination in rainforest ecosystems largely depends on pollen vector abundance, distribution and the flight behaviour of flying insects (House, 1992; House, 1993; Stacy et al., 1996; Aguilar et al., 2008). Mutualistic plant-pollinator interactions help to govern and maintain the rich biodiversity in tropical rainforests (Nishida, 2001). A high diversity of potential animal pollinators exists within tropical rainforests, partly because of the rich plant diversity (Hill and Hill 2001; Fine et al., 2008; (Boulter et al., 2006a). Generalised pollination syndromes may be common in tropical rainforests, presumably because generalisation is favoured in species that promote outcrossing and have no reproductive assurance (Bawa et al., 1985; Corlett, 2004; Vamosi et al., 2013).

Pollinator assemblages vary across tropical rainforest communities geographically among vertical forest strata within forests. For example, trees beneath the canopy in Costa Rica received more visits by pollinators such as hummingbirds and beetles, than did canopy trees that received more medium- to large-sized bees (Bawa, 1990). Bee assemblages are also known to differ between strata in the Brazilian tropical Atlantic rainforest (Ramalho, 2004). Most of the floristic diversity is found beneath the canopy of tropical rainforests (Bawa et al., 1985a; Hubbell, 2005; Ashley, 2010). The differences in plant species composition as well as temporal availability of floral resources among the vertical strata result in a complex functional relationship with pollinator diversity (Kitching et al., 2007). The rainforest environment below the canopy is markedly different to the forest canopy where the high light energy input results in a greater floral resource availability and allows tree species to produce highly productive flowers (Appanah, 1991; Roubik, 1993). The total radiation reaching the understory in closed tropical rainforests is 2-3 % of the incident light energy compared to the canopy (Mabberley, 1992). In addition, the rainforest the microclimate beneath the canopy has high relative humidity, dense evergreen foliage that creates physical barriers for pollinator movement, stable diurnal air temperatures, and low wind speeds or turbulent wind patterns compared to the

canopy. These conditions pose challenges for flying insects and can reduce the effectiveness of aerial pollen transport (Kato, 1996; Corlett, 2004).

Most of the floristic diversity is found beneath the canopy of tropical rainforests (Bawa et al., 1985a; Hubbell, 2005; Ashley, 2010). Despite this, studies of flower visitors to tree species beneath the rainforest canopy are generally underrepresented compared to canopy species. Of the relatively few studies on subcanopy trees or shrubs available, a wide variety of taxa have been documented as flower visitors and pollinators (Kato, 1996; Momose et al., 1998b; Devy and Davidar, 2006; Aguirre et al., 2011; Berecha et al., 2015; Borchsenius et al., 2016). In the Palaeotropic region, two studies in lowland dipterocarp forests, recorded mammal, bird, social and solitary (*Amegilla*) bees, and generalist insects including moths, wasps, butterflies, flies and beetles as flower visitors or pollinators (Kato, 1996; Momose et al., 1998b). Social bees, flies and generalist insects were the most common flower visitors in wet evergreen forest in the southern Western Ghats of India (Devy and Davidar, 2006). The few published records in the AWT also suggest the potential pollinators of subcanopy tree species are small unspecialised insects belonging to the Orders Diptera, Coleoptera and Hymenoptera (Crome and Irvine, 1986; House, 1989; Webber et al., 2008). Studies of subcanopy species in the Neotropics have been dominated by palm species (Listabarth, 1993; Knudsen et al., 1999; Otero-Arnaiz and Oyama, 2001; Tschapka, 2003; Luna et al., 2005; Aguirre et al., 2011; Borchsenius et al., 2016). Again, a variety of beetles, bees, and flies, and orthopterans were attracted to the flowers of many palm species (Listabarth, 1993; Knudsen et al., 1999; Luna et al., 2005; Aguirre et al., 2011; Borchsenius et al., 2016). One species is pollinated by bats (Tschapka, 2003) and three others appear to be mainly anemophilous (Listabarth, 1993; Otero-Arnaiz and Oyama, 2001; Luna et al., 2005). Thus, many flower visitors are small insects that have low energy requirements and are typically short ranging, polylectic species *(*Appanah, 1991).

Plant traits including flower colour, shape, size, scent and food rewards such as pollen, nectar, floral tissues, stigmatic secretions, and other floral exudates are all involved in pollinator attraction (Barrett and Harder, 1996; Momose et al., 1998a; Boulter et al., 2006a; Williams and Adam, 2010). Tree species that occupy the rainforest beneath the canopy often possess small, white or green flowers, small amounts of pollen and/or nectar and may offer poor floral rewards that reflect the low

energy needs of pollinators in a low light energy environment (Appanah, 1991). Flower colour is one of the principal forms of visual attraction in angiosperms that aids recruitment of potential pollinators. However, in the shaded rainforest subcanopy, colour may be less important than in canopy species and is not likely to draw pollinators from long distances (Williams and Adam, 2010). In the environment beneath the tropical rainforest canopy, species are known to produce valuable fragrant compounds. Examples of this include vanillin from cured pods of the vanilla orchid, *Vanilla planifolia* (Toth et al., 2019); agarwood from the fungal infected wood of *Aquilaria* spp. (Persoon et al., 2008) and; ylang ylang distilled from the flowers of *Cananga odorata* (Jin et al., 2015). Volatile organic compounds produced by flowers can serve as both attractants to pollinators and seed dispersers as well as defensive cues to defend plants against herbivores and pathogens (Dudareva et al., 2006; Junker and Blüthgen, 2010). Many insect pollinators have acute olfactory senses that have the potential to act over long distances before any visual cues are apparent at close range. Thus, scent, rather than flower colour, may be a strong attraction signal and chemical signals can aid in finding food over greater areas (Kite, 1998). Currently, the lack of literature means that generalisations between the main scent constituents and their relative composition in plants and pollination by different functional groups of insects can be difficult to render (Raguso, 2008a; Cordeiro et al., 2019).

Fontainea picrosperma C.T. White (Euphorbiaceae) is a dioecious, subcanopy tree endemic to upland tropical rainforests on the Atherton Tablelands, Queensland, Australia. The Atherton Tablelands is located within the Australian Wet Tropics (AWT) bioregion of northern Queensland. The AWT makes a significant contribution to global biodiversity, harbouring 16 of the 28 near-basal (or 'primitive') lineages of flowering plants with a high level of species endemism (Metcalfe and Ford, 2009). Our understanding of the pollination system of species in these rainforests is generally sparse. There are few published studies of flower visitors in the AWT bioregion and even fewer empirical assessments of successful pollinators (Boulter et al., 2009). Moreover, few studies globally have focused on plant-animal interactions in subcanopy rainforest species, an environment that is markedly different to the rainforest canopy. Knowledge of pollinator behaviour becomes economically important when plants reliant on pollination for reproduction are introduced into cultivation. As apomixis is not a significant means of reproduction in *F. picrosperma*

(Grant et al., 2017; Chapter 2), it is important to understand how pollen is delivered to the stigma.

The aim of this study is to identify the floral attractants and subsequent flower visitors of *F. picrosperma*. The pollination biology of *F. picrosperma* is of considerable commercial interest because its fruit contains the small molecule natural product, tigilanol tiglate (Boyle et al., 2014). The compound is an effective treatment for cancer having been approved for use as a therapy for canine mast cell tumours by the European Medicines Agency (Miller et al., 2019; QBiotics, 2020) and being developed for the treatment of human head and neck squamous cell carcinoma (Boyle et al., 2014; Panizza et al., 2019). Tigilanol tiglate is not amenable to a commercial total synthesis, so production of the drug on a commercial-scale relies on raw material harvested from the seed of *F. picrosperma*. An interest in the pollination system of *F. picrosperma* is due to the economic importance of these ecosystem services. We asked (1) What are the pollination modes of *F. picrosperma*? (2) What are the main floral attractants in a subcanopy rainforest species? (3) What animals are attracted to the flowers in a subcanopy environment? Specifically, we ascertained if wind is a potential pollination method, determined the reproductive success of nocturnal and diurnal pollinators, investigated floral attractants and characterised the floral scent profile. We carried out flower observations, collected in-flower visitors and used scent lures to trap insects attracted to the main compounds present in *F. picrosperma*'s floral bouquet.

3.3 MATERIALS AND METHODS

3.3.1 Study species and study site

Fontainea picrosperma is a sub-canopy tree that grows up to 25 m tall (Jessup and Guymer, 1985) and is endemic to the complex mesophyll and notophyll vine forests on the Atherton Tablelands, north Queensland, Australia. The species possesses small, white flowers that have an unspecialised structure and an open access receptacle (Grant et al., 2017; Chapter 2). Flowering occurs simultaneously between individuals of subpopulations from September to November. The red drupaceous fruit (up to 3 cm diameter) ripen in December and January and are primarily dispersed by

gravity (Lamont et al., 2016). Natural stands of *F. picrosperma* therefore are not uniformly distributed, they form small, isolated but dense clumps (2-10 m inter-tree spacing) with ~50:50 male: female ratios (Lamont et al., 2016; Grant et al., 2017; Chapter 2).

Fontainea picrosperma has a geographically restricted natural range and its distribution has been heavily influenced by both natural, and anthropogenic habitat fragmentation (Lamont et al., 2016). The populations included in this study were Boonjie, and Evelyn Highlands as described in Lamont et al. (2016). The subpopulations of "Evelyn Highlands 1" and "Evelyn Highlands 2" are from the same refugial population approximately 3.5 km apart. Boonjie, and Evelyn Highlands are the two largest, continuous rainforest areas where *F. picrosperma* occurs. Potted plants housed in the glasshouses at the University of the Sunshine Coast (USC), Sippy Downs, Australia, were used to characterise the scent profiles of *F. picrosperma* flowers. Data were collected over three flowering seasons in 2014, 2015 and 2016.

3.3.2 Pollination

To determine if wind contributes to pollination, microscope slides smeared with a thin layer of Vaseline the size of a cover slip (300 mm^2) were hung from *F. picrosperma* trees 1.5 to 3 meters from the ground in Evelyn Highlands 2. Slides were installed in three male and three female trees (three slides per tree) for 24 h before removal. New slides were installed each day for three replicate days. Once the slides were removed, the area of Vaseline was stained with Calberla's solution to detect pollen. The number of *F. picrosperma* pollen grains within the area of the cover slip were counted using a compound light microscope (Leica M125, Leica Microsystems, Wetzlar, Germany, X 40).

Pollinator exclusion experiments were carried out in the natural population at Evelyn Highlands 2 to assess the reproductive success rate of diurnal versus nocturnal pollinators. There were three treatments: (1) control where inflorescences were open to flower visitors; (2) diurnal, where inflorescences were open to insect visitors during the day (0600 h – 1800 h); and (3) nocturnal, where inflorescences were open to insect visitors during the night (1800 h – 0600 h). Inflorescences were enclosed at bud stage in a fine mesh (0.5 mm x 1.0 mm) bag fastened with a clothes peg to exclude flower

visitors and the number of initial starting buds were counted. Five trees each with two to three replicate inflorescences for each treatment were bagged. Bags were removed or replaced each morning and evening. Flowers were removed to count the number of pollen grains on the stigma and stored in fixative (25 % glacial acetic acid, 75 % ethanol; v,v) when the stigmas and petals began to shrivel and brown or were otherwise removed when subject to treatment for 10 d. The number of pollen grains present on the stigma were counted for open, diurnal and nocturnal treatment groups using a fluorescence microscope (Leica DM5500B, Leica Microsystems, Wetzlar, Germany) after staining with decolourised aniline blue (Kho and Bear, 1968; Shepherd, 2000).

3.3.3 Floral attractants

Five female trees and four male trees were sampled for nectar from the natural population, Evelyn Highlands 2. Individual flowers were tested for nectar rewards using a wash method following Morrant et al. (2008). This method is recommended for species with low floral nectar volumes and can measure nectar that accumulates from trichomal nectaries. Unopened buds were tagged and then bagged to exclude flower visitors for the duration of the experiment. One flower per tree was measured on each day of sampling, when the flower was 0, 2, 3, and 5 days old. Each flower was placed into a vial containing 0.5 mL of distilled water. The sample was manually agitated for five to 10 minutes. The sugar concentration was measured using a hand-held BRIX refractometer (ATAGO PAL-S). This is a destructive method, therefore, a flower sampled at day 5 after opening for example, was allowed 5 days to accumulate nectar.

We identified volatile compounds emitted from *F. picrosperma* flowers from potted plants in the glasshouse where environmental conditions including light, temperature and moisture were constant for each plant. Whole flowers were picked from *F. picrosperma* plants for ease of headspace volatile collection. This does however, increase the risk of obtaining higher levels of green leaf volatiles that are characteristic of wounded foliage (e.g. Grison et al., 1999; Arimura et al., 2001). Six male and six female trees were used in the experiment. Flowers were sampled at different ages (male d 0, 1, 2, 3; female d 0, 1, 2, 3, 5, 7, 9, 11) and between three and six replicates were sampled across the flower age range for each sex. Flowers were removed between 0900 h and 1500 h and placed in a 10 ml septum cup vial to allow

volatile odours to equilibrate for 1 h at room temperature (22°C). A background control of the glasshouse and sample vials were also sampled each day of floral volatile testing. Volatiles from each sample were then collected using a solid-phase microextraction (SPME) holder (Augusto and Luiz Pires Valente, 2002). For each sample, the Supelco fibre coated with polydimethylsiloxane (PDMS, 100 µm) was manually inserted into the headspace chamber and exposed for 25 minutes at 22°C.

Volatile analysis was performed on a PerkinElmer Clarus 580 GCMS. The column used was an Elite-5MS (30 m × 0.25 mm × 0.25 µm). The helium carrier gas had a constant flow of 1 mL/min. The SPME fibre was manually inserted into the injection port fitted with a splitless liner at 200°C and maintained for 2 minutes to allow the adsorbed odours to thermally desorb onto the GC column. The split ratio was shut from -0.5 to 2 min, then open at 30:1. The temperature program was operated at 40°C for 2 min, ramping at 10°C/min until 210°C and holding for 1.5 min. Mass spectrometer analysed a mass range from 40 to 250 (m/z), from 1 to 20.5 min at 70 eV. Compounds were identified by comparison of retention times to authentic standards, retention index (AI) and comparison of mass spectra against National Institute of Standards and Technology (NIST 08) Mass Spectral library match.

Compounds present at similar abundance in the control samples were considered to be contaminants and were excluded from the analysis. Proportional abundance (relative amounts with respect to aggregate peak areas, excluding contaminants) of the individual constituents was calculated based on peak area (mAU) measurements and expressed as a percent of the sum total peak area.

3.3.4 Flower visitors

Two *F. picrosperma* populations, Evelyn Highlands 2 and Boonjie, were surveyed for diurnal flower visitors. Three male trees and three female trees for each population were each observed for a 10 minute period every two hours (0820 h – 1720 h). Observations were conducted on sunny days with light winds conducive to insect activity for a total of 25 h (5 d) in the natural population at Evelyn Highlands 2 and 20 h (4 d) at Boonjie. Where possible, individual visitors were collected and stored in 70 percent ethanol. Means and standard errors were calculated for each order of insect that visited male and female flowers during the observation time period.

'In-flora' visitors were collected by enclosing individual inflorescences in sealable plastic bags and clipping the stem at the point of closure of the bag to capture whole inflorescences. Specimens in the samples were later identified. The number of open flowers and the number of buds were recorded for each inflorescence. The mean number of Thysanoptera (thrips) per inflorescence was calculated for each sex.

We trapped insects using a scent lure to ascertain what types of insects were attracted to the floral scent compounds emitted by *F. picrosperma* flowers. Field trap experiments were carried out during the flowering season in Evelyn Highlands 1. Two types of insect traps were used to target a range of insects. Black plastic coloured collision traps (Sankei Chemicals Co. Ltd.; 25 cm diameter, 42 cm height) and white sticky traps (9 cm x 15 cm) were installed together as pairs in trees approximately two metres above ground. Collision traps were treated with a fluropolymer, Fluon, to enhance their efficiency (Graham et al., 2010). Sorbic acid (1 g/L) was added to the collision trap to prevent decay of the insects and a surface-active agent (neutral detergent) was also added to the water trap in small quantities. Scent baits were attached with string to the panel traps so that they hung just above the water surface and the scent bait was attached to the top of one side of the sticky trap. Control traps did not have a scent lure. Six pairs of traps were baited with a scent lure and six pairs of traps without a scent lure (control) were installed in non-flowering trees for one week. The distance between each pair of traps was a minimum of 15 m and a minimum of 10 m from the nearest flowering *F. picrosperma*.

A synthetic mix of the main floral scent compounds detected in the SPME scent analysis were used as a scent lure. The compounds included in the scent lure were: *cis* 3-Hexen-1-ol acetate; Benzyl alcohol; Ocimene isomer mixture (3,7-Dimethyl-1,3,6-octatriene); Methyl benzoate; 4-oxoisophorone (2,6,6-Trimethyl-2-cyclohexene-1,4-dione); and Benzyl acetate all obtained from Sigma-Aldrich, Inc. The compounds were added to the scent lure according to the average proportional abundance (%) that was detected in the GC-MS analysis based on a female flower at day three of opening. The scent lure compound mixture was tested on the GC-MS to confirm the scent composition relative to female flower scent profile. A scent lure was devised for dispensing scent chemicals using mini snap lock bag containing two filters that were impregnated with 500 µL using 1:10 dilution of scent solution with paraffin oil.

Captured insects were preserved in a vial containing 70 % ethanol for later identification.

3.3.5 Statistical analysis

We tested for differences in the sum total (mAU) of all detected compounds emitted between different flower ages within sex using a generalised linear model (GLM) assuming a negative binomial distribution with a log link function (package 'MASS'; Venables and Ripley, 2002). Where differences were detected we applied a Tukey HSD test. For analyses of semi-quantitative (i.e. relative amounts of scent compounds within a flower) differences in scent between sex, total peak area (mAU) of compounds were fourth-root transformed before analysis. We calculated the Bray-Curtis similarity index and performed a permutational multivariate analysis of variance fitted to the distance matrices (PERMANOVA) (999 permutations) using the Adonis function (package 'vegan'; Oksanen et al., 2015). We first analysed the combined ages 0-3 d and then compared each age separately to test for differences between sex as the flower aged (package 'pairwise.adonis'; Martinez, 2019). Analyses were performed in R v.3.5.1 (R Development Core Team).

We tested for treatment effects on the number of pollen grains detected on a stigma using a GLM with a negative binomial distribution to correct for overdispersion due to inflated zero's in the data (package 'MASS'; Venables and Ripley, 2002). Differences between treatments were assessed using Tukey's HSD post hoc test (package 'multcomp'; Hothorn et al., 2008). Analysis were performed in R (v.3.5.1).

We conducted a Mann-Whitney U test (SPSS v 24) to determine if there were significant differences ($P < 0.05$) between the number of individuals of each morphospecies in the control and scent lure traps. Only morphospecies with more than 10 individuals in the insect traps were included in the analysis (n = 29). Morphospecies with significant differences between treatment and control (n = 5) were identified to genus level. We also combined the number of individuals of each morphospecies within an order and tested for significant differences between treatments ($P < 0.05$, Mann-Whitney U test). Analyses were performed using IBM SPSS v 24.

3.4 RESULTS

3.4.1 Pollination

To track the movement of pollen we tested for the potential of wind pollination and the effectiveness of day and night pollinators. Few airborne pollen grains were captured in the natural population (Table 3.1).

Table 3.1 Mean number of airborne pollen grains detected in a 24 h period on the microscope slides in the natural population. Standard error in parenthesis.

Sex	Number of airborne pollen grains
Male	1.18 (0.30)
Female	1.85 (0.40)

Pollination treatments affected pollen deposition in the natural population, Evelyn Highlands 2 (Figure 3.1). Significantly more pollen grains were found on open (control) flowers than on flowers that were only exposed to nocturnal visitors (GLM, $P = 0.005$, Figure 3.1). There was no significant difference in the number of pollen grains between open flowers and flowers that were exposed to visitors during the day (GLM, $P = 0.373$, Figure 3.1) or between flowers that were only open to visitors during

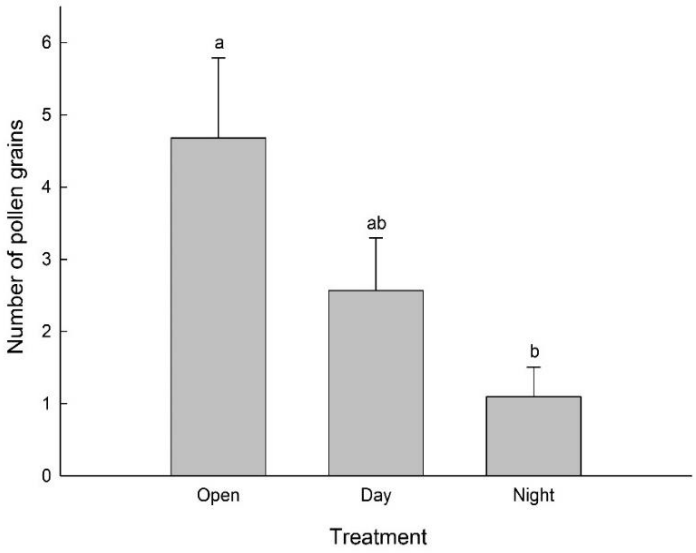

Figure 3.1 Mean number of pollen grains counted on the stigma of female *Fontainea picrosperma* flowers in a 12 h period. Open, inflorescences not bagged (n = 28); Day, inflorescences open to pollinators during the day (06:00-18:00; n = 23); Night, inflorescences open to pollinators during the night (18:00-06:00; n = 21). Different letters indicate significant differences (GLM, $P < 0.05$).

the day and flowers only open to visitors during the night (GLM, $P = 0.183$, Figure 3.1).

3.4.2 Floral attraction

Nectar was not present in *F. picrosperma* flowers. There was no visible accumulation of liquid on the petals, stamens or pistils, nor in the base of the receptacle of any of the flowers examined.

Benzyl alcohol and 4-oxoisophorone (2,6,6-Trimethyl-2-cyclohexene-1,4-dione) were the two relatively most abundant scent compounds present in both male and female flowers (Table 3.2). The next relatively most abundant compounds were cis-β-Ocimene; Methyl benzoate (Benzoic acid, methyl ester); cis-3-Hexen-1-ol acetate. Trace amounts (generally ≤1 %) of compounds that were detected in *F. picrosperma*'s floral scent bouquet included 1,1-Dimethyl-3-methylene-2-vinylcyclohexane; 1,4-Cyclohexanedione; β-Cyclocitral; Benzyl acetate (Acetic acid, phenylmethyl ester); Benzaldehyde; Methyl salicylate (Methyl 2-hydroxybenzoate); Hexyl acetate (Acetic acid, hexyl ester) and; Geranyl isovalerate ((E)-3,7-Dimethyl-2,6-octadienyl 3-methylbutanoate) (Table 3.2).

Male flowers emitted significantly less scent at day 0 than male flowers at age 1, 2 or 3 based on sum of total peak area (GLM, $P < 0.05$; Figure 3.2). The senescing stage of female flowers was characterised by a drop in overall volatile emissions (Figure 3.2). Older female flowers (> 5 days) emitted significantly less scent than flowers aged 0-5 (GLM, $P < 0.05$; Figure 3.2).

Table 3.2 Floral volatile compounds detected by GC-MS of male and female flowers aged from 1 – 3 d of *Fontainea picrosperma*. Scent compounds are listed according to compound class. Mean relative percent of each compound is presented using data from male and female flowers aged from 0 – 3 d. Standard error in parenthesis.

Compound class	Compound name	Relative %	
		Male	Female
Terpenes			
Monoterpenes			
	cis-β-Ocimene**	3.06 (0.5)	4.07 (0.5)
Irregular terpenes (*Apocarotenoids and related compounds*)			
	4-Oxoisophorone**	43.24 (3.4)	31.87 (2.4)
	2,2,6-Trimethyl-1,4-cyclohexanedione	0.643 (0.08)	1.03 (0.4)
	β-Cyclocitral*	0.18 (0.0)	0.21 (0.0)
	Geranyl isovalerate	0.09 (0.0)	0.10 (0.0)
	1,1-Dimethyl-3-methylene-2-vinylcyclohexane	0.014 (0.0)	0.108 (0.04)
Benzoic acid-related compounds			
	Benzyl acetate**	0.90 (0.1)	1.32 (0.1)
	Benzyl alcohol**	31.28 (2.3)	49.26 (2.0)
	Benzaldehyde*	1.06 (0.1)	1.06 (0.1)
	Methyl benzoate**	17.91 (2.8)	6.938 (1.3)
	Methyl salicylate*	0.23 (0.0)	1.32 (0.6)
Aliphatics (*Volatile fatty acid derivatives*)			
	cis-3-Hexen-1-ol acetate**	1.12 (0.2)	2.40 (0.3)
	Hexyl acetate*	0.271 (0.05)	0.31 (0.0)

Compounds marked with asterisks (**) were identified based on GC retention times and mass spectra of standard compounds purchased from Sigma-Aldrich, USA. Compounds marked with asterisks (*) were identified using the mass spectrum and AI (arithmetic retention index) (Adams, 2007). The remaining compounds were tentatively identified according to their mass spectral and retention indexdata in the National Institute of Standards and Technology (NIST 08) Mass Spectral library.

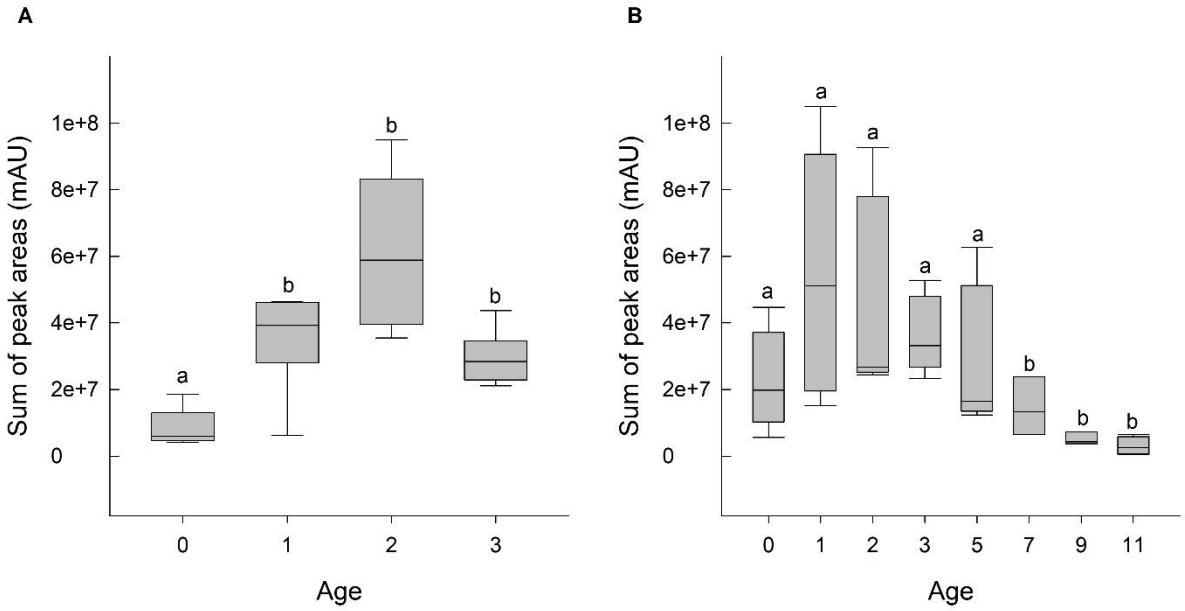

Figure 3.2 Emission patterns of the sum total of aggregate peak areas of all *Fontainea picrosperma* floral volatile compounds detected by GC-MS across the age of (A) male flowers and (B) female flowers.

Pairwise comparisons revealed no significant differences in the bouquet of floral volatile emissions (peak area, mAU) between male and female flowers when combined across ages of 0-3 days (PERMANOVA, R^2 = 0.053, P = 0.082). When the different daily ages of flowers were analysed individually, pairwise comparisons revealed significant differences between male and female flowers at age 0 (PERMANOVA, R^2 = 0.218, P = 0.045) and at age 3 (PERMANOVA, R^2 = 0.372, P = 0.002). There were no significant differences between male and female flowers at age 1 (PERMANOVA, R^2 = 0.172, P = 0.181) and 2 (PERMANOVA, R^2 = 0.265, P = 0.071). The aggregated peak area of individual compounds differed over the age of the flower (Figure 3.3).

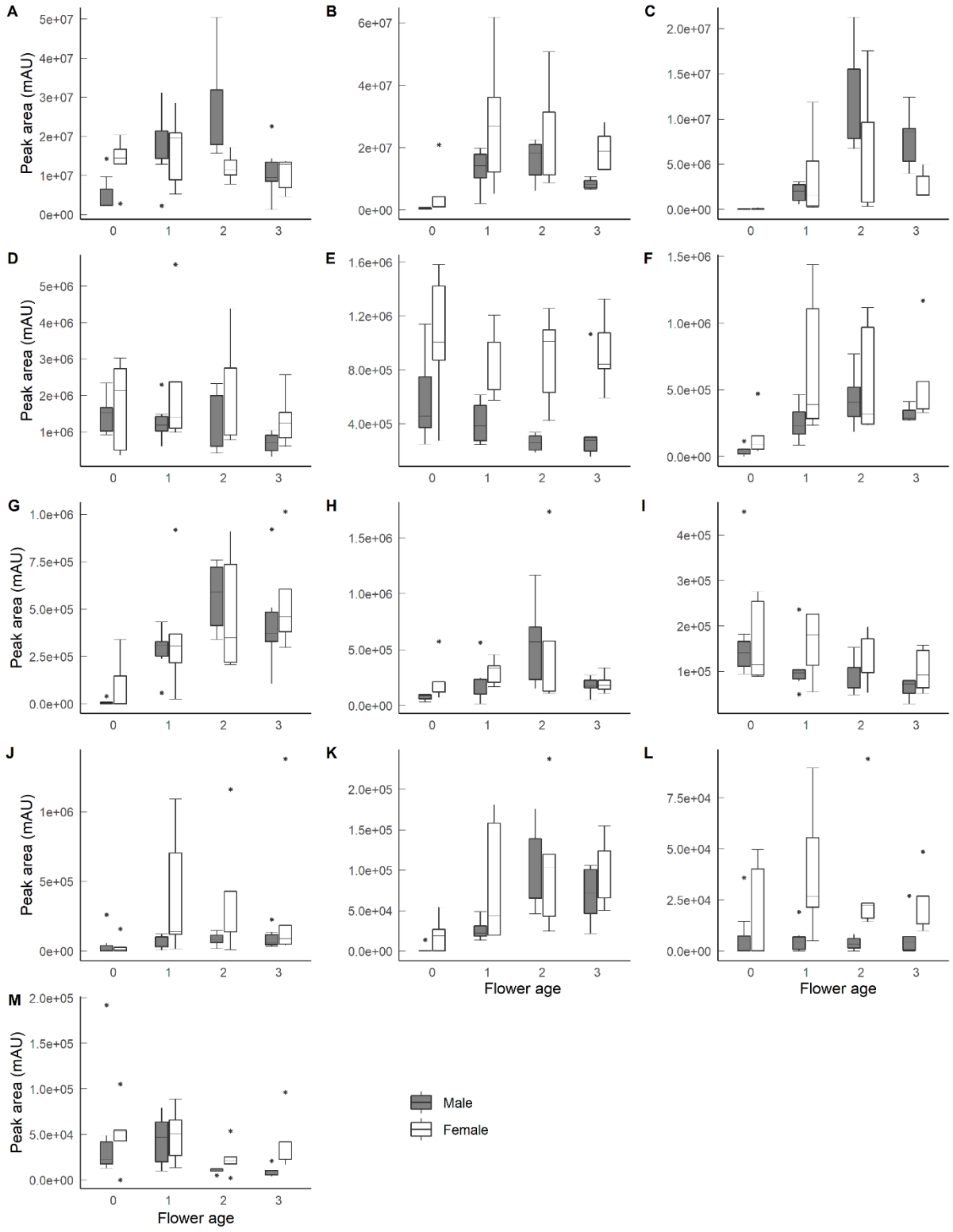

Figure 3.3 Peak area of the detected floral volatile compounds from male and female flowers of *Fontainea picrosperma* for each age 0 – 3 d. (A) 4-oxoisophorone (B) Benzyl alcohol (C) Methyl benzoate (D) *cis*-β-Ocimene (E) *cis*-3-Hexen-1-ol acetate (F) Benzyl acetate (G) Benzyl aldehyde (H) 2,2,6-Trimethyl-1,4-cyclohexanedione (I) Hexyl acetate (J) Methyl salicylate (K) β-Cyclocitral (L) 1,1-Dimethyl-3-methylene-2-vinylcyclohexane (M) Geranyl isovalerate. For each compound the graphs show the median (quartiles, minimum–maximum) of the total peak area (n = 5-6 for male flowers and; n = 3-5 for female flowers).

3.4.3 Flower visitors

Flowers were most frequently visited by insects from the orders, Thysanoptera (thrips) and Coleoptera (beetles) (Figure 3.4). The most abundant insects captured within sampled inflorescences were thrips (Table 3.3). A range of small 'in flora' flower visitors from whole inflorescences were also present (Table 3.4).

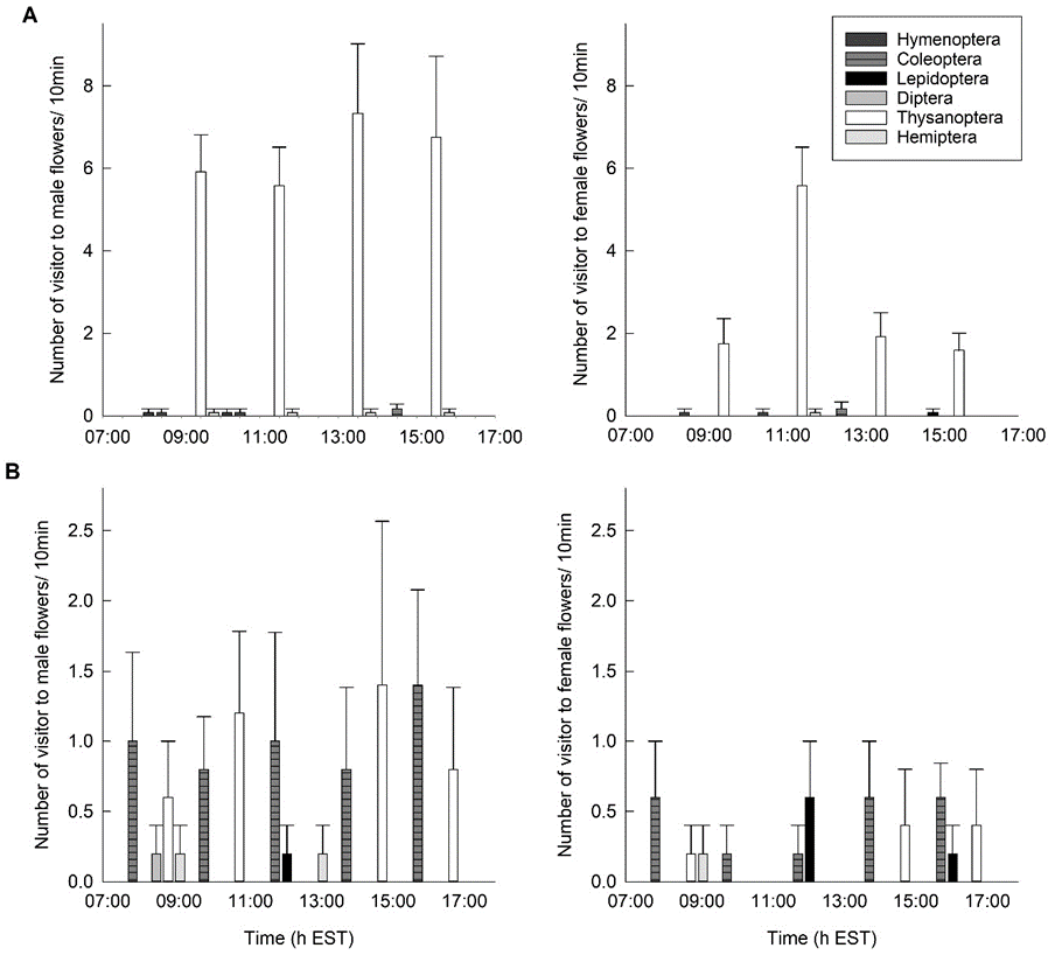

Figure 3.4 Diurnal variation in number of flower visitors to *Fontainea picrosperma* in (A) Boonjie and (B) Evelyn Highlands 2. Mean and standard error for number of visits for each observation period in male trees and female trees.

Table 3.3 Mean number of Thysanoptera captures from in-flora collections of *Fontainea picrosperma* inflorescences in the natural populations at Boonjie, Evelyn Highlands 1 and Evelyn Highlands 2 per inflorescence. Standard error shown in parenthesis.

Population	Number of inflorescences		Number of Thysanoptera	
	Male	Female	Male	Female
Boonjie	12	12	4.9 (1.3)	7.2 (2.2)
Evelyn Highlands 1	9	3	2.9 (0.8)	2.0 (1.1)
Evelyn Highlands 2	5	1	0.2 (0.2)	0

Table 3.4 Flower visitors of *Fontainea picrosperma*: Insect captures during observations and in-flora collections of insects and mites in the natural populations at Boonjie, Evelyn Highlands 1 and Evelyn Highlands 2. The number of flowers present on each inflorescence is stated.

Location	Sex	Number of flowers	Order	Identification*	Common name
Flower visitors captured within inflorescences					
Boonjie	F	6	Hemiptera	Fulgoroidea	Leaf hopper nymph
Boonjie	F	3	Hemiptera	Coccoidea	Scale insect
Boonjie	M	8	Coleoptera	Nitidulidae	Sap beetle
			Coleoptera	Curculionidae	Weevil
Boonjie	M	8	Hymenoptera	Encyrtidae	Parasitic wasp
Boonjie	M	9	Sarcoptiformes	Oribatida	Mite
Evelyn Highlands 1	M	3	Hymenoptera	Scelionidae	Parasitic wasp
Evelyn Highlands 1	M	4	Diptera	Culicidae	Mosquito
Evelyn Highlands 1	M	5	Hymenoptera	Platygastridae	Parasitic wasp
			Collembola	-	Springtail
Flower visitors captured during observations					
Evelyn Highlands 2	M	-	Coleoptera	Mordellidae	Pin tail beetle
Evelyn Highlands 2	F	-	Coleoptera	Curculionidae	Weevil
Evelyn Highlands 2	M	-	Coleoptera	Chrysomelidae: galerucinae: alticini	Flea beetle
Evelyn Highlands 2	F	-	Lepidoptera	-	Caterpillar

* Levels of identification range from suborder (Oribatida) and superfamily to tribe.

Five genera of insects were found in significantly greater numbers in the scent lure traps compared to the control traps in the Evelyn Highlands 1 population ($P < 0.05$, Mann Whitney U test; Figure 3.5). Three were beetles including *Aethyssius* (Tenbrionidae: Alleculinae); *Ictistygna* (Anthicidae: Eurygeniinae) and another alleculine tentatively identified as *Euomma*. The other two genera were a minute (body length less than 1 mm) thrips parasitoid wasp that was either a species of *Ceranisus* or *Thripobius* (Eulophidae: Entedoninae) and *Drosophila* (Drosophilidae).

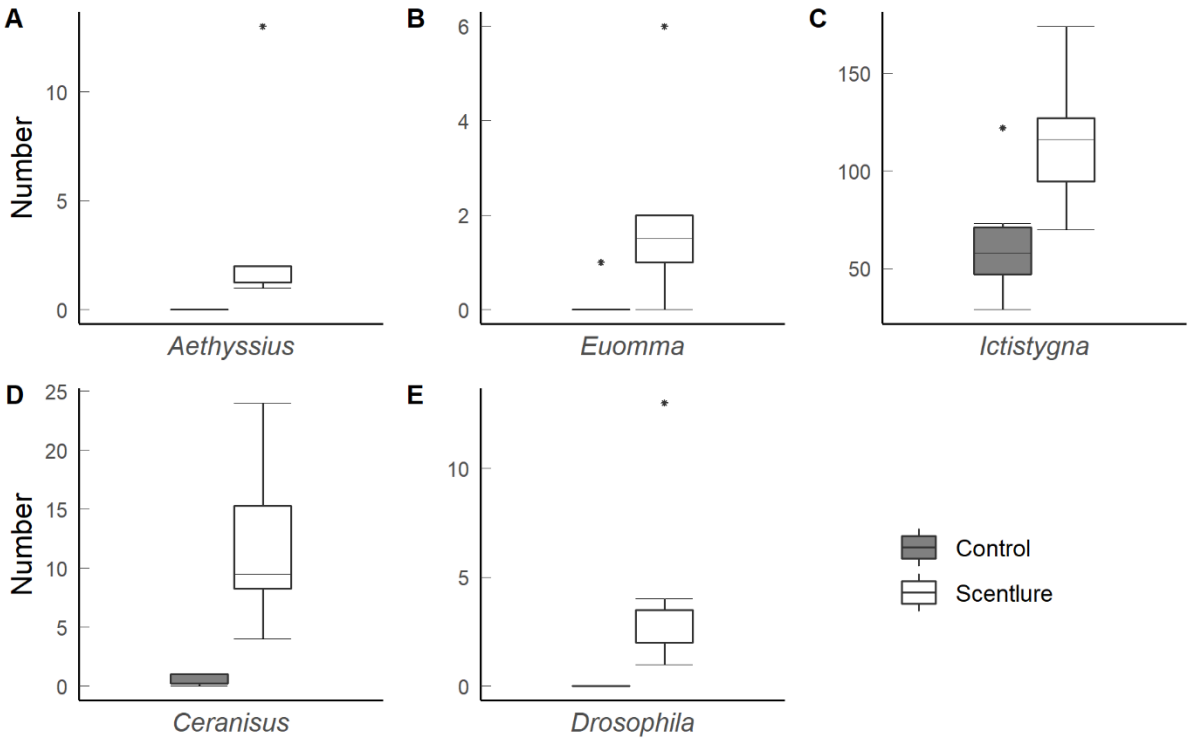

Figure 3.5 Insects (identified to genus level) found in significantly larger numbers in the scent lure traps containing volatile compounds from flowers of *Fontainea picrosperma* compared to the control traps with no volatile compounds ($P < 0.05$, Mann-Whitney U test).

There were no significant differences between scent lure and control traps in any other taxa. When the number of individuals in each order were combined, there was a significant difference in the number of Lepidoptera between treatments ($P = 0.034$), with more present in the scent lure traps. There was no significant difference in any other order tested.

3.5 DISCUSSION

Our results found that in *F. picrosperma*, floral scent is the most important floral attractant for recruiting pollinators. Male and female flowers do not produce nectar and thus female flowers are pollinated by deceit. The unspecialised structure of *F. picrosperma* flowers (Grant et al., 2017; Chapter 2) and the low frequency with which the wide variety of small insect taxa were observed visiting flowers of both sexes indicates that pollination occurs from the ever-present pool of low-energy, generalist insects found in the tropical rainforest. Female flowers remain receptive for a long time (Grant et al., 2017; Chapter 1) raising the chances of pollination by these unreliable visitors. This finding also reflects the very short pollen dispersal distances (Grant et al., 2019; Chapter 4) that characterises gene flow in *F. picrosperma* and emphasises that the environment beneath the canopy is challenging for pollinators that forage on species that offer poor floral resources, in low light levels and crowded with dense foliage.

3.5.1 Floral attraction

Pollinators are likely deceived by female flowers that offer no obvious reward due to their perceptual similarity to pollen-offering male flowers (Grant et al., 2017; Chapter 2) and pollination is the consequence of foraging errors made by insects as they move between *F. picrosperma* trees. The bouquet of floral volatile emissions did not differ between male and female flowers when combined across ages of 0-3 days ($P = 0.082$). Bakerain mimicry (where female flowers mimic male flowers and cheat pollinators out of a reward) occurs because female flowers offer no pollen or nectar reward and floral volatile compounds provide a cost-efficient way to attract pollinators, particularly in the dark, dense rainforest subcanopy where visual cues are less important (Appanah, 1991; Knudsen et al., 1999; Williams and Adam, 2010). This occurs in other tropical species such as the understorey palm *Geonoma macrostachys* in which nectarless female flowers mimic rewarding male flowers (Borchsenius et al., 2016). Pollination is achieved by deceit insofar as female flowers offer no obvious reward (i.e. pollen or nectar). However, pollinators also forage on flowers for other rewards (Renner, 2006). Field observations suggest that floral tissues may provide some food reward targeted by chewing insects such as beetles that are known to visit

flowers to feed on pollen, various flower parts and other floral exudates (Endress, 1996).

We found that the bouquet of volatiles differed between male and female flowers at age 0 and 3 days. Female flowers last significantly longer than do male flowers, by age 3, male flowers are beginning to senesce, and female flowers are at the peak of receptivity (Grant et al., 2017; Chapter 2). In comparison, male anthers dehisce approximately one day after opening (Grant et al., 2017; Chapter 2). The sum total of floral scent was highest in male flowers when anthers are fully open at day two of opening. In addition, the senescing stage of female flowers was characterised by an overall drop in the relative total of volatile emissions. Female flowers do not set fruit beyond 9 d (Grant et al., 2017; Chapter 2). Thus *F. picrosperma* decreases scent production when pollen is not available or the stigma is not receptive and potentially regulates pollinator behaviour across time to maximise pollination success, like has been shown in other species (Delle-Vedove et al., 2017).

The two relatively most abundant compounds in the floral scent profile of *F. picrosperma* were the irregular terpene, 4-oxoisophorone and the benzenoid, benzyl alcohol which comprosed approximately 78 % of the relative emissions from male and female *F. picrosperma* flowers (based on flowers aged from 1-3 days old). 4-oxoisophorone is the main floral volatile compound of *Buddleja* spp., a genus that is well known to attract butterflies and other insects (Chen et al., 2012; Chen et al., 2014; Chen et al., 2015). The compound also evokes antennal responses in moths, bees and flies (Andersson 2003; Guédot et al., 2008). However, antennal responses from insects do not necessarily indicate that the species is an effective pollinator (Chen et al., 2014), and terpenes may be produced to repel nectar thieves and pests (Junker and Blüthgen, 2010).

Benzyl alcohol is amongst the most common compounds in floral scent, occurring in 56 % of families studied (Knudsen et al., 2006). Benzyl alcohol is also known to attract *Apis mellifera* (honey bees) (Dötterl and Vereecken, 2010 and references therein). Other benzenoids present in the floral bouquet of *F. picrosperma* such as benzaldehyde also have a widespread distribution and are thought to be functionally attractive to pollinators (Schiestl, 2010). Benzyl alcohol, as well as several of the other main floral compounds of *F. picrosperma* such as benzaldehyde, benzyl acetate and methyl salicylate are known to dominate the floral scent profile of

plants with nocturnal anthesis and are associated with moth, nocturnal bee, beetle and other generalist insect pollination (Knudsen et al., 1993; Jurgens, 2002; Jürgens et al., 2002; Dotterl et al., 2012; Prieto-Benítez et al., 2015; Cordeiro et al., 2019). Methyl salicylate is one of the most common compounds in floral scent (Knudsen et al., 2006). Methyl salicylate is also emitted in high amounts by some species of Araceae that are pollinated by nocturnal beetles (Etl et al., 2016) and hawkmoths (Raguso et al., 1996). Assuming that *F. picrosperma* emits these compounds during the night-time, these compounds could be responsible for attracting nocturnal insects as we determined the occurrence of pollen on flower stigmas only open to nocturnal visitors in this study.

The monoterpene, *cis*-β-ocimene, and the benzenoid, methyl benzoate comprised a further 16 % of the floral scent profile of *F. picrosperma* (based on the peak area of compounds from male and female flowers aged from 1-3 days old). β-ocimene is the most commonly encountered scent compound in generalist plant-pollinator interactions (Dobson, 2006) and *cis*-β-ocimene has been shown to stimulate foraging behaviour in bees (Eltz et al., 2006; Gong et al., 2015). Methyl benzoate is known to attract bees (Dudareva et al., 2000; Schiestl and Roubik, 2003) and moths (Raguso and Light, 1998).

The green leaf volatile 3-hexen-1-ol actetate could be present simply because the flower sample was harvested from the plant and leaf tissue was damaged. This compound is considered a typical, wound response signal but it can also be indicative of the scent of flowers pollinated by settling moths (Dobson, 2006). Methyl salicylate also signals a wound response, but it is not normally emitted until hours after the beginning of herbivore damage (Dudareva et al., 2006). Wound volatiles have also been hypothesised to be used as kairomonal attractants by florivores whose visitation subsequently results in incidental pollination (Knudsen et al., 2006). The floral bouquet of *F. picrosperma* showed small relative amounts of 1,1-Dimethyl-3-methylene-2-vinylcyclohexane. This compound has been found in beech leaf and/or wood volatiles and is a putative kairomone of the beech leaf mining weevil (*Orchestes fagi*) (Mayo et al., 2016).

These results are one of only a few studies on a species of Euphorbiaceae, which remains a poorly sampled family with regards to floral chemistry (Knudsen et al., 2006). A review of floral scent emissions included only two species of Euphorbiaceae, both from the genus *Dalechampia* (Knudsen et al., 2006) and both pollinated by

euglossine bees (Armbruster et al., 1989; Whitten et al., 1986). The lack of literature renders comparisons of floral scent composition within the Euphorbiaceae difficult. Pollinators are known to have flexible olfactory sensory responses (Svensson et al., 2010) and compounds can play equal and interchangeable roles in pollinator attraction and learning (Raguso, 2008a). It is unclear whether insect pollinators use only a few compounds present in a scent for floral identification, or whether they use information from all the compounds present in the floral bouquet (Dudareva *et al.*, 2006). This complicates our understanding of the role of floral scent in plant-pollinator relationships and pollination syndromes more broadly (Levin et al. 2003). We studied variations in floral emissions between sex and over time and we acknowledge that within a species the level of floral emissions can change according to endogenous diurnal rhythms, which was not studied here (Dudareva *et al.*, 2004). Nevertheless, the floral bouquet of *F. picrosperma* contains compounds with a widespread distribution that are known to attract a wide variety of insect pollinators.

3.5.2 Flower visitors

We determined that *F. picrosperma* flowers are visited by a diverse set of small, generalist insects. This is concordant with many other woody plants in Australian tropical and subtropical rainforest communities (Williams and Adam, 1994; Boulter et al., 2005; Worboys and Jackes, 2005). The floral traits of *F. picrosperma* suggest little adaptation to exploit specific pollinators (Grant et al., 2017; Chapter 2). *Fontainea picrosperma* flowers, like those of many species in rainforest communities elsewhere, e.g. lowland dipterocarp forest in Sarawak (Momose et al., 1998b), have no morphological mechanisms to exclude any flower visitors and are visited by an array of generalist insects. Small generalist pollinators have low energy requirements and often move shorter distances compared to specialised insects, larger insects, or vertebrates that occur in the canopy (Appahna, 1991; Dick et al., 2008). This aligns with pollen gene flow data for *F. picrosperma*, where almost two thirds of pollination events occurred within 30 m of the mother tree (Grant et al., 2019; Chapter 4) and reflects the low light environment and dense foliage that occurs in the subcanopy.

More pollen is deposited on the stigma during daylight hours, however, both day and night visitors contributed to successful pollination. The individual constituents present in the floral bouquet of *F. picrosperma* are known to attract nocturnal

pollinators in other plant species (Knudsen et al., 1993; Raguso et al., 1996; Dotterl et al., 2012; Cordeiro et al., 2019). Diurnal observations of *F. picrosperma* flowers documented very few insect visitors other than Thysanoptera (thrips) compared to other studies (Boulter et al., 2005; Webber et al., 2008). The dearth in insect observations builds on the findings in our previous study that *F. picrosperma* is pollen limited (Grant et al., 2017; Chapter 2). Few insects were observed in this study visiting the flowers of both male and female trees, which was due in part to the lower level of pollinator discrimination against female flowers.

Thrips were among the greatest number of insects observed and collected from both male and female *F. picrosperma* flowers. However, the most frequent visitors may not be the most effective for plant fitness because of their differential role in pollen transfer. No thrips were captured in the scent lure traps and it is possible that these in-flora flower visitors move very little between trees (Boulter et al., 2006b). Thrips are known to dwell in the flowers and feed on the internal wall of the receptacle and pollen and contribute very little to cross pollination (Kondo et al., 2016). Their small body size means their movements can bypass the central stigma of female flowers (Irvine and Armstrong, 1990) and, combined with a weak flying ability, this could preclude thrips from efficient pollen transport because they are only able to carry small quantities of pollen. *Fontainea picrosperma* pollen grains are 40 µm in diameter (Grant et al., 2017; Chapter 2) and pollen grain size in plants pollinated by thrips is < 34 µm (Sakai, 2001). However, thrips were the most common flower visitor observed in this study and by sheer numbers could contribute to pollination and successful reproduction in *F. picrosperma*. In addition, thrips may indirectly contribute to pollination by attracting predators or parasites (Kondo et al., 2016). In this study, the specialist thrips parasitoid wasp, either a species of *Ceranisus* or *Thripobius* (Eulophidae), was significantly more attracted to the scent lure traps than the control traps with no scent. These wasps however are less than 1 mm in body length, and therefore may not contribute substantially to pollination. Other small parasitoid wasps from the families Encyrtidae, Scelionidae and Platygastridae were collected during our sampling of in-flora visitors. Scelionidae are often idiobionts and Platygastridae are koinobiont endoparasitoids that parasitise a variety of insects including those from the Coleoptera, Hemiptera and Diptera (Austin et al., 2005).

We observed and collected insects from other orders including Coleoptera, Lepidoptera, Diptera and Hemiptera. After Thysanoptera, beetles (Coleoptera) were the second most commonly observed and collected visitors to *F. picrosperma* flowers. Beetles play an important role in Australian rainforest pollination throughout the vertical strata (Worboys and Jackes, 2005; Kitching et al., 2007; Webber et al., 2008; Wardhaugh et al., 2015) and could pollinate up to one quarter of Australian tropical rainforest species (Irvine and Armstrong, 1990). *Fontainea picrosperma* flowers themselves may represent a resource for these taxa. For example, in other plant species beetles are known to feed on pollen as well as stigmatic secretions and other floral exudates, floral tissue, small arthropods and sometimes visit flowers to mate and/or lay eggs (Momose et al., 1998; Dobson, 2006). In our samples, we collected beetles from a variety of families visiting *F. picrosperma* flowers including Chrysomelidae, Mordellidae, Nitidulidae and two different species of Curculionidae. Three species of beetles were lured to the scent traps in this study, indicating they were attracted to the main scent constituents (or a combination thereof) of *F. picrosperma* flowers. Thus far, no reliable pattern in floral scent chemistry unifies plants pollinated by tropical beetles (Dobson, 2006). *Ictistygna* sp. (Anthicidae: Eurygeniinae) was the most abundant species captured in the scent lure traps. Adult anthicid beetles are omnivorous, being known to consume small arthropods and pollen and some species within the Family are known predators of stink bugs (Hemiptera: Pentatomidae) (Athey et al., 2019) and *Diaphania* spp. (Lepidoptera: Crambidae) (Júnior et al., 2012), though little is known about eurygeniine biology (CSIRO, 2019). The two other beetle species belonged to the Tenebrionidae, a family that characteristically feed on dead vegetable or animal matter and living plant tissue, although a few normally predacious species are known (Watt, 1974).

Tropical rainforests of north Queensland are rich in dipteran species (Austin et al., 2004) and flies are capable of transporting significant quantities of pollen, usually short distances (House, 1989; Weiss, 2001). Significantly more drosophilid flies were captured in the scent baited traps than in the control traps. However, it is unlikely that these small insects are efficient transporters of *F. picrosperma* pollen. In other plant species studied where drosophilid flies were among the most abundant insect visitors, they carried little or no pollen (Borchsenius et al., 2016). However, floral scent is a complex phenotypic trait, with diverse chemical compositions and relative amounts,

and each factor may affect the relationships between floral scent and pollinator (Raguso, 2008b). Interactions could be the result of the presence of single scent components, or combinations of scent compounds, in an additive or synergistic context. Finally, it is worth noting that the compounds in the scent lure did not include the full complement of compounds identified in the floral bouquet of *F. picrosperma*, which could have potentially biased our results.

This study provides further evidence for the assertion that generalised potential pollinators are relatively common in the subcanopy of the tropical rainforest (Momose et al., 1998b; Devy and Davidar, 2006). Other than thrips, insects that were observed visiting the flowers of *F. picrosperma* were small and infrequent. This is congruent with findings in our previous study of *F. picrosperma* that determined the species is pollen limited (Grant et al., 2017; Chapter 2) and pollen transfer in *F. picrosperma* is predominantly confined to neighbouring trees whereby almost two thirds of pollination events occurs with males within 30 m of the mother tree (Grant et al., 2019; Chapter 4). The clumped spatial distribution of *F. picrosperma* trees and random distribution of the sexes increases the chance that movements of the small and unspecialised pollinators between the trees will occur.

3.5.3 Conclusion

From our study we determined that *F. picrosperma* is likely pollinated by an array of small, generalist insects. Thrips and beetles were a common feature of the flower visitor assemblage. This is consistent with many other woody plants in Australian tropical and subtropical rainforest communities that are pollinated by small generalist insects and visitor assemblages in the subcanopy of rainforest communities elsewhere. The rainforest subcanopy environment presents a unique microclimate for pollinators. In this low light, low energy environment, female flowers of *F. picrosperma* exhibit cost saving strategies by not producing nectar and mimicking the smell of reward offering male flowers. Male and female flowers displayed the same pattern of floral scent emission, characterised by maximal emission at male flower dehiscence, maximal emission during female flower receptivity, and a decrease in the amount of scent compounds produced as the flower aged. Scent therefore is likely to regulate pollinator behaviour and is the primary attractant to the species in an environment characterised by darkness. The main scent constituents found in

F. picrosperma are ubiquitous in plant floral bouquets and likely makes them indiscriminate to specific taxonomic groups. Although thrips were the most abundant flower visitors, they are unlikely to contribute substantially to pollen transfer due to their small body size. However, the taxa could indirectly contribute to pollination by attracting thrip-feeding predators.

Fontainea picrosperma is pollen limited (Grant et al., 2017, Chapter 2), a conclusion supported by a dearth of insect visitors to flowers observed in this study. The species has low genetic diversity (Lamont et al., 2016), owing in part to predominant near neighbour mating (Grant et al., 2019, Chapter 4). Pollinators preferentially travel short distances between conspecific trees likely due to the opportunistic feeding patterns of small generalist insects in the challenging environment below the rainforest canopy with low light levels, low or turbulent wind speeds, relatively high humidity and physical barriers for pollinator movement due to dense foliage.

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Chapter 4: Short Distance Pollen Dispersal and Low Genetic Diversity in a Subcanopy Tropical Rainforest Tree, *Fontainea picrosperma* (Euphorbiaceae)

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4.1 ABSTRACT

Gene flow via pollen movement affects genetic variation in plant populations and is an important consideration in plant domestication. *Fontainea picrosperma* is a subcanopy rainforest tree that is of commercial interest because it is the source of tigilanol tiglate, a natural product used for the treatment of solid tumors. We identify patterns of pollen-mediated gene flow within natural populations of *F. picrosperma* and estimate genetic parameters and genetic structure between adult and juvenile groups using microsatellite markers. Our results show pollination events occur over much shorter distances than reported for tropical canopy species. At least 63 % of seeds are sired by male trees located within 30 m of the mother. On average, 27 % of the local male population contributed to successful reproduction of *F. picrosperma* with most fathers siring a single seed, however, the contributions to reproduction were uneven. Larger male trees with more flowers had greater reproductive success than those with less flowers ($P < 0.05$). There were comparatively low levels of genetic variation across the species ($H_E = 0.405$ for adult trees and 0.379 for juveniles) and we found no loss of genetic diversity between adult and juvenile trees. Short distance pollen flow and low genetic diversity is theoretically a prelude to genetic impoverishment, however *F. picrosperma* has persisted through multiple significant climatic oscillations. Nevertheless, the remaining low genetic diversity is of concern for domestication programs which require maximal genetic diversity to facilitate

efficient selective breeding and genetic improvement of this commercially significant species.

Key words: blushwood, understorey, paternity, gene flow, CERVUS

4.2 INTRODUCTION

Genes move within and among plant populations through pollen and seed dispersal as well as physical movement of vegetative plant material. Plant mating patterns in tropical rainforests, mediated by gene movement of pollen, is an important determinant in the level of genetic variation within and among populations. Gene flow can counteract the potentially detrimental effects of genetic drift and may be a source of new alleles within populations (Burczyk et al., 2004). However, when gene flow is restricted, inbreeding or biparental inbreeding (mating with close relatives) can occur and ultimately lead to a loss of genetic diversity, directional selection and genetic drift (Ellstrand and Elam, 1993). Tropical forest ecosystems are experiencing high rates of habitat destruction and forest fragmentation that can negatively impact on genetic variation within species (Bradshaw et al., 2009; Eckert et al., 2010). The modification of habitat can disrupt natural patterns of gene flow by creating environments that are stressful for pollinator survival and activity (Eckert et al., 2010). Though, the impacts to mating patterns vary between species and context (Hamrick, 2004; Lowe et al., 2015) and can be affected by a species life history, reproductive biology and the mobility of pollinators (Breed et al., 2015; Rymer et al., 2015; Vinson et al., 2015).

Most woody tropical rainforest species are strongly outcrossed and rely on insects for pollination (Bawa, 1992; Ollerton et al., 2011). The density of flowering conspecifics, including factors such as spatial distribution and the distance to the nearest pollen source can influence pollinator foraging behaviour (House, 1993; Ghazoul, 2005). Commonly, pollinator flight distances tend to increase with lower plant densities and decrease when flowering plants exhibit a clumped distribution or occur at high densities (House, 1992; Stacy et al., 1996; Hardy et al., 2006; Born et al., 2008; Ashley, 2010; Silva et al., 2011; Naoki et al., 2012; Duminil et al., 2016). This is because near-neighbour mating increases the foraging economy of the pollinating insect by maximising the net energy gained by the pollen or nectar source (Levin and Kerster, 1974; Degen and Sebbenn, 2016). There is strong empirical evidence that

suggests that tropical canopy species with low population densities exhibit long distance pollen dispersal (Akihiro et al., 2000; Kenta et al., 2004; Ward et al., 2005; Hardesty et al., 2006; Born et al., 2008; Carneiro et al., 2009; Ottewell et al., 2012; Monthe et al., 2017). While there are relatively few studies describing gene flow within understorey species, pollen has been found to disperse shorter distances in tropical or subtropical woody taxa, particularly in species with high local densities (Lasso et al., 2011; Castilla et al., 2016; Hahn et al., 2017).

Pollen-mediated gene flow, measured in terms of successful reproduction, is affected by other density measures such as phenology and its synchronicity, as well as the size of the nearest pollen source (O'Connell et al., 2018). Variation in these factors influence pollination distances, pollen dispersal patterns and levels of genetic isolation (Castilla et al., 2017). For example, large male trees (measured by species specific differences in diameter at breast height) can contribute disproportionately to seed production (Hoebee et al., 2007; Naoki et al., 2012; Tambarussi et al., 2015; Monthe et al., 2017; Younginger et al., 2017). Thus, the size of nearest pollen source becomes important because a large but more distant male may contribute more pollen than a smaller, closer one. Determining variations in male fecundity within populations is important because mating partners can influence genetic diversity and population fitness (Breed et al., 2012a). Unfit combinations of pollen and ovules are more likely to occur in offspring when fewer males contribute to reproduction. With more mating partners, there is a smaller probability that recessive deleterious alleles will be involved in reproduction (Breed et al., 2012b).

Historical gene flow can be inferred through the distribution of genetic diversity between generations of individuals (Slatkin, 1987; Young et al., 1996; Lowe et al., 2005). Genetic diversity and differentiation between age cohorts within populations are critical parameters that impact population genetics and structure, particularly in landscapes where habitat has been modified. This is because altering the number of reproductive individuals in the population can negatively impact genetic diversity and inbreeding levels in progeny due to disruptions in pollen diversity and pollinator mobility (Breed et al., 2015). Mating systems also contribute to the structure of genetic diversity (Hamrick and Godt, 1996) and while most tropical trees are facultative outcrossers, bi-parental inbreeding can occur in species with limited gene dispersal capabilities (Ellstrand and Elam, 1993; Collevatti et al., 2001a; Castilla et al., 2017).

Genetic variation, measured in terms of heterozygosity, is often significantly correlated with fitness (Reed and Frankham, 2003; Nutt et al., 2016). It is therefore important to assess both contemporary and historic gene flow patterns to identify the strength and spatial scale at which evolutionary forces act upon populations (Stockwell et al., 2003). This will aid management of species in the face of anthropogenic habitat modification and exploitation of forest resources.

Fontainea picrosperma C.T. White (Euphorbiaceae) is a dioecious, subcanopy tree endemic to upland tropical rainforests on the Atherton Tablelands, Queensland, Australia. The species is locally common but has a restricted natural range. The region has been subject to natural habitat fragmentation during the climatic fluctuations of the Plio-Pleiestocene that led to rainforest species retreating to moist refugia when climate conditions cooled and recolonised surrounding areas once climate conditions improved (Kershaw et al., 2007). *Fontainea picrosperma*'s current discontinuous distribution is due to anthropogenic habitat fragmentation primarily as a result of agricultural expansion, but also due to urban settlements. *Fontainea picrosperma* is the source of tigilanol tiglate (Boyle et al., 2014; Linkliter et al., 2015), a small molecule, natural product used for the local treatment of solid tumors in humans and companion animals (Linkliter et al., 2015; Panizza et al., 2019; Miller et al., 2019). Tigilanol tiglate is not amenable to a commercial total synthesis, so production of the drug on a commercial scale relies on raw material harvested from plantations of *F. picrosperma*. It is critical to understand the scale of realised gene flow across generations as well as the overall genetic diversity of the species throughout its natural range to optimise production through selective breeding and genetic improvement of planting stock.

Many studies have examined gene flow and population genetic structure in rainforest canopy species, however understorey species remain underrepresented. This study follows on from the work conducted by Lamont et al. (2016) who studied the population genetics of *F. picrosperma* across the species distribution. Here, we identified localised patterns of gene flow and estimated the population genetic structure between subpopulations of adults and juveniles in natural populations of *F. picrosperma* using microsatellite markers. Specifically, we asked (1) what is the distance of pollen-mediated gene flow and what proportion of seeds are sired by local males? (2) How many males contribute to progeny for each mother tree and how does

male reproductive fitness relate to paternal tree characteristics including flowering effort and location (direction and distance) relative to the mother tree? (3) What are the levels of genetic diversity in adult trees and juveniles in the population and what are the levels of genetic differentiation between generations? (4) Are individuals growing near to each other more related than expected from mating two random individuals?

4.3 MATERIALS AND METHODS

4.3.1 Study species and site

Fontainea picrosperma is a subcanopy tree to 25 m (Jessup and Guymer, 1985) endemic to the complex mesophyll and notophyll vine forests on the Atherton Tablelands, north Queensland, Australia. The species possesses small, white and fragrant flowers that have an unspecialised structure and an open access receptacle that are likely to be pollinated by small generalist insects (Grant et al., 2017). Flowering occurs simultaneously between individuals within subpopulations from September to November. The red drupaceous fruits (up to 3 cm diameter) ripen in December and January and are dispersed primarily by gravity. Secondary long-distance seed dispersal can occur either by hydrochory along drainage lines or zoochorous vectors (Cooper, 2004; Lamont et al., 2016). Natural stands of *F. picrosperma* therefore are not uniformly distributed within appropriate habitat, but rather form small, but dense clumps or clusters (2-10 m inter-tree spacing) with ~50:50 male: female ratios (Lamont et al., 2016; Grant et al., 2017). These clumps or clusters are often isolated from neighbouring clumps with no conspecific individuals found in between.

Data in this study were collected from trees in discrete populations from across the natural range of the species. The collection locations are labelled according to place names and are described in detail by Lamont et al. (2016). The populations included in this study were Boonjie, East Barron, Malanda, Topaz, Gadgarra, Towalla and Evelyn Highlands. We use the term subpopulation when more than one site, representing one clump or cluster, was sampled within a population.

4.3.2 Sample collection

Pollen-mediated gene flow and male fitness

We estimated pollen movement by direct paternity analysis using seedling cohorts of selected mother trees from across the *F. picrosperma* geographic range. Mother trees were selected based on the number of fruit that had matured and fallen at the time of sampling as well as their physical location within the discrete clump or cluster. A representative sample of fruit were collected from the base of 10 female trees (mother trees) from four populations (A156; A336; B27; B283; B595; B706; E17; J15; J169 and; J424) during the 2014/15 reproductive season. Very few seedlings survived in the nursery from two female trees and therefore we re-collected from two mother trees (A336 and B27) during the 2015/16 reproductive season (Table 4.1). The subpopulations of 'Evelyn Highlands 1' and 'Evelyn Highlands 2' are from the same refugial population approximately 3.5 km apart. Locations of all males within a 30 m radius of each of the mother trees were mapped using a compass and tape measure. This spatial range was selected because it represents the typical approximate scale of the local density estimates of discrete clumps or clusters of *F. picrosperma*. By sampling a 30 m radius around the mother tree, we estimated that we captured at least 90 % of individuals located within the clump. We sampled to 35 m around one mother tree, B283, to capture three males tree located just outside the sampling radius. Mother tree E17 was from a small, isolated population, Topaz, where every male individual was sampled and mapped (16 m radius).

Table 4.1 Sampling method used for each mother tree

Mother	*Population*	*Year fruit collected*	*Number of males sampled*	*Number of seedlings*
A156	Evelyn Highlands 1	2014/15	32	13
A336	Evelyn Highlands 1	2015/16	31	26
B27	Boonjie	2015/16	43	45
B283	Boonjie	2014/15	17	19
B595	Boonjie	2014/15	34	17
B706	Boonjie	2014/15	16	15
E17	Topaz	2014/15	6	19
J15	Evelyn Highlands 2	2014/15	40	20
J169	Evelyn Highlands 2	2014/15	44	20
J424	Evelyn Highlands 2	2014/15	37	20
Total			300	214

A leaf was sampled from each male and the mother tree for genetic analysis (Table 4.1). The height, diameter at breast height (dbh) and flowering effort were recorded for each male sampled. Flowering effort was determined by the number of inflorescences per tree and measured on a scale from 1-5 (1 = <10; 2 = 10-20; 3 = 20-50; 4 = 50-75; 5 = >75). Trees that were not flowering because they were juveniles were not considered as candidates and were not sampled. Twenty to 60 seeds (according to permit limitations) from each mother tree were sown in the nursery at the University of the Sunshine Coast (USC, Sippy Downs, QLD, Australia) where 13 to 44 seeds per individual germinated (Table 4.1). Leaf tissue from each of the germinated seedlings was collected for genetic analysis.

Genetic diversity and differentiation in adult trees and juveniles

We examined the genetic diversity of *F. picrosperma* and genetic differentiation between age cohorts using 187 adult trees (height > 2.5 m) and 122 juveniles (height ≤ 2.5 m) from nine *F. picrosperma* populations in the 2012-2013 reproductive season (Table 4.2). For each sampling site, a focal point was randomly selected, and trees were sampled in an expanding radius circling the focal point. The radius expanded to a maximum of 50 m, more typically 30 m, which captured at least 90 % of all trees within the clump or cluster. Each sampling site represents one subpopulation. The number of samples from each subpopulation were dependent on-site characteristics including the numbers and density of individuals present. Three subpopulations (focal points) were chosen from across the Boonjie population (Table 4.2) because Boonjie is the largest population of *F. picrosperma*. The subpopulations, 'Boonjie 1 and Boonjie 2' are approximately 400 m apart. 'Boonjie 3' is approximately a further 3.5 km east. The three subpopulations are within continuous rainforest but represent discrete clumps.

Table 4.2 Number of adult and juvenile *Fontainea picrosperma* individuals sampled from each population

Population	A_i	J_i
Evelyn Highlands	24	4
Boonjie 1	26	17
Boonjie 2	38	27
Boonjie 3	17	14
Malanda	12	19
Topaz	13	6
Gadgarra	14	16
East Barron	24	6
Towalla	19	13
Total	163	118

Ai is the number of adults (> 2.5 m) sampled, Ji is the number of juveniles (< 2.5 m) sampled in each population

4.3.3 DNA extraction and microsatellite analysis

Genomic DNA was extracted from silica-dried leaf tissue using the DNeasy™ 96-well kit or the DNeasy™ Plant Mini Kit (Qiagen, Valencia, California, USA, Hilden, Germany) following the manufacturer's instructions. Eleven polymorphic microsatellite loci (between 2 and 7 alleles per locus), previously developed and optimised for *F. picrosperma* (Agostini et al., 2013), were used to genotype all sampled individuals following the method detailed in Lamont et al. (2016). A total of 281 *F. picrosperma* individuals (adults and juveniles) were genotyped for the genetic diversity and differentiation study using the 11 loci. An additional six microsatellite loci with consistent PCR amplification, clear allelic variation, and clarity of electrophoretic signatures were developed for *F. picrosperma* and used in the paternity analysis (Supplementary Data Table 4.1). These loci were developed to increase the discriminatory exclusion power of the paternity analysis. A total of 524 individuals comprised of mother trees, seedlings and candidate father trees were genotyped for the paternity analysis.

The forward primer of each locus was direct-labelled with a fluorescent dye (VIC, PET, FAM, NED). Two multiplex PCR pools (Pool 1: FP38, FP68, FP84; Pool 2: FP66, FP69, FP82) were amplified using the Multiplex PCR Plus Kit (Qiagen). Forward and reverse primers for each multiplex pool were combined in a 10× primer mix. Reactions, with volumes adjusted to 10 μL, each contained 1.25 μL of 10× primer premix, 6.25 μL of Qiagen Multiplex Buffer, 3 μL of ddH2O, and 2 μL of template

gDNA. Where samples had to be repeated, single PCR reactions, with volumes adjusted to 10 μL, each contained 0.3 μL forward and 0.3 μL reverse primer, 8.425 μL of ddH2O, 1.5 μL PCR reaction buffer, 1.2 μL dNTP, 1.2 μL MgCl2, 0.075 μL Taq DNA Polymerase and 2.0 μL of template gDNA. Amplification for the microsatellite loci was performed using an Eppendorf Mastercycler (Hamburg, Germany) with cycling conditions as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s with a final extension at 72 °C for 10 min. PCR products were separated by capillary electrophoresis on an AB 3500 Genetic Analyser (Applied Biosystems). Fragment sizes were determined relative to an internal lane standard (GS-600 LIZ; Applied Biosystems) using GENEMARKER v. 2.4.0 (SoftGenetics LLC, PA, USA) and double-checked manually. A subset of samples were run a second time to assure accuracy of genotype reads and minimise the risk of non-amplifying alleles. Individual loci with low intensity or missing peaks were also amplified and genotyped a second time, after which, if they failed to amplify, they were included as missing data. 97.19 % and 99.76 % of all loci was successfully amplified and scored for the paternity analysis and for the genetic diversity and differentiation study, respectively.

4.3.4 Statistical analysis

Pollen-mediated gene flow and male fitness

We used CERVUS 3.0.3 (Kalinowski et al., 2007) to perform paternity analysis on seedling cohorts from 10 mother trees using 17 loci. CERVUS uses maximum likelihood for statistical evaluation of progeny-parent pairs (Oddou-Muratorio et al., 2003). Offspring genotypes that conflicted with the assumed mother tree genotypes were excluded before assigning paternal parents. These conflicts arose because seeds were collected from under the canopy of the presumed mother trees and in some instances, the presence of proximate female conspecifics led to field-based misallocation of maternal parents. We ran the program based on the multilocus genotypes of each mother tree and their associated seedlings and the candidate father trees. Simulations on paternity were run using the following parameters: 100,000

simulated offspring, the proportion of mistyped loci was set at 0.01, and the proportion of candidate fathers sampled was estimated at 0.90.

Critical Delta values were obtained from simulations and used as a criterion for parentage assignment. We compared trio Delta scores to assign the father with 'strict' (> 80 %) and 'most likely' (< 80 %) confidence levels. If the seedling received a negative LOD score, no paternal parent was sampled, and the seedling's father was left unassigned. Trio LOD scores were used to determine if there was more than one equally-likely male candidate (equal LOD scores). Two male candidates for each of two seedlings from the mother tree J15 received an equal LOD score and thus were assigned joint paternity. These fathers were allocated a score of 0.5 each for the sired seedling and allocated the 'most likely' confidence level. We manually checked the CERVUS assignments and none of the paternity assignments ('strict' or 'most likely') had greater than one mismatch between parent pairs and offspring genotypes (i.e. no greater than one trio mismatches).

Null alleles and allelic drop out are likely to occur in microsatellite studies (Ashley, 2010). However, simulation studies have shown that parentage assignment using likelihood-based parentage techniques are robust against Type II errors, that is, when a true parent is excluded due to mistyping at one or more loci (Oddou-Muratorio et al., 2003). Nevertheless, MICRO-CHECKER v2.2.3 (van Oosterhout et al., 2004) was used to check for scoring errors, homozygote excess, large allele dropout and potential null alleles based on 1000 bootstraps. There was no evidence of homozygote excess, scoring errors, large allele dropout or null alleles.

We calculated the maximum pollen immigration rate as the percent of progeny that could not be assigned a father at any confidence level (negative LOD score). We then calculated the conservative minimum pollen immigration rate as the percent of progeny that could not be assigned a father with 'strict' (> 80 %) confidence. *Fontainea picrosperma* is a dioecious species and so the parentage analysis can determine the pollen dispersal distance for each mating event. The spatial position of all candidate fathers was recorded and used to estimate the average distance of pollen dispersal. We compared the frequency distribution of the distances among putative male parents with the frequency distribution of the realised pollination using the Kolmogorov-Smirnov test (K-S test; Sokal and Rohlf, 1995) implemented in 'R' (R

Development Core Team, 2013) to determine if mating success was a function of distance between male trees and mother trees.

We assessed the relationship between paternal tree characteristics and male (reproductive) fitness. Individual male fitness was determined by the proportion of seeds sired by a given male on a mother tree. We used both 'strict' (n = 135) and all CERVUS assignments (n = 176) in two separate analyses. Paternal characteristics: height, dbh and flowering effort were significantly autocorrelated (Spearman's Rank correlation; Height x dbh $_{rs}(309) = 0.747$, P = 0.001; Height x Flower count $_{rs}(309) = 0.729$, P = 0.001; dbh x Flower count $_{rs}(309) = 0.789$, P = 0.001). Therefore, we tested for differences in number of seeds sired between categories of flowering effort using a Kruskal-Wallis H test with Bonferroni correction for multiple comparisons. The distances between the mother tree and candidate father trees were grouped into five metre intervals (0-5; 6-10; 11-15; 16-20; 21-25; 26-35 m). We then used a Spearman's Rank correlation to determine the relationship between male fitness and distance to the mother. To determine if the movement of pollinators is influenced by prevailing winds the direction of the candidate male to the mother tree was grouped into eight categories (representing 45°) and then analysed for each mother tree separately using a Spearman's Rank correlation. All inferential analyses were performed using SPSS (IBM SPSS, 2016).

Genetic diversity and differentiation in adult trees and juveniles

The original 11 microsatellite loci reported by Lamont et al. (2016) were used in the genetic diversity and differentiation analysis of adult and juvenile subpopulations. GenAlex 6.5 (Peakall and Smouse, 2012) was used to calculate the mean number of alleles per locus (N_A) and expected heterozygosity (H_E) at Hardy-Weinberg equilibrium for adults and juveniles from nine subpopulations. Allelic richness (A_R) and private allelic richness (P_{AR}) was estimated using HP RARE (Kalinowski et al., 2005) using a minimum sample size of eight. The average pair-wise level of genetic differentiation (F_{ST}) was calculated using multilocus comparisons based on 999 permutations to quantify the partitioning of genetic differentiation between adult trees and juveniles in each subpopulation using GenAlex 6.5 (Peakall and Smouse, 2012).

Statistical comparisons were carried out in IBM SPSS 24 (IBM SPSS, 2016) to determine whether there were significant differences in diversity (H_E, A_R, P_{AR}) between the adult trees and juveniles for each subpopulation. All data sets did not meet the assumptions of parametric tests and were subsequently compared using Mann-Whitney U tests.

We calculated mean within group pairwise genetic relatedness (r) for each subpopulation of adults and juveniles sampled using Lynch and Ritland (1999) (lxy) estimator in GenAlex (Peakall and Smouse, 2012). Significant differences in mean relatedness were tested using 9999 permutations and 9999 bootstrap resamplings to calculate the upper and lower 95 % confidence intervals for the expected range of lxy based on the sampled population and within subpopulation estimates of mean relatedness. Subpopulation lxy values that fall above the 95 % expected values from permutations indicate a higher degree of relatedness that expected from random mating across the sampled population.

4.4 RESULTS

4.4.1 Pollen-mediated gene flow and male fitness

From the sampled population, 135 (63.1 %) of the 214 individual seedlings tested could be assigned paternity with 'strict' confidence (Table 4.3). An additional 41 (19.2 %) seedlings were assigned a father when considering the 'most likely' CERVUS assignments (Table 4.3). The males assigned as the 'most likely' father had zero (87 %) or one (13 %) loci mismatch. The 'most likely' father could not be assigned paternity with 'strict' confidence because the second (or more) 'most likely' father also had zero or one mismatches and thus the delta score was close to zero. Therefore, we believe that false mismatches have occurred and up to 82.3 % of fathers could be assigned to offspring when accounting for all CERVUS assignments ('strict and most likely'). However, our sampling method of a 30 m radius surrounding the mother tree can potentially downwardly bias the results of the 'most likely' father assignments towards short distance pollen flow. As such, the results of the 'most likely' CERVUS assignments must be received with caution.

Table 4.3 Results of the CERVUS analysis of pollen dispersal for the sampled *Fontainea picrosperma* mother trees, showing the number of offspring that had fathers assigned with "strict" (> 80 %) and "most likely" (< 80 %) confidence levels, and those that were unable to be assigned. Number of fathers for assigned seedlings of each *F. picrosperma* mother tree showing the number of male candidates which could be assigned paternity under conditions of "strict" confidence and the total number able to be assigned ("strict" and "most likely")

Mother	*n*	*Number of seedlings assigned parentage*			*Number of fathers*	
		Strict (%)	*Most likely (%)*	*Unassigned (%)*	*Strict*	*All assignments*
A156	13	6 (46.1)	3 (23.1)	4 (30.8)	5	7
A336	26	19 (73.1)	3 (11.5)	4 (15.4)	13	16
B27	45	34 (75.6)	0	11 (24.4)	18	18
B283	19	13 (68.4)	0	6 (31.6)	7	7
B595	17	15 (88.2)	0	2 (11.8)	10	10
B706	15	15 (100)	0	0	5	5
E17	19	13 (68.4)	0	6 (31.6)	4	4
J15	20	7 (35)	12 (60)	1 (5)	7	16
J169	20	4 (20)	16 (80)	0	4	14
424	20	9 (45)	7 (35)	4 (20)	8	14
Total	214	135 (63.1)	41 (19.2)	38 (17.7)	81	111

Percentages of each category in relation to the total number of seedlings sampled from each mother tree is shown in parentheses. n is the number of seedlings

The total mean pollen immigration rate from greater than 30 m was 36.9 % (maximum pollen immigration rate; n = 79; Figure 4.1a) when considering fathers that could be assigned with 'strict' confidence and 17.7 % (minimum pollen immigration rate; n = 38) when considering all CERVUS assignments (Figure 4.1b).

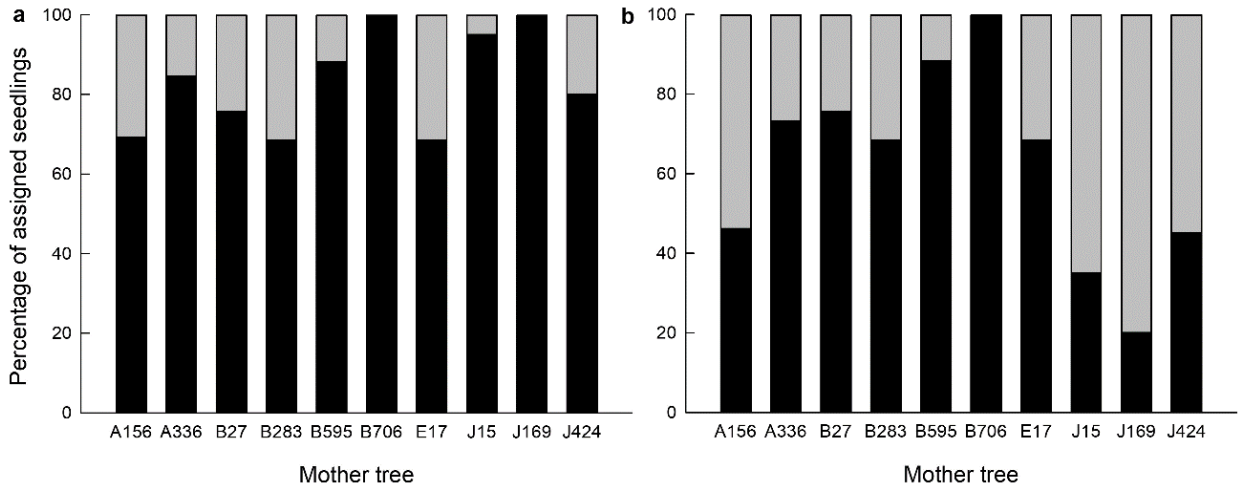

Figure 4.1 Percentage of local and immigrant pollinations for each mother tree (number of seedlings per tree: A156 = 13; A336 = 26; B27 = 45; B283 = 19; B595 = 17; B706 = 15; E17 = 19; J15 = 20; J169 = 20; J424 = 20). (a) All assignments ('strict and most likely') are shown in black and unassigned seedlings (minimum pollen immigration rates) are shown in grey. (b) 'Strict' assignments (seedlings assigned with > 80 % confidence) are shown in black and seedlings assigned with < 80 % confidence (maximum pollen immigration rates) are shown in grey.

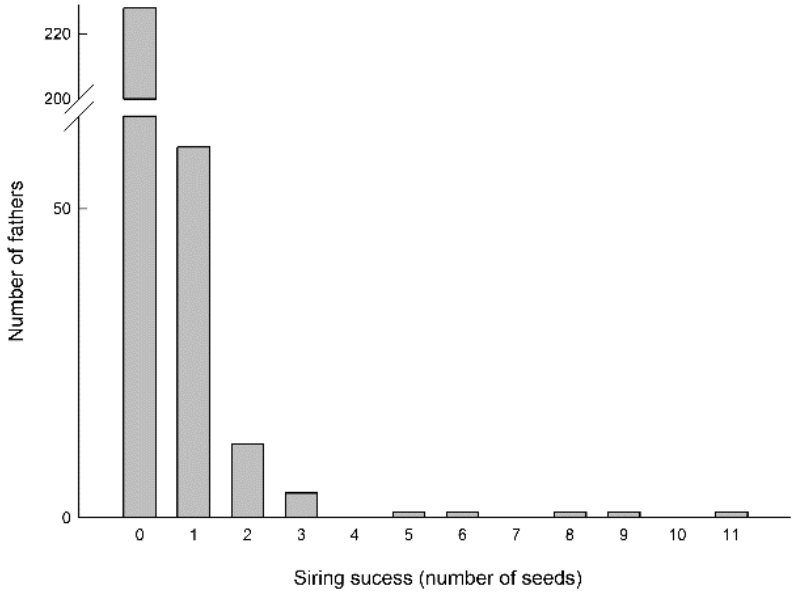

Figure 4.2 Total number of candidate fathers vs number of seedlings assigned with 'strict' confidence.

We found that 27 % (n = 81) of the 300 candidate fathers sampled, sired seedlings from the 10 mother trees tested (Table 4.3), when using 'strict' CERVUS assignments (cf. 37 % of candidate fathers for 'all' CERVUS assignments). Mother tree E17 had one of the lowest number of fathers for the seedling cohort sampled due to the low number of available candidate fathers in the small, isolated population. Most of the assigned fathers sired only a single seed (Figure 4.2), and these seeds represented 74.1 % of the total assigned offspring (cf. 74.3 % for 'all' CERVUS assignments). In contrast, one or two males sired greater than 10 % of the total progeny for each mother tree (Figure 4.2).

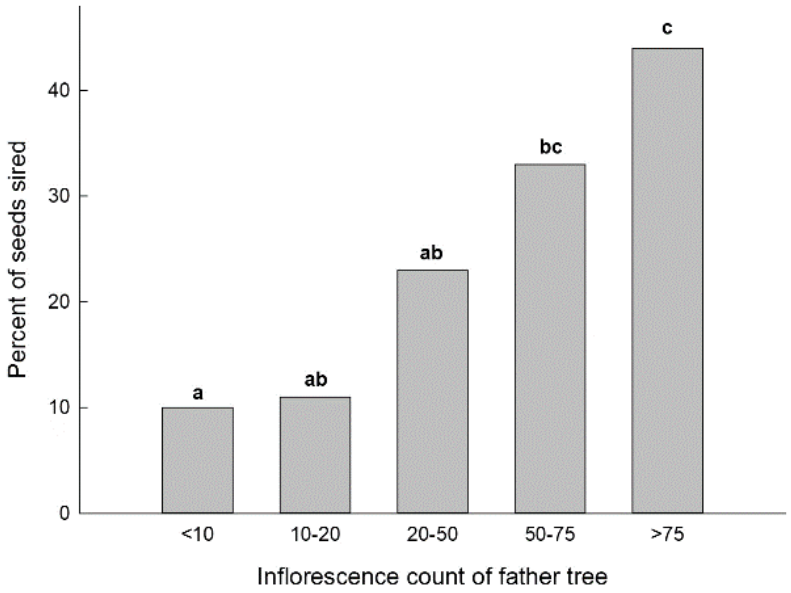

Figure 4.3 Percentage of seeds per mother tree sired by fathers relative to male inflorescence number (assigned with 'strict' confidence). Categories with different letters are significantly different ($P < 0.05$, Kruskal-Wallis H test, Stepwise step-down Bonferroni correction).

Siring success was significantly higher for candidate fathers with more flowers (X^2 (4) = 16.74, P = 0.002, Figure 4.3). No significant relationship existed between the direction of the assigned fathers to the mother trees ($P > 0.05$) for all but one mother tree, J424 ($_{rs}(37) = -0.351$, P = 0.033), thus wind direction is unlikely to influence the movement of pollinators that results in successful reproduction. The relationship between the number of offspring sired by pollen donors and the distance between the

maternal and paternal trees was not significant ($_{rs}(363) = -0.080$, P = 0.127) for 'strict' assignments. The frequency curve of pollen dispersal was significantly different to the frequency curve of distance measured among all male trees relative to the respective 10 mother trees (Kolmogorov–Smirnov test (D = 0.14743, P = 0.03367) suggesting a non-random distribution of pollen distances (Figure 4.4). The median pollen distance was 15 m for both 'strict' and 'all' CERVUS paternity assignments (Figure 4.4).

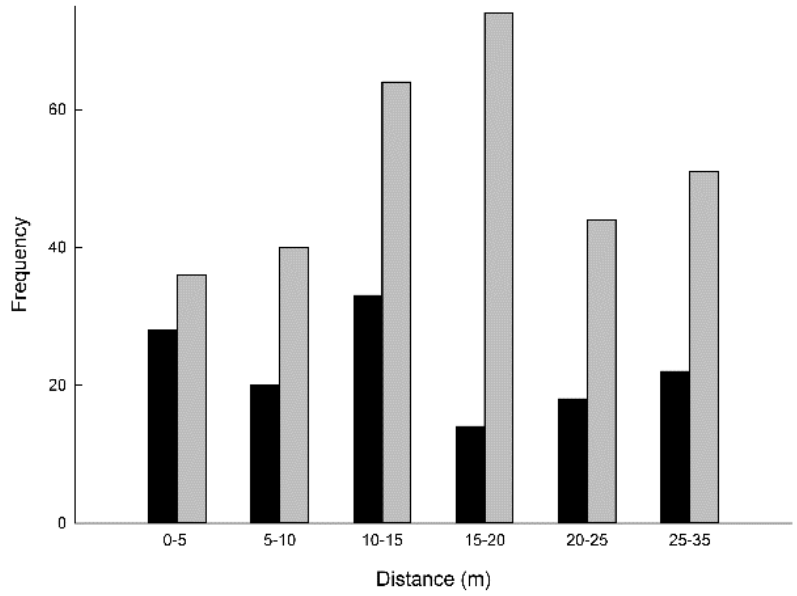

Figure 4.4 Frequency distributions of pollen dispersal distances for seeds assigned with 'strict' (> 80 %) paternity. Black bars represent inter-tree distances between the mother tree and male trees with successful pollination events. Grey bars represent the distance between the mother tree and all candidate male trees that were sampled.

4.4.2 Genetic diversity and differentiation in adult trees and juveniles

Moderately low levels of genetic diversity were detected, with a total of 43 and 41 alleles resolved across the 11 microsatellite loci used in the analysis of the 187 adults and 122 juveniles, respectively. Mean number of alleles per locus per subpopulation was 2.687 for adult trees and 2.384 for juveniles (Table 4.4). Mean expected heterozygosity (H_E) for adult trees was 0.405 and 0.379 for juveniles (Table 4.4).

No significant differences were found in the mean expected heterozygosity (H_E), allelic richness (A_R) and private allelic richness (P_{AR}) between adult and juvenile subpopulations ($P > 0.05$). Pairwise population F_{ST} values also displayed negligible genetic differentiation between generations (Table 4.4).

We found that the mean pairwise relatedness (r) of individuals within adult and juvenile subpopulations were significantly higher ($P > 0.05$) than the simulated confidence intervals for all subpopulations. This indicates that individuals within subpopulations have significantly higher measures of relatedness than expected from mating from two random individuals.

Table 4.4 Comparison of summary genetic measures between 187 adult and 122 juvenile *Fontainea picrosperma* sampled from nine subpopulations

Subpopulation	*n*	N_A	H_E	A_R	PA_R	F_{ST}	*r*
Adult subpopulations							
Evelyn Highlands 1	24	2.636 (0.31)	0.367 (0.06)	2.1	0.05	0.023	0.179
Boonjie 1	29	3.182 (0.35)	0.517 (0.04)	2.52	0.03	0.018	0.042
Boonjie 2	38	3.000 (0.27)	0.476 (0.03)	2.29	0.1	0.015	0.059
Boonjie 3	17	2.727 (0.20)	0.424 (0.04)	2.2	0.01	0.025	0.140
East Baron	24	2.818 (0.35)	0.405 (0.06)	2.14	0.09	0.001	0.155
Malanda	12	2.545 (0.28)	0.448 (0.05)	2.26	0	0	0.120
Topaz	13	2.818 (0.38)	0.373 (0.05)	2.2	0.02	0.011	0.159
Gadgarra	14	2.091 (0.21)	0.257 (0.06)	1.73	0	0.003	0.285
Towalla	19	2.364 (0.28)	0.377 (0.05)	2.03	0	0	0.133
Mean		**2.687 (0.11)**	**0.405 (0.02)**	**2.16**	**0.03**	**0.011**	-
Juvenile subpopulations							
Evelyn Highlands 1	4	1.818 (0.24)	0.307 (0.08)	1.82	0	-	0.224
Boonjie 1	17	2.545 (0.21)	0.447 (0.03)	2.15	0.03	-	0.160
Boonjie 2	27	2.909 (0.25)	0.453 (0.04)	2.26	0.04	-	0.061
Boonjie 3	14	2.545 (0.21)	0.386 (0.05)	2.09	0	-	0.119
East Baron	6	2.000 (0.19)	0.340 (0.06)	1.91	0	-	0.189
Malanda	19	2.545 (0.25)	0.436 (0.06)	2.23	0.01	-	0.092
Topaz	6	2.455 (0.34)	0.360 (0.07)	2.17	0.05	-	0.164
Gadgarra	16	2.364 (0.24)	0.326 (0.06)	1.9	0.02	-	0.211
Towalla	13	2.273 (0.19)	0.359 (0.06)	1.96	0	-	0.166
Mean		**2.384 (0.11)**	**0.379 (0.02)**	**2.05**	**0.02**	**-**	**-**

n is the number of individual plants; N_A is the mean number of alleles per locus, H_E is the expected heterozygosity, A_R is the allelic richness, P_{AR} is the private allelic richness, F_{ST} is the genetic differentiation among populations, *r* is the average pairwise relatedness within subpopulations

4.5 DISCUSSION

4.5.1 Pollen-mediated gene flow and male fitness

Our study has detected short distance pollen flow in the subcanopy rainforest tree, *F. picrosperma*. We observed that many males contributed to reproduction and most fathers sired a single seed on the mother tree. Large males with high flowering intensity had a disproportionally higher reproductive success and at least two thirds of successful mating events occurred with male trees located within a 30 m radius of the mother tree.

Our findings demonstrate that pollen dispersal in *F. picrosperma* in the subcanopy occurs over short distances compared to many insect-pollinated canopy trees (Akihiro et al., 2000; Kenta et al., 2004; Ward et al., 2005; Hardesty et al., 2006; Born et al., 2008; Monthe et al., 2017). The long dispersal distances reported for canopy species are partly because these taxa generally occur at low population densities. If only distant trees are flowering, pollinators must travel long distances to locate resources. While the breakdown of nearest-neighbour mating can occur (Dick et al., 2008), pollen dispersal patterns of many insect-pollinated tropical trees are influenced by preferential visitations to close neighbouring trees (Silva et al., 2011; Theim et al., 2014; Noreen et al., 2016). The short distance pollen dispersal we observed for *F. picrosperma* can be partly attributed to the species clumped distribution and synchronous flowering. Pollinators are preferentially visiting trees within the clump of *F. picrosperma* such that on average, two-thirds of the successful reproductive events occurred within a 30 m radius of the mother tree. Other studies of rainforest understorey and subcanopy species have also found a high proportion of short distance pollination events, for example, in *Piper* shrub spp. that have high density, aggregated populations (Lasso et al., 2011), and *Rhododendron simsii*, that have highly synchronous flowering events (Hahn et al., 2017). Many males contributed to successful reproduction of individual *F. picrosperma* females with approximately 75 % of the assigned fathers siring a single seed. Together, these findings conform to the theory of density-dependent animal pollination which assumes that tree species occurring at low densities receive pollen from fewer individuals than trees in denser populations, where dispersal distances are lower (Murawski and Hamrick, 1991; Bianchi and Cunningham, 2012; Castilla et al., 2017).

Spatial considerations as well as the attractiveness of floral displays of individual trees are important factors in determining pollinator-assisted gene flow (Barrett and Harder, 1996; Degen and Roubik, 2004; Duminil et al., 2016). Our results show that while many male trees sired seeds, the contributions to reproduction were uneven. Consistently, only one or two males were responsible for a proportionally greater number of successful fertilisation events across all 10 mother trees. As expected, large males displaying high intensity flowering had significantly greater reproductive success than males with less flowers. Flower count was autocorrelated with tree stem diameter size (dbh) in our study, this finding is congruent with other studies of tropical trees where dbh size class was positively correlated with mean individual fecundity (Latouche-Hallé et al., 2004; Naoki et al., 2012; Monthe et al., 2017). Greater biomass has been found to result in greater male fitness in many plant species (Younginger et al., 2017), and may help to explain the skewed number of fathers found to be siring progeny in *F. picrosperma*.

In addition, plant-pollinator relationships can influence pollen dispersal distances. The floral structure of *F. picrosperma* suggests that it is likely to be pollinated by small generalist insects (Grant et al., 2017). The predominant floral visitors in Australian tropical forests are small insects, particularly beetles, flies, small bees and thrips (Irvine and Armstrong, 1991; Gross, 2005). This class of generalist pollinators are known to visit unspecialised flowers and often move shorter distances compared to specialised insects, larger insects, or vertebrates (Dick et al., 2008). The 18-37 % pollen immigration rate suggests that some pollinators of *F. picrosperma* are able to transport pollen greater than 30 m. However, it remains unclear how far pollen can travel beyond this radius. We used direction to the mother tree as a proxy for prevailing wind conditions and did not find any significant correlation between mother trees and the direction of the pollen donors within the sampled plot area. This is presumably because of the lack of wind and/or turbulent wind patterns under the dense rainforest canopy. Gene flow in *F. picrosperma* is also limited by the transport of pollen by pollinators (Grant et al., 2017), which further reduces opportunities for long-distance pollen flow.

While the mean pollen dispersal distance herein is potentially underestimated due to our sampling method that aligned with the approximate clump size, we could confidently assign 63 % of successful pollen donors from within the plot. Distance

between the mother tree and the pollen source was not significantly correlated. This statistic is somewhat confounded by the fine (~30 m) scale of this study and given the localised pollination rate, distance, when analysed over a larger scale, is likely to be an important factor in reproductive success. Particularly given that *F. picrosperma* is dioecious and pollen-mediated gene flow is not restricted by self-pollination. Our results implying predominantly short distance pollen dispersal suggests that cultivation of the fruit will require a significant number of males within the carefully designed plantation to increase efficient pollination.

We believe the maximum pollen immigration rate (37 % greater than 30 m) observed in this study is conservative due to the low genetic diversity of *F. picrosperma* and subsequently low discriminatory power of the microsatellite markers used in this study. False negatives are likely to have occurred as a great majority (87 %) of the males assigned the 'most likely' father had zero loci mismatches with the offspring when accounting for the mother's genotype. Thus, localised pollination events could be up to 82 % when accounting for all paternity assignments. However, it is important to highlight that the fine scale of our study has the potential to downwardly bias the calculated dispersal range estimates when accounting for fathers assigned as 'most likely'. Moreover, our results are reflective of a single reproductive year and we acknowledge that pollinator composition can change over space and time (Dick et al. 2003; Kenta et al. 2004) and that natural flower and seed production can be influenced by natural climatic variations between years, all of which can produce different patterns of gene flow.

4.5.2 Genetic diversity and differentiation in adult trees and juveniles

The genetic diversity measures reported in this study are congruent with similar populations of *F. picrosperma* reported by Lamont et al. (2016) and for the related species *F.* rostrata (Conroy et al., 2019). Summary measures of genetic diversity were almost identical for both *F. picrosperma* adult and juvenile cohorts (H_E, A_R, P_{AR}; $P > 0.05$). Measures of diversity such as overall allelic richness (2.16 in adult trees and in 2.05 juveniles) and expected hetrozygosity (H_E) (0.405 for adult trees and 0.379 for juveniles) are relatively low when compared to microsatellite-based studies on tropical rainforest tree species reported elsewhere. For example, reports of H_E in outcrossing rainforest taxa range between 0.732 and 0.907 (Collevatti et al., 2001b; Naito et al.,

2005; Carneiro et al., 2009; Sebbenn et al., 2011; Melo and Franceschinelli, 2016; Monthe et al., 2017) while self-compatible species (H_E = 0.629 - 0.797; Latouche-Hallé et al., 2004; Tani et al., 2009), and species surviving in highly fragmented populations (H_E = 0.662 - 0.701; Gaino et al., 2010; Wang et al., 2014) are still considerably higher than *F. picrosperma*. From the limited studies available, the genetic diversity of *F. picrosperma* is more akin to other Australian rainforest tree species including *Elaeocarpus angustifolius* (H_E = 0.61) and *E. largiflorens* (H_E = 0.54; Rossetto et al., 2007) and species with reported low genetic diversity due to mechanisms such as asexual reproduction (Rossetto et al., 2004; Rossetto and Kooyman, 2005; Thurlby et al., 2012).

Genetic variation is often significantly related to population fitness and hence, the evolutionary potential of a species (Reed and Frankham, 2003). Yet *F. picrosperma* has successfully persisted through historical environmental changes with low genetic diversity (Lamont et al., 2016). Euphorbiaceae is a family that exemplifies the post-Cretaceous diversification of the Australian rainforest flora and *Fontainea* first appears in the fossil record in the early Tertiary period (Williams and Adam 2010). Expansion and contraction of *F. picrosperma* populations during the climatic oscillations of the Pleistocene over the last 230,000 years (Kershaw et al., 2007) has likely reduced the level of genetic variation within the species, as found in this study and by Lamont et al. (2016), compared to other plant species in biomes with more diverse topography and greater elevation range (Broadhurst et al., 2017). These processes have led to low genetic diversity in other upland taxa in the Australian tropics, such as *Elaeocarpus* spp. (Rossetto et al., 2009). Species with natural restricted geographical range are also usually less genetically diverse than more widespread species (Arguilar et al., 2008). Habitat fragmentation and degradation that has occurred in the region since European settlement could also have contributed to a loss of genetic diversity due to a reduced number of local and immigrant pollen sources in some populations (Sork and Smouse, 2006).

Spatially limited pollen and seed gene dispersal is known to increase the likelihood of similar genotypes mating with each other (Seidler and Plotkin, 2006; Ellstrand, 2014). We found evidence that individuals were significantly more related than is expected between two random individuals within the adult and juvenile subpopulations studied. These results are concordant with the predominantly short

distance pollen flow indicated from our paternity analysis. Theoretically, selfing and mating between close relatives will increase differentiation among populations (FST) by increasing inbreeding (Duminil et al., 2009) and reducing genetic variation at the population level (Loveless and Hamrick, 1984). However, we found a lack of genetic differentiation between *F. picrosperma* adults and juveniles (represented by a low F_{ST} value), despite the adult cohort representing a larger sample of the total available genetic diversity due to a greater number of overlapping generations present than in the juvenile group. The 18-37 % pollen immigration rate (greater than 30 m) estimated by paternity assignments could contribute to the lack of differentiation between age cohorts. Only low levels of gene flow are necessary to counteract opposing mutation, drift and selection (Ellstrand, 2014). Immigrant pollen can connect populations through gene flow and some empirical evidence has suggested that long distance pollination events attenuate genetic decline due to drift and inbreeding in isolated populations (Ashley, 2010). While we do not know how far pollen can travel, Lamont et al. (2016) found recent bottlenecks with subsequent founder effects in two isolated populations of *F. picrosperma*, Malanda (centrally located) and Gadgarra (North-East). This implies that pollen may not travel long distances from the refugial populations of Boonjie and Evelyn Highlands, which are located in the east and west peripheries of the species natural distribution (Lamont et al., 2016).

The lack of genetic differentiation between adult and juvenile groups observed in this study remains consistent with Lamont et al. (2016) who found negligible levels of inbreeding within adult populations of *F. picrosperma*, which is expected in a dioecious species. Deleterious alleles may be purged through an increase in mortality in inbred individuals and survivorship of those composed of half or unrelated siblings (Hufford et al., 2003; Naito et al., 2005; Tambarussi et al., 2017). This is a common pattern in long-lived species (Duminil et al., 2009), such as *F. picrosperma*. In addition, the large number of males contributing to reproduction of a single seed found in this study suggests that heterogenic pollen pools are received on flowering females. This can maintain variability, reduce the occurrence of full sibling progeny arrays and dilute the effects of kin mating (Breed et al., 2012b). This result may reflect the synchronous flowering and high density of available fathers surrounding the mother tree. However, not all candidate fathers sired offspring. We acknowledge that

inbreeding depression can affect initial seed set as well as plant growth (references within Angeloni et al., 2011), which was not studied here.

More than half of the mating events in *F. picrosperma* occur over very limited spatial scales. Population genetic theory suggests that restricted gene flow among populations results in population differentiation and allows populations to evolve independently in response to genetic drift or local natural selection (Slatkin, 1987; Ellstrand, 1992), which has been demonstrated empirically in some tropical understorey species (Lasso et al., 2011; Theim et al., 2014). In comparison, low genetic differentiation among tropical tree populations has been interpreted as evidence of continuous long-distance, historical gene flow (Dick et al., 2008). *Fontainea picrosperma* is a species with low interpopulation F_{ST} (Lamont et al., 2016), however, in the context of a low genetic diversity, the short distance pollen dispersal is likely to have contributed to the significant, albeit weak population structuring across *F. pirosperma*'s natural distribution. The observed low levels of genetic differentiation and genetic diversity between adult and juvenile cohorts, and the determination that proximate plants are significantly related to each other suggests that this species is highly adapted to its environment, short distance pollen flow does not affect the species capacity to persist in the environment, and there is sufficient long-distance gene flow to keep the level of genetic diversity stable across the species distribution. Future cultivation of the species however, may benefit from mixing genetically dissimilar stocks from across *F. picrosperma*'s natural distribution as a means of increasing allelic diversity.

4.5.3 Conclusion

Our results reveal spatially limited gene dispersal in the subcanopy species, *F. picrosperma*. Size and flowering effort are more important than distance in determining male fitness at the fine-scale focus in this study. The species' short-distance gene dispersal and skewed success rate of pollen donors is potentially a prelude to genetic impoverishment. However, adult and juvenile subpopulations have similar multilocus genotypes and there is no evidence of an intergenerational loss of diversity. Furthermore, *F. picrosperma* has survived several significant climatic oscillations through the Pleistocene, likely by persisting in refugia offering a more stable environment and is likely that the low genetic diversity observed in

F. picrosperma is an indication of its significant adaption to local environmental conditions. *Fontainea picrosperma* is geographically confined to a region that has been subjected to intense anthropogenic habitat fragmentation since European settlement. It may be that the species' natural clumped distribution coupled with predominantly short distance pollen dispersal helps to attenuate population genetic pressures due to habitat fragmentation. Despite the species' ongoing resilience, low overall genetic diversity may compromise *F. picrosperma*'s ability to adapt to changing environmental conditions and extreme stochastic events. Therefore, it is important to conserve the remaining populations of *F. picrosperma* to ensure that the current level of genetic diversity is maintained for both conservation and domestication of this commercially significant species.

4.6 ACKNOWLEDGEMENTS

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Supplementary Data Table 4.1 Characterisation of six microsatellite loci isolated from 564 individuals of *Fontainea picrosperma* calculated using CERVUS 3.0.3 (Kalinowski et al., 2007).

Locus and GenBank Accession No.	Repeat Motif	Primer sequences (5' – 3')	Size range (bp)	*PIC*	N_A	H_O	H_E	F_{IS}
FP38 (MK305870)	$(GAAGAG)_7$	F:ATGAAGTTATTGCAAGGGCG R:TCCTGTAGGGTTGTCTTCCG	150-168	0.244	4	0.231	0.283	0.102
FP66 (MK305865)	$(AT)_{25}$	F:CCGAATCGAATCGTACAGAA R:GGTGGAAAGAACTTTACTTTTGG	104-140	0.594	14	0.523	0.64	0.106
FP68 (MK305866)	$(AT)_{19}$	F:CCTAATCAACATCATCAATTCGT R:TGATGTGATATAGTTGTATTTCGTCTG	102-106	0.216	4	0.201	0.227	0.076
FP69 (MK305867)	$(AT)_{13}$	F:TTGTGATGCCCAAGTCTCTT R:TCACAATATACAAGGACAAAGAACA	131-133	0.278	2	0.352	0.334	-0.027
FP82 (MK305868)	$(AT)_{11}$	F:TGCTTAAATATTCATTGCTAACCATT R:CGGAAGGCGATTCAGTATGT	161-165	0.326	2	0.282	0.41	0.185
FP84 (MK305869)	$(TA)_{11}$	F:TGGATCAAGTTTCAAGCTGC R:ATGCAACGTGAAGAAGGTGT	96-98	0.236	2	0.263	0.274	0.019

Samples were collected from four locations in the Atherton Tablelands. *PIC* is the polymorphic information content, N_A is the number of alleles, H_O is the observed heterozygosity, H_E is the expected heterozygosity, F_{IS} is the inbreeding coefficient.

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Chapter 5: Synopsis

5.1 SUMMARY OF FINDINGS

The studies in this thesis investigated the ecology of a plant species that has pharmaceutical potential, *Fontainea picrosperma* (Euphorbiaceae). *Fontainea picrosperma* is a dioecious, subcanopy tropical rainforest tree endemic to the Australian Wet Tropics (AWT). Rainforests in this region are notable for their distinctive Gondwanan taxa (Webb et al., 1984), especially in the uplands (Boulter et al., 2009) where populations of *F. picrosperma* are geographically confined. The species is of considerable commercial interest following the discovery of tigilanol tiglate, a novel epoxy-tigliane, which has been approved for the treatment of canine mast cell tumours by the European Medicines Agency (QBiotics, 2020) and is being developed as a therapy for human head and neck squamous cell carcinoma. Tigilanol tiglate cannot be synthesised on a commercial scale and it is therefore manufactured by extraction and purification from the seed of *F. picrosperma*.

This thesis characterised the floral, reproductive and pollination biology of *F. picrosperma* (Section 5.1.1; Chapter 2 and 3). Further, this thesis outlines the patterns of contemporary and historical gene flow within natural populations of *F. picrosperma* using genetic markers (Section 5.1.2; Chapter 4). This understanding of the ecological aspects of the species will help to secure sustainable seed production for commercial manufacture of tigilanol tiglate. Moreover, this thesis improves our understanding of fine-scale ecological interactions within a poorly studied tropical rainforest community, the AWT, and helps to highlight the challenges and conservation strategies for this dioecious, subcanopy species. The rainforest environment beneath the canopy presents a unique microclimate for pollinators, yet subcanopy tropical rainforest species remain poorly represented in the literature.

5.1.1 Reproductive and pollination biology of *F. picrosperma*

This was the first study that examined the reproductive and pollination biology of species in the genus *Fontainea* (Chapter 2 and 3). Apomixis is not a significant means of reproduction in *F. picrosperma* (Chapter 2), and individual fecundity relies

on pollen being delivered to the stigma. I used several methods to elucidate the mode of pollen delivery from male to female flowers (Chapter 2 and 3). The combination of sampling techniques allowed me to detect a more diverse array of fauna than would otherwise be detected using only one sampling method (Boulter et al., 2006).

Male flowering phenology ensured that pollen was available across the entire female flowering period and encouraged pollinator movement between inflorescences within the population (Chapter 2) (House, 1993; Barrett and Harder, 1996; Osunkoya, 1999; Moog et al., 2002; Yamasaki and Sakai, 2013). Female *F. picrosperma* flowers remained open and continued to be receptive for long periods post-anthesis, suggesting an adaptation to low pollinator activity (Stratton, 1989; Bawa, 1990; Devaux and Lande, 2010). Indeed, results from two reproductive seasons clearly showed that *F. picrosperma* was pollen limited (Chapter 2). Dioecy can be highly beneficial for species by allowing sex-specific resource allocation and promoting outcrossing, but it can also lead to pollen limitation and reduced fruit set (Larson and Barrett, 2000; Knight et al., 2005; Aguilar et al., 2006; Davila et al., 2012), as was observed for *F. picrosperma* in this study. Pollen limitation is common in tropical rainforests where temporal variability in pollination services are often encountered because pollinator abundance and competition from co-flowering species is highly unpredictable (Williams and Adam, 2010; Vamosi et al., 2013). Moreover, no nectar was found in *F. picrosperma* flowers (Chapter 3). Consistent with this study, nectarless flowers are more likely to be pollen-limited than nectariferous flowers in self- incompatible species (Larson and Barrett, 2000; Wilcock and Neiland, 2002).

The floral morphology and flowering phenology of *F. picrosperma* (Chapter 2) were typical of many dioecious plants found in rainforest communities that are often associated with generalist entomophilous pollination (Bawa and Opler, 1975; Hansman, 2001; Carpenter et al., 2003; Machado and Lopes, 2004; Rosas-Guerrero et al., 2014). *Fontainea picrosperma* flowers were visited by an array of small, generalist insects including beetles, flies, predatory wasps and thrips (Chapter 3). This is congruent with many other woody plants in Australian tropical rainforest communities that are pollinated by small generalist insects (Williams and Adam, 1994; Boulter et al., 2005; Worboys and Jackes, 2005) and consistent with what is known of visitor assemblages in the subcanopy of rainforest communities elsewhere (Bawa et al., 1985; Kress and Beach, 1994; Devy and Davidar, 2006). Thrips and beetles were commonly

part of the visitor assemblage that visited or were captured within *F. picrosperma* flowers. Although thrips were the most abundant flower visitors, they are unlikely to contribute substantially to pollen transfer due to their small body size. The taxa could however, indirectly contribute to pollination by attracting thrip-feeding predators, as has been found in other tropical rainforest species that were previously thought to be thrip-pollinated (Kondo et al., 2016). Predatory wasps, including one specific thrip predator, and omnivorous beetles were either found in the inflorescence or trapped using a scent lure that contained the six most relatively abundant scent compounds present in the floral bouquet of *F. picrosperma* (Chapter 3).

My results found that floral scent is likely the most important floral attractant in *F. picrosperma* to recruit pollinators in the low light environment beneath the canopy. Total floral scent emission of male and female flowers was characterised by maximal emission at male flower dehiscence, maximal emission during female flower receptivity, and a decrease in the amount of scent compounds produced as the flower aged. Scent therefore is likely to regulate pollinator behaviour. In this dark, low energy environment, female flowers of *F. picrosperma* exhibit cost-saving strategies by not producing nectar and mimicking the smell of reward offering male flowers. Pollination by deceit, where female flowers offer no obvious reward (i.e. pollen or nectar) like female *F. picrosperma* flowers, is also common in tropical, dioecious species that are insect-pollinated (Renner and Feil, 1993). The main scent constituents found in *F. picrosperma* are ubiquitous in plant floral bouquets and likely makes them indiscriminate to specific taxonomic groups. This is consistent with our findings that the species possesses a generalist, entomophilous pollination system (Chapter 2 and 3). This research integrates pollination biology and floral scent chemistry and contributes to an underdeveloped field of study in the sensory ecology of plant-pollinator interactions (Dötterl and Vereecken, 2010). Moreover, isolation of these compounds could provide a means of managing insect populations in agricultural and artificial environments (Irvine and Armstrong, 1991).

Sexual reproduction of *F. picrosperma* by wind pollination is limited and incidental (Chapter 3). *Fontainea picrosperma* pollen has a sculptured exine surface (Chapter 2), which typically characterise pollen transported by insects (Faegri and van der Pijl, 1979; Williams and Adam, 1999). Wind pollination is not common in tropical

rainforests, particularly in the understory with less wind, or more turbulent wind patterns (Irvine and Armstrong, 1991; Turner, 2001; Machado and Lopes, 2004).

Fontainea picrosperma is pollen limited (Chapter 2), a conclusion supported by a dearth of insect visitors to flowers (Chapter 3). The unspecialised structure of *F. picrosperma* flowers (Chapter 2) and low frequency with which the wide variety of small insect taxa were observed visiting both sexes indicates that pollination occurs from the ever-present pool of low-energy, generalist insects found beneath the canopy in the tropical rainforest (Chapter 3). Female flowers remain receptive for a long time (Chapter 2) raising the chances of pollination by these unreliable visitors. Pollinators preferentially travel short distances between conspecific trees, most likely due to the opportunistic feeding patterns of small generalist insects and leads to the short pollen dispersal distances that characterises pollen mediated gene flow in *F. picrosperma* (Chapter 4). The species has low genetic diversity, owing in part to this predominant near neighbour mating (Chapter 4). Together, these findings emphasise that the environment beneath the canopy is challenging for pollinators that forage on species that offer poor floral resources, in low light levels, with less wind and/or turbulent wind patterns, high relative humidity and dense foliage that act as physical barriers for pollinator movement.

5.1.2 Population reproductive genetics of *F. picrosperma*

This thesis determined the spatial pattern of gene flow that occurs due to insect vectors moving pollen from male to female flowers and measured levels of genetic diversity between adult and juvenile subpopulations (Chapter 4). Many studies have examined gene flow and population genetic structure in rainforest canopy species. However, understorey species remain under represented. In this study I revealed spatially-limited gene dispersal in the subcanopy species, *F. picrosperma*.

Approximately two thirds of successful reproductive events in *F. picrosperma* occurred within a 30 m radius of the mother tree. This finding is contrary to the frequent, long-distance pollen dispersal events reported for many insect-pollinated canopy trees (Akihiro et al., 2000; Kenta et al., 2004; Ward et al., 2005; Hardesty et al., 2006; Born et al., 2008; Monthe et al., 2017). Pollen dispersal patterns of many insect-pollinated tropical trees are influenced by preferential visitations to close

neighbouring trees (Silva et al., 2010; Theim et al., 2014; Noreen et al., 2016). The long dispersal distances reported for canopy species are partly because these taxa generally occur at low population densities (Born et al., 2008; Naoki et al., 2009; Duminil et al., 2016). Conversely, the short distance pollen dispersal I observed for *F. picrosperma* can be partially attributed to the species' clumped distribution and synchronous flowering. Other studies of rainforest understorey species have also found a high proportion of short distance pollination events, for example, in *Piper* shrub spp. that have high density, aggregated populations (Lasso et al., 2011). Spatially-limited pollen and seed gene dispersal are known to increase the likelihood of similar genotypes mating with each other (Seidler and Plotkin, 2006; Ellstrand, 2014). Concordant with this theory, I found evidence that individuals within a clump were significantly more related than is expected between two random individuals.

Plant-pollinator relationships can also influence pollen dispersal distances. *Fontainea picrosperma* is likely to be pollinated by small, generalist insects (Chapter 2 and 3). This class of generalist pollinators often move shorter distances compared to specialised insects, larger insects, or vertebrates (Dick et al., 2008). Gene flow in *F. picrosperma* is influenced by its pollinators preferentially visiting trees within the clump. Gene flow is also limited by the transport of pollen by pollinators (Chapter 2), which further reduces opportunities for long-distance pollen flow.

This study adds to the literature that the attractiveness of floral displays of individual trees are also important factors in determining pollinator-assisted gene flow (Barrett and Harder, 1996; Degen and Roubik, 2004; Duminil et al., 2016). Many males contributed to successful reproduction of individual *F. picrosperma* females but the contributions to reproduction were uneven. Most of the assigned fathers sired a single seed while only one or two larger males, with relatively large numbers of flowers, had significantly greater reproductive success. Flower count was autocorrelated with tree stem diameter size (dbh) in this study. Size class has been positively correlated with mean individual fecundity in other tropical tree species (Latouche-Hallé et al., 2004; Naoki et al., 2012; Monthe et al., 2017; Younginger et al., 2017) and may help to explain the skewed number of fathers found to be siring progeny in *F. picrosperma*.

The species' short-distance gene dispersal and skewed success rate of pollen donors is theoretically a prelude to genetic impoverishment (Loveless and Hamrick,

1984; Duminil et al., 2009). However, I found that adult and juvenile subpopulations have similar multilocus genotypes and there is no evidence of an intergenerational loss of diversity. This finding remains consistent with Lamont et al. (2016) who found negligible levels of inbreeding within adult populations of *F. picrosperma*. Deleterious alleles may be purged through an increase in mortality in inbred individuals and survivorship of those composed of half or unrelated siblings (Hufford et al., 2003; Naito et al., 2005; Tambarussi et al., 2017). This is a common pattern in long lived species (Duminil et al., 2009). In addition, the large number of males contributing to reproduction of a single seed found in this study reflects the high density of available fathers surrounding the mother tree and suggests that heterogenic pollen pools are received on flowering females. This can maintain variability, reduce the occurrence of full sibling progeny arrays and dilute the effects of kin mating (Breed et al., 2012).

The 18–37 % pollen immigration rate (greater than 30 m) estimated by paternity assignments could also contribute to the lack of differentiation between age cohorts. Only low levels of gene flow through long distance pollination events are necessary to counteract opposing mutation, drift and selection in isolated populations (Ashley, 2010; Ellstrand, 2014). Pollen immigration suggests that some pollinators of *F. picrosperma* can transport pollen greater than 30 m. While we do not know how far pollen can travel beyond this radius, Lamont et al. (2016) found recent bottlenecks with subsequent founder effects in two isolated populations of *F. picrosperma*, Malanda (centrally located) and Gadgarra (North-East). This implies that pollen may not travel long distances from the refugial populations of Boonjie and Evelyn Highlands, which are located in the east and west peripheries of the species natural distribution (Lamont et al., 2016).

The genetic diversity measures reported in this study are congruent with similar populations of *F. picrosperma* reported by Lamont et al. (2016) and for the related species *F. rostrata* (Conroy et al., 2019). Measures of diversity are relatively low when compared to microsatellite-based studies on tropical rainforest tree species reported elsewhere (Collevatti et al., 2001; Naito et al., 2005; Carneiro et al., 2009; Sebbenn et al., 2011; Melo and Franceschinelli, 2016; Monthe et al., 2017), including in self-compatible species (Latouche-Hallé et al., 2004; Naoki et al., 2009), and species surviving in highly fragmented populations (Gaino et al., 2010; Wang et al., 2014).

From the limited number of studies available, the genetic diversity of *F. picrosperma* is more akin to other Australian rainforest tree species (Rossetto et al., 2007), though some of these species with reported low genetic diversity is due to asexual reproductive mechanisms (Rossetto et al., 2004; Rossetto and Kooyman, 2005; Thurlby et al., 2012).

Genetic variation is often significantly related to population fitness and hence, the evolutionary potential of a species (Reed and Frankham, 2003), yet *F. picrosperma* has successfully persisted through historical environmental changes with low genetic diversity (Lamont et al., 2016). Expansion and contraction of *F. picrosperma* populations during the climatic oscillations of the Pleistocene over the last 230,000 years (Kershaw et al., 2007) has likely reduced the level of genetic variation within the species, as found in this study and by Lamont et al. (2016). These processes have led to low genetic diversity in other upland taxa in the Australian tropics (Rossetto et al., 2009) and it is likely that the low genetic diversity observed in *F. picrosperma* is an indication of its significant adaption to local environmental conditions.

Fontainea picrosperma is geographically confined to a region that has been subjected to intense anthropogenic habitat fragmentation since European settlement that could also have contributed to a loss of genetic diversity due to a reduced number of local and immigrant pollen sources in some populations (Sork and Smouse, 2006). It may be that the species' natural clumped distribution coupled with predominantly short distance pollen dispersal by small generalised insects (Chapter 3) helps to attenuate population genetic pressures due to habitat fragmentation. However, in the context of low genetic diversity, the short distance pollen dispersal is likely to have contributed to the significant, albeit weak population structuring across the species natural distribution (Lamont et al., 2016).

The observed low levels of genetic differentiation and genetic diversity between adult and juvenile cohorts, and the determination that proximate plants are significantly related to each other suggests that this species is highly adapted to its environment, short distance pollen flow does not affect the species capacity to persist in the environment, and there is sufficient long-distance (> 30 m) gene flow to keep the level of genetic diversity stable across the species distribution. Despite the species' ongoing resilience, low overall genetic diversity may compromise the ability of *F. picrosperma* to adapt to changing environmental conditions and extreme stochastic events.

Moreover, the remaining low genetic diversity is of potential concern for domestication programs, which require maximal genetic diversity to facilitate efficient selective breeding and genetic improvement of this commercially significant species.

5.2 RECOMMENDATIONS AND FURTHER RESEARCH

5.2.1 Applied aspects of the research: Domesticating *F. picrosperma*

A reliable and economical supply of the Active Pharmaceutical Ingredient, in this case tigilanol tiglate, is a key element in the development of a therapeutic agent. As such, domestication of *F. picrosperma* to supply raw materials for the manufacture of tigilanol tiglate is crucial.

Flower size is a potentially significant commercial trait as it has been correlated with pollinator visitation (Klinkhamer and van der Lugt, 2004), fruit set (Wetzstein et al., 2013) and fruit size (Johnson et al., 2011). Relationships between flower diameter and fruit set or fruit size in *F. picrosperma* warrant further investigation to ensure that fruit yield is maximised through plantation management practices or genotypic selections that optimise flower quality.

Optimising seed production of *F. picrosperma* could also depend upon improving pollen transfer between male and female trees by hand pollination or by managing natural or introduced pollinators. I determined that *F. picrosperma* was limited by pollen transfer under natural conditions over two reproductive seasons using a representative sample of inflorescences per tree (Chapter 2). However, it is unknown if maternal resources are also a limiting factor in fruit-set. The availability of maternal resources to support seed development may affect the retention, size and tigilanol tiglate content of fruit. For example, higher frequencies of fruit set associated with hand pollination may result in compensatory effects that reduce average seed size or seed quality including tigilanol tiglate content.

Pollination is an important, free ecosystem service that wild pollinators provide to facilitate or enhance seed production in the agricultural landscape (Garibaldi et al., 2013). The potential pollinators of *F. picrosperma* are small, unspecialised insects including beetles, thrips, flies and predatory wasps. Management techniques for these

pollinating taxa do not exist and plantation designs and conditions may not favour the behaviour or life cycle of the pollinator. It may be important to have a healthy, native forest near future plantations to increase fruit production and seed set (Irvine and Armstrong, 1991; Ricketts, 2004; Blanche and Cunningham, 2005; Garibaldi et al., 2011). Intact forests act as refugia and increase the presence of native pollinators by allowing them to complete their life cycle in a suitable habitat. Adopting these management strategies may be a more effective and sustainable solution to hand pollination or managed beehives. Further, I observed flower visitors in natural populations, and it is recommended that observations be carried out in a plantation setting to confirm if pollination services are sufficient in this environment.

Individual compounds discovered in the floral bouquet of *F. picrosperma* are known to attract honey bees (*Apis mellifera*) (Dobson, 2006; Dötterl and Vereecken, 2010). Introducing managed honey bee hives into a plantation could enhance fruit set in this pollen limited species. However, it is currently unknown whether the entire floral bouquet or individual compounds act to attract insects and therefore honey bees. Further, the female flowers do not produce an obvious floral reward for nectar or pollen foraging bees that are able to distinguish between pollen offering flowers and non-rewarding flowers within some species (Dötterl and Vereecken, 2010). Thus, honey bees have the potential to learn avoidance behaviour despite male and female flowers being perceptually similar. The floral olfactory responses of native eusocial bees (*Tetragonula* spp.) is, to my knowledge, unknown. Further research into the effectiveness of managed honey bee hives, or alternatively, native bee hives in a *F. picrosperma* is therefore required.

Seed production for the extraction of tigilanol tiglate could be enhanced by improving pollen transfer from flowers from male trees to female trees. This could be achieved by increasing the ratio of male to female trees in plantations and optimising the placement of male trees around female trees. My results identified predominantly short distance pollen dispersal, which suggests that cultivation of the fruit will require a significant number of male trees within a carefully designed plantation to increase efficient pollination. Only female trees bear the fruit necessary to extract tigilanol tiglate and the optimal ratio of male and female trees needs to be such that space within the plantation is not unnecessarily allocated to male trees, whilst still ensuring that enough pollen is available within the plantation to optimise fruit set. The results of

the paternity analysis presented in this thesis are reflective of flower and seed production in the rainforest. The species' natural habitat has different environmental conditions compared to plantation settings that have more available light and are highly fragmented areas of forest. These conditions have the potential to attract a different assemblage of pollinating insects. Therefore, it is recommended that experimental plantations, with differing ratios of male and female trees be established to measure gene flow and assess fruit set in conjunction with pollinator observations. This would also provide insights into pollen flow in a plantation setting and help to achieve a specific optimal value for the ratio of male-to-female trees for fruit cultivation. Moreover, my results highlight that larger males, with a more intense floral display have greater reproductive success than smaller males with less flowers. Overall, it may be advantageous to allow elite male trees the space within a plantation to grow large, thereby attracting pollinators to the environment.

Finally, future cultivation of the species may benefit from mixing genetically dissimilar stocks from across *F. picrosperma's* natural distribution as a means of increasing allelic diversity. Genetic variability provides the material for breeding programs in response to environmental and demographic changes. Therefore, it is important to conserve the remaining populations of *F. picrosperma* to ensure that the current level of genetic diversity is maintained for domestication of this commercially significant species.

5.2.2 Ecological aspects of the research: *Fontainea picrosperma* in the wild

Fontainea picrosperma is a long-lived species with low population genetic diversity (Lamont et al., 2016). After persisting through multiple significant climatic oscillations during the Plio-Pleistocene era the species now persists in a region heavily impacted by anthropogenic habitat fragmentation due to agricultural expansion. Species that exhibit pollen limitation, like *F. picrosperma*, are vulnerable to habitat fragmentation and loss when individual population sizes are reduced (Kearns et al., 1998; Knight et al., 2005; Memmott et al., 2007). Despite the species' ongoing resilience, it is possible that subtle impacts on population genetic diversity have occurred due to altered gene flows (Lamont et al., 2016).

The likely pollinators of *F. picrosperma* are typically weak flyers that travel relatively short distances compared to larger bodied or canopy pollen vectors. Ensuring the ongoing continuance of pollen immigration into disjunct populations of *F. picrosperma* through conservation of remaining habitat in which the species resides is critically important because it contributes to the maintenance of genetic diversity in subsequent generations of the species (Chapter 4). Moreover, low overall genetic diversity may compromise the ability of *F. picrosperma* to adapt to changing environmental conditions and extreme stochastic events that are currently impacting global ecosystems. Therefore, it is important to conserve the remaining populations of *F. picrosperma* to ensure that the current level of genetic diversity is maintained for conservation of this commercially significant species.

I identified the floral visitors of *F. picrosperma* (Chapter 3) but was unable to ascertain which visitors were effective pollinators. Pollination leading to successful fertilisation is not always guaranteed by flower visitors and further research is required to determine the pollination efficacy of different flower visitors to *F. picrosperma*. Nevertheless, *F. picrosperma* is likely pollinated by an array of small generalist insects such as beetles, thrips, flies and predatory wasps. Pollinators are of critical importance in natural ecosystems (Garibaldi et al., 2013). Appreciating the value of the ecosystem services that wild pollinators provide to maintain biodiversity and to enhance seed production in crops reinforces the conservation value of remnant tropical rainforest vegetation in the heterogenic landscape where *F. picrosperma* persists.

Rainforest habitat in the AWT is rich in evolutionary history because it contains more primitive linages of flowering plants than any other ecosystem in the world (Metcalfe and Ford, 2009). In addition, the AWT is mega-diverse and contains a high level of endemism which makes the region a unique reservoir for the discovery of novel natural products. *Fontainea picrosperma* is the focus of this thesis because it contains a natural product within its seed with anti-cancer activity, which has the potential to positively impact people's lives. Priority should be given to conserving these remaining habitats, especially in the context of an unprecedented rate of climate change in a heavily modified landscape.

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