Evidence for Genetic Decline Within Afromontane Forest Fragments on the Mambilla Plateau, Nigeria

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CHAPTER 1. Introduction

1.1 The Value of Genetic Diversity

1.1.1 Genetic diversity as a component of biodiversity.

Habitat destruction is arguably the most significant cause of biodiversity loss across the globe (Wilson *et al.* 2016). The majority of deforestation has occurred as the result of global urbanization and expanding agricultural activities in order to accommodate rapidly growing human populations (Adedire 2002; Vranckx *et al.* 2012). However, without biodiversity and the ecosystem services that it provides, the fate of the human species is uncertain. This fact is now widely acknowledged, and extensive efforts have been made in recent decades to stem the loss of biodiversity throughout the planet. Many biodiversity conservation efforts have focused on the preservation and/or restoration of ecosystem diversity and species diversity. However, there is growing recognition that conservation must also incorporate a third aspect of biodiversity; genetic diversity (Aguilar *et al.* 2006).

Darwin's theory of natural selection is contingent on the presence of variation among individuals within a species (Violle *et al.* 2012). Without variation in the genetic make-up of all living things, adaptation is not possible and we would not observe the endless assortment of species and habitats throughout the Earth (Ratnam *et al.* 2014). The existence of species, and therefore ecosystems, are a result of genetic diversity, and their future preservation may also be contingent upon it. While ecological factors influencing the survival of species have been widely studied for many years, genetic factors affecting their evolutionary potential and fitness, and therefore on-going survival, have only relatively recently become available to study through the development of molecular genetic techniques (Lande 1988; Frankham 2005). A central risk to genetic diversity within populations is reduced population size and fragmentation of populations through habitat destruction.

There are two major genetic implications of habitat fragmentation; inbreeding and genetic drift (Frankham 2005; Aguilar *et al.* 2008). Firstly, the reduced size and increased isolation among populations increases the frequency of inbreeding (mating of close relative or self-fertilisation), resulting in increased homozygosity (Frankham *et al.* 2002; Höglund 2009). Inbreeding increases the likelihood of exposing mildly deleterious recessive alleles, thereby reducing individual fitness, increasing mortality (inbreeding depression), and reducing genetic diversity through selection (Aguilar *et al.* 2008; Höglund 2009). Secondly, genetic drift leading to the stochastic fixation of alleles in small populations will act to decrease heterozygosity. Erosion of genetic variation may affect the ability of a species to cope with pathogens, predation and other disturbances, and their capacity to adapt to environmental change (Lande 1988; Ellstrand & Elam 1993; Aguilar *et al.* 2008). The lone or combined impacts of inbreeding and genetic drift in fragmented populations highlights the importance of genetic diversity for sustaining vulnerable species in both the short-term, through maintaining

individual fitness, and in the long-term, by retaining adaptive potential (Frankham *et al.* 2002).

The loss of genetic diversity in small, isolated populations is frequently illustrated by the "extinction vortex" (Figure 1.1). In this cycle, the reduced productivity and increased mortality caused by genetic factors in turn leads to further reductions in population size. It follows that without intervention small populations may be driven to extinction due to evolutionary factors. Therefore, a failure to consider genetic factors in conservation management of small populations may underestimate their risk of extinction.

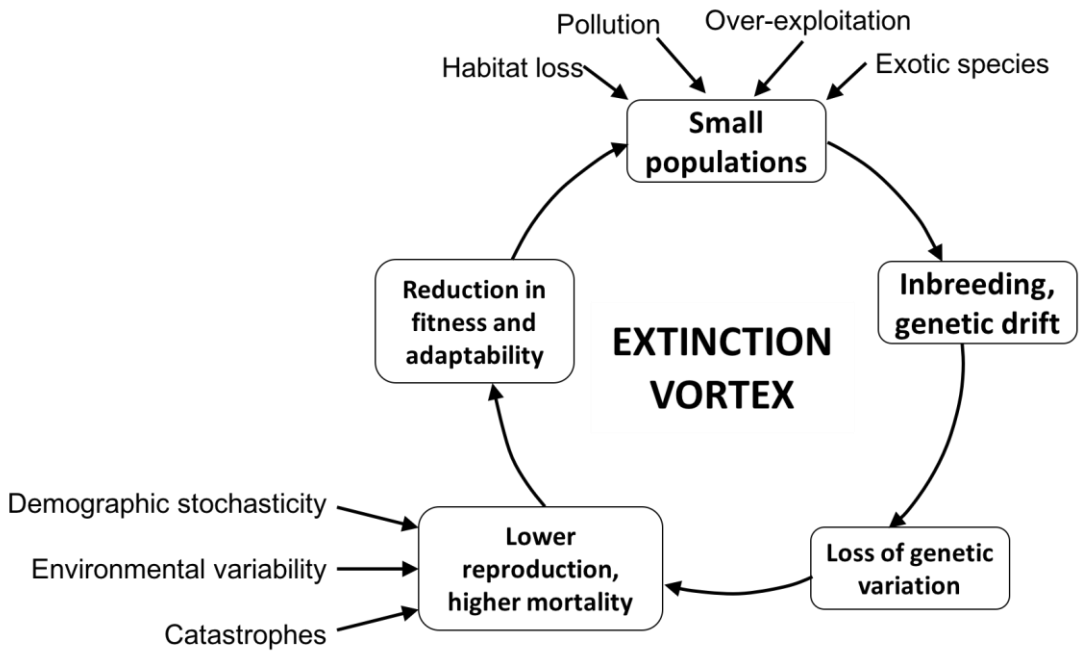

Figure 1.1: Representation of the extinction vortex. A conceptualisation of the effect of genetic diversity on the viability of small, isolated populations. Adapted from Frankham et al. (2002) and Hoglund (2009).

Genetic diversity can impact many factors beyond the demography of rare species (Hughes *et al.* 2008). Diverse genotypes within a population may not only ensure their resilience and long-term viability, but can also promote species productivity through niche partitioning, facilitation, or sampling effects. Above-ground net primary productivity of *Solidago altissima* has been found to increase with increasing number of genotypes due to greater opportunity for niche complementarity (Crutsinger *et al.* 2006). The effects of genetic diversity can also extend beyond the species level (Figure 1.2), but research is limited due the considerable complexity of community and ecosystem interactions (Whitham *et al.* 2006; Jacquemyn *et al.* 2012). High within-species genetic diversity may lead to increased species diversity, both within and across trophic levels (Hughes *et al.* 2008).

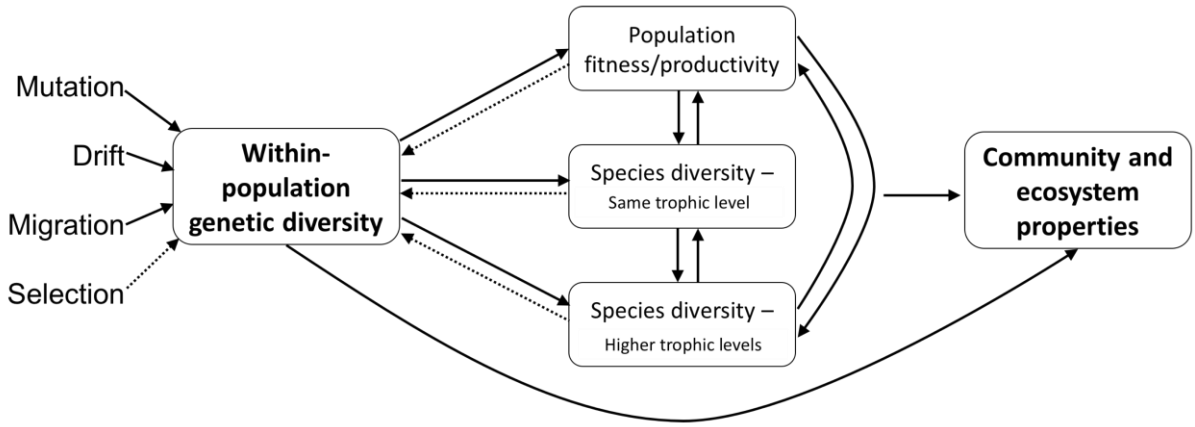

Figure 1.2: Illustration of effects of genetic diversity on ecological processes. Solid lines indicate direct/indirect effects, dotted lines indicate effects due to selection. Adapted from Hughes et al. (2008).

Booth and Grime (2003) showed, through manipulation of the genetic diversity of long-lived herbaceous plants, that communities with a higher diversity of genotypes resulted in higher levels of plant species diversity. Lankau and Strauss (2007) showed that genetic diversity leading to variation in the production of secondary compounds by *Brassica nigra* populations was fundamental for coexistence of congener species. This effect can also translate to higher taxonomic levels, with several studies showing that higher genetic diversity within plant species leads to greater diversity of arthropod species (Wimp *et al.* 2004; Johnson *et al.* 2006; Crutsinger *et al.* 2006).

The effect of genetic diversity on the productivity and viability of populations, as well as its influence on species diversity, is thus anticipated to have extensive effects on community and ecosystem level processes. For example, genetic diversity has been seen to strongly influence nutrient and energy fluxes. Madritch and Hunter (2002) showed that the diversity of genotypes of leaf litter from *Quercus laeris* trees in South Carolina, USA had a significant influence on carbon and nitrogen levels within the soil. It was found that 34% of the variation in chemistry of leaf litter was due to variation in genotypes. Furthermore, genetic diversity may also contribute to greater community stability, making habitats more resilient to disturbances (Prieto *et al.* 2015). It is evident that genetic diversity can have a profound effect on multiple aspects of productivity, species diversity, community processes, and ultimately on ecosystem functioning. Therefore, conserving genetic diversity within species in habitats affected by degradation and fragmentation, particularly in biodiversity hotspots, may be vital to stemming the current "biodiversity crisis".

1.1.2 Conservation genetics beyond rare species.

To date, the majority of studies of genetic diversity and population structure in fragmented habitats have focused on the rarest species in these ecosystems (Honnay & Jacquemyn 2007). This is unsurprising, as these are species that are in the most urgent need of conservation attention. It has been considered by many that common species have a sufficiently high population size, even within habitat fragments, to protect them from the negative effects of

inbreeding and genetic drift illustrated by the extinction vortex (Figure 1.1). This expectation is supported in a number of studies that directly compare genetic diversity of rare species with a common congener (Gitzendanner & Soltis 2000). However, it is possible that even common species may suffer significant genetic deterioration in fragmented landscapes (Honnay & Jacquemyn 2007). Prober and Brown (1994) observed a significant negative relationship between population size and allozyme diversity of the dominant tree species *Eucalyptus albens* in Southeastern Australia. A decrease in genetic diversity in small populations of the common woodland herb species *Trillium camschatscense* in Hokkaido, Japan has also been identified (Tomimatsu & Ohara 2003). The decline in the genetic diversity of these prevalent plant species following extensive habitat fragmentation highlights the potential for common species to be impacted by evolutionary factors. A review of such studies by Honnay and Jacquemyn (2007) noted that common species actually exhibit a stronger correlation (effect size) between expected heterozygosity and population size than rare species. Additionally, there was found to be no significance difference between common and rare species in the effect size for the measures of percent polymorphic loci and number of alleles per locus. These results show that even species that are considered to be common can experience erosion of genetic diversity in fragmented habitats.

The primary focus of genetic assessments of rare species is the ongoing survival of the focal species, and will have little effect on community or ecosystem-level processes (Wimp *et al.* 2004). However, alterations to genetic diversity in common species is anticipated to have far-reaching impacts on ecosystems through the processes illustrated in Figure 1.2 (Hughes *et al.* 2008; Ratnam *et al.* 2014). Therefore, maintenance of genetic diversity in common species may be fundamental to the conservation of natural habitats.

"Conserving genetic diversity in dominant plant species may just as important as conserving genetic diversity of rare and endangered species"
- *(Wimp* et al. *2004)*

The increased productivity of plants due to diversity of genotypes is expected to be more substantial in common plant species. This is because intraspecific competition for resources is greater at high densities, therefore the potential for niche partitioning or facilitation is greater (Violle *et al.* 2012). The resulting increase in primary productivity can then be expected to influence multiple ecosystem processes (Hughes *et al.* 2008). Several studies have illustrated the pervasive community and ecosystem consequences of genetic diversity in common species associated with habitats dominated by cottonwood hybrids (*Populus fremontii* x *P. augustifolia)* in the Western United States (Whitham *et al.* 2006). For example, Wimp *et al* (2004) found that 60% of diversity in arthropod communities in these habitats was due to genetic variability of *Populus* stands. Also, Schweitzer *et al.* (2005) revealed that mixed genotype litter of *Populus* trees had faster rates of decomposition and nutrient release than litter from a single genotype. They suggest that genetic diversity within a species may have as much influence on nutrient and energy fluxes as species diversity. The *Populus* study system provides evidence that maintaining genetic diversity is vital to conservation of dependent

animal communities, and that common species are likely to be more important to overall ecosystem function than rare species (Whitham *et al.* 2006; Hughes *et al.* 2008).
Establishing the level of genetic diversity in abundant species may also be a key method to estimating the overall wealth of genetic diversity within a habitat. If erosion of genetic diversity due to habitat fragmentation is observed in such prolific species then it is likely that many other species with lower abundances are also suffering losses of genetic diversity. Therefore, assessments of genetic diversity and population differentiation of common species may be used as an archetype for all plant species and act as indicators of dispersal routes and particularly vulnerable habitat fragments.

1.1.3 Microsatellites in population genetics

The scientific field of molecular ecology is one which is rapidly evolving. Genetic diversity can be measured by many molecular genetic techniques evaluating DNA of nuclear or organelle genomes, or protein sequences, as a proxy of overall genetic diversity (Ljungqvist *et al.* 2010). Microsatellite genetic markers, also referred to as simple sequence repeats (SSRs), are hyper-variable sites within either the chloroplast/mitochondrial or nuclear genome. These highly polymorphic loci are sites at which short sequences (1-6 units) are repeated many times successively (Abdel-Mawgood 2012). The number of repetitions is variable between individuals due to a high rate of mutation, thought to be due to slippage by polymerase enzymes or incorrect crossing over during DNA replication (Kalia *et al.* 2010; Guichoux *et al.* 2011; Abdel-Mawgood 2012). This high variability between individuals makes microsatellites the marker of choice when assessing genetic diversity at a small scale (Selkoe & Toonen 2006). Advantages of the use of microsatellites over other comparable methods include high variability, repeatability, low running costs, ability to use small amount of low quality DNA, and their speed and efficiency (Selkoe & Toonen 2006; Kalia *et al.* 2010; Guichoux *et al.* 2011; Abdel-Mawgood 2012). However, as with any method, there are some drawbacks of microsatellites. One of the primary disadvantages is the high cost and lengthy development time of primers associated with microsatellites (Arif *et al.* 2010; Guichoux *et al.* 2011). The hyper-variable regions of the microsatellites are flanked by highly conserved regions that are used as the primers for replicating and conveying the length of the microsatellite. The development of these primers is necessary in order to gain data regarding the microsatellite variability, and the determination of the nucleotide sequence of the primers requires expensive genome sequencing (Arif *et al.* 2010; Abdel-Mawgood 2012). The resulting primers are also often species specific, making microsatellite data difficult to use for comparisons between species. Another widely cited criticism of the use of microsatellite markers is that they may not accurately reflect the actual genetic diversity across the genome because they are so variable. However, several studies suggest that this is not the case, and that the use of hyper-variable markers such as microsatellites will in fact give a more accurate representation of genome wide variation than makers with low variation such as single nucleotide polymorphisms (SNPs) (Ljungqvist *et al.* 2010). Also, because they are selectively neutral, levels of genetic diversity of microsatellite loci do not have any ecological consequences

(Hughes *et al.* 2008). Therefore, assessments employing neutral markers only allow for an approximation of diversity at ecologically relevant traits.

Nuclear DNA is less conserved and has a higher mutation rate than chloroplast DNA, making nuSSRs (nuclear Simple Sequence Repeats) highly polymorphic and generally making them more suitable for fine scale studies of genetic diversity (Wolfe *et al.* 1987; Abdel-Mawgood 2012). Nuclear microsatellites also differ from chloroplast microsatellites in that they are co-dominant and follow a Mendelian inheritance model (Abdel-Mawgood 2012). However, because they are less conserved, nuclear microsatellites are not as easily transferable between species, making it more difficult to make direct comparisons of genetic diversity between taxa (Barbará *et al.* 2007).

A small number of highly conserved cpSSRs (chloroplast Simple Sequence Repeats) have been identified, which can be readily used for assessment of genetic diversity in any angiosperm species exhibiting a high level of variability at these loci (Provan *et al.* 2001). This is a major advantage of the use of the universal chloroplast microsatellites over species-specific nuclear microsatellites, as their lower species specificity allows for their use in previously unevaluated species, avoiding the high costs of primer development. It also allows direct comparisons of genetic diversity between two unrelated plant species. However, a disadvantage of chloroplast microsatellites is that the chloroplast DNA is uni-parentally inherited, so only assesses gene flow within the maternal line (Abdel-Mawgood 2012). Therefore, chloroplast microsatellites cannot be used in parentage analysis or estimates of pollen flow. However, because they are haploid, chloroplast microsatellites have half the usual effective population size, making them useful for detecting historical bottlenecks, founder effects, and genetic drift (Provan *et al.* 2001).

While microsatellite loci may not be a definitive indicator of ecologically relevant genetic diversity, it is apparent that they are an effective method of producing an approximation of genetic diversity and population structure for a broad range of conservation applications. A combination of both nuSSRs and cpSSRs can be used to thoroughly assess genetic diversity within species, and can allow determination of the relative contribution of pollen and seed dispersal to the maintenance of gene flow in fragmented habitats (Provan *et al.* 2001; Kartzinel *et al.* 2013).

1.2 Afromontane Forests

1.2.1 Biodiversity of the Afromontane archipelago

The discontinuous montane regions of Africa are frequently referred to as the Afromontane archipelago, due to the analogy of their distribution with scattered ocean islands (White 1983). These regions, between c. 1,500-2,000 metres elevation, were created by tectonic activity occurring since the Miocene epoch (~ 23-5.3 Mya) (Grimshaw 2001; Linder 2014). Afromontane habitats are primarily distributed throughout East Africa, from Ethiopia to South Africa, including Madagascar (Figure 1.3). More isolated montane regions also exist in

Western Africa in both the Guinean and Cameroonian highlands. The 'islands' of the Afromontane archipelago are separated from one another by thousands of kilometres, yet retain similar habitat characteristics and share many endemic taxa that are distinctly different from the rest of lowland Africa.

Figure 1.3: The Afromontane archipelago. Approximate distribution of montane habitats in Africa. Adapted from White (1983).

The Afromontane archipelago is defined not by geography, but by the distinct floristic composition of tropical forests found in these regions (White 1981). The biodiversity of these forests has a complex evolutionary history created by cycles of glaciation and deglaciation. During glacial periods, many species across Africa evolved resilience to cold, wet conditions, and during interglacial periods these adapted species retreated to montane refuges (Kadu *et al.* 2013). Currently, high altitude of montane areas results in a cooler, more humid environment than lowland areas of Africa, and these climatic conditions maintain the unique suite of flora and fauna that evolved (Lambi & Neba 2009). Afromontane forests are often referred to as "hotspots" of biodiversity (Brooks *et al.* 2002; Cronin *et al.* 2014). It is estimated that there are around 4,000 plant species found in Afromontane regions, of which ~75% are endemic (White 1981). Two plant families, Curtisiaceae and Oliniaceae, and about one-fifth of tree genera in these regions are Afromontane endemics (Grimshaw 2001). In the Eastern Afromontane region, rates of endemicity are particularly high; 119 genera are endemic (Mittermeier *et al.* 2011). However, information about the biodiversity and ecology of the Afromontane archipelago is limited compared to other biodiversity hotspots around the world (Grimshaw 2001). Further research is necessary to decipher the complex process that have shaped the biota of these forests, and to ensure their long-term persistence.

1.2.2 Ecosystem services

While Afromontane forests cover only a limited land area, they offer high intrinsic value and contribute significantly to the health and prosperity of local communities through the provision of ecosystem services. One of the most important contributions of Afromontane forests is to watershed function (Doumenge *et al.* 1994; Grimshaw 2001). It is proposed that the structure of vegetation of montane forest makes then disproportionate contributors to the capture of atmospheric water in Africa (Doumenge *et al.* 1994). Therefore, these forests are fundamental to the provision of water to communities within these catchments. This provides significant economic benefit due to increased productivity in surrounding agricultural areas. Additionally, Afromontane forests prevent soil erosion, thereby maintaining water quality and preventing flooding. They also play a key role in carbon storage and sequestration and have additional economic benefits that include agricultural pest control and pollination, and tourism values (Ndenecho 2010).

Afromontane forests also have high value to local people as a source of goods, food and traditional medicine. The majority of rural African communities are reliant on firewood as their sole means of cooking and heating (Adedire 2002). Forests provide timber for building infrastructure and tools, and food is provided by hunting of animals and collection of fruits from forest plant species (Lambi & Neba 2009). Non-timber forest products, such as honey, are also provided by Afromontane forests. Afromontane forests, like many forests throughout Africa, provide considerable cultural value, with many forests protected by local people as sacred spiritual groves (Doumenge *et al.* 1994; Ndenecho 2010). Tropical plant species have a vast array of medicinal properties, and are frequently used in traditional medicine to treat a range of ailments (Ndenecho 2010). Traditional medicine is the primary health service available to many rural communities of Africa, therefore a great number of people are reliant on plant species within indigenous forests (Fokunang *et al.* 2011). Afromontane plant species are likely to be a key reservoir for the development of pharmaceuticals in the future (Ndenecho 2010).

1.2.3 Threats and conservation

Despite their high biodiversity, economic and social value, Afromontane forests are among the most highly threatened ecosystems in the world (Toledo-Aceves *et al.* 2011). Relatively little is known about rates of deforestation in Sub-Saharan Africa, though it is estimated to be lower than in other tropical regions, around 0.05% per year (Rudel 2013). However, rates in Afromontane regions are likely to be much higher than elsewhere in the Afrotropics (Doumenge *et al.* 1994). The rate of deforestation in Southwest Ethiopian Afromontane forests is estimated to be 0.38% per year (Getahun *et al.* 2013). In West African forests, which includes the Western Afromontane forests, it is estimated that forest cover is lost at a rate of 1.21% per year (Brooks *et al.* 2002). Rapidly increasing human populations throughout the continent have created a strong need for additional food production, and have therefore driven deforestation, degradation, and fragmentation of forest habitats. While measures of deforestation rates in Africa are limited, it is apparent that Afromontane habitats are in

decline (Grimshaw 2001). Montane forests across Africa now typically linger only as a mosaic of fragments among an agricultural/grassland matrix (Aerts *et al.* 2006; Linder 2014; Newmark & McNeally 2018).

Deforestation of Afromontane regions is driven by immense social, economic and political pressures. Direct clearing of forests occurs due to logging of trees for timber, or clearing for grazing or agricultural land. It is estimated that 60% of the population of Africa is employed in agricultural production (Collier *et al.* 2008). Nigeria is reported to have the 11th highest level of biodiversity among African nations, but also has the highest rate of deforestation in the world (Borokini *et al.* 2012). As much as 80% of Nigerian forests that have been cleared were converted to provide additional agricultural land (Adedire 2002). Agriculture in Africa frequently occurs at high elevations up to 2,400m, leading to extensive clearing of natural montane habitats (Doumenge *et al.* 1994). Additional harvesting of plant material for firewood and non-timber forest products, and hunting of animals for bushmeat, further degrades these forests (Cronin *et al.* 2014). Also, encroachment of cattle into forest results in degradation of forest undergrowth, preventing effective regeneration (Lambi & Neba 2009; Wassie *et al.* 2009). Afromontane forests are typically highly susceptible to fire, and therefore are frequently lost through slash-and-burn agricultural practices (Adedire 2002).

Biodiversity hotspots, such as Afromontane forests, are frequently associated with high rates of poverty (Fisher & Christopher 2007; Mittermeier *et al.* 2011). In the Eastern Afromontane region, 38% of children are malnourished, one of the highest rates in the world (Mittermeier *et al.* 2011). In West African Afromontane regions poverty rates are also high; in Nigeria 60% of the population lives below the 'poverty line' (Fisher & Christopher 2007). Extreme poverty, and a lack of alternative resources, is the primary driver of the exploitation of African natural habitats. With so many people struggling to provide the necessities of life, biodiversity conservation is frequently disregarded (Grimshaw 2001; Lambi & Neba 2009). However, the conservation of Afromontane forests in the long-term may provide the key to alleviating poverty in the future (Korndoerfer 2010).

> *"Throughout Africa resource managers face the dilemma of sustainable development – how to meet the needs of current populations without jeopardizing the ability for future generations to meet theirs" – (Glaesel Frontani 2010).*

Impending climate change is another key threat to the long-term preservation of Afromontane forests. Temperature increases in Sub-Saharan Africa are predicted to be particularly pronounced (Collier *et al.* 2008). In fact, mean annual temperatures are already seen to be rapidly increasing in some regions, corresponding to a decrease in rainfall (Ndenecho 2011). It is predicted that globally 30% of species will be driven to extinction due to climate change, with a large proportion belonging to tropical habitats (Cronin *et al.* 2014). Sosef et al. (2017) predict that up to 90% of Sub-Saharan species will lose a large part, if not all, of their adapted ranges by 2085 due to climatic changes. The specialised adaptations and limited ranges of montane species makes them particularly vulnerable to climatic changes

(Ndenecho 2011; Toledo-Aceves *et al.* 2011). Increasing temperatures throughout Africa will result in considerable range contraction of montane species, which will be further affected by range shifts of lowland species to higher elevations (Cronin *et al.* 2014). These factors indicate that Afromontane species will be disproportionately affected by climate change. Maintenance of high levels of genetic diversity in Afromontane species may be vital in ensuring their long-term survival in the face of climate change (Pauls *et al.* 2013).

Government protection of montane forests throughout Africa has been largely ineffective, primarily due to inadequate funding and a lack of political will (Cronin *et al.* 2014). Few government directed conservation initiatives directly seek to preserve biodiversity. Instead, the primary focus tends to be on attracting international funding or promoting tourism values (Ndenecho 2011). Community-based conservation initiatives are emerging as a potentially effective protection method by prioritising biodiversity conservation alongside the needs of local communities (Newmark & Hough 2000; Borokini *et al.* 2012). In such undertakings, local communities are encouraged to take guardianship of natural habitats, incorporating traditional belief systems alongside modern scientific knowledge. Berkes *et al*. (2007) suggest that neither institutionalised government-run or community-based conservation methods are ideal for achieving biodiversity, livelihood, or economic goals. They suggest that community-based conservation may lead to the application of a generalised strategy across different areas and ecosystems without the consideration of context. However, community-based conservation may represent the only opportunity in many areas of Africa for cooperation of authorities, scientists, and local people (Adams & Hulme 2001). With limited funding availability, there is an apparent need for low-cost techniques that can be used for diagnosis of vulnerable habitats in Africa and to assess the efficacy of conservation management practices.

1.2.4 Conservation genetics of African trees

It has proven difficult to produce empirical evidence for reduced genetic diversity in tropical forest tree in small, isolated populations. Kramer *et al*. (2008) refer to this occurrence as the "paradox of forest fragmentation genetics". There are two likely reasons for this; firstly, the edges of forest fragments do not necessarily delineate the edges of populations due to efficient long distance dispersal mechanisms of many tree species, and secondly, the generation times of trees tend to be much longer than many other plants or animals, slowing the loss of genetic diversity (Finkeldey & Hattemer 2007). Therefore, African tree species may be inherently more resistant to the genetic impacts of fragmentation relative to species in temperate regions, as they have evolved at low densities necessitating longer dispersal distances (Kramer *et al.* 2008).

A study of genetic variation within *Adansonia digitate,* the iconic African baobab tree, found that extensive habitat degradation did not result in reduced genetic diversity in Sudanese populations (Wiehle *et al.* 2014). Ouinsavi *et al.* (2010) also detected high levels of genetic diversity within fragmented populations of the threatened tree species *Milicia excelsa* in Benin. In contrast, populations of the endangered member of the Proteaceae, *Leucodendron*

argenteum population in South Africa have very low levels of diversity (Heelemann *et al.* 2013). This loss of genetic diversity may be attributed to the cessation of natural fynbos fire regimes preventing regeneration. It is suggested that sampling methodologies may be key to revealing effects of fragmentation on forest genetics; sampling of juvenile plants may reveal recent fragmentation impacts (Aguilar *et al.* 2008; Vranckx *et al.* 2012).

Several Afromontane species have been studied at a large scale to compare genetic diversity between mountain ranges in order to determine historic migration routes. For example, Ethiopian populations of the important timber tree *Prunus africanus* on the east and west of the Rift Valley are strongly divergent from one another, with populations to the west being more closely related to populations in the Cameroon Highlands in Western Africa (Kadu *et al.* 2013). The same phylogeographic trend exists in the Afromontane herb *Canarina eminii* (Mairal *et al.* 2017). Studies such as these give insight into the historic processes that resulted in the floristic characteristics of Afromontane forests. However, studies at smaller scales allow an understanding of how forest species are changing as a result of recent and rapid habitat fragmentation. In Eastern Afromontane populations of the Afromontane endemic tree *Hagenia abyssinica*, microsatellite analysis revealed high within population genetic diversity despite extensive fragmentation and reduction in population size (Gichira *et al.* 2017). *P. africanus* in Kenyan forest fragments also revealed high genetic diversity despite selective logging of the species (Farwig *et al.* 2007). However, microsatellite variation was found to be significantly lower in seedlings than in adults within the same forest, giving evidence for decreasing genetic diversity of an Afromontane tree as a result of human activities (Farwig *et al.* 2007).

Mixed empirical signals regarding the vulnerability of Afromontane forests to the evolutionary impacts of habitat fragmentation highlight the need for further research into the genetic characteristics of species within this ecoregion. However, limited funding has often restricted the capacity to undertake conservation science throughout many African nations (Newmark & Hough 2000). The high cost of many molecular genetic studies, along with a lack of easily perceivable conservation outcomes, may be a further limitation to the implementation of conservation genetics (Taylor *et al.* 2017). Substantial evidence suggests that genetics have a significant role to play in conservation of African species, and therefore ecosystems, so there is an apparent need for further research in this field. Cross-amplifying microsatellite loci may offer an opportunity for rapid, low-cost genetic analysis, allowing conservation managers to develop a more comprehensive understanding of the multifaceted threats facing Afromontane forests.

1.3 This Thesis

1.3.1 Mambilla Plateau

A large proportion of West African montane habitats can be found in the Cameroon Highlands. This extensive mountain range was created around 100 million years ago due to

volcanic activity (Cronin *et al.* 2014). The Mambilla Plateau is the highest plateau of the Cameroon Highlands, at an elevation of ~1600 metres above sea level, situated on the southeastern border of Nigeria (Borokini *et al*. 2012) (Figure 1.4; Figure 1.6A).

Figure 1.4: Landscape of the Mambilla Plateau. (Photo credit: H. Chapman)

Rainfall in the region is plentiful relative to other areas of Sub-Saharan Africa, with c. 1800mm during the wet season between March and October (Chapman & Chapman 2001; Oruonye & Adebayo 2013). The Mambilla Plateau is also one of the coldest locations in Nigeria, with a mean annual temperature of 21°c (Oruonye & Adebayo 2013). In contrast to elsewhere in Nigeria, a trend of decreasing mean annual temperature and increasing rainfall has been observed on the Mambilla Plateau over the last 35 years (Oruonye & Adebayo 2013). Primary occupation of the plateau's inhabitants, like the majority of Nigerians, is farming. The current human population size of plateau is unclear, however in 1980 it was estimated the population had increased from 16,000 to greater than 126,000 over a 50 year period (Hurault 1998).

The livelihoods of all Mambilla's communities is highly dependent of the ecosystem services provided by the Afromontane forest fragments that can be found scattered across the plateau. However, the presence of these people is also the leading detriment to these forests (Chapman & Chapman 2001; Korndoerfer 2010). It is apparent that the Mambilla Plateau has historically consisted of grasslands, with forests mainly restricted only to the steep escarpments and river verges (H. Chapman, pers. comm.). It is also clear that, like many other terrestrial ecosystems worldwide, these forests are suffering significant deforestation in recent times (Chapman *et al.* 2004). The forests of the Mambilla Plateau face extensive destruction due to a multitude of factors, including subsistence farming practices, high population densities, and cattle grazing (Chapman *et al.* 2004). Recently, an upsurge in

sapphire mining on the plateau has been implicated in the destruction of riverine forest fragments (Oruonye 2015). Furthermore, the influx of miners from across Africa and beyond has increased the population of Mambilla, placing additional pressure on forests and agricultural land. Another threat to Mambilla's forests is the impending construction of Nigeria's largest hydrological dam on the Donga river which crosses the plateau (Oruonye 2015). This dam is expected to flood thousands of hectares of the plateau, submerging many forest fragments. Dam construction may also become a significant barrier to plant and animal dispersal across landscapes (Kang *et al.* 2017).

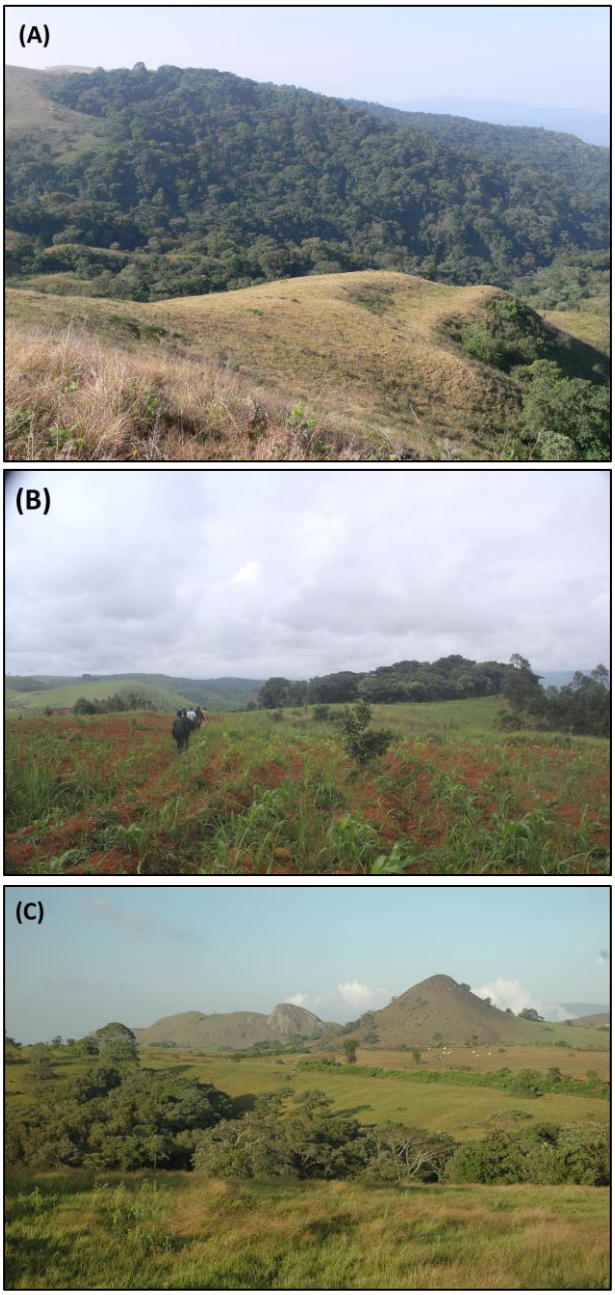

Figure 1.5: Forest fragment types seen on the Mambilla Plateau. A: Large forest reserve (Ngel Nyaki), B: Sacred kurmi forest fragment (Yana), C: Vegetation patches typical of those around Mbamnga and Tamnyar. (Photo credits: H. Chapman).

Several of the small forest fragments that remain on the plateau are sacred forests, protected by local communities for cultural and religious reasons (Figure 1.5B). Sites such as these are often referred to as 'kurmi' (plural: kurame) and it is likely that such forests only remain due to their cultural protection (Jones 1963; Chapman & Chapman 2001). However, as economic pressures increase and cultural beliefs are degraded, sacred kurame may face also become subject to deforestation.

The socioeconomic forces driving deforestation and fragmentation, and therefore biodiversity loss, in the Cameroon Highlands is expected to become particularly acute given the large and rapidly expanding population of this region (Cronin *et al.* 2014). Early in the 20th century the Mambilla Plateau was host to a large diversity of fauna, including elephants, lions and leopards, as well as large numbers of primates, including chimpanzees (Korndoerfer 2010). However, current mammal communities of the plateau are significantly less diverse; elephants, lions, and leopards are no longer present, and only one small troop of chimpanzee remain. However, the region still boasts high diversity in other taxonomic groups, in particular birds, plants, invertebrates, and amphibians (Dowsett-Lemaire 1989; Chapman & Chapman 2001; Borokini *et al.* 2012). Extensive conservation efforts will be required in order to preserve this unique and endangered biodiversity.

Genetic analysis has previously been carried out for several different taxa, assessing diversity within the largest remaining forest fragment on the plateau, Ngel Nyaki. Amphibian diversity at AFLP loci was found be moderate within this forest, with average expected heterozygosity of frog species varying between 0.170 and 0.219 (Arroyo Lambaer 2015). Thia *et al*. (2016) assessed the genetic variation of three rare tree species, *Cordia millenii, Entandrophragma angolense* and *Lovoa trichilioides,* at Ngel Nyaki. It was found that seven conserved chloroplast loci showed extremely limited polymorphism. A single locus, ccmp6, showed genetic diversity within *L. trichilioides* samples, resulting in two haplotypes for this species. The other two species exhibit only one haplotype each in all analysed samples. The lack of genetic diversity observed in these species was suggested to be due to either long-term isolation or recent colonization of this forest, along with the small population size of these rare tree species. This study has suggested cause for concern about genetic diversity of plant species across the plateau, prompting the need for further research.

1.3.2 Sampling sites

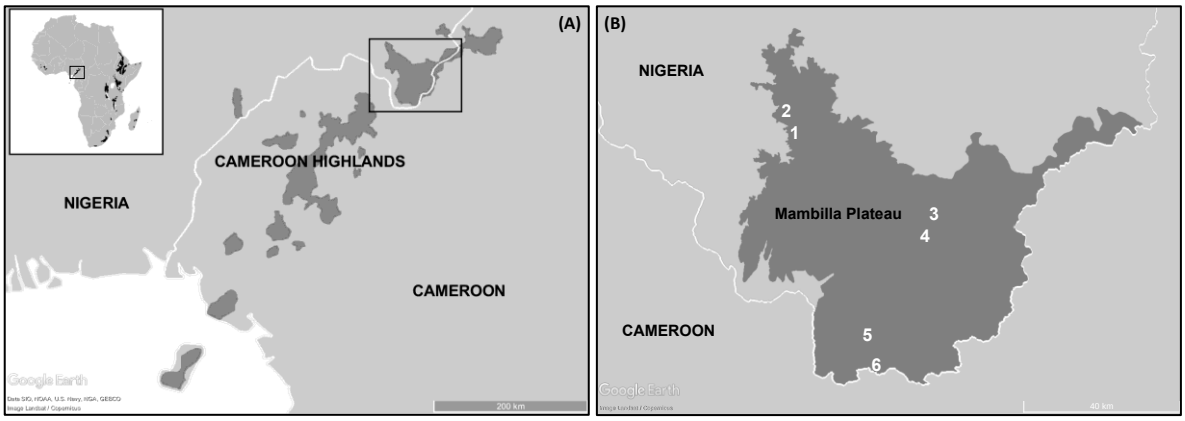

Figure 1.6: Study location. A. Approximate distribution of Afromontane habitats in the Cameroon highlands and location of the Mambilla Plateau (Insert: location of Cameroon highlands in relation to other montane regions). B. Location of study sites across the Mambilla Plateau. 1=Ngel Nyaki, 2=Kurmin Danko, 3=Kuma, 4=Yana, 5=Mbamnga, 6=Tamnyar.

The following study surveys the genotypes of *Albizia gummifera* (Fabaceae) and *Clausena anisata* (Rutaceae) (hereafter *Albizia* and *Clausena* respectively) samples collected from six forest fragments across the Mambilla Plateau, over an area of c.94,000ha (Figure 1.6B):

1. At 720ha Ngel Nyaki is the largest forest fragment on the Mambilla Plateau, and is also the most species diverse (Dowsett-Lemaire 1989; Chapman & Chapman 2001; Figure 1.5A). The forest is located on the Northwestern escarpment of the plateau at 7°04'45.9" N, 11°03'04.1" E and has a site elevation ranging from c. 1200m-1700m. Ngel Nyaki forest falls within a 46km^2 Local Authority Forest Reserve that was established in 1969 (Ihuma *et al.* 2011) and is currently managed by Taraba State Government and the Nigerian Conservation Foundation, and is supported by the Nigerian Montane Forest Project (NMFP). This forest is home to a remnant population of Nigerian-Cameroon chimpanzee *(Pan troglodytes ellioti)*, and contains four Red Data List tree species; *Entandrophragma angolense, Lovoa trichilioides, Millettia conraui,* and *Pouteria altissima (Chapman & Chapman 2001)*.
2. Kurmin Danko forest is the northern-most forest fragment (7°07'08.3" N, 11°01'54.3" E) sampled in this study, and has and approximate area of 230ha. Like Ngel Nyaki, it is located on the northwestern escarpment and covers a broad range of elevation from 1200-1735m. Chapman and Chapman (2001) reported that Kurmin Danko was significantly impacted by farming practices until the establishment of the Local Authority Forest Reserve in 1969.
3. Kuma village kurmi (6°53'24.6" N, 11°22'19.1" E) is located 8km southwest of the large village of Mayo-Ndaga to the east of the plateau (Figure 1.5B). This forest covers an area of approximately 25ha across an elevation of 1450-1550m. The spiritual status of this forest appears to have kept it intact despite the high intensity of farming in the area, and the forest border is strongly defined (Chapman & Chapman 2001).
4. Yana forest is another well preserved village kurmi in the east of the plateau (6°50'50.4" N, 11°21'05.0" E) and is located ~800m from the village of Yana. It is the

smallest forest fragments included in this study (15ha), and covers an elevation range of 1450-1550m. This small forest has remained well protected through cultural traditions of the local people, and also has a strongly defined border.

5. The Mbamnga population is represented by a number of degraded forest patches and riverine fragments to the south of the village of Mbamnga. Sampling was undertaken over an area of roughly 50ha along the roadside and river verges. This population is located toward the south of the plateau (6°36'44.0" N, 11°12'03.9" E), with altitude ranging from 1500-1600m. Within the centre of Mbamnga village is a very small kurmi, only ~0.3ha in size. No samples were taken from within the village kurmi, as access was denied by the village leaders.
6. Tamnyar is the farthest south sampling location (6°31'50.5" N, 11°13'27.4" E), consisting of degraded forest patches located within and around the village of Tamnyar, on the border between Nigeria and Cameroon. Samples were taken from an area of approximately 30ha over a range of 1600-1650m elevation.

1.3.3 Thesis outline

The following thesis investigates the genetic diversity and population structure of *Albizia* and *Clausena,* two tree species prevalent throughout the forest fragments of the Mambilla Plateau. A combination of conserved chloroplast microsatellite loci (cpSSRs) and nuclear microsatellite loci (nuSSRs) are employed in this analysis. These species were selected because of their high abundance, and because they employ different dispersal mechanisms. *Albizia* seeds are wind-dispersed, whereas the fruits of *Clausena* are animal dispersed. This allows assessment of how habitat fragmentation has affected gene flow of species employing alternate methods of seed dispersal. Genetic measures are also compared between adult and juvenile cohorts within these species to reveal changes in genetic diversity and structure over generations.

The following chapters explore several alternate applications for studies of genetic characteristics within species. In Chapter 2 the genetic diversity and differentiation of *Albizia* is assessed, and discussed in the context of its prospective use as a restoration species, and the potential need for genetic rescue. Chapter 3 investigates the genetic diversity and differentiation of *Clausena* and discusses the biodiversity value of culturally protected forests. In Chapter 4, diversity of conserved chloroplast loci is directly compared between the study species, to determine how species employing different dispersal mechanisms may be differentially affected by habitat fragmentation. Finally, Chapter 5 synthesises the findings within this thesis, compares these results with previous findings, and discusses the future prospects for the Afromontane forests of the Mambilla Plateau. The primary objective of this thesis is to provide insight into how habitat fragmentation on the Mambilla Plateau has affected the genetic characteristics of two dominant tree species, and to collect baseline data for assessing changes in the future. These finding can be applied to conservation management for the purpose of ensuring enduring protection of these forests for future generations. Furthermore, this work identifies suitable microsatellite loci for the rapid assessment of

genetic characteristics of the focal species in other threatened montane habitats across Africa.

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CHAPTER 2. Genetic Diversity and Population Structure of *Albizia gummifera*.

Abstract

Throughout the planet, ecological restoration has been used in an attempt to mitigate the current "biodiversity crisis". However, few restoration endeavours have considered genetic factors alongside ecological factors in order to achieve maximum conservation outcomes. In this study, characterisation of genetic diversity of *Albizia gummifera* within the Afromontane forest fragments of the Mambilla Plateau is used to advise restoration strategies and to assess potential sites for genetic rescue. Analysis of cpSSR and nuSSR loci showed substantial differences in genetic diversity among forest fragments, however, minimal population structure is observed. Comparisons between adult and juvenile cohorts suggest that genetic diversity of *A. gummifera* is declining in all forest fragments. Use of *A. gummifera* in restorative planting at Ngel Nyaki forest is advised as a means to maintain genetic diversity within the species, and to promote successful restoration. The small, isolated forest fragments of Kuma and Yana are identified as sites in need of genetic rescue. The depauperate diversity of *A. gummifera* within these forests displays the potential for abundant species to incur erosion of genetic diversity in fragmented landscapes. Therefore, the effects of habitat fragmentation on population genetics may more pervasive than many believe.

2.1 Introduction

2.1.1 Population genetics in ecological restoration

The global threat of deforestation to ecological, economic and social well-being has motivated many forest restoration endeavors. Ecological restoration aims to restore degraded or destroyed habitats to their previous state of diversity and ecosystem function (Bowles & Whelan 1996; Kettenring *et al.* 2014). Restoration techniques are frequently employed in fragmented landscapes with the objective of reducing the ecological consequences of habitat fragmentation. However, evolutionary consequences are not often considered, and assessments of the genetic characteristics of existing forest fragments are rarely incorporated into restoration efforts. The same evolutionary principles that are applied to fragmented habitats (discussed in Chapter 1) are applicable to habitat restoration (Thomas *et al.* 2014). A failure to restore genetic diversity alongside species and functional diversity may result in vulnerable habitats. Firstly, restored populations harbouring high genetic diversity will be more resistant to pathogens, predation, and other perturbations (Aerts & Honnay 2011). Additionally, restoration projects should aim to maintain genetic variation in

order to preserve evolutionary potential, as this will ensure long-term persistence (Bowles & Whelan 1996).

The short-term success and long-term sustainability of ecological restoration in fragmented habitats may be contingent on preserving any remaining genetic diversity, and it is therefore vital that conservation managers consider the genetic implications of the approaches being employed (Reynolds *et al.* 2012; Thomas *et al.* 2014). An increasing number of studies show that genetic diversity can have a significant influence in the restoration of natural ecosystems (Aerts & Honnay 2011; Reynolds *et al.* 2012; Kettenring *et al.* 2014). These studies suggest that in order to achieve maximum restoration outcomes high genetic diversity is desirable (Aerts & Honnay 2011; Thomas *et al.* 2017).

Both passive and active restoration techniques may act to undermine their own purpose by allowing further loss of genetic variation already degraded through habitat fragmentation (Aerts & Honnay 2011). Passive restoration techniques, whereby ecosystems are allowed to naturally regenerate after the cessation of environmental stressors, may allow further erosion of genetic diversity through natural evolutionary processes of genetic drift and inbreeding. Alternatively, active restoration, usually in the form of restoration planting, may also reduce the genetic diversity of species within the habitat through selective planting of particular genotypes (Kettenring *et al.* 2014). Source seeds may come from a limited parental source, and as a result will restore a limited amount of genetic diversity. When growing seedlings for planting, these plants may also be subject to artificial selection in a nursery, resulting in a loss of genetic variation (Thomas *et al.* 2014). In some cases, seeds or seedlings may be sourced from further afield and may introduce undesirable traits to the population, resulting in outbreeding depression, and therefore increased mortality (Vander Mijnsbrugge *et al.* 2010). These issues highlight the importance of incorporating an understanding of current levels of genetic diversity and population structure in existing habitats when undertaking restoration within or adjacent to these areas.

2.1.2 Ecological restoration at Ngel Nyaki

The high richness of species and community complexity of tropical forests makes the task of restoring these habitats challenging. In Africa, restoration efforts have often focused on restoring the economic services of forests, and typically have involved the establishment of plantations of exotic species (i.e. *Eucalyptus sp.*) (Lamb *et al.* 2005). However, this technique is now recognized to be unable to provide the variety of ecosystem services that are provided by indigenous forests (Lambi & Neba 2009; Telila *et al.* 2015). Current restoration practices, such as grazing exclusion, reinstatement of natural fire regimes, or planting of indigenous species, incorporate a greater understanding of ecosystem assembly and ecosystem function (Aerts *et al.* 2007; Heelemann *et al.* 2013). A combination of active and passive restoration has been implemented for the regeneration of Ngel Nyaki forest on the Mambilla Plateau, Nigeria (Barnes & Chapman 2014). The 'nurse-species' technique, whereby fast-growing, short-lived native trees are planted to rapidly form a canopy to promote colonization of other species, has been employed by conservation managers to aid rapid restoration at Ngel Nyaki

since 2006 (Barnes & Chapman 2014). Seeds collected from within the forest are germinated in a nursery, then planted out in focal areas. Passive restoration is promoted in other areas by the exclusion of cattle grazing and agricultural activities from buffer zones surrounding the core forest to encourage natural successional processes. Species diversity and functional recovery have primarily motivated restoration at Ngel Nyaki, and have also been used as a measure of their success (Ihuma *et al.* 2011). However, to date, genetic factors have been given little consideration in this restoration effort.

2.1.3 Genetic rescue

An assessment of plateau-wide genetic diversity and connectivity provides a potential aid for the preservation and restoration of forest fragments beyond Ngel Nyaki, and this restoration may not always entail expansion of the forest borders. In situations where genetic diversity is depauperate, such as in small, isolated populations, genetic diversity itself may require restoration, and can be increased through augmentation of gene flow (Richards & Ritland 2000; Ingvarsson 2001). Artificial supplementation of genetically impoverished populations with individuals/genetic material from another population is referred to as genetic rescue or genetic restoration (Hedrick 2005; Finger *et al.* 2011). The potential for genetic rescue for tropical tree species has not been extensively investigated in wild populations (Finger *et al.* 2011). Conservation mangers are generally reluctant to carry out transfers for the purpose of genetic rescue due to the fact that introduction of individuals from outside populations may result in outbreeding depression, where co-adapted gene complexes are broken down due to intra-specific hybridisation between different ecotypes (Richards & Ritland 2000; Willi *et al.* 2007; Frankham *et al.* 2011). In addition, translocations of genetic material between populations may result in an overall decrease in genetic diversity due to homogenisation of populations (Whiteley *et al.* 2015). However, it is suggested that in many cases the benefits of genetic rescue will outweigh the impact of outbreeding when severe genetic erosion has occurred (Willi *et al.* 2007; Frankham *et al.* 2011). Genotypes of immigrants may in fact spread quickly through a population due to heterosis, an effect whereby the mixing of genomes for different ecotypes results in hybrid vigor (Willi *et al.* 2007; Pickup *et al.* 2013). Frankham (2015) found that in 93% of genetic rescue efforts there was a beneficial effect of outcrossing. Furthermore, it has been seen that benefits frequently extend beyond the F1 generation; outcrossing has been found to remain highly beneficial in the F3 generation and beyond (Frankham 2016). Frankham *et al*. (2011) proposed a decision tree to be used by conservation managers to determine the risk of outbreeding depression, and therefore the potential for genetic rescue of genetically depauperate populations. They consider that in cases where there is resolved taxonomy, no fixed chromosomal differences, gene flow among populations has occurred within the last 500 years, and environmental differences are minimal, then outbreeding depression following translocations is unlikely.

Willi *et al*. (2007) showed that small populations of *Ranunculus reptans* in Central Europe suffered high rates of inbreeding, resulting in reduced fitness. It was found that crossing of individuals from separate populations had a positive effect on plant fitness due to heterosis.

For the critically endangered tree species *Medusagyne oppositifolia,* artificial cross-pollination was found to result in significantly greater seed production, suggesting potential for genetic rescue (Finger *et al.* 2011). Bossuyt (2007) found that interpopulation cross-pollination of *Parnassia palustris* resulted in significantly higher seed set than intrapopulation crosses. An increasing body of research suggests that genetic rescue of small, genetically impoverished populations may be key to their conservation, and apprehension for the implementation of genetic rescue is unjustified (Whiteley *et al.* 2015). Genetically depauperate populations on the Mambilla Plateau may necessitate genetic rescue in order to restore genetic diversity and consequently encourage long-term persistence.

2.1.4 Study species

Albizia gummifera (J.F.Gmel.) C.A.Sm. (Fabaceae: Mimosoideae) (hereafter *Albizia*) is a common deciduous canopy tree in the upland forests of Africa, ranging from West Africa to Madagascar (Keay 1989; Maroyi 2007). It is fast growing, reaching a height of up to 30m, and grows to elevations of 2500m (Keay 1989; Mullah *et al.* 2014). *Albizia* is used by people for a diverse range of purposes (Maroyi 2007). The species is not a major target of logging, but the timber can be used in light construction such as furniture and canoe building, and is often used for firewood (Hines & Eckman 1993). *Albizia* is frequently planted to shade crops such as coffee plantations, and is used as an ornamental plant in gardens (Teklay & Malmer 2004). *Albizia* has been attributed to a wide range of properties in traditional medicines throughout Africa. Such uses include treatment of malaria, headache and other pain relief, and the treatment of scabies (Maroyi 2007).

The tree develops nitrogen fixing nodules, and has been found to live in association with arbuscular fungi (Kadiata *et al.* 1996; Wubet *et al.* 2003). Leaves are large, alternate pinnately compound, and the crimson flowers exhibit the distinctive characteristic of other Mimosoideae species; numerous prominent stamen and inconspicuous petals. The flowers are bisexual and entomophilic (insect-pollinated) (Hines & Eckman 1993; Maroyi 2007). While the nature of the mating system is not confirmed for this species, high rates of outcrossing and self-incompatibility have been reported for other species in the same genus (Dunphy & Hamrick 2005; Pardini & Hamrick 2007). Seeds (~10x8mm) are contained within a pod (100-210mm), and do not detach when the pod desiccates, allowing the pod and seeds to be dispersed by the wind as a whole unit (Maroyi 2007; Mullah *et al.* 2014; Barnes & Chapman 2014).

Albizia gummifera is a fast growing, early-successional tree with potential for a significant role in both active and passive restoration of Afromontane forests. The species has been noted for mid-distance seed dispersal and consequential germination beyond forest edges into grasslands (Mullah *et al.* 2012; Barnes & Chapman 2014). It is therefore likely that, when deforestation threats are excluded from these areas, *Albizia* will be among the first forest species to become established and may facilitate the passive regeneration of other forest species (Mullah *et al.* 2012, 2014). *Albizia* has been found to have a positive effect on diversity of other plant species in both secondary and transitional forests in Kenya (Mullah *et al.* 2014).

The nitrogen-fixing properties of the species are likely to aid in habitat restoration by providing a significant proportion of the available nitrogen within the soil (Kadiata *et al.* 1996; Mullah *et al.* 2014). Also, the rapid growth of the species allows ease of use for planting projects. In Madagascar, other *Albizia sp.* have shown very high rates of survival and growth in restoration programs (Manjaribe *et al.* 2013).

The high abundance of *Albizia* in upland African forests and riparian zones has led to the assumption that it is unlikely to experience genetic erosion following habitat fragmentation as populations should be able to maintain high levels of gene flow (Maroyi 2007; Honnay & Jacquemyn 2007). However, increasing awareness of the potential for loss of genetic diversity in common species (discussed in Chapter 1) suggests *Albizia* may not be as resilient as previously thought. In fact, initial investigation of this species has already revealed the potential for an acute loss of genetic variation. Nantango *et al*. (2009) used RFLP (Random Amplified Fragment Polymorphisms) chloroplast regions to assess genetic diversity of *Albizia* in Uganda, Kenya, and Madagascar. This study revealed substantial genetic structuring among the populations, and extremely low haplotype diversity in Kenya, which was attributed to a greater intensity of habitat destruction in the region. This suggests that intense habitat fragmentation on the Mambilla Plateau may have left some *Albizia* populations vulnerable to erosion of genetic diversity. Therefore, characterisation of the genetic diversity of *Albizia* may aid in the ecological restoration efforts at Ngel Nyaki, and highlight populations in need of genetic rescue across the Mambilla Plateau.

2.1.5 Research goals

In this chapter, current levels of genetic diversity and population structure of *Albizia* are identified in remnant forest fragments on the Mambilla plateau and differences in diversity between generations is assessed. This reveals the vulnerability of *Albizia* to the genetic fallout of habitat degradation and fragmentation. It also gives an indication as to the rate at which genetic diversity is being altered in these fragments through a comparison of different demographic cohorts. These results are used to assess the suitability of *Albizia* for ecological restoration, and identifies populations in need of genetic rescue.

The primary research goals are as follows:

1. Identify chloroplast and nuclear microsatellite loci that can be used for rapid assessment of genetic characteristics of *Albizia*.
2. Determine the current level of genetic diversity and population structure exhibited by established *Albizia* trees in forest fragments across the Mambilla Plateau.
3. Assess differences in genetic diversity and population structure between adult and juvenile cohorts of *Albizia*.

2.2 Methods

2.2.1 Study sites and sample collection

Details of sampling locations are given in Section 1.3.2. Adult leaf tissues of *Albizia* were collected by NMFP staff in August 2016 from the six forest fragments across the Mambilla plateau. Juvenile samples were collected in June 2017 from Ngel Nyaki, Kurmin Danko, Kuma, and Yana forest fragments. Approximately 30 samples were taken throughout each of the forest fragments. Sampling entailed the removal of leaves from adult trees (>10m) or juvenile seedlings (~0.5m), which were stored in silica gel. Samples were then exported to the School of Biological Sciences, University of Canterbury, New Zealand, where they were stored at -20°c prior to DNA extraction.

2.2.2 DNA extraction

DNA extraction was carried out using a modified CTAB method from Weising *et al*. (1995). A small sample of dry leaf tissue (~15mg) was placed in a 1.7ml ependorf tube with two 3.2mm diameter stainless steel ball bearings. The tissue was ground to a fine powder in a Retsch® mixer mill. 800µl of 2x CTAB buffer with 0.5% β-mercaptoethanol was added to the sample. The CTAB buffer contains 0.1M Tris (pH 8.0), 1.4M NaCl, 20mM EDTA (pH 8.0), and 2% CTAB (Cetyl trimethylammonium bromide). The samples were briefly vortexed and then incubated on a heat block at 60°c, with occasional mixing. After incubation for 15-30 minutes, the samples were cooled, and 600µl of chloroform-isoamyl (24:1) was added, vortexed, and centrifuged for 5minutes at 13000rpm. The supernatant of the sample was pipetted into a new 1.7ml tube. Again, 600µl chloroform-isoamyl (24:1) was added to the supernatant, vortexed, and centrifuged 5 minutes at 13000rpm. The supernatant was then pipetted into a 1.7ml tube to which 4µl of 5mg/ml RNase is added. The samples were incubated for 30 minutes at 37°c. Then, 500µl of the cold isopropanol (-20°c) was added, and the tubes inverted several times. The samples were then chilled to -20°c for 30minutes-24hours.

The samples were centrifuged at 4°c for 10 minutes at 13000rpm. The supernatant was discarded and the pellet washed with 300µl of 70% ethanol, ensuring that that pellet had been loosened from the side of the tube. The samples were again centrifuged at 13000rpm for 10 minutes and the supernatant discarded. The tubes were then left open and upside down to allow the pellet to thoroughly dry (30-60 minutes). The DNA pellet was dissolved in 200µl of TE buffer by being left overnight at 4°c. The quality and quantity of the DNA extraction was then determined using a Nanodrop® ND-1000 spectrophotometer. DNA extracts were stored at -20°c until required.

2.2.3 Microsatellite optimisation.

44 primer pairs were tested for amplification in samples of *Albizia*. 30 of these pairs were chosen from the available "universal" chloroplast microsatellites, intended to amplify in all angiosperm species (Weising & Gardner 1999; Chung & Staub 2003; Cheng *et al.* 2006). The

remaining 14 primer pairs were sourced from nuclear microsatellites previously developed for species of close relation to *Albizia.* No studies were found to use microsatellite loci for species belonging to the same genus as *Albizia.* Instead, selections were restricted to those species belonging to the same subfamily; Mimosoideae. Nine pairs were taken from studies of *Enterolobium cyclocarpum* (Peters *et al.* 2008), two primer pairs originated from a study of *Acacia brevispica* (Otero-Arnaiz *et al.* 2005), and three of *Pithecellobium elegans* (Dayanandan *et al.* 1997). Ccmp primers (Weising & Gardner 1999) were assessed with fluorescently labelled primers. All other loci were designed for the attachment of M13 fluorescently labelled primers.

All primer pairs were tested for amplification on four randomly selected DNA samples. A standard PCR procedure was tested at annealing temperatures 45°c, 50°c and 55°c (see below for details). The products were then run on 1% agarose gel and examined for the presence of distinct bands. Those primers which amplified successfully were further tested with the inclusion of fluorescently labelled primers or M13 fluorescent tags (6-FAM, VIC, NED), and if bands were once again observed, clear amplification at these loci was assessed by genotyping. Those primer pairs with clear allele peaks were tested on additional samples from multiple populations in order determine which loci were polymorphic.

2.2.4 Microsatellite amplification and genotyping

PCR protocol used a 15μl reaction volume consisting of 1x PCR buffer (Bioline), 2mM MgCl2, 0.08mM dNTP, 0.08mM forward primer, 0.33uM reverse primer, 0.33uM M13 primer (of appropriate dye colour), 0.6U BiotaqTM DNA polymerase (Bioline), and 1μl of DNA extract. For primer pairs where florescent tags had already been attached (ccmp primers) the M13 primer was not used, and 0.33mM of the forward primer was used. The following reaction cycle conditions were used: Initial denaturation at 94°c for 5min, followed by 30 cycles of denaturation at 94°c for 1 min, annealing at 50°c or 1min, and extension at 72°c for 1 minute, and then a final extension at 72°c for 8 minutes. The resulting PCR product was tested on 1.4% agarose gel with SYBR Safe®. 5μl of the PCR product was mixed with 2μl of loading dye, and the gel run for 50 minutes at 80 volts. When bands were present, PCR products were then analysed by genotyping using an Applied Biosystems 3130xl Genetic Analyser, using 12μl of HiDiTM Formamide (Applied Biosystems) and 0.3μl of GeneScanTM 500 Liz® Size Standard (Applied Biosystems). The amount of PCR product used was subjectively decided based on the intensity of the band produced on agarose gel and ranged between 0.25μl and 1.5μl.

2.2.5 Data analysis

Fragment sizes were determined using the program Gene Marker v2.6.1 (SoftGenetics LLC®) and are recorded as either haploid (chloroplast) or codominant (nuclear) genotype data. The alleles at all polymorphic chloroplast loci for an individual are combined to give the individual's chloroplast haplotype. The non-recombining nature of the chloroplast genome allows genotypes across various regions to be analysed as a single multilocus haplotype.

Diversity measures for chloroplast loci are determined from haplotype frequencies, whereas nuclear loci diversity is calculated from allele frequencies. All differentiation indices are calculated from allele frequencies. Samples that have missing data are excluded from haplotype analysis, but are included in all other calculations.

Allele and haplotype frequencies, along with percent polymorphic loci (%P), were calculated using GenAlEx (Genetic Analysis in Excel) version 6.5 (Peakall & Smouse 2006, 2012). GenAlEx was then used to determine the number of effective haplotypes/alleles (n_e), and unbiased haplotype/allelic diversity (uH_E) within each population, using the following equations:

$$n_e = \frac{1}{\sum_i p_i^2}$$

(Equation 2.1)

$$uH_E = \frac{N}{N-1} \times \left(1 - \sum_i p_i^2\right)$$

(Equation 2.2)

Where p_i is the frequency of the *i*-th haplotype/allele within a population and *N* is the number of individuals sampled in that population. Regression analysis was used to asses any correlation in the diversity indices (n_e and uH_E) between the chloroplast and nuclear loci. Regression analysis was also performed to compared the uH_E of each population to the corresponding fragment size.

Overall Φ_{PT} (an analogue of F_{ST}) to determine the extent of population differentiation was calculated using the Analysis of Molecular Variance (AMOVA) function in GenAlEx. Pairwise Φ_{PT} ($\Phi_{PT}P$) and unbiased Nei's genetic distance (uNeiP) were used to compare the differentiation between pairs of populations. Nei's genetic distance is calculated as follows:

$$uNeiP = -ln\left(\frac{J_{xy}}{\sqrt{\left(\hat{J}_x \hat{J}_y\right)}}\right)$$

$$\text{Where, } \hat{J}_x = \frac{(2NJ_x - 1)}{2N_x - 1}, \hat{J}_y = \frac{(2N_y J_y - 1)}{2N_y - 1}$$

$$\text{And, } J_{xy} = \sum_{i=1}^{k} p_{ix} p_{iy},\ J_x = \sum_{i=1}^{k} p_{ix}^2,\ J_y = \sum_{i=1}^{k} p_{iy}^2.$$

(Equation 2.3)

p_{ix} and p_{iy} are the frequency of the *i*-th allele in populations *x* and *y,* and N_x and N_y is the corresponding sample size of those populations. When calculating for multiple loci, the average of j_{xy} , $\hat{J}_x$ and , $\hat{J}_y$ is used in the calculation of uNeiP. Mantel testing in GenAlEx is used to test for correlation between adult and juvenile uNeiP and $\Phi_{PT}P$ values. Isolation-by-distance is also assessed through a Mantel test of the pairwise Φ_{PT} matrix and the geographic

distance (km) between populations. All Mantel tests undergo 999 permutations. Regression and Mantel analyses are used to assess the congruence of chloroplast and nuclear loci at genetic diversity and differentiation measures.

Genotype assignment was performed in STRUCTURE version 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003; Hubisz *et al.* 2009) in order to test for population genetic structure. Assignment tests used an admixture model within correlating allele frequencies, because gene flow among populations was considered to be likely, and used sampling locations as prior information to assess for migrants among sampling locations. 10,000 repeats for burn-in was followed by 100,000 repeats of MCMC. For chloroplast loci, genetic structure is assessed by combining both adult and juvenile data to see if different cohorts are assigned to the same cluster. The Evanno method, which can be used to define the optimal number of populations, cannot validate K=1 or the maximum K run in Bayesian analysis (Evanno *et al.* 2005). Therefore, genotype assignments should exceed the maximum number of likely populations. For cpSSR loci, the total number of populations is ten if different cohorts are considered as separate populations, so 11 was the maximum K value that was run in STRUCTURE. K values between 1 and 7 are tested for the nuclear loci, as only samples from six adult populations were assessed. K values were tested with 10 repeats each. Optimal K (number of populations) was subjectively determined from plots of the mean Ln probability of K vs. K and ΔK vs. K (Evanno *et al.* 2005) developed in Structure Harvester (Earl & vonHoldt 2012).

2.3 Results

2.3.1 Chloroplast loci

Twenty-two universal chloroplast loci showed successful amplification for *Albizia* (Table 2.1). However, genotyping of at least 8 samples from across a minimum of 4 populations revealed that 17 of these loci were monomorphic. The five loci that were found to be polymorphic were tested on all adult and juvenile samples and used to assess genetic diversity and population structure of *Albizia*. Locus NTCP40 was the only polymorphic locus that resulted in missing data, at a rate of 4.38%.

For *Albizia* adult samples, fourteen alleles from five polymorphic chloroplast loci ranged in size from 201-428 base pairs, and the number of alleles per locus across all populations ranged from 2-5 (Table 2.2). The average number of alleles was 2.8 per locus. Juvenile samples showed 11 alleles with a size range of 201-412, a range of 1-3 alleles per locus, and an average of 2.2 alleles per locus (Table 2.3). Only one private allele was identified within adult populations; ccSSR5 allele 284 occurred at a frequency of 0.14 in Kurmin Danko. Among juvenile populations there were four private alleles; one each in Ngel Nyaki and Kurmin Danko, and two within Yana. Four alleles that were present in *Albizia* adults were absent from juvenile samples. Only one allele occurred in juvenile populations that had not been observed among adults (ccSSR5 allele 283).

Table 2.1:Successfully amplifying chloroplast loci for Albizia. *[a] Y (= C or T), R (= A or G), K (= T or G), W (= A or T) and S (= C or G). [b] T_a = annealing temperature. * ccmp loci used fluorescently labelled forward primers, all other primer pairs were designed for the attachment of a M13 florescent primer to the forward primer. Loci in bold indicate polymorphic loci that were used for further analysis.*

Primer	Source	Location	Repeat motif	Primer sequence [a]	Dye/Colour	T_a [b]	Sample size	Approx. Allele size (bp)
ccmp2*	(Weising & Gardner 1999)	5' to *trnS*	$(A)_{11}$	F: GATCCCGGACGTAATCCTG R: ATCGTACCGAGGGTTCGAAT	6-FAM/Blue	50°c	20	279
ccmp3*	(Weising & Gardner 1999)	*trnG* intron	$(T)_{11}$	F: CAGACCAAAAGCTGACATAG R: GTTTCATTCGGCTCCTTTAT	PET/Red	50°c	24	130
ccmp4*	(Weising & Gardner 1999)	*atpF* intron	$(T)_{13}$	F: AATGCTGAATCGAYGACCTA R: CCAAAATATTBGGAGGACTCT	6-FAM/Blue	50°c	24	134
ccmp5*	(Weising & Gardner 1999)	3' to *rps2*	$(C)_7(T)_{10}$	F: TGTTCCAATATCTTCTTGTCATTT R: AGGTTCCATCGGAACAATTAT	VIC/Green	50°c	24	137
ccmp7*	(Weising & Gardner 1999)	*atpB-rbcL* intergenic	$(A)_{13}$	F: CAACATATACCACTGTCAAG R: ACATCATTATTGTATACTCTTTC	PET/Red	50°c	24	157
ccmp10*	(Weising & Gardner 1999)	*rpl2-rps19* intergenic	$(T)_{14}$	F: TTTTTTTTTAGTGAACGTGTCA R: TTCGTCGDCGTAGAAATAG	VIC/Green	50°c	24	135
ccSSR5	**(Chung & Staub 2003)**	**Rps2-RpoC2**	**$(T)_{10}$**	**F: TCTGATAAAAAACGAGCAGTTCT R: GAGAAGGTTCCATCGGAACAA**	**NED/Black**	**50°c**	**274**	**282-285**
ccSSR7	(Chung & Staub 2003)	PsbC-TrnS	$(T)_{11}$	F: CGGGAAGGGCTCGKGCAG R: GTTCGAATCCCTCTCTCCTTTT	6-FAM/Blue	50°c	12	370
ccSSR8	**(Chung & Staub 2003)**	**Ycf3**	**$(T)_5C(T)_{17}$**	**F: TTGATCTTTACGGTGCTTCCTCTA R: TCATTACGTGCGACTATCTCC**	**NED/Black**	**50°c**	**274**	**260-265**
ccSSR9	(Chung & Staub 2003)	PsbC-TrnScf3	$(A)_{13}$	F: GAGGATACACGACAGARGGARTTG R: CCTATTACAGAGATGGTGYGATTT	6-FAM/Blue	50°c	12	186
ccSSR11	(Chung & Staub 2003)	Rpl20-ClpP	$(T)_6C(T)_{14}$	F: TTGGCTACTCTAACCTTCCC R: ACCATAGAAACGAWGGAACCCACT	VIC/Green	50°c	16	177
ccSSR12	(Chung & Staub 2003)	PsbB-PsbT	$(A)_8$	F: CCAAAAACTTGGAGATCCAACTAC R: TTCCATAGATTCGATCGTGGTTTA	6-FAM/Blue	50°c	30	250
ccSSR15	(Chung & Staub 2003)	Rpl2-Rpl23	$(T)_9$	F: GCTTATGACCTCCCCCTCTATGC R: TGCATTACAGACGTATGATCATTA	VIC/Green	50°c	30	284
ccSSR16	(Chung & Staub 2003)	TrnL	$(T)_7C(T)_2$	F: TACGAGATCACCCCTTTCATTC R: CCTGGCCCAACCCTAGACA	NED/Yellow	50°c	19	376
ccSSR18	(Chung & Staub 2003)	Ycf5	$(A)_8$	F: TCGTTGGATTTCTTCDGGACATTT R: CCCAATATCATCATACTTACRTGC	NED/Yellow	50°c	11	290
ccSSR19	(Chung & Staub 2003)	Ycf5	$(T)_8$	F: CTATGCAGCTCTTTTATGYGGATC R: TCCARGTAATAAATGCCCAAGTT	6-FAM/Blue	50°c	30	382
ccSSR20	(Chung & Staub 2003)	NDHD-PSAC	$(A)_8$	F: CCGARATATTGGAAAAACWACAA R: GCTAARCAAATWGCTTCTGCTCC	NED/Yellow	45°c	8	356
ccSSR21	(Chung & Staub 2003)	TrnR-Rrn5	$(T)_{13}$	F: CCACCCCGTCTCSACTGGATCT R: AAAAATAGCTCGACGCCAGGAT	6-FAM/Blue	50°c	30	293
ccSSR22	**(Chung & Staub 2003)**	**TrnL-16SrRNA**	**$(T)_8$**	**F: CCGACCTAGGATAATAAGCYCATG R: GGAAGGTGCGGCTGGATC**	**6-FAM/Blue**	**50°c**	**274**	**201-202**
NTCP9	(Cheng *et al.* 2006)	**trnG/trnR**	**$(T)_{10}$**	**F: CTTCCAAGCTAACGATGC R: CTGTCCTATCCATTAGACAATG**	**VIC/Green**	**50°c**	**274**	**412-429**
NTCP40	**(Cheng *et al*. 2006)**	**Rps12/trnH**	**$(A)_{14}$**	**F: GATGTAGCCAAGTGGATCA R: TAATTTGATTCTTCGTCGC**	**VIC/Green**	**50°c**	**262**	**294-302**
ARCP5	(Cheng *et al*. 2006)	*trnL/trnF* intergenic	$(T)_{13}$	F: GGCCATAGGCTGGAAAGTCT R: GTTTATGCATGGCGAAAAGG	6-FAM/Blue	50°c	12	206

Non-amplifying loci: ccsmp1*, ccmp6*, ccmp8*, ccmp9*, ccSSR2, ccSSR4, ccSSR17, & ARCP2.

Table 2.2: Allele sizes for polymorphic cpSSR loci, and their frequencies in populations of adult Albizia*. *Indicates private alleles. n= number of samples.*

Locus	Allele frequency						
	Ngel Nyaki	Kurmin Danko	Kuma	Yana	Mbamnga	Tamnyar	Total
ccSSR5	282 (0.94) 285 (0.06)	282 (0.76) 284 (0.14) * 285 (0.10)	282 (1.00)	282 (1.00)	282 (1.00)	282 (1.00)	282 (0.94) 284 (0.03) 285 (0.03) *
ccSSR8	260 (0.94) 265 (0.06)	260 (0.90) 265 (0.10)	260 (1.00)	260 (0.93) 265 (0.07)	260 (0.79) 265 (0.21)	260 (1.00)	260 (0.92) 265 (0.08)
ccSSR22	201 (0.06) 202 (0.94)	201 (0.10) 202 (0.90)	202 (1.00)	201 (0.07) 202 (0.93)	201 (0.21) 202 (0.79)	202 (1.00)	201 (0.08) 202 (0.92)
NTCP9	412 (0.94) 428 (0.06)	412 (0.90) 428 (0.10)	412 (1.00)	412 (0.93) 428 (0.07)	412 (0.79) 428 (0.21)	412 (1.00)	412 (0.92) 428 (0.08) *
NTCP40	294 (0.30) 295 (0.52) 296 (0.12) 302 (0.06)	294 (0.08) 295 (0.88) 296 (0.04)	293 (0.03) 294 (0.03) 295 (0.93)	295 (1.00)	293 (0.16) 294 (0.21) 295 (0.42) 302 (0.21)	296 (1.00)	293 (0.03) * 294 (0.12) 295 (0.73) 296 (0.07) 302 (0.04) *
n	33	29	29	30	24	5	150

Table 2.3: Allele sizes for polymorphic cpSSR loci, and their frequencies in populations of juvenile Albizia*. *Indicates private alleles. n= number of samples.*

Locus	Allele frequency				
	Ngel Nyaki	Kurmin Danko	Kuma	Yana	Total
ccSSR5	282 (0.88) 284 (0.12)	282 (0.93) 283 (0.03) * 284 (0.03)	282 (1.00)	282 (1.00)	282 (0.95) 283 (0.01) * 284 (0.04)
ccSSR8	260 (1.00)	260 (1.00)	260 (1.00)	260 (0.97) 265 (0.03) *	260 (0.99) 265 (0.01)
ccSSR22	202 (1.00)	202 (1.00)	202 (1.00)	201 (0.03) * 202 (0.97)	201 (0.01) 202 (0.99)
NTCP9	412 (1.00)	412 (1.00)	412 (1.00)	412 (1.00)	412 (1.00)
NTCP40	294 (0.06) * 295 (0.94)	295 (0.87) 296 (0.13)	295 (1.00)	295 (0.97) 296 (0.03)	294 (0.02) 295 (0.94) 296 (0.04)
n	34	30	30	30	124

Alleles present in adult samples translated to 7 different haplotypes, whereas juvenile samples possessed 6 different haplotypes. The allelic composition and frequencies of haplotypes in each population is given in Table 2.4. Haplotype A4 was the most frequent haplotype for both adults and juveniles. The number of haplotypes within a population ranged between 1 and 4 haplotypes for both adults and juveniles. Four haplotypes occurred in juveniles that had not previously been observed in adults in those populations. Among adults one private haplotype was observed in each of the populations Ngel Nyaki and

Mbamnga. For juveniles, there were three private haplotypes found in Ngel Nyaki, Kurmin Danko and Yana.

Table 2.4: Haplotype frequencies within populations for cpSSR loci among samples of adult and juvenile Albizia*. In populations where adults and juveniles were sampled their values are indicated by 'adults'/'juveniles'. *Indicates private haplotypes.*

Haplotype	Allelic composition	Haplotype frequency						
		Ngel Nyaki	Kurmin Danko	Kuma	Yana	Mbamnga	Tamnyar	Total
A1	201/265/285/302/428	0.06*/0						0/0.01
A2	202/260/282/293/412			0.03/0		0.16		0.01/0
A3	202/260/282/294/412	0.30/ 0.06*	0.08/0	0.03/0		0.21		0.01/0
A4	202/260/282/295/412	0.52/ 0.82	0.72/ 0.80	0.93/ 1.00	1.00/ 0.93	0.42		0.01/0
A5	202/260/282/296/412	0.12/0	0.04/ 0.13		0/0.03		1.00	0.13/0.1
A6	202/260/282/302/412					0.21*		0.51/ 0.77
A7	202/260/284/295/412	0/0.12	0.16/ 0.03					0.04/ 0.04
A8	201/265/282/295/412				0/ 0.03*			0.01/0
A9	202/260/283/295/412		0/0.03*					0/0.01

Measures of genetic diversity varied greatly among populations and age classes (Table 2.5). Among adult populations, percent polymorphic loci was highest in Ngel Nyaki and Kurmin Danko at 100%. However, polymorphism at all loci did not correspond to the highest effective number of haplotypes or highest haplotype diversity, which was observed in Mbamnga, with 80% polymorphic loci, 3.44 effective haplotypes, and haplotype diversity of 0.75. The population of Tanmyar showed no genetic diversity as all loci were monomorphic in these samples. However, the small sample size taken within this population gives low confidence in these results. Despite relatively high %P in Yana, when samples with missing data are excluded from haplotype analysis only a single haplotype remains in this population, and therefore no haplotype diversity. Low genetic diversity was also observed in Kuma, where only one of the five loci was polymorphic, effective number of haplotypes was 1.15 and haplotype diversity was 0.14. There was no significant correlation between haplotype diversity and fragment size (r^2=0.311, p=0.250).

All populations except Yana also showed a reduction in the number of polymorphic loci in juveniles compared to adults, whereas %P in Yana did not change (60%). Yana was also the only population that did not show a reduction in n_e and uH_E in juveniles compared with corresponding adults. Two additional haplotypes occur in juveniles in this population that were not among adults. However, genetic diversity of juveniles at Yana is still very low.

Juveniles from Kuma exhibited no genetic diversity. There was no significant correlation between haplotype diversity and fragment size (r^2=0.305, p=0.255).

Table 2.5: Genetic diversity indices for polymorphic chloroplast microsatellite loci in populations of Albizia. *N= number of samples taken from the specified populations, %P = percent polymorphic loci, n_e = number of effective haplotypes, uH_E = unbiased haplotype diversity. In populations where adults and juveniles were sampled their values are indicated by 'adults'/'juveniles'.*

Population	N	%P	n_e	uH_E
Ngel Nyaki	33/34	100/40	2.66/1.44	0.64/0.31
Kurmin Danko	29/30	100/40	1.81/1.52	0.47/0.35
Kuma	29/30	20/0	1.15/1.00	0.14/0.00
Yana	30/30	60/60	1.00/1.15	0.00/0.13
Mbamnga	24	80	3.44	0.75
Tamnyar	5	0	1.00	0.00
Total	138/124	100/100	3.45/1.63	0.72/0.39

AMOVA analysis revealed a moderate level of genetic differentiation among populations of *Albizia* adults (Φ_{PT}=0.142, p=0.010). Pairwise Φ_{PT} comparisons between adult populations range between 0.022 (Kuma-Yana) and 0.886 (Kuma-Tamnyar). Unbiased Nei's genetic distance between Kuma and Yana was also the lowest (0.001) and the highest uNeiP occurred between Yana and Tamnyar (0.234) (Table 2.6). There was no evidence for isolation-by-distance (r^2=0.029, p=0.230; Figure 2.1). Overall genetic differentiation was low yet significant among juvenile populations with a Φ_{PT} of 0.036 (p=0.012). In all cases the Φ_{PT}P and uNeiP was lower in juvenile samples than in corresponding adults. Φ_{PT}P values between juvenile populations were found not to be significant, indicating that none of these pairs of populations are genetically distinct from one another. Mantel test for correspondence finds that there is significant correlation between adults and juvenile pairwise Nei's genetic distance (r^2=0.330, p=0.041), however there is not a significant correlation of pairwise Φ_{PT} values (r^2=0.603, p=0.070).

Plots of mean of estimated Ln probability of data vs. K (Figure 2.2) and ΔK vs. K (Figure 2.3) both identify the optimal number of populations as two. This is determined by the levelling off by Ln probability values at K=2 in Figure 2.2, and a clear peak in ΔK at K=2 in Figure 2.3. The structure plot for K=2 (Figure 2.4) shows that the majority of samples are assigned to a single cluster (displayed in blue). All samples in Kuma and Tamnyar populations are assigned this cluster. 12 adult samples across the other four populations are primarily assigned (>50%) to the second cluster (orange). A single juvenile sample from Yana was also assigned to this cluster. Additional samples in Mbamnga population have low assignment (10-50%) to the second cluster. The structure plot shows no evidence for real genetic structure among all populations, but suggests there are some individual samples throughout the populations that possess several rare alleles and may represent migrants from untested populations. Overall,

the structure results show that fragments of *Albizia* are representative of a single, plateau wide population with high levels of gene flow throughout.

Table 2.6: Pairwise Φ_{PT} and unbiased pairwise Nei's genetic distance of populations of Albizia *as calculated in GenAlEx (Peakall & Smouse 2006, 2012). Φ_{PT}P values are given above the diagonal, uNeiP below the diagonal. In populations where adults and juveniles were sampled their values are indicated by 'adults'/'juveniles'. * indicates statistically significant (p<0.05) differentiation between population pairs (Φ_{PT}P) based on 999 permutations.*

	Ngel Nyaki	Kurmin Danko	Kuma	Yana	Mbamnga	Tamnyar
Ngel Nyaki	-	0.049/0.022	0.161*/0.063	0.114*/0.037	0.057*	0.321*
Kurmin Danko	0.024/0.002	-	0.090*/0.075	0.028/0.021	0.078*	0.363*
Kuma	0.023/0.002	0.005/0.002	-	0.022/0.00	0.263*	0.886*
Yana	0.033/0.002	0.003/0.001	0.001/0.000	-	0.162*	0.632*
Mbamnga	0.010	0.044	0.046	0.046	-	0.261*
Tamnyar	0.128	0.221	0.209	0.234	0.173	-

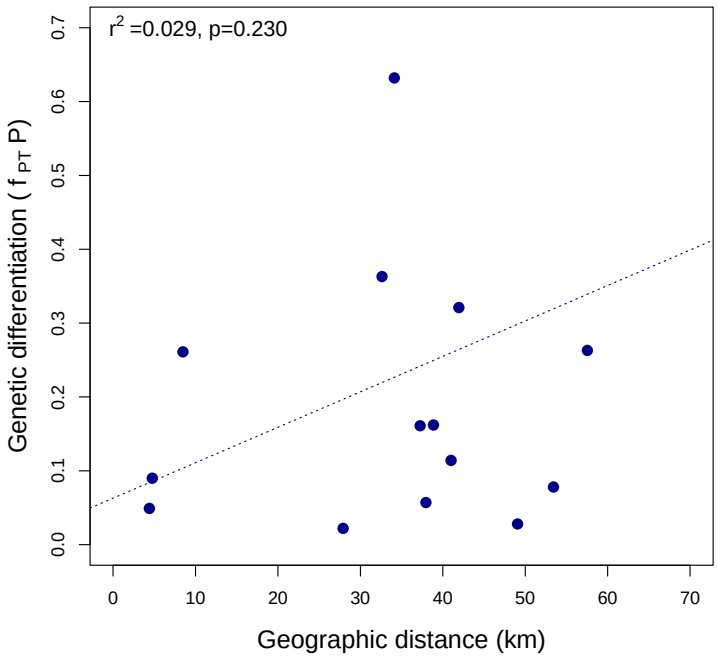

Figure 2.1: Isolation-by-distance plot for adult Albizia *samples using cpSSRs. Mantel test in GenAlEx (Peakall & Smouse 2006, 2012) used to assess significance (α=0.05).*

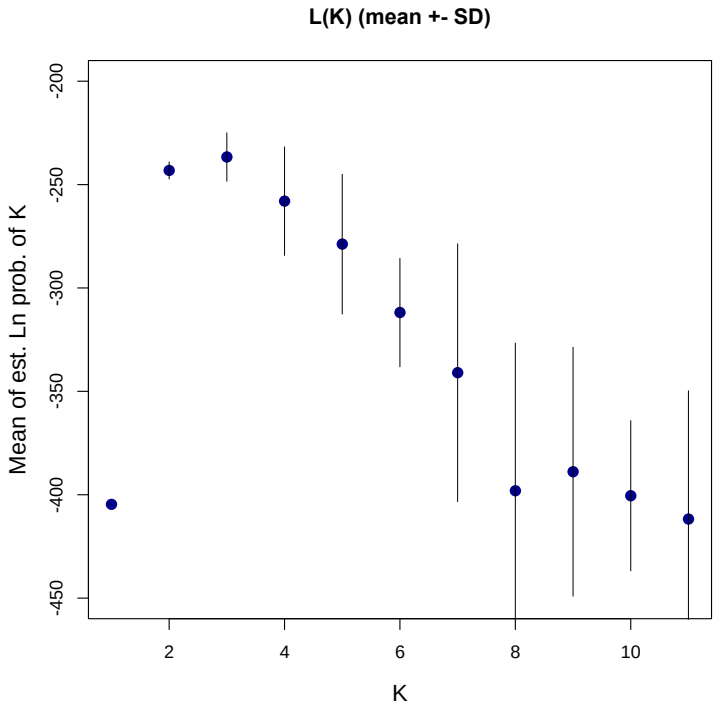

Figure 2.2: Mean of estimated Ln probability of data vs. K (number of populations) for Albizia *using cpSSRs. Determined from 10 replicate/K of genotypes assignments performed in STRUCTURE (Pritchard et al., 2000) and summarised in Structure harvester (Earl & vonHoldt, 2012).*

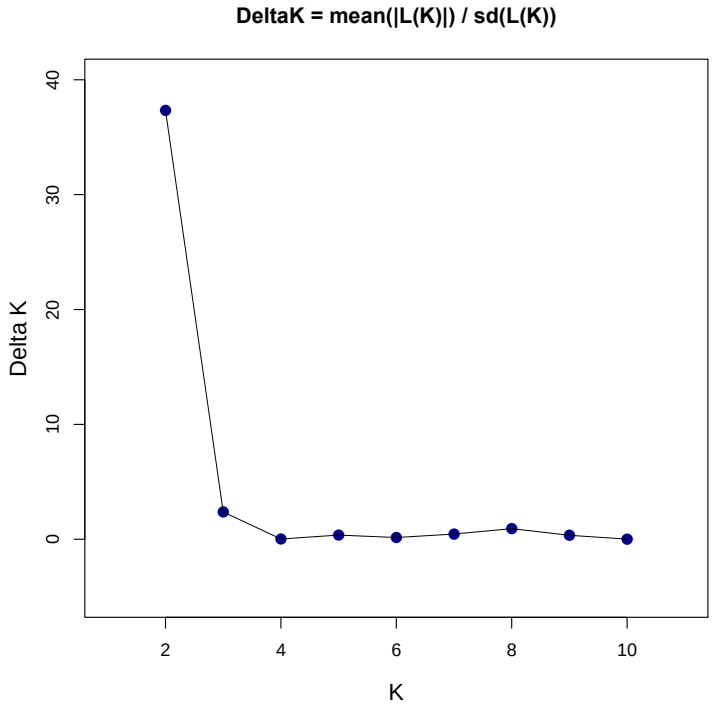

Figure 2.3: Optimal number of populations shown by ΔK vs. K calculated from 10 replicates per K of genotype assignments performed in STRUCTURE (Pritchard et al. 2000) on adult samples of Albizia *using cpSSRs. ΔK calculated using method of Evanno et al. (2005) in Structure Harvester (Earl & vonHoldt 2012).*

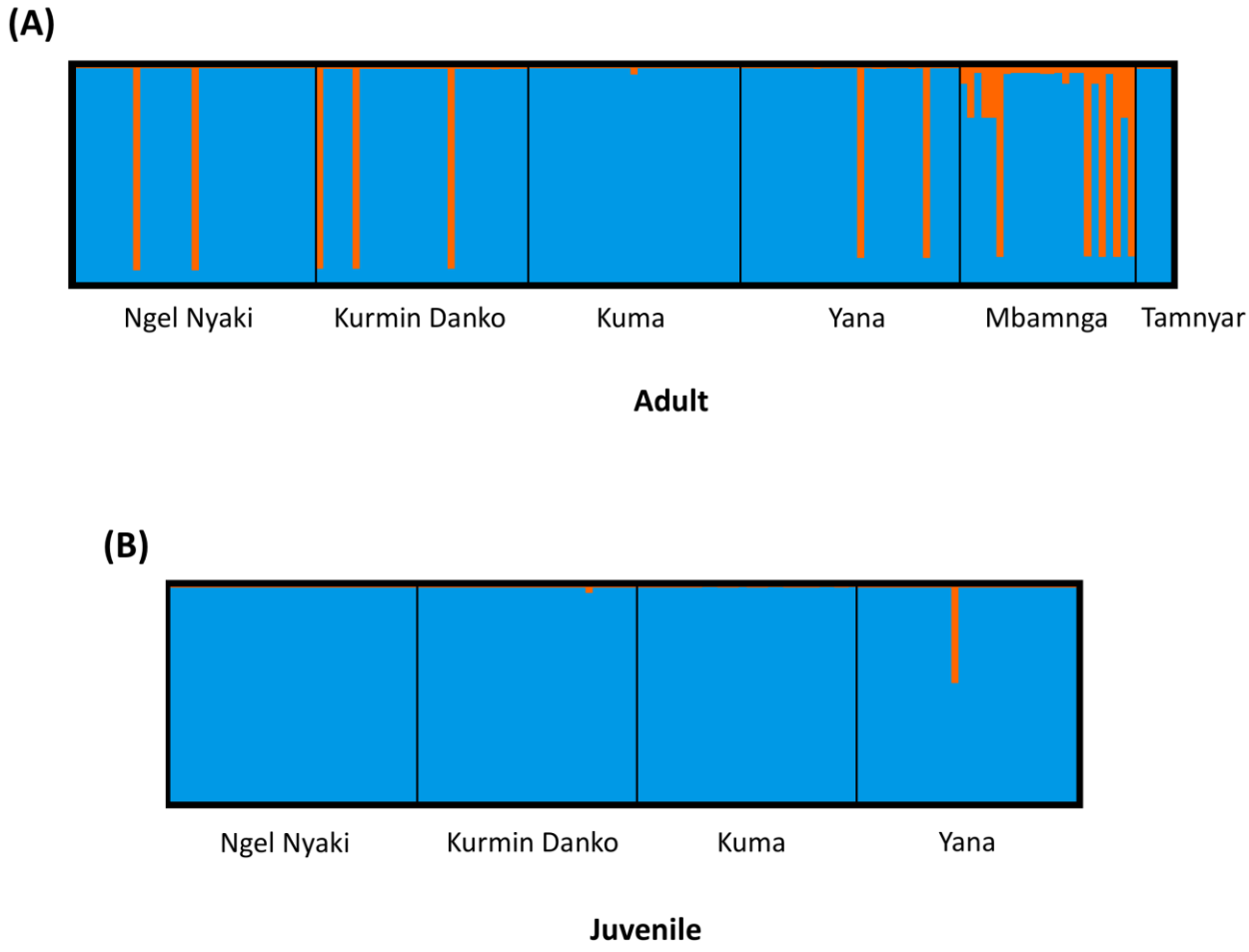

Figure 2.4: Structure plot from Bayesian cluster analysis by STRUCTURE for 2 populations (K=2) of Albizia *by cpSSRs for A. Adult samples and B. Juvenile samples. Each bar represents a single individual's Q-value (probability of assignment), and their true population is indicated at the base of the plot.*

2.3.2 Nuclear loci

Of the 14 nuclear loci examined, it was found that only two loci produced distinguishable peaks when genotyped (Table 2.7:). Ency-21 (Peters *et al.* 2008) exhibited a fixed allele at 228bp for 8 samples tested across multiple populations. Ency-17 (Peters *et al.* 2008) showed polymorphism, and was genotyped for all adult samples to assess its congruence with the results from the chloroplast loci. Four of the 150 samples did not produce a clear peak when genotyped, and were therefore excluded from further analysis.

Locus Ency-17 produced 15 different alleles ranging from 267-286 base pairs. All populations exhibited polymorphism at this locus. Genetic diversity indices are summarized in Table 2.9 and compared by population to cpSSR loci in Figure 2.5. The highest effective number of alleles is identified in Kurmin Danko, which also had the highest allelic diversity, equal with Ngel Nyaki. The lowest n_e and uH_E was found within Kuma. There is found to be no significant correlation between allelic diversity and fragment size (r^2=0.302, p=0.258).

Table 2.7: Successfully amplifying nuclear microsatellite loci for Albizia. *[a]T_a = annealing temperature.*

Primer	Source	Source species	Repeat motif	Primer sequence	Dye/Colour	T_a[a]	Sample size	Approx. Allele size (bp)	Polymorphic (Y/N)
Ency-17	(Peters *et al.* 2008)	***Enterolobium cyclocarpum***	**$(GTTT)_6$**	**F: GTTTATTAGGAGCCTCGACTGTTA R: ACCTGCACTTCCAACATAGT**	**VIC/Green**	**50°c**	**146**	**267-286**	**Y**
Ency-21	(Peters *et al.* 2008)	*E. cyclocarpum*	$(AC)_8(AC AT)_4$	F: GTTTGTCCAAAAGGTAGTA R: TAGGCTCATGTTCAGATA	NED/Yellow	50°c	8	228	N

Non-amplifying loci: Ency-4, Ency-8, Ency-9, Ency-13, Ency-22, Ency-24, Ency-33, AY843537, AY843557, Pel2, Pel3, and Pel6.

Table 2.8: Ency-17 allele sizes and frequencies within populations of adult Albizia.

Allele	Allele frequency						
	Ngel Nyaki	Kurmin Danko	Kuma	Yana	Mbamnga	Tamnyar	Total
267	0.02*						<0.01
269		0.02*					<0.01
270	0.02*						<0.01
273	0.03	0.25	0.17	0.18	0.31		0.17
274	0.06		0.10		0.02	0.30	0.05
275	0.25	0.30	0.60	0.43	0.29		0.36
276		0.09	0.02	0.02	0.02		0.03
277		0.04		0.02			0.01
278	0.13	0.16	0.10	0.25	0.10	0.20	0.15
279				0.02*			<0.01
280	0.02	0.02		0.02	0.08		0.02
281	0.33	0.11		0.07	0.08	0.50	0.14
282	0.03	0.02					0.01
285	0.13*						0.03
286					0.08*		0.01
n	32	28	29	28	24	5	124

Table 2.9: Genetic diversity indices for nuSSR Ency-17 in populations of Albizia*. N= number of samples taken from the specified populations, n_e = number of effective alleles, uH_E = allelic diversity.*

Population	N	n_e	uH_e
Ngel Nyaki	32	4.81	0.81
Kurmin Danko	28	4.95	0.81
Kuma	29	2.41	0.60
Yana	28	3.52	0.73
Mbamnga	24	4.65	0.80
Tamnyar	5	2.63	0.69
Total	146	3.68	0.73

Regression analysis finds that the correlation between nuSSR and cpSSR genetic diversity across populations was not significant (n_e: r^2=0.569, p=0.083; uH_E: r^2=0.541, p=0.096; Figure 2.5). However, like the chloroplast loci, this locus reveals high levels of diversity in Ngel Nyaki, Kurmin Danko and Mbamnga relative to Kuma, Yana and Tamnyar. The overall Φ_{PT} value for the nuclear locus was found to be 0.142 (p=0.001), in agreement with the moderate level of population differentiation identified by the chloroplast loci (Φ_{PT}=0.142, p=0.011). The Mantel test also revealed a strong significant correlation between the nuclear and chloroplast loci for uNeiP (r^2=0.707, p=0.021) and Φ_{PT}P (r^2=0.731, p=0.008). Again, there is no support for isolation-by-distance (r^2=1.5×10^{-4}, p=0.429; Figure 2.6). These results show that the single nuclear locus reflects trends of genetic diversity and population differentiation that are similar to the chloroplast loci.

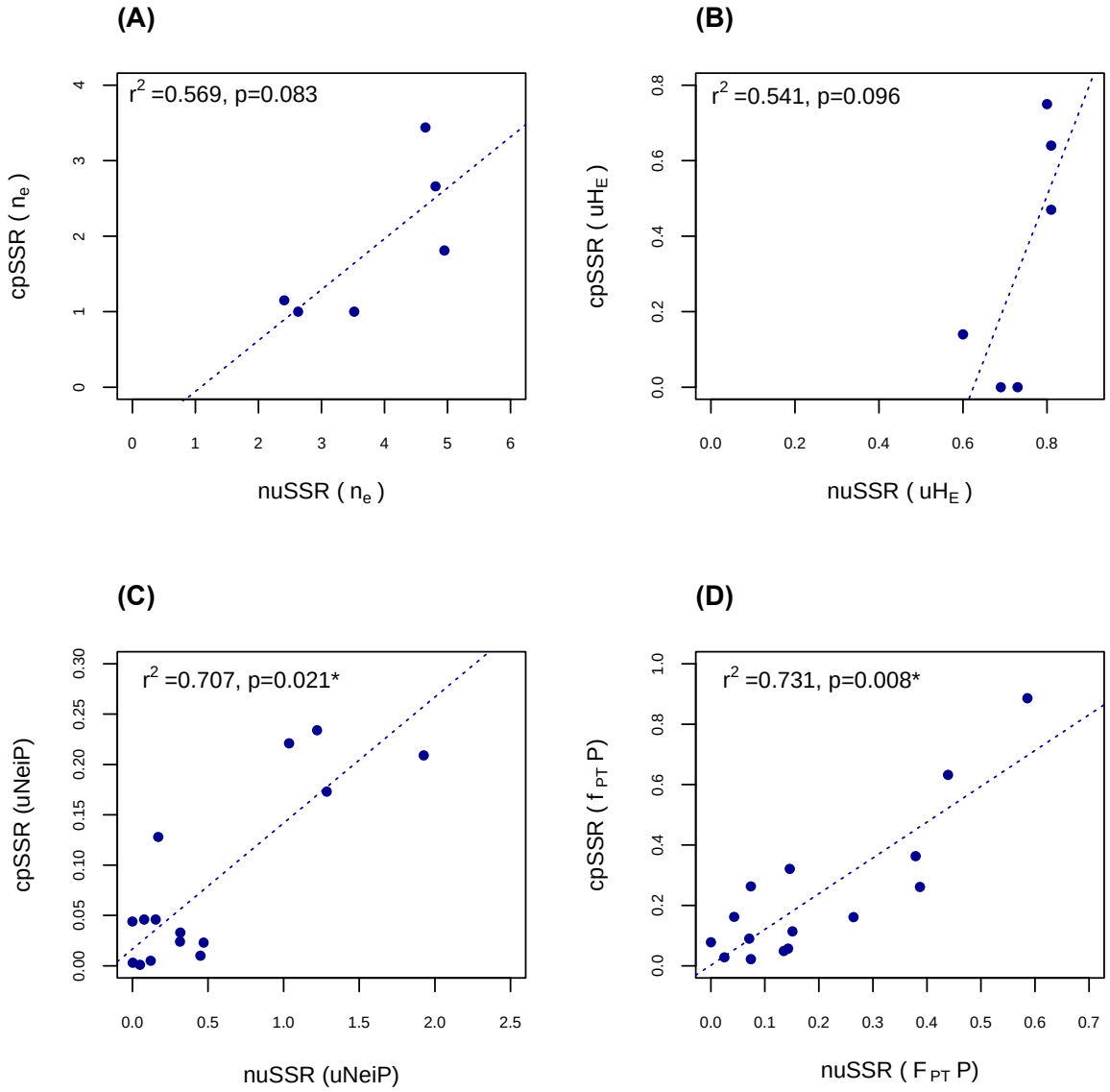

Figure 2.5: Regression analysis to assess correlation between cpSSR and nuSSr loci for Albizia. *A. Effective number of haplotypes/ alleles (n_e). B. Unbiased haplotype/allelic diversity (uH_E). C. Unbiased pairwise Nei's genetic distance (uNeiP). D. Pairwise population differentiation (Φ_{PT}P). * represents significant correlation (α=0.05).*

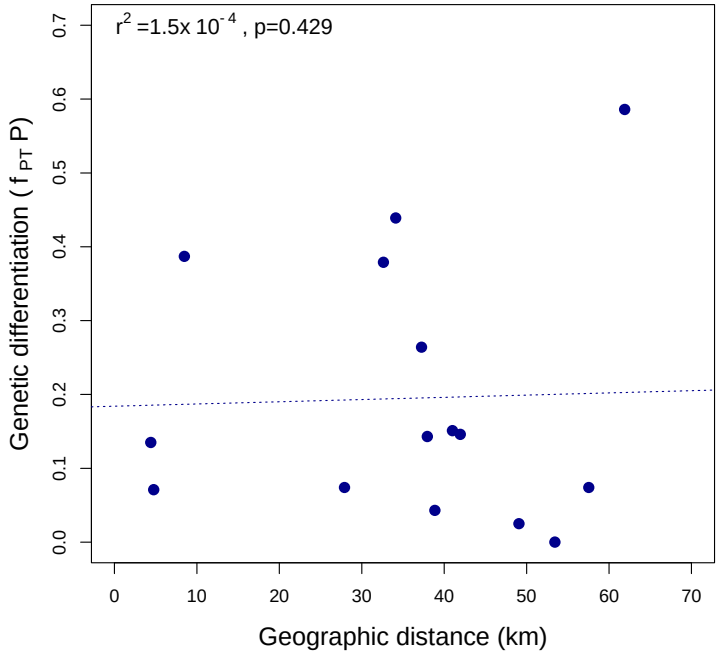

Figure 2.6: Isolation-by-distance plot for adult Albizia *samples using nuSSR loci ENCY-17 (Peters* et al. *2008). A Mantel test in GenAlEx (Peakall & Smouse 2006, 2012) is used to assess significance (α=0.05).*

Analysis in STRUCTURE using the Ency-17 locus suggests that the optimal number of populations may be one or two (Figure 2.7A&B), in congruence with the lack of genetic structure observed for the chloroplast loci. However, the structure plot for K=2 reveals population structure that was not evident from the chloroplast loci (Figure 2.7C). Individuals in Ngel Nyaki and Tamnyar are primarily assigned to cluster 2 (orange) and most individuals in all other populations are assigned to cluster 1 (blue). Most individuals are not assigned solely to one cluster, suggesting high gene flow throughout the plateau.

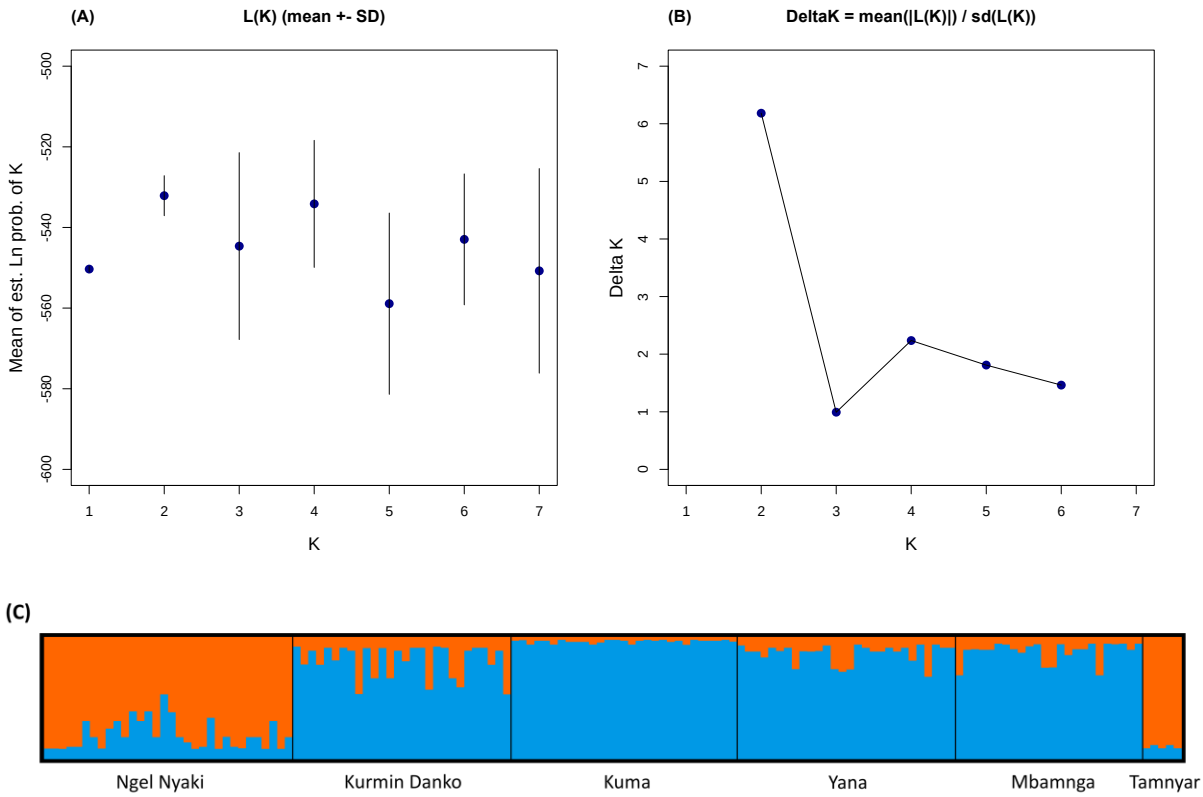

Figure 2.7: Summary results of structure analysis of Albizia *using nuSSR Ency-17 (Peters* et al. *2008). A. Mean of estimated Ln probability vs. K, B. Optimal number of populations shown by ΔK vs. K, C. Structure plot of Q-values per sample for 2 populations (K=2).*

2.4 Discussion

2.4.1 Identification of microsatellite loci

Our investigation found a limited number of microsatellite loci are currently available for analysis of genetic characteristics of *Albizia*. While many of the chloroplast microsatellite loci did amplify in our samples, a very low proportion of these were found to be polymorphic (23%). Low levels of intraspecific variation are often observed for 'universal' loci such as these (Weising & Gardner 1999). This exemplifies the need to examine a high number of loci in order to obtain a sufficient number of polymorphic loci. The high number of monomorphic loci obtained may be found to exhibit variation in *Albizia* if assessed across a greater spatial scale.

The rate of cross amplification of nuclear loci found in this investigation is lower than anticipated. Barbará *et al*. (2007) summarized rates of interspecies transfer of nuclear microsatellites and found that an average of 60% of loci cross-amplify between species within the same family. Only two (14%) of the nuclear loci examined cross-amplified to *Albizia.*

While a limited number of loci were identified in this investigation, the consistencies between chloroplast and nuclear loci suggest that genetic diversity and structure inferred from chloroplast loci are accurate. Analysis of few highly polymorphic loci can still be useful for determining genetic diversity or population structure (Arthofer *et al.* 2017). These five chloroplast loci appear to be satisfactory for rapid and low cost assessment of *Albizia*.

2.4.2 Genetic diversity

The results from analysis of polymorphic cpSSR loci show that genetic diversity of *Albizia* is not consistent across the six forest fragments sampled. Ngel Nyaki, Kurmin Danko, and Mbamnga populations exhibit considerably higher n_e and uH_E relative to the other three populations. The single polymorphic nuclear locus analysed exhibited higher levels of genetic diversity than the chloroplast loci, but show agreement with the trends of genetic diversity observed across populations. In the previous assessment of *Albizia* in Eastern Africa using allozyme variation, the estimated haplotype diversity within populations ranged between 0.19 and 0.77 (Nantongo *et al.* 2009). The differences in diversity observed in different populations in the present study confirms that *Albizia* is capable of considerable losses of genetic variation despite their high abundance within Afromontane forests.

Few studies are available for comparison of genetic diversity in tropical tree species. Contrasts are also confounded by high variability in geographic scale of studies, number of samples per population, number of loci used, and the type of loci used. An analysis of genetic diversity in *Dalbergia monticola*, an endangered tree species in Madagascar, using universal chloroplast microsatellites revealed the average effective number of haplotypes was 2.01, and average haplotype diversity range was 0.40 (Andrianoelina *et al.* 2007). These values are similar to the genetic diversity calculated from the chloroplast loci in the present study (mean n_e=1.84, mean uH_E=0.33). Fontaine *et al*. (2004) showed an average expected number of haplotypes of 1.185 and haplotype diversity of 0.10 in *Vitellaria paradoxa* populations in Sub-Saharan Africa, which is classified by the IUCN as vulnerable. While average diversity in the present study is higher than this, these values are in line with the within-populations diversity observed at some sites. These studies support the opinion that diversity values obtained in the present study are indicative of high current genetic diversity in Ngel Nyaki, Kurmin Danko, and Mbamnga populations, while the populations of Kuma, Yana, and Tamnyar are genetically depauperate.

These findings support the proposal that both common and rare species are susceptible to the genetic effects of habitat fragmentation. Despite no significant correlation being found between genetic diversity and fragment size, results do suggest that the smaller forest fragments are at greater risk of a loss of genetic diversity; the three populations exhibiting lowest genetic diversity are small fragments between 15 and 30 hectares. Ngel Nyaki and Kurmin Danko populations exhibit higher genetic diversity, and are substantially larger than other sites. This is aligned with the expectation that large populations will retain higher genetic diversity. While the Mbamnga population is much smaller than Ngel Nyaki and Kurmin Danko, it is larger than the other forests, so it is unsurprising that this site also

exhibited high genetic diversity. Yana and Tamnyar populations exhibited extremely low levels of genetic diversity. Yana forest is noted for having remained culturally protected despite high social and economic pressure to undertake logging (Chapman & Chapman 2001). This suggests that Yana forest has existed as a small, isolated forest for an extensive period, and this is a likely explanation for the low genetic diversity observed in this forest. These results highlight the need to consider deforestation history, rather than fragment size alone, when predicting losses of genetic diversity. The lack of genetic diversity observed at Tamnyar is likely due to the small sample size at this site (Hale *et al.* 2012). While the size of the sampling area at Tamnyar is not the smallest of this study, few samples of *Albizia* were able to be collected from this location due to the sparsity of forest habitat.

The reduced genetic diversity in juvenile populations relative to adults suggests that populations of *Albizia* are undergoing erosion of genetic diversity. Few studies have observed decline in genetic diversity between generational cohorts in tropical forests following habitat fragmentation (Lowe *et al.* 2005). None appear to show a reduction to the extent observed in the present study. A reduction in expected heterozygosity of seedlings has been observed in populations of *Prunus africana* in Kenya, with the greatest change in H_E of -0.09 (10.8%) (Farwig *et al.* 2007). Dayanandan *et al*. (1999) found that the greatest loss of expected heterozygosity in seedling vs. adult cohorts of *Carapa guianensis* in Costa Rica was 0.06 (9.8%). André al. (2008) also report significant loss of expected heterozygosity (-0.18) in a population in post-logging cohorts of *Swietenia macrophylla* in Amazonian forest. In the present study, the change in uH_E at Ngel Nyaki was -0.33, equating to a 51.6% change in haplotype diversity between the generations. This rapid loss of genetic diversity suggests changes may not be solely attributed to the typical evolutionary impacts of habitat fragmentation (inbreeding and genetic drift). One possible explanation may be that the seedlings sampled represent the progeny from only a small subset of the adult trees which were successful in reproduction for the limited period which the seedlings represent (André *et al.* 2008). Genetic diversity may be higher in adult cohorts because they represent many overlapping generations from multiple mating events (Hall *et al.* 1994). Further sampling across a greater variety of age classes may aid in more accurately determining if genetic diversity is in decline.

2.4.3 Genetic differentiation and population structure

Current levels of differentiation across the Mambilla Plateau are consistent with the anticipated levels for wind-dispersed species of 0.142 reported by Hamrick et. al. (1993). Pairwise differentiation measures vary between low to moderate, with Tamnyar population showing consistently higher differentiation. This suggests that this small population, located at the southern border of the plateau, is likely the most isolated population and may contribute to the low genetic diversity observed at this site due to drift. Alternatively, high differentiation of Tamnyar may also be due to introgression of genetic material from an unidentified congener species that was also observed in this vicinity. Analysis of additional

samples at this site would be of high value to establishing the genetic characteristics of this site.

Pairwise differentiation measures suggest that despite there being no evidence for isolation-by-distance, populations in close proximity to one another tend to be less differentiated. For example, Kuma and Yana are located 4.8km apart and are the least differentiated populations. Ngel Nyaki and Kurmin Danko are also located 4.4km from one another, and show no significant differentiation. The moderate level of overall genetic differentiation determined in adult samples suggests that at the time of their production gene flow across the plateau was relatively high. This level of genetic differentiation is slightly lower than that previously observed for *Albizia* by Nantongo *et al*. (2009) (Φ_{PT}=0.209), however with their study covering a much greater geographic scale, higher isolation among populations is to be expected.

Genetic theory predicts that as habitat fragmentation increases, genetic differentiation among fragments increases due to diminishing rates of gene flow. In the present study, juvenile samples exhibit minimal genetic differentiation, significantly lower than was observed for adult samples. These results suggest that despite increasing geographical isolation among forest fragments on the plateau, *Albizia* gene flow has increased. Most studies of demographic genetic structure observe the predicted increase in differentiation in juvenile cohorts (Aldrich *et al.* 1998). One study by Hall *et al*. (1994) did find that genetic differentiation (F_{ST}) of *Pentaclethra macroloba* in Costa Rica was lower in seedlings (0.038) than adult (0.019) cohorts. They suggest that lower genetic differentiation observed in seedling cohorts is due to a lower effective sample size than adults, because fewer mating events are represented. This may explain the observed decrease in differentiation in the present study.

It is possible that extensive land clearing around forest fragments on the Mambilla Plateau may allow further dispersal of *Albizia* genetic material. Additionally, as riverine habitats are further degraded, the colonizing ability of *Albizia* may allow it to thrive in such areas, and creates corridors for gene flow. However, high levels of gene flow would also likely allow *Albizia* to maintain high genetic diversity in all populations, which was not observed in the present study. An alternative possibility is that low population differentiation simply reflects the fact that all populations are becoming more similar to each other as rare alleles are lost from each population, and rates of gene flow among them may actually be minimal. This is supported by the lack of genetic structure observed among adults for the cpSSR loci. While two clusters were identified from Bayesian analysis, these clusters did not correspond with the true populations, but rather with individuals possessing rare genotypes. The lack of any population structure in juvenile analysis suggests those rare alleles have since been lost, and may explain the high gene flow that was inferred. Population structure was more evident for the nuSSR loci, which assigned individuals Ngel Nyaki and Tamnyar to a different cluster. Greater genetic structure of nuclear markers is anticipated due to their higher variability and larger effective population size (Provan *et al.* 2001). Genotyping of juvenile samples for the Ency-17 nuSSR loci may confirm low gene flow if increasing genetic structure is observed.

Additionally, direct measures of pollen movement and seed dispersal may be needed to elucidate the rates of gene flow.

2.4.4 Restoration implications

The results of this analysis have two primary implications for the restoration of Afromontane forest fragments on the Mambilla Plateau:

1. Genetic diversity of *Albizia* at Ngel Nyaki is relatively high at present, but may be in decline as indicated by reduced genetic diversity in juvenile samples. Using only passive techniques to restore *Albizia* may be detrimental to the species, and therefore restoration efforts, by allowing further decline in genetic diversity as few adults may be contributing genotypes to seedlings in regeneration zones. If regeneration is limited to this method, genetic diversity may continue to decline, and will not only threaten the success of restoration, but may result in deterioration throughout the established forest core. It would be of high value to carry out further sampling of *Albizia* seedlings in regenerating areas to compare with samples collected in the established forest. This may confirm the expectation that naturally regenerating *Albizia* are restoring a limited number of genotypes.

   The relatively high current level of genetic diversity of *Albizia* at Ngel Nyaki suggests the species can be more widely used as a nurse tree if seeds are taken from a variety of sources within the existing forest. This will aid in maintaining genetic diversity within the current forest, and in promoting genetic and species diversity in the restored areas. The genetic characteristics, alongside ecological functions, promote *Albizia* as a suitable species for active restoration at Ngel Nyaki.
2. Some of the smaller forest fragments are genetically impoverished, indicating a need for genetic rescue. Kuma and Yana populations exhibit low genetic diversity in adult samples, and juvenile samples suggest that Kuma has suffered further reductions to the extent that all individuals are identical. Further samples need to be analysed from the population of Tamnyar to clarify current levels of genetic diversity and assess the need for genetic rescue.

   Based on current data, Kuma and Yana populations are strong candidates for translocation of *Albizia* with the aim of genetic restoration. Ngel Nyaki and Mbamnga are the most suitable source populations as they possess the lowest frequency of the A4 haplotype, which has a very high frequency within the receiving populations. Outbreeding depression following translocations is unlikely, given the relatively low levels of differentiation between donating and receiving populations (Φ_{PT}P: 0.114-0.263). Based on the decision tree produced by Frankham *et al*. (2011), *Albizia* on the Mambilla Plateau likely meets the criteria for reestablishment of gene flow with a low probability outbreeding depression, without requiring further assessments of adaptive variation.

2.4.5 Conclusions

This investigation lends strong support to the growing notion that the genetic impacts of habitat fragmentation are not solely limited to rare species. We have shown that extensive fragmentation across the Mambilla Plateau has resulted in losses of genetic diversity of abundant *Albizia* in some of the small forest fragments. Furthermore, comparison of demographic genetic structure suggests that genetic diversity in all forest fragments may be in decline. This highlights an urgent need for habitat restoration across the plateau. I recommend the use of *Albizia* in active restoration planting currently underway at Ngel Nyaki forest in an effort to retain the genetic diversity of the species, and promote successful ecological restoration. Additionally, there is an apparent need for genetic rescue of *Albizia* at Kuma and Yana forest fragments in order to improve their genetic diversity. Translocation of genetic material may aid in ensuring the resilience and long-term viability of these forests. However, effective implementation of these recommendations is likely to be challenging due to resource limitations.

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CHAPTER 3. Genetic Diversity and Population Structure of *Clausena anisata*.

Abstract

Many sacred forests throughout the world have persisted despite extensive deforestation, and are reported as inadvertent refugees of biodiversity. The typically small size of such forests suggests they may be particularly susceptible to erosion of genetic diversity due to inbreeding and genetic drift. However, few studies have attempted to assess this hypothesis. In this study, the genetic diversity and population structure of *Clausena anisata* within sacred 'kurame', protected forest reserves, and degraded forests on the Mambilla Plateau is assessed using cpSSR and nuSSR loci. Genetic diversity of Kuma kurmi was found to be high and comparable to significantly larger protected forest reserves. Alternatively, Yana kurmi presented minimal genetic diversity. Assessment of juvenile cohorts also suggests declining diversity within Kuma kurmi, while diversity remained stable in large forest reserves. On the Mambilla Plateau sacred forests represent the only intact primary Afromontane forests outside of existing forest reserves. Low and declining diversity within the kurame shows that urgent action needs to be taken to conserve these forests.

3.1 Introduction

3.1.1 Biodiversity value of sacred forests

Worldwide many forest fragments have been retained in the face of habitat destruction, without formal protection, due to their significant cultural value. 'Sacred forests' may have been protected with the intention of preserving social, cultural, or religious practices, but have inadvertently resulted in the conservation of biodiversity (Dudley *et al.* 2010; Onyekwelu & Olusola 2014). These forests may represent the very first, and most widespread, form of community-based conservation, and their long-term protection has often resulted in vestiges of unique and high biodiversity (Dudley *et al.* 2009; Bossart & Antwi 2016). However, the conservation value of sacred sites around the world is poorly understood (Dudley *et al.* 2010). Studies that have assessed the biodiversity value of sacred forests have primarily focused on species diversity (Bossart & Antwi 2016). In this chapter, the genetic diversity and population structure of the common Afromontane species *Clausena anisata* (hereafter *Clausena*) is investigated among forest fragments on the Mambilla Pleateau, Nigeria. These results are then discussed in the context of comparing the biodiversity value of sacred sites, degraded forests and forest reserves.

Sacred forest fragments, particularly common throughout Africa and Asia, are often cited as refuges of endangered and endemic species (Dudley *et al.* 2010). In some regions, it is reported that sacred forests may harbour higher species diversity than designated state forest

reserves (Dudley *et al.* 2010). Sacred groves are also likely to have a significant contribution to the health and prosperity of proximate communities through the provision of ecosystem services (Babalola *et al.* 2014; Daniel *et al.* 2016). However, the decay of religious beliefs and increasing economic pressures and human populations in recent decades threatens the long-term survival of such forests, and as a result the critical and unaccounted biodiversity within may be lost (Bhagwat & Rutte 2006; Onyekwelu & Olusola 2014). It is therefore important the biodiversity of sacred forests is evaluated, and additional protection put in place to prevent their destruction.

Sacred forests are widespread throughout the African continent, and a small number of studies have assessed their biodiversity value, typically regarding species diversity. In Tanzania, sacred groves were found to contribute a significant proportion of regional biodiversity of *Miombo* woodlands (Mgumia & Oba 2003). Similarity of species in sacred sites versus formally protected areas was 45%, suggesting that much of the diversity harboured within sacred sites is unique. Onyekwelu and Olusola (2014) assessed the species diversity of sacred, primary, and degraded forest in Osun and Ondo States in Nigeria. They found that the biodiversity value varied greatly between the two sacred sites assessed. Osun-Osogbo sacred grove reported the greatest number of endangered species and the highest species diversity of all sites evaluated, whereas Igbo-Olodumare sacred grove had the fewest endangered species and lowest species diversity. Low biodiversity of Igbo-Olodumare was attributed to its relatively low level of 'sacredness', which has allowed encroachment into this forest. In Ethiopian montane regions, sacred forests were found to be highly vulnerable to declines of soil nutrients due to their small size and relative isolation (Cardelús *et al.* 2013).

The typically small size of sacred groves means that such sites can be expected to be particularly susceptible to erosion of genetic diversity (as discussed in Chapter 1) (Bossart & Antwi 2016). However, while assessments of species diversity in sacred groves are becoming more frequent, few studies have undertaken the task of evaluating their genetic diversity. A study of butterfly diversity in sacred groves and nearby forest reserves of Ghana showed that species diversity and genetic diversity are not strongly correlated (Bossart & Antwi 2016). Therefore, one cannot assume high genetic diversity of these fragments when species diversity is high, or vice-versa, and multiple aspects of biodiversity should be considered when prioritising areas for conservation management. Bossart and Antwi (2013, 2016) also found that the genetic diversity within butterfly species was not associated with fragment size; some of the sacred grove sites were seen to have levels of genetic diversity comparable to considerably larger forest reserves. This reinforces the idea that sacred forests may contribute disproportionately high biodiversity value (Bossart & Antwi 2013).

Determining the genetic diversity of sacred groves may aid in ensuring their long-term persistence, thereby preserving their value as providers of ecosystem services alongside their biodiversity value. Assessments of genetic diversity can highlight forest fragments of conservation importance due to high diversity or unique genetic characteristics, or sites notably lacking in genetic variability that may require augmentation. Sacred forests are often present in heavily degraded landscapes, representing once extensive habitat types of these

regions. Therefore, these forest also possess the genetic resources required for future habitat restoration (Dudley *et al.* 2010).

3.1.2 Sacred forests of the Mambilla Plateau

In Nigeria, forests are often referred to as 'kurmi' (plural: kurame), derived from the Hausa language, and many kurame are protected as 'sacred' or 'fetish' groves due to religious reverence or taboos (Jones 1963; Chapman & Chapman 2001). Spiritual kurame are often closely associated with small villages, providing vital resources to these local communities. When assessing species diversity of forests on the Mambilla Plateau, Chapman and Chapman (2001) noted that individual kurmi did not possess high species diversity or high levels of species rarity. However, when all kurame were combined they revealed a significant contribution to species diversity in the area. This observation is consistent with the expectation that alone, small forests have little conservation value. However, when combined, they may possess a large proportion of local biodiversity (Bhagwat & Rutte 2006). Therefore, ensuring long-term viability of each individual forest fragment is fundamental to maintaining overall biodiversity.

Two study sites on the Mambilla Plateau are considered sacred kurame; Kuma and Yana forests. These are small but well established forest islands amongst agricultural fields to the east of the plateau. They are characterized by strongly defined borders, making them particularly conspicuous in this region (Figure 3.1). Mbamnga and Tamnyar sampling sites represent unprotected, degraded forests, as samples from these sites were taken from riverine forest patches and verges surrounding the local villages, which are not culturally protected. Ngel Nyaki and Kurmin Danko populations represent two local authority forest reserves formally protected since 1969 (Borokini *et al.* 2012).

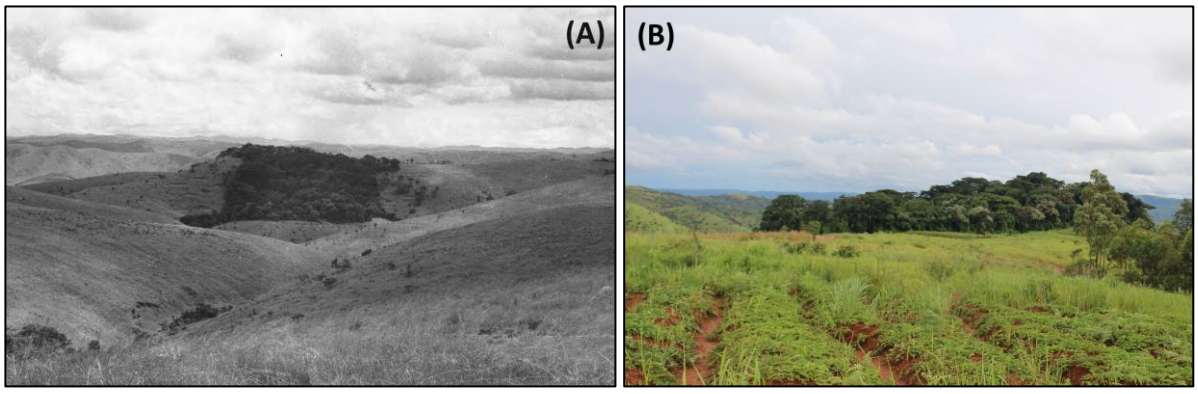

Figure 3.1: Images of Yana kurmi past and present. A: 1973 (Photo credit: J. Chapman). B: 2017 (Photo credit: H. Chapman)

3.1.3 Study species

Clausena anisata (Willd.) Hook. F. ex Benth (Rutaceae: Aurantioideae) is a small shrub or tree up to 10m high, distributed throughout the wetter regions of Central Africa and Southeast Asia from sea level to 3000m altitude (Arbab *et al.* 2012). The tree is fast-growing, particularly in the shade of forest margins (Tchinda 2011; Lawal & Olagoke 2016). The small, creamy white

flowers are bisexual and insect pollinated (Tchinda 2011). Fruits are a fleshy berry around 10mm, dark red/purple in colour, and are often consumed by local people. The fruit is primarily dispersed by birds, but also by mammals, particularly primates (Tchinda 2011). *Clausena* has a wide variety of uses in traditional medicines, including treatment of gastrointestinal disorder, fever, pneumonia, pain relief, and as an antiseptic (Tchinda 2011; Lawal & Olagoke 2016). A review of the phytochemical and pharmacological properties of *Clausena* indicates high levels of anticancer, antimicrobial, and antimalarial secondary metabolites (Arbab *et al.* 2012). Stems and leaves of *Clausena* are also used in traditional religious ceremonies and strong wood is often used for building tools handles, implements and walking sticks (Tchinda 2011).

The Aurantioidae subfamily contains many species of commercial importance (Morton 2009). Their value is largely culinary or medicinal, particularly those species in the genus of *Citrus.* As a result, many Aurantioidae species have undergone extensive cultivation and artificial selection (Chen *et al.* 2014). A multitude of studies have investigated genetic diversity of domesticated crop plants within the Aurantioidae or examined the phylogenetic relationships among its members (Uzun *et al.* 2009; Luro *et al.* 2011). However, very few studies have assessed the genetic diversity of wild populations of species within this subfamily.

ZhenShi *et al*. (2016) investigated the genetic diversity of *Clausena indica* in China through the use of ISSR (Inter-Simple Sequence Repeat) loci, and found that there was high similarity among individuals, but that there was distinct differences among regions. It was found that average percent polymorphism was relatively low, 55% across all populations. Another assessment of genetic variation using ISSR, DAMD (Directed Amplification of Minisatellite DNA) and RAPD (Random Amplified Polymorphic DNA) loci in *Murraya paniculata*, a close relative of Clausena, found that genetic diversity of wild populations was higher than that of cultivated populations, with Nei's diversity of 0.27 and 0.23 respectively (Verma *et al.* 2009). A study of *Murraya koenigii* wild populations in India found Nei's diversity to vary between 0.15 and 0.24 based on ISSR loci (Verma & Rana 2011). AMOVA analysis revealed moderate differentiation among these populations (11%) and regions (9%). Verma and Rana (2013) also compared of wild and cultivated populations of *M. koenigii* using ISSR, DAMD, and RAPD molecular markers, and found genetic diversity was marginally higher in the cultivated samples. Genetic diversity estimates of three *Atalantia* species in South India ranged between 0.19 and 0.25 using DAMD and RAPD methods (Ranade *et al.* 2009). Microsatellite loci have been used to assess the genetic diversity of few wild Aurantioidae species. Nuclear microsatellite analysis of *Citrus japonica* accessions from Southern China revealed an average expected heterozygosity of 0.46 (Chen *et al.* 2014). Sharma and Sharma (2015) detected an effective number of alleles of 1.88, and expected heterozygosity of 0.41 for *Aegle marmelos* samples using nuclear microsatellite loci. Neither of these studies use the nuSSRs to assess the impact of habitat fragmentation on genetic diversity of Aurantioideae species. High genetic diversity observed in these studies is likely due to a wide sampling area, as well as the high variability of many microsatellite markers.

3.1.4 Research goals

In the following chapter a combination of chloroplast and nuclear microsatellite loci are used to determine the genetic diversity and structure among populations of *Clausena* within sacred forests, protected forest reserves, and unprotected forest fragments on the Mambilla Plateau. This initial assessment is used as an indication of the susceptibility of *Clausena* to the erosion of genetic diversity, to evaluate the degree of isolation among forest fragments, and to assess the biodiversity value of sacred forests on the Mambilla Plateau.

The primary objectives of this chapter are as follows:

1. Identify chloroplast and nuclear microsatellite loci that can be used for rapid assessment of genetic characteristics of *Clausena*.
2. Determine the current level of genetic diversity and population structure exhibited by established *Clausena* trees in forest fragments across the Mambilla Plateau.
3. Assess differences in genetic diversity and population structure between adult and juvenile cohorts of *Clausena*.

3.2 Methods

3.2.1 Study sites, sample collection and DNA extraction

Details of sampling locations are given in Section 1.3.2. Methods for sample collection and extraction of DNA are as given in Sections 2.2.1 and 2.2.2 respectively. Samples were collected from adult shrubs/trees around 2m tall, and juvenile seedlings around 0.5m high.

3.2.2 Microsatellite optimisation

A total of 36 microsatellite loci primer pairs were tested for amplification in samples of *Clausena*. These loci included the same 30 'universal' chloroplast loci assessed in Chapter 2 (Weising & Gardner 1999; Chung & Staub 2003; Cheng *et al.* 2006). An additional 6 nuclear loci were tested for amplification, sourced from loci effective in *Citrus* species, of which *Clausena* is a close relative (Kijas *et al.* 1997; Ahmad *et al.* 2003; Barkley *et al.* 2006). Amplification of all primers was tested using the same methods detailed in Section 2.2.3.

3.2.3 Microsatellite amplification, genotyping, and data analysis

PCR and genotyping procedures are detailed in Section 2.2.4. Methods for data analysis are as per Section 2.2.5. However, nuclear loci are coded as dominant binary data for each allele in order to include samples expressing more than two alleles at a locus. Each allele is coded as either present (1) or absent (0), because the true frequency the alleles is unknown; the same allele may be present multiple times in the same sample. The binary matrix is analysed as a dominant (diploid) data type in GenAlEx (Peakall & Smouse 2006, 2012).

3.3 Results

3.3.1 Chloroplast loci

Examination of 30 conserved chloroplast microsatellite loci revealed 19 loci that successfully amplified in *Clausena* samples. Genotyping of PCR products determined that 10 of these loci were polymorphic, and these loci were used for genotyping of all samples collected. Rates of missing data at a single polymorphic locus vary between 0% and 2.05%. Details of all successfully amplifying loci are given in Table 3.1. The 10 polymorphic loci produced a range in allele sizes of 97-370 base pairs, and each locus possessed 4-13 alleles, with an average of 7.1 alleles. Allele frequencies by population are given in Table 3.2 (adults) and Table 3.3 (juveniles). Nineteen alleles were found only in adult samples, and 15 alleles occurred only in juvenile samples. Twenty-five private alleles were identified among adult populations, and 26 private alleles among juvenile populations. Mbamnga was the only population that did not possess any private alleles.

Allele combinations resulted a total of 45 different haplotypes, with 22 of these haplotypes occurring more than once (Table 3.4). 27 different haplotypes were identified among adult samples, with populations possessing between 1 and 9 haplotypes. In the juvenile samples, there was also found to be 27 different haplotypes, with 1-11 haplotypes within a population. A very high proportion of haplotypes were private haplotypes; 93% among adult populations and 96% among juvenile populations. The most common haplotype was not consistent across populations or across age cohorts within a population. Haplotype C17 was unique to the Yana population, and was the only haplotype observed among adult and juvenile samples in this population.

Genetic diversity was seen to be highly variable among adult populations. Percent polymorphic loci varied between 40% in Kuma and 100% in Mbamnga and Tamnyar. Despite relatively high percent polymorphic loci (60%) in the Yana adult population, no diversity is observed when samples with missing data are excluded. Diversity measures in all other populations were found to be high, with haplotype diversity of 0.64-0.75. Kuma produced the highest effective number of haplotypes (3.60) and haplotype diversity (0.75). There was no correlation found between haplotype diversity and fragment size (r^2=0.086, p=0.573).

Genetic diversity was seen to be relatively consistent between adult and juvenile samples within populations. While percent polymorphism (%P) increased in the juvenile relative to adult populations at Ngel Nyaki, Kurmin Danko and Kuma, no polymorphism was detected at any loci within the juvenile population of Yana. Ngel Nyaki and Kurmin Danko juveniles both showed an increase in effective number of alleles (n_e) and unbiased haplotype diversitu (uH_E), whereas Kuma was the only population that showed a reduction in diversity in juvenile samples (adult uH_E=0.75, juvenile uH_E=0.67). Kurmin Danko showed the highest levels of diversity in juvenile samples (n_e=5.73, uH_E=0.86). As with adult samples, juvenile samples from Yana exhibited no haplotype diversity.

Table 3.1: Successfully amplifying chloroplast loci for Clausena. [a] *Y (= C or T), R (= A or G), K (= T or G), W (= A or T) and S (= C or G).* [b] *Ta = annealing temperature. * ccmp loci used fluorescently labelled forward primers, all other primer pairs were designed for the attachment of a M13 florescent primer to the forward primer. Loci in bold indicate polymorphic loci that were used for further analysis.*

Primer	Source	Location	Repeat motif	Primer sequence	Dye/Colour	Ta[b]	Sample size	Approx. Allele size (bp)
ccmp2*	**(Weising & Gardner 1999)**	**5' to *trnS***	**$(A)_{11}$**	**F: GATCCCGGACGTAATCCTG R: ATCGTACCGAGGGTTCGAAT**	**6-FAM/Blue**	**50°c**	**291**	**204-212**
ccmp3*	(Weising & Gardner 1999)	*trnG* intron	$(T)_{11}$	F: CAGACCAAAAGCTGACATAG R: GTTTCATTCGGCTCCTTTAT	PET/Red	50°c	24	133
ccmp4*	**(Weising & Gardner 1999)**	***atpF* intron**	**$(T)_{13}$**	**F: AATGCTGAATCGAYGACCTA R: CCAAAATATTBGGAGGACTCT**	**6-FAM/Blue**	**50°c**	**289**	**106-142**
ccmp5*	(Weising & Gardner 1999)	3' to *rps2*	$(C)_7(T)_{10}$	F: TGTTCCAATATCTTCTTGTCATTT R: AGGTTCCATCGGAACAATTAT	VIC/Green	50°c	22	128
ccmp7*	(Weising & Gardner 1999)	*atpB-rbcL* intergenic	$(A)_{13}$	F: CAACATATACCACTGTCAAG R: ACATCATTATTGTATACTCTTTC	PET/Red	50°c	24	147
ccmp10*	**(Weising & Gardner 1999)**	**rpl2-rps19 intergenic**	**$(T)_{14}$**	**F: TTTTTTTTTAGTGAACGTGTCA R: TTCGTCGDCGTAGAAATAG**	**VIC/Green**	**50°c**	**292**	**97-125**
ccSSR4	(Chung & Staub 2003)	TrnR-AtpA	$(T)_8$	F: AGGTTCAAATCCTATTGGACGCA R: TTTTGAAAGAAGCTATTCARGAAC	6-FAM/Blue	50°c	12	324
ccSSR5	**(Chung & Staub 2003)**	**Rps2-RpoC2**	**$(T)_{10}$**	**F: TCTGATAAAAAACGAGCAGTTCT R: GAGAAGGTTCCATCGGAACAA**	**6-FAM/Blue**	**50°c**	**292**	**263-285**
ccSSR7	**(Chung & Staub 2003)**	**PsbC-TrnS**	**$(T)_{11}$**	**F: CGGGAAGGGCTCGKGCAG R: GTTCGAATCCCTCTCTCTCCTTTT**	**6-FAM/Blue**	**50°c**	**291**	**346-370**
ccSSR8	**(Chung & Staub 2003)**	**Ycf3**	**$(T)_5C(T)_{17}$**	**F: TTGATCTTTACGGTGCTTCCTCTA R: TCATTACGTGCGACTATCTCC**	**VIC/Green**	**50°c**	**287**	**260-282**
ccSSR9	**(Chung & Staub 2003)**	**PsbC-TrnScf3**	**$(A)_{13}$**	**F: GAGGATACACGACAGARGGARTTG R: CCTATTACAGAGATGGTGYGATTT**	**6-FAM/Blue**	**50°c**	**293**	**172-216**
ccSSR19	(Chung & Staub 2003)	Ycf5	$(T)_8$	F: CTATGCAGCTCTTTTATGYGGATC R: TCCARGTAATAAATGCCCAAGTT	VIC/Green	50°c	9	377
ccSSR20	(Chung & Staub 2003)	NdhD-PsaC	$(A)_8$	F: CCGCARATATTGGAAAAACWACAA R: GCTAARCAAATWGCTTCTGCTCC	6-FAM/Blue	50°c	8	388
ccSSR21	(Chung & Staub 2003)	TrnR-Rrn5	$(T)_{13}$	F: CCACCCCGTCTCSACTGGATCT R: AAAAATAGCTCGACGCCAGGAT	VIC/Green	50°c	12	300
ccSSR22	(Chung & Staub 2003)	TrnL-16SrRNA	$(T)_8$	F: CCGACCTAGGATAATAAGCYCATG R: GGAAGGTGCGGCTGGATC	VIC/Green	50°c	30	202
NTCP9	(Cheng *et al.* 2006)	***trnG/trnR* intergenic**	**$(T)_{10}$**	**F: CTTCCAAGCTAACGATGC R: CTGTCCTATCCATTAGACAATG**	**VIC/Green**	**50°c**	**292**	**270-333**
NTCP40	(Cheng *et al*. 2006)	Rps12/trnH	$(A)_{14}$	F: GATGTAGCCAAGTGGATCA R: TAATTTGATTCTTCGTCGC	VIC/Green	50°c	18	407
ARCP2	**(Cheng *et al*. 2006)**	***trnS/trnG* intergenic**	**$(A)_{13}$**	**F: TGGAGAAGGTTCTTTTTCAAGC R: CGAACCCTCGGTACGATTAA**	**NED/Black**	**50°c**	**290**	**168-171**
ARCP5	**(Cheng *et al*. 2006)**	***trnL/trnF* intergenic**	**$(T)_{13}$**	**F: GGCCATAGGCTGGAAAGTCT R: GTTTATGCATGGCGAAAAGG**	**NED/Black**	**50°c**	**292**	**205-233**

Non-amplifying loci: ccmp1, ccmp6, ccmp8, ccmp9, ccSSR2, ccSSR11, ccSSR12, ccSSR16, ccSSR17, ccSSR18, NTCP4.

Table 3.2: Allele sizes for polymorphic cpSSR loci, and their frequencies in populations of adult Clausena. ** Private alleles. Private alleles in the 'Total' column indicate alleles exclusive to adult samples. n= number of samples.*

Locus	Allele frequency						
	Ngel Nyaki	**Kurmin Danko**	**Kuma**	**Yana**	**Mbamnga**	**Tamnyar**	**Total**
ccmp2	204 (0.23) 205 (0.77)	204 (0.63) 205 (0.37)	204 (1.00)	204 (0.96) 213 (0.04) *	204 (0.97) 205 (0.03)	204 (0.91) 212 (0.09) *	204 (0.76) 205 (0.23) 212 (0.01) 213 (0.01) *
ccmp4	124 (0.14) * 125 (0.54) * 130 (0.06) 131 (0.20) 132 (0.03) 142 (0.03) *	127 (0.20) 128 (0.17) * 130 (0.43) 131 (0.20)	132 (0.39) 133 (0.45) * 134 (0.16) *	136 (1.00) *	130 (0.15) 131 (0.62) 132 (0.24)	106 (0.08) * 127 (0.08) 131 (0.42) 132 (0.42)	106 (0.01) * 124 (0.03) 125 (0.11) 127 (0.04) 128 (0.03) 130 (0.12) 131 (0.23) 132 (0.15) 133 (0.08) 134 (0.03) 136 (0.16) 142 (0.01) *
ccmp10	115 (0.46) 125 (0.54)	115 (0.80) 125 (0.20)	115 (1.00)	109 (0.03) 115 (0.97)	109 (0.06) 115 (0.94)	097 (0.08) * 109 (0.08) 115 (0.83)	097 (0.01) * 109 (0.02) 115 (0.83) 125 (0.15)
ccSSR5	265(0.97) 285 (0.03) *	265 (1.00)	263 (0.16) * 265 (0.84)	265 (0.96) 283 (0.04) *	265 (0.97) 282 (0.03)	265 (0.83) 275 (0.08) * 282 (0.08)	263 (0.03) 265 (0.94) 275 (0.01) * 282 (0.01) * 283 (0.01) * 285 (0.01) *
ccSSR7	369 (1.00)	369 (1.00)	369 (1.00)	346 (0.03) * 369 (0.97)	369 (0.97) 370 (0.03)	361 (0.08) * 369 (0.83) 370 (0.08)	346 (0.01) * 361 (0.01) * 369 (0.98) 370 (0.01)
ccSSR8	280 (0.53) 281 (0.29) 282 (0.18)	280 (0.17) 281 (0.83)	281 (0.65) 282 (0.35)	281 (1.00)	260 (0.03) 280 (0.79) 281 (0.18)	260 (0.09) 274 (0.09) * 280 (0.45) 281 (0.27) 282 (0.09)	260 (0.01) * 274 (0.01) * 280 (0.33) 281 (0.54) 282 (0.11)
ccSSR9	196 (0.03) 197 (0.97)	197 (1.00)	198 (1.00)	172 (0.03) * 196 (0.03) 197 (0.93)	185 (0.03) 196 (0.03) 197 (0.77) 198 (0.17)	185 (0.08) 197 (0.58) 198 (0.25) 216 (0.08) *	172 (0.01) * 185 (0.01) * 196 (0.02) * 197 (0.73) 198 (0.23) 216 (0.01) *
NTCP9	274 (0.17) * 275 (0.29) 276 (0.54)	275 (0.80) 276 (0.20)	275 (1.00)	277 (1.00) *	270 (0.03) 275 (0.94) 276 (0.03)	270 (0.08) 275 (0.67) 276 (0.17) 333 (0.08) *	270 (0.01) * 274 (0.04) 275 (0.62) 276 (0.16) 277 (0.16) 333 (0.01) *
ARCP2	168 (0.54) 169 (0.06) 170 (0.17) 171 (0.23)	168 (0.20) 169 (0.63) 171 (0.17)	168 (0.16) 169 (0.42) 170 (0.42)	168 (1.00)	168 (0.18) 170 (0.82)	168 (0.73) 169 (0.09) 170 (0.18)	168 (0.43) 169 (0.21) 170 (0.29) 171 (0.08)
ARCP5	232 (0.46) 233 (0.54)	232 (0.80) 233 (0.20)	232 (1.00)	222 (0.03) * 239 (0.97) *	205 (0.03) 232 (0.97)	205 (0.09) 232 (0.91)	205 (0.01) * 222 (0.01) 232 (0.67) 233 (0.15) 239 (0.16)
n	35	30	31	29	35	12	172

Table 3.3: Allele sizes for polymorphic cpSSR loci, and their frequencies in populations of juvenile Clausena*. * Private alleles. Private alleles in the 'Total' column indicate alleles exclusive to juvenile samples. n= number of samples.*

Locus	Allele frequency				
	Ngel Nyaki	**Kurmin Danko**	**Kuma**	**Yana**	**Total**
ccmp2	204 (0.35) 205 (0.65)	204 (0.43) 205 (0.53) 212 (0.03) *	203 (0.03) * 204 (0.93) 231 (0.03) *	204 (1.00)	203 (0.01) * 204 (0.68) 205 (0.30) 212 (0.01) 231 (0.01) *
ccmp4	124 (0.03) * 125 (0.26) * 130 (0.32) 131 (0.35) 132 (0.03)	127 (0.32) * 128 (0.25) * 130 (0.21) 131 (0.21)	121 (0.13) * 132 (0.67) 133 (0.17) * 134 (0.03) *	136 (1.00) *	121 (0.03) * 124 (0.01) 125 (0.07) 127 (0.08) 128 (0.06) 130 (0.13) 131 (0.14) 132 (0.18) 133 (0.04) 134 (0.01) 136 (0.25)
ccmp10	109 (0.03) 115 (0.74) 125 (0.23)	109 (0.07) 115 (0.69) 125 (0.24)	115 (1.00)	115 (1.00)	109 (0.03) 115 (0.86) 125 (0.12)
ccSSR5	264 (0.03) * 265 (0.97)	259 (0.03) * 265 (0.97)	263 (0.03) * 265 (0.83) 268 (0.13) *	265 (1.00)	259 (0.01) * 263 (0.01) 264 (0.01) * 265 (0.94) 268 (0.03) *
ccSSR7	369 (0.97) 370 (0.03)	369 (0.97) 370 (0.03)	369 (0.90) 375 (0.10) *	369 (1.00)	369 (0.96) 370 (0.02) 375 (0.03) *
ccSSR8	280 (0.26) 281 (0.71) 282 (0.03)	271 (0.03) * 280 (0.27) 281 (0.67) 282 (0.03)	281 (0.27) 282 (0.63) 283 (0.10) *	281 (1.00)	271 (0.01) * 280 (0.13) 281 (0.66) 282 (0.17) 283 (0.02) *
ccSSR9	197 (0.97) 198 (0.03) *	188 (0.03) 197 (0.97)	188 (0.13) 197 (0.87)	197 (1.00)	188 (0.04) * 197 (0.95) 198 (0.01)
NTCP9	274 (0.03) * 275 (0.71) 276 (0.23) 277 (0.03)	254 (0.03) * 275 (0.70) 276 (0.27)	263 (0.13) * 275 (0.87)	277 (1.00)	254 (0.01) * 263 (0.03) * 274 (0.01) 275 (0.57) 276 (0.12) 277 (0.26)
ARCP2	166 (0.03) 168 (0.19) 169 (0.35) 171 (0.06)	168 (0.27) 169 (0.47) 171 (0.17)	166 (0.13) 168 (0.03) 169 (0.67)	168 (1.00)	166 (0.04) * 168 (0.37) 169 (0.37) 170 (0.16) 171 (0.06)
ARCP5	232 (0.71) 233 (0.29)	222 (0.03) * 223 (0.03) * 232 (0.67) 233 (0.27)	218 (0.13) * 232 (0.87)	239 (1.00) *	218 (0.03) * 222 (0.01) 223 (0.01) * 232 (0.56) 233 (0.14) 239 (0.25)
n	31	30	30	30	121

Table 3.4: Within population frequencies of haplotypes occurring more than once in all samples of Clausena*. In populations where adults and juveniles were sampled their values are indicated by 'adults'/'juveniles'. *Indicates private haplotypes.*

Haplotype	Allelic composition	Haplotype frequency						
		Ngel Nyaki	Kurmin Danko	Kuma	Yana	Mbamnga	Tamnyar	Total
C3	263/282/198/115/168/ 232/204/134/369/275			0.16*/0				0.03/0
C8	265/280/197/115/168/ 232/204/131/369/275						0.56*	0.03/0
C9	265/280/197/115/170/ 232/204/130/369/275					0.15*		0.03/0
C10	265/280/197/115/170/ 232/204/131/369/275					0.58*		0.12/0
C11	265/280/197/115/170/ 232/204/132/369/275					0.06*		0.01/0
C13	265/280/197/125/168/ 233/205/125/369/276	0.53*/ 0.16*						0.11/ 0.04
C14	265/280/197/125/168/ 233/205/127/369/276		0.17*/ 0.19*					0.03/ 0.04
C17	265/281/197/115/168/ 239/204/136/369/277				1.00*/ 1.00*			0.16/ 0.26
C18	265/281/197/115/169/ 232/204/130/369/275	0.03/ 0.29	0.45/ 0.23					0.09/ 0.13
C19	265/281/197/115/169/ 232/204/131/369/275		0.21*/ 0.23*					0.04/ 0.05
C20	265/281/197/115/169/ 232/204/132/369/275			0/ 0.10*				0/0.03
C22	265/281/197/115/170/ 232/204/133/369/275			0/ 0.17*				0/0.04
C24	265/281/197/115/170/ 232/205/131/369/275	0.18*/ 0.29*						0.04/ 0.08
C26	265/281/197/115/171/ 232/205/128/369/275		0.17*/ 0.15*					0.03/ 0.03
C28	265/281/197/115/171/ 232/205/132/369/275	0.03*/ 0.03*						0.01/ 0.01
C29	265/281/198/115/168/ 232/204/132/369/275					0.15	0.22	0.04/0
C31	265/281/198/115/169/ 232/204/132/369/275			0.19*/ 0				0.04/0
C33	265/281/198/115/170/ 232/204/133/369/275			0.42*/ 0				0.08/0
C36	265/282/197/115/169/ 232/204/132/369/275			0/0.52*				0/0.14
C39	265/282/197/115/171/ 232/204/124/369/274	0.12*/ 0.03*						0.02/ 0.01
C42	265/282/198/115/169/ 232/204/132/369/275			0.19*/0				0.04/0
C44	268/283/188/115/166/ 218/204/121/375/263			0/0.07*				0/0.02

Genetic diversity was seen to be relatively consistent between adult and juvenile samples within populations. While percent polymorphism (%P) increased in the juvenile relative to adult populations at Ngel Nyaki, Kurmin Danko and Kuma, no polymorphism was detected at

any loci within the juvenile population of Yana. Ngel Nyaki and Kurmin Danko juveniles both showed an increase in effective number of alleles (n_e) and unbiased haplotype diversity (uH_E), whereas Kuma was the only population that showed a reduction in diversity in juvenile samples (adult uH_E=0.75, juvenile uH_E=0.67). Kurmin Danko showed the highest levels of diversity in juvenile samples (n_e=5.73, uH_E=0.86). As with adult samples, juvenile samples from Yana exhibited no haplotype diversity.

Table 3.5: Genetic diversity indices for polymorphic chloroplast microsatellite loci in populations of Clausena*. N= number of samples taken from the specified populations, %P = percent polymorphic loci, n_e = mean number of effective haplotypes, uH_E= haplotype diversity. In populations where adults and juveniles were sampled their values are indicated by 'adults'/'juveniles'.*

Population	N	%P	n_e	uH_E
Ngel Nyaki	35/31	90/100	3.03/4.93	0.70/0.82
Kurmin Danko	30/30	70/100	3.30/5.73	0.72/0.86
Kuma	31/30	40/90	3.60/2.83	0.75/0.67
Yana	29/30	60/0	1.00/1.00	0.00/0.00
Mbamnga	35	100	2.61	0.64
Tamnyar	12	100	2.61	0.69
Total	162/116	100/100	12.65/8.32	0.93/0.89

Population differentiation as measured by AMOVA was very high among the six adult populations, with Φ_{PT} of 0.461 (p=001). This level of differentiation is comparable with observations for species with a selfing breeding system, rather than predominantly outcrossing species (Hamrick & Godt 1996). This indicates that populations of *Clausena* are genetically isolated from one another, with little gene flow occurring among them. There is no evidence for isolation-by-distance between adult populations (r^2=-0.048, p=0.141) (Figure 3.2). Φ_{PT}P values vary between 0.138 and 0.731 and all statically significant, while uNeiP values range from 0.044 and 0.657 (Table 3.6). Mbamnga and Tamnyar populations show the lowest degree of differentiation between them, suggesting there is a higher rate of gene flow between them relative to other population pairs. Kuma and Yana populations show the strongest differentiation between them.

Genetic differentiation among the four populations from which both adult and juvenile samples were taken shows a slight reduction in overall Φ_{PT} of juveniles (Φ_{PT}=0.422, p=0.001) compared with adults (Φ_{PT}=0.511, p=0.001). However, this level of differentiation is still indicative of a very high level of population structure. Kuma and Yana remain the most strongly differentiated populations, and Ngel Nyaki and Kurmin Danko populations are the only population pairs found not to be significantly differentiated from one another in juvenile samples (Φ_{PT}P=0.016). Mantel testing finds a significant correlation between adult and juveniles for both uNeiP (r^2=0.533, p=0.036*) and Φ_{PT}P (r^2=0.875, p=0.037*).

Table 3.6: Pairwise Φ_{PT} (Φ_{PT}P) and unbiased pairwise Nei's genetic distance (uNeiP) of populations of Clausena *as calculated in GenAlEx (Peakall & Smouse 2006, 2012). Φ_{PT}P values are given above the diagonal, uNeiP below the diagonal. In population where adults and juveniles were sampled their values are indicated by 'adults'/'juveniles'. * indicates statistically significant (p<0.05) differentiation between population pairs (Φ_{PT}P) based on 999 permutations.*

	Ngel Nyaki	Kurmin Danko	Kuma	Yana	Mbamnga	Tamnyar
Ngel Nyaki	-	0.250*/ 0.016	0.513*/ 0.268*	0.552*/ 0.615*	0.395*	0.240*
Kurmin Danko	0.207/ 0.011	-	0.406*/ 0.237*	0.582*/ 0.582*	0.329*	0.190*
Kuma	0.625/ 0.208	0.266/ 0.189	-	0.731*/ 0.714*	0.427*	0.286*
Yana	0.560/ 0.462	0.434/ 0.435	0.657/ 0.555	-	0.700*	0.559*
Mbamnga	0.338	0.192	0.224	0.596	-	0.138*
Tamnyar	0.278	0.141	0.154	0.408	0.044	-

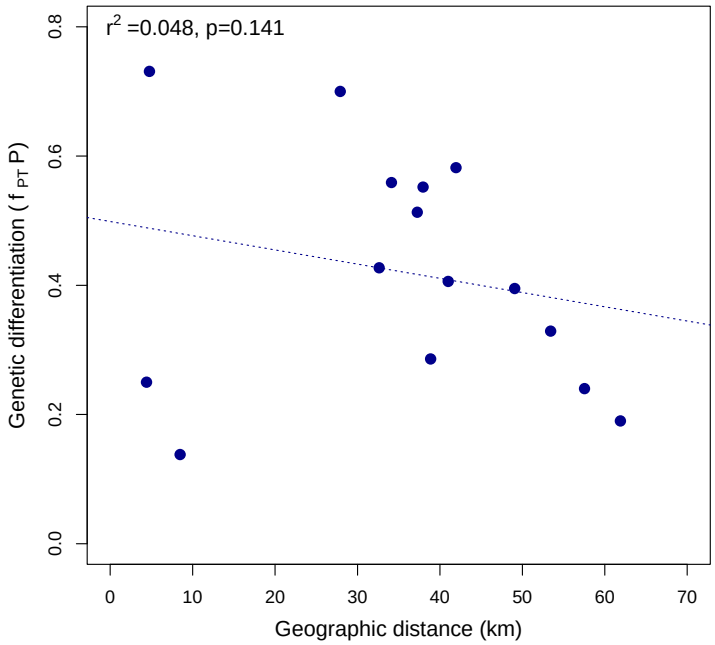

Figure 3.2: Mantel test for isolation-by-distance of adult Clausena *samples using cpSSRs. Mantel test in GenAlEx (Peakall & Smouse 2006, 2012) used to assess significance (α=0.05).*

Bayesian population structure analysis performed in STRUCTURE (Pritchard *et al.* 2000; Falush *et al.* 2003; Hubisz *et al.* 2009) suggests the optimal number of populations for all samples is six (Figure 3.3A). While deltaK calculated using the Evanno method clearly indicates that K=3 is strongly supported, there is a second peak at K=6, suggesting hierarchical structure (Figure

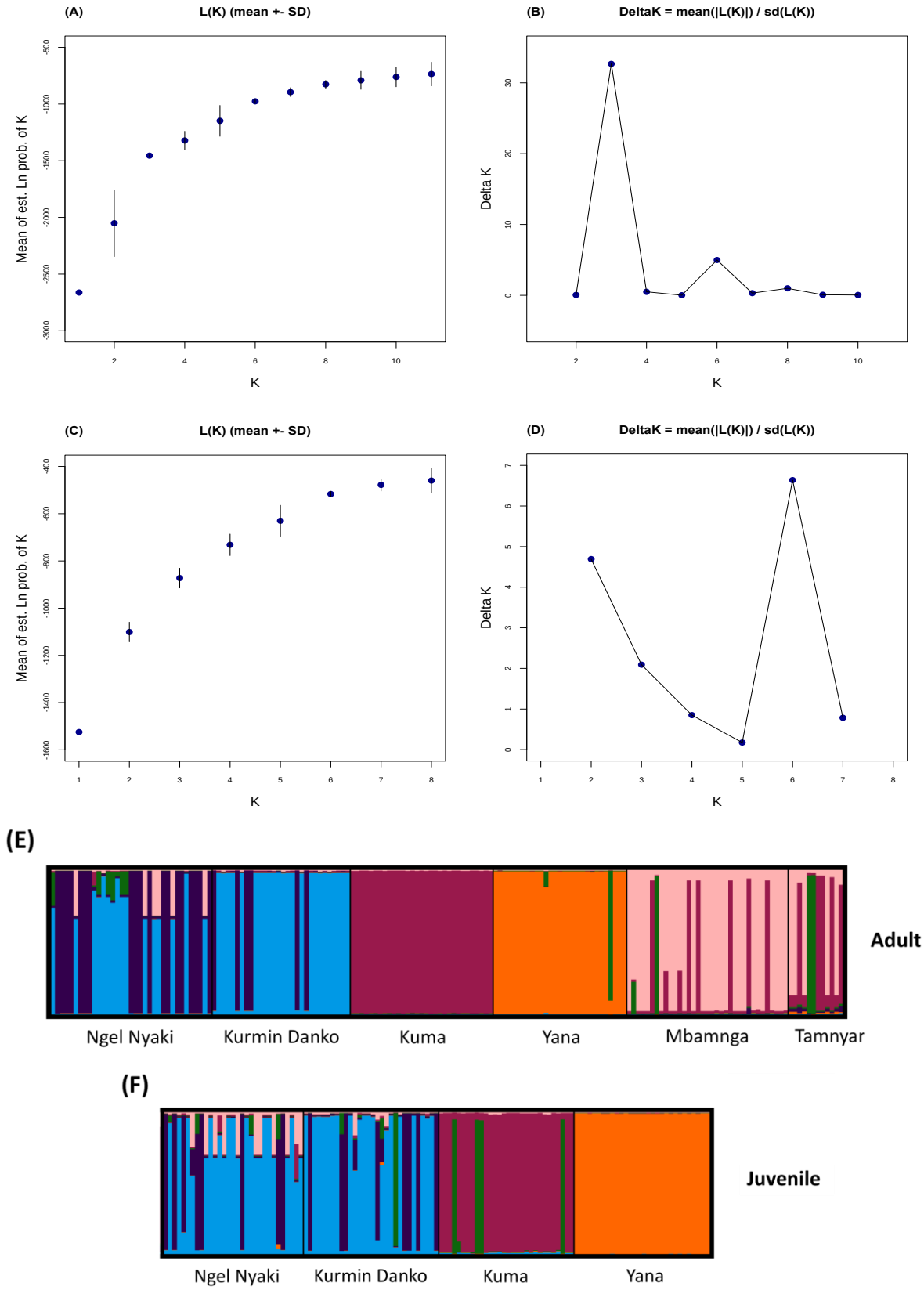

Figure 3.3: Genetic structure analysis of Clausena samples in STRUCTURE (Pritchard et al., 2000). A. Mean of estimated Ln probability of data vs. K. for all Clausena *samples B. Optimal number of populations shown by ΔK vs. K for all* Clausena *samples, calculated from 10 replicates per K. C. Mean of estimated Ln probability of data vs. K. for adult* Clausena *samples D. Optimal number of populations shown by ΔK vs. K for adult* Clausena *samples, calculated from 10 replicates per K. E. Structure plot of Q-values of adult samples for K=6. F. Structure plot of Q-values of juvenile samples for K=6.*

Figure 3.3B). Six clusters are confirmed by the plot of mean Ln probability of K vs. K, which shows a levelling off of L(K) at K=6 (Figure 3.3A). This is further supported by structure analysis of adult samples alone, which clearly shows the optimal K to be 6 (Figure 3.3C&D). The structure assignment plot shows that adult and juvenile cohorts within a population are assigned to the same clusters. Shared clusters between the populations of Ngel Nyaki and Kurmin Danko (purple and blue) as well as Mbamnga and Tamnyar (red and pink) suggests admixture within these population pairs. This shows relatively high levels of gene flow occurring between them, as was indicated by uNeiP and Φ_{PT}P measures. All adult samples and the majority of juvenile samples from Kuma are assigned to the same cluster (red). This cluster is also observed in Mbamnga and Tamnyar populations, suggesting moderate gene flow with these other populations. The majority of individuals in Yana are assigned to the same cluster (orange), and this cluster does not occur in any other populations, indicating that this forest is genetically isolated.

3.3.2 Nuclear loci

Of the six nuclear chloroplast microsatellite loci tested for *Clausena,* only two loci successfully amplified (Table 3.7). Locus TAA15 produced a single monomorphic band at 157 base pairs in all ten samples tested. Locus CMS-4 produced up to four bands for a single sample, around 229-251 base pairs in length. Genotyping of this locus was carried out for all adult samples to test for congruence with the chloroplast loci. 13 samples were excluded from analysis as they did not produce any clear allele peaks when genotyped.

CMS-4 loci produced 10 different alleles, ranging in size from 229-251 base pairs (Table 3.8). Between two and six alleles were present within a population. Five private alleles were observed across populations, one each in Ngel Nyaki, Kuma, and Mbamnga, and two within Tamnyar. Yana exhibited the lowest effective number of alleles and allelic diversity (1.02 and 0.02 respectively). High n_e and uH_E values were observed in Ngel Nyaki, Kuma and Tamnyar. Regression analysis reveals that genetic diversity measures (n_e and uH_E) within adult populations are significantly correlated between chloroplast and nuclear loci (n_e: r^2=0.838, p=0.010; uH_E: r^2=0.923, p=0.002) (Figure 3.4A&B).

Table 3.7 Successfully amplifying nuclear microsatellite loci for Clausena. *[a] Ta = annealing temperature.*

Primer	Source	Source species	Repeat motif	Primer sequence	Dye/Colour	T_a[a]	Sample size	Approx. Allele size (bp)	Polymorphic (Y/N)
CMS-4	(Ahmed *et al.* 2003)	***Citrus sp.***	**$(CT)_{11}(AT)_6$ $(CA)_6$**	**F: CCTCAAACCTTCTTCCAATCC R: CTGTAAAGTACATGCATGTTGG**	**6-FAM/Blue**	**50°c**	**159**	**229-251**	**Y**
TAA15	(Kijas *et al.* 1997)	*Citrus sp.*	(TAA)	F: AGGTCTACATTGGCATTGTC R: ACATGCAGTGCTATAATGAATG	NED/Yellow	50°c	10	157	N

Non-amplifying loci: CMS-26, TAA41, GT03, and AG14.

Table 3.8: CMS-4 allele sizes and frequencies within populations of adult Clausena*. *Indicates private alleles.*

Allele	Allele frequency						
	Ngel Nyaki	Kurmin Danko	Kuma	Yana	Mbamnga	Tamnyar	Total
229					0.74*		0.16
235	0.25*						0.05
237	0.53	0.75	0.10	0.20	0.65	0.30	0.45
239	0.56	0.57	0.83	1.00	0.35	0.60	0.64
241	0.13	0.21	0.33			0.20	0.14
243	0.03		0.33				0.07
245	0.63	0.18	0.67		0.03	0.10	0.30
248						0.20*	0.01
250						0.60*	0.04
251			0.23*				0.04
n	32	28	30	25	34	10	159

Table 3.9: Genetic diversity indices for nuSSR CMS-4 in populations of Clausena*. N= number of samples taken from the specified populations, N_e = effective number of alleles, uH_E = allelic diversity. Brackets "()" indicate standard error values.*

Population	N	n_e	uH_e
Ngel Nyaki	35	1.30 (0.12)	0.18 (0.07)
Kurmin Danko	30	1.23 (0.12)	0.14 (0.06)
Kuma	31	1.31 (0.12)	0.19 (0.06)
Yana	29	1.02 (0.02)	0.02 (0.02)
Mbamnga	35	1.24 (0.13)	0.14 (0.07)
Tamnyar	12	1.27 (0.11)	0.17 (0.06)
Total	159	1.25 (0.10)	0.16 (0.05)

Kuma and Mbamnga were the most strongly differentiated population pairs, with uNeiP of 0.216 and Φ_{PT}P of 0.469. Pairwise measures of population differentiation are not correlated between the different types of loci (uNeiP: r^2=0.042, p=0.315; Φ_{PT}P: r^2=0.163, p=0.202) (Figure 3.4C&D). No evidence was found for isolation-by-distance at the CMS-4 loci (r^2=0.021, p=0.225) (Figure 3.5).

AMOVA analysis revealed high overall population differentiation at the CMS-4 locus (Φ_{PT}=0.329, p<0.001), in agreement with the results of the chloroplast loci. This confirms that some *Clausena* populations are genetically isolated from one another and that there may be little gene flow occurring among fragments. This is reiterated by the cluster analysis, which identified the optimal number of populations as 5 (Figure 3.6A). The ΔK plot shows strong support for K=2. However, the plot of ln(K) shows a levelling off at K=5, so 5 was selected as the optimal number of clusters. This is supported by the fact that the structure plot of K=5 exhibits similar trend of genetic structure to the analysis of chloroplast loci. The plot shows individuals within Ngel Nyaki and Kurmin Danko have similar assignment probabilities to

several clusters (Figure 3.6C), indicating that Ngel Nyaki and Kurmin Danko samples represent a highly admixed population. The other four populations are primarily assigned to separate clusters. However, the presence of the blue cluster across all populations suggests a small amount of gene flow occurring among all populations is likely. Unlike the analysis of chloroplast loci, the populations of Mbamnga and Tamnyar were assigned to different clusters. This was also reflected in a higher level of population differentiation ($\Phi_{PT}P$) than was observed for cpSSR loci.

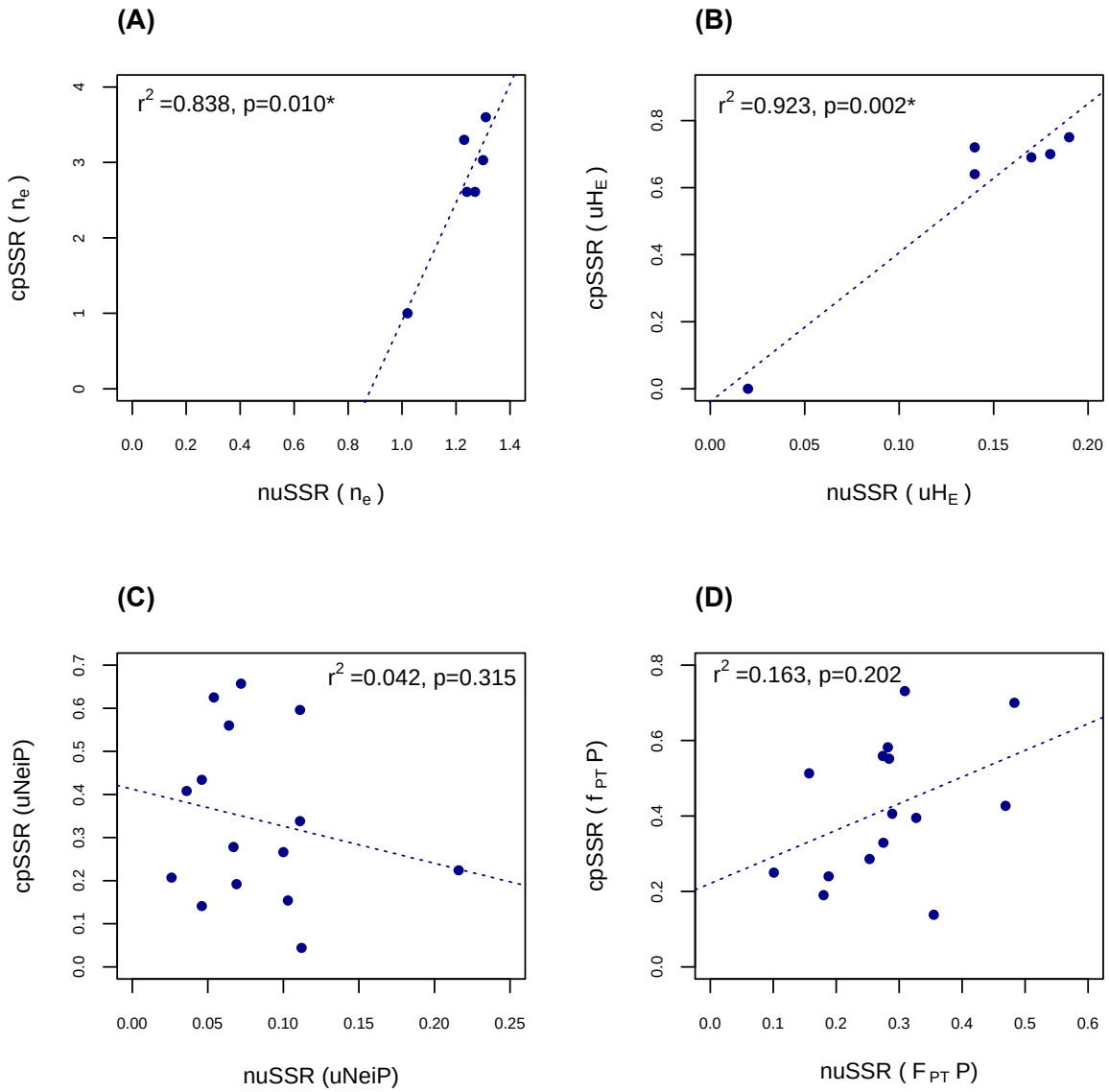

Figure 3.4: Correlation between cpSSR and nuSSr loci for diversity indices for Clausena. *A. Effective number of haplotypes/alleles (n_e). B. Pairwise unbiased haplotype/allelic diversity (uH_E). C. Unbiased pairwise Nei's genetic distance (uNeiP). D. Pairwise population differentiation ($\Phi_{PT}P$).*

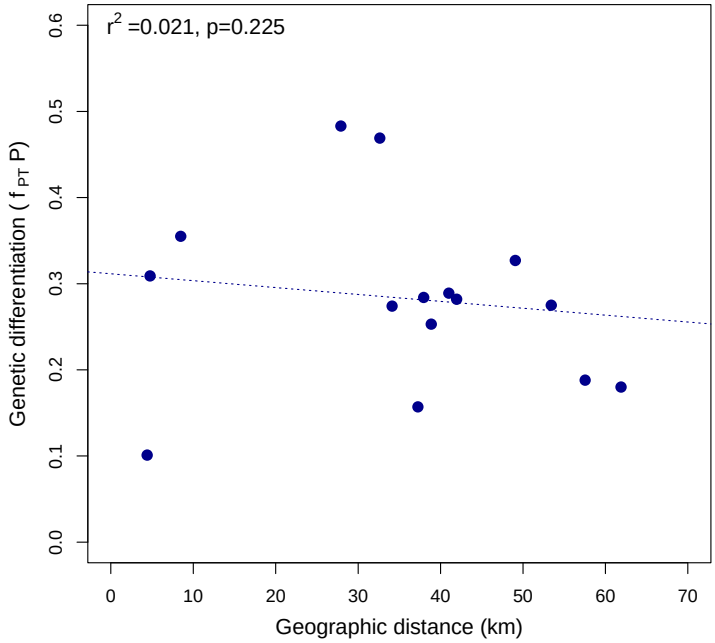

Figure 3.5: Isolation-by-distance plot for adult Clausena *samples using nuSSR loci CMS-4. A Mantel in GenAlEx (Peakall & Smouse 2006, 2012) was used to assessed significance (α=0.05).*

AMOVA analysis revealed high overall population differentiation at the CMS-4 locus (Φ_{PT}=0.329, p<0.001), in agreement with the results of the chloroplast loci. This confirms that some *Clausena* populations are genetically isolated from one another and that there may be little gene flow occurring among fragments. This is reiterated by the cluster analysis, which identified the optimal number of populations as 5 (Figure 3.6A). The ΔK plot shows strong support for K=2. However, the plot of ln(K) shows a levelling off at K=5, so 5 was selected as the optimal number of clusters. This is supported by the fact that the structure plot of K=5 exhibits similar trend of genetic structure to the analysis of chloroplast loci. The plot shows individuals within Ngel Nyaki and Kurmin Danko have similar assignment probabilities to several clusters (Figure 3.6C), indicating that Ngel Nyaki and Kurmin Danko samples represent a highly admixed population. The other four populations are primarily assigned to separate clusters. However, the presence of the blue cluster across all populations suggests a small amount of gene flow occurring among all populations is likely. Unlike the analysis of chloroplast loci, the populations of Mbamnga and Tamnyar were assigned to different clusters. This was also reflected in a higher level of population differentiation (Φ_{PT}P) than was observed for cpSSR loci.

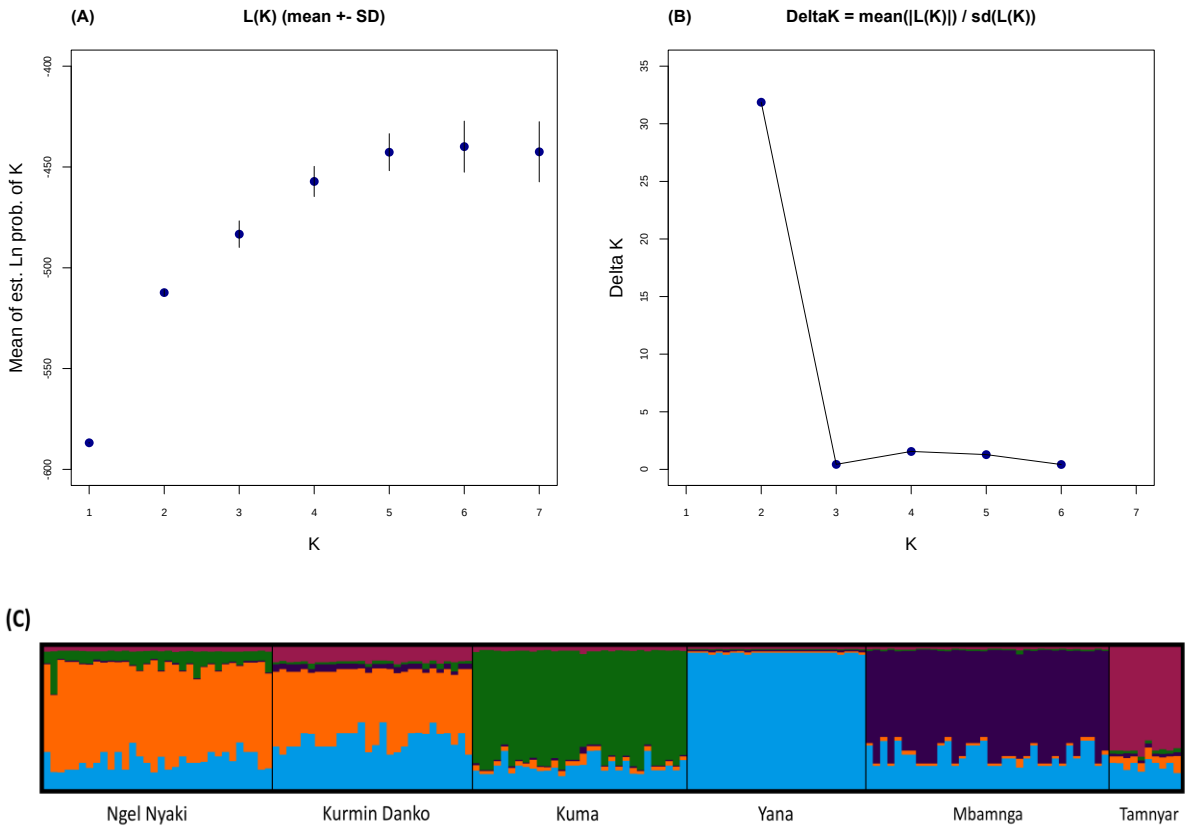

Figure 3.6: Population genetic structure of Clausena *indicated by nuSSR loci CMS-4 using STRUCTURE (Pritchard et al., 2000). A. Mean of estimated Ln probability of data vs. K for all samples. B. Optimal number of populations shown by ΔK vs. K calculated from 10 replicates per K for all samples. C. Structure plot of Q-values per sample for K=5.*

3.4 Discussion

3.4.1 Identification of microsatellite loci

Investigation of conserved cpSSRs revealed a high number of polymorphic loci suitable for genetic analysis of *Clausena*. Previous studies have used as few as one polymorphic conserved locus to assess genetic diversity (Mohanty *et al.* 2002; Thia *et al.* 2016). Other studies have found no polymorphism of conserved cpSSR within a species (Rendell & Ennos 2002; Thia *et al.* 2016). Angioi *et al*. (2009) assessed genetic diversity of *Phaseolus vulgaris* and identified seven polymorphic loci among eleven of the conserved chloroplast loci from Weising and Gardner (1999) and Chung and Staub (2003). The rate of polymorphism of conserved chloroplast microsatellite loci observed in this study (53%) appears to be high in comparison with other studies of a wide variety of angiosperm species.

Nuclear microsatellite locus CMS-4 exhibited up to four alleles present within a single individual, suggesting this species may have undergone either genome duplication, or

localized gene duplication. The ploidy of *Clausena* is undetermined, and the ploidy of other species within the *Clausena* genus is somewhat disputed (Mou & Zhang 2012). However, most species within this genus are considered to be diploid, making it likely that *C. anisata* is also diploid. Therefore, the excess alleles observed at the CMS-4 locus is not likely to be due to genome duplication. Alternatively, the CMS-4 locus may be a site of gene duplication, resulting in the amplification of up to four alleles (Zhang & Rosenberg 2007). In this case, the primers pairs of CMS-4 may be amplifying two different regions of the genome, resulting in the presence of up to four different alleles, potentially conveying higher genetic diversity than is present at a single gene locus.

3.4.2 Genetic diversity and population structure

The single polymorphic nuclear locus produced levels of genetic diversity substantially lower than the chloroplast loci. This is surprising, because diversity of nuSSR loci is anticipated to be higher than cpSSR because the effective population size is effectively double that of the chloroplast genome, resulting in faster rates of evolution (Wolfe *et al.* 1987; Provan *et al.* 2001). Gandhi *et al*. (2005) found that average expected heterozygosity of *Aegilops sp.* at nuclear microsatellites was typically more than 2x higher than that of chloroplast microsatellites. Andrianoelina *et al*. (2009) also found that within-population diversity of *Dalbeergia monticola* at nuclear loci was significantly higher than chloroplast loci. The low diversity of CMS-4 nuSSR locus in *Clausena* populations compared to cpSSR loci highlights the need to assess multiple loci in order to get an accurate representation of genetic diversity. Low diversity may also be an artefact of data analysis using dominant data, which may obscure genetic diversity. Therefore, it would also be useful to assess nuSSR loci that do not exhibit gene duplication, so that codominant analysis can be performed. Regardless, the diversity indices of *Clausena* population were strongly correlated between cpSSR and nuSSR loci. Therefore, while the diversity of the nuSSR locus alone is not appropriate for basing genetic recommendations on, it's congruence with the cpSSR provides confirmation of genetic characteristics of *Clausena* populations.

The overall genetic differentiation was high and similar between chloroplast and nuclear loci, suggesting low levels of gene flow among forest fragment on the plateau. However, pairwise differentiation measures were not congruent across the different types of loci. Chloroplast loci typically exhibit higher population differentiation than nuclear loci (see review in Muller *et al.* 2009), likely due to their smaller effective population size making them more susceptible to drift (Andrianoelina *et al.* 2009). However, in the case of *Clausena,* differentiation between population pairs at the nuclear locus was typically higher than the chloroplast loci. Again, this is likely due to the use of a single nuclear locus analysed as dominant data.

Analysis suggests that overall *Clausena* is not susceptible to erosion of genetic diversity following habitat fragmentation. The relatively large, protected forests of Ngel Nyaki and Kurmin Danko as well as the unprotected fragments of Mbamnga and Tamnyar showed substantial diversity at chloroplast and nuclear loci. However, sacred forest fragments do appear to be negatively affected. Yana sacred forest exhibited no haplotype diversity of

chloroplast loci, and minimal diversity at the nuclear locus. Yana population represents the smallest forest fragment sampled in this study, therefore evolutionary theory predicts that this population will possess the lowest level of genetic diversity. However, the extremely depauperate diversity observed at this site may be indicative of a historical population bottleneck. Extremely low effective population size in the past may have necessitated inbreeding and accelerated genetic drift, leading to erosion of genetic diversity. Isolation of Yana forest likely prevented replenishment of alleles due to restricted gene flow.

Adult samples from Kuma sacred forest exhibited high genetic diversity among adult samples, comparable with the formally protected forests. However, Kuma was the only population that showed a reduction of genetic diversity in juvenile samples, suggesting that diversity within this small forest fragment may be in decline. The 10.7% reduction in haplotype diversity that was observed at Kuma was consistent with demographic changes in diversity observed in other studies (Dayanandan *et al.* 1997; André *et al.* 2008). Hall *et al*. (1994) observed a decrease by 13.7% in mean heterozygosity of the common tropical tree species *Pentaclethra macroloba* in fragmented populations. The results of the present study suggest that the small population size of Kuma cannot sustain high genetic diversity, and alleles are slowly being lost within this population due to drift. Without intervention, genetic diversity within Kuma may degrade to the extent observed at Yana.

The unprotected sites of Mbamnga and Tamnyar exhibited relatively high genetic diversity, marginally lower than the formally protected forests and Kuma sacred forest. Kramer *et al*. (2008) suggest that erosion of genetic diversity following habitat degradation is not always evident in tropical tree species because forest edges do not define population boundaries and genetic effects may take substantial time to manifest. In regard to population boundaries, it is apparent that in the vicinity of Mbamnga and Tamnyar there are many gullies and streams that harbor small pockets of forest vegetation. Populations of *Clausena* likely extend well beyond the bounds of the sampling area, resulting in a large effective population size, minimising inbreeding and genetic drift. This may be an explanation for the high genetic diversity observed among samples collected in Mbamnga and Tamnyar. However, because these forests are unprotected, increasing economic pressures are likely to strongly impact these populations. Increasing intensity of logging, agriculture and farming may have already reduced these populations below the necessary threshold to sustain genetic diversity. The adult samples examined from these populations may represent historically large populations, when these forests were less degraded. Analysis of juvenile samples from these sites may reveal changes of diversity in recent cohorts.

The present study revealed very strong genetic differentiation of *Clausena* populations across the plateau that did not correspond to distance between fragments. However, the proximate populations of Ngel Nyaki and Kurmin Danko (4.4km) were not strongly differentiated. Structure analysis of both cpSSR and nuSSR loci suggest that these sites represent a single, admixed population. The populations of Kuma and Yana were the most strongly differentiated populations in the assessment of chloroplast loci, despite these populations being located relatively close to one another (4.8km). This suggests that the grassland matrix surrounding

these forest fragments is more inhospitable than elsewhere on the plateau, preventing the movement of biotic dispersal agents between these two sites. Structure analysis also showed that these populations are strongly divergent from one another. The impact of habitat fragmentation on movement of biotic dispersers is discussed extensively in Chapter 4.

3.4.3 Conservation implications

Despite there being no significant correlation between forest size and genetic diversity, our results show that the smallest forest fragment sampled on the Mambilla Plateau, Yana, is genetically depauperate despite large populations size of the study species and apparent strong social protection of this forest. Additionally, Kuma, the second smallest population, exhibits signs of decreasing genetic diversity. This indicates that the small, isolated sacred forest groves on the Mambilla Plateau are at high risk of erosion of genetic diversity in a common and inherently resilient species.

The lack of genetic diversity observed in Yana kurmi closely parallels the findings of Bossart and Antwi (2013, 2016). In their assessment of genetic diversity of butterflies of Ugandan sacred and formally protected forests it was found that the smallest forest, Kajease sacred forest (6ha), possessed the lowest haplotype diversity for two of three study species. Low diversity at this site was considered not to be solely due to forest size, but also because of the site's proximity to heavy urbanization and a degradation of traditional belief systems, leading to exploitation of this forest. Genetic diversity of several other sacred sites was comparable to the large, formally protected forest reserves. Furthermore, these sacred forests often harboured a high proportion of unique haplotypes.

Low genetic diversity within Yana forest may be attributed to several factors aside from small population size. Declines in diversity are not likely to be due to encroachment, as the religious value of this has remained high in recent decades (H. Chapman, pers. comm.). However, the history of the sacred kurame of the Mambilla Plateau is unclear. In Ghana, it is suggested that sacred groves are not relics of previous extensive forest cover, but were created by human settlement (Chouin 2002). Oral traditions in Guinea also suggest that forest fragments were planted by the original founders of associated settlements (Fairhead & Leach 1996). If sacred groves on the Mambilla Plateau are anthopogenic creations, then it is possible the forests were planted, and therefore the low and unique genetic diversity observed may be rationalised by founder effects. A survey of mythology and oral traditions surrounding these forests may help to elucidate their history. Low diversity in Yana may also be due to its position amongst high intensity agriculture, which is likely to discourage the movement of animal dispersal agents, thereby preventing gene flow and leading to a decline of genetic diversity.

Kuma sacred forest exhibited high levels of genetic diversity, comparable with large, government protected forests, despite being only 10 ha larger than Yana. This indicates that the isolation of Kuma forest fragment may be more recent than Yana, or these forests may have different origins. High genetic diversity within the adult *Clausena* population at Kuma may be because genetic effects can take to time to manifest (Kramer *et al.* 2008; Bossart &

Antwi 2016). The declining in genetic diversity exhibited by juvenile samples suggest that habitat fragmentation has had a significant impact at this site, and expected evolutionary impacts may be beginning to take effect. Moreover, cultural and religious reverence at Kuma is in decline, allowing increased encroachment and harvesting within the forest (H. Chapman, pers. comm.).

Importantly, Kuma and Yana sacred forests both exhibit entirely unique haplotypes. Therefore, if diversity were to decline in Kuma to the extent observed in Yana, much of the unique genetic diversity within this site would be lost. As a result, there would be a significant decline of overall genetic diversity on the Mambilla Plateau. It is apparent from this assessment that there is a need for conservation efforts to decrease the isolation of sacred forests on the Mambilla Plateau. Promotion of biotic disperser movement in the grassland/agricultural matrix may aid in reviving the genetic diversity of Yana kurmi, and prevent further losses of diversity at Kuma kurmi.

There is an apparent need for conservation management strategies for sacred forests on the Mambilla Plateau in order to prevent losses of biodiversity and ensure their protection in perpetuity. This poses a complex challenge; how to integrate these forest into the national network of protected forests without disrupting the cultural and religious reverence by local communities. Reclassifying these forest as state reserves and policing their use would likely increase rates of degradation due to alienation of local people (Ormsby 2011). Therefore, local communities must retain ownership of their sacred forests, and enforcement of long-standing cultural laws may need to be supported by national legislation. Crucially, there is a need for official demarcation of forest boundaries and fostering of traditional belief systems (Babalola *et al.* 2014). I recommend that the biodiversity value of Kuma and Yana sacred forests needs to be more thoroughly assessed and local communities need to be educated about the actions they can take to minimise ecological and evolutionary risks to the long-term survival of these forests.

3.4.4 Conclusions

The results of the present study contribute to a growing body of research confirming that sacred forests are important contributors to global biodiversity. It has also confirmed that biodiversity within such forests on the Mambilla Plateau may be under threat due to evolutionary factors. Kuma sacred forest showed high genetic diversity among adult *Clausena* samples, comparable with the diversity observed in much larger government protected forest reserves. However, genetic diversity of this forest appears to have declined in recent generations. Alternatively, Yana sacred forest was depauperate of genetic diversity in both adult and juvenile cohorts, suggesting there may be long-standing isolation of this small forest fragment, which poses a risk to its long-term viability.

The high and temporally stable genetic diversity observed within Ngel Nyaki and Kurmin Danko populations confirms that large, protected forest habitats remain the cornerstone of biodiversity conservation. Legislated protection of other such forests should be prioritised in Afromontane habitats to provide the strongest opportunity for conserving the unique

biodiversity of this ecoregion. However, on the Mambilla Plateau, no other substantial forests remain that can be set aside for conservation purposes. Sacred kurame, that have persisted as the landscape around them has been dramatically altered, represent the only other intact forests that remain on the plateau, and therefore the only other opportunity for preserving biodiversity.

Sacred forests are particularly vulnerable to declines of both species and genetic diversity due to their inherently small size. The erosion of genetic diversity poses a high threat to the continued survival of these forests, particularly in the face of imminent climatic changes in Sub-Saharan Africa. Most evident from this study is the need to promote gene flow through grassland/agricultural matrixes to reduce the isolation of sacred forests. Therefore, the sacred forests of the Mambilla Plateau should be incorporated into conservation strategies in order to better manage and protect the unique biodiversity that they possess. In light of increasing human populations and ethnic conflicts in the region, these forest will likely be increasingly threatened if no action is taken. The question remains of how to incorporate these sacred forests into national conservation policies while respecting their cultural and spiritual value.

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CHAPTER 4. Does Dispersal Mechanism Influence Genetic Structure in Two Common Tree Species?

Abstract

Gene flow via seed dispersal is an important force for mitigating the ecological and evolutionary impacts of habitat fragmentation on plant populations. However, when habitat fragmentation is severe, dispersal vectors may not be capable of bridging the gaps among fragments. The type of dispersal vector is important in determining dispersal distances and frequencies, and therefore for anticipating the effects of habitat fragmentation on genetic characteristics of plant populations. Conserved chloroplasts microsatellite loci offer the opportunity to make direct genetic comparisons of species employing different dispersal mechanisms. In this study, differences in genetic diversity and population differentiation for common Afromontane species, *Albizia gummifera* and *Clausena anisata*, on the Mambilla Plateau was assessed. It was found that bird/primate dispersed *C. anisata* exhibited substantially higher differentiation among populations than wind dispersed *A. gummifera*. This is consistent with the expectation that adverse effects of habitat fragmentation will be more significant for species employing abiotic dispersal mechanisms. Minimal rates of gene flow for both species likely contributed to the low genetic diversity observed in small forest fragments.

4.1 Introduction

4.1.1 Seed dispersal in fragmented landscapes

Gene flow, the migration of alleles among populations, is one of the four mechanisms of evolutionary change (Ellstrand & Elam 1993; Ellstrand 2014). Following habitat fragmentation, gene flow acts to maintain genetic diversity by uniting populations, replenishing alleles as they are lost and preventing divergence among populations (Jacquemyn *et al.* 2012; Ellstrand 2014; Ratnam *et al.* 2014). Extensive habitat fragmentation can lead to the breakdown of gene flow among populations, and this may have severe evolutionary and ecological implications for small, isolated forest fragments (Ellstrand & Elam 1993). While low migration rates may act to increase overall genetic diversity due to divergence among populations, diversity is expected to decline within small fragments due to the effects of inbreeding and genetic drift (as discussed in Chapter 1). Alternatively, high levels of gene flow may result in complete homogenisation of populations, and prevent the development of unique genetic characteristics associated with local adaptation (Ellstrand

2014). Metapopulations represent an equilibrium whereby gene flow counteracts the effects of inbreeding and genetic drift while still allowing for local identity (Ouborg *et al.* 1999).
Gene flow primarily occurs among plant populations through the movement of haploid pollen and diploid seeds (Wang *et al.* 2011; Kartzinel *et al.* 2013). Both pollen and seed dispersal are mediated by either biotic (i.e. animal ingestion, adhesion, or scatterhoarding) or abiotic vectors (i.e. wind, water, or gravity) (Howe 1982; Hughes *et al.* 1994; Nathan *et al.* 2008). The type of dispersal vector has a significant role in the efficiency and spatial scale of dispersal (Aguilar *et al.* 2008). In the past it has been thought that pollen dispersal is the primary means of long-distance gene dispersal, and is therefore expected to be most significant for maintaining gene flow in fragmented landscapes (Bacles *et al.* 2006). However, some types of seed dispersal may result in effective long-distance dispersal events that can have a significant impact in maintaining genetic connectivity among forest fragments (Ouborg *et al.* 1999; Soons & Ozinga 2005; Bacles *et al.* 2006). Plant species adapted for dispersal by birds, large mammals, or wind may have particularly high rates of gene flow, potentially allowing them to overcome the negative effects of habitat fragmentation (Clark *et al.* 2005; Pearson & Dawson 2005; Nathan *et al.* 2008). Efficient long-distance seed dispersal mechanisms have been cited as a probable cause of the 'paradox of forest fragmentation'; the observation that many tropical tree species do not exhibit the anticipated declines of genetic diversity following habitat fragmentation (Kramer *et al.* 2008).

4.1.2 Estimating gene flow

Measures of genetic differentiation calculated from molecular markers can be used to provide indirect estimates of gene flow among populations (Whitlock & McCauley 1999). The amount of gene flow occurring among populations can have a strong influence on the amount of diversity within and across populations. However, rates of genetic diversity and population differentiation of species is dependent on a wide array of factors, such as effective population size, deforestation history, and dispersal ability as well as the type and number of molecular markers used, number of samples analysed, and age class of samples (Loveless & Hamrick 1984; Ouborg *et al.* 1999; Kramer *et al.* 2008). This makes predicting genetic characteristics and making comparisons between species, notoriously difficult. Hamrick *et al*. (1993; 1996) first tried to summarise typical levels of allozyme diversity and population differentiation associated with particular life history traits (Table 4.1). However, no publications that I am aware of have attempted to incorporate the findings of more recent studies employing a wider range of species and more modern molecular markers. While the data of Hamrick *et al*. (1993) cannot be used for direct comparisons with the cpSSR loci employed in the present study, it can however, provide expected values for different life history traits relative to one another, and is therefore useful for predicting differences in genetic characteristics of the present study species. Hartl and Clark (1997) also attempted to categorise levels of genetic differentiation. They reported four classes of F_{ST} as follows: <0.05=little genetic differentiation, 0.05-0.15=moderate genetic differentiation, 0.15-0.25=high genetic differentiation, >0.25=very high genetic differentiation. Frankham *et al*. (2002) consider

genetic differentiation greater than 0.15 to represent significant differentiation. However, these categories of genetic differentiation are subjective and context dependent, so should be applied with caution.

Table 4.1: Mean and distribution of allozyme diversity and genetic differentiation for wind and animal dispersed plant species, from Hamrick et al. (1993). Parentheses '()' indicate standard error.

Dispersal mechanism	H_T	H_E	G_{ST}
Wind	0.292 (0.021)	0.241 (0.011)	0.143 (0.020)
Animal (ingested)	0.394 (0.022)	0.305 (0.020)	0.223 (0.033)

Mean dispersal distances of wind dispersed seeds are typically lower than some biotic methods (i.e. bird or large mammal), however the ability for infrequent long-distance dispersal events may effectively maintain connectivity between fragments (Clark *et al.* 2005; Soons & Ozinga 2005; Bacles *et al.* 2006). In contiguous forests, trees with wind dispersed seeds are anticipated to have moderate levels of genetic diversity. When habitats become fragmented, these species are expected to maintain their genetic diversity due to infrequent long-distance dispersal. This is because the frequency and distance of abiotic dispersal by wind is not expected to be significantly reduced by habitat fragmentation (Loveless & Hamrick 1984). Wind dispersal distances may actually increase following forest fragmentation due to the increased openness of habitat leading to changes in wind dynamics (Bacles *et al.* 2006; Damschen *et al.* 2014). These effects likely allow wind dispersed species to maintain sufficient gene flow across fragmented landscapes, thereby conserving genetic diversity.

Species dispersed by large mammals and birds are anticipated to have long dispersal distances, as these dispersal agents can readily move over large distances. Therefore, in continuous habitats, animal dispersed tree species are expected to exhibit moderate levels of genetic diversity and low levels of population differentiation (Loveless & Hamrick 1984). In marginally degraded habitats animal dispersed plant species are expected to maintain high gene flow, and therefore low population differentiation, due to the movement of their disperser over large ranges (Loveless & Hamrick 1984; Hamrick *et al.* 1993). However, in severely fragmented landscapes, animal dispersed tree species are predicted to become extensively differentiated among habitat fragments because dispersal agents will be unwilling to traverse the inhospitable gaps among fragments (Loveless & Hamrick 1984; Cramer *et al.* 2007). The composition of the matrix among forest fragments can have a strong influence on habitat permeability for animals (Anderson *et al.* 2007; Aben *et al.* 2012). Furthermore, the anthropogenic activities that have led to habitat fragmentation are also expected to have led to declines of animal populations, reducing the number of potential dispersers (Aguilar *et al.* 2008). The survival of populations of animal species will be limited by their ability to cross habitat gaps. Lees and Peres (2009) found that patch size, quality, degree of isolation, and permeability of the agricultural matrix of Amazonian forests were all important determinants of bird species assemblages. Primates are expected to be particularly affected by habitat

fragmentation. In Brazil it is found that fragment size, matrix permeability, and visibility are a major determinants of primate abundance (Silva *et al.* 2015).

A combination of low numbers of dispersal agents, as well as an inability for them to move between forest fragments, is anticipated to lead to a low level of gene flow among forest fragments for animal dispersed plant species. This is expected to manifest as high genetic differentiation between habitat fragments as plant populations diverge from one another. This divergence may lead to an increase in genetic diversity across populations. However, within populations genetic diversity is expected to decline, due to inbreeding and genetic drift, which may affect population viability (Loveless & Hamrick 1984). Animal dispersed species are expected to encounter greater declines of genetic connectivity, and therefore genetic diversity, following extensive habitat fragmentation than abiotically dispersed species (Aguilar *et al.* 2008).

4.1.3 Genetic comparisons between species

Cross-amplifying genetic markers facilitate comparisons of genetic characteristics between multiple species, and can therefore be used for comparative studies of genetic variability of plant species employing alternate dispersal mechanisms (Barbará *et al.* 2007). However, such loci can be difficult to come by. Barbará *et al.* (2007) demonstrated the limited transferability of nuclear microsatellite loci across different plant families, diminishing the potential for their use in comparative studies of broad groups of taxa. However, the 'universal' applicability of conserved chloroplast microsatellites allows the opportunity to directly compare genetic diversity among distantly related angiosperm species (Weising & Gardner 1999; Chung & Staub 2003; Cheng *et al.* 2006). Because the chloroplast genome has a slower rate of mutation than the nuclear genome and does not undergo recombination, it is more highly conserved. Therefore, flanking primer regions around microsatellites are more likely to be maintained across taxa (Provan *et al.* 2001). However, because the chloroplast genome is maternally inherited, conserved cpSSR loci can only be used to reveal rates of gene flow via seed dispersal, not pollen dispersal (Provan *et al.* 2001; Abdel-Mawgood 2012).

The conserved chloroplast microsatellite loci employed for analysis of *Albizia gummifera* (*Albizia*) and *Clausena anisata* (*Clausena*), in Chapters 2 and 3 respectively, can be used for a direct comparison of genetic diversity and population differentiation between these species. This may be used to reveal how habitat fragmentation on the Mambilla Plateau has differentially affected tree species employing alternate seed dispersal mechanisms. *Albizia* and *Clausena* share many ecological similarities; both are predominantly outcrossing with bisexual flowers and are common, fast-growing, Afromontane species with high colonising abilities (Maroyi 2007; Tchinda 2011). Particularly, both species are insect pollinated, and therefore the effect of habitat fragmentation on pollen dispersal is anticipated to be similar for these species. These species also face similar harvesting threats, as both are not targeted by logging, but are valued for firewood, light construction, and have multiple uses in traditional medicine. However, *Albizia* and *Clausena* employ different seed dispersal mechanisms, wind and animal respectively, and therefore levels of gene flow via seed

dispersal is expected to have been differentially affected by deforestation and increasing habitat fragmentation on the plateau.

The wind dispersal method of *Albizia* is atypical, and specific to the family of Fabaceae; dry seed pods disperse as a whole unit with seeds contained within (Shaanker *et al.* 1988; Mullah *et al.* 2014). The wind dispersed single-seeded pod of *Platypodium elegans* may disperse up to 100metres from the parent tree (Augspurger 1983; Hamrick *et al.* 1993). Alternatively, pods of *Dalbergia sissoo* containing 1-4 seeds have been found to disperse minimal distances, up ~10metres (Ganeshaiah & Shaanker 1988). Low seed numbers are often observed within pods of wind dispersed Fabaceae species because it is expected that numerous seeds will limit the dispersal ability due to increased 'wing-loading' (weight to surface area ratio) (Shaanker *et al.* 1988; Ganeshaiah & Shaanker 1988, 1991). Typical pods of *Albizia* may contain 9-12 seeds, suggesting their dispersal ability may be limited (Maroyi 2007). A previous study found low abundances of *Albizia* seeds dispersed beyond the border of Ngel Nyaki forest, which is consistent with the expectation that long distance dispersal is a rare event (Barnes & Chapman 2014). This study only recorded seed dispersed up to 30m in to surrounding grasslands, so potential dispersal distances of *Albizia* may be greater. Seeds of *Albizia julibrissin* have been found to readily disperse up to 90m, and potentially much farther in high winds (Pardini & Hamrick 2008).

Seeds of *Clausena* are primarily dispersed through the ingestion of fruits by generalist frugivore birds and primates (Snow 1981; Tchinda 2011). Generalist seed dispersing bird species present on the Mambilla Plateau include *Pogoniulus bilineatus* (Yellow-rumped Tinkerbird)*, Arizelocichla tephrolaemus* (Western Greenbul)*, Sylvia borin* (Garden Warbler) and *Pycnonotus barbatus* (Common Bulbul) (Dowsett-Lemaire 1988; University of Canterbury 2003; Ihuma *et al.* 2011). The capability of these species to move among isolated forest fragments is unknown. It is suggested that the small size of these bird species may allow them to maintain high population sizes and promote long distance gene flow (Cramer *et al.* 2007). However, given the extreme isolation of some forest fragments on the Mambilla Plateau, necessary dispersal distances may exceed the ranges of many small bird species. *Clausena* seeds are unlikely to be dispersed by large bodied bird species such as *Turaco sp.* which may be capable of sustained flight between distant fragments (Babalola *et al.* 2012). However, small migratory bird species, such as *S. borin*, are likely capable of dispersal over large distances, so may provide opportunity from gene flow of *Clausena* among the forest fragments (Dowsett-Lemaire 1988; Nathan *et al.* 2008).

Potential primate dispersers of *Clausena* fruits are not well documented. The Javan lutung, *Trachypithecus auratus sonaicus*, has been found to extensively consume seeds of another species of *Clausena* (*C. excavata*) in Indonesia, indicating that *Clausena sp.* are a desirable food source for some primate species (Kool 1993). Chimpanzees have been shown to feed on the fruits of *Clausena,* so may provide long distance dispersal where chimpanzee are abundant and have large ranges (Houle *et al.* 2010). However, on the Mambilla Plateau only a single troop of chimps is present, restricted to the forest of Ngel Nyaki (Beck & Chapman 2008). Therefore, chimpanzee will not provide long distance dispersal of *Clausena* among

forest fragments in this region. Tantalus monkeys (*Chlorocebus aethiops*) are likely the only primate species capable of migrating through the agricultural/grassland matrix among forest patches of the plateau (Agmen *et al.* 2010). However, this species has not previously been reported to consume the fruits of *Clausena*. In a survey of Tantalus diets at Ngel Nyaki, *Clausena* was not identified as a dietary component (Grassham 2012). However, in this study many seeds remained unidentified and may have included *Clausena.*

On the Mambilla Plateau, declines in bird and primate diversity and abundances have been documented in recent decades (Chapman *et al.* 2004). It is therefore anticipated that *Clausena* populations will have experienced declines in long-distance seed dispersal events due the decline in potential dispersal vectors and the increasingly inhospitableness of the agricultural lands among forest fragments on the plateau. This is expected to manifest as a high degree of genetic differentiation among forest fragments in comparison to wind dispersed *Albizia.* High genetic differentiation among forest fragments is then expected to lead to declines in genetic diversity within small populations because gene flow may be insufficient to counteract inbreeding and genetic drift. In this chapter, cross-amplifying conserved chloroplast microsatellite loci are used to compare the genetic characteristics of *Albizia* and *Clausena* on the Mambilla Plateau. The focal species of this thesis are directly compared to evaluate how species employing different seed dispersal mechanisms have been differentially affected by forest fragmentation.

4.2 Methods

To directly compare the genetic diversity and differentiation between the study species, adult genotyping data from the chloroplast microsatellite loci that amplified in both species and exhibited polymorphism in at least one of the species, were used in the analysis. The software GenAlEx (Peakall & Smouse 2006, 2012) was used to determine percent polymorphic loci (%P), effective number of haplotypes (n_e), and unbiased haplotype diversity (uH_E) within populations of each species. A paired t-test was used to compare the measures of genetic diversity between species. Unbiased pairwise Nei's genetic distance (uNeiP) was calculated for each species in GenAlEx. ANOVA analysis was used to determined overall population differentiation (Φ_{PT}) and pairwise population differentiation ($\Phi_{PT}P$) using 999 iterations in GenAlEx. A paired t-test was used to assess if uNeiP and $\Phi_{PT}P$ are significantly different between species, and a Mantel test (999 permutations) was used to assess if indices are correlated between species.

4.3 Results

Eleven of the 30 universal chloroplast loci tested in Chapters 2 and 3 fitted the criteria for direct comparison between *Albizia* and *Clausena*. Three loci were polymorphic in both species (ccSSR5, ccSSR8 and NTCP9), two loci were polymorphic among *Albizia* samples and

monomorphic for *Clausena* (ccSSR22 and NTCP40), and the remaining six loci were polymorphic only within *Clausena* (ccmp2, ccmp4, ccmp10, ccSSR7, ccSSR9, and ARCP5). Because all polymorphic loci of *Albizia* amplified in *Clausena*, diversity and genetic differentiation indices for *Albizia* are the same as reported in Chapter 2. However, one of the original polymorphic loci of *Clausena* was excluded (ARCP2) because it did not amplify for *Albizia.* Therefore, diversity and differentiation measures of *Clausena* differs from Chapter 3. NTCP9 showed the most notable disparity of allele sizes between species, with *Albizia* alleles around 412 base pairs and *Clausena* around 275 base pairs. Disparity among allele sizes of NTCP40 was also relatively high between the species, with ~294 base pairs for *Albizia* and 407 base pairs for *Clausena*. Locus ccSSR22 showed the highest similarity between the species, with two allele sizes for *Albizia* of 201 and 202 base pairs, and a single allele of 202 base pairs for *Clausena*.

Ngel Nyaki and Kurmin Danko exhibited the highest percent polymorphism for *Albizia* (45.5%), while %P of *Clausena* was highest in Mbamnga and Tamnyar (81.8%) (Figure 4.1A). Population effective number of haplotypes range between 1.00 and 3.44 for *Albizia* and 1.00-3.30 for *Clausena* (Figure 4.1B). The haplotype diversity ranges between 0.00 and 0.75 for *Albizia* and 0.00-0.72 for *Clausena* (Figure 4.1C). There is no significant difference between the two species for either diversity measure (N_e: p=0.154, uH_E: p=0.133). However, diversity measures were higher for *Clausena* at four of the six populations. At Yana, there was no diversity reported for either study species, and therefore no difference between them. At Mbamnga the diversity of *Albizia* was slightly higher than *Clausena,* with uH_E of 0.75 and 0.62 respectively. Diversity was most disparate between species in Tamnyar; no diversity was observed among *Albizia*, yet *Clausena* was most diverse in this population. Despite there being no significant pairwise difference in genetic diversity of populations, total diversity across all populations was considerably higher for *Clausena* than for *Albizia*. The total effective number of alleles for *Albizia* was 1.93, whereas for *Clausena* it was 11.00, and haplotype diversity was 0.49 and 0.92 respectively.

Population differentiation across all populations of *Albizia* was moderate (with respect to Hartl and Clark (1997)), with Φ_{PT} of 0.142 (p=0.012). Population differentiation was very high among *Clausena* populations (Φ_{PT}=0.474, p<0.001). Pairwise differentiation indices are compared in Figure 4.2A&B. The mean pairwise genetic distance (uNeiP) is 0.034 and 0.237, and mean pairwise differentiation (Φ_{PT}P) of 0.232 and 0.424, for *Albizia* and *Clausena* respectively. Maximum uNeiP for *Albizia* was 0.098 between Yana and Tamnyar, and maximum Φ_{PT}P occurred between Tamnyar and Kuma (0.886). For *Clausena*, the maximum differentiation occurred between Kuma and Yana populations (uNeiP=0.459, Φ_{PT}P=0.759). There was no significant correlation of pairwise differentiation between *Albizia* and *Clausena* for both uNeiP (r^2=0.183, p=0.130; Figure 4.2A) and Φ_{PT}P (r^2=0.057, p=0.272; Figure 4.2B). uNeiP values were found to be significantly higher in *Clausena* than *Albizia* population pairs (p<0.001). The difference in Φ_{PT}P values was not found to be significant (p=0.054).

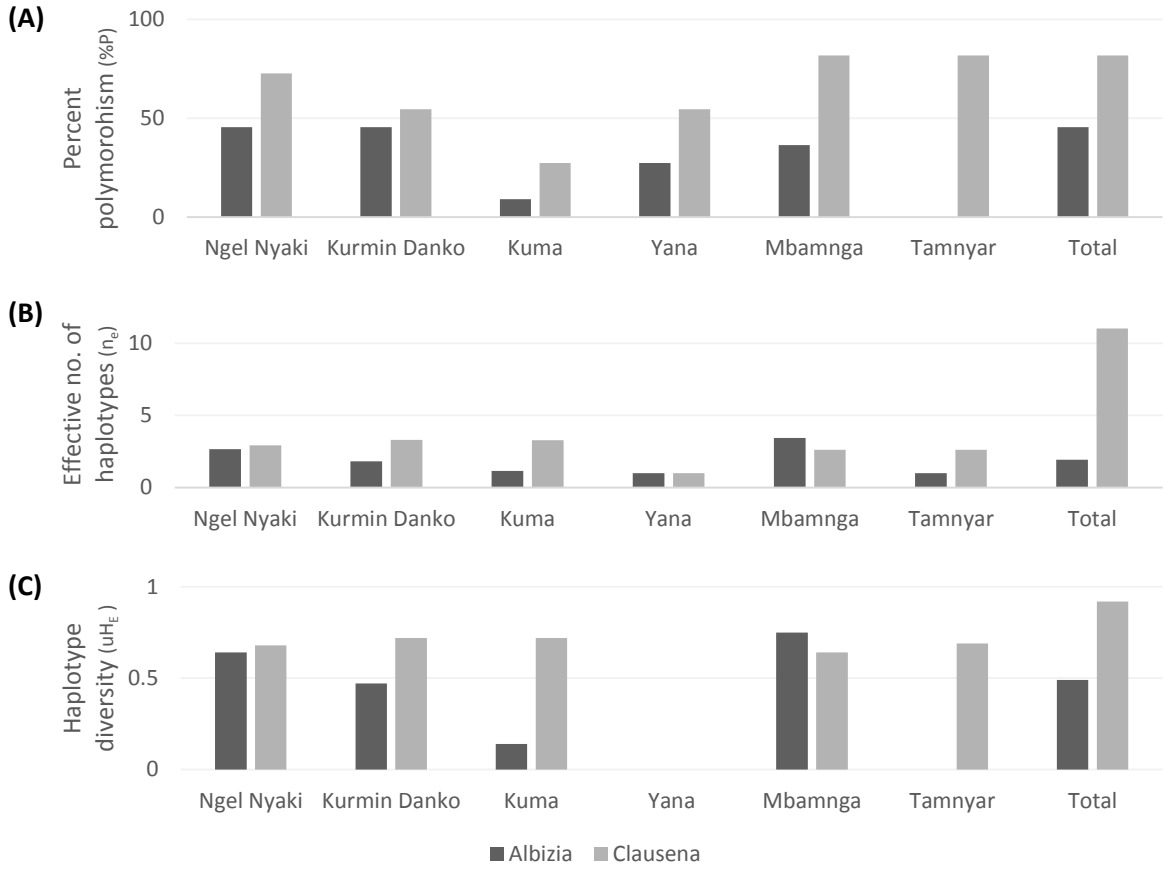

Figure 4.1: Diversity indices for Albizia *(dark grey) and* Clausena *(light grey) populations and total diversity across populations. A. Percent polymorphism of loci (%P), B. Effective number of haplotypes (n_e), C. Unbiased haplotype diversity (uH_E).*

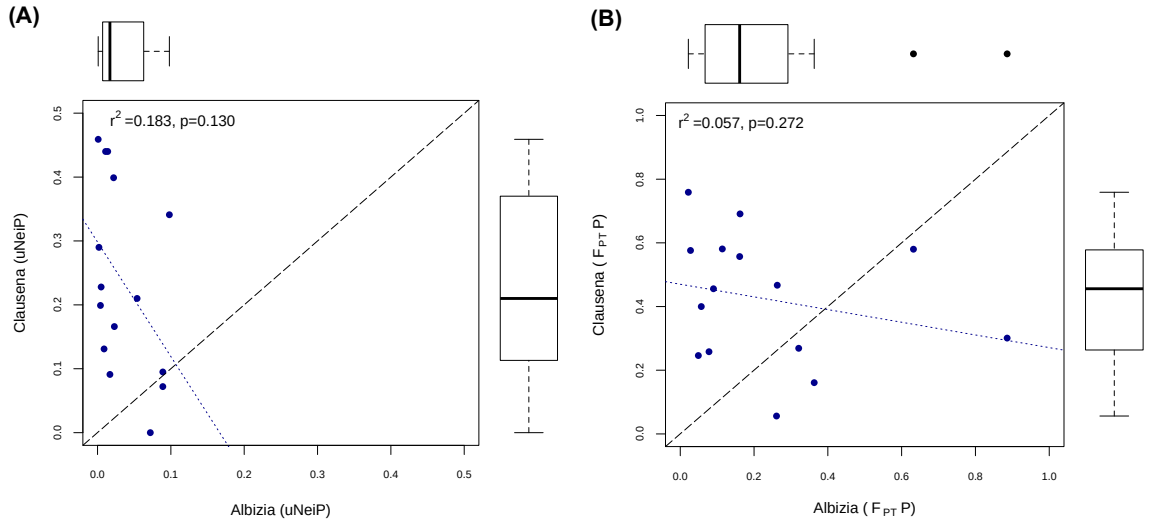

Figure 4.2: Pairwise distance and differentiation comparison between Albizia *and* Clausena *populations. A. Distribution of unbiased Nei's pairwise genetic distance (uNeiP) values. B. Distribution of pairwise differentiation ($\Phi_{PT}P$) values. Dashed diagonal line shows 1:1 ratio. Dotted line shows regression.*

Overall, genetic diversity for bird/primate dispersed *Clausena*, both within populations and across all populations, is seen to be higher than for wind dispersed *Albizia* (Table 4.2). The difference in total genetic diversity is considerable. Population differentiation is also substantially higher for *Clausena.* Diversity measures are all higher than anticipated by Hamrick *et al*. (1993) for the relevant dispersal method, which is an expected consequence of the use of microsatellite loci instead of allozymes (Table 4.1). Population differentiation of *Albizia* populations (Φ_{PT}=0.142) is comparable to typical allozyme diversity observed for wind dispersed species (G_{ST}=0.143), whereas population differentiation of *Clausena* (Φ_{PT}=0 .474) is substantially higher than typically observed for animal ingested species (G_{ST}=0.223).

Table 4.2: Summary statistics of genetic diversity and population differentiation of Albizia *and* Clausena*. Dispersal mechanism refers to the method of seed dispersal. H_T= total haplotype diversity of all samples. H_E= average haplotype diversity across six populations. Φ_{PT}= overall genetic differentiation calculated by AMOVA.*

Study Species	Dispersal mechanism	H_T	H_E	Φ_{PT}
Albizia	Wind	0.486	0.333	0.142
Clausena	Bird/Primate	0.915	0.575	0.474

4.4 Discussion

4.1.4 Comparison of seed dispersal mechanisms

Analysis of conserved chloroplast microsatellite loci presented both congruent and contrasting levels of genetic diversity within populations of *Albizia* and *Clausena* on the Mambilla Plateau. High relative genetic diversity was observed in the populations of Ngel Nyaki, Kurmin Danko, and Mbamnga for both study species. This is aligned with the expectation of evolutionary theory, which predicts that large populations will maintain higher levels of genetic diversity (Lowe *et al.* 2005). Consistently low levels of genetic diversity was also observed within the population of Yana, which was also the smallest population assessed in this study. However, Kuma and Tamnyar populations showed highly contrasting levels of genetic diversity between the two study species; diversity was high in *Clausena* samples and depauperate of diversity in *Albizia* samples at both these sites. Disparities in diversity observed at Tamnyar is likely due to the small sample size. Low levels of diversity observed for *Albizia* from Kuma may be indicative of a greater susceptibility to erosion of genetic diversity than *Clausena*. While, overall, differences in diversity were not found to be significant, most *Clausena* populations did show higher genetic diversity than *Albizia.* The Mbamnga population was the exception; at this site samples of *Albizia* showed marginally higher diversity than *Clausena.*

Comparison of rates of differentiation found that *Clausena* populations were more differentiated than *Albizia.* The moderate level of overall differentiation between *Albizia* populations suggests that populations are somewhat isolated, yet maintain connectivity by occasional long distance dispersal events, as is anticipated for wind dispersed species. Wind

dispersed tree species can exhibit extensive long-distance dispersal; seed of *Populus sp.* may dispersed up 30km (Hughes *et al.* 1994). However, such long distances are typically achieved by extremely small, light seeds with adaptions such as plumes or hairs to increase air resistance. *Albizia* exhibits an atypical wind dispersal syndrome for which little research has been undertaken. However, it appears unlikely that the pods of *Albizia* are capable of being dispersed across the entire distance (>4.4km) between any pair of forest fragments I measured on Mambilla. Low genetic diversity observed in Kuma and Yana *Albizia* populations, despite the apparent high levels of gene flow with other populations, suggests that gene flow is not sufficient to maintain diversity within these small forests. It appears that genetic distance/differentiation is a poor indicator of gene flow when populations are dominated by a single genotype. The loss of rare genotypes due to drift acts to make populations more similar, giving the appearance of high gene flow among some sites. These results caution against reliance on genetic differentiation measures for estimating gene flow. Parentage analysis using codominant markers may provide a more accurate estimation of migration rates (Ouborg *et al.* 1999).

The high level of differentiation and strong population structure of *Clausena* populations suggests that animal dispersers of this species infrequently move between distant forests. Ngel Nyaki and Kurmin Danko, and Mbamnga and Tamnyar are the only population pairs that appear to have high levels of gene flow between them. It is likely the close proximity of these populations, and relatively low intensity of agriculture in the areas between them, allows dispersing species to readily move among forests, thereby aiding gene flow. This is aligned with the expectation that bird/primate dispersed species will incur greater reductions of gene flow than wind dispersed plant species in highly fragmented landscapes (Loveless & Hamrick 1984). Bird and primate diversity and movement in Afromontane habitats is strongly influenced by habitat fragmentation. Aben *et al*. (2012) found that bird species in the Taita Hills, Kenya, were highly selective of habitats in fragmented matrices. Habitat selectivity may be due to the non-random distribution of resources, or may be the result of variability in predation risk. In the KawaZula-Natal midlands, South Africa, it was found that smaller forest patches contained fewer bird species (Wethered & Lawes 2003), which will likely correspond to reduced seed dispersal. Anderson *et al*. (2007) found that colobus monkeys (*Colobus angolensis palliatus*) in Kenya frequently travelled up to 4.2km from forest borders, but that they infrequently occupied cropland or grassland habitats, preferring to travel through degraded forests and shrublands. These studies showed that that matrix composition is fundamental to determining the movement of birds and primates among forest fragments. It is likely that few animal frugivores will move among isolated forest fragments on the Mambilla Plateau, providing explanation for the observed high genetic differentiation of *Clausena* populations. Encouraging establishment of indigenous forests in gullies and riparian margins may provide corridors for movement of dispersing species. Linear dispersal corridors or 'stepping stone' fragments of vegetation can provide the necessary habitat cover for animals to move among forests, thereby increasing seed dispersal (Pearson & Dawson 2005; Lees & Peres 2009). However, in reality, increasing native forest cover on the Mambilla

Plateau is likely to be challenging due to economic constraints. Indigenous forest cover in Afromontane habitats is projected to incur further decreases as the increasing demand for arable land increases isolation among fragments (Aben *et al.* 2012).

Alternatively, establishment of more exotic plantations may aid in promoting movement of bird dispersers without compromising economic productivity. Wethered and Lawes (2003) showed that in the Afromontane forests of South Africa, smaller forest patches contained fewer bird species than larger forests. It was also found that small forest patches (<50ha) in close proximity to exotic plantation boasted significantly higher bird species diversity than small forests surrounded only by grasslands. However, plantations surrounding large forest fragments did not encourage greater species diversity. This suggests that the establishment of plantations near the small fragments of Kuma and Yana may promote greater bird species diversity, which in turn may encourage dispersal among fragments. Many *Eucalyptus* plantations are distributed across the plateau, however, plantations in the vicinity of Kuma and Yana are small in size and may be insufficient to attract birds. Therefore, increasing the size of these plantations may provide a means to promote bird diversity, and therefore biotic seed dispersal.

Both *Albizia* and *Clausena* exhibited extremely low genetic diversity in the population of Yana. This suggests that Yana forest has been significantly impacted by habitat fragmentation, or has been isolated for a long time, and there is a high likelihood that other species in this forest also possess low genetic diversity. The cause of low diversity may be due to the small size (15ha) of this forest, and long-standing isolation from other forests. This may have led to high rates of inbreeding and genetic drift, resulting in declines of genetic diversity. It is likely that this forest existed as a small fragment prior to agicultural intensification on the plateau, however, it's true origins are unknown (H. Chapman, pers. comm.). Evidence for the anthropogenic creation of small forest fragments in Ghana and Guinea suggests that plant populations within Yana may have different origins from other forests on the plateau (Fairhead & Leach 1996; Chouin 2002). Founder effects may have created a population bottleneck that resulted in a loss of genetic diversity, as well as the distinctly unique genotypes that are observed.

The genetically depauperate populations of *Albizia* and *Clausena* within Yana kurmi generates high concern for the long-term persistence of this forest. Low genetic diversity is predicted to result in reduced fitness, potentially resulting in extirpation of affected populations. Empirical evidence for local extinctions of natural populations due to loss of genetic diversity is limited. However, meta-analysis by Spielman *et al*. (2004) reported that in many cases genetic factors may have a significant role in population extinctions. Saccheri *et al*. (1998) showed that wild butterfly populations with low diversity had a higher risk of extinction than genetically diverse populations. Among populations of the annual plant *Clarkia pulchella* it has been found that 69% of populations possessing low genetic diversity became extinct within 3 years, whereas only 25% of genetically diverse population became extinct (Newman & Pilson 1997). Extirpation of the study species from Yana forest will result in a decline in species diversity and may contribute to declines in aspects of ecosystem function, such as forest productivity

and nutrient cycling (Hughes *et al.* 2008). This forest is also unlikely to possess appropriate genetic variability to ensure its survival in the face of inevitable environmental changes (Pauls *et al.* 2013). If low genetic diversity is pervasive throughout tree species in this forest, severe environmental perturbations may result in rapid deterioration of the entire forest as multiple taxa become extirpated. Given the high value of this forest to proximate villages due to ecosystem services, the loss of this forest will likely be highly detrimental to the well-being of local communities (Dudley *et al.* 2010; Babalola *et al.* 2014).

4.1.5 Conclusions

This study emphasizes the utility of conserved chloroplast microsatellite loci for direct comparisons of genetic diversity and differentiation between distantly related taxa. This allows the opportunity to evaluate how habitat fragmentation has differentially affected tree these study species employing alternate seed dispersal vectors. Also, this comparison can be used to identify forest fragments of high conservations value. Consistencies in genetic diversity across species can provide a useful archetype for other tree species within the forest fragments of the Mambilla Plateau; where diversity is seen to be low in both study species it can be anticipated that a great number of other unassessed species are also likely affected by genetic drift and inbreeding following habitat fragmentation. The ease of use, high rates of cross-amplification and relatively high congruence between study species promotes the use of the conserved cpSSR loci published by Weising and Gardner (1999), Chung and Staub (2003), and Cheng *et al*. (2006) in rapid, low cost assessments of genetic characteristics.

Direct comparison of genetic diversity and differentiation of *Albizia* and *Clausena* exhibited results consistent with previous meta-analysis and the expectations of evolutionary theory (Loveless & Hamrick 1984; Hamrick *et al.* 1993). Bird/primate dispersed *Clausena* exhibited higher diversity and population differentiation than wind dispersal *Albizia.* These results suggested extremely limited movement of birds and primates among forest fragments across the plateau. However, there may be a lack of gene flow for both study species. It appears that the genetic diversity of large forest fragments is not adversely affected by this lack of gene flow. Alternatively, isolation is a likely contributing cause to the low genetic diversity observed in small forest fragments. This suggests a high need to promote gene flow among forest fragments on the Mambilla Plateau to maintain and restore genetic diversity in small populations.

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CHAPTER 5. Discussion

5.1 Synthesis of results

The primary aim of my research was to provide a prelimina ry assessment of the genetic diversity within, and differentiation among, the forest fragments of the Mambilla Plateau, Nigeria, to determine the impact of forest fragmentation. This assessment is used to identify forest fragments of conservation interest due to either relatively high or relatively low diversity, or the presence of unique genotypes on the Mambilla Plateau. This work also catalogues baseline data on the genetic characteristics of the forest fragments for evaluating changes in the future. Populations that possess high genetic diversity are of high value because they have the greatest potential for long term survival and fostering of high biodiversity across multiple levels (Hughes *et al.* 2008). Alternatively, genetically depauperate populations are of interest because they represent populations that have likely been significantly affected by fragmentation and are consequently at greater risk of extirpation (Frankham *et al.* 2002; Höglund 2009). Populations possessing unique or rare genotypes must also be conserved because they disproportionately contribute to diversity across all populations, and may possess optimal traits for persistence following environmental perturbations (Hannah *et al.* 2014).

I found that *Albizia* adult populations presented very low to moderate levels of genetic diversity within populations, with moderate differentiation among populations. Analysis of juvenile samples indicated genetic diversity of this species has declined in more recent generations, suggesting a high susceptibility to erosion of diversity, and generating concern for the long-term viability of this species in all forest fragments. In contrast, adult samples of *Clausena* exhibited high genetic diversity in all populations except Yana, and high differentiation among populations. Many unique genotypes were present within *Clausena* populations. Juvenile samples suggested genetic diversity has remained stable in all populations except Kuma, where are decline in diversity was observed. These results suggest that *Clausena* may be more resistant to the evolutionary implications of habitat fragmentation than *Albizia.* However, when fragmentation is severe, *Clausena* is still susceptible to inbreeding and genetic drift. While no significant correlation between fragment size and diversity was obtained for either species, it was consistently observed that the three largest sampling sites, Ngel Nyaki, Kurmin Danko and Mbamnga, possessed high relative levels of haplotype diversity. Also, Yana forest, the smallest and most isolated forest fragment, exhibited the lowest level of diversity in both study species. Low genetic diversity observed within small, isolated populations of *Albizia* and *Clausena* provides confirmation that common tree species are capable of significant losses of genetic diversity following habitat fragmentation (Honnay & Jacquemyn 2007). *Clausena* populations showed higher genetic differentiation of than *Albizia* population, which is consistent with the expectation that species that have their seeds dispersed by animals will be more significantly affected by

severe forest fragmentation than those dispersed by wind (Loveless & Hamrick 1984; Aguilar *et al.* 2008).

Providing evidence for the anticipated losses in genetic diversity of tropical tree species following habitat fragmentation has proved elusive for many researchers (Lowe *et al.* 2005; Kramer *et al.* 2008). In this thesis, I present evidence that genetic diversity and gene flow of *Albizia* and *Clausena* have been adversely affected by extensive habitat fragmentation on the Mambilla Plateau. It has been suggested that naturally low abundances of many tropical tree species relative to temperate species promotes long-distance dispersal abilities that prevent inbreeding and genetic drift (Kramer *et al.* 2008). However, *Clausena* populations exhibited a very high degree of differentiation among them, which may have resulted in inbreeding and genetic drift within small populations. This indicates that biotic dispersers are unable to maintain gene flow among the forest fragments. Kramer *et al*. (2008) also suggest that genetic erosion of tropical tree species is infrequently observed due to sampling of adult trees that represent relics from the previously undisturbed habitat. This theory is supported in this thesis by the observed low genetic diversity of *Albizia* juvenile cohorts compared to adult cohorts.

5.2 The utility of cpSSR and nuSSR loci

My research has demonstrated the effectiveness of conserved chloroplast microsatellite loci for rapid assessment of genetic characteristics of plant species. The cpSSR loci exhibited appropriate variability that could be explained by evolutionary theory and further supported by relative congruence of nuSSR loci. A high number of cpSSR loci were seen to amplify in both study species, with a moderate number exhibiting polymorphism (5 of 22 for *Albizia,* 10 of 19 for *Clausena*). Given the relatively small spatial scale of the present study, it may be unwise to ignore monomorphic loci because polymorphisms may be observed in these if the study is broadened across a wider geographic scale. The conserved cpSSR loci have provided the opportunity to collect baseline data on the genetic diversity and differentiation of *Albizia* and *Clausena* populations across the Mambilla Plateau. These data can be used in the future to determine changes in diversity within these species over time, to use in comparisons with other species within these forests, or compare with other Afromontane forests.

The observed low rate of cross-amplification of nuSSR from closely related species was not unexpected, and confirms the limited utility of such loci. In order to gain a sufficiently meaningful number of nuclear microsatellites for these species it is apparent that species-specific loci must be developed. An initial objective when undertaking this study was to assess diversity and differentiation of the study species using both cpSSR and nuSSR loci in order to determine the relative contributions of pollen and seed dispersal to gene flow among fragments (Provan *et al.* 2001). However, with only one polymorphic nuSSR locus obtained for each species, such comparisons were not possible. Instead, nuSSR loci were used to affirm the results of cpSSR loci. Comparison of the indices from the single nuSSR locus with those of the cpSSR loci for each study species found relatively high congruence. For *Albizia,* the Ency-

17 nuSSR locus exhibites higher diversity than cpSSR, as is expected due to the greater rate of mutation in the nuclear genome. I found that cpSSR and nuSSR differentiation indices for *Albizia* were significantly correlated, whereas within-population diversity indices were not significantly correlated. The nuclear locus exhibited additional population genetic structure that was not evident from chloroplast loci. For *Clasuena,* the CMS-4 nuSSR locus exhibited lower diversity than the cpSSR loci, likely due to the use of dominant binary data analysis. This locus exhibited up to four alleles within a single sample, suggesting that the gene has previously undergone duplication. Diversity of *Clausena* populations for nuSSR and cpSSR loci were found to be significantly correlated, while differentiation was not. Like *Albizia*, *Clausena* samples showed additional population genetic structure at the nuSSR locus.

Previously, Thia *et al*. (2016) employed the conserved cpSSRs developed by Weising and Gardner (1999) to assess the genetic diversity of three threatened tree species, *Cordia millenii* (Boraginceae), *Entandrophragma angolense* (Meliaceae), and *Lovoa trichilioides* (Meliaceae), within Ngel Nyaki forest (Table 5.1). They found that seven of the ten loci amplified in all three study species. However, only one locus was polymorphic (ccmp6), and only for the species *L. trichilioides*. Thia *et al*. (2016) concluded that these species were extremely depauperate of genetic diversity due to their small population size within the forest.

Of the ten conserved cpSSR loci of Weising and Gardner (1999) six loci amplified for *Albizia* and *Clausena. Albizia* exhibited no polymorphism at any of the ccmp loci, whereas *Clausena* was polymorphic at three loci (Table 5.1). As a result, diversity of *Albizia* at Ngel Nyaki was zero, equal to that observed for *C. millenii* and *E. anngolense* by Thia *et al*. (2016). However, *Albizia* exhibited a moderate level of diversity when assessed at a greater number of loci in Chapter 2, showing that the ccmp loci alone were not sufficient to encapsulate levels of genetic diversity. These results exemplify the need to examine a high number of loci in order to obtain loci which are polymorphic. If these species evaluated by Thia *et al* (2016) were to be reexamined at other conserved chloroplast loci, such as those from Chung and Staub (2003) and Cheng *et al*. (2006), some polymorphic loci may be obtained, and therefore genetic diversity may be found to be higher than was previously determined.

Table 5.1: Genetic diversity measures for five tree species within Ngel Nyaki forest using ccmp loci of Weising and Gardner (1999). Indices for Albizia *and* Clausena *are from the present study. Indices for* Cordia millenii*,* Entandrophragma angolense*, and* Lovoa trichilioides *were obtained from Thia et al. (2016). N= sample size, %P=percent polymorphic loci, n_e=effective number of haplotypes, uH_E= unbiased haplotype diversity.*

Study Species	Population density	N	%P	n_e	uH_E
Albizia	High	33	0	1.00	0.00
Clausena	High	35	50	2.78	0.66
C. millenii	Low	37	0	1.00	0.00
E. angolense	Low	26	0	1.00	0.00
L. thichilioides	Low	25	14	1.17	0.15

5.3 Conservation implications for Afromontane forests of the Mambilla Plateau

5.3.1 Ngel Nyaki and Kurmin Danko

I observed high genetic diversity relative to the other sites in this study for both study species in the relatively large, formally protected forest of Ngel Nyaki and Kurmin Danko. These forests were designated as a Local Authority Forest Reserve in 1969, and since then have been subject to a concerted conservation and restoration effort (Ihuma *et al.* 2011; Barnes & Chapman 2014). Despite these efforts, *Albizia* juvenile samples showed evidence that genetic diversity of this species is in decline. Ecological restoration projects are likely to have an important influence in maintaining or further degrading genetic diversity (Reynolds *et al.* 2012; Thomas *et al.* 2014). Ngel Nyaki represents the largest intact primary forest remaining in this region and is highly species diverse; maintaining high genetic diversity may be vital to retaining this status. Therefore, efforts to expand Ngel Nyaki forest through ecological restoration must also consider genetic factors.

Analysis of genetic diversity of *Albizia* in Chapter 2 showed high potential for the use of this species in active restoration efforts. The nitrogen-fixing abilities of the species promotes its use as the 'nurse' species in planting projects, and previous studies have also shown that *Albizia* facilitates regeneration of high species diversity (Kadiata *et al.* 1996; Mullah *et al.* 2014). At present, *Albizia* is not being often used in regeneration efforts at Ngel Nyaki (H. Chapman, pers. comm.). However, the high relative genetic diversity of *Albizia* found at Ngel Nyaki promotes more extensive use of the species. High and stable diversity of *Clausena* populations at Ngel Nyaki and Kurmin Danko establishes these forests as strongholds of genetic diversity for this species. The conservation of genetic and species diversity at Ngel Nyaki and Kurmin Danko may be vital for the provision of seed sources for genetic rescue of other populations or future restoration endeavors.

5.3.2 Kuma and Yana

This study found evidence that sacred kurame forests on the Mambilla Plateau may be under significant threat due to evolutionary factors. The Kuma and Yana populations of *Albizia,* and *Clausena* at Yana, were found to be genetically depauperate. Additionally, *Clasuena* juvenile samples from Kuma were observed to have lower genetic diversity than corresponding adults, indicating that diversity is declining in recent generations within this population. High rates of genetic drift and inbreeding within Kuma and Yana forests is likely due to their small size and long-standing isolation from other fragments (Frankham *et al.* 2002).

The depauperate genetic diversity of Kuma and Yana forests emerges from this assessment as the most significant and immediate conservation concern. Without genetic diversity, these populations are unlikely to possess resilient genotypes that may survive pathogen attack or environmental perturbations. Additionally, without variation, these populations will be unable to adapt to environmental changes in the future. In the face of impending climate

change in Sub-saharan Africa, there is significant concern for the long-term survival of these populations. The lack of genetic diversity observed in abundant species within these forest fragments may result in pervasive community and ecosystem level ecological effects (Hughes *et al.* 2008). Also, given the evidence for low, or decreasing, genetic diversity of two tree species with differing life history traits within Kuma and Yana forests, it is likely that other species have also been affected by the evolutionary impacts of habitat fragmentation. Therefore, perturbations or environmental change may not simply result in extirpation of a single species, but may lead to breakdown of the entire forest community. Despite their small size, the high ecosystem service value of sacred kurame forests means that their loss would have severe implications for the health and prosperity of associated communities, and efforts must be made to ensure their long-term viability.

In Chapter 2, the potential for genetic rescue of *Albizia* populations at Kuma and Yana forests was discussed. I concluded that this species presented a promising opportunity for genetic rescue of *Albizia* using translocations. However, if erosion of genetic diversity is pervasive throughout tree species in these forest fragments, genetic restoration of a single species will do little to ensure long-term sustainability of the entire forest. The unique genotypes present in *Clausena* populations at Kuma and Yana brings into question the suitability of this species as a candidate for genetic rescue, as it appears that the potential for outbreeding depression is greater than for *Albizia*. Reinstating genetic diversity at Kuma and Yana sacred forests presents a complex management issue. Because genetic rescue is species specific, further assessment of genetic diversity across multiple tree taxa is needed to confirm if erosion of diversity is pervasive throughout these forest fragments. If so, genetic rescue will have limited utility. Furthermore, the communities protecting their kurame may be resistant to the idea of introducing foreign material to these sacred forests.

I propose that promoting migration of biotic dispersers into these forests may be a more effective method of increasing gene flow. Increasing disperser movement will also increase gene flow for other plant species employing animal dispersal, thereby enhancing diversity across multiple taxa. Two methods to enhance biotic disperser movement were presented in Chapter 4; establishment and protection of riverine and gallery forests to create dispersal corridors, or the establishment exotic forestry plantations in close proximity to small forest fragments to attract bird species.

Establishment of dispersal corridors may aid in restoring gene flow for a higher number of species with a variety of life history traits. However, configuration of dispersal corridors is vital to achieving positive outcomes, so will require additional research and funding in order to be effective (Pearson & Dawson 2005; Segelbacher *et al.* 2010). Also, further restricting areas of arable land in agricultural regions is likely to create conflict with local communities. Elsewhere in Africa, establishment of biodiversity corridors has been successfully achieved with positive community involvement (Roe *et al.* 2009; Manjaribe *et al.* 2013). However, on the Mambilla Plateau, conflicts over land-use rights and existing hostility towards conservation managers means the establishment of dispersal corridors is likely to be extremely difficult.

Alternatively, promotion of animal disperser migration through exotic plantations, such as Eucalyptus, may promote genetic diversity in a greater number of species (Wethered & Lawes 2003), but is still limited to plant species utilizing large mammals or birds for dispersal. Well-managed plantations can also promote indigenous plant diversity with their understory, which can in turn promote primate abundance (Chapman & Chapman 1996; Bonilla-Sánchez *et al.* 2012; Telila *et al.* 2015). Therefore, plantations may provide an alternative means for seed dispersal across fragmented landscapes. However, establishment of plantations requires high initial capital and it may take a long time to observe positive effects. Additionally, in order to recover costs, plantations will eventually be logged, potentially erasing positive benefits unless an effective management strategy is in place. There are already many established *Eucalyptus* plantations in place across the Mambilla Plateau, and it would be of high value to assess their potential contribution in promoting indigenous biodiversity in this region.

5.3.3 Mbamnga and Tamnyar

High genetic diversity was typically observed in the populations of Mbamnga and Tamnyar, suggesting limited evolutionary effects at these sites. These sites consisted of heavily degraded riverine and gallery forests. The patchy but widespread distribution of vegetation within and beyond the sampling area indicates the populations of these species may be extensive, allowing them to maintain high gene flow and diversity. Wind dispersal of *Albizia* is likely to be frequent among patches. Additionally, bird diversity, and therefore gene flow of *Clausena*, may be supported by the presence of *Eucalyptus sp.* plantations in the vicinity (Wethered & Lawes 2003). Juvenile samples were not collected from Mbamnga and Tamnyar, so changes in diversity in recent generations cannot be reported. However, in these heavily degraded habitats I suggest that ecological factors are of greatest concern to population persistence. Because these forests are unprotected, human and cattle encroachment is likely to be high. Human disturbances likely includes logging of established trees which will alter ecosystem functioning, while livestock grazing may prevent regeneration in the understory (Wassie *et al.* 2009; Aerts & Honnay 2011). The sporadic distribution of vegetation also likely creates strong edge effects (Aerts & Honnay 2011). Regardless of the high genetic diversity contained within Mbamnga and Tamnyar populations, the prospects for long-term persistence of these forests are poor.

5.4 Future opportunities for research

1. Low genetic diversity of juvenile samples in comparison to adults samples is interpreted as an indicator of eroding genetic diversity. However, differences in diversity may be an artefact of sampling. Adult tree samples likely represent a greater number of years than juvenile samples, and therefore more reproductive events. Different genotypes may be more favourable in different years, and therefore the relative contribution to new generations will differ among years. Sampling across a

restricted number of reproductive events may not encapsulate important genotypes that reproduce infrequently. I suggest that further sampling across a greater number of age classes may provide a more accurate insight into changes in diversity over time, and may provide confirmation of erosion of genetic diversity.

2. It would be of high value to explore erosion of genetic diversity in other tree species in the populations of Kuma and Yana to confirm that other tree species are experiencing evolutionary impacts of habitat fragmentation in these sacred forests. I propose that such an assessment could be carried out using the same conserved cpSSR loci used in the present study. Samples should be collected for multiple species, with varying life history traits, throughout the fragments of Kuma and Yana, and also from Ngel Nyaki and Kurmin Danko in order to provide context. If diversity is seen to be low in the smaller forest fragments for multiple species, then a scheme to restore diversity needs to be developed.
3. Low genetic diversity of Albizia was observed at Tamnyar, and analysis of differentiation suggests that this site was distinct from other populations. It is proposed that low diversity was due to the limited samples size (5), and differentiation due to hybridization with another species of *Albizia*. Further samples need to be collected and assessed from Tamnyar in order to increase the sample size (Hale *et al.* 2012). Another important step would be to identify the congener species with which *Albizia* is hybridising at Tamnyar to establish if it is a naturally occurring hybrid, and to determine the extent of the hybrid zone.
4. *C. millenii*, *E. angolense*, and *L. trichilioides* samples from within Ngel Nyaki should be reassessed at a greater number conserved cpSSR loci. The conserved cpSSR loci from Chung and Staub (2003) and Cheng *et al*. (2006) should be evaluated for cross-amplification and polymorphism in these species. If polymorphic loci are obtained, the genetic diversity of these species should be reassessed.

5.5 Final remarks

In this study I have emphasised the utility of conserved chloroplast mircosatellite loci for assessments of genetic characteristics in distantly related angiosperm species, and allowing direct comparisons among species. This promotes the incorporation of conserved cpSSR loci analysis into a standard toolkit of methodologies for rapid assessment of the ecological and evolutionary implications of habitat fragmentation in previously unassessed species or locations. These loci emerge as particularly useful in study locations such as Sub-Saharan Africa, where genetic studies are typically limited due to funding constraints.

I have demonstrated that results from analysis of cpSSR loci can be applied to several alternate applications. Chapter 2 explored the importance of assessments of genetic characteristics when undertaking ecological restoration projects. It was found that passive restoration methods may be insufficient to sustain diversity of *Albizia* at Ngel Nyaki, and it is recommended that the species be used more extensively in restoration planting. Futhermore,

it is suggested that genetic rescue of *Albizia* at Kuma and Yana may be necessary to restore genetic diversity within these forests. Analysis of *Clausena* in Chapter 3 supported the hypothesis that culturally protected forest are important contributors to global biodiversity. It was found that sacred kurame forests on the Mambilla Plateau possess entirely unique haplotypes, and were strongly isolated from other forest fragments. Kuma forest exhibited levels of diversity comparable to the large, formally protected forests. In contrast, Yana forest was found to be depauperate of diversity, suggesting that sacred forests are particularly susceptible to the impacts of habitat fragmentation. The cross-amplifying cpSSR loci also facilitated interspecies comparisons of genetic characteristics of *Albizia* and *Clausena* in Chapter 4. This has allowed a direct comparison of the effects of habitat fragmentation on the genetic characteristics of species employing alternate seed dispersal mechanisms. This comparison has led me to the conclusion that gene flow among forest fragments on the Mambilla Plateau is inadequate in bird/primate dispersed species, but may also be limited for wind dispersed species.

Genetic diversity comprises an important component of this biodiversity, and evolutionary factors should be considered alongside ecological factors when evaluating the impacts of habitat fragmentation and determining the long-term sustainability of biodiversity resources. Assessments of genetic diversity of abundant tree species in Afromontane habitat may be vital to ensuring their resilience (Aguilar *et al.* 2008). Throughout this thesis, I have presented evidence to support the growing notion that common species are capable of significant losses of genetic diversity following habitat fragmentation. This may have important ecological implications, not only for the productivity and persistence of populations of these species, but also for community and ecosystem level processes (Hughes *et al.* 2008). Conserving genetic diversity of these common species may be vital to the conservation of habitats and the success of ecological restoration endeavors. There are also major evolutionary implications, as depauperate populations may not possess the variability to adapt to future environmental change. Therefore, maintaining genetic diversity of common tree species may be vital for the maintenance of global biodiversity. Afromontane forests at among the most highly threatened ecosystems in the world, yet they have high biodiversity, economic, and social value. Evidence for erosion of genetic diversity in small forest fragments of the Mambilla Plateau promotes the need to incorporate genetic studies into the conservation management of Afromontane habitats in order to ensure enduring protection of these forests for future generations.

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