GENETIC CONSIDERATIONS AND MOLECULAR TOOLS FOR FOREST CONSERVATION AND RESTORATION: EMPHASIZING *Afrocarpus gracilior* (Pilg.) C. N. Page IN SOUTHERN ETHIOPIA

PhD DISSERTATION

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HAWASSA UNIVERSITY, HAWASSA, ETHIOPIA

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A DISSERTATION SUBMITTED TO THE SCHOOL OF PLANT AND HORTICULTURAL SCIENCE, COLLEGE OF AGRICULTURE, SCHOOL OF GRADUATE STUDIES
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We, the undersigned, members of the Board of Examiners of the final open defense by **Nigussu Begashaw Abate**, have read and evaluated his PhD thesis entitled "**Genetic Considerations and Molecular Tools for Forest Conservation and Restoration: Emphasizing *Afrocarpus gracilior* (Pilg.) C. N. Page in Southern Ethiopia**", and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy (PhD) in Plant Biotechnology.

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DECLARATION

I hereby declare that this PhD dissertation is my original work and has not been presented for a degree in any other university, and all sources of materials used for this dissertation have been duly acknowledged.

Name: Nigussu Begashaw Abate
Signature: _____
Date: _____

DEDICATION

This dissertation is dedicated to my beloved parents, Begashaw Abate and Belay Asfaw, whose unwavering support and sacrifices ensured I had access to the light of education amidst the challenges of rural Ethiopia, where illiteracy could have been my fate.

BIOGRAPHICAL SKETCH

Nigussu Begashaw Abate was born in Haik, South Wollo, Ethiopia. He attended Kulumbi Elementary and Haik Secondary schools for his primary and secondary education, respectively. He began his higher education at Mekelle University, where he earned a BSc degree in Land Resources Management and Environmental Protection in 2005. Following graduation, he was employed at Mekelle University, serving as a Graduate Assistant and Assistant Lecturer for three years.

In 2008, Nigussu was awarded an Erasmus Mundus scholarship, allowing him to pursue MSc studies in Europe. By 2010, he had earned a joint Master's degree in Sustainable Forest and Nature Management from the University of Copenhagen in Denmark and Bangor University in the UK. Upon returning to Ethiopia, he took on lecturer roles at Dilla University and Hawassa University, where he taught courses and conducted research in Forestry, Agroforestry, and Natural Resources Management. Throughout his career, Nigussu also participated in various academic programs and short-term trainings at other international institutions, including the University of Padova (Italy), Kwame Nkrumah University of Science and Technology (Ghana), and the Norwegian University of Life Sciences.

In 2018, he embarked on a PhD study in Plant Biotechnology at the School of Plant and Horticultural Science, Hawassa University, an endeavor that culminated in a successful public defense of his dissertation on April 3, 2025.

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ABBREVIATION AND ACRONYMS

AFLP	Amplified Fragment Length Polymorphism
AFR100	African Forest Landscape Restoration Initiative
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
A_R	Allelic Richness
BIC	Bayesian Information Criterion
BSA	Bulked Segregant Analysis
CLD	Compact Letter Display
CRD	Completely Randomized Design
CTAB	Cetyltrimethylammonium bromide
DAPC	Discriminant Analysis of Principal Components
DArTseq	Diversity Arrays Technology sequencing
DNA	Deoxyribonucleic acid
EEFRI	Ethiopian Environment and Forest Research Institute
EFD	Ethiopian Forestry Development
ENA	Ethiopian News Agency
FAO	Food and Agriculture Organization of the United Nations
FDRE	Federal Democratic Republic of Ethiopia
F_{IS}	Inbreeding Coefficient
F_{ST}	Fixation Index
GBS	Genotyping-by-Sequencing
H_E	Expected Heterozygosity
H_O	Observed Heterozygosity
HSD	Honestly Significant Difference
HWE	Hardy-Weinberg Equilibrium
ICRAF	International Centre for Research in Agroforestry
ISSR	Inter Simple Sequence Repeat
iTOL	Interactive Tree Of Life
ITS	Internal Transcribed Spacer
IUCN	International Union for Conservation of Nature
LD	Linkage Disequilibrium
MAF	Minor Allele Frequency
MEFCC	Ministry of Environment, Forest and Climate Change
MLG	Multilocus Genotypes

N_A	Number of Alleles
NGS	Next-Generation Sequencing
N_m	Effective Number of Migrants
N_{PA}	Number of Private Alleles
PATSPO	Provision of Adequate Tree Seed Portfolios in Ethiopia
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PIC	Polymorphic Information Content
PPL	Percentage of Polymorphic Loci
RADseq	Restriction-site Associated DNA sequencing
RAPD	Random Amplified Polymorphic DNA
REDD+	Reducing Emissions from Deforestation and Forest Degradation plus
RFLP	Restriction Fragment Length Polymorphism
SCAR	Sequence Characterized Amplified Region
SI	Self-Incompatibility
SNNPR	Southern Nations, Nationalities, and Peoples' Region
SNPs	Single Nucleotide Polymorphisms
SSR	Simple Sequence Repeats, also known as Microsatellites
Ts/Tv	Ratio of Transitions to Transversions
uH_E	Unbiased Expected Heterozygosity
UN	United Nations
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

ARTICLES/MANUSCRIPTS

Chapter II

Genetic Considerations for Species Choice and Seed Procurement in Ethiopia's Forest Restoration Initiatives

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Chapter III

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Chapter IV

Genetic Consequences of Forest Fragmentation: Inbreeding Depression Revealed Through Reduced Progeny Fitness in *Afrocarpus gracilior*

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Chapter V

Identification of a Sex-Linked RAPD Marker in the Dioecious Conifer *Afrocarpus gracilior* for Early-Stage Sex Determination

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Large-scale tree planting initiatives and ambitious global forest restoration commitments aim to mitigate the impacts of deforestation. Ensuring the long-term success of these efforts requires integrating genetic principles into restoration practices. This study aims to enhance forest conservation and restoration by generating molecular genetic insights and tools, using *Afrocarpus gracilior*, a native dioecious conifer, as a case study. Specifically, it evaluates the extent to which genetic principles are considered in species selection and seed procurement, investigates the genetic consequences of population fragmentation, assesses inbreeding depression, and develops molecular markers for early sex identification. To achieve these objectives, the study combined a survey of tree nurseries and seed vendors with genetic analyses using DArTseq-generated SNPs to assess genetic diversity and population differentiation. Additionally, *in vitro* germination and early progeny growth experiments were conducted to evaluate inbreeding depression, while RAPD markers and bulk-segregant analysis were used for sex identification.

Assessment of genetic considerations in forest restoration revealed that crucial guidelines are frequently overlooked in species selection and seed procurement, with exotic species dominating while native species remain underrepresented. Seed collection practices often neglect essential measures for preserving genetic diversity, heightening the risk of inbreeding and reducing adaptive potential. Notably, 84% of seed collectors sourced seeds indiscriminately, 87% of nurseries received seeds without passport data, 97% of seed collectors failed to meet the minimum required number of mother trees per collection event, and 88% ignored recommended spacing between selected mother trees. Genetic diversity analysis of *A. gracilior* populations showed overall low genetic diversity ($H_e < 0.1$), with progeny cohorts exhibiting even lower diversity than adults. Progeny from isolated or few mother trees had the lowest genetic diversity, indicating heightened genetic drift and inbreeding. In contrast, sacred forests and larger remnant patches harbored relatively higher genetic diversity, indicating their importance for *in situ* conservation. Further assessment of inbreeding depression in fragmented populations revealed significant reductions in progeny fitness, including 53% lower germination rates, 33% reduced acclimatization, 30% and 41% slower growth in diameter and height, respectively, and a 62% increase in leaf scorch. Screening for sex-linked markers identified OPD-18 (5'-GAGAGCCAAC-3') as a 600 bp male-specific RAPD marker, providing a foundation for early sex determination in *A. gracilior*.

The results highlight the critical role of genetic considerations in successful forest restoration, yet current practices often overlook these principles, increasing the risk of inbreeding and loss of adaptive potential. Very low genetic diversity was recorded across all fragmented populations of *A. gracilior*, with progeny from isolated or few mother trees exhibiting the lowest genetic diversity and significantly reduced fitness. These findings indicate increased genetic erosion, drift, and inbreeding depression, jeopardizing the species' long-term survival. Recommendations include establishing and enforcing policies that promote the use of native species and genetic standards in seed procurement; sourcing seeds from diverse, larger populations; prioritizing *in situ* conservation of sacred sites; and further developing robust molecular markers to improve the reliability of early-stage sex identification in the dioecious *A. gracilior*. Implementing these measures would enhance the long-term success of restoration initiatives, fostering resilient forest ecosystems that support biodiversity conservation and sustainable land management.

Key words: dioecy, forest restoration, fragmentation, gene flow, genetic differentiation, genetic diversity, inbreeding, progeny fitness, seed procurement, sex-linked marker

CHAPTER ONE

1. General Introduction

1.1. Background Information

1.1.1. Deforestation and Forest Fragmentation

Deforestation has profoundly altered landscapes worldwide, diminishing biodiversity, disrupting ecosystem functions, and threatening essential services such as climate regulation and water cycling. Although human-driven forest loss has occurred throughout the Holocene (i.e., the current geological epoch beginning approximately 11,700 years ago), recent decades have seen a shift from small-scale subsistence farming to large-scale industrial activities, including commercial agriculture, logging, mining, and infrastructure expansion (Laurance & Useche, 2009; Bhagwat, 2014). Globalization has further accelerated these processes, with rising demand for commodities driving land conversion, particularly in tropical regions where deforestation rates remain high (Laurance, 2014). While some temperate and boreal regions have seen forest recovery through afforestation and natural regeneration, these secondary forests often lack the structural complexity and ecological functions of old-growth forests (Lindenmayer et al., 2000, 2012; Laurance, 2014). Despite a decline in the annual rate of net forest loss—from 7.8 million hectares in the 1990s to 4.7 million hectares in the 2010s—global forest cover continues to shrink, with a net loss of 178 million hectares since 1990 (FAO, 2020).

Discussions of deforestation often focus solely on habitat loss. However, remaining forest areas are increasingly degraded and fragmented (Laurance, 2014). Forest fragmentation occurs when once-continuous forests are reduced to smaller, isolated patches surrounded by non-forest land (Young & Boyle, 2000; Schlaepfer et al., 2018). This process intensifies edge effects, disrupts species movement, and increases vulnerability to human disturbances such as overhunting, fire, and selective logging (Aguilar et al., 2019; Mengist et al., 2022). Fragmented landscapes often experience further degradation, compounding biodiversity loss and ecological instability (Brook et al., 2008; Laurance & Useche, 2009). Addressing deforestation and its consequences requires

integrated conservation strategies that prioritize habitat preservation and ecological restoration to maintain biodiversity and sustain ecosystem services.

1.1.2. Genetic Consequences of Forest Fragmentation

Forest fragmentation alters ecological processes and plant-animal interactions, influencing the demography and genetic composition of plant populations through reduced gene flow, increased inbreeding, and genetic drift (Schlaepfer et al., 2018; Aguilar et al., 2019). Over the past two decades, molecular marker studies have investigated the genetic consequences of fragmentation, revealing mixed outcomes (Lowe et al., 2005; Kramer et al., 2008; Finger et al., 2014; Lowe et al., 2015; Vinson et al., 2018). While population genetics theory predicts that fragmentation leads to reduced genetic diversity, increased genetic structure, and heightened inbreeding, early reviews found that these patterns were not universally observed, a phenomenon termed the "paradox of forest fragmentation genetics" (Kramer et al., 2008). This led to the notion that trees, due to their longevity, flexible mating systems, and extensive gene flow, might be resilient to fragmentation (Lowe et al., 2015; Vinson et al., 2018). However, other studies suggest that susceptibility to genetic erosion is highly species- and context-dependent, influenced by life history traits, pollinator mobility, dispersal mechanisms, and time since fragmentation (Bacles and Jump, 2011; Schlaepfer et al., 2018; Aguilar et al., 2019).

The primary genetic consequences of fragmentation in vulnerable tree species include restricted gene flow, increased genetic structure, reduced genetic diversity, and lower progeny fitness (Cascante et al., 2002; Dubreuil et al., 2010; Ismail et al., 2014; Rymer et al., 2015; Duminil et al., 2016; Qin et al., 2021). Small, isolated populations are particularly prone to inbreeding, genetic drift, and the fixation of deleterious alleles, which can lower seed viability, germination rates, and adaptive potential (Lowe et al., 2005; Finger et al., 2014; Ghazoul, 2015). While inbreeding depression and genetic erosion present long-term risks, their severity varies across species. Some species are buffered from genetic decline due to their dispersal capacity or ability to persist in small patches, whereas others—particularly outcrossing species—are more vulnerable to fragmentation-driven genetic deterioration (Finger et al., 2012, Aguilar et al., 2019). Given these complexities, identifying species-specific traits that confer vulnerability or resilience to the genetic consequences of fragmentation is critical for developing nuanced conservation and management strategies.

1.1.3. Genetic Concerns in Forest Restoration

The global momentum for forest restoration has grown significantly, driven by international commitments such as the Bonn Challenge, the African Forest Landscape Restoration Initiative (AFR100), and the UN Decade on Ecosystem Restoration (2021–2030) (Stanturf et al., 2019). These initiatives aim to restore degraded landscapes, enhance biodiversity, and mitigate climate change through large-scale tree planting efforts (Brancalion & Holl, 2020). Two billion hectares of land is estimated to benefit from restoration initiatives worldwide (Bozzano et al., 2014). However, the success of such programs depends not only on the number of trees planted but also on the selection of appropriate species and genetically diverse seed sources, which are crucial for long-term ecosystem resilience and adaptability (Broadhurst et al., 2008; Boshier et al., 2015). Failure to integrate genetic considerations in restoration planning can lead to maladaptation, reduced ecosystem services, and increased vulnerability to environmental stressors (Bozzano et al., 2014).

Despite these ambitious efforts, many restoration projects rely heavily on exotic species or genetically poor germplasm, often sourced from narrow or non-local populations (Thomas et al., 2014). The use of non-native trees can disrupt ecosystem dynamics, outcompete native flora, and fail to support local fauna (Bozzano et al., 2014). Furthermore, seed sources with low genetic diversity may result in reduced adaptability, inbreeding depression, and poor long-term survival (Jalonen et al., 2018). Such genetic bottlenecks can compromise restoration success, highlighting the urgent need for science-based seed sourcing strategies that prioritize locally adapted and genetically diverse planting material (Bozzano et al., 2014; Boshier et al., 2015). Addressing these challenges is essential to ensure that afforestation and reforestation initiatives contribute meaningfully to biodiversity conservation, climate resilience, and sustainable ecosystem restoration.

1.1.4. Dioecy and Its Implications for Forest Conservation and Restoration

Dioecy, the presence of separate male and female individuals within a species, is relatively uncommon among flowering plants, accounting for approximately 6% of angiosperms (Charlesworth, 2016; He & Hörandl, 2022). In gymnosperms, however, dioecy is more prevalent, occurring in about 64.6% of species (Walas et al., 2018). A significant challenge in the conservation and restoration of dioecious species is the difficulty in distinguishing male and female

plants during their juvenile stages, as they often lack distinct morphological differences until they reach reproductive maturity (Heikrujam et al., 2015; Razumova et al., 2023). This limitation is particularly critical in long-lived tree species, where reproductive maturity may take years or even decades to manifest, complicating conservation, restoration, and commercial breeding efforts. The delay in sexual differentiation can impede effective management strategies, as the sex ratio within a population plays a crucial role in its reproductive success and genetic diversity.

Identifying the sex of plants at early developmental stages is vital for several reasons. In conservation efforts, understanding the sex ratio is essential for assessing population viability, especially since male and female plants may exhibit different sensitivities to environmental stressors, leading to skewed sex ratios and potential population decline (Munné-Bosch, 2015; Hultine et al., 2016; Emprin et al., 2024). In forestry and agriculture, sex determination is also economically important, as yield and productivity in species like date palm (*Phoenix dactylifera*), sea buckthorn (*Hippophae rhamnoides*), and poplar (*Populus spp*.) are often sex-dependent (Ainsworth, 2000; Kresten et al., 2017; Heikrujam et al., 2015). In restoration projects, ensuring a balanced sex ratio is critical for successful reproduction and long-term sustainability of plantings. Given that cytogenetic sex markers are rare in plants, molecular tools provide a promising avenue for early and precise sex identification, offering practical applications for conservation, breeding programs, and sustainable forest management (Heikrujam et al., 2015; Keresten et al., 2017; Leite Montalvão et al., 2021; Razumova et al., 2023).

1.1.5. Molecular Markers for Forest Conservation and Restoration

Molecular markers have become powerful tools for assessing genetic diversity, population structure, and gene flow in forest ecosystems, aiding conservation and restoration efforts (Porth & El-Kassaby, 2014; Vinson et al., 2018). A molecular marker, also known as a genetic marker, is a DNA fragment associated with a specific location in the genome. These markers serve as genetic flags, enabling the identification of genetic variation linked to particular traits or loci (Nadeem et al., 2018). Various molecular marker systems have been developed, each with distinct advantages and limitations.

The most commonly used marker types include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs or

microsatellites), and single nucleotide polymorphisms (SNPs) (see reviews by Porth & El-Kassaby, 2014; Allendorf, 2017; Vinson et al., 2018). Among these, SSRs and SNPs are widely used in population genetic studies due to their high resolution, codominant inheritance, and genome-wide coverage. Recent advances in next-generation sequencing (NGS) technologies have enabled genome-wide SNP discovery without the need for prior genomic information or a reference genome. Genotyping by sequencing (GBS) approaches such as RADseq (Davey et al., 2011; Andrews et al., 2016) and DArTseq (Kilian et al., 2012; Abate et al., 2024) use restriction enzymes to reduce genome complexity, sequencing a representative subset of the genome. These technologies have improved the efficiency and cost-effectiveness of molecular markers, expanding their applications to non-model species such as trees (Andrews et al., 2016; Vinson et al., 2018). These advances have broadened the scope of molecular markers in conservation genetics, particularly for forest ecosystems.

When applied in forest conservation and restoration, molecular markers provide detailed insights into genetic diversity, connectivity among populations, and the effects of human activities such as logging and habitat fragmentation. They help identify genetically distinct populations in need of conservation, optimize seed sourcing for reforestation, assess hybridization between species, and evaluate inbreeding levels that may compromise population viability (Muchugi et al., 2008; Vinson et al., 2018). Additionally, molecular markers aid in identifying forest refugia—areas that have historically served as genetic reservoirs during climatic fluctuations—ensuring the preservation of diverse genetic lineages for future adaptation and resilience (Muchugi et al., 2008). Integrating molecular markers into forest conservation and restoration initiatives strengthens the genetic resilience of tree populations, supporting sustainable management and adaptation to environmental changes. As sequencing technologies continue to advance, molecular markers will remain essential tools for safeguarding the genetic integrity of forest ecosystems worldwide.

Molecular studies on forest populations in Ethiopia remain scant, with research conducted on only a few species. Sertse et al. (2011) used AFLP markers to assess the genetic diversity and population structure of *Juniperus procera*, revealing extensive gene flow among populations. Similarly, Derero et al. (2011) examined 22 populations of *Cordia africana* using AFLP markers and found high genetic diversity, likely maintained through efficient pollen and seed dispersal. Microsatellite markers have also been employed in a few other studies. Yineger et al. (2014) used

SSR markers to analyze *Prunus africana* populations in fragmented forest patches in Northwest Ethiopia and reported significant genetic structuring. Addisalem et al. (2016) investigated *Boswellia papyrifera* and found that substantial genetic variation persists even in highly degraded remnant populations. Kassa et al. (2017) explored genetic diversity and gene flow in *Olea europaea* subsp. *cuspidata* across church forests of varying sizes and found that larger, intact forests harbored higher genetic diversity compared to smaller, fragmented ones. These studies demonstrate the value of molecular markers in guiding forest management and conservation efforts. Expanding such research to other indigenous tree species in Ethiopia is crucial for understanding their genetic resilience or susceptibility amid severe deforestation and habitat fragmentation.

1.2. Rationale of the Study

The age-old deforestation in Ethiopia has led to the fragmentation of its natural forests, reducing them to small, isolated patches on steep hills and inaccessible valleys (Dessie & Kleman, 2007; Wassie et al., 2010; Teketay, 2011; Yineger et al., 2014; Mengist et al., 2022). Such fragmentation exposes tree populations to genetic consequences, including reduced genetic diversity, inbreeding, and compromised reproductive success (see Section 1.1.2). Among the indigenous trees facing these threats is *Afrocarpus gracilior* (see Section 1.3 for species description). As one of the most unwisely exploited trees in Ethiopia, *A. gracilior* was heavily logged for its high-quality timber, with records indicating that it accounted for approximately 60% of felled trees in the 1940s (Negash, 2010). Overexploitation, particularly the selective extraction of mature mother trees, has left only heavily depleted remnant populations. Today, *A. gracilior* persists mainly in relic forest patches (Negash, 2003, 2010; Teketay., 2011) and as 'refugees' in church forests (Aerts et al., 2016: Sahle et al. 2021) and other culturally sacred sites (Doffana, 2014). However, the remaining individuals no longer exhibit the towering stature described in historical records, raising concerns about their genetic and reproductive viability (Negash, 2010).

The genetic consequences of forest fragmentation have profound implications not only for the long-term survival of *A. gracilior* but also for its artificial regeneration in nurseries. When seeds are collected from isolated trees or small, fragmented populations, they are more likely to be inbred, leading to poor seedling performance and reduced fitness in reforestation efforts. This issue is especially a concern with *A. gracilior*, a dioecious and wind-pollinated species, as its obligate

outcrossing nature makes it highly dependent on the proximity of male and female trees to achieve successful pollination. Fragmented populations may suffer from inefficient pollen transfer, resulting in unpollinated female cones that produce empty seeds (Negash, 2010; Orwa, 2009). This could explain the species' historically poor germination success in nurseries (Negash, 2010; Getachew, 2011).

Despite these critical concerns, no molecular marker-based studies on *A. gracilior*—including assessments of genetic diversity, population structure, or molecular sex determination—have been conducted in Ethiopia or elsewhere. Available DNA sequence data for the species consists primarily of short ribosomal and chloroplast DNA sequences, including the Internal Transcribed Spacer 2 (ITS2) (Barker et al., 2004; Biffin et al., 2011), the trnL-trnF intergenic spacer (Biffin et al., 2011), the RuBisCO large subunit (rbcL), and the NEEDLY intron 2 (Knopf, 2012). These sequences, along with the complete chloroplast genome sequence (Sudianto et al., 2019), have been used to refine genus-level phylogenetic relationships within Podocarpaceae, supporting the taxonomic reclassification of the species from *Podocarpus* to *Afrocarpus*. However, while these sequences aid broader phylogenetic studies, they are inadequate for intraspecific genetic diversity research. Their limitations stem from their short length, insufficient genome-wide coverage, highly conserved nature, slower evolutionary rate, or maternal inheritance, which restricts variability detection within species. Moreover, phenotypic studies have yet to evaluate how habitat fragmentation and small population size affect key reproductive traits, including seed viability, germination rates, and progeny performance. As a result, the extent to which *A. gracilior* populations exhibit the expected genetic consequences of fragmentation remains unknown.

This study aims to fill this critical knowledge gap by integrating molecular and phenotypic approaches to assess the genetic diversity, reproductive success, and early progeny vigor of *A. gracilior*. A molecular marker-based investigation could reveal whether some fragmented populations still harbor sufficient genetic diversity, while others have become genetically impoverished. The phenotypic assessment of seed production, viability, and early seedling growth could complement the genetic analysis, providing insights into how fragmentation affects the species' reproductive fitness and regeneration potential. Together, these findings would help identify genetically viable *A. gracilior* populations, which may serve as priority conservation targets and reliable seed sources for restoration efforts.

This research is particularly timely given Ethiopia's ongoing large-scale tree-planting initiatives, including the 'Green Legacy' campaign, which aims to restore degraded landscapes. While these efforts have primarily focused on fast-growing exotic species, ensuring the successful propagation of valuable native species like *A. gracilior* is vital for ecological restoration. Moreover, as a dioecious species, *A. gracilior* presents an additional challenge: male and female trees cannot be distinguished at the seedling stage, and phenotypic identification is only possible once trees reach reproductive maturity (Negash, 2010). This poses a concern because artificially established populations may fail to reproduce naturally if only same-sex seedlings are planted together by chance. Developing a molecular sex identification method would provide a practical tool for ensuring balanced sex ratios in planted populations, significantly enhancing the species' long-term conservation and restoration success.

By addressing these pressing issues, this study would contribute valuable scientific knowledge and practical guidelines for the conservation, sustainable management, and restoration of *A. gracilior*, an ecologically and economically important species now under serious threat.

1.3. The study species – *Afrocarpus gracilior* (Pilger) C. N. Page

1.3.1. Taxonomy

Afrocarpus gracilior (Pilger) C. N. Page (synonym: *Podocarpus gracilior* Pilger) is an elegant evergreen conifer in the family Podocarpaceae that once dominated the canopy of Ethiopia's Afromontane forests (Negash, 2003, 2010; Teketay, 2011). It is one of only two native conifer species in Ethiopia, along with *Juniperus procera* (Gebrehiwot et al., 2024). The species is known by various vernacular names, including East African yellow wood (English), Zigba (Amharic, Gurage), Birbirsa (Afaan Oromo), Chido (Kafanono), Elta (Afan Konso), Dagucho (Gumuz, Sidaamu Afoo), and Ziga (Wolaitta) (Teketay, 2011).

There has been long-standing taxonomic confusion surrounding *A. gracilior* due to historical classification under the genus *Podocarpus* (Page, 1989; Barker et al., 2004). As a result, much of the Ethiopian literature refers to the species as *Podocarpus* (e.g., Negash, 2003, 2010; Strobl et al., 2011; Teketay, 2011; Krepkowski et al., 2012; Tadele & Fetene, 2013). However, morphological and molecular evidence (Barker et al., 2004) supported elevating *Afrocarpus* to a distinct genus, as originally proposed by Page (1988). More recent phylogenetic studies using DNA sequences

(Knopf et al., 2012; Migliore et al., 2020; Khan et al., 2023) and transcriptome data (Chen et al., 2022) further confirmed *Afrocarpus* as a separate genus, more closely related to *Nageia* and *Retrophyllum* than to *Podocarpus*. Additionally, earlier cytological studies identified a key distinction in chromosome number—*Afrocarpus* (n = 12) versus African *Podocarpus* species (n = 11) (Page, 1988, 1990). Despite this taxonomic clarification, even recent publications (e.g., Asefa et al., 2020; Negash, 2021; Gebrehiwot et al., 2024) continue to use the outdated nomenclature and refer to *A. gracilior* as *Podocarpus falcatus*, a different South African species that itself has been reclassified as *Afrocarpus falcatus (*Barker et al., 2004*)*. Nonetheless, since *A. gracilior* is the only podocarp conifer found in Ethiopia (Farjon & Filer, 2013, pp 465-467), works discussing *Podocarpus* in the Ethiopian context are generally assumed to be referring to *Afrocarpus*, despite the misclassification.

1.3.2. Habitat and Geographic Distribution

A. gracilior is primarily found in Afromontane Forest regions where it is observed as a dominant species (e.g., in Podocarp Forest) or as one of the co-dominant species (e.g., in Juniper-Podocarp Forest) (Teketay, 2011). It predominantly occurs in the Dry Evergreen Afromontane Forest and grassland complex (DAF) but also extends into the zones of the Moist Evergreen Afromontane Forest (MAF) vegetation types of Ethiopia (Friis et al., 2010). Studies based on pollen and charcoal analysis of sediment cores (Bonnefille & Mohammed, 1994; Darbyshire et al., 2003) indicated that it used to be the dominant species in the ancient vegetation of Ethiopia, distributed throughout the Afromontane regions. Nowadays, it often persists in relic forest patches, such as along gullies or in church forests, and is frequently found as a single tree left in grasslands and farmlands when there is sufficient rainfall (Teketay, 2011). It commonly grows in the altitudinal range 1500 – 2800 m and annual rainfall 1000 – 2000 mm. According to the floristic regions used in the Flora of Ethiopia and Ertrea (Hedberg et al., 2009), its geographical distribution in Ethiopia is widespread, including Tigray (TU), Gondar (GD), Gojam (GJ), Wollo (WU), Shewa (SU), Arsi (AR), Ilubabor (IL), Keffa (KF), Gamo Gofa (GG), Wellega (WG), Sidama (SD), Bale (BA), and Harerghe (HR) regions (Demissew and Friis, 2009). Various authors (e.g., Sharew, 1997; Tesfaye et al., 2002; 2010; Negash, 2003; Teketay, 2011; Woldearegay & Bekele, 2020) reported its presence in Munesa-Shasemene, Gera-Ades, Arba-gugu, Dindin, Chilimo, Belete gera, Harenna, Dodola, Menagesha-suba, Wof-washa, Megada, and Tiro natural forests in central, southern, and eastern

parts of Ethiopia. Moreover, outside Ethiopia, its natural occurrence is reported in Burundi, Congo, Kenya, Rwanda, South Sudan, Tanzania, and Uganda (Farjon, 2013; Khan et al., 2023).

1.3.3. Botanical Description

Detailed botanical description of the species (in its inaccurate nomenclature *Podocarpus falcutus*) is found in Orwa et al. (2009), Negash (2010, pp 67-98), and Farjon (2017, pp 143-145). What is presented here is only a brief excerpt from these pieces of writing.

Figure 1.1. *Afrocarpus gracilior* in pictures. (A) The trunk of a tall, mature *A. gracilior* tree, which makes it a valuable timber species; (B) A large *A. gracilior* tree with a partial view of its crown;(C) Male cones of *A. gracilior*, showing spirally arranged sporophylls responsible for releasing pollen grains; (D) Female cones or "fruits" of *A. gracilior* with their conspicuous fleshy outer covering called the *epimatium*; the cones are green when unripe and turn yellowish upon ripening.

A. gracilior is a tall evergreen tree that may reach 45 meters in height in nature (Figure 1.1), with historical records indicating it could grow even taller (see section 1.3.6). The dense canopy, with its drooping branches, contributes to the tree's imposing stature. Its cylindrical bole features a thin, pale-grey to dark-grey bark that fissures both horizontally and longitudinally, peeling off in roughly square flakes. The leaves vary in arrangement, sometimes spiralling, while at other times forming two opposite or sub-opposite ranks. The petioles are linear to linear-oblong, narrowing

abruptly to a sharp or blunt apex and basally to a slightly twisted short stalk. The adult leaf is 3-5 x 0.3-0.5 cm; the midrib of the adult leaf is not prominent above but is well-marked beneath. Mature leaves are tougher and dark-green in color, while juvenile leaves are thinner, longer, softer and light green, marking a distinctively attractive bright-green flush to the tree's canopy.

A. gracilior is a dioecious species, i.e., the male and female trees are separate individuals. The male cones (male strobili) are axillary and usually occur in twos or threes. Their color is yellowish, which turns to brownish when mature. The male cones consist of spirally arranged sporophylls which, when blown by wind, release pollen and fertilize the ovules of the female cones (Figure 1.1C). The female cones/strobili (or the 'fruits') are spherical, green when not ripe, and become yellowish when ripe (Figure 1.1D). The cones/fruits consist of an epimatium—a fleshy outer covering—a tough sclerotesta, which forms the seed coat, and a relatively large female gametophyte (or a megagametophyte) enclosed within the brownish sclerotesta. Cones/fruits measure about 1.4 to 2.6 cm in length and about 1.2 to 2.0 cm in diameter.

1.3.4. Reproductive Biology

A. gracilior is a dioecious species that is pollinated by wind. Inefficient pollination (when the male and female trees are not in proximity or due to the longer time it takes for the pollen to mature) commonly results in the development of unpollinated female cones that produce empty seeds (Orwa et al., 2009; Negash, 2010). Such fruits are termed *parthenocarp* fruits, in which fruits develop without having their ovules fertilized by pollen grains (Negash, 2010). Reduction of population density due to selective logging/deforestation is thus expected to increase the chance of getting more *parthenocarp* fruits. The species has seed masts (i.e., high seed production) at 2 – 4-year intervals (Orwa et al., 2009). Since the fleshy *epimatium* is edible, the seeds are dispersed by different types of animals such as birds, monkeys, and bats (Orwa et al., 2009; Teketay, 2011). A study on the phenology of the species at Munesa- Shashemene forest (Tesfaye et al., 2011) reported peak flowering and fruiting dates of 1 November and 1 January, respectively. The hard sclerotesta (the woody seed coat) hampers germination (Negash, 2010; Teketay, 2011). As a result, naturally, germination is likely to occur over a long period. However, since the seeds are intermediate/semi-recalcitrant (Tesfaye et al., 2016) and dormancy is absent, soil seed banks in the forest are lacking (Teketay, 2011). Artificial regeneration (like when seedlings need to be raised in

nurseries) requires breaking the sclerotesta with a mechanical scarification method (Negash, 2010; Teketay, 2011).

1.3.5. Uses

A. gracilior is a multipurpose tree valued both economically and ecologically. Its yellowish-white softwood timber is of superior quality with high demand in international markets (Negash 2003,2010; Teketay, 2011). Its wood is straight-grained, very fine, featureless, and non-resinous (Orwa et al., 2009). Its timber is widely used in construction, particularly for inside work such as floors, doors, wall paneling, carpentry and joinery, and furniture making (Orwa et al., 2009; Farjon, 2013). In certain localities in Ethiopia, edible oil is extracted from its seeds, which is also therapeutically used to treat gonorrhea (Teketay, 2011). In some cultures, such as the Sidama in Ethiopia, it is considered a sacred tree (Doffana, 2014). Phytochemicals that have cytotoxic effects (i.e., anticancer activity) have been extracted from its leaves (Stahlhut et al., 1998; Faiella et al., 2012; Kamal et al., 2019) that would potentially make the species a highly demanded medicinal tree. Its huge stature with evergreen dense canopy stabilizes land, protects soil erosion, and helps develop water springs (Negash 2003, 2010). It also supports biodiversity as many birds and primates feed on its fleshy seeds (Negash, 2003; Teketay, 2011).

1.3.6. Conservation Status

A. gracilior has long been a target of illegal logging due to its high-quality timber (Teketay, 2011). In the 1940s, it was the most commercially exploited tree species in Ethiopia, contributing to about 60% of all felled trees (Negash, 2010). Extensive logging has led to the selective extraction of the best, mature trees, severely depleting populations. Negash (2010) laments this loss, referencing Russ' (1944) description of *A. gracilior* as a giant tree, reaching heights of 50 meters with diameters of 3 meters, and his reports to the imperial government about vast podocarp forests in Arsi, areas near Lake Awassa, Sidamo (Megada and Jemjem), Wellega, and southwestern Ethiopia. Russ also noted that sawmills located in Chelenko, Chilalo, and Shashemene exclusively processed *Afrocarpus* timber, with tree depletion near Chelenko prompting recommendations to relocate the sawmill (Negash, 2010).

Today, *A. gracilior* persists mainly in relic forest patches (Negash, 2003, 2010; Teketay, 2011) and as 'refugees' in church forests (Aerts et al., 2016) and culturally protected sites (Doffana,

2014), though the remaining individuals no longer exhibit the towering stature described in historical records (Negash, 2010). Occasionally, reports still emerge of exceptionally large remnant trees, reminiscent of the historic grandeur. For example, Pankhurst (2000) describes a massive remnant tree named 'Awliyaw,' meaning it's considered sacred, with a diameter of about 4 meters and a height of about 65 meters in Anabe Forest, South Wollo. Similarly, large and old remnant trees exceeding 3 meters in diameter were observed in the present study at some sacred sites, known as *gudumales*, in Sidama (see Appendix 3.4).

Despite this declining status, the IUCN classifies *A. gracilior* as a species of 'Least Concern' (Farjon, 2013), citing its broad distribution, including areas outside Ethiopia. However, its primary habitat in Ethiopia lies in the Eastern Afromontane biodiversity hotspot—one of the world's 35 global biodiversity hotspots, where ecosystems are both species-rich and highly threatened (Myers et al., 2000). Given the intense pressure from selective logging and deforestation, *A. gracilior* is considered threatened in Ethiopia and is among the few native trees prioritized for conservation efforts in the country (Vivero et al., 2005; Kalinganire et al., 2021).

1.4. Objectives of the Study

1.4.1. General Objective

To support forest conservation and restoration efforts in Ethiopia and beyond by generating molecular genetic insights and tools for *Afrocarpus gracilior*, a native dioecious conifer.

1.4.2. Specific Objectives

1. To evaluate the extent to which genetic principles are integrated into tree species selection and seed procurement for forest restoration initiatives in Ethiopia.
2. To investigate the genetic consequences of population fragmentation in *A. gracilior* using *de novo* developed molecular markers.
3. To assess the extent of inbreeding depression in progeny derived from fragmented populations of *A. gracilior*.
4. To develop sex-linked molecular markers for early sex identification in the dioecious *A. gracilior*.

1.5. Organization of the Dissertation

This dissertation is organized in a manuscript format, where each specific objective is addressed in a separate published or publishable manuscript, presented as an individual chapter. The first chapter provides a general introduction, outlining the background, study rationale, and objectives. The final chapter presents a synthesis of findings, drawing overarching conclusions and recommendations from all preceding chapters. Chapters 2 through 5 correspond to the four specific objectives of the study, each structured as a stand-alone manuscript. While efforts have been made to minimize redundancy, some overlap in background information and references may occur.

Chapter 2 assesses the extent to which genetic principles are considered in species selection and seed procurement for forest restoration initiatives in Ethiopia. Based on surveys conducted in selected tree nurseries and among tree seed vendors, as well as secondary data from tree seed centers, this chapter discusses key challenges associated with germplasm sourcing in Ethiopian forestry.

Chapter 3 investigates the genetic consequences of population fragmentation in *Afrocarpus gracilior*. Using SNP markers generated via DArTseq—the first molecular markers developed for this species—this chapter examines genetic diversity, population structure, and differentiation among populations. The implications of these genetic insights for conservation and restoration strategies are discussed.

Chapter 4 builds on the findings of Chapter 3 by assessing whether genetic erosion observed at the molecular level translates into inbreeding depression in progeny. Seeds collected from the same genotyped populations were evaluated for physical seed traits, *in vitro* germination, seedling acclimatization, and early progeny growth under controlled conditions. The findings provide insights into seed sourcing strategies and their conservation and restoration planning implications.

Chapter 5 focuses on developing a sex-linked molecular marker for the dioecious *A. gracilior*, enabling early-stage sex identification. It discusses the challenges posed by dioecy in conservation and restoration efforts and the potential of molecular marker technologies to improve restoration planning and population management.

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CHAPTER TWO

2. Genetic Considerations for Species Choice and Seed Procurement in Ethiopia's Forest Restoration Initiatives

Abstract

Global commitments to large-scale tree planting and forest restoration are increasing to help restore degraded ecosystems. Ethiopia has pledged to restore 22 million hectares of degraded land, undertaking massive forest restoration campaigns under the umbrella of the "Green Legacy Initiative," with billions of tree seedlings reportedly planted annually. Genetic and ecological research underscores that using native tree species with high genetic diversity is essential for restoration success. This study assessed the consideration of genetic principles in species choice and seed procurement in Ethiopia's restoration initiatives. Data were gathered through surveys of seed vendors and nurseries, complemented by secondary data from tree seed centers within the national tree seed network. The findings indicated that genetic considerations in species choice and seed procurement were often overlooked in ongoing large-scale restoration practices. Species selection was mainly dominated by a few exotics—*Grevillea robusta, Eucalyptus camaldulensis, Acacia decurrens,* and *Cupressus lusitanica*—leaving native species underrepresented. Moreover, seed collection practices frequently disregard guidelines critical for preserving genetic diversity. Notably, 84% of seed collectors source from any available tree, 87% of nurseries received seeds without passport data, 97% of seed collectors did not consider a minimum number of mother trees for a single collection event, and 88% ignored the required distances between selected mother trees, risking inbred seed collection. These gaps threaten the evolutionary resilience and adaptive capacity of planted seedlings, impacting the long-term success of restoration efforts. To improve outcomes, the Ethiopian Forestry Development (EFD) and other relevant authorities leading the restoration initiatives should devise policies that promote native species and enforce genetic standards in seed procurement.

Key words: forest restoration, germplasm, genetic diversity, seed procurement, tree nurseries, tree seed

2.1. Introduction

Deforestation, land degradation, climate change, desertification, and biodiversity loss continue to pose significant challenges to ecosystems worldwide. One widely adopted strategy to mitigate these environmental crises is forest ecosystem restoration. This imperative has spurred large-scale tree planting initiatives and ambitious global reforestation commitments (Broadhurst et al., 2008; Thomas et al., 2014; Lamb, 2018; Fagan et al., 2020). Notable examples include the Green Wall of China, spanning 4,500 km and covering 35 million hectares since its inception in 1978, and the Great Green Wall of Africa, which aims to plant a 7,775 km-long tree belt across the Sahel. Initiatives such as the Bonn Challenge and the African Forest Landscape Restoration (AFR100) aim to restore 350 million hectares of degraded land globally, 100 million hectares of which are in Africa, by 2030 (Bozzano et al., 2014; Pistorius et al., 2017; Verdone and Seidl, 2017). To further inspire and accelerate these global restoration initiatives, the United Nations (UN) has designated 2021–2030 as the UN Decade on Ecosystem Restoration(https://www.decadeonrestoration.org/), followed by the World Economic Forum's 1 Trillion Trees Initiative in support of the UN's goals (Aronson et al., 2020). However, many reforestation projects around the globe have faced limited success, often due to mismatches between planting material and site conditions, poor genetic quality of planting stock, and inadequate management practices (Thomas et al., 2014; Méndez-Toribio et al., 2021).

In Ethiopia, deforestation has been a persistent challenge, dating back to 500 BC (Darbyshire et al., 2003) and re-intensifying since the 16th century (Pohjonen & Pukkala, 1990; Pankhurst, 1995). Natural forest cover continues to decline (Reusing, 2000; Dessie & Kleman, 2007; Demissie et al., 2017; Etefa et al., 2018), with recent losses confirmed by the FAO (2020). A rapidly growing population exceeding 120 million has intensified pressures on forests, driven by agricultural expansion and wood extraction for household energy (Stebek, 2008; Kindu et al., 2015), resulting in land degradation, erosion, biodiversity loss, and forest fragmentation (Kassa et al., 2017; Mengist et al., 2022). Efforts to combat deforestation began in the 1890s with the introduction of eucalyptus and expanded in the 1970s with large-scale plantations of fast-growing exotic species of Eucalyptus, Cupressus, and Pinus (Ayana et al., 2013; Lemenih & Kassa, 2014). Recent approaches, such as the "climate-resilient green economy strategy," aim to double forest cover by 2025 and cut greenhouse gas emissions by 50% by 2030 (FDRE, 2011; MEFCC, 2018). Ethiopia

has also pledged to restore 22 million hectares of degraded land through global programs like the Bonn Challenge and AFR100 (Pistorius et al., 2017; Kassa et al., 2022). Massive tree-planting campaigns, including the "Green Legacy Initiative," are central to these efforts, with billions of tree seedlings reportedly planted annually to restore degraded landscapes (Fikreyesus et al., 2022; Kassa et al., 2022).

While plantations of exotic species in Ethiopia have supported fuelwood and timber supplies, their role in ecosystem restoration remains questionable. Exotics often underperform in maintaining soil quality (Lemenih et al., 2004; Demessie et al., 2012), water-use efficiency (Gindaba et al., 2004; 2005), biodiversity (Abiyu et al., 2011), and pest resistance (Demeke, 2018), with some becoming invasive (Shiferaw et al., 2004, 2019). Whether exotic species still dominate large-scale planting efforts or if native species are now prioritized remains unclear. Restoration success also depends on the genetic quality of germplasm, crucial for resilient and self-sustaining populations capable of adapting to environmental challenges (Broadhurst et al., 2008; Thomas et al., 2014). The extent to which tree seed collections in Ethiopia follow established guidelines, such as those from the World Agroforestry Centre, ICRAF (Kindt et al., 2006; Lillesø et al., 2021), and the Royal Botanical Gardens, Kew (2003), recommending sampling from at least 30 trees spaced 50 – 100 meters apart to avoid inbreeding, is largely unstudied. A few available studies highlight gaps in the tree seed system and guidelines being overlooked, with collections often relying on limited mother trees or fragmented populations, leading to reduced genetic diversity (Dedefo et al., 2016; Mehari et al., 2024). As many tree species are naturally outbreeding and carry a genetic load of deleterious recessive alleles (Lowe et al., 2005; Broadhurst & Boshier, 2014), seed collection from isolated trees often results in inbred progeny with slower growth, higher susceptibility to stresses, and reduced resilience (White et al., 2007; Tata et al., 2023). This jeopardizes long-term sustainability as today's plantations often serve as future seed sources. Ensuring genetically diverse germplasm is thus essential for restoration initiatives to yield viable, self-sustaining, and resilient landscapes.

Formal evaluations of the large-scale tree planting campaigns are rare, yet anecdotal reports suggest that seedling survival rates are low, with some estimates indicating that less than 40% of seedlings survive beyond the initial establishment phase (ENA, 2019). A recent study in Tigrai, northern Ethiopia, reported a survival rate of only 53% for planted seedlings, with overall plantation success deemed unsatisfactory (Berhe et al., 2024). However, even these short-term

survival rates may not accurately predict long-term restoration outcomes. Effective landscape restoration requires that initial seedling establishment be accompanied by long-term indicators such as growth, maturation, and reproductive capacity (Le et al., 2011; Bozzano et al., 2014; Thomas et al., 2014). Ethiopia's 10-year national forest development program document (MEFCC, 2018) acknowledges that "… billions of seedlings are planted each year in the country but hardly grow to become a forest." While poor site conditions and inadequate silvicultural management are often blamed for these failures (MEFCC, 2018; Magaju et al., 2020), the role of genetic quality of the germplasm in planting success remains largely overlooked.

This study aims to assess the extent to which genetic considerations are factored into tree species selection and seed procurement for ongoing forest restoration initiatives in Ethiopia. The results will have substantial practical implications for restoration authorities and practitioners, informing future efforts to enhance the genetic quality of planting material and improve the long-term sustainability of restoration projects.

2.2. Materials and Methods

2.2.1. Data sources and types

Both primary and secondary data were utilized in this study (Table 2.1). Primary data were collected through a survey of 24 tree nurseries and 23 seed suppliers or vendors (Figure 2.1). The nurseries and vendors were identified in consultation with the former South Nations, Nationalities, and Peoples Region (SNNPR) Forest and Environment Bureau, the SNNPR Tree Seed Center, and the SNNPR REDD+ Coordination Office. At the time of sampling, the region had not yet been divided; however, it has since been split into four new regions, with the sampled districts now falling within three of them—Sidama, South Ethiopia, and Central Ethiopia. Initially, 14 active tree seed cooperatives, organized by the SNNPR Tree Seed Center and distributed across 11 districts (woredas), were identified and included in the survey.

Private seed vendors, as well as public tree nurseries operating in these districts, were also included in the survey. Since no formal registry of private seed vendors existed, a snowball sampling approach was used to identify them. Additionally, five tree nurseries managed by the regional REDD+ Coordination Office were incorporated into the survey.

Table 2.1. Survey entities used as data sources for this study

Survey entities	Data type	Source	No. of entries
National tree seed network			3
EEFRI	secondary	https://t.me/Ethiopian_TSN	
Dimma tree seed center	secondary	https://t.me/Ethiopian_TSN	
Bahirdar tree seed center	secondary	https://t.me/Ethiopian_TSN	
Nurseries			
Government/public	primary	own survey	17
NGO/project	primary	own survey	5
Private	primary	own survey	2
Seed suppliers/ vendors			
Cooperatives	primary	own survey	14
Private	primary	own survey	9

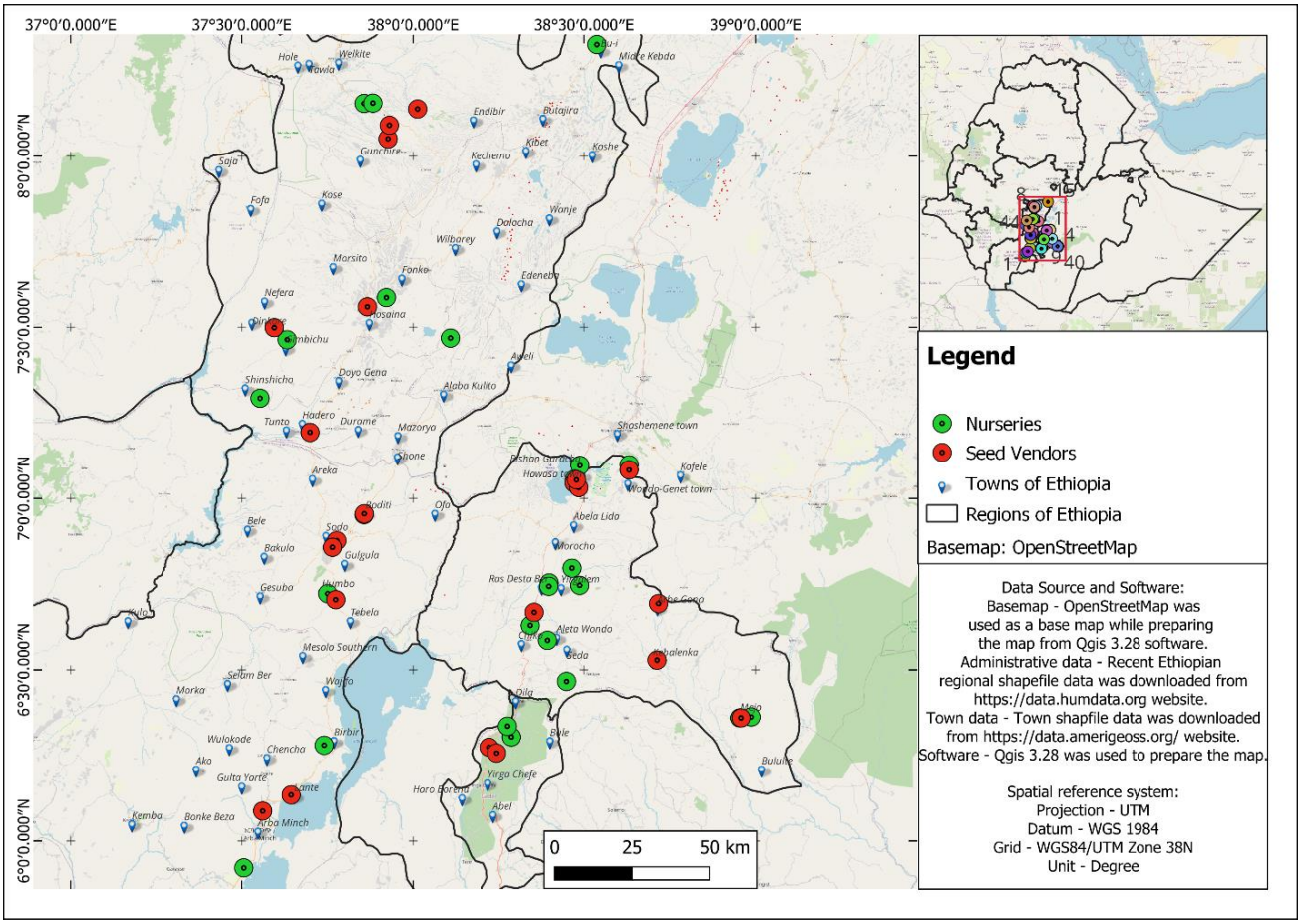

Figure 2.1. Map showing the locations of surveyed tree nurseries and seed vendors

Once the nurseries and seed vendors were identified, data were gathered through interviews with nursery foremen, cooperative leaders, and individual seed vendors using a semi-structured

questionnaire. Key informant interviews were also conducted with the heads of the regional Tree Seed Center and the REDD+ Coordination Office.

Secondary data were sourced from the Telegram page of the Ethiopian Tree Seed Network (https://t.me/Ethiopian_TSN). These data comprised monthly seed availability reports from three tree seed centers that regularly posted seed balance updates between January 2020 and August 2022. These centers were the Ethiopian Environment and Forest Research Institute (EEFRI) in Addis Ababa, the Dimma Tree Seed Center in Sebeta (Oromia Region), and the Bahir Dar Tree Seed Center (Amhara Region).

2.2.2. Data analysis

The survey data were coded and initially entered into MS Excel. After organizing the data, it was imported into the R statistical software for further analysis and visualization. Most analyses involved summarizing the data using the dplyr package (Wickham et al., 2023), followed by graphical visualization with the ggplot2 package (Wickham, 2016). Additional R packages employed included xlsx (Dragulescu & Arendt, 2020) for importing Excel files and patchwork (Pedersen, 2024) for combining multiple plots. The results were presented as tables and graphs.

2.3. Results

2.3.1. Composition and ranking of tree species distributed by vendors and raised in nurseries

Figure 2.2A presents the average seed quantity reported per species in a single monthly entry from the three seed centers. The data reveal that exotic species dominate the largest seed stocks, with the top six species being exotics. Among the top ten, only two native species—*Cordia africana* and *Olea africana*—are represented. *Afrocarpus gracilior* (*P. falcatus* in the figure), the focal species of this dissertation, ranks 33rd, placing it in the lower half of the distribution. A larger average seed quantity may reflect frequent restocking or a large initial acquisition that remains in stock without significant distribution.

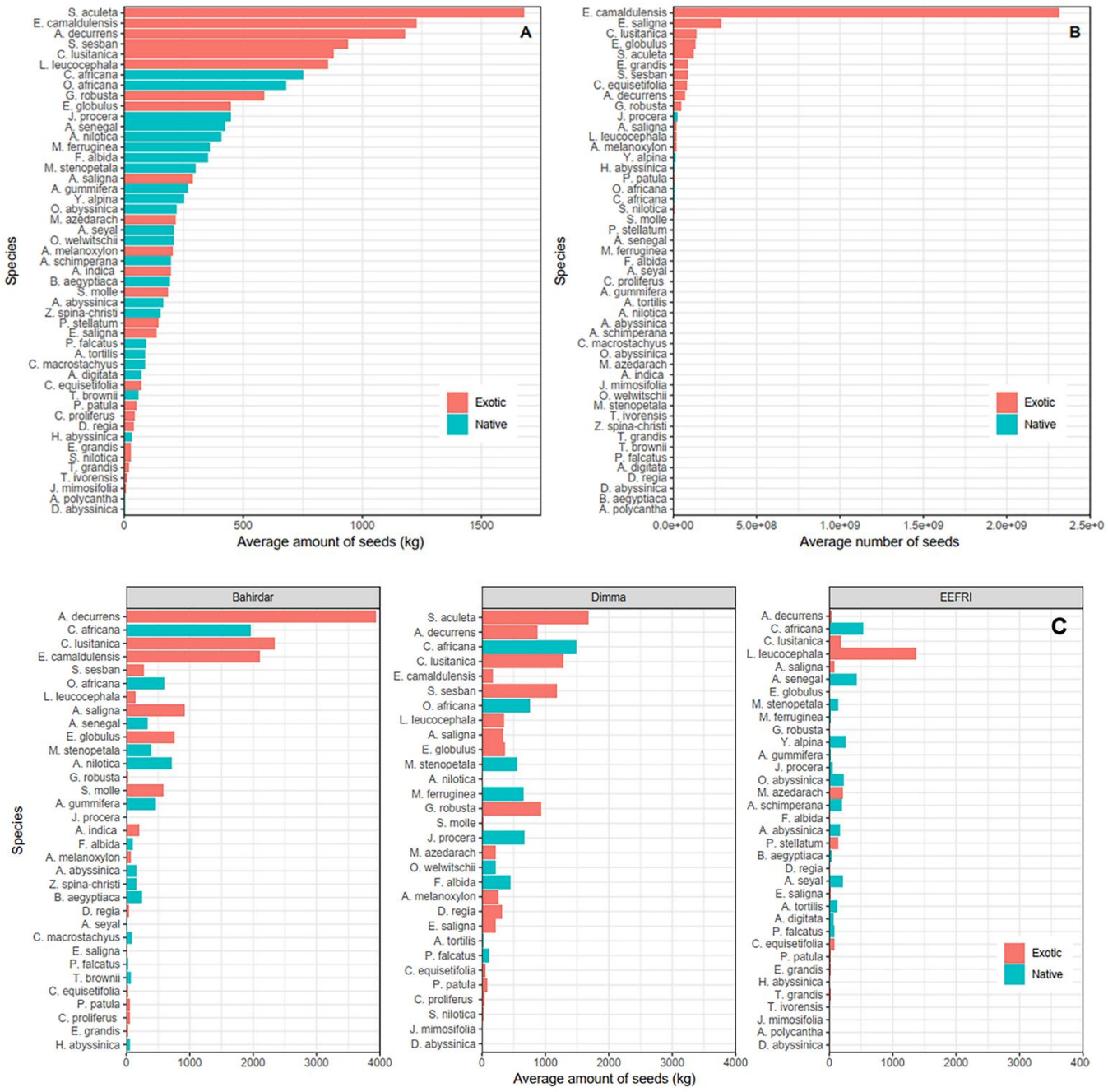

Figure 2.2. Tree seed availability per species reported by three centers of the national tree seed network of Ethiopia. A) Total amount of seed (in kg) for each species, averaged across all monthly reports.; B) Estimated average number of seeds per species, derived from the average seed amounts reported in A; C) Average seed amounts (in kg) reported by each center (Bahir Dar, Dimma, and EFFRI) for each species. Species are categorized as either exotic (shaded red) or native (shaded blue).

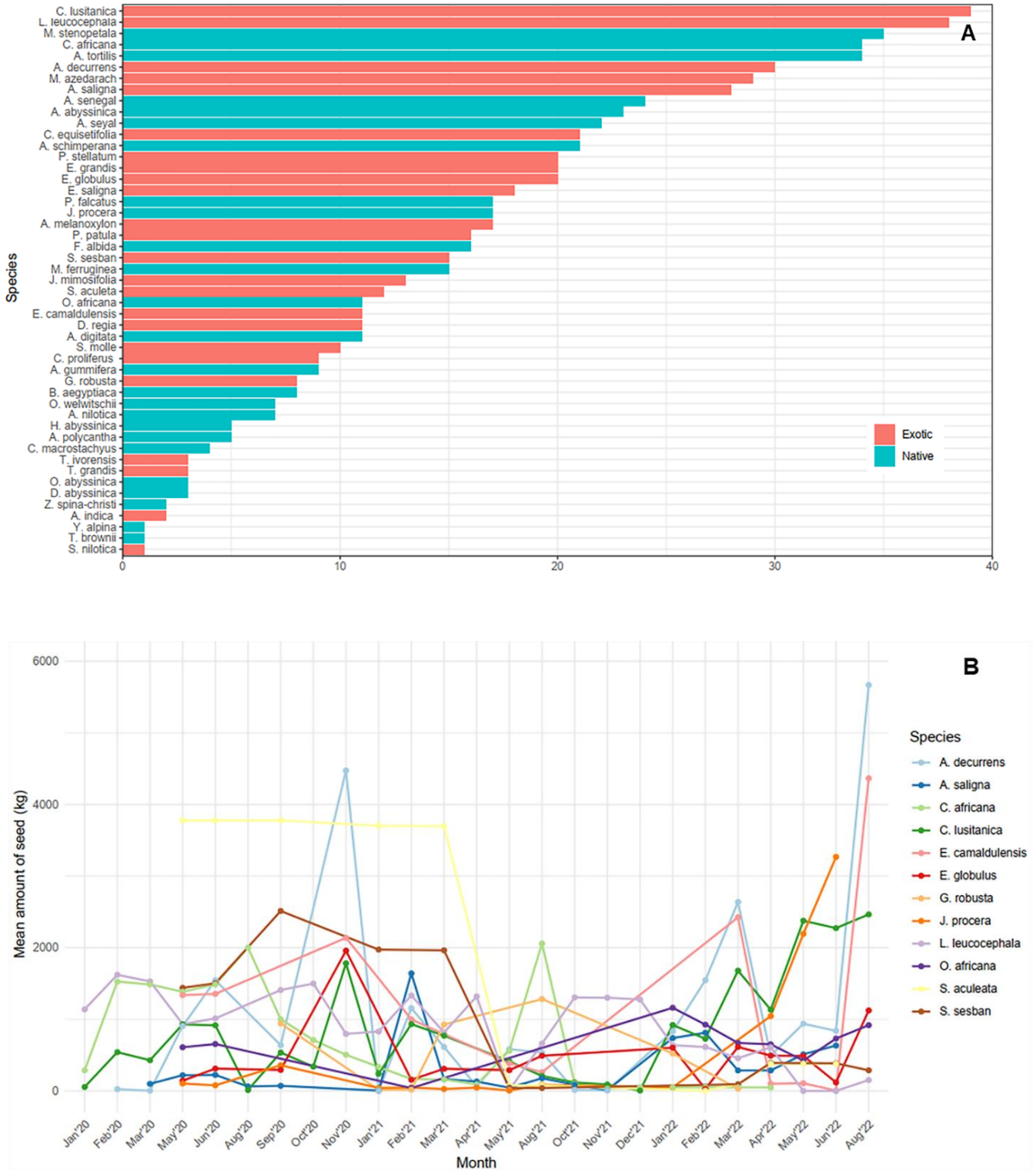

Figure 2.3. Commonality of Species in the Tree Seed Balance Report. A) The frequency of each species reported in the seed balance by the three seed centers; B) The trend of 12 common species reported over a 32-month period.

This dynamic is further explored in Figure 2.3, where Figure 2.3A shows the frequency with which species were reported as available, and Figure 3B tracks seed quantity trends over a 32-month period for the 12 most commonly reported species. *Sesbania aculeata*, for example, ranked highest in seed quantity (Figure 2.2A), but was not frequently reported, ranking 26th in frequency (Figure 3A). Figure 3B reveals that *S. aculeata* was acquired in bulk in May 2020, remained stable in stock until March 2021, and was fully distributed by May 2021, with no further significant reports thereafter. In contrast, species like *Acacia decurrens*, *Eucalyptus camaldulensis*, and *Cupressus lusitanica* exhibited periodic fluctuations, indicating more frequent acquisitions and distributions over the entire period.

In Figure 2.2B, seed availability is ranked by the estimated number of seeds per species, further highlighting the dominance of exotic species over native ones. The number of seeds, which develop into potential seedlings in nurseries, is disproportionately higher for exotics. This dominance is even more evident in Figure 2.2B, where native species are nearly invisible when plotted on the same scale as the exotics. The disparity can be attributed to the finer seeds of exotic species, which yield significantly more seeds per kilogram compared to native species. For instance, exotic species like *Eucalyptus camaldulensis* (1,887,507 seeds/kg), *Eucalyptus saligna* (2,101,282 seeds/kg), and *Cupressus lusitanica* (156,739 seeds/kg) far outnumber the native species *Cordia africana* (6,141 seeds/kg) and *Olea africana* (10,020 seeds/kg), despite these natives being reported in relatively higher quantities in Figure 2.2A.

Figure 2.2C breaks down the average amount of seed reported for each species by the three seed centers. Exotic species dominate in the Bahirdar and Dimma seed centers, while the Ethiopian Environment and Forest Research Institute (EEFRI) center reported a relatively higher presence of native species. The Bahirdar and Dimma centers also reported higher seed quantities for most of the species compared to EEFRI. Notably, *Acacia decurrens*, *Sesbania aculeata*, and *Leucaena leucocephala* have the highest seed quantities at Bahirdar, Dimma, and EEFRI, respectively.

Lastly, Figure 2.4 presents the species preference rankings based on primary survey data from tree nurseries and seed vendors in southern Ethiopia. *Grevillea robusta* ranked first in the volume of seeds and seedlings distributed, followed by *Cupressus lusitanica*, both of which are exotic species. Among the native species, *Cordia africana* and *Afrocarpus gracilior* were relatively preferred, but the gap between them and the exotics, particularly *G. robusta*, is substantial. Overall,

the results from both primary and secondary data sources indicate that exotic species dominate germplasm distribution for tree planting activities in Ethiopia.

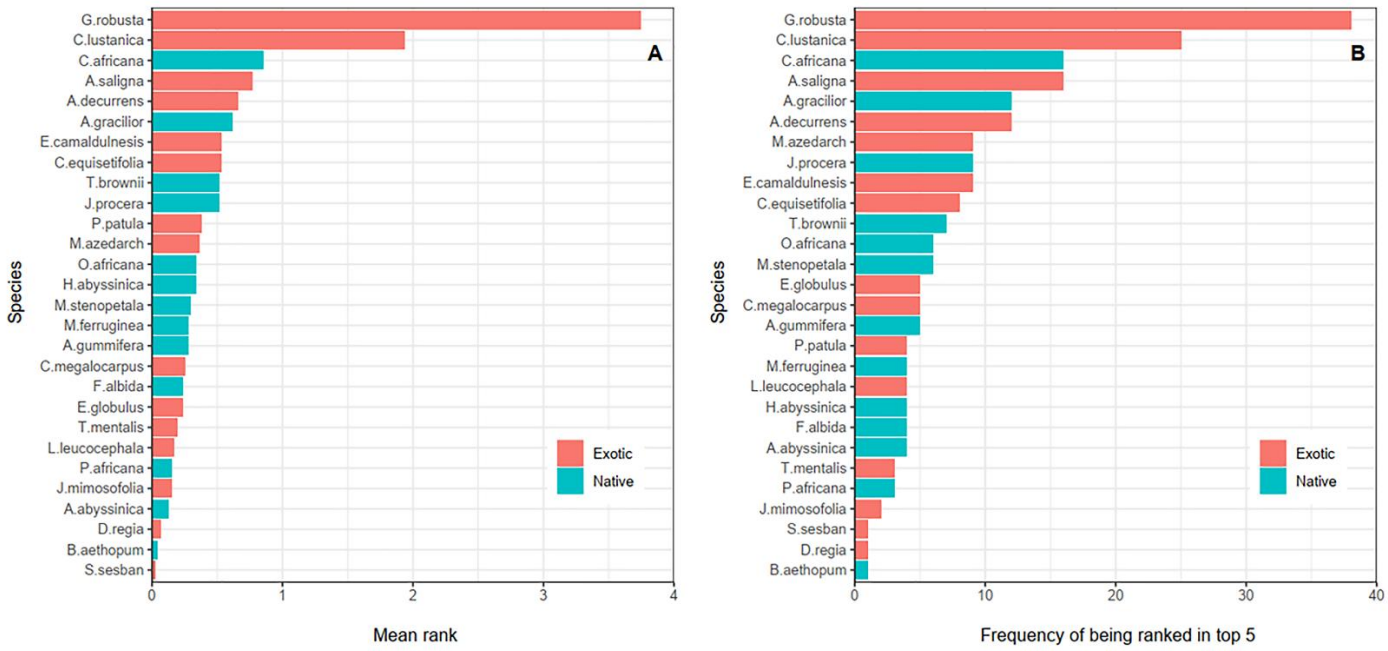

Figure 2.4. Rank of species preference based on surveyed nurseries and seed vendors. A) Mean rank (on a scale of 1 to 5, with 5 being the highest) for each species, with unranked species scored as 0 in the calculation.; B) The frequency of each species being ranked among the top 5.

2.3.2. Seed procurement practices by nurseries and seed vendors

Figures 2.5, 2.6 and 2.7 illustrate tree seed procurement practices of nurseries and seed vendors. Half of the nurseries surveyed fully outsource their seed requirements, while an additional 46% combine outsourcing with their own seed collection (Figure 2.5A). This means that 96% of the nurseries rely on some form of outsourced seed to meet their production needs. When outsourcing, 52% of nurseries acquire seed through an open bidding process, 22% purchase directly from local vendors, and another 22% use a combination of both methods (Figure 2.5B). Among the seed vendors, 65% are cooperatives whose members participate in seed collection, whereas 26% of vendors fully outsource their seed supply, purchasing seeds only after winning bids to provide nurseries (Figure 2.7B). Regarding seed populations, 84% of seed collectors utilize any available tree or population as a seed source, 10% focus exclusively on natural forests, and only 3% each collect seeds from designated seed production areas and plantations or provenances (Figure 2.6A).

Moreover, only 59% of seed collectors select mother trees based on phenotypic superiority, while the remaining 41% collect from any tree as long as it bears seeds (Figure 2.6B).

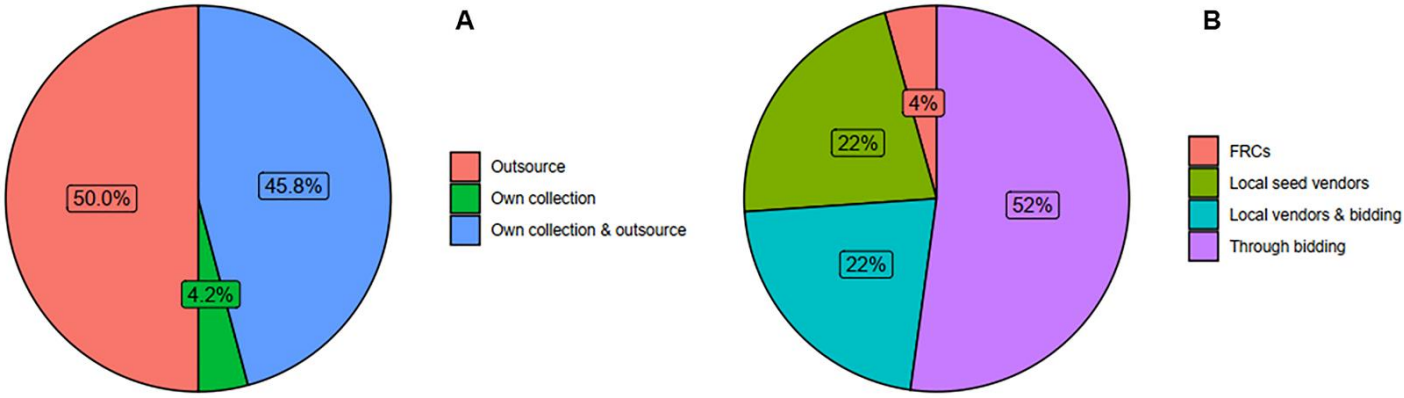

Figure 2.5. Tree seed procurement approaches by the surveyed nurseries. A) Whether nurseries conduct their own seed collection or outsource their seed supply; B) Methods used by nurseries for outsourcing seed procurement.

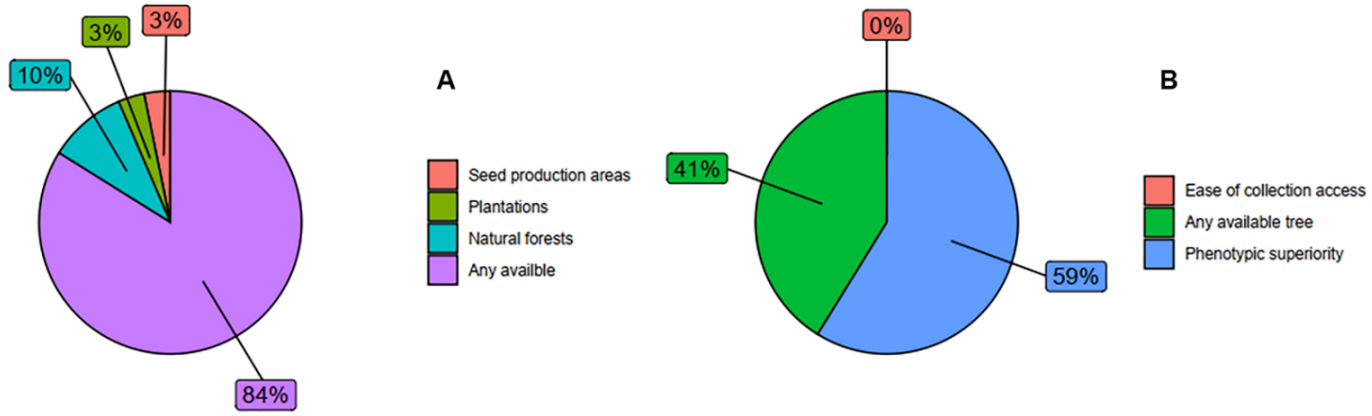

Figure 2.6. Survey responses on seed source population and mother tree selection. A) Types of populations from which seeds are collected; B) Criteria used for selecting specific mother trees

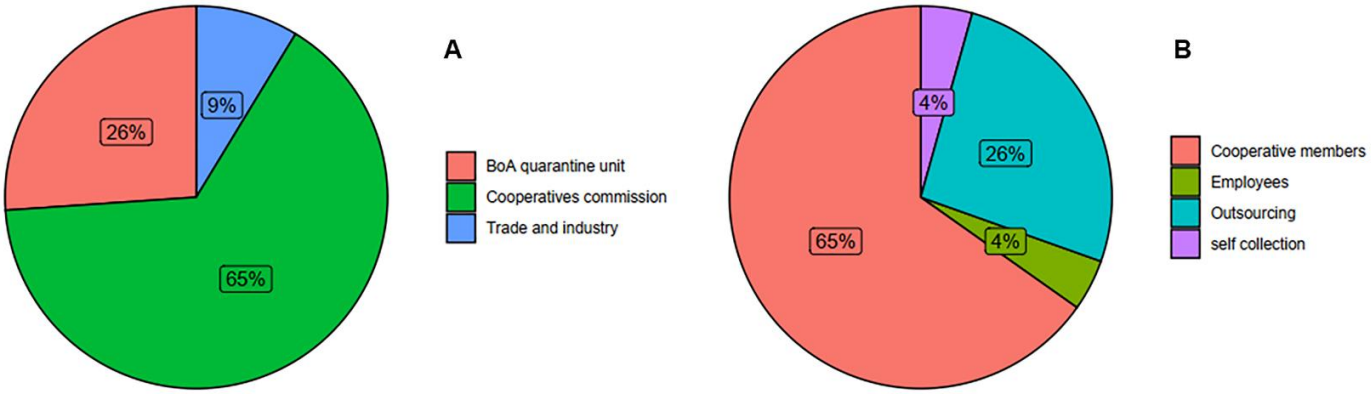

Figure 2.7. Licensing authorities and seed procurement methods of seed vendors. A) Authorities responsible for issuing licenses to seed vendors; B) Methods used by seed vendors to procure seeds

Table 2.2. Survey responses on giving genetic consideration during seed sourcing

No.	Questions	Responses		Proportion		Total responses
		Yes	No	Yes (%)	No (%)	
1	Do nurseries receive pertinent information (passport data) on the seed they outsource (e.g., location, climate, altitude, test results)?	3	20	13	87	23
2	Do nurseries face germination issues with outsourced seeds?	20	3	87	13	23
3	Do seed collectors consider the minimum number of mother trees when collecting seeds?	1	33	3	97	34
4	Do seed collectors account for minimum distances between mother trees during collection?	4	30	12	88	34
5	Do seed collectors follow established guidelines for seed collection?	19	10	66	34	29
6	Have seed collectors received training in seed handling?	24	10	71	29	34
7	Do seed vendors have the required license to operate?	22	1	96	4	23

The majority of tree seed vendors (96%) reported having the required license to operate in this business (Table 2.2). However, when examining the authorities responsible for issuing these licenses (Figure 2.7A), the forestry sector is notably absent from the process. Instead, licenses are granted by the Cooperative Commission (65%), the Bureau of Agriculture (26%), and the Trade

and Industry Commission (9%), primarily as a legal formality to run the business like any other commodity, without accounting for the peculiarities of the tree seed system.

2.3.3. Genetic considerations in seed sourcing

Table 2.2 summarizes the responses regarding factors affecting the genetic quality of procured tree seeds. A significant proportion of nurseries (87%) reported that the seeds they purchase do not come with labels (or passport data) providing critical information such as the location, seed source population, collection date, or seed test results. The same percentage also noted experiencing germination issues with their outsourced seeds. Furthermore, 97% of seed collectors do not consider a minimum number of mother trees when collecting seeds, and 88% do not consider minimum distances between mother trees, which increases the risk of collecting genetically-related seeds, leading to inbreeding. While 66% of seed collectors claimed to follow established guidelines for tree seed collection, none of the surveyed vendors actually possessed written guidelines. Instead, they relied on training they had received, using their 'knowledge' from these trainings as a substitute for formal guidelines. About 70% of seed collectors indicated they had received some level of training in tree seed handling.

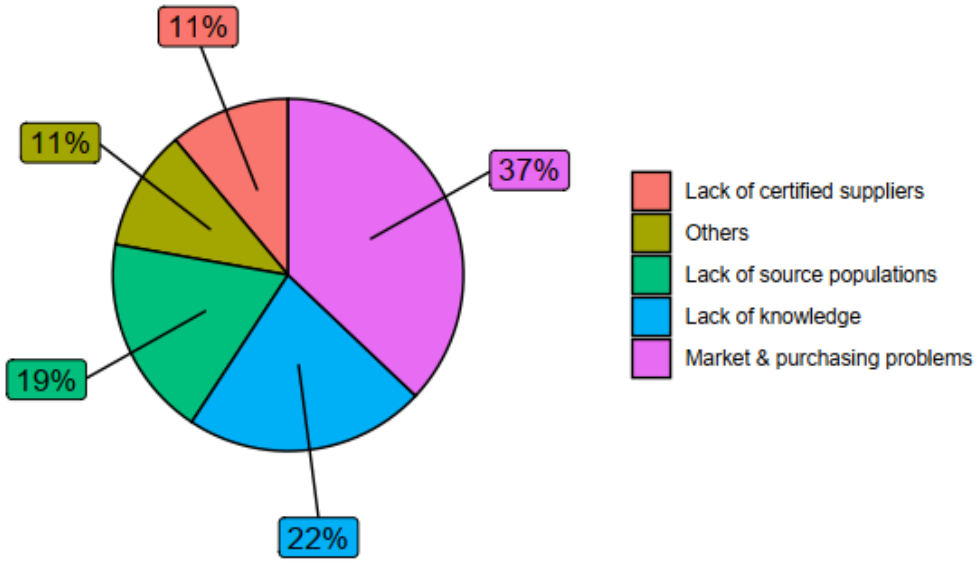

Figure 2.8. Major seed procurement challenges identified by tree nurseries and seed vendors

Figure 2.8 highlights the major challenges perceived by survey participants in procuring high-quality tree seeds. The most frequently cited challenge (37%) was the lack of market access, primarily related to the government's bidding-based purchasing system. This was followed by a lack of knowledge and awareness in tree seed collection and handling (22%), and a shortage of good source populations for seed collection (19%). Regarding market issues, respondents expressed concerns that the bidding system allows a few private dealers to dominate the market. These dealers, despite not collecting their own seeds, win bids and then procure any available seed afterward. Respondents attributed this to the government's open bidding system, which doesn't prioritize vendors offering higher-quality seeds but who cannot compete with private dealers on price. They also voiced concerns about corruption, noting that a few regular dealers, leveraging their financial influence, repeatedly manipulate the system to secure winning bids.

2.4. Discussion

2.4.1. Native versus exotic tree species

The predominance of exotic species in germplasm distribution for tree planting (Figures 2.1 – 2.4) reflects a longstanding preference for fast-growing non-native species over native trees—a trend persisting in current large-scale forest restoration initiatives as well. This preference is largely due to the rapid growth rates typical of exotic species, contrasting with the slower growth and limited silvicultural knowledge available for many native species (Lemenih & Kassa, 2014; Zeleke & Vidal, 2020; Negash, 2021). Although substantial knowledge gaps existed in the domestication, propagation, and management of native trees when exotic species were historically introduced, recent advances in native tree species propagation techniques now provide alternatives (Negash, 2010; 2021). Yet, nurseries and restoration practitioners still primarily rely on a limited range of exotic species, hindering the practical application of native species. There is no clear direction or legislative support in the country's 10-year national forest development program (MEFCC, 2018) or the Green Legacy Initiative (Fikreyesus et al., 2022; Beyene & Shumetie, 2023) regarding the extent or specific locations for native tree planting. From a restoration perspective, however, native species should be prioritized for their adaptability to local environmental conditions, support for biodiversity, and enhancing essential ecosystem functions (Thomas et al., 2014; Negash, 2021).

The present study also revealed regional and seed center differences in species preferences and distributed germplasm (Figures 2.2 and 2.4). For instance, the Bahirdar seed center's large distribution of Acacia decurrens corresponds to its increasing expansion in northwestern Ethiopia, where it is widely used for charcoal making to meet urban energy needs (Nigussie et al., 2020; Chanie et al., 2021). Similarly, larger distributions of *Sesbania* (at Dimma) and *Leucaena* (at EEFRI) seeds likely result from their roles in agroforestry, particularly as fodder and green manure (Lupwayi et al., 1999; Mengistu et al., 2002; Oosting et al., 2011; Lebrazi & Fikri-Benbrahim, 2021).

In southern Ethiopia, *Grevillea robusta* stands out as the most preferred species for seed distribution and nursery cultivation (Figure 2.4). Its popularity stems from its versatile uses as an ornamental and commercial tree, found along roadsides, in parks, homesteads, farm boundaries, and woodlots during restoration efforts. Unlike eucalyptus (described below), *G. robusta* has not been associated with severe ecological impacts, making it a favored agroforestry species despite being a nonlegume. It is commonly intercropped with maize and beans on smallholder farms and acts as a shade tree for coffee and tea in East Africa and Asia (Lott et al., 2009; Kehlenbeck et al., 2011; Nesper et al., 2017). However, assessments of its intercropping performance yield inconsistent results; some report minimal competition with crops (e.g., Bucagu et al., 2013), while others note significant below-ground competition with herbaceous crops (Smith et al., 1999; Lott et al., 2009). Nevertheless, *G. robusta* remains highly valued for its rapid growth and commercial appeal—its wood is sought after for fuelwood, construction, and timber (Niyomfura et al., 2022; Bhandari et al., 2023), while its role in carbon sequestration makes it attractive for environmental service payment schemes like REDD+ (Kiyingi et al., 2016; Owate et al., 2017). Given its widespread planting in Ethiopia, further research into its agroforestry interactions, ecosystem restoration potential, and timber quality could maximize its economic and ecological benefits.

Another notable finding in this study is the distribution of *Eucalyptus* germplasm (*E. camaldulensis, E. globulus, E. saligna*, and *E. grandis*), which was minimal across seed centers except at Bahirdar (Figure 2.2). Eucalyptus species were neither highly ranked in species preferences by surveyed nurseries and seed vendors in southern Ethiopia (Figure 2.4) nor commonly found in public nurseries. The cultivation and distribution of eucalyptus seedlings appears discouraged in public nurseries, apparently due to ecological concerns surrounding the species. Nonetheless, farmer preference for eucalyptus plantations remains strong, especially in

northern Ethiopia (Jenbere et al., 2012; Molla et al., 2022; Yimam et al., 2024). Most seedlings for these plantations are either produced by the farmers themselves or supplied by private nurseries (MEFCC, 2018; Tesfaw et al., 2022). Eucalyptus's seeds, which lack hard coats and germinate readily without pretreatment or special nursery facilities, enable easy seedling growth and contribute to the widespread plantings. Additionally, the seeds are very small, allowing large numbers of seedlings to be produced from a small quantity of seed (Figure 2.2B).

Since its introduction to Ethiopian forestry over a century ago, eucalyptus has been both celebrated and criticized (Jagger & Pender, 2002; Abebe & Tadesse, 2014; Jaleta et al., 2016; Negash, 2021). Critics emphasize ecological concerns, including aggressive water and nutrient consumption that outcompetes other vegetation and allelopathic chemicals that suppress plant growth beneath its canopy, often leaving the ground barren, described as "green on top, but Sahara beneath" (Negash, 2021). Advocates, however, highlight its economic importance in meeting Ethiopia's growing wood demand, alleviating poverty, and protecting remnant natural forests. Its rapid growth, resilience, and capacity to provide consistent cash flow make it a favorite among farmers, with some calling it a "living bank account" (Zerga et al., 2021). Consequently, eucalyptus plantations are expanding rapidly, particularly in the Amhara region, where districts like Mecha and Sinan have experienced substantial increases (Tesfaw et al., 2022; Molla et al., 2022; Yimam et al., 2024). Despite its benefits, this unregulated expansion poses risks to farmland productivity, food security, and ecological balance, underscoring the need for policies to balance economic gains with environmental sustainability (Bazzana et al., 2021; Alemayehu & Melka, 2022).

2.4.2. Seed procurement practices and genetic considerations

The survey results revealed that the tree seed procurement system is plagued by noncompliance with quality standards, with most nurseries relying on outsourced seeds with little passport data. Open bidding prioritizes low cost over quality, allowing private dealers to outcompete cooperatives whose members are trained in seed collection. Additionally, licenses issued by non-forestry authorities fail to address the unique needs of tree seed supply, resulting in unregulated participation by uncertified suppliers. This lack of regulation undermines the delivery of high-quality, certified tree seeds essential for successful restoration initiatives.

The results of this survey also indicated that seed dealers frequently overlook essential guidelines for genetic diversity in seed collection, such as those from ICRAF (Kindt et al., 2006; Lillesø et al., 2021) and the Royal Botanical Gardens, Kew (2003). These standards recommend collecting seeds from a minimum of 30 mother trees spaced 50–100 meters apart to reduce inbreeding and ensure genetic diversity. Although it is generally advised to collect small amounts of seeds from many trees rather than large quantities from a few (Rogers and Montalvo, 2004; Bozzano et al., 2014), this practice was not observed among the surveyed vendors. The neglect of these principles may stem from inadequate training or intentional oversight, as regulatory or incentive mechanisms for promoting high-quality seed collection are absent.

Germplasm collected through such practices is often inbred or lacks genetic diversity, which is essential for successful restoration. Seeds of low genetic diversity tend to produce seedlings with limited adaptation, potentially leading to higher mortality, slower growth, and reduced reproductive success over time (Rogers & Montalvo, 2004; Broadhurst et al., 2008; Broadhurst & Boshier, 2014; Thomas et al., 2014). Restoration projects that rely on germplasm from a few parent- trees risk establishing founder populations prone to inbreeding, ultimately reducing fitness in subsequent generations (Broadhurst & Boshier, 2014). Maintaining higher genetic diversity is especially important for restoration sites, which often face challenges like poor soil or low moisture, where inbreeding depression may intensify under such stressful conditions (Fox & Reed, 2010; Thomas et al., 2014; Sandner et al., 2021).

Current large-scale forest landscape restoration initiatives in Ethiopia (Kassa et al., 2022) are likely to face these challenges due to germplasm sourcing practices that capture potentially inbred and low-quality seed. This should be critically evaluated as the use of low genetic quality germplasm affects the success of both current and future restoration attempts. Forests established today are likely to become seed sources for future restoration activities. The Ethiopian Forestry Development (EFD) and other authorities leading restoration initiatives should devise policies, action plans, and regulatory frameworks to ensure the use of high-quality germplasm in restoration projects.

Some initiatives are currently being taken to tackle these challenges. For example, the Environment and Forestry Development (EFD) and the Provision of Adequate Tree Seed Portfolios (PATSPO) project (https://worldagroforestry.org/project/PATSPO-II), funded by the Norwegian

government and implemented by ICRAF in Ethiopia, are actively involved. The EFD and PATSPO have collaborated to form the national Tree Seed Network (TSN) (https://tss.epa.gov.et/ts-devt) to establish an effective tree seed system that ensures the supply of high-quality tree seed for restoration efforts in Ethiopia. However, the implementation of standards promoted by the TSN has largely not reached the grassroots level among seed vendors, nurseries, and other participants in the tree seed system. For example, the TSN has developed seed standards for about 30 tree species, specifying requirements for inspecting source populations, seed collection, physical characteristics, seed test results, and labeling. Unfortunately, stakeholders surveyed in this study were often unaware of these standards. Additionally, although TSN intends for seed provision to be done by network members, these practices were not widely followed in the seed procurement processes surveyed. For instance, seed cooperatives included in this survey, which were organized by the Southern Ethiopia tree seed center (a TSN member), were largely outcompeted by private seed dealers in the market despite providing better quality seed.

To ensure that high-quality germplasm reaches nurseries and that robust seedlings capable of adapting and sustaining on restoration sites are planted, the EFD and PATSPO should work to implement these standards at the grassroots level. Seeds should be collected and procured following these standards and provided to the market only by certified seed suppliers who are TSN members. Additionally, introducing niche or specialty markets exclusively for certified suppliers could incentivize quality, offering a better alternative to the current open bidding system. This approach aligns with a recent study by Kindt et al. (2023), which suggests that investor funding for tree planting should be based on comprehensive explanations of how tree seeds and seedlings will be sourced, ultimately incentivizing the procurement of higher-quality tree seeds and seedlings.

2.5. Conclusion

This study found that genetic principles in species selection and germplasm procurement are largely neglected in Ethiopia's large-scale forest landscape restoration initiatives. Survey results showed that few exotic species, such as *Grevillea robusta, Acacia decurrens*, *Eucalyptus spp*., and *Cupressus lusitanica,* dominate seed distributions and nursery preferences, with native species notably underrepresented. Seed procurement practices also fall short of adhering to standards and guidelines that ensure the capture of intraspecific genetic diversity available in seed source

populations. Tree seeds are often collected randomly from any available tree, ignoring standard requirements such as the minimum number of mother trees needed for a single seed collection and the minimum isolation distance between two adjacent mother trees to avoid inbred seed collection. The seeds also lack proper labels (passport data), making it impossible to trace back to the source populations and match the afforestation sites with seed provenances. These practices risk the loss of evolutionary adaptive potential associated with genetically diverse seeds, which is crucial for survival and long-term sustainability of restoration projects.

These suboptimal practices in sourcing genetic material for forest restoration initiatives call for the EFD and other relevant authorities leading the restoration initiatives to devise policies and regulatory instruments that increase the share of native species in restoration projects and ensure the procurement of high-quality tree seeds. For seed sourcing, this includes strict inspection to ensure seeds are collected and distributed following set standards, and making sure only certified seed dealers participate in the seed market. To facilitate this, the EFD should seek authorization to license and regulate seed dealers, enabling stricter enforcement of compliance and greater control over seed quality. Additionally, the EFD needs to undertake assessments on the performance of restoration projects, including survival, growth, and genetic diversity studies.

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CHAPTER THREE

3. Genetic Diversity and Population Structure in Fragmented Populations of *Afrocarpus gracilior* in Southern Ethiopia Using DArTseq-Generated SNPs

Abstract

Forest fragmentation can have severe genetic impacts that threaten the long-term viability of tree species, though susceptibility varies by species. This study investigates the genetic effects of fragmentation on *Afrocarpus gracilio*r populations in traditional agroforestry systems and relict forest patches. We used single-nucleotide polymorphism (SNP) markers generated by the DArTseq platform to assess genetic diversity and population structure in adult and progeny cohorts. Our findings indicate overall low genetic diversity ($H_E < 0.1$) across all *A. gracilior* populations studied, with progeny cohorts showing even lower diversity than adults. Progeny from isolated or few mother trees exhibited the lowest genetic diversity, suggesting heightened genetic drift and inbreeding. Genetic differentiation between populations ranged from little ($F_{ST} < 0.05$) to moderate ($0.05 < F_{ST} < 0.15$), with progeny cohorts from smaller populations showing relatively higher differentiation and significant index of association scores. A Mantel test found no significant correlation between genetic and geographic distances. Analysis of molecular variance revealed that most genetic variation occurred within populations (57–61%), rather than between populations (1.07–4.93%) or individuals (about 38%). Clustering analysis using the discriminant analysis of principal components (DAPC) method classified the genotypes into five groups, whereas phylogenetic analysis identified three major clusters with further subgrouping. Overall, the study highlights low genetic diversity in *A. gracilior* populations, with significant inbreeding and genetic drift affecting progeny from small, isolated populations. These findings are essential for guiding conservation, restoration, and genetic rescue efforts.

Key words: *circa situm*, fragmentation, genetic differentiation, gene flow, inbreeding, sacred tree

3.1. Introduction

Anthropogenic habitat destruction has been besetting the Earth since the Holocene (Bhagwat, 2014). Forest fragmentation occurs when large, intact, and continuous forests are converted into small and isolated patches separated by non-forest land (Young and Boyle, 2000; Schlaepfer et al., 2018; Taubert et al., 2018; Aguilar et al., 2019; Mengist et al., 2022). Over time, these patches shrink in size, become more isolated, and develop more edges exposed to increased human-land use interactions (Aguilar et al., 2019; Mengist et al., 2022). Research (Schlaepfer et al., 2018; Aguilar et al., 2019; Ony et al., 2020) has shown that these changes in forest patches affect ecological processes and plant-animal interactions that influence the demography of plant populations through processes such as reduced gene flow, increased inbreeding, and genetic drift.

The genetic effects of forest fragmentation have been a major area of research over the past two decades, particularly with the advancement of molecular marker technologies (Lowe et al., 2005; Kramer et al., 2008; Vranckx et al., 2012; Finger et al., 2014; Lowe et al., 2015; Schlaepfer et al., 2018; Vinson et al., 2018; Sen and Ravikanth, 2022). The prevailing theory on the genetics of small populations following fragmentation suggested that reduced gene flow, genetic drift, inbreeding, low genetic diversity, and increased genetic structure would occur. However, early reviews of molecular marker-based genetic diversity studies of fragmented forest populations revealed that these expectations were met in only a few instances (Lowe et al., 2005; Kramer et al., 2008). This lack of empirical evidence to support population genetics expectations was labeled by Kramer et al. (2008) as "the paradox of forest fragmentation genetics." This led to the belief that trees might be resilient to the genetic effects of fragmentation due to their longevity, flexible mating systems, and extensive gene flow (Lowe et al., 2015; Vinson et al., 2018).

Nevertheless, subsequent studies have indicated that tree species are not completely resilient to fragmentation, but rather that the impact depends on the species being investigated and the context of the study (Bacles and Jump, 2011; Lowe et al., 2015). Previous research that led to the mentioned 'paradox' focused primarily on adult populations, which was criticized as 'looking in the wrong place' (Lowe et al., 2015). This is because the alleles present in adults may not accurately represent current gene flow. If present, the genetic effects of fragmentation would be more apparent

in progenies sired in such landscapes, as they carry more recent gene flow. Following this realization, research on the topic has become more diverse, incorporating adult and offspring cohorts in genetic studies and linking the findings to the mating habits of the species. Consequently, more recent reviews (Schlaepfer et al., 2018; Vinson et al., 2018; Aguilar et al., 2019; Sen and Ravikanth, 2022) have indicated that the resilience or susceptibility of tree species to fragmentation depends on various factors, including the species' life history, mating patterns, dispersal mode, pollinator mobility, time since fragmentation, and ecological landscape.

The primary genetic effects of forest fragmentation reported in susceptible tree species include limited gene flow, increased inbreeding, reduced genetic diversity, increased genetic structure, and reduced progeny fitness (Cascante et al., 2002; Dubreuil et al., 2010; Ismail et al., 2012, 2014; Yineger et al., 2014; Ganzhorn et al., 2015; Rymer et al., 2015; Duminil et al., 2016; Ony et al., 2020; Qin et al., 2021). Reduced population size and restricted gene flow typically result in genetic isolation by distance. Reproductively isolated populations are more susceptible to frequent inbreeding and the loss of rare alleles, which can impact seed production, seed germination, and progeny vigor.

For example, Cascante et al. (2002) reported that seedlings from continuous forest populations exhibited higher germination rates, larger leaf area, and greater biomass than seedlings from isolated trees. Similarly, Jolivet et al. (2013) noted lower genetic diversity in seedlings than in adults and observed biparental inbreeding depression, leading to reduced offspring performance in the self-incompatible wild cherry tree, *Prunus avium*. A comparative study on pollination and progeny fitness (Rymer et al., 2015) on *Pachira quinata* trees from continuous forests and those scattered in pastures revealed higher levels of inbreeding in pasture trees than in continuous forests. A global synthesis report (Aguilar et al., 2019) revealed overall genetic erosion, as well as decreased germination, survival, and growth in progeny sired in fragmented habitats compared with progeny from continuous populations. This poses a threat to the long-term viability of species and populations in the face of climate change, where genetic diversity is essential for adaptation.

Chronic deforestation in Ethiopia (Teketay, 1992; Reusing, 2000; Darbyshire et al., 2003; Dessie and Kleman, 2007; Teketay, 2011; Mengist et al., 2022) has made the remnant natural forests small, nonconnected patches located on slopy hills and in inaccessible valleys. These patches are likely

susceptible to the effects of habitat fragmentation and isolation described thus far. These impacts have been observed in *Prunus africana* (Yineger et al., 2014) and *Olea europaea* subsp. *cuspidata* (Kassa et al., 2017), two indigenous tree species found in these fragmented landscapes. A lower genetic diversity was recorded in small patches than in relatively larger, intact forests, as well as in seedlings than in adult populations. Another indigenous tree species facing a similar threat, but not yet studied, is the East-African yellowwood (*Afrocarpus gracilior* (Pilg.) C.N.Page), hereafter referred to as *A. gracilior*.

A. gracilior is an evergreen conifer native to the Afromontane forests of Ethiopia, renowned for its economic and ecological significance. It is a dioecious, wind-pollinated species that naturally propagates by seed (Negash, 2003, 2010). Historically, it was a dominant canopy tree and is valued for its superior-quality timber (Negash, 2010; Teketay, 2011), edible oil from seeds used medicinally (Teketay, 2011), cytotoxic phytochemicals with anticancer properties (Stahlhut et al., 1999; Faiella et al., 2012; Kamal et al., 2019), and its role in soil and water conservation (Negash, 2003, 2010). Additionally, it holds sociocultural importance as a sacred tree (Doffana, 2014; Doda and Abuelgasim, 2019). However, its high-quality timber has led to illegal logging, making it one of Ethiopia's most unsustainably logged species (Teketay, 2011). Many remnant forests of *A. gracilior* are heavily depleted, with selective extraction of mature trees. Historical records describe it as a giant tree reaching up to 50 meters in height and 3 meters in diameter (Russ, 1944, cited in Negash, 2010). Today, *A. gracilior* is primarily found in relict forest patches, church forests (Aerts et al., 2016), and other culturally sacred areas (Doffana, 2014; Doda and Abuelgasim, 2019), though these trees are smaller than those described historically (Negash,2010). Due to its wide distribution, the IUCN Red List categorizes the species as 'least concern' (Farjon, 2013). Nonetheless, its primary habitat in the Eastern Afromontane biodiversity hotspot is threatened by selective logging and deforestation, highlighting the need for conservation. As one of the most charismatic native tree species in Ethiopia, *A. gracilior* is also valuable for the country's ongoing forest restoration initiative known as the "green legacy" (Fikreyesus et al., 2022; Beyene and Shumetie, 2023; Lambert and Deyganto, 2024).

The species is, however, relatively abundant in the Gurage and Sidama regions in southern Ethiopia, where this study was conducted. In Gurage, *A. gracilior* populations are found mainly as natural remnant patches of various sizes away from villages and farmlands. However, in Sidama,

populations are part of the enset-coffee traditional agroforestry systems. These home gardens are thought to have originated from forests, where farmers keep the upper-storey trees, whereas removing the lower vegetation to make room for growing enset, coffee, and various other crops (Abebe and Bongers, 2012). This setup has enabled the system to hold a diversity of indigenous tree species, remnants of the old natural forests that have been converted to agroforestry home garden systems. *A. gracilior* is one of the indigenous tree species maintained as such in the system. The Sidama people hold this tree species in high regard, as it is culturally considered sacred (Doffana, 2014; Doda & Abuelgasim, 2019). As a result, it is common to find isolated individuals or a small group of trees (less than 10 in number) in the front yards of houses and along farm boundaries; a relatively larger group (10 to 50 individuals) in graveyards and public gathering places locally known as *gudumales*; and hundreds of individuals in small remnant natural patches and sacred places (i.e., places of worship).

Agroforestry systems like these are considered important for the *circa situm* conservation of indigenous trees, where they are maintained in modified agricultural landscapes where the original forests containing these trees once thrived (Boshier et al., 2004; Dawson et al., 2013; Pinard et al., 2014). Can the small and fragmented populations of *A. gracilior* serve this purpose and become germplasm sources for the restoration of the species? Or are they too small and vulnerable to the genetic effects of forest fragmentation? These questions are particularly pertinent for *A. gracilior* because it is a dioecious species pollinated by wind, making it an obligate outcrossing species. As a result, inefficient pollination is reported to commonly result in the development of unpollinated female cones that produce empty seeds (Negash, 2010), which may be one of the reasons why the seeds give poor germination results when artificial regeneration is attempted in nurseries (Negash, 2010; Getachew, 2011).

Using DArTseq-generated SNP markers, the first set of molecular markers developed for the species, this study explored whether the genetic consequences of small populations are evident in *A. gracilior*. The specific questions we aimed to answer were: 1) What is the genetic diversity of *A. gracilior* at the study sites? 2) How does genetic diversity differ between small, isolated populations and larger, continuous populations, as well as along different life stages of *A. gracilior*? 3) Does current gene flow connect the populations, or are they genetically differentiated? 4) Are remnant *A. gracilior* trees in traditional agroforestry systems genetically

impoverished, or do they function as *circa situm* reservoirs of genetic diversity? The results will have important practical applications in the conservation and restoration of this species.

3.2. Materials and methods

3.2.1. Sampling sites and plant material

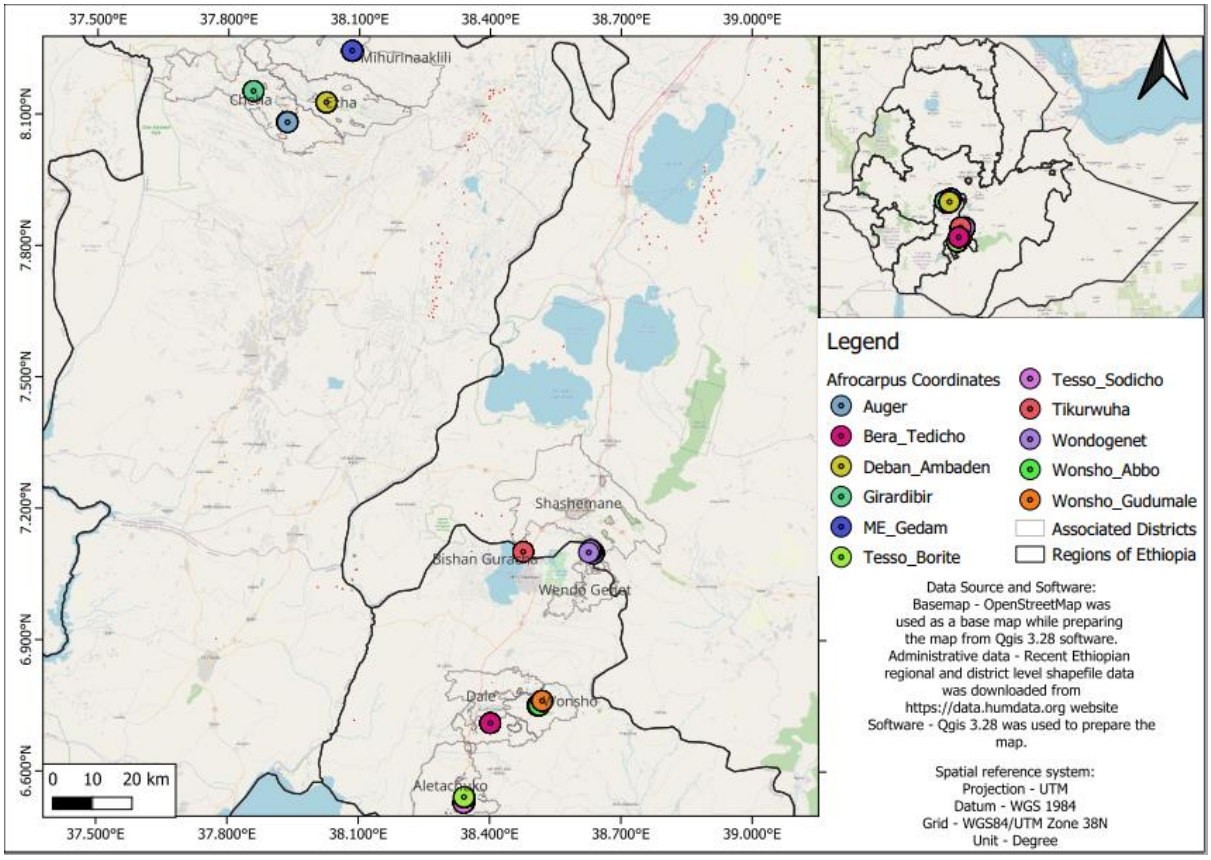

Figure 3.1. Location map of the sampling populations in southern Ethiopia. The northern populations belong to the Gurage, whereas southern populations belong to the Sidama regions. The site descriptions and corresponding population codes are indicated in Table 3.1.

Young leaf samples of *A. gracilior* were collected from 11 sites in southern Ethiopia, specifically in the Gurage and Sidama regions (Figure 3.1). From 9 of the sites, both parent and progeny samples were collected, whereas the remaining two sites were nurseries where only seedling (progeny) samples were taken (Table 3.1). The collected leaf samples were immediately placed in zip-lock plastic bags with silica gel and left to dry until DNA extraction. The number of samples

genotyped for each population is indicated in Table 3.1. Differences in sample sizes were due to the requirement to fit to 2 plates, a maximum of 188 samples, sent for genotyping.

Table 3.1. Description of the study populations from which samples were collected for genotyping. The '-p' in the population codes indicates that the population is a parent or adult cohort, otherwise, it is a progeny

Site/Population	Region	Population codes	Number of samples	Altitude (m.a.s.l)	Description
Auger	Gurage	AUG AUG-p	10 10	2226	A remnant mixed *Afrocarpus-Juniperus* natural forest patch, relatively large and intact; 138 ha
Bera-Tedicho	Sidama	BTD BTD-p	10 10	1807	A small group of old (>100 years) remnant trees in a village; 54 trees, 0.5ha patch area
Deban Amba-den	Gurage	DAD DAD-p	9 9	2353	A high hill *Afrocarpus* dominated natural remnant patch: about 15 ha
Girar dibir	Gurage	GDB	10	1943	Nursery seedlings regenerated from seeds; the source is nearby but not exactly known
Mihur Eyesus gedam	Gurage	MEG MEG-p	9 9	2330	An old monastery; some very old trees (> 200 years); intermediates and juveniles also present; 16 ha
Tesso-Borite	Sidama	TBO TBO-p	9 9	1930	A small natural remnant patch on a hilltop, about 10 ha, many juveniles, few old trees
Tesso-Sodicho	Sidama	TSO TSO-p	9 9	1795	A few adult trees planted in front-yard of houses in a village; a single isolated female tree (> 200 m from nearest adult males), a heavy seeder, is the mother for the progeny here
Tikurwuha	Sidama	TKW	10	1693	Nursery seedlings from wildlings brought from around; exact source unknown
Wondogenet college	Sidama	WGC WGC-p	9 9	1900	Scattered remnant trees from a natural forest, partially converted to a plantation of different species; 150ha
Wonsho-Abbo	Sidama	WAB WAB-p	10 10	2060	Lower half of culturally protected, worship site; about 75 ha
Wonsho-Gudumale	Sidama	WGD WGD-p	9 9	2110	About 30 huge and old trees (some > 200 years); site is a communal gathering for cultural events; about 0.5 ha; progeny sampled from an adjoining homegarden

3.2.2. DNA extraction and genotyping

Approximately 10–15 mg of each silica-dried sample was placed in two plates (188 samples), securely capped, and sent to SEQART AFRICA (https://www.seqart.net) located at the International Livestock Research Institute in Nairobi, Kenya, for DNA extraction and genotyping. DNA was extracted using the Nucleomag Plant Extraction Kit (Macherey-Nagel, Düren, Germany), based

on CTAB for tissue lysis, following the manufacturer's protocol. The concentration of the extracted genomic DNA ranged from 50 to 100 ng/µl, and its quality and quantity were further confirmed with 0.8% agarose gel electrophoresis.

For genotyping, sequencing libraries were prepared using the DArTseq™ technology (https://www.diversityarrays.com/services/dartseq), which combines DArT's proprietary genome complexity reduction method with next-generation sequencing (Wenzl et al., 2004; Sansaloni et al., 2011; Kilian et al., 2012). The technology primarily employs PstI, a rare-cutting, methylation-sensitive enzyme, to selectively reduce genome complexity by excluding heavily methylated repetitive DNA. PstI is used in combination with one or more frequent-cutter enzymes to generate DNA fragments suitable for short-read sequencing. For this study on *A. gracilior*, three common-cutting enzymes (MseI, HpaII, and AlwI) were tested, with MseI proving to be optimal based on call rate, reproducibility, and polymorphic information content. Barcoded adapters complementary to the sticky ends of the PstI enzyme were ligated to allow only fragments with a PstI end to be present in the genomic representation.

Only fragments with a PstI end on one side and an MseI end on the other (PstI-MseI) were selectively amplified through 30 cycles of PCR using *DArT PstI* primers (5'-GAT GGA TCC AGT GCA G-3'). The PCR conditions included an initial denaturation at 94°C for 1 minute, followed by 30 cycles at 94°C for 20 seconds (denaturation), 58°C for 30 seconds (annealing), and 72°C for 45 seconds (extension), with a final extension at 72°C for 7 minutes. The amplified products were sequenced on the HiSeq2500 platform (Illumina, San Diego, USA) using single-read sequencing for 77 cycles. The sequences generated were processed through DArT's analytical pipeline (Ren et al., 2015; Egea et al., 2017), which included filtering poor-quality sequences, collapsing identical sequences into "FASTQCOLL," and scoring markers.

Markers were scored using DArTsoft14, DArT's proprietary scoring pipeline, which applies C-means clustering and analysis of variance (Wenzl et al., 2004) to identify allelic differences. Two types of markers were generated: silicoDArT markers and SNP markers. SilicoDArTs are dominant markers scored as binary (1 and 0 for presence and absence, respectively of the restriction fragment in the genomic representation). SNP markers, which are biallelic codominant markers, were scored as 0 for reference allele homozygotes, 1 for alternative allele homozygotes,

and 2 for heterozygotes. Since *A. gracilior* lacks a reference genome, SNP markers were generated *de novo* by identifying pairs of stacks (FASTQCOLL files) with a single base-pair mismatch, which are considered to represent alternative alleles of a locus or an error (Catchen et al., 2013; Lu et al., 2013). These candidate SNPs were further subjected to quality checks and filtering to ensure accuracy.

3.2.3. Data analysis

SNP quality assessment and data filtering

Two types of data generated with the DArTseq genotyping platform were received from SEQART Africa: SNP and SilicoDArT. Only the SNP markers were utilized in this analysis because the SilicoDArT markers, which are dominant, are considered less informative for population genetics studies (Gapare et al., 2021).

A two-row format SNP data with 10,219 SNP loci with 38.49% missing data (scored as NA) was imported into R (version 4.3.3) as a genlight object using adegenet (Jombart, 2008; Jombart and Ahmed, 2011) and dartR (Gruber et al., 2018; Mijangos et al., 2022) packages. The quality SNPs were assessed using the *gl.report* function of the dartR package, which evaluates both the loci and individual metrics of the genlight object. SNP filtering was performed using the *gl.filter* function, where loci with a call rate below 70% and individuals with a call rate below 50% were excluded. Additionally, loci with reproducibility lower than 95% were removed, and filtering on the basis of read depth (lower threshold = 5, upper threshold = 30) was applied. Monomorphic loci and those with entirely NA scores were also filtered out.

For population structure and phylogenetic analyses, which are more sensitive to missing data than genetic diversity analyses, additional stringent filtering was implemented. The call rate threshold was increased to 75% for loci and 60% for individuals, secondaries were removed, and filtering by allele frequency (threshold = 1/ (2 × 2 N), where N is the number of individuals) was applied to eliminate singleton SNPs (Linck and Battey, 2019). Missing data were subsequently imputed using the *gl.impute* function. The cleaned SNP dataset was saved as an RData file for further genetic diversity and population structure analyses.

Various marker quality and diversity parameters, such as call rate, reproducibility, minor allele frequency, and polymorphic information content, were generated from the filtered SNP data and summarized. Information on SNP types (i.e., transitions and transversions) was extracted from the filtered genlight object and analyzed. The transition to transversion ratio (Ts/Tv) was then calculated. The SNP loci were tested for significant deviations from HWE using the function *hw.test* of the pegas package (Paradis, 2010).

Analysis of genetic diversity

Before proceeding with the genetic diversity analyses, the dataset was checked for duplicate genotypes, which could introduce bias in downstream analyses, and to determine if clone correction was necessary. This examination was conducted using the *mlg* function of the poppr package (Kamvar et al., 2014). The poppr function from the same package was then used to calculate the number of unique multilocus genotypes (MLGs) and the index of association (I_A) for each population. The genetic diversity within populations were subsequently assessed using various indices. The total number of alleles (N_A) and the number of private alleles (N_{PA}) were calculated using the *popgenreport* function of the PopGenReport package (Adamack and Gruber, 2014). Allelic richness (A_R) was also calculated with rarefaction correction for sample size, based on the lowest sample size of 7, equivalent to 14 alleles. The percentage of polymorphic loci (PPL), observed heterozygosity (Ho), expected heterozygosity (He), unbiased expected heterozygosity (uHe), and inbreeding coefficient (F_{IS}) were obtained using the *gl.report.heterozygosity* function of the dartR package (Gruber et al., 2018; Mijangos et al., 2022).

Population differentiation

A pairwise population differentiation (F_{ST}) matrix was generated using the *gl.ibd* function of the dartR package. Gene flow (in terms of the relative number of migrants, Nm) between pairs of populations was calculated using the *divMigrate* function of the diveRsity package (Keenan et al., 2013). Population-specific F_{ST} values were generated using the *fs.dosage* function of the hierfstat package (Goudet and Jombart, 2022). Population-specific F_{ST}, as defined by Weir and Goudet (2017), measures the probability of two alleles being identical by descent within a population relative to alleles from different populations. This metric is crucial for understanding population differentiation and offers deeper insight into population history. Kitada et al. (2017) also highlighted its value, noting that whereas pairwise F_{ST} indicates current population structure,

population-specific F_{ST} provides a historical perspective by tracing evolutionary divergence from an ancestral population.

For easier visualization, the pairwise F_{ST} and gene flow matrices were combined into a single heatmap plot, using ggplot2 (Wickham et al., 2016) and ggnewscale (Campitelli, 2024) packages. This plot displayed two symmetric half-matrices with the population-specific F_{ST} value along the diagonal. Differentiation among populations was further assessed by analyzing molecular variance, AMOVA (Excoffier et al., 1992), in the poppr package (Kamvar et al., 2014) using the *ade4* method (Dray and Dufour, 2007; Thioulouse et al., 2018). AMOVA was conducted considering different population hierarchies, such as the original population assignments based on sampling sites and geographic regions, as well as population clustering based on DAPC analysis (described below). Additionally, a Mantel test (Mantel, 1967) was performed using the *gl.ibd* function of the dartR package (Gruber et al., 2018; Mijangos et al., 2022) to determine whether there was a significant correlation between pairwise geographic distance and genetic distance matrices of the individual samples.

Population Structure and Phylogenetic Analysis

Two complementary approaches were used to infer the population structure of the adult and progeny samples of *A. gracilior*. These methods included discriminant analysis of principal components (DAPC), a model-free method based on k-means clustering (Jombart et al., 2010), and principal component analysis (PCA), a dimensionality reduction method that simplifies the data for clustering (Wold et al., 1987; Patterson et al., 2006; Greenacre et al., 2022). STRUCTURE (Pritchard et al., 2000), a model-based clustering method commonly used for inferring population structure, was not used in this study because it assumes that loci are at Hardy-Weinberg equilibrium (HWE) and are not linked, whereas our data showed both linkage and significant deviations from HWE.

DAPC was conducted after determining the optimal number of principal components to retain using the *optim.a.score* function of the adegenet package (Jombart, 2008; Jombart and Ahmed, 2011). The optimal number of clusters for the DAPC was determined by running k-means clustering with the *find.clusters* function of the adegenet package. The k-means algorithm iteratively increases the number of clusters (k) and compares different clustering solutions using

the Bayesian information criterion, BIC (Neath and Cavanaugh, 2012). The optimal k value was selected on the basis of the lowest BIC value on a plot of BIC values against each k (Jombart and Collins, 2017). The *dapc* and *scatter* functions of the adegenet package were then used to perform the DAPC and generate a scatterplot of the discriminant functions, along with scree plots of the discriminant and principal component analyses. Bar plots showing the posterior assignment of individuals to the new clusters were created using the *ggplot* function of the ggplot2 package (Wickham et al., 2016) to produce structure-like plots from the DAPC analysis.

The PCA was run using the *dudi.pca* function of the ade4 package (Adamack and Gruber, 2014). The row coordinates (principal components) of the PCA analysis were then plotted using the *ggplot* function of the ggplot2 package.

Phylogenetic analysis (Cavalli-Sforza and Edwards, 1967; Nei, 1996; Horiike, 2016) was conducted using an unweighted pair group method with arithmetic mean (UPGMA) tree for the populations and a neighbor-joining tree for the individual genotypes. This analysis was performed using the *aboot* function from the *poppr* package. The population tree was based on a genpop data format, whereas the tree for individual samples used a genlight format. Nei's distance method was applied for the population tree, and the bitwise distance was used for the individual tree, with a bootstrap cutoff threshold of 0.5 following 1,000 resampling iterations. The resulting dendrograms were saved in Newick format and uploaded to the Interactive Tree of Life (iTOL) website, an online platform for advanced phylogenetic tree customization and annotation (Letunic and Bork, 2021).

3.3. Results

3.3.1. SNP marker quality

After thorough filtering of the raw SNP data, which initially included 10,219 SNPs and 185 individuals, 1,820 SNPs and 183 individuals were retained for genetic diversity analyses, and 948 SNPs and 173 individuals were retained for population structure analyses. Among the 1,820 SNPs, 52.5% were transition mutations, with the remaining being transversions, resulting in a transition-to-transversion ratio (Ts/Tv) of 1.1 (Appendix 3.1a). A summary of other quality and diversity parameters for the 1,820 SNPs is provided in Appendix 3.1b. Among the quality parameters, call rates ranged from 0.7 to 1, with a mean of 0.774, and reproducibility ranged from 0.95 to 1, with

a mean of 0.994. For marker diversity, the polymorphic information content (PIC) ranged from 0.006 to 0.494, with a mean of 0.074, whereas the minor allele frequency (MAF) ranged from 0.0023 to 0.496, with a mean of 0.045. An exact test indicated that 74% of these loci deviated significantly ($p < 0.05$) from HWE, with individual populations showing significant deviations ranging from 3% to 10% (supplementary material in the published manuscript, not annexed here because it is a large spreadsheet). The genotype accumulation curve, which assesses the marker's power to distinguish unique multilocus genotypes (MLGs) for a random sample of loci, is also presented in supplementary material (appendix 3.2).

3.3.2. Genetic diversity

Table 3.2 presents various indices commonly used to assess genetic diversity within each sampling population. An initial check for duplicate genotypes (Kamvar et al., 2015) revealed that all 183 samples represented unique multilocus genotypes. The MLG and eMLG columns also show values equal to the number of samples (N) for each population, adding up to 183 confirming the uniqueness of each sample and eliminating the need for clone correction before further analyses. The highest index of association (I_A) score was recorded for WGD (7.56), with TKW showing the lowest score. The rbarD, a standardized form of I_A, indicated significant values ($p < 0.01$) for BTD, GDB, MEG, TSO, TSO-p, WGC, and WGD, with all but TSO-p belonging to progeny cohorts. The WAB-p population exhibited the highest number of alleles (2440). Considerable variation in N_{PA} was also observed, ranging from 15 (GDB) to 64 (WAB-p).

Further differences were revealed in allelic richness (A_R), corrected for sample size through rarefaction (Kalinowski, 2004). The lowest A_R score (1.19) was recorded for GDB and TSO, whereas WAB-p had the highest score (1.28). The percentage of polymorphic loci (PPL) ranged from 22.7% (WGD-p) to 34.1% (WAB-p). The heterozygosity estimates also varied between populations, with particular attention given to unbiased expected heterozygosity (uH_E), a corrected measure for uneven sample sizes. uH_E scores ranged from 0.055 (GDB and TSO) to 0.081 (WAB-p). The highest inbreeding coefficient (F_{IS}) was observed for TBO-p (0.645), whereas GDB had the lowest (0.386). The last column indicates the percentage of loci that significantly deviated from HWE ($p < 0.05$, exact test). Both the adult and the progeny populations of the WAB showed the highest deviation (10%), whereas the lowest deviation (3%) was recorded in the WGC-p population.

Table 3.2. Indices* that indicate genetic diversity within the study populations

Population	N	MLG	eMLG	IA	rbarD	p.rD	N_A	N_{PA}	A_R	PPL	H_O	H_E	uH_E	F_{IS}	HWE
AUG	10	10	10	0.232	0.000	0.181	2364	32	1.245	29.89	0.030	0.067	0.071	0.463	6.5
AUG-p	10	10	10	0.456	0.001	0.055	2367	43	1.247	30.05	0.022	0.068	0.072	0.581	8.8
BTD	10	10	10	1.342	0.003	0.001	2305	25	1.225	26.65	0.026	0.066	0.070	0.533	7.4
BTD-p	8	8	8	0.489	0.001	0.088	2324	19	1.245	27.69	0.025	0.072	0.077	0.557	4.5
DAD	9	9	9	0.112	0.000	0.315	2320	31	1.237	27.47	0.024	0.069	0.073	0.573	5.6
DAD-p	9	9	9	0.377	0.001	0.103	2314	29	1.236	27.14	0.022	0.069	0.073	0.605	6.9
GDB	10	10	10	1.742	0.004	0.001	2253	15	1.190	23.79	0.026	0.052	0.055	0.386	4.5
MEG	9	9	9	4.911	0.013	0.001	2243	17	1.204	23.24	0.019	0.061	0.065	0.604	6.8
MEG-p	9	9	9	0.558	0.001	0.051	2278	18	1.218	25.16	0.019	0.063	0.067	0.617	6.4
TBO	9	9	9	0.674	0.001	0.029	2353	36	1.251	29.29	0.029	0.073	0.077	0.512	6.4
TBO-p	9	9	9	0.320	0.001	0.147	2350	36	1.254	29.12	0.020	0.075	0.079	0.645	8.7
TKW	10	10	10	-0.040	0.000	0.504	2376	30	1.247	30.55	0.033	0.067	0.071	0.425	5.6
TSO	9	9	9	1.467	0.004	0.001	2242	22	1.191	23.19	0.026	0.052	0.055	0.398	3.2
TSO-p	9	9	9	1.538	0.003	0.001	2348	64	1.245	29.01	0.022	0.070	0.074	0.577	6.5
WAB	10	10	10	0.085	0.000	0.325	2379	39	1.254	30.71	0.025	0.073	0.076	0.546	10.0
WAB-p	10	10	10	0.646	0.001	0.027	2440	60	1.278	34.07	0.026	0.077	0.081	0.550	10.1
WGC	9	9	9	1.872	0.004	0.001	2373	57	1.257	30.38	0.028	0.072	0.076	0.525	5.4
WGC-p	8	8	8	0.295	0.001	0.165	2348	32	1.254	29.01	0.028	0.072	0.077	0.538	3.1
WGD	9	9	9	7.559	0.017	0.001	2294	30	1.226	26.04	0.031	0.069	0.073	0.494	4.8
WGD-p	7	7	7	0.747	0.002	0.034	2233	24	1.211	22.69	0.022	0.067	0.072	0.599	4.5
Total	183	183	10	1.158	0.001	0.001	46504	659	-	-	-	-	-	-	-
Mean	9.15			-	-	-	2325.2	32.95	1.24	27.76	0.025	0.068	0.072	0.536	6.3

* N: Number of individuals in the population that remained after data filtering; MLG: number of multilocus genotypes; eMLG: Expected number of MLGs at the lowest common sample size; I_A: Index of Association for each population; rbarD: Standardized Index of Association, p.rD: p-value for rbarD after 999 reshuffles; N_A: Total number of alleles; N_{PA}: Number of private alleles; A_R: Mean allelic richness; PPL: Percentage of polymorphic loci; H_O: Observed heterozygosity; H_E: Expected heterozygosity; uH_E: Unbiased expected heterozygosity; F_{IS}: Fixation index (inbreeding coefficient); HWE: Percentage of loci (out of 1820 loci in total) that significantly deviated from Hardy-Weinberg equilibrium (exact test, $p < 0.05$). The population codes correspond to the descriptions in Table 3.1.

3.3.3. Population differentiation

Different indices that show the level of genetic differentiation between populations are presented in Figure 3.2. The GDB and TSO populations had relatively high population-specific F_{ST} values. The pairwise F_{ST} is commonly interpreted using the categories suggested by Wright (1978), as cited in Hartl & Clark (2007), p 283, which state that $0 < F_{ST} < 05$ indicates little genetic differentiation, $0.05 < F_{ST} < 0.15$ indicates moderate genetic differentiation, $0.15 < F_{ST} < 0.25$ indicates great genetic differentiation, and $F_{ST} > 0.25$ indicates very great genetic differentiation. Following this interpretation, the pairwise F_{ST} values in the present study (below the diagonal in Figure 3.2) showed little to moderate levels of genetic differentiation between pairs of populations. In all instances, it was observed that there is little genetic differentiation between the adult and progeny cohorts at the same site.

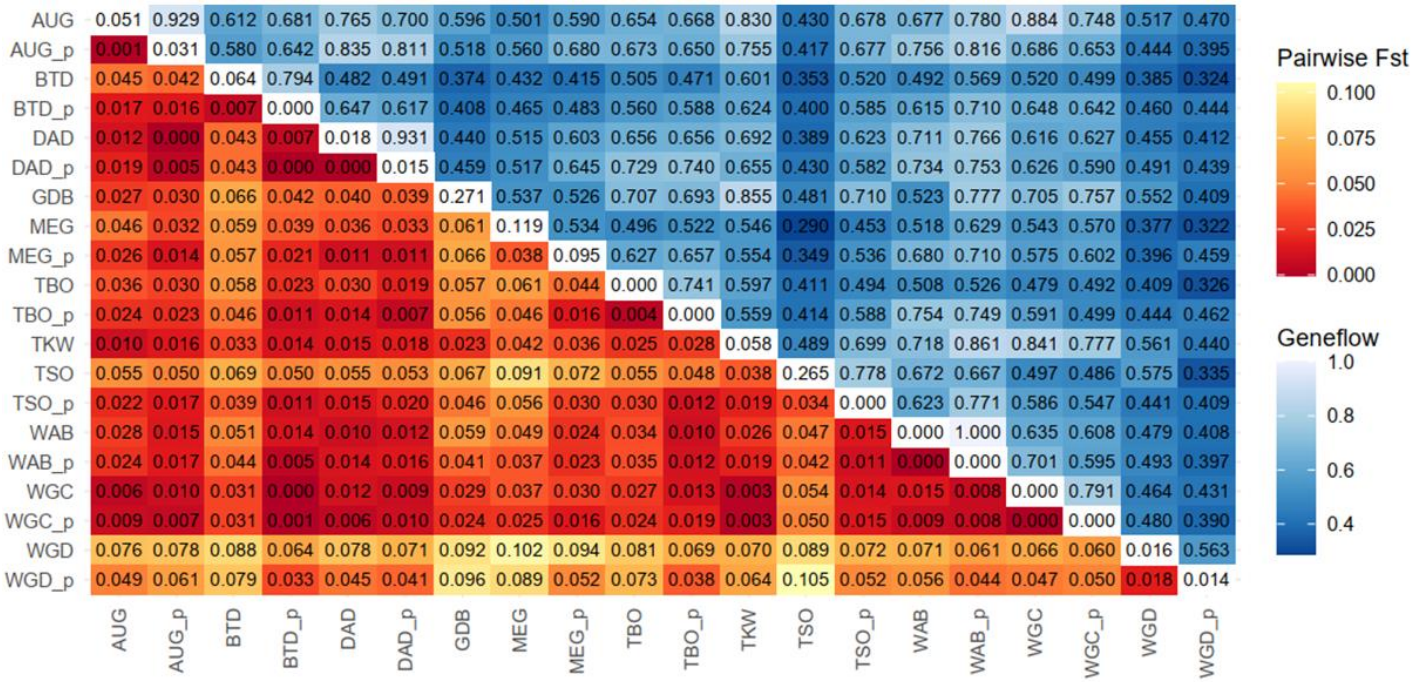

Figure 3.2. Indices that show the level of population differentiation. The diagonal values display the population-specific F_{ST} (Weir & Goudet, 2017). The values below the diagonal indicate Wright's fixation index (Wright, 1965; Nei, 1977), the pairwise FST between the specified populations. Values above the diagonal indicate gene flow in terms of the relative number of migrants (Nm) between pairs of populations (Sundqvist et al., 2017). Darker shades represent lower values, whereas lighter shades represent higher values. Populations AUG, AUG-p, DAD, DAD-p, GDB, MEG, and MEG-p represent the Gurage region, whereas BTD, BTD-p, TBO, TBO-p, TKW, TSO, TSO-p, WAB, WAB-p, WGC, WGC-p, WGD, and WGD-p belong to the Sidama region.

No noticeable trends in pairwise F_{ST} were observed on the basis geographic distance or regional differences. The WGD population exhibited the largest differences, being moderately different from all other populations except WGD-p, which is the adult cohort from the same site. This was followed by the TSO population (with 14 out of 19 possible pairings), the WGD-p population (with 11 pairings), and the GDB population (with 9 pairings). Notably, all of these populations except the WGD-p were progeny cohorts with small patch sizes (Table 3.1).

For each population, higher relative migration rates were observed between pairs of adult and progeny cohorts than pairing with any other population (Figure 3.2, above the diagonal). For example, the highest relative migration rate (1.000) was recorded between WAB-p and WAB, followed by DAD-p and DAD (0.931) populations. The only exception to this pattern was the score between the adult and progeny cohorts of the MEG population (0.534), which was not the highest pairwise score relative to pairings with other populations. Compared with those of the other populations, lower gene flow values (mostly less than 0.5) were observed for the WGD and WGD-p populations.

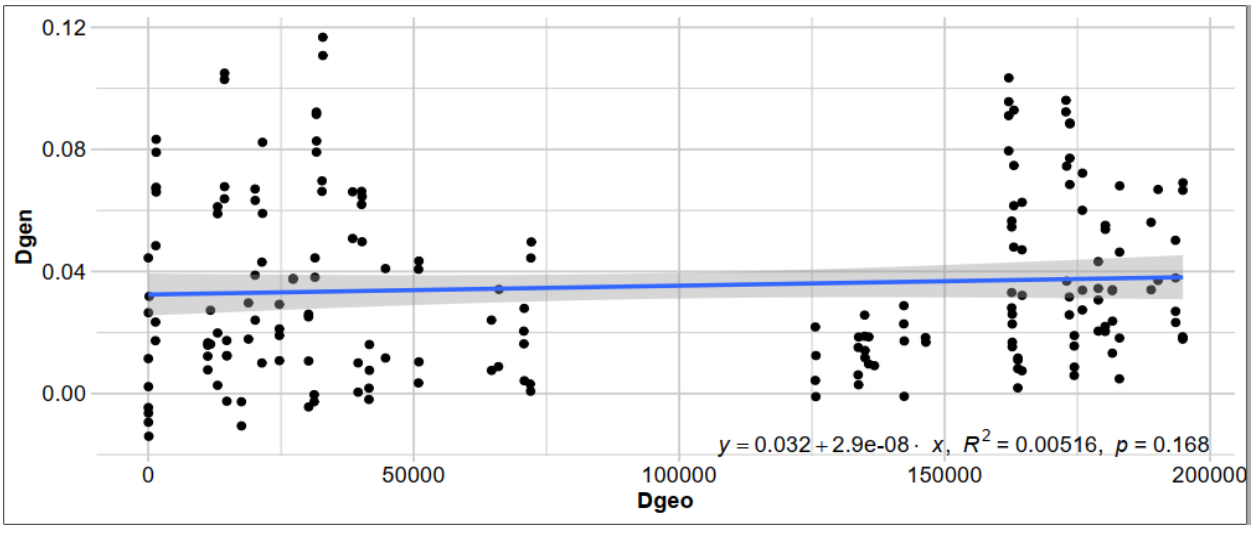

Figure 3.3. Correlation between genetic and geographic distances. The x-axis represents geographic distance(m) and the y-axis represents genetic distance (F_{ST}). The R^2 and p values are the results of a Mantel-test (Mantel statistic, r = 0.07182; significance (p = 0.168) after 999 permutations).

It was also noted that, apart from between adult and progeny cohorts of the same site, the geographic proximity of the populations did not necessarily imply increased gene flow. For example, the geographic distance between the TBO and TSO populations (about 1.3 km) as well as the WAB and WGD populations (about 1.5 km) were the two shortest distances between any two sites (Appendix 3.3). However, the gene flow between populations of these sites was among the lowest (Figure 3.2). Similarly, a Mantel-test did not reveal a significant correlation between the geographic and genetic distance matrices (Figure 3.3).

Table 3.3. Analysis of molecular variance (AMOVA) based on different factors for grouping the samples. The grouping factors were taken from the description of the populations in Table 3.1 and the DAPC cluster assignments

Source of variation	Df	Sum Sq	Mean Sq	Sigma	%	Phi(Φ)	p value*
Based on prior population assignments							
Between pops	19	2983.29	157.02	2.58	3.54	0.04	0.01
Between samples Within pop	153	17199.45	112.41	42.14	57.84	0.60	0.01
Within samples	173	4867.00	28.13	28.13	38.62	0.61	0.01
Total	345	25049.74	72.61	72.85	100		
Based on administrative region (Sidama vs Gurage)							
Between regions	1	241.48	241.48	0.03	1.07	0.01	0.01
Between samples within region	171	19941.26	116.62	44.24	60.47	0.61	0.01
Within samples	173	4867.00	28.13	28.13	38.45	0.61	0.01
Total	345	25049.74	72.61	73.16	100		
Based on DAPC clusters							
Between Clusters	4	1373.72	343.43	3.63	4.93	0.05	0.01
Between samples Within Cluster	168	18809.02	111.96	41.91	56.89	0.60	0.01
Within samples	173	4867.00	28.13	28.13	38.19	0.62	0.01
Total	345	25049.74	72.61	73.67	100		

*after 10,000 permutations

The results of the AMOVA are presented in Table 3.3. The samples were grouped according to various factors, including prior population assignments based on administrative regions, specific collection sites, and *de novo* clustering assignments of samples using the DAPC analysis results. Small but significant molecular variation between groups was found for all these factors. Among the prior group assignments, the highest between-group variation (3.54%) was observed between populations, followed by between regions (1.07%). *De novo* cluster assignment using DAPC revealed slightly greater between-group variation (4.93%) than the prior population assignments.

In all the cases, the variation between samples within each group (57–61%) was the greatest, followed by the variation within samples (about 38%).

3.3.4. Population Structure and Phylogenetic Analysis

Figure 3.4 shows the results of the principal component analysis of individual genotypes. The first two principal components accounted for only 5.4% of the variation in the data. Most of the samples clearly clustered together, with the exception of WGD and a few samples from the WGD-p populations, which formed a distinct cluster to the left of the biplot.

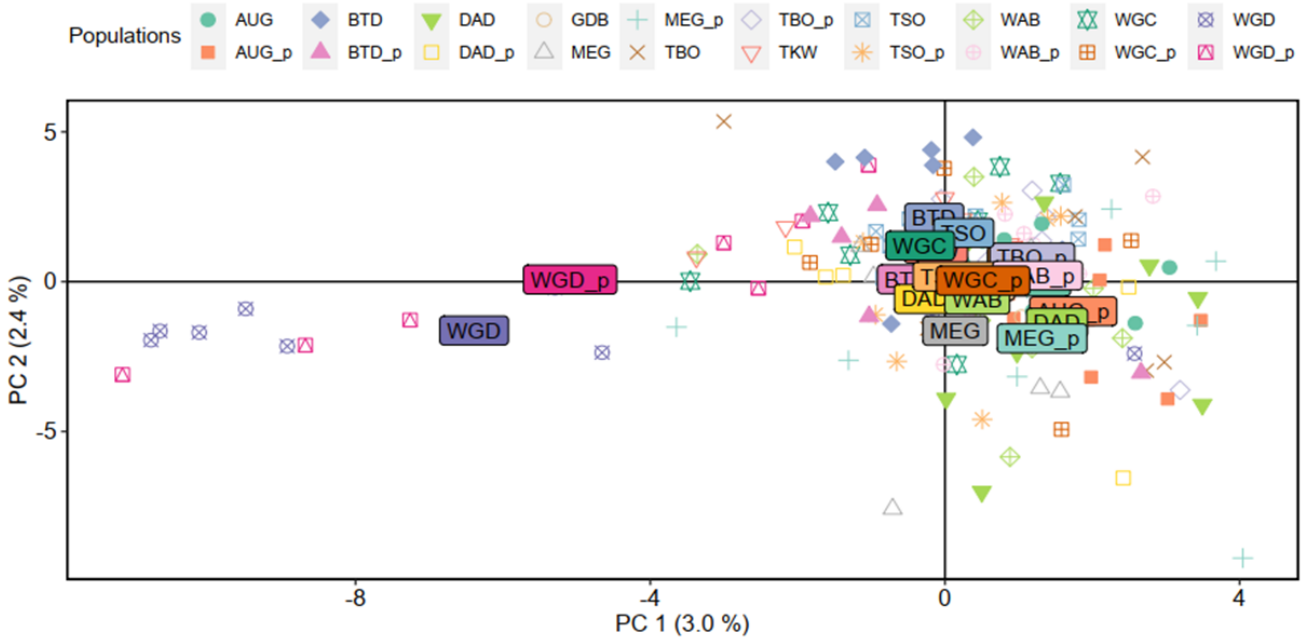

Figure 3.4. A biplot of the first two principal components (PC 1 and PC 2) of *A. gracilior* genotypes. Different colors represent different prior population assignments. Population codes are as described in Table 3.1.

The DAPC classified the genotypes into 5 clusters (Figure 3.5) on the basis of the minimum BIC value observed at K = 5. In total, 28 (16%) of the genotypes were assigned to the first cluster, 34 (20%) to the second cluster, 30 (17%) to the third cluster, 11 (6%) to the fourth cluster, and 70 (40%) to the fifth cluster. Notably, 10 out of the 11 genotypes in the fourth cluster were from either the WGD or WGD-p populations. Some adult populations exhibited greater genetic structure than their corresponding progeny populations did (Figure 3.5C), a pattern particularly evident in the

TSO-p versus TSO and BTD-p versus BTD population pairs. Among these populations, the TSO population displayed the least admixture, followed by BTD and GDB, all of which are progeny populations.

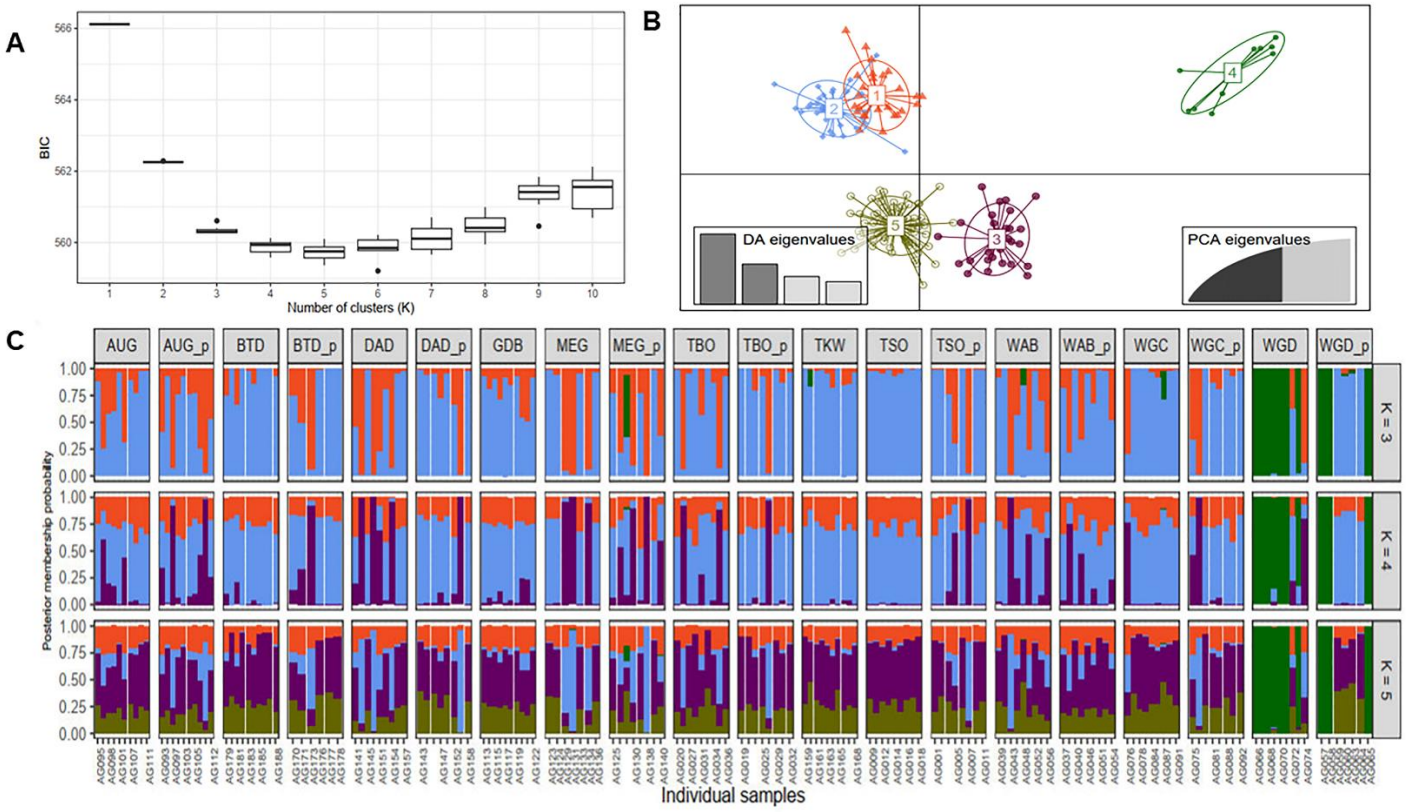

Figure 3.5. Results of the DAPC analysis. A) Boxplots of BIC values against the number of clusters used to determine the optimum number of clusters, K; B) Scatter plot of the DAPC analysis displaying 5 clusters; C) Structure-like bar-plots of individual samples on the basis of their posterior membership probability from the DAPC analysis. The population codes are as described in Table 3.1.

Figure 3.6 shows the neighbor-joining tree of individual genotypes, initially forming 3 main clusters (colored red, blue, and green). The blue and green clusters further subdivide into two clusters each, resulting in a total of 5 distinct clusters. Figure 3.7 presents a UPGMA phylogenetic tree of the populations, with the WGD populations showing the earliest divergence, supported by a 100% bootstrap value. The Sidama populations, highlighted in green, appear to have evolved before the Gurage populations, except for the MEG populations, which are positioned in between. At the top of the tree, the GDB and TKW populations represent the most recent evolutionary branches.

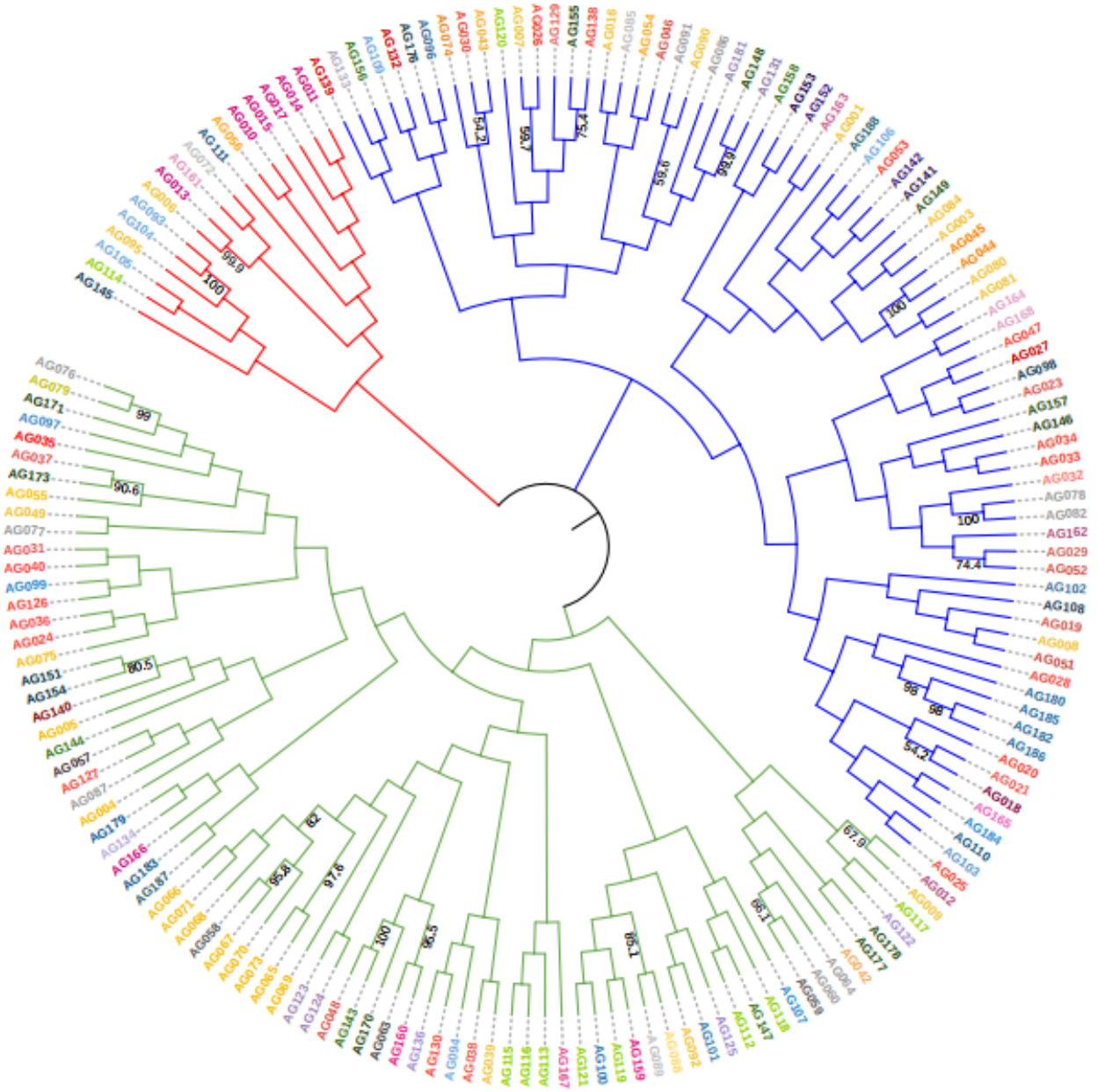

Figure 3.6. A neighbor-joining dendrogram showing the genetic relationships of individual *A. gracilior* genotypes sampled in this study. The branch colors indicate distinct clusters. The labels at branch tips refer to the codes of the individual genotypes; individuals with similar colors belong to the same population. Node labels show bootstrap support (with 50% cutoff) after 1000 replicates

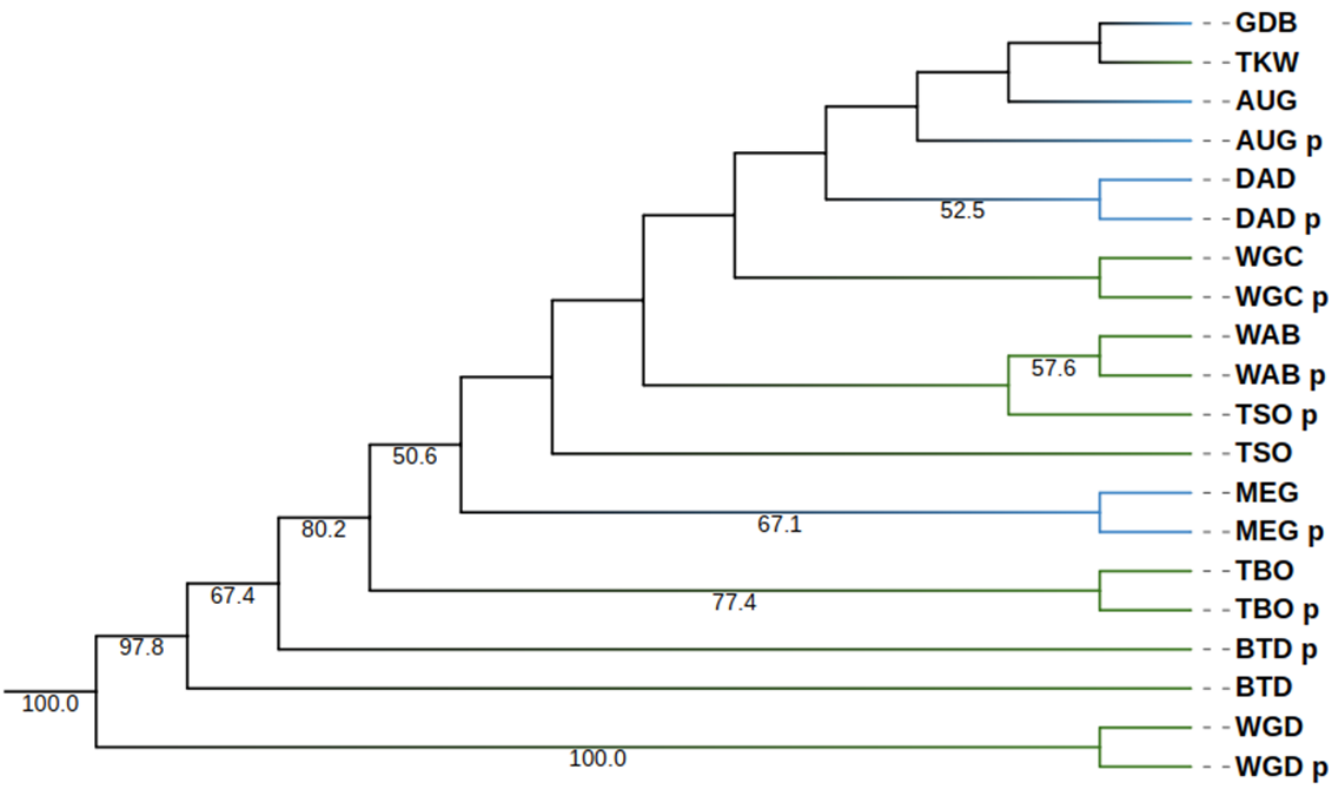

Figure 3.7. An unweighted pair group method with arithmetic mean (UPGMA) tree showing the genetic relationships of the study populations using Nei genetic distance. Node labels display bootstrap support (with 50% cutoff) after 1000 replicates. The labels at the tip of each branch (leaf) refer to the population codes (Table 3.1). Leaves colored blue belong to the 'Gurage' populations, whereas those colored green belong to the 'Sidama' populations.

3.4. Discussion

Marker quality information

Prior to genetic analysis, it was crucial to filter the DArTseq-generated SNP data to retain only high-quality and informative markers. After filtering, only 18% of the original 10,219 SNPs remained for genetic diversity analysis. The 1.1 transition-to-transversion ratio (Ts/Tv) was consistent with expectations as transitions are more common because of their lower energy requirements and preference by natural selection (Stoltzfus and Norris, 2016; Cote-L'Heureux et al., 2023). This ratio indicated acceptable SNP quality, which was further supported by a genotype accumulation curve showing sufficient markers to distinguish multilocus genotypes.

The average polymorphic information content (PIC) was 0.0735, which Serrote et al. (2020) categorized as low. However, over 25% of the SNPs fell into the medium to very high informativeness range. The generally low PIC values likely reflect low heterozygosity in the sampled genotypes rather than poor marker quality, as high PIC values are associated with high polymorphism in the samples genotyped (Huanget al., 2022). The significant deviations from HWE were also likely due to factors such as nonrandom mating, population structure, inbreeding, and genetic drift in small, fragmented populations rather than genotyping error (Waples, 2015; Chen and Cole, 2017). For this reason, we opted not to filter out loci that showed significant deviation from HWE. This is supported by Pearman et al. (2022), who showed that commonly applied HWE-based filtering can affect population structure inference. Furthermore, poor-quality samples or genotyping errors that might cause significant HWE deviations were likely filtered out by the applied criteria, such as the call rate and reproducibility thresholds.

Overall, some quality concerns cannot be fully ruled out due to the use of *de novo* SNP calling (Torkamaneh et al., 2016) and the absence of prior SNP-based research on this species for comparison. However, the SNPs proved valuable for the study, as confirmed by the genotype accumulation curve and consistent patterns in the results of downstream genetic analyses.

Genetic diversity

The overall lack of genetic diversity revealed in the *A. gracilior* populations sampled in the present study can be attributed to several factors. First, these populations were primarily fragmented, small, and isolated (Table 3.1). As a result, they may have been subjected to the genetic consequences typical of such populations, including nonrandom mating, reduced gene flow, inbreeding, and genetic drift, all of which can reduce genetic diversity (Lowe et al., 2005; Dubreuil et al., 2010; Schlaepfer et al., 2018; Minter et al., 2021; Sen and Ravikanth, 2022). Second, similar to most conifers, which have slower life cycles and specialized environmental adaptations (Buschiazzo et al., 2012; De La Torre et al., 2017), the evolutionary rate of *A. gracilior* might be slow, resulting in lower intraspecific diversity. Third, the low genetic diversity may be caused by a founder effect, where new environments are colonized by a small number of individuals (founders) from another population (Lef`evre et al., 2004; Parisod et al., 2005). The effect would be particularly strong if the founding populations were small and isolated. The populations in the

present study might have descended from ancestral populations that persisted in small numbers for generations, such as the WGD population, which diverged first in the UPGMA tree (Figure 3.7). Even the adult populations from this site had similarly low genetic diversity scores. Consequently, founding populations with low genetic diversity may have played a significant role in the overall low genetic diversity observed in the *A. gracilior* populations.

The significant index of association (I_A) observed in seven of the study populations indicates the presence of multilocus linkage disequilibrium (LD), with higher scores reflecting greater disequilibrium (Agapow and Burt, 2001). LD occurs when alleles at different loci in the genome are nonrandomly associated, which implies processes such as nonrandom mating and clonal propagation (Slatkin, 2008; Qanbari, 2020). In fragmented populations, this often includes selfing or biparental inbreeding. In randomly mating populations, both I_A and rbarD are expected to have values close to zero (Aboul-Naga et al., 2022), with significant deviations from zero indicating nonrandom mating and clonal propagation. Thus, the significant LD observed in the seven populations likely results from nonrandom mating and inbreeding due to fragmentation. A more pronounced index of association in progeny than in adults, observed in six of the seven populations, suggests that the genetic consequences of fragmentation intensify with each generation. The adult cohorts might have regenerated from seed dispersals of relatively intact, randomly mating populations, except for the TSO-p cohort, which also showed significant LD. This exception may be explained by the trees being planted rather than naturally regenerated, potentially from inbred seedling sources.

The I_A results align with other genetic diversity metrics, such as allelic richness (A_R) and unbiased expected heterozygosity (uH_E), both of which are commonly used to evaluate within-population genetic diversity. The GDB and TSO populations recorded the lowest scores for these indices, whereas WAB-p had the highest scores. In the TSO population, the wildling progeny samples originated from a single, isolated mother tree, with only a few adult male trees located over 200 meters away (Table 3.1). Both the mother tree and the likely father trees were planted in village front yards, which may have had a narrow genetic base, resulting in limited pollination opportunities and increased biparental inbreeding.

The GDB samples were collected from a tree nursery where the seedlings were grown from seeds of unknown origin. It is likely that the seeds came from a single isolated tree or just a few mother trees, as seed-sourcing practices in Ethiopia are generally poor (Derero, 2011; Dedefo et al., 2017; Mehari et al., 2024; Abate & Abebe, 2024). In contrast, nursery seedlings sourced from wildlings in the TKW population showed genetic diversity levels comparable to those of larger populations. This suggests that wildlings may be better sources of germplasm for afforestation or restoration efforts of *A. gracilior* than seeds of unknown origin or those collected from a limited number of mother trees. Other studies have also supported the use of wildlings as acclimatized and cost-effective germplasm alternatives in forest restoration (Tolentino, 2008; Reis et al., 2021; Bailey et al., 2024).

The WAB population, located at a culturally protected worship site, is one of the larger populations studied (Table 3.1). Trees at this site are considered sacred, and cutting them is believed to bring misfortune (Doda and Abuelgasim, 2019). This strict protection has allowed for a dense canopy of trees, which likely facilitates gene flow and contributes to the relatively high genetic diversity observed. This finding is consistent with other research in southern Ethiopia, where *Olea europaea* subsp. *cuspidata* trees in church forests (another type of sacred site) exhibited greater genetic diversity than those at adjoining fragmented and disturbed sites (Kassa et al., 2017).

The high positive inbreeding coefficient (F_{IS}) values observed across all populations provide direct insight into the level of inbreeding within populations. These high values indicate a deficit of heterozygosity, indicating that observed heterozygosity is lower than expected, which implies that inbreeding is present in all populations. However, the F_{IS} scores are somewhat inconsistent with the findings from other genetic diversity metrics discussed previously. Surprisingly, in most cases, the F_{IS} values for the progeny cohorts were lower than those for the adult cohorts. Additionally, populations with the lowest diversity scores, such as GDB and TSO, also exhibited the lowest F_{IS} values, suggesting that they are the least inbred, which is inexplicable.

On the other hand, population-specific F_{ST} scores align more closely with the genetic diversity results. Notably, the GDB (0.271) and TSO (0.265) populations recorded the highest population-specific F_{ST} values. These scores suggest that these populations have a greater likelihood of alleles being identical by descent and indicate significant genetic drift from an ancestral population.

Therefore, population-specific F_{ST} may serve as a more reliable indicator of inbreeding levels than F_{IS}. A meta-analysis on the genetic effects of habitat fragmentation (Schlaepfer et al., 2018) supports this, showing that F_{IS} is the genetic diversity index least affected by fragmentation. Furthermore, Kardos et al. (2016) advised against relying solely on F_{IS} as a measure of inbreeding within populations, as it can be biased by factors such as population size and allelic frequency differences between male and female samples.

Similar to the findings for F_{IS}, the results of the HWE tests did not consistently align with the patterns observed in other genetic diversity indices. Although significant deviations from HWE are typically expected in small and inbred populations experiencing genetic drift (Waples, 2015; Lachance, 2016), the percentage of loci showing significant deviations in each population did not follow this pattern. This inconsistency is likely due to differences in allele frequency between the sexes, as *A. gracilior* is a dioecious species. These differences can produce an excess of heterozygotes, which is more pronounced in smaller populations, and can bias HWE test results (Waples, 2015).

Population structure and differentiation

The relatively higher population-specific F_{ST}, as well as higher pairwise F_{ST} and lower gene flow scores for the progeny populations TSO and GDB, indicate that they are genetically more differentiated (i.e., they have drifted away) from the given set of populations. This is likely due to genetic drift as a result of inbreeding, as these progeny samples originated from a single, isolated mother tree or only a few mother trees (as explained under the genetic diversity section above). However, the explanation for the WGD populations, which showed highest pairwise Fst and lowest gene flow scores despite their genetic diversity scores not being as low as those of the TSO and GDB populations, would not be as straightforward.

The WGD site is an ancient *Gudumale*, a cultural community gathering site in Sidama, with only a few old trees. The trees in the adult cohort themselves might have descended from a few very ancient trees (2 trees on the site look distinctively very old, probably older than 200 years, Appendix 3.4). The site does not support natural regeneration as the ground is often trampled for gatherings. However, nearby farmers spread seeds from the few old mother trees in their backyard. The progeny samples from this site were collected from one such backyard. Thus, the most likely

reason why the adult and progeny populations from this site showed greater genetic differentiation, despite not showing low genetic diversity, is that those few old trees retained the ancient gene pool. This is also reflected by the fact that the WGD trees were the first to diverge in the UPGMA tree (Figure 3.7), which shows the evolutionary relationship between the populations. This supports the theory that the long-lived nature of trees and overlapping generations on the same site delay the loss of genetic diversity (Lowe et al., 2015). This argument corroborates the findings of a recent genotyping study on Sicilian monumental olive trees over 400 years old (Marchese et al., 2023), which revealed high genetic diversity and hailed the old olive trees as valuable gene reservoirs.

The pairwise F_{ST} and gene flow scores, along with Mantel and AMOVA tests, suggest that *A. gracilior* populations in the Gurage and Sidama regions show little genetic differentiation, despite being separated by the Great Rift Valley with distances of at least 125 km between them. This lack of differentiation is surprising given the absence of *A. gracilior* in the Rift Valley, which can connect the populations in the two regions through a 'stepping-stone' type of gene flow. Whereas wind pollination between these regions is highly unlikely, occasional long-distance seed dispersal by frugivorous animals could theoretically maintain some genetic connectivity. However, this possibility is not strongly supported, as some populations within the same region, separated by less than 2 km, exhibited higher pairwise F_{ST} scores than those between the regions.

The genetic similarity between the regions may be attributed to the slow rate of neutral mutation in conifers (e.g., Buschiazzo et al., 2012; De La Torre et al., 2017) rather than ongoing gene flow. If populations in these regions share a common ancestral population and/or were isolated recently (in evolutionary terms), there may not have been enough time for significant genetic divergence. Additionally, *A. gracilior's* narrow ecological range, between altitudes of 1500 and 2500 m, might lead to stabilizing selection (Lieberman and Dudgeon, 1996; Rolhauser and Pucheta, 2017), favoring the same alleles across all populations, further reducing genetic differentiation.

The relatively high genetic differentiation between geographically proximate populations within a region is likely driven by isolation due to environmental adaptation. Natural selection in landscapes with diverse ecological features promotes local adaptation, as established in various studies (Holderegger et al., 2006; Orsini et al., 2013; Teixeira and Huber, 2021). This adaptation

minimizes effective gene flow among ecologically divergent habitats, as migrants from new environments often fail to establish and survive, leading to genetic isolation by adaptation. This contrasts with the more common genetic isolation by distance, where reduced gene flow is due to increasing geographic distance. A study on two alpine conifers (Mosca et al., 2014) highlighted that genetic structure is influenced by both geographic distance and local environmental adaptation, emphasizing the dual impact of these factors on genetic differentiation.

The greater molecular variance observed among individuals within populations than between populations, as revealed by the AMOVA, aligns with common findings in molecular marker-based studies of forest trees, where typically less than 10% of the variation occurs among populations (Danusevi˘cius et al., 2024). This pattern is largely due to gene flow and genetic diversity being shaped by fine-scale genetic structuring within populations (Danusevi˘cius et al., 2024; Sandurska et al., 2024). Fine-scale genetic structure refers to the nonrandom distribution of genotypes within a forest, often resulting from gravity-driven seed dispersal, which causes seeds to fall near the parent tree. Consequently, neighboring trees are genetically related, leading to biparental inbreeding across generations. In *A. gracilior,* which has large, gravity-dispersed seeds, this fine-scale structuring likely contributes to greater within-population genetic variation. Additionally, as a dioecious species and obligate out-crosser, *A. gracilior* may tend to maintain higher heterozygosity within populations. This is supported by studies showing that dioecy is associated with increased genetic diversity (Muyle et al., 2021) and spatial genetic structure (Nazareno et al., 2013) in plant populations.

The clustering analyses grouped the genotypes into 3–6 clusters, which appeared consistent across the different approaches. According to Jombart and Collins (2017), the optimal number of clusters (K value) typically corresponds to the lowest BIC value. However, in practice, the 'elbow' of the BIC curve, where it first turns, is often selected as the optimal K value. In this study, the lowest BIC value was observed at K = 5, with the elbow at K = 3 (Figure 3.5a). Additionally, BIC values at K = 4 and K = 6 were nearly identical to the minimum, making the selection of 3–6 clusters logical across different approaches. The neighbor-joining phylogenetic tree (Figure 3.6) showed that the genotypes initially formed three main clusters, which later subdivided into five. However, individuals from different populations exhibited a mixed clustering pattern, with weak bootstrap support at higher-level clusters, indicating a lack of strong genetic differentiation among the study

populations. The PCA, DAPC, and UPGMA analyses all consistently identified the WGD population as a divergent group.

The UPGMA tree suggests a directional evolutionary trend from Sidama to Gurage, with Sidama populations likely evolving first. The MEG population (of Gurage), however, disrupts this pattern. Notably, MEG is an ancient monastery site that harbors very old trees (Table 3.1). The WGD population, an ancient *Gudmale* (a cultural gathering site preserved for generations), diverged early from the Sidama populations, while the MEG population diverged from the Gurage populations. This pattern indicates that these ancient sites have preserved unique genetic lineages, reflecting ancient gene flow. These findings emphasize the critical role of sacred places in conserving genetic diversity, as highlighted by previous studies (Wassie et al., 2010; Aerts et al., 2016; Klepeis et al., 2016; Kassa et al., 2017; Doda and Abuelgasim, 2019; Sahle et al., 2021; Teku et al., 2024).

3.5. Conclusion

This study employed DArTseq-generated SNP markers to examine the genetic diversity and population structure of *A. gracilior*, marking the first molecular marker-based investigation for this species. The findings revealed that genetic diversity is generally low across the sampled populations, with progeny from isolated or few mother trees exhibiting the lowest diversity and highest genetic drift. These results suggest that *circa situm* conservation in small and isolated agroforestry populations is unlikely to maintain viable genetic diversity in *A. gracilior*; larger patch sizes are essential. The higher and sometimes unique genetic diversity observed in sacred sites highlights their significance as vital gene pools for the species' restoration, emphasizing the need for robust *in situ* conservation. Furthermore, nursery seedlings from seeds showed low genetic diversity, whereas those from wildlings were more genetically diverse, similar to populations from larger patches, highlighting the limitations of current seed sourcing practices.

Given these findings, it is recommended that seed and wildling collection from a few isolated mother trees, such as those in front yards, should be discouraged, even if these trees are prolific seeders. Instead, seeds should be sourced from diverse, larger populations, particularly from

culturally protected sites and natural remnant patches, which have demonstrated higher genetic diversity. Additionally, mixing seeds from different populations could mitigate the effects of inbreeding and genetic drift. Since this study did not encompass all populations within the species' native range in Ethiopia or other East African countries, the results should not be generalized to the entire range. Future research should incorporate a broader range of populations to develop a more comprehensive understanding of *A. gracilior*'s genetic diversity.

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CHAPTER FOUR

4. Genetic Consequences of Forest Fragmentation: Inbreeding Depression Manifested through Reduced Progeny Fitness in *Afrocarpus gracilior*

Abstract

Human activities such as agriculture, urbanization, and logging have caused widespread destruction of forests, leading to forest fragmentation. Fragmentation has been shown to reduce genetic diversity and increase inbreeding in forest populations, potentially leading to inbreeding depression manifested through decreased reproductive success and progeny vigor. However, the severity of these impacts varies among species and is largely influenced by their mating systems. This study examines the effects of forest fragmentation on *Afrocarpus gracilior*, a dioecious, wind-pollinated conifer, by assessing genetic diversity, reproductive success, and early progeny fitness. Genetic diversity was assessed using DArTseq-generated SNP markers, while reproductive success and progeny fitness were evaluated through on-site assessments, physical seed quality tests, *in vitro* germination tests, and early growth monitoring in a lathhouse. Our analysis revealed alarmingly low genetic diversity and high genetic drift, especially in small and isolated populations. Consistent with these findings, the *in vitro* germination test and early progeny vigor assessment in a lathhouse revealed reduced progeny fitness. Small populations exhibited 53% lower germination rates, 33% reduced acclimatization, 30% slower diameter growth, 41% reduced height growth, and an 80% increase in leaf scorch. Correlation analysis further confirmed a strong relationship between the genetic diversity and progeny fitness traits. These findings suggest that inbreeding depression severely affects the fitness of progeny from small and isolated populations of *A. gracilior*, posing a serious threat to their long-term survival. The implications for conservation and restoration efforts are immense, underscoring the need to prioritize genetically diverse populations for conservation and strategically procure seeds to support the survival of this species.

Key words: gene flow, genetic diversity, inbreeding, *in vitro* germination, progeny vigor

4.1. Introduction

Human activities such as agriculture, urbanization, and logging have caused widespread destruction of forests, leading to forest fragmentation. Fragmentation occurs when large, intact, and continuous forests are broken into smaller, isolated patches separated by non-forest land (Young & Boyle, 2000; Bacles & Jump, 2011; Schlaepfer et al., 2018). In these fragmented forest patches, the reduction in population size and increased isolation limit the number of available pollen donors and restrict pollinator movement. This results in a decrease in both the quantity and diversity of pollen being deposited (Cunningham, 2000; O'Connell et al., 2006; Seltman et al., 2007; Broadhurst, 2015). The restricted gene flow also leads to mating between related individuals and self-pollination (in self-compatible species), which causes inbreeding, a loss of genetic diversity, and genetic drift (Ellstrand & Elam, 1993; Aguilar & Galetto, 2004; Bacles & Jump, 2011; Lloyd et al., 2018). The reduction in genetic diversity and the prevalence of inbreeding diminishes a population's ability to adapt to environmental changes. This is evidenced by reduced progeny fitness, known as inbreeding depression, which results from the expression of deleterious mutations in homozygous inbred offspring (Charlesworth & Willis, 2009; Eckert et al., 2010). Inbreeding depression is manifested through decreased reproductive success and lower progeny vigor (Cascante et al., 2002; Seltman et al., 2007; Aguilar et al., 2019; Aguilar-Aguilar et al., 2023), which can ultimately threaten the long-term survival of populations and potentially lead to local extinction (Young et al., 1996; Frankham, 2005; Reed, 2005; Schlaepfer et al., 2018; Phang et al., 2024).

Over the past three decades, the genetic and demographic impacts of forest fragmentation have been the focus of extensive research, supported by advancements in molecular marker technologies (reviewed by Young et al., 1996; Lowe et al., 2005; Kramer et al., 2008; Vranckx et al., 2012; Finger et al., 2014; Schlaepfer et al., 2018; Aguilar et al., 2019; González et al., 2020). The findings of these studies have been diverse. Some have demonstrated that fragmentation negatively impacts genetic diversity and progeny fitness in trees (e.g., Cascante et al., 2002; Aguilar & Galetto, 2004; Hirayama et al., 2005; Jump & Peñuelas, 2006; Seltmann et al., 2009; Lloyd et al., 2018; Aguilar-Aguilar et al., 2023; Phang et al., 2024). For instance, Cascante et al. (2002) found that seedlings of *Samanea saman* from isolated trees exhibited lower germination rates, reduced leaf area, and biomass compared to those from continuous forest populations. Similarly, Seltman et al. (2009)

reported reduced vigor (lower N-metabolism capacity) and high mortality in *Polylepis australis* seedlings from short-distance pollination crosses compared to those from long-distance crosses. Lloyd et al. (2018) highlighted limited pollen dispersal distances and biparental inbreeding in *Vallisneria americana*, exacerbated by habitat fragmentation. Recently, Phang et al. (2024) identified genetic erosion, marked by reduced heterozygosity and increased inbreeding in juvenile populations compared to adult populations of *Palaquium obovatum*.

In contrast, other studies suggest that gene flow in some tree species remains largely unaffected by habitat fragmentation, indicating potential resilience (e.g., White et al., 2002; O'Connell et al., 2007; Silva et al., 2008; Kamm et al., 2009; Lander et al., 2010; Ashworth et al., 2015; Broadhurst, 2015; Guidugli et al., 2016). This resilience, against the genetic expectations for small and fragmented populations, has been described as "the paradox of fragmentation genetics" (Kramer et al., 2008). However, Lowe et al. (2015) argued that this phenomenon is "no longer a paradox," emphasizing that earlier studies primarily focused on adult populations, which often fail to capture current gene flow dynamics in long-lived trees. They described this narrow focus as "looking in the wrong place" and called for more comprehensive approaches. In response, recent research has adopted a broader approach, incorporating both adult and progeny cohorts in genetic diversity assessments, conducting gene flow and paternity analyses, and examining diverse mating systems and pollination mechanisms. This shift has provided deeper insights into the interplay between forest fragmentation and genetic diversity, offering a more nuanced understanding of tree species' responses to environmental changes.

A common shift in mating systems involves reduced outcrossing and increased selfing, which may result in inbreeding depression (Aguilar et al., 2006; Broadhurst, 2015). Inbreeding depression typically results in reduced seed production and less vigorous progeny. While most tropical trees are primarily outcrossing (Cascante et al., 2002; Ward et al., 2005), their hermaphroditic and self-compatible nature makes them facultative out-crossers (Eckert et al., 2010). In fragmented habitats, a reduction in the number of mating trees disrupts pollinator movement, hindering effective pollination (Wilcock & Neiland, 2002; Breed et al., 2015). Pollinators adapt by shortening their foraging distances and spending more time on individual trees (Breed et al., 2015). Consequently, trees dependent on these pollinators are forced to lower their outcrossing rates and increase selfing (Aguilar et al., 2006; Breed et al., 2015; Broadhurst, 2015; Aguilar et al., 2019).

According to Aguilar et al. (2019), the severity of inbreeding depression due to selfing varies with a species' mating history. Populations with a long history of selfing are less prone to severe inbreeding depression, as continuous selfing over generations tends to purge deleterious alleles. In contrast, species that predominantly outcross but shift to selfing due to habitat fragmentation are likely to suffer significant inbreeding depression, particularly in early fitness traits such as germination and survival.

Dioecious and self-incompatible species are obligate out-crossers, meaning that they cannot shift to selfing (Aguilar et al., 2006; Broadhurst, 2015; Cristóbal-Pérez et al., 2021; Aguilar-Aguilar et al., 2023). Dioecism serves as an adaptation to prevent self-fertilization and enhance heterozygosity by involving multiple parents in reproduction (Arruda et al., 2015). Consequently, selfing-induced inbreeding is inherently impossible. Nonetheless, biparental inbreeding can still occur, involving mating between closely related individuals (Broadhurst, 2015; Arruda et al., 2015; Vinson et al., 2018). Dioecious species are particularly susceptible to the effects of habitat fragmentation (Aguilar et al., 2006; Cristóbal-Pérez et al., 2021; Aguilar-Aguilar et al., 2023, Aguilar et al., 2024), as decreases in population size and density can influence several ecological factors that affect reproductive success. These factors include the sex ratio, the spatial distribution of male and female trees, and the foraging behavior of pollinators.

Dioecious species typically rely on small insects and wind for pollination (Cristóbal-Pérez et al., 2021; Aguilar-Aguilar et al., 2023). Habitat fragmentation can restrict the mobility of small insect pollinators, making long-distance foraging inefficient (Breed et al., 2015), which reduces pollen availability for female dioecious trees. This pollen limitation often results in lower seed set (Wilcock & Neiland, 2002; Ohya et al., 2007) and may ultimately disrupt reproduction in fragmented populations. In contrast, wind pollination is commonly expected to facilitate long-distance gene flow (Seltmann et al., 2007; Provan et al., 2008; Ashley, 2010, 2021; Dubreuil et al., 2010; Broadhurst, 2015; Zeng & Fischer, 2019; Aguilar-Aguilar et al., 2023), potentially reducing the vulnerability of wind-pollinated species to the genetic impacts of fragmentation.

Nonetheless, evidence suggests that even wind-pollinated species are not immune to fragmentation-induced genetic challenges, with several studies documenting declines in reproductive success and progeny fitness due to inbreeding. Examples include Quercus *douglasii*

(Knapp et al., 2001), *Fagus sylvatica* (Jump & Peñuelas, 2006), Picea glauca (O'Connell et al., 2006), *Araucaria nemorosa* (Kettle et al., 2007, 2008), *Juniperus communis* (Provan et al., 2008), *Polylepis australis* (Seltmann et al., 2009), *Taxus baccata* (Dubreuil et al., 2010), and *Brosimum alicastrum* (Aguilar-Aguilar et al., 2023). For instance, Jump and Peñuelas (2006) demonstrated that habitat fragmentation in *Fagus sylvatica* caused genetic bottlenecks, disrupted breeding systems, and significantly elevated inbreeding, population divergence, and reduced genetic diversity—even in this widely distributed, wind-pollinated species. Similarly, Aguilar-Aguilar et al. (2023) found that fragmentation altered mating patterns in *Brosimum alicastrum*, a dioecious, wind-pollinated tree, resulting in progeny with reduced fitness, evidenced by fewer leaves with smaller foliar areas, shorter heights, and lower biomass compared to progeny from continuous forests. These mixed findings suggest that the impact of fragmentation on wind-pollinated species is complex and species-specific, underscoring the need for further research to better understand the genetic and ecological dynamics of trees in fragmented landscapes.

In this study, we explored the effects of fragmentation on the reproductive success and early progeny fitness of *Afrocarpus gracilior* (Pilg.) C. N. Page, a member of the Podocarpaceae family. The species is known by several common names, such as East African yellowwood, African fern pine, or weeping Podocarpus. This wind-pollinated, dioecious conifer is native to Ethiopia and other parts of East and Central Africa. Once dominant in the canopy of Afromontane forests across Ethiopia, *A. gracilior* has long been threatened by illegal logging for its high-quality timber (Negash, 2010; Teketay, 2011). Today, it is primarily found in inaccessible relict forest patches (Negash, 2003; Negash, 2010), some church forests (Aerts et al., 2016), and other culturally sacred sites (Doffana, 2014; Doda & Abuelgasim, 2019). Although classified as 'Least Concern' on the IUCN Red List due to its wide distribution, including areas outside Ethiopia, *A. gracilior* is considered threatened within Ethiopia and is among the few native trees prioritized for conservation efforts in the country (Vivero et al., 2005; Kalinganire et al., 2021). In the Sidama and Gurage regions of southern Ethiopia, however, *A. gracilior* remains relatively abundant, persisting in fragmented populations such as isolated individuals, small groups in front yards, graveyards, sacred sites, and natural forest remnants. The impact of fragmentation on the genetic diversity of these populations and the long-term viability of offspring from their seeds has not yet been examined.

A companion study (Abate et al., 2024, presented in chapter 3) assessed genetic diversity, using DArTseq-generated SNP markers, in adult and progeny cohorts from fragmented populations of varying sizes. This study revealed very low genetic diversity and high genetic drift, especially in progeny from isolated and small populations. The overall mean expected heterozygosity (He) was 0.072, and the mean allelic richness (Ar) was 1.24, with the maximum for SNP markers (which are biallelic) being 2. The mean He and Ar values for the adult populations were 0.075 (range: 0.067-0.081) and 1.243 (range: 1.218-1.278), respectively, while for the progeny populations, these values were 0.069 (range: 0.055–0.076) and 1.229 (range: 1.19-1.257), respectively. For He and Ar, smaller scores were recorded in small/isolated populations, and larger scores were recorded in relatively larger/intact populations. Similarly, progeny in small/isolated populations exhibited higher population-specific Fst values, indicating significant drift from ancestral populations.

Building on these findings, the present study aimed to explore whether the genetic erosion observed at the molecular level manifests as inbreeding depression, characterized by reduced reproduction and progeny fitness. Specifically, this study sought to: 1) determine whether populations of different sizes differ in physical seed quality traits; 2) evaluate differences in germination rates among seeds from these populations; and 3) assess variation in seedling survival and early progeny vigor. These insights are crucial for the conservation and restoration of *A. gracilior* in Ethiopia and other regions where the species is native.

4.2. Materials and Methods

4.2.1 Description of the Seed Source Populations

Seeds of *A. gracilior* were collected from selected populations in the Sidama and Gurage regions of southern Ethiopia (Figure 2). In these regions, the size distribution of *A. gracilior* populations varies significantly. Isolated trees or small groups of fewer than 10 individuals are commonly found in front yards, whereas groups of 10-50 individuals occur in graveyards and communal gathering places, known locally as *gudumales*. Conversely, remnant natural forest patches and sacred sites, such as churches and traditional worship places, host relatively large populations (Table 4.1, Figure 4.3). Populations for this study were chosen following a reconnaissance survey,

ensuring representation from all population types. Based on their size, populations were categorized as 'small' (area < 1 ha), consisting of isolated individuals or groups not more than approximately 50 trees; 'intermediate' (1 ≤ area ≤ 50 ha); and 'large' (area > 50 ha).

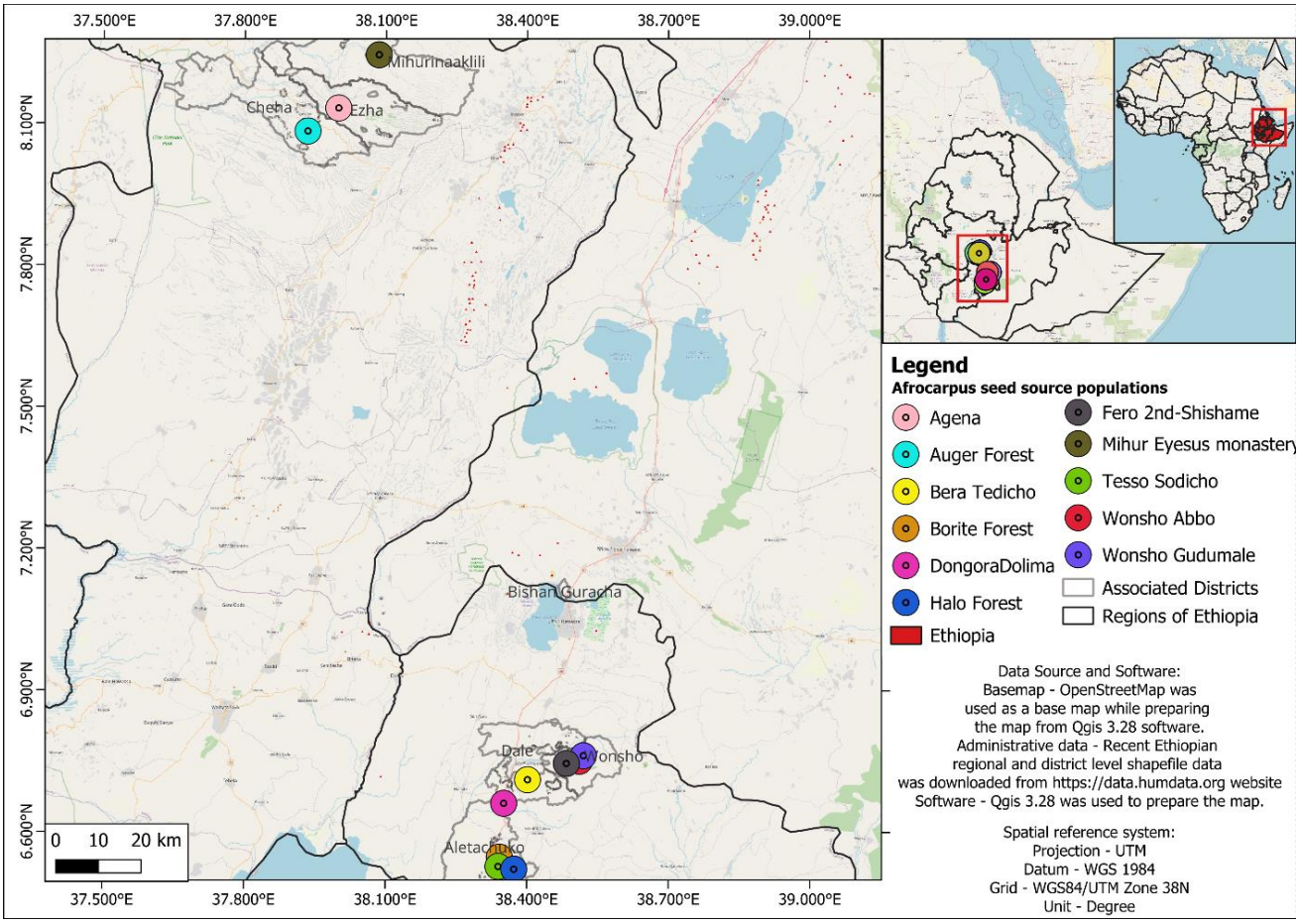

Figure 4.1.The locations of the sampling populations of *A. gracilior* are shown on the map of Ethiopia. Southern populations represent the Sidama region, whereas those in the north belong to the Gurage.

Table 4.1. Description of the *A. gracilior* populations examined in this study. The Agena, Fero 2nd-Shishame, Dongora-Dolima, and Halo Forest populations were not included in the genetic diversity analysis; they were only part of the reproduction and early progeny vigor assessments. Consequently, data from these populations are not included in the genetic diversity summary (Table 4.2) or the correlation analysis (Figure 4.9).

Site/Population	Region/Zone	Altitude (m.a.s.l.)	Population size class	Description
Agena	Gurage	2344	Small/isolated	An old female tree situated along a roadside in a town stands isolated, with no nearby conspecifics to act as pollen donors. Although seed production is relatively high, there is no observed regeneration beneath its crown.
Auger forest	Gurage	2226	Large/intact	A remnant natural forest patch featuring a mixed *Afrocarpus-Juniperus* community is relatively large and intact, covering 138 hectares with a density of approximately 250 trees per hectare. Although many trees produce seeds, the seed output per tree is relatively low. Nevertheless, there is healthy regeneration beneath the canopy.
Bera-Tedicho	Sidama	1807	Small/isolated	A small group of old remnant trees in a village comprises 54 trees within a 0.5-hectare area. The canopy is open, and seed production per tree is relatively high. While no regeneration is observed under the canopy, it is observed in nearby farms .
Mihur Eyesus monastery	Gurage	2330	Intermediate	This old monastery is situated on a plateau, featuring a front yard with several ancient *A. gracilior* trees, some over 200 years old. The plateau gradually slopes down into a dense, mixed-species forest spanning 16 hectares, with an *A. gracilior* density of approximately 100 trees per hectare. The forest exhibits a range of tree sizes, from juveniles to intermediate-sized trees, indicating robust regeneration. While the few ancient trees in the monastery's front yard produce a high number of seeds per tree, seed production is minimal in the dense canopy of the forest below.
Borite forest	Sidama	1930	Intermediate	This small natural remnant patch, located on a hilltop and covering about 10 hectares, has dense regeneration with numerous juvenile trees but relatively few adults (about 75 trees per hectare). The forest features an open canopy and exhibits relatively high seed production per tree.
Fero 2nd-Shishame	Sidama	1903	Small/isolated	wo old trees, one male and one female, are situated at the edge of a 0.5-hectare communal front yard. These trees are isolated, with no other conspecifics visible

				nearby. The female tree produces a substantial number of seeds. No regeneration beneath canopy.
Dongora-Dolima	Sidama	1800	Small/isolated	This communal graveyard, situated in the front yards of several houses, spans about 1 hectare and contains 53 adult trees, some of which are quite old. While regeneration is not observed beneath the canopy, it is present along fences by the road and in adjacent home gardens. The trees are widely spaced, and each tree produces a relatively high amount of seeds.
Halo forest	Sidama	1974	Intermediate	This segment of a relatively large natural remnant forest, situated on a continuous hilltop bordering three kebeles (rural counties), is facing encroachment due to farmland expansion and illegal timber harvesting. Seeds of *A. gracilior* were collected from the foothill on the Dara side of the forest, which covers about 6 hectares. The tree density here is sparse, approximately 100 trees per hectare. Regeneration is good at some spots, with a variety of age classes present. The canopy, dominated by a few old trees, is open, and seed production per tree is relatively high.
Tesso-Sodicho	Sidama	1795	Small/isolated	In the front yards of houses in a village, a few adult *A. gracilior* trees are present. A single isolated female tree, located over 200 meters from the nearest male trees, is the primary seed bearer in this area. This tree produces a very high quantity of seeds. Regeneration has been observed along fences and in adjacent home gardens.
Wonsho-Abbo	Sidama	2060	Large/intact	This population occupies the lower half of a culturally protected worship and traditional court site. The site encompasses 75 hectares of mixed forest with numerous indigenous tree species. The lower segment, predominantly composed of *A. gracilior*, is relatively dense, with 250 trees per hectare, and exhibits good regeneration. However, the seed production per tree is low.
Wonsho-Gudumale	Sidama	2110	Small/isolated	The site features 30 very large and old trees, some over 200 years old. This area, used for centuries as a communal gathering space known locally as a *gudumale* for cultural events, spans approximately 0.5 hectares. Only a few of these trees are seed-bearing, producing a high quantity of seeds. While there is no regeneration beneath the canopy, some regeneration is observed in the adjoining fences and home gardens.

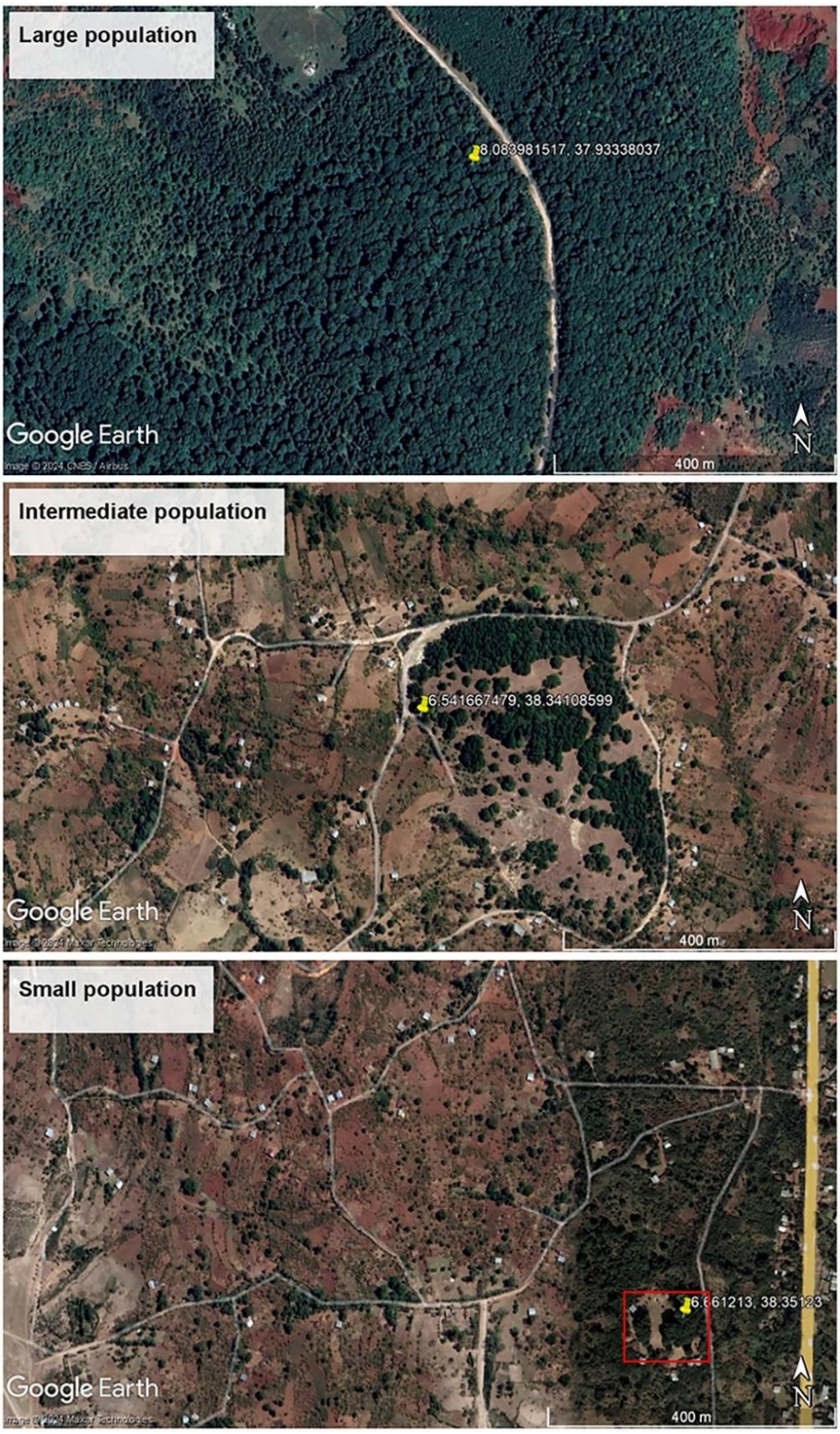

Figure 4.2. Google Earth images displaying examples of the different population types of *A. gracilior* included in this study. From top to down, there is a relatively large and intact remnant of an *A. gracilior*-dominated natural forest patch in Gurage (Auger in Table 4.1), an intermediate open natural remnant patch in Sidama (Borite in Table 4.1), and a small population consisting of a few *A. gracilior* trees at a communal graveyard, highlighted by the box (Dangora–Dolma in Table 4.1).

4.2.2 Genetic Diversity

Detailed methods for the genetic diversity analysis, including sampling, genotyping, and data processing, are outlined in the companion paper (Abate et al., 2024). Briefly, young leaf tissue samples were collected from adult and progeny populations of *A. gracilior* across 11 sites in southern Ethiopia. Two plates (188 samples) of silica-dried leaf tissues were sent to SEQART Africa in Kenya for DNA extraction and genotyping. DNA was extracted using the CTAB-based Nucleomag Plant Extraction Kit (Macherey-Nagel, Düren, Germany), and genotyping was conducted using DArTseq™ technology, which integrates a proprietary genome complexity reduction method with next-generation sequencing. The genotyping yielded two types of markers: silicoDArT markers, which are dominant and scored as binary, and SNP markers, which are biallelic and codominant. Only the SNP markers were utilized for the genetic diversity analyses due to their higher informativeness for population genetics studies compared to the dominant silicoDArT markers. After rigorous filtering of the raw SNP dataset, which initially contained 10,219 SNPs and 185 individuals, 1820 high-quality, informative SNPs and 183 individuals were retained for downstream analysis.

4.2.3 Population Inventory and Seed Collection

Inventories were conducted on the selected populations to determine population sizes, tree density, size-class distribution, estimated age of the dominant trees, and seedling recruitment (availability and density of seedlings and saplings under the canopy of adult trees). For the small populations, the inventory was based on a total census. We used 10-15 randomly distributed 10-meter radius circular plots for the intermediate and large populations. Seedling recruitment and density observations were carried out using four 1m × 1m quadrats nested within each sampling plot. Since *A. gracilior* is a dioecious species, an assessment was also made on the availability of male individuals near female individuals to ensure adequate pollination. Seed setting by female trees (those bearing female cones) was observed, and estimates of seed yield were noted. Mature female cones were collected from the crowns and from those that had fallen to the ground. In smaller populations, cones were collected from all cone-bearing female trees, while in larger populations, cones from 15–20 female trees were collected, depending on availability. Equal amounts of cones collected from each tree in each population were then combined into a single bag representing that population.

Mature female cones collected in this manner were then transported to the forest seed laboratory of the College of Agriculture at Hawassa University. Seed extraction was performed following the procedure outlined in Negash (2010). Briefly, the process involved soaking the cones in water for 2 days, gently squashing them with a wooden mortar and pestle, and washing them to remove the fleshy pulp from the inner seeds (Figure 4.3). The de-pulped seeds, which still had a hard seed coat or sclerotesta, were air-dried in the shade for 2–3 days, placed in seed bags, and stored in a refrigerator at 4°C until they were used for subsequent *in vitro* germination.

4.2.4 Physical Seed Quality Assessment and *In Vitro* Germination

The sclerotesta of an *A. gracilior* seed is a hard, woody structure that encases and protects the true propagule, which is enlarged by a large megagametophyte, also known as the female gametophyte (Negash, 2010). For germination to occur, the sclerotesta has to be cracked, allowing the inner propagules to be released (Figure 4 B & C). When the sclerotesta cracks, it may release clean, intact seeds. However, some seeds may be damaged (immature, shriveled, or infested with microorganisms) while others may be empty. In this study, we assessed physical seed quality using the proportions of intact, empty, and damaged seeds, along with seed weight. We randomly selected 100 seeds (5 replicates of 20 seeds each), cracked them, counted the number of intact, empty, and damaged seeds, and determined the percentage proportion of each. After that, we took 100 intact seeds (5 replicates of 20 seeds each), measured their weight on a digital balance, and calculated 1000 seed weights.

Prior to *in vitro* germination, the seeds were surface sterilized by soaking in a solution of 5% sodium hypochlorite followed by 70% ethanol for 10 minutes each. The seeds were subsequently rinsed in distilled and sterilized water three times to remove disinfectants. The disinfected seeds were then placed in a 10^{-4} M solution of gibberellic acid-3 (GA_3) to stimulate germination. Seeds recovered from this solution (Figure 4D) were placed in tissue culture jars (Figure 4E), with 5 replicates of 10 seeds per jar, containing 50 ml of 20 g/L melted and autoclaved agar. The jars were kept in the culture room of the tissue culture laboratory at Hawassa University, College of Agriculture in a completely randomized design (CRD). The room was maintained with 16 hours of light from fluorescent lamps, providing a photon fluorescence rate of 100 µmol m^{-2} s^{-1} at a temperature of 25°C. The *in vitro* germination rate

was monitored, and data recording was performed every three days over a 10-week experimental period.

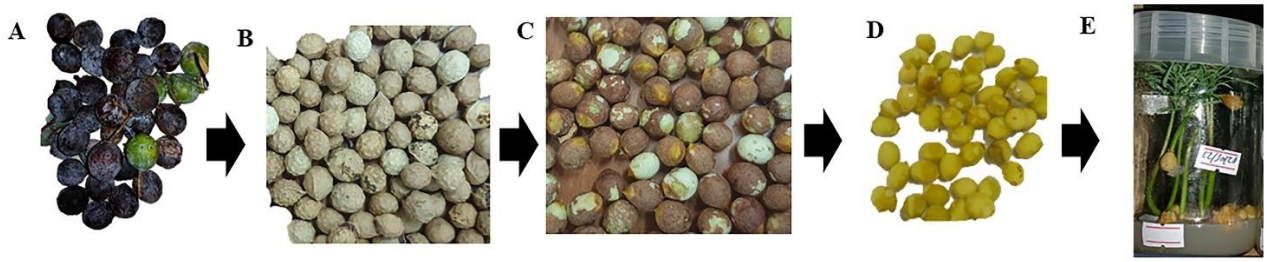

Figure 4.3. Process of *A. gracilior* seed collection to *in vitro* germination. A) Ripe female cones after collection; B) Seeds showing the hard seed coat (sclerotesta) after removal of the fleshy pulp (epimatium); C) The released true seeds after cracking the sclerotesta; D) Surface sterilized seeds ready for *in vitro* germination; and E) *In vitro* germinated *A. gracilior* seedlings in a tissue culture jar.

4.2.5 Assessment of Early Progeny Vigor in a Lathhouse

After the 10-week experiment in the tissue culture lab, the *in vitro* germinated seedlings were transferred to a lathhouse for acclimatization and early progeny vigor monitoring. The lathhouse was a shade house covered with a plastic roof and mesh wire ventilation. The seedlings were grown in 3-liter plastic pots filled with mixed growing media composed of local agricultural soil, compost, and sand at a 2:1:1 ratio, and arranged in a CRD. All *in vitro* germinated seedlings in a single jar were initially transferred to a single pot. Later, after acclimatization, the seedlings were singled out and transferred to other pots so that only a single seedling would grow in a single pot (Figure 4.4).

The survival of the seedlings in the lathhouse environment (i.e., their acclimatization) was assessed one month after they were transferred. Monitoring of the seedlings continued until the end of the 8-month experiment, with periodic measurements of seedling growth in terms of root collar diameter (using a Vernier caliper) and height (using a graduated ruler) taken. Additionally, the percentage of leaf scorch (of each plant) was estimated as an indicator of susceptibility to heat stress in the lathhouse. Given that the temperature in the lathhouse occasionally becomes very hot (reaching over 30°C), this was considered a coincidental stress treatment against which the susceptibility of the seedlings was evaluated. Acclimatization and leaf scorch scores were taken as measures of the survival/mortality of the seedlings in the lathhouse environment.

Figure 4.4. *Acclimatization of in vitro germinated seedlings in the lathhouse; A) top view of 2-month seedlings in a group of pots; B) a closer view at a single pot; and C) a single seedling per pot at the end of the experiment*

4.2.6. Data Analysis

A detailed analysis of genetic diversity, population structure, and phylogenetic relationships is provided in the companion study (Abate et al., 2024). For this study, key genetic diversity metrics from that study—allelic richness (Ar), unbiased expected heterozygosity (uHe), and population-specific Fst—were summarized to link genetic patterns with the seed quality and progeny fitness results of the present study. Expected heterozygosity (He), also known as Nei's gene diversity, reflects the average proportion of heterozygotes expected per locus under Hardy-Weinberg equilibrium and is a widely recognized metric for assessing genetic diversity (Nei, 1973; Harris & DeGiorgio, 2017; Barrandeguy & García, 2021). Unbiased expected heterozygosity (uHe) adjusts for biases from small sample sizes, ensuring more accurate comparisons across populations (Harris & DeGiorgio, 2017). Allelic richness (Ar), which measures the number of alleles per locus and can be standardized using rarefaction for populations of varying sizes, is particularly valuable for conservation prioritization (Petit et al., 1998; Barrandeguy & García, 2021). While both He and Ar are sensitive to shifts in allele frequency, Ar is more responsive in long-lived trees and self-incompatible species (González et al., 2020; Barrandeguy & García, 2021). The inbreeding coefficient (Fis), calculated from discrepancies between observed and expected heterozygosity, is another common parameter. However, it is often less sensitive to habitat fragmentation (Schlaepfer et al., 2018) and is considered less reliable as a standalone measure of inbreeding (Kardos et al., 2016). Population-specific Fst, a measure of the probability of two alleles being identical by descent

within a population relative to alleles from different populations, has been suggested as a more robust indicator of inbreeding and genetic drift than Fis (Weir and Goudet, 2017; Kitada et al., 2021; Abate et al., 2024).

The data from the physical seed quality assessment, *in vitro* germination, acclimatization in a lathhouse, and early progeny vigor assessment were subjected to one-way analysis of variance (ANOVA), with populations grouped by population size classes taken as independent variables. For the *in vitro* germination analysis, a one-way ANOVA was performed on the final germination data at the end of the 10-week experiment. The cumulative germination data, collected every three days, were plotted in line graphs with error bars to indicate significant differences between groups, illustrating the germination trend over the test period. The ANOVA models were fitted in R4.3.3 statistical software (R Core Team, 2024) via the *aov* function. The results of the models were visualized via the *summary* function, and variances were considered significant when the *p* values were less than 0.05. The assumptions of homoscedasticity (i.e., constancy of variance) and a normal distribution were checked by plotting the models in R via the *plot* function and visualizing the standardized vs fitted and normal Q–Q plots of the residuals (Schützenmeister et al., 2011). The data were considered homoscedastic when the residuals were randomly scattered around zero and the red line representing the mean of the residuals was horizontal and centered on zero in the residuals vs. fitted plot. Similarly, the data were considered normally distributed when points lay close to the diagonal line, which represents the true normal quantiles, in the Q–Q plot. The skewness level of the data was also checked using the *skewness* function of the moments package (Komsta & Novomestky, 2022). For traits in which the data appeared to violate the assumptions of homoscedasticity and normality, data transformation was applied prior to performing the ANOVA tests.

Post hoc mean comparisons were conducted via Tukey's honestly significant difference (HSD) test, with the *Tukey HSD* function in R, at a significance level of $p < 0.05$. To present the results of Tukey's test in tables or bar plots, a data summary of each independent variable was first created via the *group_by* function of the dplyr (Wickham et al., 2023) and plotrix (Lemon, 2006) R packages. A compact letter display (CLD) was then generated via the *multcompLetters4* function of the multicompview R package (Graves et al., 2023) to indicate significantly different means with different letters. The results in boxplots and line graphs were plotted via the *ggplot* function of the ggplot2 package (Wickham, 2016).

A correlation analysis was conducted to determine the relationships between genetic diversity parameters of the populations (specifically, allelic richness, expected heterozygosity, and population-specific Fst, extracted from Abate et al., 2024) with physical seed quality, *in vitro* germination, acclimatization, and seedling growth traits of seeds collected from these populations. The results of the correlation analysis are displayed in a correlation plot, which includes both numerical and graphical representations of the correlation intensity, generated using the *corr_coef* function of the metan package (Olivoto & Lúcio, 2020).

4.3. Results

4.3.1. Summary of Genetic Diversity Analysis

The genetic diversity of *A. gracilior* populations, categorized by population size and developmental stage, is summarized in Table 4.2.

Table 4.2. Mean genetic diversity indices* summarized by population type and developmental stage, based on 1,820 SNP markers generated using the DArTseq™ platform from 183 sampled genotypes. The values of these indices for the individual populations, with further details about the SNP markers, are available in the companion paper (Abate et al., 2024, presented in Chapter 3).

Population type	Developmental stage	N	N_A	N_{PA}	A_R	H_O	uH_E	F_{IS}	F_{ST}
Large/intact	Adult	28	2385	45	1.260	0.025	0.077	0.556	0.010
	Progeny	29	2372	43	1.252	0.028	0.074	0.511	0.017
	Mean		2378.5	44	1.256	0.027	0.075	0.534	0.014
Intermediate	Adult	27	2314	28	1.236	0.020	0.073	0.622	0.037
	Progeny	27	2305	28	1.231	0.024	0.072	0.563	0.046
	Mean		2310	28	1.233	0.022	0.072	0.593	0.041
Small/isolated	Adult	24	2302	36	1.234	0.023	0.074	0.578	0.005
	Progeny	28	2280	26	1.214	0.028	0.066	0.585	0.115
	Mean		2291	31	1.224	0.026	0.070	0.582	0.060

*N: number of individuals in the population that remained after data filtering; N_A: total number of alleles; $N_{PA:}$ number of private alleles; A_R: mean allelic richness; Ho: observed heterozygosity; uH_E: unbiased expected heterozygosity; F_{IS}: Fixation index (inbreeding coefficient); F_{ST}: population-specific Fst

The results revealed a clear trend of decreasing genetic diversity as population size diminishes and as populations transition from adult to progeny cohorts. This decline is more consistent and pronounced in the progeny cohorts. For instance, allelic richness (Ar) values for progeny cohorts drop from 1.252 in large populations to 1.231 in intermediate populations and 1.214 in small/isolated populations. Similarly, unbiased expected heterozygosity (uHe) values decreased from 0.074 in large progeny populations to 0.072 in intermediate and 0.066 in small/isolated populations. Large populations also exhibited higher mean numbers of private alleles (N_{PA}) compared to intermediate and small populations. While fixation indices (F_{IS}) were high across populations, they did not display a consistent pattern relative to population size or developmental stage. Conversely, population-specific Fst values showed an inverse relationship with He and Ar, increasing as population size decreased, particularly among progeny cohorts.

4.3.2. Physical Seed Quality Characteristics

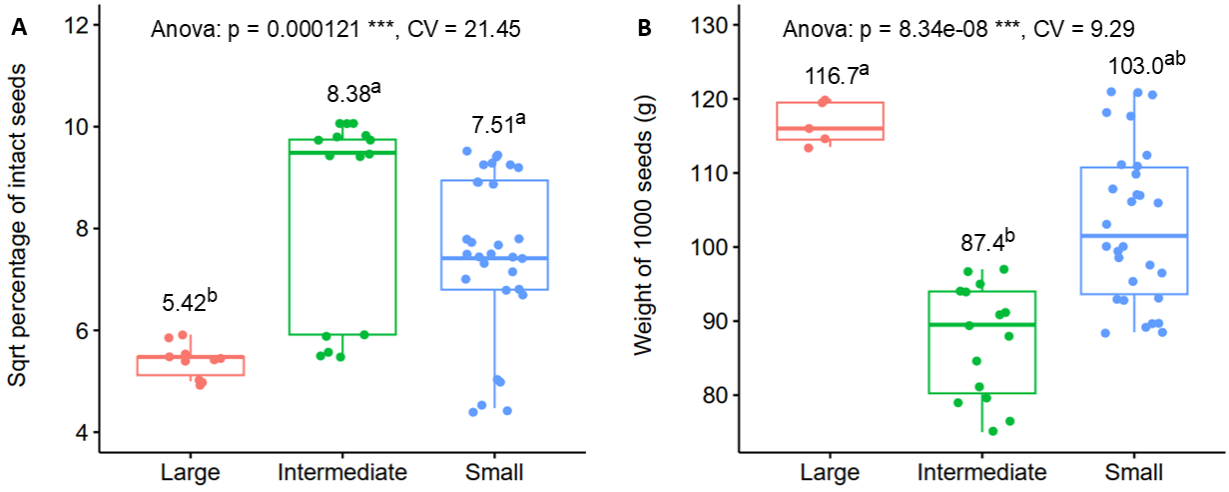

Figure 4.5. Physical seed quality comparison of *A. gracilior* seeds from different populations, grouped by population size categories (A) Percent of intact seeds by population size class. (B) Weight of 100 seeds by population size class. The p-values and coefficients of variation (CV) from the ANOVA tests are provided at the top of the plots. Mean values for each population size class are shown above the boxplots, accompanied by letters representing Tukey's HSD mean separation (p = 0.05).

Analysis of variance (ANOVA) revealed significant variations ($p < 0.001$) among population size categories for all physical seed quality traits, such as the percentage of intact seeds and the

weight of 1000 seeds (Appendix 4.11a-b, Appendix 4.2). Figure 4.5 presents boxplots comparing physical seed quality traits among population size classes. The intermediate population size class exhibited the highest percentage of intact seeds (sqrt-transformed mean of 8.38), whereas the large population size class showed the lowest percentage, with a sqrt-transformed mean of 5.42 (Figure 4.5A). For the weight of 1000 seeds, the large population size class had the highest score (mean of 116.7 g), while the intermediate class had the lowest score, with a mean of 87.4 g (Figure 4.5B).

4.3.3. *In vitro* Germination

The results of the ANOVA for *in vitro* germination revealed significant differences ($p < 0.001$) among the population size classes (Appendix 4.1c, Appendix 4.2). Figure 4.6 shows the germination trend over the 10-week *in vitro* test period, comparing the three population size categories throughout the germination period and at the end, which is typically considered the final germination percentage. The 'large' group had the highest germination scores, especially from the 3rd week onward, with a mean final germination score of 68%. In contrast, the 'small' group consistently presented the lowest scores, with a mean final germination of 32%.

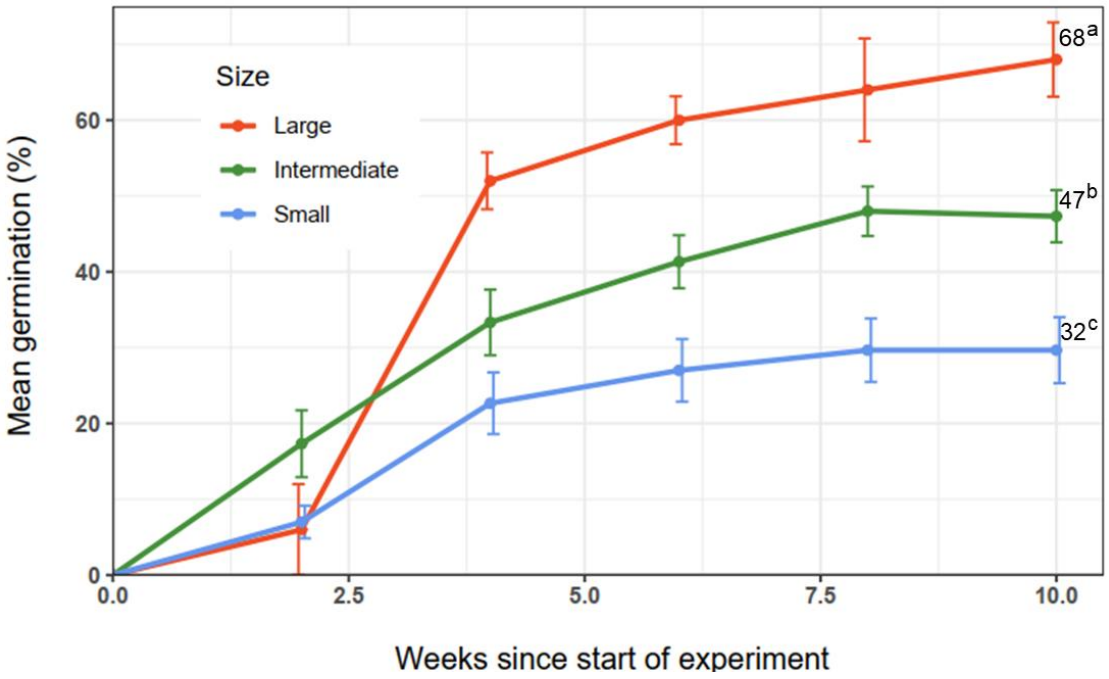

Figure 4.6. *In vitro* germination trend of *A. gracilior* seeds grouped by population size over 10 weeks. The error bars indicate the means ± SE; the numbers and letters at the ends of the lines indicate Tukey's HSD mean separation of the final in vitro germination at week 10 (p = 0.05).

4.3.4. Acclimatization and Growth in a Lathhouse

The results of the ANOVA (Appendix 4.1d-g, Appendix 4.2) revealed significant differences in acclimatization ($p < 0.05$), leaf scorch ($p < 0.001$), and growth traits ($p < 0.001$) among the population size classes. Figure 4.7 shows boxplots comparing the population size classes with respect to acclimatization and leaf scorch, which could be taken as measures of survival in the lathhouse after one month and at the end of the experiment, respectively. The 'large' population size group had the highest acclimatization score (mean of 91%) and lowest leaf scorch score (log-transformed mean of 1.12), whereas the 'small' population size group had the lowest acclimatization score (mean of 61%) and highest leaf scorch score (log-transformed mean of 1.81). The 'intermediate' group had acclimatization scores comparable to both the 'large' and 'small' groups, while its leaf scorch scores were comparable to the 'small' group. In Figure 4.8, a comparison of population size classes regarding seedling growth in terms of diameter and height in the lathhouse is presented. The 'large' population size class exhibited the highest seedling growth scores for both diameter (mean of 3.41 cm) and height (mean of 25.4 cm). Conversely, both the 'intermediate' and 'small' population categories had significantly lower growth performance compared to the 'large' category in terms of both height and diameter.

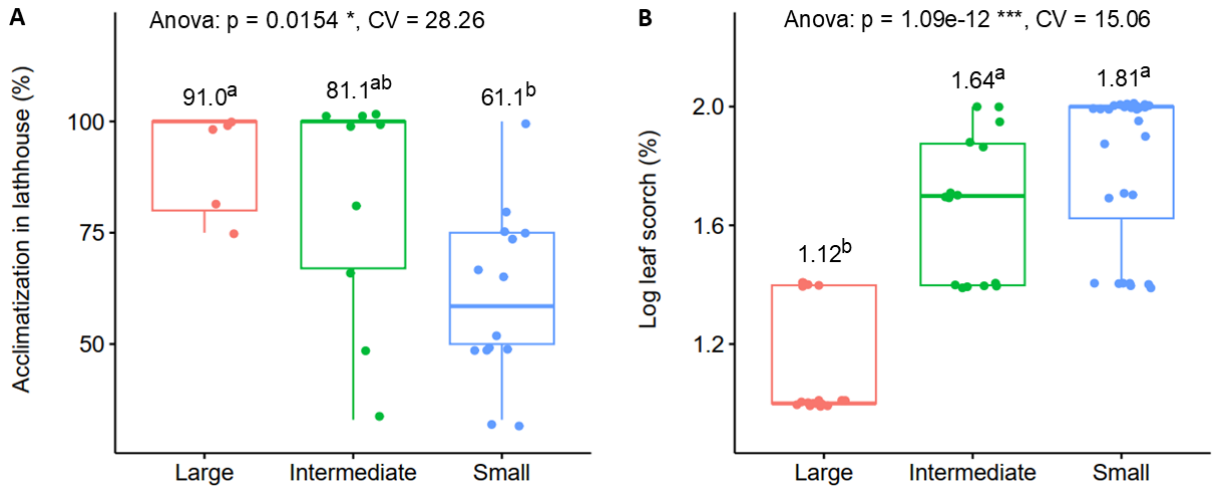

Figure 4.7. Acclimatization in the lathhouse and susceptibility to heat stress (leaf scorch scores) of *in vitro* germinated *A. gracilior* seedlings. A) Survival of transferred seedlings after a 1-month stay in the lathhouse, grouped by population size class, and B) leaf scorch at the end of the 8-month experiment, grouped by population size classes. The p-values and coefficients of variation (CV) from the ANOVA tests are provided at the top of the plots. Mean values for each population size class are shown above the boxplots, accompanied by letters representing Tukey's HSD mean separation ($p = 0.05$).

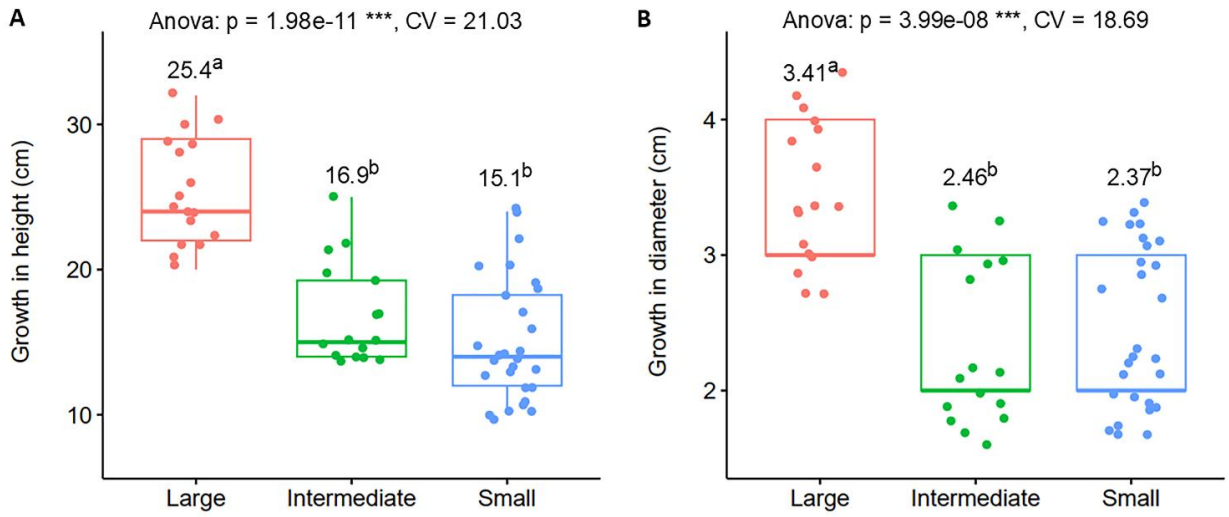

Figure 4.8. Growth performance 8 months after the *in vitro* germinated *A. gracilior* seedlings were transferred to the lathhouse. A) Height growth, grouped by population size classes, and B) diameter growth, grouped by population size classes. The p-values and coefficients of variation (CV) from the ANOVA tests are provided at the top of the plots. Mean values for each population size class are shown above the boxplots, accompanied by letters representing Tukey's HSD mean separation (p = 0.05).

4.3.5. Relationships between Genetic Diversity and Fitness Traits

Figure 4.9 displays the correlations between genetic diversity, physical seed quality, *in vitro* germination, acclimatization, and seedling growth traits. Following some suggested guidelines (Mukaka, 2012; Schober et al., 2018), the correlation coefficients were interpreted as follows: negligible (0–0.1), weak (0.1–0.39), moderate (0.4–0.69), strong (0.7–0.89), and very strong (0.9–1.0). Notably, highly significant ($p < 0.001$), moderate to strong correlations were observed between the genetic diversity parameters (Ar and He) and *in vitro* germination, acclimatization, and seedling growth in diameter and height. The population-specific Fst also showed moderate to strong negative correlations with these germination and early-progeny performance parameters. Seed weight had weak correlations with *in vitro* germination, acclimatization, and seedling growth. Seed intactness had a moderate negative correlation with seedling growth. Leaf scorch was negatively correlated with most other traits. *In vitro* germination showed a moderate positive correlation with acclimatization and seedling growth and a moderate negative correlation with leaf scorch.

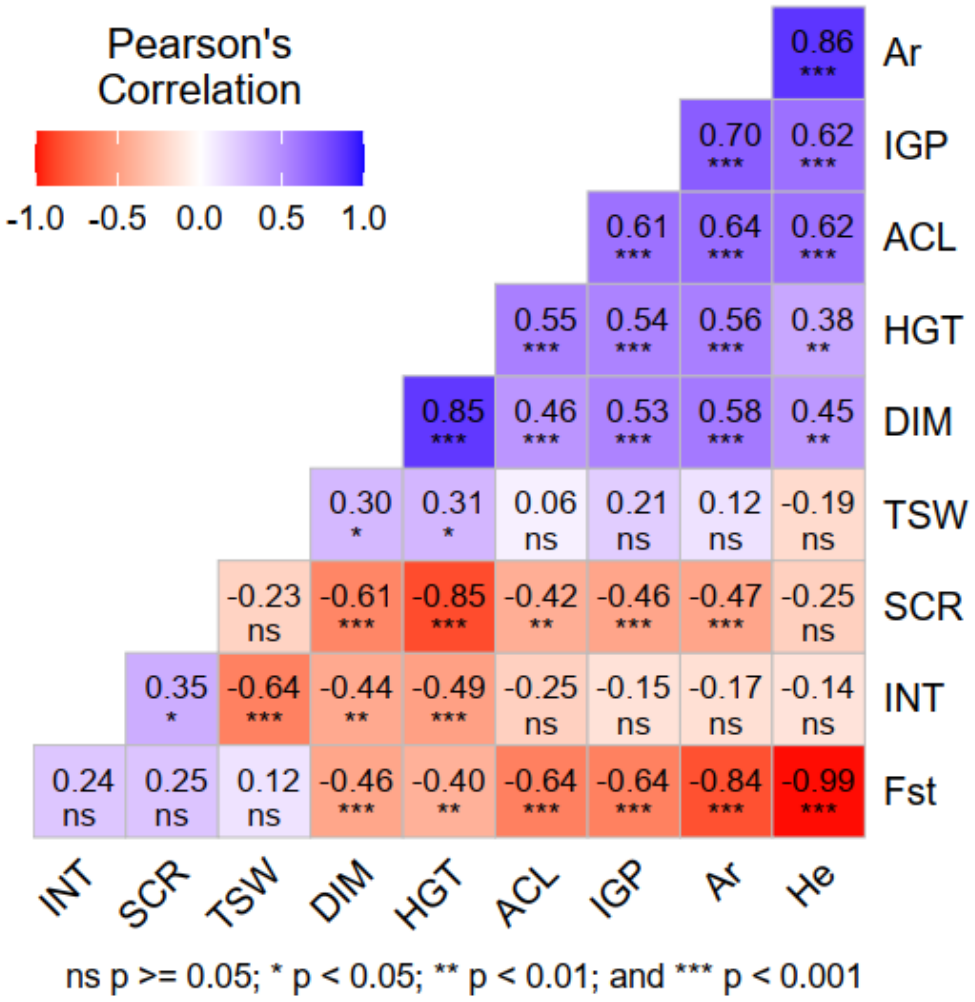

Figure 4.9. Correlations among genetic diversity, physical seed quality, *in vitro* germination, survival, and growth traits of *A. gracilior* seeds and seedlings from different populations. Each square displays Pearson's correlation coefficient, with the corresponding significance level, for the respective trait pairs. Positive correlations are shaded blue, while negative correlations are shaded red, with color intensity reflecting the strength of the correlation. Abbreviations: Ar = allelic richness; He = expected heterozygosity; Fst = population-specific Fst; INT = percentage of intact seeds; TSW = weight of 1000 seeds; IGP = *in vitro* germination percentage; ACL = acclimatization; DIM = diameter; HGT = height; SCR = leaf scorch.

4.4. Discussion

4.4.1. Genetic Diversity

Results of the genetic diversity analysis revealed a loss of genetic diversity (genetic erosion) in small or isolated populations of *A. gracilior* compared to relatively large, intact populations across fragmented landscapes. This decline was more pronounced along life-stage transitions

from adults to progeny. The reduction in genetic diversity was reflected in terms decreased allelic richness (Ar) and unbiased expected heterozygosity (uHe), as well as an increase in population-specific Fst, an indicator of inbreeding and genetic drift (Table 4.2). Both Ar and uHe were adversely impacted by fragmentation, even though a recent meta-analysis (González et al., 2020) indicates that self-incompatible, long-lived trees tend to lose allelic richness more rapidly than heterozygosity following fragmentation. This is explained by the faster loss of rare alleles that affects Ar, whereas changes in heterozygosity require more time to manifest in long-lived organisms like trees (Guidugli et al., 2016; González et al., 2020). The simultaneous decline of both Ar and uHe across population sizes and life stages in the present study, despite *A. gracilior* being dioecious and long-lived, may reflect the long time elapsed since fragmentation events, given the long history of forest disturbance in Ethiopia (Teketay, 1992; Darbyshire et al., 2003), and overlapping generations in adult populations. In support of this, the companion study (Abate et al., 2024, chapter 3 in this dissertation), based on phylogenetic analysis, attributed the overall low genetic diversity in the studied *A. gracilior* populations to a likely founder effect, where populations were suspected to have descended from ancestral populations that persisted in small numbers for generations.

Another notable result of the genetic diversity analysis is that while the fixation index (F_{IS}) scores were high across populations, indicating the presence of inbreeding, they did not follow a consistent pattern relative to population size or developmental stage, unlike Ar and uHe. This is consistent with the findings of a review article (Schlaepfer et al., 2018), which reported that F_{IS} is typically the least sensitive genetic diversity parameter to fragmentation. Moreover, Kardos et al. (2016) caution against relying solely on F_{IS} as a measure of inbreeding, as it can be biased by factors such as population size and allele frequency differences between sexes. In contrast, population-specific Fst values exhibited inverse patterns to He and Ar, increasing as population size decreased, particularly in progeny populations. This suggests that population-specific Fst may be a more robust indicator of inbreeding and genetic drift than F_{IS}, providing insights into evolutionary divergence from an ancestral population (Weir and Goudet, 2017; Kitada et al., 2021), making it a useful metric for measuring genetic drift.

The negative effects of forest fragmentation on the genetic diversity of *A. gracilior* observed in this study are consistent with findings in other wind-pollinated and/or dioecious tree species, such as *Fagus sylvatica* (Jump & Peñuelas, 2006), Picea glauca (O'Connell et al., 2006), *Araucaria nemorosa* (Kettle et al., 2007, 2008), *Juniperus communis* (Provan et al., 2008),

Polylepis australis (Seltmann et al., 2009), *Taxus baccata* (Dubreuil et al., 2010), *Spondias purpurea* (Cristóbal-Pérez et al., 2021) and *Brosimum alicastrum* (Aguilar-Aguilar et al., 2023). These studies consistently report consequences such as restricted gene flow (O'Connell et al., 2006; Provan et al., 2008; Cristóbal-Pérez et al., 2021), heightened genetic structure or differentiation (Jump & Peñuelas, 2006; Provan et al., 2008; Seltmann et al., 2009), reduced genetic diversity and bottlenecks (Jump & Peñuelas, 2006; Kettle et al., 2007, 2008; Cristóbal-Pérez et al., 2021), elevated inbreeding levels (Jump & Peñuelas, 2006; Kettle et al., 2007; Dubreuil et al., 2010; Cristóbal-Pérez et al., 2021), correlated paternity (Cristóbal-Pérez et al., 2021; Aguilar-Aguilar et al., 2023), and diminished reproductive success and progeny fitness (O'Connell et al., 2006; Kettle et al., 2008; Seltmann et al., 2009; Aguilar-Aguilar et al., 2023) in fragmented compared to continuous populations. The population genetics paradigm underpinning these impacts (Young et al., 1996; Frankham, 2005; Lowe et al., 2005; Bacles & Jump, 2011; Aguilar et al., 2019) explains them as consequences of reduced population size and increased isolation, which lead to allele loss, increased genetic drift, and restricted gene flow. These changes also elevate (biparental) inbreeding, resulting in higher homozygosity and the expression of deleterious alleles, culminating in inbreeding depression. This, in turn, reduces fecundity, seedling recruitment, and survival, ultimately threatening the long-term viability of fragmented populations.

Conversely, studies on other species such as *Swietenia humilis* (White et al., 2002), *Fraxinus excelsior* (Bacles et al., 2005), *Araucaria angustifolia* (Bittencourt & Sebbenn, 2007), *Sorbus domestica* (Kamm et al., 2009), *Gomortega keule* (Lander et al., 2010), *Eucalyptus leucoxylon* and *Eucalyptus camaldulensis* (Ottewell et al., 2010), *Dysoxylum malabaricum* (Ismail et al., 2012), *Allocasuarina verticillata* (Broadhurst, 2015), *Cariniana estrellensis* (Guidugli et al., 2016), and *Quercus species* (Ashley, 2021) demonstrated that fragmented populations can remain functionally connected through extensive gene flow despite spatial isolation. Some of these studies have even suggested that small, isolated populations can act as genetic buffers or stepping stones connecting broader populations (White et al., 2002; Lander et al., 2010). Intriguingly, others pointed out that isolated trees in agroforestry or fragmented landscapes often receive pollen from genetically diverse, unrelated trees in multiple directions, serving as valuable sources of outbred seeds for forest restoration efforts (Ottewell et al., 2010; Ismail et al., 2012). These findings defied theoretical expectations in population genetics, prompting the characterization of forest fragmentation genetics as a 'paradox' (Kramer et al., 2008).

This apparent resilience of trees to fragmentation has been attributed to mechanisms such as extensive pollen and seed dispersal, long lifespans, overlapping generations, and flexible mating systems (Kramer et al., 2008; Dubreuil et al., 2010; Lowe et al., 2015). However, more recent insights (Bacles & Jump, 2011; Lowe et al., 2015; Aguilar et al., 2019; González et al., 2020) emphasize that not all tree species are equally immune to the genetic consequences of fragmentation. The degree of resilience or susceptibility varies depending on factors such as differences in mating systems (e.g., monoecy vs. dioecy, selfing vs. outcrossing), pollination mechanisms (wind vs. animal), pollinator mobility and specialization, seed dispersal modes, and the time elapsed since fragmentation occurred. In the present study, the long history of forest disturbance in Ethiopia, combined with the dioecy in *A. gracilior* and a potential founder effect (explained in Chapter 3 & Abate et al., 2024), likely contributed to the observed low genetic diversity and inbreeding depression in progeny from small/fragmented populations. These findings underline the necessity of species-specific approaches when evaluating genetic responses to habitat fragmentation.

4.4.2. Reproduction and Early Progeny Vigor

Our study revealed significant variation ($p < 0.05$) among individual populations in physical seed quality, *in vitro* germination, survival, and early progeny growth traits. Notably, the "large" population size class consistently performed better ($p < 0.05$) across all the traits, except for the percentage of intact seeds, than did the smaller size classes. This disparity is likely due to the impact of inbreeding depression, which tends to be more pronounced in smaller and more fragmented populations than in larger, more intact populations. Consistent with this, the genetic diversity analyses revealed lower genetic diversity and greater inbreeding and genetic drift in the progeny from smaller populations (Table 4.2 and discussion in section 4. 4.1). Furthermore, the genetic diversity indices, specifically Ar, uHe and Fst, exhibited strong positive correlations with the progeny fitness traits assessed in this study (Figure 4.9).

The observation of a lower percentage of intact seeds, alongside a greater proportion of empty and aborted seeds in larger populations, is contrary to our initial expectations. We anticipate that smaller, more isolated populations would exhibit greater inbreeding depression, leading to reduced fertilization and seed set and, consequently, a greater incidence of empty cones (parthenocarpy). Indeed, the genetic diversity analyses of the present study revealed lower genetic diversity and greater inbreeding in these small, isolated populations, supporting this expectation.

Fragmented populations often experience limited seed set due to pollen limitation from increased isolation between mating individuals and embryo abortion from inbreeding depression (Offord et al., 1999; Kettle et al., 2008; Ahlinder et al., 2021). Consistent with this, higher percentages of empty seeds in small and fragmented populations than in larger populations have been reported in other conifers, such as *Pinus strobus* (Rajora et al., 2002), *Picea glauca* (O'Connell et al., 2006), and *Araucaria nemorosa* (Kettle et al., 2008). Despite these findings, our seed quality assessment results contradict these reports and our genetic diversity analysis. This discrepancy suggests that while inbreeding depression is expected to affect seed set, its impact might not be evident at the seed setting stage for *A. gracilior*. Inbreeding depression can manifest at various life stages, potentially influencing seed set and germination early on or becoming apparent later during growth and adult survival (Husband & Schemske, 1996; Hill et al., 2006; Ahlinder et al., 2021). Consequently, the effects of inbreeding depression in *A. gracilior* may only become apparent in later developmental stages. The lower percentage of intact seeds in larger populations observed in our study may be due to pollen movement being obstructed by the dense canopy of surrounding trees, leading to pollen limitation. Previous studies have demonstrated that plant density and the presence of other tree species in mixed forests can impact wind pollination by reducing the wind velocity and impeding pollen flow, which in turn decreases the effective dispersal distance of pollen (Whitehead, 1969; Biitencourt & Sebbenn, 2007; Hardy, 2009; Millerón et al., 2012; Piotti et al., 2012). In this study, it appeared that the dense canopy in larger populations hinders pollen flow, potentially reducing the amount of pollen reaching the mother trees. Despite this obstruction, larger populations seemed to benefit from a more diverse range of pollen than smaller and isolated populations. In contrast, mother trees in small and isolated populations may receive a quantity of pollen but experience reduced genetic diversity due to the limited number of mating individuals, leading to inbreeding. These observations align with the genetic diversity analysis and progeny fitness traits assessed in this study.

In the present study, seed weight did not appear to be influenced by inbreeding. Although the 'large' population size class exhibited the highest 1000-seed weight, the lowest value was unexpectedly observed in the 'intermediate' class. If inbreeding had been a significant factor, the lowest seed weight would be expected in the 'small' class. This suggests that other factors may have contributed to the observed variation in seed weight. During data collection, we noted that the soil in the two 'intermediate' populations (Borite and Halo in Table 4.1) was shallow and situated on rocky outcrops. This indicates that environmental factors, particularly soil

resource limitations, likely played a role in reducing seed weight in these populations. This finding aligns with O'Connell et al. (2016), who noted that environmental resource limitations can obscure the effects of inbreeding on seed weight. They suggested correcting for this by dividing seed weight by cone mass.

Another important observation is that seed production was greater in isolated trees and small populations with open canopies than in large populations with closed canopies (as described in Table 4.1). This is likely due to the full exposure of the crown to sunlight, which enhances seed production. These results, together with the data on intact, empty, and aborted seeds mentioned earlier, suggest that inbreeding depression due to habitat fragmentation may not be apparent during the seed-setting stage in *A. gracilior*.

There is no consistent trend in the literature regarding the effect of inbreeding on seed weight. Negative impacts of inbreeding on seed weight have been reported in species such as *Plantago coronopus* (Koelewijn et al., 2005), *Shorea acuminata* (Naito et al., 2007), *Hymenaea courbaril* (Pereira et al., 2020), and *Pinus massoniana* (Wei et al., 2024). Conversely, in species such as *Silene latifolia* (Teixeira et al., 2009), *Pinus sylvestris* (Mullin et al., 2019), and *Pinus yunnanensis* (Li et al., 2024), inbreeding did not significantly affect seed weight. Additionally, Baskin and Baskin (2015) reported, in a review of 216 case studies, that the mean mass of inbred seeds was greater than that of outbred seeds in 12.5% of cases, equal in 38% of cases, and less in 49% of cases. In some cases, there may be a tradeoff between seed size and number, where pollen-limited females produce fewer but larger seeds that perform better during germination to compensate for reduced seed production (Labouche et al., 2017). When inbreeding depression is not apparent in seed weight, it may be due to the purging of inbred individuals early during the seed development stage (O'Connell et al., 2006; Hill et al., 2006; Li et al., 2024).

The significantly greater *in vitro* germination observed in the 'large' population suggests that inbreeding depression has impacted the growth and development of *A. gracilior* at this life stage. The absence of a strong correlation between *in vitro* germination and both seed intactness and seed weight further supports the idea that the differences in these traits between populations were not caused by inbreeding. This implies that the larger values of these traits do not represent genetic gains that would translate into enhanced progeny vigor in terms of germination, growth, and survival. Moreover, the 'large' population had significantly greater values for lathhouse survival and seedling growth traits, along with significantly lower values for leaf scorch,

indicating that the effects of inbreeding depression also extend to later stages of survival and growth. This assertion is further reinforced by the strong positive correlations observed between genetic diversity parameters (Ar, uHe, and Fst) and the germination, survival, and growth metrics.

The carry-over effect of inbreeding from early to later life stages may be attributed to genes influencing early development, which can have cumulative effects as cell lineages multiply, as well as to pleiotropy, where a single gene affects multiple traits or functions (Husband & Schemske, 1996). This finding aligns with the general theoretical model that suggests that inbreeding depression manifests in later life stages for selfing species and in both early and later stages for self-incompatible and dioecious species (Husband & Schemske, 1996; Ahlinder et al., 2021). Additionally, the significantly higher leaf scorch scores observed in the 'small' populations imply that the occasionally elevated temperatures in the lathhouse may have exacerbated the effects of inbreeding depression. This observation is consistent with the literature, indicating that inbreeding depression is more pronounced in stressful environments than in benign environments. Stress can increase the expression of deleterious recessive alleles, which may eventually lead to their purging (Armbruster & Reed, 2005; Fox & Reed, 2010; Sandner et al., 2021).

4.4.2. Concluding Remarks and Management Implications

This study demonstrated that population fragmentation leads to inbreeding depression in *A. gracilior*. Progeny from fragmented populations, comprising isolated individuals or small groups, exhibited significantly reduced *in vitro* germination, growth, and survival compared to progeny from larger populations. These findings are consistent with the genetic diversity analyses, which revealed lower genetic diversity and increased genetic drift in fragmented populations. Notably, inbreeding depression was not evident during the seed-setting stage, as there were no significant differences in seed weight or the percentage of intact seeds. This suggests that for *A. gracilior*, the impacts of inbreeding depression manifest predominantly during later life stages, particularly germination and recruitment.

While this study provides robust insights, certain limiting factors should be considered when interpreting the results. As the first molecular marker-based investigation of genetic diversity in *A. gracilior*—and, to the best of our knowledge, the genus—there is no baseline for comparison, particularly regarding the exceptionally low genetic diversity observed.

Additionally, the study did not include gene flow or parentage analyses, which could have provided a more detailed understanding of the genetic consequences of habitat fragmentation. Furthermore, due to the unequal availability of population types (large, intermediate, and small) across study sites, a fully nested experimental design (population type by site) was not feasible for assessing reproductive success and progeny vigor. This limitation restricted us from accounting for the magnitude of site differences (i.e., those not attributable to the genetic effects of population size) on the observed variation. Despite these caveats, the study uncovered significant and consistent differences among population sizes using both molecular markers and phenotypic traits on reproduction and early progeny vigor. Importantly, the strong correlations observed between genetic diversity parameters and phenotypic traits bolster the validity of our conclusions. As a pioneering investigation into the genetics of *A. gracilior*, we believe this work contributes to the broader understanding of fragmentation genetics and provides a foundation for evidence-based strategies aimed at conserving and restoring this important species.

The implications of these findings for the conservation and management of *A. gracilior* are substantial, particularly in guiding seed procurement practices. Seeds from isolated trees or small populations should be avoided, even when these populations produce abundant seeds and physical assessments suggest high seed quality. Instead, restoration efforts should prioritize sourcing seeds from larger, genetically diverse *A. gracilior* populations. To facilitate this, authorities should identify and designate these larger populations as seed sources and enforce regulations to ensure compliance. This recommendation may appear to contradict previous studies (Lander et al., 2010; Ottewell et al., 2010; Ismail et al., 2012), which highlighted the potential of isolated trees in fragmented landscapes to provide genetically diverse, outbred seeds due to pollination from unrelated trees across multiple directions. This perspective stems from claims that isolated and scattered trees and small populations function as stepping stones for pollinators and enhance genetic connectivity in fragmented landscapes (White et al., 2002; Lander et al., 2010). While this may apply to species that depend on insect and vertebrate pollinators, we could not verify whether similar mechanisms hold for the wind-pollinated *A. gracilior*, as the present study did not include gene flow or parentage analysis. Nevertheless, the results of genetic diversity analysis and progeny vigor assessments suggest otherwise, highlighting the need for cautious and species-specific seed procurement approaches in forest restoration efforts.

While our findings suggest prioritizing larger natural forest remnants and sacred sites that harbor greater genetic diversity, all populations examined showed a notable presence of private alleles (Abate et al., 2024; Table 4.2). This indicates that each population uniquely contributes to the species' gene pool and, therefore, deserves conservation attention. From a conservation perspective, no population should be dismissed as too small or insignificant. Although direct seed collection from small populations or isolated trees to be used alone is not recommended, an alternative strategy involves collecting seeds from multiple such populations, mixing them, and growing them as seedlings in nurseries. Such mixed-source germplasm approaches have been advocated as effective for genetic rescue, aiding species that suffer from genetic impoverishment and inbreeding depression (Finger et al., 2011; St. Clair et al., 2020). This strategy could enhance genetic diversity and bolster population resilience in restoration initiatives for *A. gracilior*.

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CHAPTER FIVE

5. Identification of a Sex-Linked RAPD Marker in the Dioecious Conifer *Afrocarpus gracilior* for Early-Stage Sex Determination

Abstract

Sex identification at the juvenile stage is a major challenge in breeding, economic cultivation, conservation, and restoration of dioecious plants. *Afrocarpus gracilior* (Pilg.) C. N. Page, a long-lived, dioecious conifer native to Ethiopia's Afromontane forests, is threatened by habitat loss and selective logging. The species' dioecious nature hinders restoration efforts as male and female individuals cannot be morphologically distinguished before reproductive maturity, which may take decades. This study aimed to develop a molecular marker for early sex identification in *A. gracilior* using Random Amplified Polymorphic DNA (RAPD) and convert it into a more reliable Sequence-Characterized Amplified Region (SCAR) marker. Screening of 60 RAPD primers identified 14 putative sex-linked markers in bulked segregant analysis (BSA). Further validation in individual male and female samples confirmed a single sex-linked RAPD marker: OPD-18 (5'-GAGAGCCAAC-3'), which consistently produced a 600-bp band in males but was absent in females. While this marker serves as a useful tool for early sex determination in *A. gracilior* using Polymerase Chain Reaction (PCR) and agarose gel electrophoresis, its dominant nature and reproducibility limitations highlight the need for Sequence-Characterized Amplified Region (SCAR) marker conversion. Due to resource and time constraints, this step could not be completed in the present study. However, refining the marker into a more robust, locus-specific SCAR marker and exploring high-throughput approaches is the focus of further research. These advancements will facilitate the effective management of *A. gracilior* populations, ensuring a balanced representation of both sexes in plantations, supporting breeding programs that may be envisaged for the species, and enhancing the long-term success of restoration initiatives.

Key words: dioecy, bulked segregant analysis, RAPD, SCAR, sex-linked marker, sex chromosomes

5.1. Introduction

Sexual systems in plants are remarkably diverse and more complex than in animals. While animals typically exhibit a single sexual system—gonochory, or separate male and female individuals—plants can express multiple reproductive strategies. These range from hermaphroditism, where flowers have both functional stamens and carpels, to more specialized systems such as monoecy (male and female flowers on the same individual), gynodioecy (female and hermaphroditic individuals), androdioecy (male and hermaphroditic individuals), and dioecy (male and female individuals on separate plants) (Heikrujam et al., 2015; Charlesworth, 2016; Leite Montalvão et al., 2021; Masuda and Akagi, 2022). Dioecy, a sexually dimorphic system, is relatively rare in angiosperms, occurring in approximately 6% of flowering plants, or around 14,600 species across 960 genera and 200 families (Ming et al. 2011; Renner, 2014; Charlesworth, 2016). In contrast, dioecy is the predominant mating system in gymnosperms, present in 64.6% of species, spanning 42 of 84 genera and 8 of the 12 gymnosperm families (Walas et al., 2018; Ohri & Rastogi, 2020). Notably, six gymnosperm families, *Cycadaceae, Zamiaceae, Ginkgoaceae, Welwitschiaceae, Gnetaceae*, and *Ephedraceae*, are entirely dioecious. Other families, such as Podocarpaceae (94.9% dioecious) and Taxaceae (93.7%), also show high dioecy levels, while it is less common in *Cupressaceae* (29.6%) and *Araucariaceae* (5.4%). *Sciadopityaceae* and most *Pinaceae* lack dioecy, except for *Pinus johannis* (Walas et al., 2018).

The dominance of dioecy among gymnosperms is noteworthy, especially given its rarity among other sedentary organisms (Käfer et al., 2017; Barrett, 2021). This phenomenon may stem from the absence of hermaphroditic flowers in gymnosperms. Unlike angiosperms, where more than 80% of species produce perfect flowers with both male and female reproductive organs, gymnosperms exclusively rely on unisexual structures, potentially mitigating the risk of self-fertilization in the absence of mechanisms like self-incompatibility (SI) (Walas et al., 2018). SI, a common feature in angiosperms, is present in over 100 families and about 39% of species (Igic et al., 2008; Ferrer & Good, 2012). Conversely, gymnosperms exhibit either weak or no SI mechanisms (Owens et al., 1998), which may explain the prevalence of dioecy as a strategy to prevent selfing and maintain genetic diversity (Grossenbacher et al., 2017; Muyle et al., 2021). Mixed sexual systems, such as dioecy and monoecy coexisting within the same species, are extremely rare in gymnosperms, occurring in only about 1% of species (Walas et al., 2018).

Dioecy, despite being rare in plants, offers distinct advantages and is linked to specific ecological traits. Its primary benefit is the prevention of self-pollination, reducing inbreeding and enhancing genetic diversity (Charlesworth & Charlesworth, 1978; Charlesworth, 1999; Ainsworth, 2000). Additionally, separating male and female reproductive roles allows for more efficient resource allocation, often facilitating the development of fleshy, resource-intensive fruits (Leslie et al., 2013; Walas et al., 2018). Dioecy is frequently associated with features such as woody growth forms, tropical habitats, and abiotic pollination, indicating that it may arise in response to particular environmental conditions. However, this reproductive strategy has significant drawbacks. It limits reproductive assurance, as female plants depend entirely on nearby males for fertilization, and reduces overall seed production since only females bear seeds, creating a "seed-shadow handicap" (Heilbuth et al., 2001; Charlesworth, 2016). These challenges have led to the proposition that dioecy represents an evolutionary "dead end," associated with higher extinction rates and lower diversification compared to hermaphroditic lineages (Heilbuth, 2000; Vamosi & Otto, 2002). Nonetheless, recent evidence shows that diversification rates for dioecious and hermaphroditic lineages are comparable, with occasional reversions to monoecy or hermaphroditism, particularly under domestication (Käfer et al., 2017; Barrett, 2021; Cossard et al., 2021).

Dioecious plants offer critical insights into the evolution of separate sexes, a phenomenon that evolved independently and relatively recently across multiple genera (Charlesworth, 2012; Heikrujam et al., 2015; Charlesworth, 2016; Henry et al., 2018; Masuda & Akagi, 2023). These plants are pivotal in exploring sexual dimorphism and mechanisms of sex determination, which are essential for reproductive biology and commercial applications. Dioecy involves intricate genetic interactions, with mutations in flower development genes leading to diverse reproductive strategies. Notably, two key mutations—one causing male sterility and the other female sterility—must be tightly linked on the same chromosome to form functional sex chromosomes (Charlesworth, 2016; Charlesworth & Harkess, 2024). Plants exhibit three main sex chromosome systems: XX|XY (male heterogamety, similar to mammals), ZZ|ZW (female heterogamety, as in birds), and U|V (in bryophytes, where haploid males and females are designated U and V, respectively) (Bachtrog et al., 2014; Renner et al., 2017; Masuda & Akagi, 2023). In some species, an XY “dosage system” determines sex based on the ratio of X chromosomes to autosomes (X/A ratio) (Tanurdzic & Banks, 2004). Interestingly, a unique system has been reported in some Podocarpus, where females possess four sex chromosomes (X1X1X2X2) and males have three (X1X2Y) (Walas et al., 2018). Unlike animals, plants rarely

exhibit cytologically distinct sex chromosomes, likely due to their relatively recent evolution (Charlesworth, 2016; Leite Montalvão et al., 2021). Most plants instead display homomorphic sex chromosomes, where X and Y (or Z and W) are morphologically similar. However, molecular studies reveal that sex-linked regions in plants often undergo genetic degeneration and accumulate repetitive sequences, mirroring processes observed in animals like humans and birds (Charlesworth, 2016; Charlesworth & Harkess, 2024).

The identification of plant sex at the seedling stage represents a key area where molecular genetics tools could have a transformative impact. Many dioecious plants, such as poplar (*Populus* spp.), kiwifruit (*Actinidia* spp.), asparagus (*Asparagus officinalis*), papaya (*Carica papaya*), spinach (*Spinacia oleracea*), hemp (*Cannabis sativa*), date palm (*Phoenix dactylifera*), yam (*Dioscorea* spp.), sea buckthorn (*Hippophae rhamnoides*), and pistachio (*Pistacia vera*), are economically important crops. Widely cultivated crops like hexaploid persimmon (*Diospyros kaki*), grape (*Vitis vinifera*), and strawberry (*Fragaria* × *ananassa*) also trace their domestication to dioecious relatives (Henry et al., 2018). Among trees, poplars (*Populu*s spp., willows (*Salix* spp.), gingko (*Ginko biloba)*, and most other gymnosperms are dioecious (Keresten et al., 2017; Walas et al., 2018). The economic value of dioecious crops often depends on plant sex—for instance, females are preferred for their yields in sea buckthorn, date palm, and yams, while males exhibit agronomic advantages in asparagus, poplar, and hemp (Ainsworth, 2000; Heikrujam et al., 2015). However, sex determination is challenging in juvenile plants, as they lack distinct morphological traits and cytological sex chromosomes are rare (Leite Montalvão et al., 2021; Razumova et al., 2023). In perennial species, reproductive maturity can take years to reveal gender, leading to resource inefficiencies when undesired plants are unknowingly cultivated (Heikrujam et al., 2015; Razumova et al., 2023). Early and reliable sex identification is thus essential to streamline breeding programs, optimize resource allocation, and enhance productivity. Molecular markers offer a promising solution by enabling accurate, early-stage identification of plant sex, addressing longstanding challenges in dioecious crop management.

Several molecular markers based on DNA fingerprinting have been utilized for early-stage sex identification in agronomically important dioecious plants (see reviews by Ainsworth, 2000; Milewicz & Sawicki, 2013; Heikrujam et al., 2015; Razumova et al., 2023). Commonly used markers include Random Amplified Polymorphic DNA (RAPD), Sequence-Characterized Amplified Region (SCAR), Amplified Fragment Length Polymorphism (AFLP), Restriction

Fragment Length Polymorphism (RFLP), Simple Sequence Repeats (SSR or microsatellites), and Inter Simple Sequence Repeats (ISSR). Among these, RAPD is the most widely employed due to its simplicity and lack of need for prior sequence information (Williams et al., 1990). However, RAPD has notable limitations, including poor reproducibility due to its sensitivity to amplification conditions and its dominant nature (Staub et al., 1996; Weising et al., 2005). To overcome these drawbacks, sex-linked RAPD markers have often been converted to more reliable and locus-specific SCAR markers (Paran & Michelmore, 1993; Bhagyawant, 2016). These methods are frequently combined with Bulked Segregant Analysis (BSA), which compares pooled DNA samples of individuals with contrasting traits—such as sex and pathogen resistance—while ensuring homogeneity for the trait of interest and nontarget traits varying randomly (Michelmore et al., 1991; Chen et al., 2010; Zou et al., 2016). With advancements in molecular genetics, next-generation sequencing (NGS) techniques such as restriction site-associated DNA sequencing (RAD-seq), double-digest RAD-seq (ddRAD-seq), and whole-genome sequencing have emerged as faster, more efficient, and higher-resolution alternatives. These advanced methods have recently been successfully employed in various dioecious species, including *Excoecaria agallocha* (Zhou et al., 2018), *Eucommia ulmoides* (Wang et al., 2019), *Actinidia arguta* (Guo et al., 2023), and *Hippophae tibetana* (Zeng et al., 2024).

In the present study, we aimed to develop a RAPD marker for the dioecious conifer *Afrocarpus gracilior* (Pilg.) C. N. Page to enable early sex identification at the seedling stage. *A. gracilior* is a charismatic indigenous conifer that once dominated Ethiopia's Afromontane forests and is highly valued for its high-quality timber, medicinal uses, ecological significance, and sociocultural importance as a sacred tree (Negash, 2010; Doda & Abuelgasim, 2019; Abate et al., 2024). However, its prized timber has made it a target for illegal logging, severely diminishing its presence in natural forests (Negash et al., 2010; Teketay et al., 2011). Today, the species persists mainly in inaccessible relict forest patches, traditional agroforestry systems, church forests, and other culturally protected sites (Negash, 2010; Doda & Abuelgasim, 2019; Abate et al., 2024). As a result, *A. gracilior* is considered threatened within Ethiopia and is among the few native trees prioritized for conservation and restoration (Kalinganire et al., 2021). However, its dioecious nature presents a challenge for restoration, as male and female individuals cannot be distinguished morphologically at the seedling stage. Sex-related traits, such as flowers or cones, become visible only at reproductive maturity (Figure 5.1), which may take years or even decades in conifers like *A. gracilior* due to their long juvenile phase. This

poses a risk that artificially established populations may fail to reproduce if only one sex is inadvertently planted. Developing molecular methods for early sex identification could significantly enhance restoration efforts by ensuring a balanced sex ratio in reintroduced populations. Therefore, this study aimed to develop a sex-linked RAPD marker for *A. gracilior* and convert it into a locus-specific SCAR marker to facilitate reliable molecular sex identification at seedling stage.

Figure 5.1. Reproductive features that distinguish adult male and female trees of *A. gracilior*. (A) Male cones of *A. gracilior*, showing spirally arranged sporophylls responsible for releasing pollen grains. (B) Female cones, or "fruits," displaying their conspicuous fleshy outer covering known as the *epimatium.*

5.2. Materials and methods

5.2.1. Plant tissue sampling

We sampled 20 male and 19 female adult individuals of *A. gracilior* from the College of Agriculture (7° 3' 11.2176" N, 38° 28' 19.254" E) and Main Campus (7° 3' 41.8356" N, 38° 29' 43.1736" E) of Hawassa University, Ethiopia. The sexes of the adult individuals can be easily identified through their reproductive features: adult males bear male cones, which are spirally arranged sporophylls responsible for releasing pollen grains, whereas adult females bear the female cones or "fruits" with their conspicuous fleshy outer covering called the *epimatium*. (Figure 5.1). Young leaf tissues were collected from the sampled adult male and female

individuals, immediately placed in zip-lock plastic bags with silica gel, and left to dry until DNA extraction.

5.2.2. DNA extraction

Optimization of the DNA extraction process was required as there was no protocol developed for the species prior to this study. Accordingly, we optimized the process to determine from which plant tissue we could extract high-quality DNA. We isolated DNA from silica-desiccated young leaves, cambium (inner bark), and freshly collected (non-desiccated) young leaves of the species using the CTAB method (Doyle and Doyle, 1987; 1990) at the plant cell and biotechnology laboratories of the College of Agriculture, Hawassa University. The CTAB protocol was also modified (Appendix 1) to include a repeated isolation step with chloroform and isoamyl alcohol after adding a stronger CTAB lysis buffer, as higher levels of phenolic compounds and other secondary metabolites are expected in conifer tissues (Bhardwaj et al., 2020). Although both the silica-desiccated young leaves and cambium tissues yielded good-quality DNA (Table 5.1; Figure 5.3), we used the silica-desiccated young leaves to extract DNA from the sampled male and female *A. gracilior* trees for ease of tissue collection and grinding.

The concentration and quality (purity) of the extracted DNA were checked using a Nanodrop 2000 Spectrophotometer (Thermo-Scientific, USA), which measures concentration and UV absorption ratios at A260/280 and A260/230. DNA yield and quality were also further evaluated using electrophoresis on a 1% agarose gel. When the quality of extracted DNA was found unsatisfactory, we discarded the samples and replaced them with new extractions (Appendix 5.2).

5.2.3. RAPD Analysis

The entire process of identifying sex-linked markers using RAPD primers is outlined in Figure 5.2. Before conducting the bulk segregant analysis (BSA) on pooled male and female DNA, we optimised the annealing temperatures of 60 decamer primers of arbitrary sequences (East Port, Czech Republic) in a PCR temperature gradient that ranged from 37 – 47ºC, using *A. gracilior* DNA that exhibited the best quality parameters. Thirty-nine RAPD primers that produced clear polymorphic bands were selected for the BSA with pooled male and female genomic DNA (Appendix 5.3).

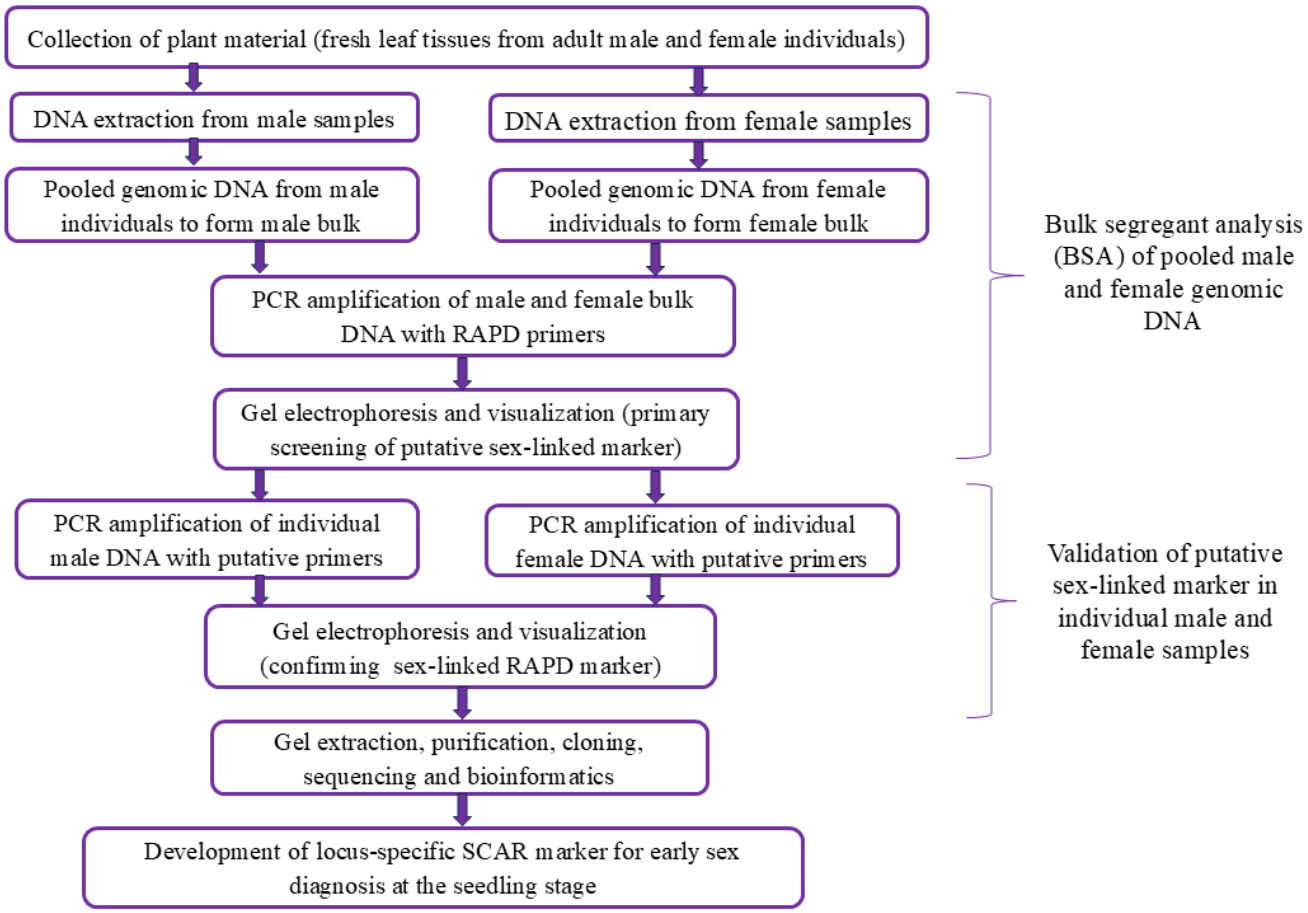

Figure 5.2. Procedure for RAPD-SCAR based sex identification in dioecious plants (adapted from Heikrujam et al., 2015)

For each identified primer, genomic DNA from the two pools (20 males in one pool and 19 females in a second pool) were used for PCR. PCR amplifications were performed in a final volume of 10 µL reaction mixture containing 5 µL of PPP Master mix (Top-Bio, Czech Republic) which contains Taq DNA polymerase, deoxyribonucleotides, reaction buffer components and additives, 1 µLof 10 pmol L^{-1} primer, 2µl of 20 ng/µL template DNA, and 2 µL of ddH2O. The PCR reactions were run in a T100 Thermal cycler (Bio-Rad, USA) programmed as follows: initial denaturation for 1 minute at 95 °C, followed by 35 cycles of denaturation for 30 seconds at 94°C, annealing for 1 minute at optimal temperature ranging from 37.4 – 46°C depending on for the respective primer (Appendix 3), extension for 1 minute at 72 °C and final extension for 10 minutes at 72 °C.

Amplified fragments were separated by size using agarose gel electrophoresis. Two µL of each PCR product were mixed with 2 µL of loading dye (Thermo Fisher Scientific, USA) and loaded

onto a 1.2% agarose gel stained with ethidium bromide (EtBr). The length of the amplified fragments for each RAPD primer was assessed based on their migration relative to a 100 bp DNA ladder (Thermo Fisher Scientific, USA). The electrophoresis was run for 75 minutes at 60 V in a 1× TBE buffer solution. Subsequently, the gels were visualized and photographed under UV light using a UVP 310 GelDoc-It^2 Imager (Fisher Scientific, USA).

Primary screening of the 39 RAPD primers using bulk male and female DNA helped us identify putative sex-linked markers, i.e., bands that appeared in either the male or female bulk but failed to amplify in the other bulk. The putative sex-linked primers obtained by screening with bulk DNA samples were then used with individual DNA samples of male and female plants for reproducibility testing and validation of the sex-specific marker/s.

5.3. Results and Discussion

5.2.1. Optimizing DNA Extraction

Since there was no DNA extraction protocol developed for *A. gracilior* prior to this study, we optimized the process to determine from which plant tissue we could extract high-quality DNA. Readings from a Nanodrop spectrophotometer and an image of agarose gel electrophoresis indicating the concentration and quality of DNA obtained from the optimization process are shown in Table 5.1 and Figure 5.2, respectively. The silica-desiccated young leaf tissue samples yielded DNA of high concentration and reasonably good purity, except for sample L11, which was affected by the leakage of contents in the centrifuge from a broken Eppendorf tube. The cambium tissue samples also yielded DNA of good concentration and purity. The missing band in the gel image for sample C22, despite comparable concentration and purity in the Nanodrop readings, is likely due to a pipetting error causing an insufficient amount to be loaded onto the gel. The non-desiccated fresh leaf samples (FL1 and FL2), on the other hand, failed to yield DNA of good concentration and purity, as demonstrated in both the gel image and the Nanodrop readings (Figure 5.2; Table 5.1).

The high quality and yield of DNA extracted from silica-desiccated young leaf tissues is consistent with the expectation that young plant tissues generally produce DNA of high quality and quantity. This can be attributed to their high proportion of actively dividing cells and lower concentrations of secondary metabolites, such as polyphenols and polysaccharides, which are

known to impede DNA extraction and compromise quality (Varma et al., 2022; Schenk et al., 2023). In contrast, the very low DNA quality and quantity obtained from non-desiccated fresh leaves, despite being young, is likely due to the activity of plant nucleases. In the absence of effective preservation techniques like desiccation or lyophilization, these enzymes remain active and degrade the DNA (Till et al., 2015).

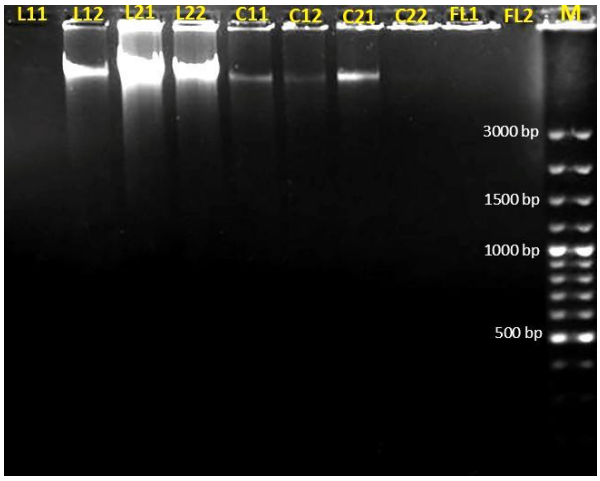

Figure 5.3. Agarose gel electrophoresis image of the DNA samples extracted during the optimization process. L11, L12, L21, and L22 refer to silica-desiccated leaf samples; C11, C12, C21, and C22 refer to cambium tissue samples; FL1 and FL2 refer to non-desiccated fresh leaf samples; M stands for a 100 bp DNA size ladder.

Table 5.1. Nanodrop readings* of DNA samples from the tissue optimization process.

Sample	Concentration (ng/µl)	A260/A280	A260/A230
L11	37.54±1.42	1.46±0.02	0.35±0.03
L12	370.28±4.47	1.85±0.01	0.89±0.02
L21	1484.6±9.54	1.96±0.03	1.71±0.03
L22	884.65±7.35	1.97±0.02	1.65±0.01
C11	401.99±5.42	1.83±0.01	0.83±0.02
C12	218.55±3.28	1.96±0.02	0.69±0.01
C21	573.71±6.16	1.88±0.03	0.93±0.02
C22	562.07±5.92	1.96±0.02	1.16±0.01
FL1	80.43±1.84	1.58±0.04	0.35±0.03
FL2	0	-	-

*The values presented are the means (± standard deviation) of three readings of the same sample

The high quality and yield of DNA extracted from cambium tissues present an excellent alternative to the commonly used leaf sampling, especially when young leaves are inaccessible. In many tropical forest tree species, accessing canopies for leaf sampling is challenging due to their height, often requiring climbing, which increases time, cost, and risk (Colpaert et al., 2005; Mangaravite et al., 2020). Furthermore, tropical tree leaves are frequently subjected to herbivory and infections, prompting the accumulation of defensive secondary metabolites such as alkaloids, cyanides, polyphenols, and terpenes (Colpaert et al., 2005). These compounds interfere with DNA extraction and can inhibit downstream processes like PCR and sequencing (Schenk et al., 2023). Additionally, leaf samples may harbor microorganisms and small insects, leading to contamination with foreign DNA (Colpaert et al., 2005). In such situations, cambium tissues offer a practical alternative, being more accessible, typically lower in secondary metabolite content, and less prone to contamination. For *A. gracilior*, a tall tree often exceeding 30 meters in natural forests, young leaves are inaccessible, not available during the dry season, and are frequently infested by insects like aphids. Consequently, cambium tissue sampling provides a reliable and versatile solution under such constraints.

5.2.2. RAPD Analysis

The first step of the RAPD analysis involved optimizing the annealing temperatures of 60 decamer primers for PCR using a temperature gradient ranging from 37°C to 47°C. This optimization identified 39 primers capable of amplifying random sequences of *A. gracilior* DNA (Appendix 5.3). Figure 5.4 presents examples of agarose gel electrophoresis images showing the PCR products generated during this optimization process. Screening 39 working RAPD primers using bulked male and female DNA in PCR identified 14 primers that showed sex-specific banding patterns, making them putative sex-linked markers. In agarose gel images, at least one band appeared exclusively in either male or female bulk DNA lanes, absent in the other. Representative gel profiles are presented in Figure 5.5 (A and B).

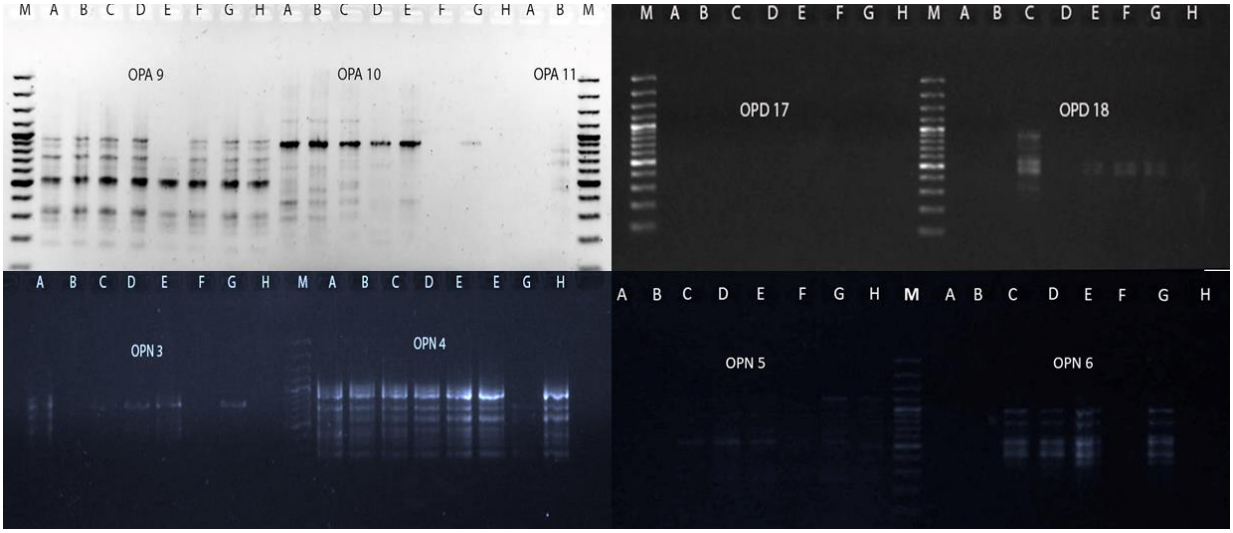

Figure 5.4. Agarose gel electrophoresis images showing PCR products from annealing temperature optimization across a gradient of 37°C to 47°C for primers OPA 9, OPA 10, OPD 17, OPD 18, OPN 3, OPN 4, OPN 5, and OPN 6. The temperature gradient along lanes A to H corresponds to 47°C, 46.2°C, 44.9°C, 43.3°C, 40.9°C, 38.8°C, 37.6°C, and 37°C, respectively. Lane M represents a 100 bp molecular size ladder.

For example, primer OPA-08 produced a 1000 bp band specific to the male bulk but absent in the female bulk (lanes A8m and A8f in Figure 5.5A). Similarly, primer OPA-09 generated an approximately 700 bp band specific to the male bulk and an approximately 400 bp band specific to the female bulk (lanes A9m and A9f in Figure 5.5A). In Figure 5.5B, primer OPD-18 (lanes A18m and A18f) produced two male-specific bands, approximately 600 bp and 1000 bp, which were absent in the female bulk. Additionally, primer OPN-18 (lanes N18m and N18f) produced an approximately 1200 bp band specific to the male bulk but absent in the female bulk. However, subsequent PCR using DNA from individual male and female samples to validate the putative sex-linked markers revealed that most of these markers lacked consistent specificity to either sex. Figure 5.6 (A and B) presents examples where primers initially identified as putative sex-linked markers failed to demonstrate consistent specificity when tested on individual DNA samples.

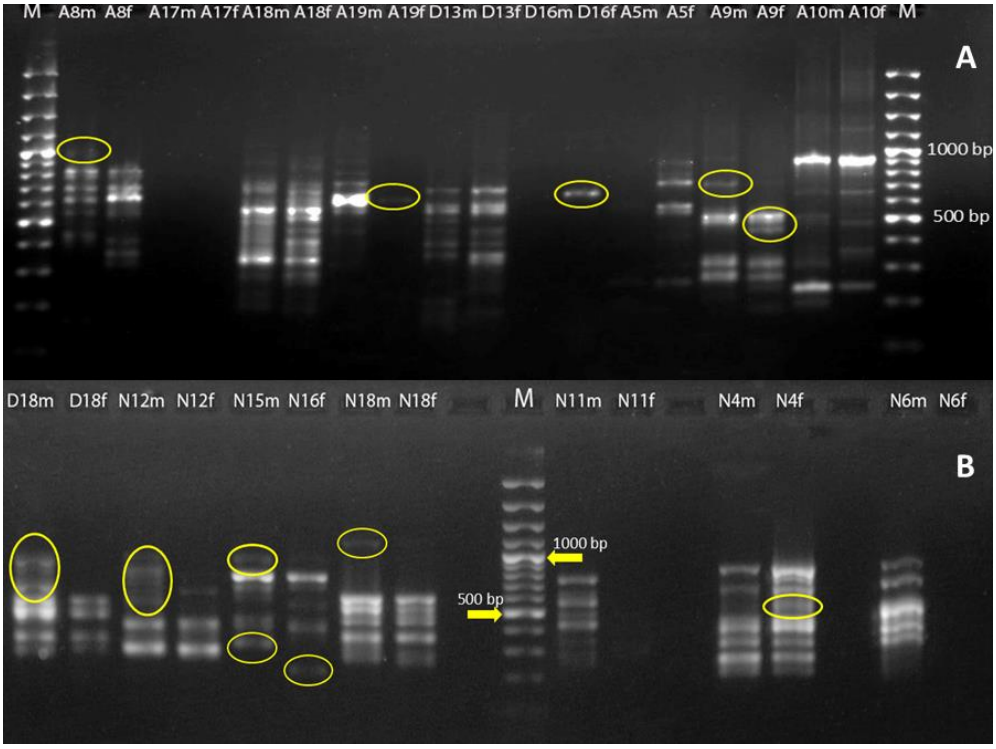

Figure 5.5. Agarose gel electrophoresis profiles highlighting putative sex markers identified with PCR screening using bulk male and female DNA (BSA). (A) RAPD primers OPA-08, OPA-19, OPD-16, and OPA-09 showing specific bands in either male or female bulk samples. (B) Male- or female-specific bands observed for primers OPD-18, OPN-12, OPN-15, OPN-18, OPN-04, and OPN-06. Lane M represents a 100 bp molecular size ladder.

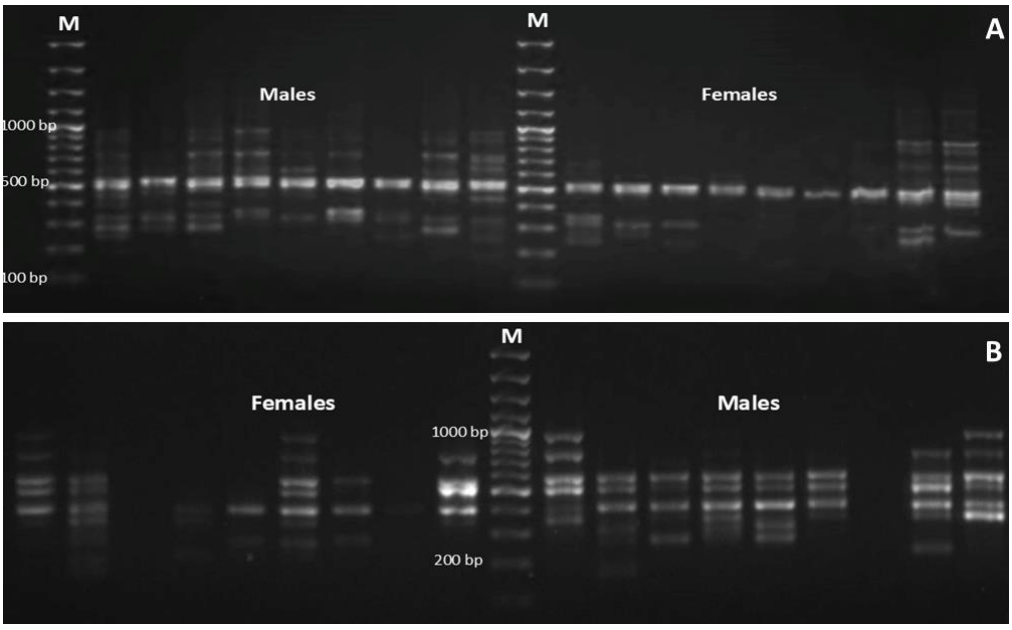

Figure 5.6. Example agarose gel profiles of PCR products using primers A) OPA-09 and B) OPN-06, which produced putative sex-linked markers during bulk segregant analysis but failed to confirm sex specificity in individual male and female samples. Lane M represents a 100 bp molecular size ladder.

In contrast, primer OPD-18 appeared to produce a 600 bp band that was consistently present in individual male samples but absent in individual females (Figure 5.7). Therefore, the RAPD primer OPD-18 (5'-GAGAGCCAAC-3') could be recognized as a male-specific sex-linked marker, enabling the molecular differentiation of male and female individuals of the dioecious *A. gracilior* at earlier life stages, without the need to wait for flowering and seed setting.

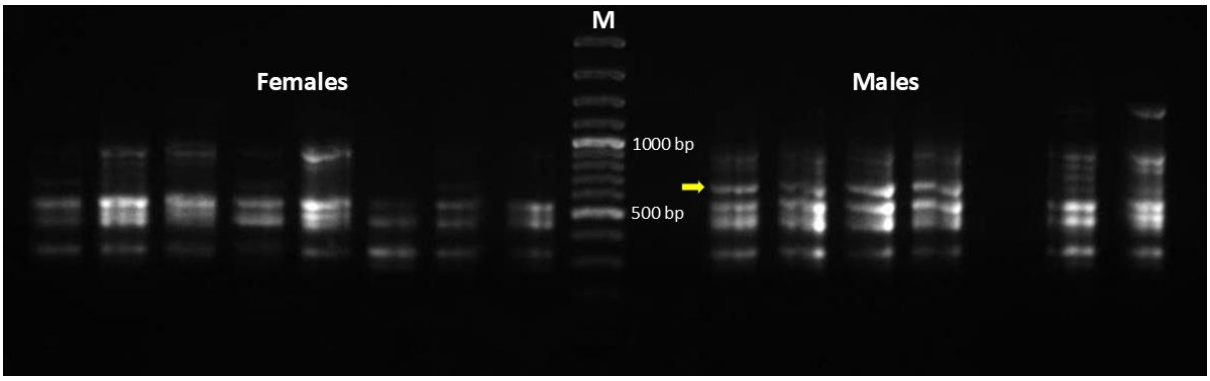

Figure 5.7. Agarose gel profiles of PCR products obtained using primer OPD-18 on DNA of individual male and female *A. gracilior* plants. The arrow highlights an approximately 600 bp band present in the male samples but absent in the female samples. Lane M represents a 100 bp molecular size ladder.

The advent of RAPD markers (Williams et al., 1990) generated significant enthusiasm in the 1990s, as they did not require prior sequence information for development. Unlike other molecular markers of the time, such as restriction fragment length polymorphism (RFLP), RAPD markers were also technically less cumbersome and easier to apply. However, their limited reproducibility (across experiments and laboratories) and dominant nature have constrained their broader applicability (Staub et al., 1996; Weising et al., 2005). The lack of reproducibility is primarily due to the high sensitivity of RAPD markers to slight changes in PCR reaction conditions, such as variations in DNA quality and concentration, Mg^{2+} and salt concentrations, thermal cycler calibration, and primer or enzyme concentrations. These variations can lead to inconsistent banding patterns between experiments (Weising et al., 2005). This high sensitivity is attributed to the non-stringent PCR conditions inherent to RAPD primers. These primers are short

oligonucleotides, typically 10 bases long, and have low annealing temperatures, which promote mismatch priming and non-specific binding.

To address the limitations of RAPD markers, a more reliable class of molecular markers, known as sequence-characterized amplified regions (SCARs), was developed (Paran & Michelmore, 1993). SCAR markers are derived by cloning and sequencing PCR-amplified RAPD fragments of interest and designing longer (24-mer) oligonucleotide primers complementary to the ends of the sequenced RAPD fragments. The use of these longer primers, combined with high annealing temperatures (i.e., stringent PCR conditions), allows for the reproducible amplification of a specific, single locus. This makes SCAR markers significantly more reliable than RAPDs, as they are less sensitive to variations in PCR reaction conditions. Additionally, SCAR markers can potentially be converted to codominant markers, further enhancing their utility in genetic studies.

RAPD-derived SCAR markers have been successfully employed for molecular sex identification in several dioecious plants. Examples include *Cannabis sativa* (Mandolino et al., 1999), *Carica papaya* (Chaves-Bedoya & Nuñez, 2007; Modi et al., 2018), *Ginkgo biloba* (Lao et al., 2009), *Momordica dioica* (Patil et al., 2012), *Hippophae rhamnoides* (Korekar et al., 2012; Zhou et al., 2018), *Piper betle* (Khadke et al., 2012), *Garcinia gummi-gutta* (Joseph et al., 2014), asparagus (Kim et al., 2014), salak palm (Li et al., 2017), and date palm (Al-Qurainy et al., 2018). While the application of SCAR markers for sex identification is specific and reliable, the process—starting from identifying RAPD or other PCR-based primers to isolating and sequencing the sex-linked band, and developing sequence-specific primers—is somewhat laborious and time-consuming (Weising et al., 2005; Wang et al., 2019). For instance, Li et al. (2017) screened 901 RAPD primers to develop a single male-specific SCAR marker for salak palm, while Jiang and Sink (1997) screened 760 RAPD primers to develop a male-specific SCAR marker for asparagus. These examples underscore how laborious and lengthy the process can be. In recent years, next-generation sequencing (NGS) technologies, RAD-seq, ddRAD-seq, and whole-genome sequencing, have emerged as faster and more efficient alternatives to conventional PCR-based methods for sex identification in dioecious plants (Zhou et al., 2018; Wang et al., 2019; Guo et al., 2023; Zeng et al., 2024).

The 600 bp male-specific RAPD marker (OPD-18) identified in this study for *A. gracilior* holds potential for conversion into a SCAR marker, offering a more robust and reliable tool for molecular sex identification in this dioecious species. Originally, we planned to advance the work on developing SCAR markers, which involves extracting and purifying the male-specific band amplified by the OPD-18 primer, Sanger sequencing, and designing locus-specific primers based on the sequence (Figure 2). However, due to resource and time constraints, we had to pause the investigation at this stage. Nevertheless, the promising results achieved so far lay a strong foundation for future efforts. We aim to resume our research by converting the RAPD marker into a SCAR marker, which will address reproducibility concerns and enhance its practical utility. Additionally, with the increasing accessibility of NGS technologies, we plan to explore high-throughput approaches, such as RAD-seq or whole-genome sequencing, to identify sex-specific loci with greater precision and efficiency in *A. gracilior*. Such advancements would not only improve molecular sex identification but also contribute to a deeper understanding of the genetic basis of dioecy in this species.

5.3. Conclusion

In this study, we identified OPD-18 as a sex-linked RAPD marker, amplifying a 600 bp band specific to male individuals of *Afrocarpus gracilior*, providing a potential tool for sex identification at the juvenile stage. However, the limitations of RAPD markers, including their dominant nature and reproducibility issues, underscore the need for further refinement. Future research should focus on converting this marker into a SCAR marker for improved reliability and exploring high-throughput sequencing approaches to enhance sex identification accuracy, as well as gaining a more nuanced understanding of sexual dimorphism at the molecular or chromosomal level. After developing and validating a reliable sex-linked marker, further research could link the molecular marker to morphological traits for easy and inexpensive applicability. Developing robust markers will facilitate the effective management of *A. gracilior* populations, ensuring a balanced representation of both sexes in plantations, supporting breeding programs that may be envisaged for the species, and contributing to the long-term success of restoration initiatives.

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CHAPTER SIX

6. Summary, Conclusions, and Recommendations

6.1. Summary and Conclusions

Deforestation has profoundly reshaped landscapes worldwide, driving biodiversity loss, disrupting ecosystem functions, and fragmenting forest habitats. Such fragmentation often reduces genetic diversity and increases inbreeding within tree populations, potentially leading to inbreeding depression characterized by diminished reproductive success and reduced progeny vigor. In response, global commitments to large-scale tree planting and forest restoration have intensified, with Ethiopia pledging to restore 22 million hectares of degraded land by 2035. However, the long-term success of these initiatives hinges on selecting appropriate species and ensuring genetically diverse seed sources, which are essential for ecosystem resilience and adaptability.

This dissertation examines key genetic considerations in forest conservation and restoration, highlighting challenges in seed and seedling quality, the genetic risks associated with forest fragmentation, and the implications of dioecy in tree management. Using *Afrocarpus gracilior*, a dioecious and wind-pollinated conifer, the study assesses genetic diversity, inbreeding levels, and the applicability of molecular sex identification techniques. The key findings described below, synthesized from chapters 2 – 5, offer valuable insights to enhance restoration efforts, maintain genetic integrity, and advance molecular tools for species conservation.

The first study assessed the extent to which genetic principles are considered in species selection and seed procurement for forest restoration in Ethiopia. The findings indicate that genetic considerations are frequently overlooked, with species selection dominated by a few exotic species, including *Grevillea robusta, Eucalyptus camaldulensis, Acacia decurrens*, and *Cupressus lusitanica*. Native species remain underrepresented, and seed collection practices often fail to follow guidelines essential for maintaining genetic diversity. Notably, 84% of seed collectors source seeds from any available tree, 87% of nurseries receive seeds without passport data, 97% of seed collectors do not adhere to a minimum number of mother trees per collection event, and

88% disregard recommended distances between selected mother trees. These practices compromise the evolutionary resilience and adaptive capacity of planted seedlings, ultimately affecting the long-term success of restoration efforts.

The second study investigated genetic diversity, population structure, and differentiation in fragmented populations of *Afrocarpus gracilior* using SNP markers generated via the DArTseq platform. Results revealed an overall low genetic diversity ($H_e < 0.1$) across all populations, with progeny cohorts exhibiting even lower diversity than adult trees. Progeny from isolated or few mother trees showed the lowest genetic diversity, suggesting increased genetic drift and inbreeding. In contrast, remnant forest patches of relatively larger size and sacred sites, such as churches and traditional worship areas, maintained relatively higher genetic diversity, underscoring their importance for *in situ* conservation. Genetic differentiation between populations ranged from low ($F_{ST} < 0.05$) to moderate ($0.05 < F_{ST} < 0.15$), with progeny from smaller populations exhibiting relatively higher differentiation. Most genetic variation was found within populations (57–61%), while variation between populations (1.07–4.93%) and among individuals (approximately 38%) was lower.

A follow-up study examined phenotypic traits related to reproduction and early progeny vigor. The findings revealed that progeny from small and isolated populations—those with the lowest genetic diversity—exhibited significantly reduced fitness. Germination rates were 53% lower, acclimatization success was reduced by 33%, diameter growth was 30% slower, height growth was 41% lower, and leaf scorch incidence increased by 80%. Strong and significant correlations between genetic diversity and progeny fitness traits confirmed that genetic erosion is a key factor driving reduced fitness. These results indicate that inbreeding depression severely affects the viability of progeny from small and isolated populations of *A. gracilior*, posing a serious threat to the long-term persistence of the species.

The final study sought to develop a molecular marker for early sex identification in *A. gracilior* using RAPD primers. Screening of 60 RAPD primers identified 14 putative sex-linked markers, of which a single marker (OPD-18) was confirmed to be sex-linked. OPD-18 consistently produced a 600-bp band in male individuals but was absent in females, making it a potential tool

for early sex identification. However, concerns regarding reproducibility highlight the need for further refinement, such as converting it into a SCAR marker or employing high-throughput approaches to enhance reliability.

Overall, the findings of this dissertation lead to the following key conclusions:

1. Genetic considerations in species selection and seed procurement are crucial for the success of forest restoration initiatives in Ethiopia. However, current practices often neglect these principles, increasing the risk of inbreeding and loss of adaptive potential. Strengthening guidelines for seed sourcing and nursery management is essential to enhance genetic diversity in restoration efforts.
2. Low genetic diversity and genetic drift in fragmented populations of *A. gracilior* pose a serious threat to the species' long-term viability. Larger remnant patches and sacred sites harbor higher genetic diversity, highlighting their importance as priority areas for *in situ* conservation.
3. Inbreeding depression significantly reduces progeny fitness in small and isolated populations of *A. gracilior*, as evidenced by lower germination rates, reduced growth performance, and higher susceptibility to stress. These findings emphasize the need for seed collection strategies that prioritize genetically diverse populations to improve restoration outcomes.
4. The identification of a sex-linked RAPD marker for early sex determination in *A. gracilior* represents a valuable step toward improving the management of dioecious tree species. However, further refinement and conversion to a SCAR marker is necessary to enhance reliability for practical applications.

6.2. Recommendations

1. Policy and Regulation: Establish and enforce policies that promote the use of native species in restoration projects while ensuring adherence to genetic standards in seed procurement. Authorities should incentivize seed dealers supplying genetically diverse and high-quality seeds while discouraging uncertified vendors.

2. Seed Sourcing: Source seeds from genetically diverse and larger populations, including culturally protected sites such as sacred groves and remnant forest patches, while avoiding collection from isolated or few mother trees. When seed shortages occur, wildlings from larger patches and sacred sites could serve as alternative germplasm sources for restoration efforts.

3. Conservation Efforts: Prioritize in-situ conservation of *A. gracilior* in sacred sites and larger forest patches to maintain genetic diversity.

4. Monitoring and Evaluation: Conduct regular assessments of restoration projects to evaluate survival, growth, and genetic diversity, ensuring that planted populations maintain genetic integrity and adaptive potential.

5. Further Research: Future studies should address the following research gaps to complement the present study:

    - Conduct genetic diversity analyses on a broader range of *A. gracilior* populations, including those outside Ethiopia, to gain a more comprehensive understanding of the species' genetic structure.

    - Perform gene flow and parentage analyses to provide a more detailed and nuanced understanding of the genetic consequences of habitat fragmentation in *A. gracilior*.

    - Continue developing robust molecular markers by refining the identified RAPD marker into a SCAR marker or exploring high-throughput methods to improve the reliability of early sex determination in dioecious *A. gracilior*.

    - Link the molecular markers to morphological traits for easy and inexpensive application in early sex identification.

APPENDICES

Appendix 3.1. Marker quality information

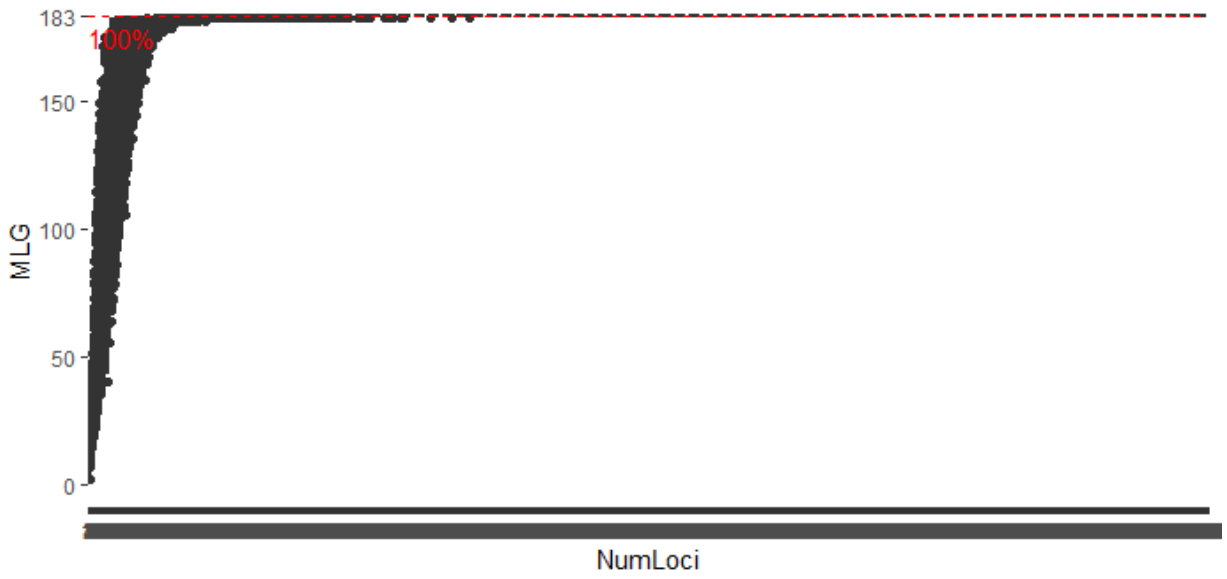
a. Transition and transversion-type SNPs of the 1820 filtered loci

Types of SNPs	Base substitutions	Number of loci	Frequency
Transitions	A/G	466	0.256
	C/T	489	0.269
	Total	955	0.525
Transversions	A/C	210	0.115
	A/T	200	0.110
	G/C	196	0.108
	G/T	259	0.142
	Total	865	0.475
Total		1820	1

b. A summary of different SNP marker quality and diversity parameters

Marker quality and diversity parameters	Min	Mean	Max
Call rate	0.699	0.774	1.000
One ratio-reference allele	0.006	0.818	1.000
One ratio-SNP allele	0.006	0.209	1.000
Reproducibility	0.950	0.994	1.000
Minor allele frequency (MAF)	0.003	0.045	0.496
Nei's genetic diversity (He)	0.006	0.077	0.500
Polymorphic information content (PIC)	0.006	0.074	0.494

Appendix 3.2. Genotype accumulation curve



S1 Figure. Genotype accumulation curve of 183 genotypes of *A. gracilior* over 1820 loci after filtering. The x-axis represents the number of loci randomly sampled (up to n - 1 = 1819) and the y-axis represents the number of multilocus genotypes (MLG) observed.

Appendix 3.3. Pairwise geographic distance matrix between populations

AUG	0																		
AUG_p	0	0																	
BTD	162.6	162.6	0																
BTD_p	162.6	162.6	0	0															
DAD	11.2	11.2	164.6	164.6	0														
DAD_p	11.2	11.2	164.5	164.5	0	0													
GDB	11.7	11.7	173.0	173.0	18.9	18.9	0												
MEG	24.7	24.7	175.9	175.9	14.8	14.8	27.3	0											
MEG_p	24.7	24.7	175.9	175.9	14.8	14.8	27.3	0	0										
TBO	178.9	178.9	20.1	20.1	181.6	181.6	188.9	193.5	193.5	0									
TBO_p	178.9	178.9	20.1	20.1	181.6	181.6	188.9	193.5	193.5	0	0								
TKW	125.6	125.6	44.7	44.7	125.7	125.7	136.8	135.7	135.7	64.6	64.6	0							
TSO	180.2	180.3	21.5	21.5	183.0	182.9	190.2	194.8	194.9	1.4	1.4	66.1	0						
TSO_p	180.2	180.2	21.4	21.4	182.9	182.9	190.2	194.8	194.8	1.4	1.3	65.9	0	0					
WAB	162.7	162.7	13.1	13.1	163.8	163.8	173.5	174.4	174.4	30.2	30.2	39.5	31.4	31.2	0				
WAB_p	162.7	162.7	13.0	13.0	163.8	163.8	173.5	174.4	174.4	30.2	30.2	39.5	31.4	31.2	0.0	0			
WGC	134.9	134.9	51.0	51.0	133.7	133.7	146.4	142.3	142.3	70.8	70.8	17.5	72.1	72.0	41.6	41.6	0		
WGC_p	135.0	135.0	50.9	50.9	133.8	133.8	146.4	142.4	142.4	70.7	70.7	17.5	72.1	71.9	41.6	41.6	0.1	0	
WGD	162.1	162.1	14.4	14.4	163.0	163.0	172.8	173.6	173.6	31.7	31.7	38.5	32.9	32.7	1.5	1.5	40.3	40.2	0
WGD_p	162.0	162.0	14.3	14.3	163.0	163.0	172.8	173.5	173.6	31.6	31.7	38.0	32.9	32.7	1.5	1.5	40.3	40.2	0.0	0
	AUG	AUG_p	BTD	BTD_p	DAD	DAD_p	GDB	MEG	MEG_p	TBO	TBO_p	TKW	TSO	TSO_p	WAB	WAB_p	WGC	WGC_p	WGD	WGD_p

Geographic distance (in kilometers) between any pair of the study populations

Population codes are described in Table 3.1 of the main text

Appendix 3.4. Trees of Wonsho Gudumale (the WGD site)

S2 Figure. Old trees at the WGD site: a) a few standing trees on the site; b & c) the oldest mother tree on the site, photo shots at the base and the crown respectively; d) the trunk of the oldest tree with an average person, for scale; e & f) measuring the circumference, which was 10.46 meters, equivalent to a diameter of 3.33 meters.

Appendix 4.1. ANOVA models and outputs

a. Percent of intact seeds by population size

```
> seedset.data$intact <- sqrt(seedset.data$intact)
> one.way_is <- aov(intact ~ Size, data = seedset.data)
> summary(one.way_is)
            Df Sum Sq Mean Sq F value   Pr(>F)
Size         2  53.88  26.938   10.78 0.000121 ***
Residuals   52 129.94   2.499
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

b. Seed weight by population size

```
> one.way_w1 <- aov(weight_1000 ~ Size, data = seedset.data)
> summary(one.way_w1)
            Df Sum Sq Mean Sq F value   Pr(>F)
Size         2   4035  2017.7   23.52 8.34e-08 ***
Residuals   47   4032    85.8
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

c. *In vitro* germination by population size

```
> germn.data$gp <- asin(sqrt(germn.data$gp))
> one.way_gp <- aov(gp ~ Size, data = germn.data)
> summary(one.way_gp)
            Df Sum Sq Mean Sq F value   Pr(>F)
Size         2 0.8154  0.4077   9.873 0.000263 ***
Residuals   47 1.9407  0.0413
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

d. Lathhouse acclimatization by population size

```
> one.way_accs <- aov(AccP ~ Size, data = acclmtzn.data) #by popn size
> summary(one.way_accs)
            Df Sum Sq Mean Sq F value Pr(>F)
Size         2   4204  2101.8   4.956 0.0154 *
Residuals   25  10602   424.1
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

e. Growth in height by population size

```
> one.way_hts <- aov(Ht ~ Size, data = growth.data) #by popn size
> summary(one.way_hts)
             Df Sum Sq Mean Sq F value   Pr(>F)
Size          2 1166.2   583.1   38.84 1.98e-11 ***
Residuals    58  870.7    15.0
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

f. Growth in diameter by population size

```
> one.way_diams <- aov(Diam ~ Size, data = growth.data) #by popn size
> summary(one.way_diams)
             Df Sum Sq Mean Sq F value   Pr(>F)
Size          2  11.86   5.928   23.18 3.99e-08 ***
Residuals    58  14.83   0.256
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

g. Leaf scorch by population size

```
> growth.data$Scorch<- log10(growth.data$Scorch)
> one.way_scr <- aov(Scorch ~ Size, data = growth.data) #by popn size
> summary(one.way_scr)
             Df Sum Sq Mean Sq F value   Pr(>F)
Size          2 0.4818 0.24091   54.55 4.71e-14 ***
Residuals    58 0.2561 0.00442
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Appendix 4.2. Tukey's HSD post-hoc mean separation of traits used in the ANOVA

Abbreviations: INT = percentage of intact seeds (data square root transformed); TSW = weight of 1000 seeds; IGP = *in vitro* germination percentage (data arcsine transformed); ACL = acclimatization; DIM = diameter; HGT = height; SCR = leaf scorch (data $\log_{10}$ transformed).

Population size class	INT (sqrt)	TSW	IGP (asin)	ACL	HGT	DIM	SCR (log)
Large	8.4±2.0[a]	116.7±2.9[a]	0.97±0.12[a]	91±5.6[a]	25.4±3.6[a]	3.4±0.5[a]	1.8±0.3[a]
Intermediate	7.5±1.6[a]	103±10.5[b]	0.76±0.14[b]	81.1±8.6[ab]	16.9±3.4[b]	2.5±0.5[b]	1.6±0.2[a]
Small	5.4±0.3[b]	87.4±7.6[b]	0.58±0.24[c]	61.1±5.1[b]	15.1±2.2[b]	2.4±0.5[b]	1.1±0.2[b]

Appendix 5.1. Modified CTAB protocol used in the present study

1. Grind tissue using mortar and pestle (with a little addition of sterilized, laboratory-grade sand)
2. Put ground tissue in a 2 ml Eppendorf tube
3. Add 800 µl of extraction buffer (Table 1), vortex well
4. Add 5 µl of Proteinase K (20 mg/ml), keep for 1 hour at 65°C, mix every 10 min, let cool at room temperature
5. Add 700 µl of chloroform: IAA (24:1) and mix for 10 min
6. Centrifuge for 10 min at 14000 RPM (at 4°C, optional)
7. Transfer the supernatant into a new 2 ml Eppendorf tube, add 55 µl of CTAB 7% and mix for 5 min, repeat steps 5. and 6.
8. Transfer supernatant into a 1.5 ml Eppendorf tube, add 700 µl of isopropanol, mix for 5 min and store at -20°C for one hour or at 4°C overnight
9. Centrifuge for 10 min at 14000 RPM. A white pellet should form at the bottom of the tube. Eliminate the supernatant and wash pellet twice with ethanol:
    i. Add 400 µl of 96% EtOH, leave for 3 min at 37°C, discard EtOH
    ii. Add 400 µl of 70% EtOH, leave for 5 min at room temperature, (centrifuge for 3 min), discard EtOH
    iii. Let pellet dry at room temperature
10. Add 100 µl of double distilled water or 1× TE buffer and 5 µl of RNase and leave at 37°C until pellet dissolves

Table 1. CTAB extraction buffer (300 ml)

Reagents	Final Concentration	Mr	Quantity for 300 ml
CTAB	2,8%		8.4 g
NaCl	1,3 M	58.44	22.8 g
EDTA	20 mM	292.24	1.75 g
TRIS-HCl (pH 8,0) 1 M	100 mM		30 ml
PVP 40	1%		3 g
Mercaptoethanol	0,2%		600 ul
Water	-		Add to 300 ml

Appendix 5.2: DNA concentration and quality of male and female *A. gracilior* samples measured using a Nanodrop spectrophotometer, and dilution details for preparing working template DNA solutions for PCR reactions

Sample ID	Nanodrop readings			Remark	Dilution (20ng/µl)		Dilution (50ng/µl)	
	Conc. (µg/ml)	A260/280	A260/230		DNA (µl)	ddH2O (µl)	DNA (µl)	ddH2O (µl)
F1	277.21	1.89	0.854		7.2	92.8	18	82
F2	207.35	1.753	0.684		9.6	90.4	24.1	75.9
F3	169.07	1.803	0.644		11.8	88.2	29.6	70.4
F4	229.21	1.809	0.961		8.7	91.3	21.8	78.2
F5	199.76	1.817	0.79		10	90	25	75
F6	561.4	1.83	0.86	resampled	3.6	96.4	8.9	91.1
F7	291.8	1.882	1.086		6.9	93.1	17.1	82.9
F8	390	1.918	1.154		5.1	94.9	12.8	87.2
F9	253.89	1.827	0.811		7.9	92.1	19.7	80.3
F10	291.9	1.854	0.809		6.9	93.1	17.1	82.9
F11	424.71	1.839	0.849		4.7	95.3	11.8	88.2
F12	425.88	1.786	0.716		4.7	95.3	11.7	88.3
F13	197.88	1.778	0.668		10.1	89.9	25.3	74.7
F14	261.64	1.785	0.742		7.6	92.4	19.1	80.9
F15	492.08	1.74	0.55	resampled	4.1	95.9	10.2	89.8
F16	745.99	1.79	0.81	resampled	2.7	97.3	6.7	93.3
F17	488.34	1.79	1.03	resampled	4.1	95.9	10.2	89.8
F18	235.99	1.795	0.755		8.5	91.5	21.2	78.8
M1	422.48	1.83	0.91	resampled	4.7	95.3	11.8	88.2
M2	328.7	1.864	1.1		6.1	93.9	15.2	84.8
M3	184.93	1.868	0.797		10.8	89.2	27	73
M4	340.28	1.87	0.787		5.9	94.1	14.7	85.3
M5	235.87	1.825	0.812		8.5	91.5	21.2	78.8
M6	436.57	1.977	1.299		4.6	95.4	11.5	88.5
M7	285.2	1.91	0.896		7	93	17.5	82.5
M8	242.11	1.791	0.6		8.3	91.7	20.7	79.3
M9	420.69	1.92	1.002		4.8	95.2	11.9	88.1
M10	491.79	1.8	0.89	resampled	4.1	95.9	10.2	89.8
M11	750.42	1.72	0.79	resampled	2.7	97.3	6.7	93.3
M12	609.85	1.82	0.74	resampled	3.3	96.7	8.2	91.8
M13	358.83	1.948	1.261		5.6	94.4	13.9	86.1
M14	391.96	1.891	0.897		5.1	94.9	12.8	87.2
M15	293.45	1.991	1.385		6.8	93.2	17	83
M16	468.13	1.961	1.35		4.3	95.7	10.7	89.3
M17	389.06	1.948	1.154		5.1	94.9	12.9	87.1
M18	258.75	1.781	1.213		7.7	92.3	19.3	80.7
M19	274.16	1.892	0.945		7.3	92.7	18.2	81.8
M20	432.49	1.77	1.01	resampled	4.6	95.4	11.6	88.4

Appendix 5.3: List of decamer RAPD primers used in bulk segregant analysis of male and female DNA samples, along with their optimized annealing temperatures

Primer	Sequence 5'-3'	Ta	Primer	Sequence 5'-3'	Ta
OPA-01	CAGGCCCTTC	37°C	OPD-10	GGTCTACACC	37°C
OPA-02	TGCCGAGCTG	40.9°C	OPD-11	AGCGCCATTG	40.9°C
OPA-03	AGTCAGCCAC	40.5°C	OPD-12	CACCGTATCC	37°C
OPA-04	AATCGGGCTG	37°C	OPD-13	GGGGTGACGA	46.2°C
OPA-05	AGGGGTCTTG	40.9°C	OPD-14	CTTCCCCAAG	45°C
OPA-06	GGTCCCTGAC	45°C	OPD-15	CATCCGTGCT	45°C
OPA-07	GAAACGGGTG	45°C	OPD-16	AGGGCGTAAG	46.2°C
OPA-08	GTGACGTAGG	46.2°C	OPD-18	GAGAGCCAAC	45°C
OPA-09	GGGTAACGCC	40.9°C	OPN-02	ACCAGGGGCA	44.3°C
OPA-10	GTGATCGCAG	40.9°C	OPN-03	GGTACTCCCC	40.9°C
OPA-11	CAATCGCCGT	45°C	OPN-04	GACCGACCCA	40.9°C
OPA-17	GACCGCTTGT	46.2°C	OPN-05	ACTGAACGCC	44.3°C
OPA-18	AGGTGACCGT	46.2°C	OPN-06	GAGACGCACA	40.9°C
OPA-19	CAAACGTCGG	46.2°C	OPN-11	TCGCCGCAAA	44.3°C
OPD-01	ACCGCGAAGG	37°C	OPN-12	CACAGACACC	45°C
OPD-02	GGACCCAACC	40.9°C	OPN-13	AGCGTCACTC	40.9°C
OPD-04	TCTGGTGAGG	37°C	OPN-15	CAGCGACTGT	45°C
OPD-05	TGAGCGGACA	40.9°C	OPN-16	AAGCGACCTG	44.3°C
OPD-07	TTGGCACGGG	45°C	OPN-18	GGTGAGGTCA	45°C
OPD-08	GTGTGCCCCA	45°C			