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Stellingen

1. It is possible to accentuate the differences between taxa with different character state definitions in such a way, that these look much more different than the taxa actually are (see Verdcourt, Verbenaceae FTEA, 1992 p. 58-59).

2. Geographical isolation of populations of a species alone is inadequate for taxonomic distinction unless it is supported by other characters, otherwise a number of ecotypes would result in endless species lists (this thesis, page 39).

3. Species cannot be fully defined, nor can it be intuitively sensed, although subjectivity is involved in decision making; a species is only as good as the knowledge and insights used in its delimitation. Certainly methodologies help, so do good sense and good judgement based on meaningful experiences, and the more the better (Crum, 1985, Bryologist 88: 221).

4. So long as the morphological, physiological and genetic characteristics of an organism do not impose limitations to its survival, evolution or speciation will be a luxury, unfortunately nature operates on necessities only (this thesis page 91).

5. To reap the investment and the potential of Meru oak in plantations of Mt. Kenya region, thinning to reduce stem density is urgently necessary.

6. The frontier of science is like a mirage, the closer you get the further it moves.

7. Enforcing good governance in developing countries by withholding aid does not work; by the time the government is brought to its knees and submits to the demand, the ordinary people would be on their bellies.

8. The decision by the Bank of England to sell of part of its gold reserves to bail out the heavily indebted, developing countries is like creating fresh wounds to temporarily relieve the pains of the old ones.

9. Waiting for something to happen is worse than when it just happens.

10. Necessity is the mother of all creation but imagination is the first step to creativity.

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Taxonomy and Genetic Structure of Meru Oak Populations, *Vitex keniensis* Turrill and *Vitex fischeri* Gürke, in East Africa

Joseph Oloo Ahenda

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Contents

Summary 3

Samenvatting 5

1 Introduction 7
1.1 Taxonomy and genetic diversity in relation to conservation of tropical forest trees 7
1.2 Genetic structure and mating systems of forest tree populations 9
1.3 The economic importance and exploitation of Meru oak 10
1.4 Project background 12
1.5 Project objectives 13
1.6 Thesis organisation 14

2 The taxonomy and geographical distribution of *V. keniensis* and *V. fischeri* . 15
2.1 Introduction 15
2.2 Conspectus of the genus *Vitex* in East Africa 15
2.3 Brief description of *V. keniensis* and *V. fischeri* 18
2.4 The ecology of the natural range of *V. keniensis* and *V. fischeri* 19
2.4.1 The mountainous forests of Mt. Kenya and Nyambene hills 19
2.4.2 The woody wet savanna at the foot of Mt. Elgon and Lake Victoria basin 21
2.5 Comparison between *V. keniensis* and *V. fischeri* herbarium specimens 22
2.6 Morphological and cytological characteristics of *V. keniensis* and *V. fischeri* 27
2.6.1 Morphological variation in *V. keniensis* and *V. fischeri* seed characteristics 27
2.6.2 Seedling leaf morphology of *V. keniensis* and *V. fischeri* 29
2.6.3 Wood characteristics of *V. keniensis* and *V. fischeri* 31
2.6.4 Cytological characteristics of *V. keniensis* and *V. fischeri* 35
2.7 Conclusion 38

3 Mating systems of *V. keniensis* and *V. fischeri* 41
3.1 Introduction 41
3.2 Floral morphology and phenology of *V. keniensis* and *V. fischeri* 42
3.3 Reproductive biology of *V. keniensis* and *V. fischeri* 46
3.3.1 Viability assessment of *V. keniensis* and *V. fischeri* pollen grains . 46
3.3.2 Evaluation of stigma receptivity of *V. keniensis* and *V. fischeri* . . 49
3.3.3 Pollination mechanisms and self-compatibility in *V. keniensis* and *V. fischeri* 51

3.3.4 Estimates of the mating parameters of *V. keniensis* and *V. fischeri* populations 54
3.3.5 Comparison of the germination vigour of seeds from cross- and self-pollination of *V. keniensis* and *V. fischeri* 55
3.3.6 Reproductive compatibility between the *V. keniensis* and *V. fischeri* populations and the vigour of the resultant seeds 58
3.4 Conclusion 59

4 The genetic structure of *V. keniensis* and *V. fischeri* populations 61
4.1 Introduction 61
4.2 Analyses of the genetic structure of *V. keniensis* and *V. fischeri* populations 63
4.2.1 Sampling and isozyme analysis methods 63
4.2.2 Staining procedures 66
4.2.3 Isozyme analyses on samples of germinating seeds of *V. keniensis* and *V. fischeri* 68
4.3 Electrophoretic analysis on polyploidy and allozyme polymorphism in *V. keniensis* and *V. fischeri* 71
4.4 Electrophoretic phenotypes in *V. keniensis* and *V. fischeri* populations . 75
4.5 Genetic diversity of *V. keniensis* and *V. fischeri* populations 80
4.5.1 Statistical analysis on the electrophoretic data 81
4.5.2 The proportion of polymorphic loci (p) 81
4.5.3 Estimates of genetic diversity of populations of *V. keniensis* and *V. fischeri* 82
4.5.4 Genetic distance and identity of populations of *V. keniensis* and *V. fischeri* 87
4.6 Conclusion 89

5. General conclusions and recommendations 91

Literature 105

Acknowledgements 117

Curriculum vitae 118

Summary

Taxonomy and genetic structure of Meru Oak populations, *Vitex keniensis* Turrill and *Vitex fischeri* Gürke, in East Africa

V. keniensis (fam. Verbenaceae), the Meru oak, is a popular tree species in East Africa valued for its high quality, termite- and fungus- resistant timber. It is fast growing thus a good choice for commercial forestry establishment. Its fruits are edible and the tree is appreciated by small-scale farmers as an agroforestry species. The species is classified among the threatened forest trees in East Africa. The benefits from the species and the risks of depletion looming over its natural populations have made it a priority species for conservation and improvement programmes. However, information on its taxonomic status, mating systems and the diversity of its existing gene-pool has been poor or lacking. Taxonomically, the distinction between the disjunct populations of *V. keniensis* of Mt. Kenya and Nyambene hills and *V. fischeri* of the Lake Victoria and Mt. Elgon regions has been obscure. Studies were therefore carried out on the species' morphology, cytology, mating systems and genetic structure to characterise its taxonomic status, delimit its populations and propose appropriate conservation measures and improvement breeding strategy.

The cytological, reproductive and genetic traits showed more similarity than distinction between the species populations. There was significant variation at $p < 0.05$ in some of the morphological traits among the populations but they did not follow the pattern of the geographical distance or ecological gradient range in which the species occurs or the current taxonomic grouping. It is therefore recommended to consider the different *Vitex* populations as one taxon. The name *V. fischeri* has priority and therefore the well-known *V. keniensis* (Meru oak) falls into synonymy.

V. fischeri (= *V. keniensis*) exhibits homogamy and high ploidy level ($2n = 96$). The flowers are pollinated by *Apis mellifera* (bees) and seeds dispersed by monkeys and hornbill birds. It is partially self-compatible, producing seeds by both selfing and outcrossing. However, in nature, outcrossing seems to predominate, thus maintaining the high heterozygosity. The germination of the seeds resulting from the different modes of pollination did not vary, an indication of the absence or suppression of inbreeding depression by the polyploidy. The different *Vitex* populations are reproductively compatible and produced viable seeds. An indication that they have not undergone reproductive isolation.

Electrophoretic analysis on progeny array of the species displayed tetrasomic inheritance and independent segregation implying that it is an autotetraploid. For the eight enzymes coding for 12 loci, the species exhibited high proportion of polymorphic loci (P) of 100%, 5.3 mean effective number of alleles and 5.1 mean number of alleles

per locus. The total genetic diversity (H_T) was 99% and mean genetic variation within the populations (H_S) was 96.5%. The high polymorphism and total genetic diversity could be a result of high outcrossing rates, seed dispersal and polyploidy. The genetic variation between the *Vitex* populations (G_{ST}) was lower than within them (H_S). The average values of D_{ST} and G_{ST} (3.5%) each were equally low among the species' populations showing low inter-population variation.

The pattern of morphological and genetic variation between the populations did not match the geographical distance and ecological variation. This further supports the similarity observed in the cytological traits and inheritance pattern in the species' populations. The inter-population similarity can be attributed to their common ancestry and lack of divergence possibly caused by the species' long lifespan, high heterozygosity, polyploidy and or mild selection pressure. The low G_{ST} (3.5%) implied that the bulk of the species' genetic variation (96.5%) is retained within each population, therefore sampling from any of the four populations would capture a sufficiently large gene pool of the species for conservation. However, care has to be taken to ensure that at least two populations each from the contrasting ecological zones are included, just in case some of unique alleles in the populations were not detectable by the method used i.e. isozymes. Being an autotetraploid with up to four alleles per locus, a minimum of a hundred dispersed mother trees at least 300 m from each other can be maintained in-situ in each of the populations. Their propagules can used for raising ex-situ conservation stands which can also serve as living gene-bank.

Samenvatting

Taxonomie en genetische opbouw van Meru Oak populaties, *Vitex keniensis* Turrill en *Vitex fischeri* Gürke in Oost Afrika

Vitex keniensis Turrill (Fam. Verbenaceae), de Meru Oak, is een populaire boomsoort in Oost Afrika, gewaardeerd om de goede kwaliteit, en de weerstand tegen termieten en schimmels van het hout. De groei van de boom is snel, waardoor de soort een goede keuze is voor commerciële houtaanplant. De vruchten zijn eetbaar en de boom wordt op prijs gesteld door kleine boeren in agro-forestry. De soort wordt gerekend tot de bedreigde bomen in Oost Afrika. Het nut van de soort en de risico's van achteruitgang in de natuurlijke populaties leidden ertoe dat *Vitex* prioriteit krijgt voor behoud en veredelingsprogramma's. Kennis over de taxonomische plaatsing, de kruisingssystemen en de diversiteit van de bestaande genenpool was tot nu toe beperkt of ontbrak. Taxonomisch was het onderscheid vaag tussen de disjuncte populaties van *V. keniensis* van Mount Kenia en de Nyambene heuvels respectievelijk *V. fischeri* Gürke van de streken rond het Victoriameer en Mount Elgon. Om deze redenen werd onderzoek verricht naar de morfologie, de cytologie, het kruisingsgedrag en de genetische structuur van de populaties om de taxonomische status vast te stellen, de populaties te karakteriseren en geëigende methoden voor conservering en veredeling te ontwerpen.

De cytologische, kruisings- en genetische eigenschappen bleken meer overeenkomst dan verschillen tussen de populaties op te leveren. De variatie tussen morfologische eigenschappen was significant ($p < 0.05$) maar er was geen samenhang te onderkennen met het patroon van geografische afstand of ecologische gradiënt, noch met de huidige taxonomische indeling. Daarom wordt aangeraden de verschillende *Vitex* populaties als een taxon te beschouwen. De naam *V. fischeri* heeft de prioriteit, zodoende vervalt de welbekende naam *V. keniensis* tot synonymie.

V. fischeri (=V. keniensis) vertoont homogamie en een hoog ploidieniveau ($2n = 96$). De bloemen worden bestoven door *Apis mellifera* bijen, de zaden worden verspreid door apen en neushoornvogels. De boom is ten dele zelfcompatibel, zaden ontstaan zowel na zelfbestuiving als na kruisbestuiving. Van nature lijkt kruisbevruchting te overheersen en houdt zo de hoge mate van heterozygotie in stand. De kiemkracht van zaden ontstaan uit beide vormen van bestuiving verschilde niet, hetgeen wijst op de afwezigheid of onderdrukking van inteeltdepressie door de polyploidie. De verschillende *Vitex* populaties zijn onderling kruisbaar en leverden levenskrachtige zaden, een teken dat zij nog geen reproductieve isolatie hebben ondergaan.

De electroforetische analyse van de soort vertoonde tetrasome vererving en onafhankelijke uitsplitsing, wijzende op autotetraploïdie. In de acht enzymsystemen

coderende voor 12 loci, vertoonde de soort een hoog aandeel polymorfe loci (P) van 100%, gemiddeld 5,3 effectieve allelen en 5,1 allelen per locus. De totale genetische diversiteit (H_T) was 99 % en de gemiddelde genetische variaties binnen de populaties (H_S) was 96.5%. De hoge graad van polymorfie en totale genetische variatie kan het resultaat zijn van de hoge mate van kruisbevruchting, zaadverspreiding en polyploïdie. De genetische variatie tussen de *Vitex* populaties (G_{ST}) was geringer dan die binnen de populaties (Hs). De gemiddelde waarde van Dst en G_{ST} (beide 3.5%) was even laag onder de populaties duidende op een geringe variatie tussen de populaties.

Het patroon van morfologische en genetische variatie tussen de populaties kwam niet overeen met de geografische afstand ertussen en de ecologische verschillen. Deze resultaten ondersteunen de overeenkomsten in cytologische kenmerken en overervingspatroon van de populaties van de Meru Oak. De overeenkomst tussen de populaties kan worden toegeschreven aan de overeenkomstige voorouders en het gebrek aan veranderingen, mogelijk veroorzaakt door de lange levensduur van de soort, hoge heterozygotie, polyploïdie en/of lage selectiedruk. De lage G_{ST} (3.5%) betekent dat het merendeel van de genetische variatie van de soort (96.5%) in iedere populatie aanwezig is, zodat bemonstering van welke van de vier populaties dan ook een genenpool van voldoende diversiteit oplevert. Desalniettemin is het aan te raden dat tenminste twee populaties van de twee contrasterende ecologische zones worden bemonsterd, voor het geval dat unieke of zeldzame allelen in de populaties niet door de gebruikte methode, isoenzymanalyse, werden ontdekt. Als autotetraploid met tot vier allelen per locus volstaat het in-situ instandhouden van een minimum van een honderdtal ver uiteenstaande bomen. Hun zaden kunnen ex-situ boomgaarden opleveren die als levende genenbank dienen.

1 Introduction

1.1 Taxonomy and genetic diversity in relation to conservation of tropical forest trees

Global concern over the conservation of biodiversity is manifested in the high political profile of the topic, and in the number of activities related to conservation strategies such as organisation of conferences and meetings, the involvement of many national and international institutions, and the increased level of scientific research and documentation. However, despite all these efforts, only a small fraction of biodiversity so far is under proper conservation, especially in the tropics. Currently, many ecosystems are threatened by mismanagement and adverse environmental as well as socio-economic changes. Tropical forests in developing countries hold many species that have not been described, unfortunately they are under increased pressure from deforestation and cultivation (Adams et al., 1980). Currently, forests are among the most threatened ecosystems in the world (National Research Council, 1991; Williams, 1991). The benefits of forests and trees, both in ecological terms and as a source of forest products cannot be overemphasised (Bawa & Krugman, 1991), thus the questions currently being raised do not concern any longer the rationale for forest conservation and improvement programmes, but rather how they can be effected. While the population of the industrialised temperate nations are expected to increase by 30 % over the next 30 years, those in the nations of the tropics will double (Ashton & Bawa, 1990). This fact has a profound implication to sustainable management of forest ecosystems.

As natural forest resources decline, there is an increasing need to resort to plantation and on-farm forestry. The main obstacle to this is lack of knowledge on the biology of most of the tropical forest trees and their ecosystems. The majority of the highly exploited tropical forest are not under any conservation or improvement breeding programme. Currently, about 500 tree species occur in well-managed forests; even fewer (approximately 60) worldwide are included in well-established tree improvement programmes (National Research Council, 1991), and the remainder, most of which are tropical species, are exploited without explicit consideration for their conservation. This trend poses a threat to the highly demanded forest tree species especially in the tropics. The amount of species the world can afford to lose is unknown, but it is certain that the tolerance to species over-exploitation and loss is limited. Thus, the need for strategies for sustainable utilisation of plant diversity and their genetic resources is urgent. This requires exploration of their natural range and definition of their taxonomic status, genetic distribution pattern and mating systems. Insufficient information on genetic structure, reproductive biology and pollination ecology have constrained the conservation of most tropical forest tree species and their inclusion in breeding programmes (National Research Council, 1991).

Population genetic theory predicts that the decrease in the genetic diversity limits a species' ability to keep pace with the changing selection pressure (e.g., Young et al. 1996). Plant species, especially the perennials such as trees, rely on the available genetic diversity for stability and survival under the ever-changing environments (National Research Council, 1991). This variability forms the base on which natural selection may act and has been exploited ever since the first domestication by man for breeding purposes. Genetic studies by Namkoong (1991), Epperson (1992), Hamrick et al. (1992) and Loveless (1992) have characterised parameters of plant genetic resources as dynamic and complex which emphasises the need for a sound approach to sampling for conservation and utilisation of species variation. The understanding of the species population structure and potential genetic resources is essential for their conservation planning and sustainable management (Sun et al., 1998). In the past most of the information was either ignored or gathered through limited inventory followed by establishment of provenance (field) trials which were both laborious and slow. Recent development of biochemical and molecular techniques to answer some of the basic questions have supplemented and hastened the process of information gathering, reducing the time taken in planning the best sampling strategies for specific purposes.

In Kenya more efforts are now being directed towards the vulnerable, highly demanded indigenous forest tree species with the aim of promoting their propagation on-farm and in plantations, natural regeneration and conservation (both *in-situ* and *ex-situ*). *V. keniensis* (commonly referred to as Meru oak) is one of the indigenous forest tree species in Kenya, rated highly for both commercial and on-farm production and, conservation. Currently, it is threatened by rapid exploitation for its quality timber. The number of individuals of the plant species remaining in the natural forests is small and is further dwindling. The species has been under selective logging for a long period of time subjecting it to genetic erosion. Thus its populations are threatened (Noad & Birnie, 1989; ICRAF, 1991). The demand for *V. keniensis* timber in Kenya outstrips the existing populations most of which are natural. Thus promotion of productive commercial plantations and on-farm planting supported by improvement breeding programmes is called for. That is expected to divert or reduce the pressure on the remaining trees in the natural forests. These improvement and commercial production efforts need to be coupled with both *ex-situ* and *in-situ* conservation strategies for the hardwood tree species.

However, the basic but essential information required for the initiation of its propagation and conservation programme such as its taxonomic status, mating system and the diversity of Meru oak genetic resource is still sparse. Taxonomy plays a critical role in species management since only formally named entities can be afforded legal protection (Avise, 1994). Moreover, taxonomic uniqueness has been promoted as a factor leading to priority conservation status. Thus, elucidation of genetic relationships among taxa may help define taxonomic entities and determine conservation priorities (Stewart et al., 1996). Despite the recent trend to place many Verbenaceae genera in Lamiaceae, for this thesis I will keep the family as recognized in the Flora of Tropical East Africa, and keep the genus *Vitex* in the Verbenaceae

family. *V. keniensis* is so closely related to *V. fischeri* that it is doubtful whether they belong to different species (Dale & Greenway, 1961). Frequent confusion is encountered in literature dealing with the "two species". Their leaves and flowers are hardly distinguishable. Up to now, the distinction between the two was obscure and their separation has been based mainly on their habitats and geographical location: *V. keniensis* has been considered a montane moist evergreen forest species and *V. fischeri* a wet savanna woodland species occasionally growing in forest margins. Figure 1.1 & 1.2 shows the natural habitats of the *Vitex* populations. The species' populations in Mt. Elgon and Lake Victoria regions have been referred to as *V. fischeri* by some authors but others have synonymised it with *V. keniensis* (Appendix 2.1). This has led to confusion in the taxonomy of species thus causing problems in determining its exact population size, distribution and consequently its gene-pool for sustainable management. The "two species" have a wide range of occurrence in East Africa, mostly concentrated in Mt. Kenya and Nyambene hills of central Kenya, Mt. Elgon and Lake Victoria regions in Kenya, Uganda and Tanzania. The study was therefore designed to clarify the taxonomic status of *V. keniensis* and *V. fischeri*, and their natural range of distribution, mating systems and the genetic structure of their populations. The information is expected to contribute to sustainable management of the species through sound conservation, improvement breeding and propagation programmes.

1.2 Genetic structure and mating systems of forest tree populations

The amount of genetic variation within a species and its distribution within and between populations may provide clues to factors that govern the maintenance of variation and geneflow (Ayres & Ryan, 1999). These factors are useful in delimiting populations, identifying potential selection domain and measuring genetic diversity. The knowledge of the genetic diversity and mating systems of a tree species gene pool is crucial to its conservation, improvement and management of its plantations. However, like in most tropical forest tree species, information on the biological characteristics *V. keniensis* is sparse. The mating system of a plant species has a direct influence on its gene flow and the genetic structure of its populations (Maki et al., 1999). The two are important factors to plant breeders and conservationists. The variation in the genetic structure and mating systems in different species and even populations are also taxonomically valuable and may be used in taxonomic characterisation. In recent years, isozyme electrophoresis techniques have been used widely to evaluate taxonomic conclusions of different authors, genetic relationships of tree populations and in the understanding of their mating systems (e.g. Chase et al. 1995; Ford et al. 1998; Williamson et al., 1999; Maki et al. 1999). Such studies have been generally employed in response to tropical deforestation and its consequences i.e. loss of biodiversity and the potential loss of genetic diversity within species (Figure 1.3 & 1.4).

The specific traits such as life form, reproductive biology, seed dispersal and geographical range of distribution can affect the level and partitioning of genetic

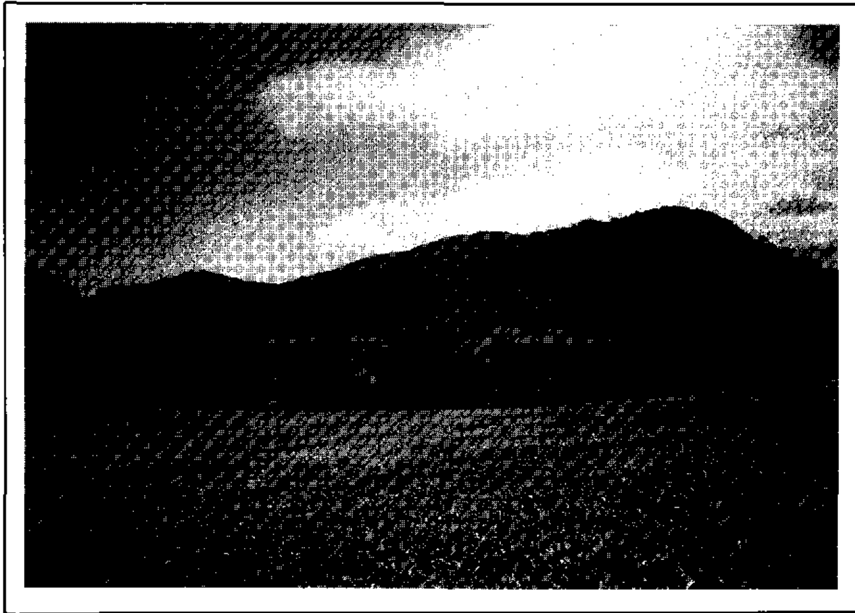


Figure 1.1. The natural habitat of *V. keniensis* in Kenya.

diversity of a tree population (Massey & Hamrick, 1998; Sun et al., 1998). Thus, they are important in the designing of sampling and management strategy for conservation programmes. Outcrossing plant species tend to maintain high total genetic diversity but with a higher proportion of it maintained within individual populations (Hamrick & Godt, 1989). Burley et al. (1986) concluded that mainly self-incompatible, out-crossing species exhibit a combination of clinal variation, in response to broad climatic factors, and discontinuous variation, when distributed over disjunct environmental factors such as soil and relief. Thus data on the genetic diversity and mating systems of Meru oak populations would be therefore be important in predicting geneflow and designing both *in-situ* and *ex-situ* conservation strategies. Gene bank managers and researchers also need such information to successfully collect, conserve and study such a threatened plant species. The results of the species' mating systems and genetic structure should also cast some light on the level taxonomic similarity and distinction between *V. keniensis* and *V. fischeri*.

1.3 The economic importance and exploitation of Meru oak

V. keniensis is one of the most popular and exploited indigenous forest tree species in East Africa. It produces high quality, termite- and fungus-resistant timber. Its timber is widely used in furniture, cabinets and joinery in East Africa (Benghou, 1971). The current demand for its timber outstrips the production level of the existing natural populations and commercial plantations of the tree species mainly in the Mt. Kenya region (Kigomo, 1985). Besides its high quality timber, the tree species is appreciated

Figure 1.2. The natural habitat of *V. fischeri* in Tanzania.

for its edible fruits and medicine for stomach ailments, the flowers are popular as bee-forage for quality honey production. These uses are still at subsistence level but they have high economic potential especially to the rural areas. The timber is rated highly because of its light weight and light brown colour with attractive wavy grain.

Meru oak is recommended for plantation establishment especially in areas prone to elephant damage. This is because its leaves and bark are unpalatable to elephants thus making foraging damage less likely. As a high value multipurpose tree species, it is appreciated by small scale farmers for its compatibility with agricultural crops. It provides the associated crops such as maize, coffee, yams, cassava, sweet potatoes, beans and bananas with shade during the hot, dry season and mulch from its shed leaves in cold, rainy periods. It is able to withstand lopping and pollarding, thus providing the farmers with fuelwood while allowing the main stem to reach merchantable size. The tree species, therefore has a substantial potential for agroforestry. Surveys on the *Vitex* populations indicated that their number is dwindling and their natural range of occurrence shrinking due to exploitation and destruction of their natural habitats (Figures 1.1 - 1.4). The causes of the exploitation vary from one region to another. In Mt. Elgon and Lake Victoria, *Vitex* populations are under threat from clearing the habitats for human settlement and plantation agriculture, mainly sugar cane and tobacco growing. In these regions the species is also exploited for charcoal and firewood for curing tobacco and domestic cooking. Its timber is also popular for making tool handles and oxen yokes. In Mt. Kenya and Nyambene regions the species is exploited for its valuable timber and habitat destruction through human settlement, agricultural activities and forest fires which are also common.

Figure 1.3. Conversion of the natural habitat of *V. keniensis* in Kenya, to plantation forestry.

1.4 Project background

The variety of uses of *V. keniensis* (Meru oak) qualifies it as a multipurpose tree species. Its wide range of distribution over different ecological zones may be an asset in its potential for large scale improvement and propagation. Any achievement in increasing the productivity of the tree species will raise the living standards of the people who depend directly on its products and other benefits. In addition to that, non-wood products such as fruits and medicine, have potential commercial value. Good progress in commercial production would also alleviate the current exploitation pressure on the remaining, dwindling natural populations of the tree species in different parts of East Africa.

Most tropical forest tree species, including *V. keniensis* have not been studied beyond taxonomic level; further, the taxonomy itself often requires a thorough revision as different forms are encountered among distinct species populations. Currently, there are difficulties in distinguishing *V. keniensis* from *V. fischeri*. An integrated approach was therefore be required to compare the possible pattern of phenotypic variation in relation to genetic diversity and possibly environmental conditions. Genetic evaluation and characterisation, and elucidation of mating systems of the species will form the basis for its conservation, improvement breeding, propagation and utilisation regionally. The information is also of more general scientific interest because of its probable contribution to the understanding of the biology of other tropical plant species.

Figure 1.4. Conversion of the natural habitat of *V. fischeri* in Tanzania, to other land uses.

1.5 Project objectives

The main objectives of the research project were to determine the taxonomic status, genetic variation and mating systems of Meru oak populations for the purpose of generating information relevant to its sustainable management through scientifically based conservation, improvement breeding and propagation strategies. This is expected to enhance the preservation of the species' existing natural populations, and its productivity in commercial plantations and small scale farms ensuring the timber demand is met from the planted trees.

Specific objectives

1. to elucidate the taxonomic status of *V. keniensis* (Meru oak) and the closely related *V. fischeri* for identification and establishment of its population size and natural distribution range.
2. to understand the mating systems of Meru oak.
3. to determine the genetic variation within and between the populations of Meru oak across its natural range.
4. to recommend possible sustainable management strategies for Meru oak and the other related forest tree species on the information gained in this research project.

1.6 Thesis organisation

The thesis is presented in 5 chapters. Chapter 1 covers the background information on the subject. Chapter 2 presents the taxonomic distinction and similarity between *V. keniensis* and *V. fischeri* based on the study of the existing literature, herbarium specimens, morphological and cytological traits. Chapter 3 covers the reproductive biology and the mating systems of the species in the different geographical locations. The genetic structure of the species' populations is presented in Chapter 4. The general conclusion, recommendations for sustainable management of the species and suggestions for further research are given in Chapter 5.

2 The taxonomy and geographical distribution of *V. keniensis* and *V. fischeri*

2.1 Introduction

The genus *Vitex* belongs to the family Verbenaceae and is composed of over 130 species. It has a wide distribution in Africa, Asia, Australia, New Zealand, South America and the Caribbean. Africa has about 60 species of *Vitex* with most of them occurring in the sub-saharan regions of West, East and Southern Africa including the Islands of the Seychelles and Madagascar. A few of them are found in the mediterranean regions of northern Africa. In Asia the genus is widespread in the tropical and sub-tropical regions of south west Asia and the Middle East. There are about 35 species of the genus in Asia while Australia and New Zealand have three *Vitex* species. South America and Caribbean Islands have over 40 species of *Vitex* spreading from Panama, Puerto Rico, Jamaica to Venezuela.

Some of the species in the genus such as *V. agnus-castus*, *V. ferruginea* Schum., *V. negundo* and *V. altissima* have a wide distribution across the world. There are about twenty different *Vitex* species occurring in East Africa. The uses of the species in the genus vary from one continent to another but they include timber, medicine, food (fruits and production of honey), mulch, shade and ornamental purposes.

2.2 Conspectus of the genus *Vitex* in East Africa

Verdcourt recently (1992) revised the genus *Vitex* for the Flora of Tropical East Africa. Not all species are sufficiently known, most do not play a major role in the timber trade. For a key, detailed descriptions, synonyms and specimens examined may be referred to his 1992 revision in FTEA (*Verbenaceae*). Shorter keys, maps and descriptions are given by Beentje (1994). The species are given in alphabetical order. Figure 2.1 shows the natural of distribution of the species in the genus *Vitex* in East Africa.

1. ***Vitex agnus-castus*** L. Deciduous ornamental shrub or spreading tree, once collected incompletely north of Dar-es-Salaam.

2. ***V. amaniensis*** G. Piep. Tree 12 to 20 m tall, Lushoto district, Morogoro district in Tanzania, forest, forest edges and relict, syn. *V. lokundjensis* sensu Moldenke, *pro parte*, non Pieper.

3. ***V. buchananii*** Gürke. Herb, shrub or branched tree 1.2 to 15 m tall, Lushoto district in Tanzania, also Malawi, Zambia, Mozambique.

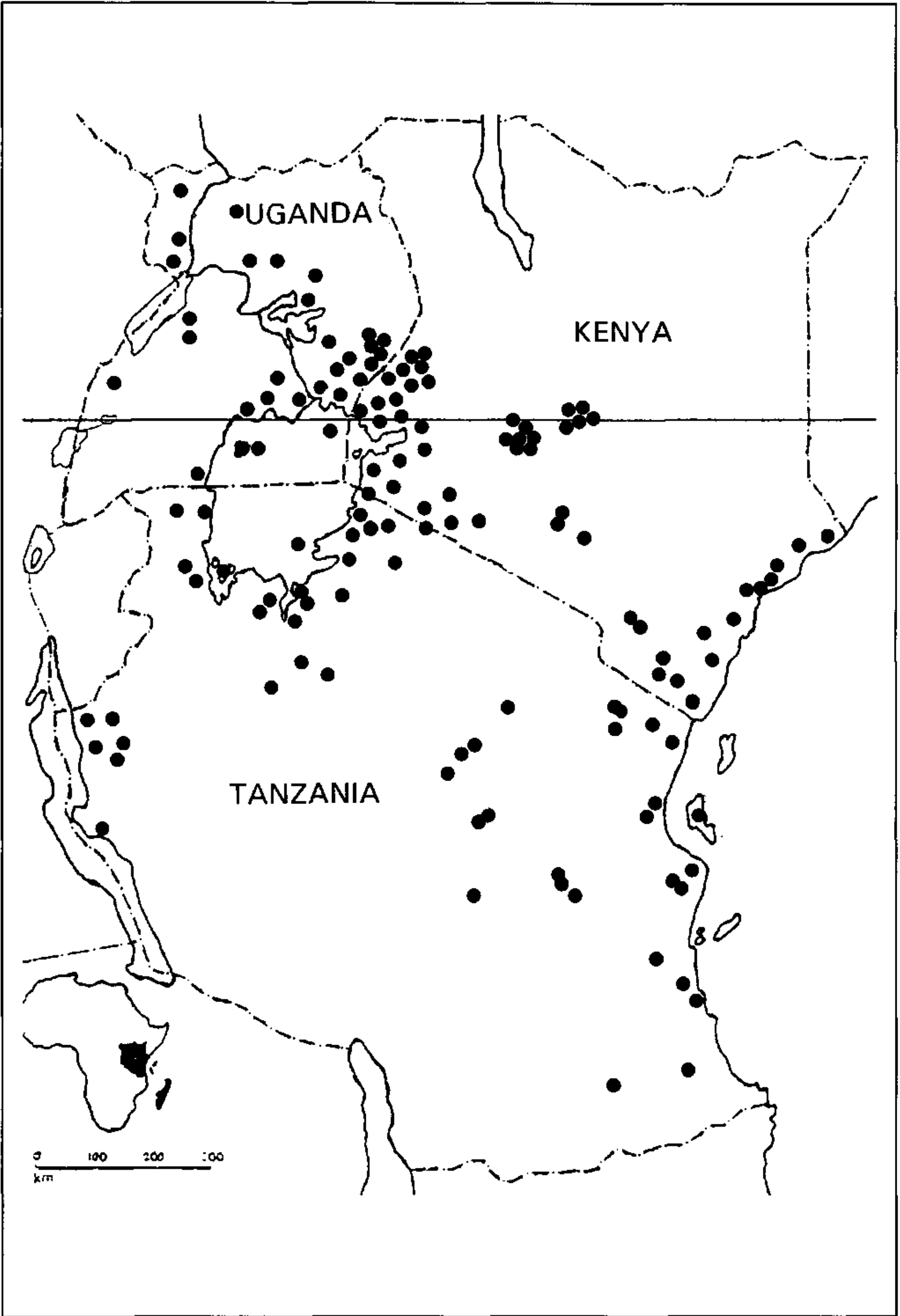


Figure 2.1. Natural distribution of the genus *Vitex* in East Africa.

4. ***V. doniana*** Sweet. Tree 3.5 to 15 m tall, deciduous, branched, in wooded grassland or forest edge, commonest savanna tree and one of the most useful member of the family, in Teso Bunyoro districts of Uganda; Mt. Elgon, South Nyanza, Kuria and Kwale districts of Kenya; Bukoba, Kondoa, Morogoro districts and Zanzibar in Tanzania. Also throughout western and southern tropical Africa. Its wood is used for making furniture, dhow ribs, canoes and house-building.

5. ***V. ferruginea*** Schum. (include *V. amboniensis* Gürke, *V. carvalhi* Gürke, *V. tangensis* Gürke). Shrub or tree 1.5 - 6 m, forest (margin), secondary bushland or thicket, evergreen coastal bushland. Verdcourt distinguishes subspecies *ferruginea* from Uganda, Tanzania and Kenya, and subspecies *amboniensis* (Gürke) Verdcourt from Tanzania, also in Somalia, Mozambique, Zambia, Zimbabwe and South Africa.

6. ***V. fischeri*** Gürke. Tree 3 - 15 m, wooded grassland less often in forest margins, its timber is used for making furniture and oxen yokes, and its fruits are edible, occurs in Teso, Busoga and Mengo districts Uganda; Trans-Nzoia, Kakamega and north Kavirondo districts of Kenya; Mwanza, Shinyanga and Kigoma districts of Tanzania, also in Zaire, Sudan, Zambia and Angola Savanna wooded grassland, syn. *V. keniensis* sensu T.T.C.L.: 643 (1949), *non* Turrill.

7. ***V. keniensis*** Turrill (syn. *V. balbi* Chiov.). Tree 12 to 35 m tall, moist evergreen forest sometimes riverine in South Nyeri, Meru, Nyambene hills, Taita hills in Kenya, cultivated in Uganda and Tanzania, produces superior termite fungi resistant timber and fruits are edible, often synonymised with *V. fischeri*, see conclusion in chapter 2.

8. ***V. madiensis*** Oliv. Shrub or small tree, 1.2 to 7.5 m tall, with rough bark, or pyrophytic herb to small shrub 0.3-1.5 m tall, forming patches 1 m wide from a massive subterranean woody rootstock. Verdcourt distinguishes subsp. *madiensis* from Uganda and many tropical African countries, subsp. *milanjiensis* (Britten) F. White var. *milanjiensis* from Tanzania, and var. *epidictyodes* (Pieper) Verdc., a pyrophytic shrubby herb from Tanzania, and Congo to Cabinda.

9. ***V. mombassae*** Vatke. Shrub or tree 1.8 to 6 m in Kwale Kenya, Tanga Mbulu districts of Tanzania, also in Democratic Republic of Congo, Burundi, Mozambique, Malawi, Zambia, Zimbabwe, Angola and South Africa.

10. ***V. mossambicensis*** Gürke. Shrub or small tree 4 to 18 m in coastal areas, Kilwa and Lindi districts of Tanzania, also in Mozambique.

11. ***V. payos*** (Lour.) Merr. var. *payos*, var. *glabrescens* (Pieper) Mold. 2 to 9 m in Machakos, Kwale districts of Kenya, Handeni, Morogoro, Kilwa districts of Tanzania, also in Mozambique, Malawi, Zimbabwe.

12. ***V. schliebenii*** Moldenke. Shrub of 2.4 to 3 m, in Tana River and Lamu districts of Kenya, Kilwa, Masasi and Lindi districts of Tanzania, also in Malawi, Mozambique.

13. ***V. strickeri*** Vatke & Hildebrandt. Erect or scrambling shrub or small tree, 1.5 to 4.5 m tall, Kiambu, Teita and Kilifi districts in Kenya; Mwanza, Tanga and Bagamayo districts in Tanzania.

14. ***V. trifolia*** L. Shrub or small tree up to 6.5 m tall, sometimes creeping, Kwale district in Kenya; Uzaramo district in Tanzania, also other parts of Africa, Asia and Australia.

15. ***V. ugogoensis*** Verdc. Shrub or tree 1.5 to 5 m tall, deciduous thicket, bushland in Kondoa and Dodoma districts of Tanzania.

16. ***V. zanzibarensis*** Vatke. Shrub or tree or liana in coastal thicket or dry forest, especially at the edges. Kwale district in Kenya, Uzaramo and Kilwa districts in Tanzania, 0 to 10 and also 360 to 600 m. Probably also in Mozambique.

17. ***V. sp. A.*** Related to *V. buchananii* shrub with older stems hollow, Morogoro district, Tanzania.

18. ***V. sp. B.*** 18 m, Uzaramo district, Tanzania, probably close to *V. mossambicensis*.

2.3 Brief description of *V. keniensis* and *V. fischeri*

V. keniensis and *V. fischeri* both belong to the Verbenaceae family. The common names of *V. keniensis* are Meru oak (English), Muhuru (Kikuyu) and Moru (Meru). Meanwhile, *V. fischeri* is commonly called Mohutu (Nandi), Jwelo (Luo), Mufutumbwe, Muhutu (Luhya), Omuhuruhuru (Taita), Mhunda (Zanaki) and Kabrampako (Kitongwe). The local names of the species tend to go by the language groups and regions. The distinction between the *Vitex* populations has been unclear (Dale & Greenway, 1961), therefore the description presented below applies to both of them.

V. keniensis and *V. fischeri*; a small to a big tree reaching a maximum height of 30 m tall and 2.3 m diameter depending on the environment, deciduous with dark brown fissured bark, sapwood light brown and heartwood dark brown, coarse-textured with well-marked growth zones, often with wavy grain figure. The wood seasons, works and nails well. Young branches and petioles are covered with orange brown velvety hairs; palmate leaves are 5-foliolated and scented; the 5 leaflets are oblong, obovate-elliptic or elliptic measuring 5 to 19 cm long, 3 to 10 cm wide, acuminate at apex, cuneate to rounded at the base, glabrous above but velvety woolly tomentose beneath with glands, venation evident, up to 15 pairs of lateral nerves; petioles 6.5 to 16.5 cm long; petiolules 1 to 8 cm long. Cymes axillary with many flowers up to 15 cm wide, peduncle 6 to 14 cm long; pedicel up to 1 mm long but of the central flower of cyme up to 12 mm long; calyx truncate or toothed up to 1 mm long; corolla white with blue or purple or mauve upper lip, yellowish white tube up to 3 mm long, the lip 3.5 mm long and the lower lobe 2.5 mm long and wide; fruit a pulpy drupe, black when ripe,

oblong-globose up to 1.3 cm long, 1 cm wide, edible; seeds are nuts with calycine cup up to 1 cm wide and 2 cm deep; containing 1 to 4 embryos. Figure 2.2 shows the leaves, flowers and fruits of *V. keniensis*. Further descriptions and comparisons made by different authors between the "two species" are presented in appendix 2.1.

2.4 The ecology of the natural range of *V. keniensis* and *V. fischeri*

The observations made during the field work indicated that the "two species" (*V. keniensis* and *V. fischeri*) occur in different habitats (Figure 1.1 & 1.2), ranging from the wet, cool, high altitudes of Mt. Kenya and Nyambene hills to low-lying drier, warmer areas around the basin of Lake Victoria and lower slopes of Mt. Elgon. These populations are disjunct. The populations in the evergreen montane forests of Mt. Kenya and Nyambene hills are commonly referred to as *V. keniensis* while those in the low lying wooded savanna of the foot of Mt. Elgon and Lake Victoria belt are considered to be *V. fischeri* (Verdcourt, 1992; Beentje, 1994). These zones have markedly different ecological conditions. The ecological zones and the habitats occupied by the different the four populations of *Vitex* are described below.

2.4.1 The mountainous forests of Mt. Kenya and Nyambene hills

The central highlands of Kenya of Mt. Kenya and Nyambene hills are mainly of tertiary and recent volcanic origin, dominantly represented by kenyte which is the parent material of the soils in this region. The soils are brown loam (ferrisols), mostly up to 175 cm deep. The streams are perennial and the valley floors have dark brown friable clay frequently mottled by seasonal flooding. The general physiography includes mountain foot ridges, upper uplands, upper scarps, plateaus and scarps, all belonging to volcanic landscape (Morgan, 1973). In this region the populations of Meru oak occur at an altitude range of 1300 to 2000 m.

In this region, the climate tends to vary with the altitude; the rainfall increases with altitude while the temperature drops. The climatic conditions of the areas where *Vitex* occurs are shown in appendix 2.2. The rainfall here is concentrated in two seasons; the first is termed as "warm" due to relatively high temperatures and lasts from March to May. The second rains fall between October and December and that is when mature fruits of *Vitex* are shed from the crown as are the dead leaves. The dry season also shows seasonality and has an influence on the plant phenology. A short cool, dry season occurs from January to February, when flowering and pollination take place and a long warm, dry season lasts from June to September when the fruits mature (the species' mating system is presented in chapter 3).

The *Vitex* of Mt. Kenya and Nyambene hills occur in association with *Prunus africana* (Hook.f.) Kalkm. (*Pygeum africanum* Hook.f.), *Ocotea usambarensis* Engl., *Celtis africana* Burm.f., *Podocarpus falcatus* Mirb. (*P. graciolor* Pilger), *Olea*

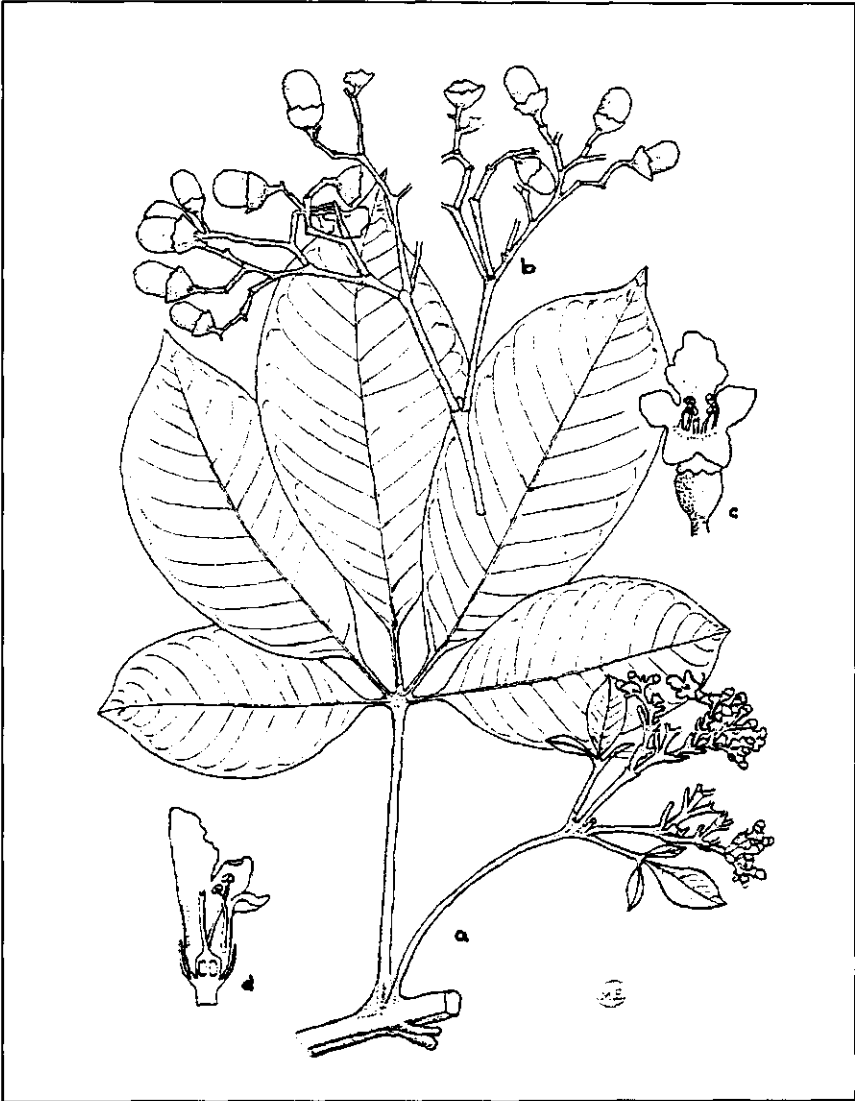


Figure 2.2. Leaves, flowers and fruits of *V. keniensis* (Dale & Greenway, 1961). a) flowering branchlet b) fruits c) flowers d) longitudinal section of flower (x 0.5).

capensis L., *Aningeria adolfi-friederici* (Engl.) Robyns & Gilb., *Fagaropsis angolense* (Engl.) Dale, *Polyscias kikuyensis* Summerh., *Antiaris toxicaria* (Pers.) Lesch., *Cassipourea malosana* (Bak.) Alston, *Tabernaemontana pachysiphon* Stapf (= *T. holstii* K. Schum.), *Strombosia scheffleri* Engl., *Premna maxima* T.C.E. Fries and *Xymalos monospora* (Harv.) Warb. The upper limit of *Vitex* is determined by the frost-line. The zone of *Vitex* in Mt. Kenya and Nyambene hills may be described as montane rain

forest. The level above the altitudinal range of *Vitex* is dominated by *Podocarpus latifolia* (Thunb.) Mirb. (*P. milanjianus* Rendle), *Podocarpus falcatus* Mirb. (*P. gracilior* Pilger), *Juniperus procera* Endl., *Hagenia abyssinica* (Bruce) J.F. Gmel. and *Arundinaria alpina* K. Schum.

The forest canopy in the *Vitex* zone is complex and multi-layered. The trees of the upper strata are 30 to 40 m tall and is dominated by *Ocotea usambarensis*. *Vitex* and the associated species form the second layer between 20 and 35 m. The shrub layer is 2 to 6 m tall, dominated by *Galiniera saxifraga* (Hochst.) Bridson. The forest floor is normally open with little undergrowth except in "gaps" arising from dead or felled trees. The forest floor vegetation consist of broad-leaved forest grasses, several ferns and regenerating tree species. Most of regeneration of *Vitex* occur in the gaps that is where the canopy is opened, most likely because of the light and warmth in such spots. The main disturbances the species is subjected to in the montane forests of Mt. Kenya and Nyambene hills are tree felling, falling dead trees and sometime forest fire. The natural regeneration of *Vitex* is generally low in the closed forest. This is probably due to shady and cold conditions on the forest floor and seed predators. In natural forests Meru oak seems to generate in canopy gaps (Konuche, 1994), because the species is a light demander (Kenya Forest Department, 1969), thus needing crown opening to regenerate. Most of *Vitex* fruits shed from the crown are eaten by the forest floor animals attracted by the sweet pulp. The depulping of the fruits by monkeys and hornbills tend to improve the germination of the resultant seeds and make the seeds less attractive to forest fungi, rodents and insect pests. Some of the animals especially insect pest burrow the seeds, damaging the embryos thus interfering with their germination reducing natural regeneration rate of the species in the nature. And since the forest floor remains shady, moist and cold most of the year, the regeneration rate remains low and most seeds do rot.

Mature trees of *Vitex* are getting fewer in these regions because of over-exploitation. In the *Vitex* zone lianas grow to a height of about 20 m. In the valleys tree ferns such as *Cyathea manniana* Hook. and wild bananas, *Ensete ventricosum* (Welw.) Cheesman are common. However, the vegetation bordering the streams was somewhat different and had a lot of ferns but almost no trees. The vegetation there is under constant disturbance by wild animals such as elephants which frequent the areas for water and low plants, thus enabling the growth of the secondary and ruderal species.

2.4.2 The woody wet savanna at the foot of Mt. Elgon and Lake Victoria basin

The regions around Lake Victoria and Mt. Elgon where *Vitex* occurs are predominated by pre-cambrian granite with a variety of volcanic tuff and lava. The soils are mainly yellow-red ferralitic, fersialitic and ferruginous tropical soils. The physiography is undulating with a number of drainage channels flowing towards Lake Victoria (Morgan, 1973). The vegetation here may be described as wet-wooded savanna which is a grassland with scattered or grouped trees and shrubs providing less

crown cover. The trees are more branched with crowns that do not form a complex deep canopy. A number of tree and shrub species here including *Vitex*, are deciduous and shed their leaves during some parts of the year. The plant species occurring alongside *Vitex* in these areas are *Combretum molle* G. Don, *Albizia gummifera* (J.F. Gmel.) C.A. Sm., *Diospyros abyssinica* (Hiern) F. White, *Commiphora africana* (A. Rich.) Engl. [*C. pilosa* (Engl.) Engl.], *Milicia excelsa* (Welw.) C.C. Berg., *Teclea nobilis* Del., *Trichilia roka* Chiov., *Terminalia brownii* Fresen.,*Vitex doniana* Sweet and *Piliostigma thonningii* (Schum.) Milne-Redh. In some riverine areas with less human activities, *Olea europea* L. ssp. *africana* (Mill.) P. Green, *Prunus africana* (Hook.f.) Kalkm. and *Warburgia ugandensis* Sprague also occur along side *Vitex*. The trees grow up to 20 m tall, with crowns just touching to form an open canopy. The *Vitex* in this region is capable of growing even taller if it were not for human activities such as pollarding and pruning. The stunted growth habit reported on the *Vitex* in Lake Victoria and Mt. Elgon region is mostly a result of human disturbance.

The forest floor in the woody wet savanna is mostly open and is dominated by grasses and herbs such as *Andropogon*, *Brachiaria*, *Chloris*, *Eragrostis*, *Themeda* and *Setaria* species. The altitude of this region reaches a maximum of 1500 m above sea level. The region is highly populated leading to rapid deforestation to pave way for settlement and farming activities, thus the wooded grassland. Many of the islands in Lake Victoria such as Lolui and Sese Islands also have forests with *Vitex* populations. The vegetation in Lake Victoria and Mt. Elgon areas is also subjected to frequent deliberate fires to promote growth of grass for livestock. Most of the areas in these regions are being cleared for growing of cash crops like sugar-cane, tobacco and cotton, and food crops such as maize, beans, sorghum, millet, sweet potatoes and cassava. The land cleared of natural vegetation is covered to a large extent by crops and post-cultivation vegetation. Appendix 2.2 shows the climatic conditions prevailing in Mt. Kenya, Nyambene hills, Lake Victoria and Mt. Elgon, the natural range of the *Vitex* tree populations.

2.5 Comparison between *V. keniensis* and *V. fischeri* herbarium specimens

The label information on sheets of *V. fischeri* and *V. keniensis* at e.g. EA revealed difficulties in the distinction between the two species. Studies on the EA sheets indicated that *V. fischeri* occurred widely in Tanzania, Uganda and Kenya. However, labels on some sheets indicate that *V. keniensis* occurred alongside *V. fischeri* in the same areas mentioned above. It was therefore difficult to distinguish the species by origin when using herbarium specimens.

The *V. keniensis* sheets at EA indicated that the collections came from Kakamega, Chogoria in Mt. Kenya, Kitale, Gogoni Forest Reserve (Kwale), Port Victoria (at the shore of Lake Victoria), Nyambene hills, Ngaongao Forest in Taita Taveta and Meru on Mt. Kenya. But also from Shinyanga in Tanzania and Serere, Teso district in Uganda. In many cases, *V. keniensis* and *V. fischeri* were used interchangeably by

collectors and some authors; an indication of difficulties faced by the collectors to distinguish the plant species on their morphology. It is clearly possible that the two are either closely related or they are the same taxon.

The map in figure 2.3 shows the natural range of distribution of the *V. keniensis* and those identified by some authors as *V. fischeri* populations identified in the three countries of East Africa. Appendix 2.2 shows data on the climatic conditions of the areas where *V. keniensis* and *V. fischeri* populations occur. They were areas around Lake Victoria basin of Kenya, Uganda and Tanzania, at the foot of Mt Elgon, central Kenya highlands around Mt. Kenya and Nyambene hills. In Tanzania most of the *Vitex* is concentrated around Lake Victoria (Musoma, Bukoba and Mwanza). According to herbarium labels they also occur in Shinyanga, Morogoro, Kigoma and Iringa regions. No *Vitex* trees were collected from the north-eastern mountains of Tanzania (Usambaras Mountains and Mt. Kilimanjaro) contrary to earlier reports. The *V. keniensis* specimens collected from Arusha and Mgamba (Lushoto) in Tanzania were not from nature but from plantations raised from seeds collected from Mt. Kenya's *Vitex* according to records and the forester in Lushoto (pers. communication).

The species was also not found in Taita and Shimba hills near the Kenyan Coast, contrary to some previous reports on exploration of Ngaongao and Ngongoni forests. The *Vitex* populations in these areas might have been depleted by over-exploitation or habitat destruction. The Kenyan coastal forests also did not have any *V. keniensis* or *V. fischeri*. However, there are scattered populations of *Vitex doniana* Sweet, *V. mombassae* Vatke, *V. strickeri* Vatke and Hildebr., *V. ferruginea* Schum., *V. trifolia var. bicolor* (Wild.) Moldenke and *V. payos* (Lour.) Merr.

According to Dale & Greenway (1961), *V. keniensis* naturally occurs in the Mt. Kenya region and collections away from that region were referable to *V. fischeri*. They further stated that the leaf and flower characteristics of the "two species" were scarcely distinguishable and that it was doubtful whether *V. keniensis* was distinct from *V. fischeri*. Verdcourt (1992), observed that *V. keniensis* is usually synonymised with *V. fischeri* but he regarded them as distinct taxa worthy of specific rank. However, the morphological characteristics such as flowers and leaves as described by Beentje (1994) and Verdcourt (1992) are in reality hardly distinguishable to rank the two species as separate entities. Appendix 2.1 compares the exact wording or lack thereof in the descriptions by the authors.

Therefore studies to determine the species' population size, mating systems and the genetic structure was preceded by investigating their taxonomy to clarify the distinction between them. It has been unclear whether the two were distinct species, ecotypes or intra-specific taxa. The geographical distribution of the species in East Africa (Figure 2.3) was studied and laboratory analysis of representative specimens from their natural populations done. Herbarium specimens and seed samples were collected from the four *Vitex* populations of Mt. Kenya, Nyambene hills, Mt. Elgon and Lake Victoria regions for further observation and analyses. The leaf and flower specimens from the sampled

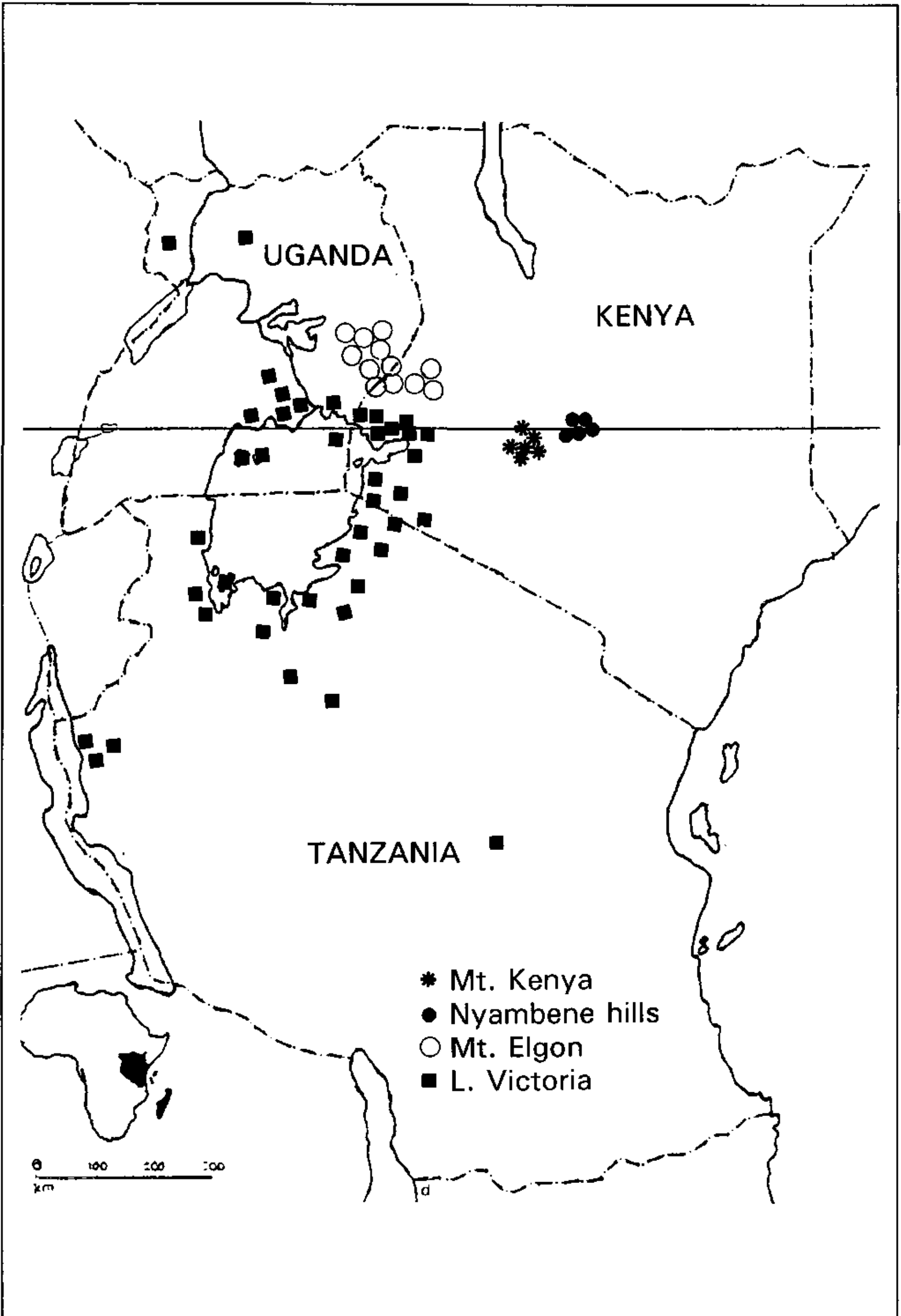


Figure 2.3. Natural range of distribution of *V. keniensis* and *V. fischeri* in East Africa.

Vitex populations were kept at EA, KEFRI (Kenya) and WAG (Netherlands). Laboratory analyses were carried out to determine the morphological, cytological and genetic relationships of the *Vitex*'s populations. Further studies were carried out on their mating systems to generate additional information relevant to its sustainable management.

The morphological characteristics of *V. keniensis* and *V. fischeri* (Appendix 2.1) used for keeping them apart are apparently not clearly distinct. Most of the traits used in separating them overlapped and did not vary between the two. This observation is in line with the scepticism expressed by Dale & Greenway (1961) although Beentje (1994) continued to consider the two as different taxa. It is therefore most likely that the two are not distinct as will be explained in the following chapters.

Most of the authors handling the two taxa as separate species have relied mainly on their geographical distribution. The dependence on geographical distribution and habitats as the principal taxonomic tool could be unreliable because trends and rates of evolution and speciation like most natural phenomena are unpredictable and are under the influence of a number of different factors. Despite the variation in their natural range of distribution, the close similarity between *V. keniensis* and *V. fischeri* casted doubts on their being separated taxonomically. Trees like *Vitex* have long life cycles and this can slow down the rate of genetic drift thus delaying speciation in isolated populations. Despite the geographical and ecological separation between the different *Vitex* populations, the varying degrees of selection pressure and the limited gene flow between them, they retained similar morphological features. This is possibly implying that there are other factors maintaining the similarity between the disjunct *Vitex* populations.

Some authors further relied on traits such as height, crown size and shape. This criterion has a high chance of leading to even a deeper confusion. That is because the above traits are to a large extent influenced by the prevailing environmental conditions, age of the plant and their assessment is normally subjective. Most of adaptive changes, involve alteration of genotypes (Stebbins, 1974), and variation in height and crown size may not necessarily imply complete genetic alteration or separation of the populations into distinct taxa.

Geographically, the four populations of *Vitex* are isolated, limiting the possibility of gene flow between them which mostly occurs through pollination and seed dispersal. Therefore, the morphological similarity between the *Vitex* population cannot be associated with inter-population gene exchange. The morphological similarity despite the geographical isolation and variable ecological conditions between the populations could be attributed to common ancestry and biological traits enabling the species' populations to withstand selection pressure in the different ecological zones.

The subsequent chapters investigate more biological characteristics that could cast more light on the taxonomy of the *Vitex* species. The study is based on additional

taxonomical traits: seed morphology, seedling morphology, wood properties and cytological characteristics, mating systems and genetic structure of the different *Vitex* populations in East Africa.

Specimens examined

KENYA: *Ahenda* JA 1 (WAG): Muguga-Nairobi; *Ahenda* JA 2 (WAG): Chuka forest; *Ahenda* JA 3 (WAG): Chogoria Forest; *Ahenda* JA 4 (KEFRI): Irangi Forest; *Ahenda* JA 5 (KEFRI): Nyambene hills. Mikinduri; *Ahenda* JA 6 (KEFRI): Kimilili; *Ahenda* JA 7 (KEFRI): Kimilili division; *Ahenda* JA 8 (WAG): Malaba; *Balbo & Balbo* 78 & 846 (EA); *Battiscombe* 960: Nandi; *Battiscombe* K484 (EA): Nyanza basin; *Battiscombe* K563 (EA): Southern Mt. Kenya; *W.R. Buch* 60/455 (EA): Isebania; *W.R. Buch* 61/190 (EA): Bungoma; *I.R. Dale* 4752 (EA): Kakamega/Kaimosi; *I.R. Dale* K3125 (EA): Kakamega; *R.B. Faden & H. Beentje* 466 (EA): Ngangao forest (Taita hills); *Fischer* 476 (EA); *L.P. Glasgow* 46/46 (EA): Port Victoria; *D.K.S. Grant* 846 (EA); *E.J. Humore* 15669 (EA): Mt. Kenya; *C.H.S. Kabuye* 152 (EA): Pangani (Nairobi); *J. Karuhanga* 877 (EA): Lowy Church (Nairibi); *Kenya Horticultural Soc.* 1 54/263 (EA): Chogoria forest; *P. Kuchar* 9496 (EA): Masai Mara; *P. Kuchar* 11654 (EA): Masai Mara; *van der Maesen & Ahenda* 6196 (WAG): Meru; *van der Maesen & Ahenda* 6202 (WAG); Nyambene Tea Estate; *van der Maesen & Ahenda* 6203 (WAG): Lake Victoria Basin. Suba-Kuria (near Migori); *van der Maesen & Ahenda* 6204 (WAG): Suba-Kuria (near Migori); *van der Maesen & Ahenda* 6205 (WAG): Suba-Kuria (near Migori); *van der Maesen & Ahenda* 6206 (WAG): Beroya-Taranganya (nea Tanzania/Kenya border); *van der Maesen & Ahenda* 6207 (WAG): Kadongo-Nyaduong (God Jope); *van der Maesen & Ahenda* 6209 (WAG): Ogwedhi- Moasai; *van der Maesen & Ahenda* 6212 (WAG): Kamasela; *J. McDonald* s.n.: Songhor; *McDonald* 2728 (EA); *J.L. Moon* 15078 (EA): Nyanza basin; *NRB* 39 (EA): Kitale; *W.B.N. Pamba* 74 (EA); *Polhill & Verdcourt* 275 (EA): Nyambene hills; *S.A. Robertson & Q. Luke* 6322 (EA): Gogoni forest (Kwale); *Stuhlman* 3576 (EA); *Trapnell* 59/62 (EA): Donyo Sabuk; *Tweedie* 2599 (EA); *G.R. Williams* 276 (EA): Kakamega; *S.H. Wimbush* 1936 (EA): Meru.

UGANDA: *Ahenda* JA 17 (KEFRI): Entebbe; *Ahenda* JA 18 (KEFRI): Iganga-Jinja; *Ahenda* JA 19 (KEFRI): Busia; *Ahenda* JA 21 (KEFRI): Serere-Lake Kyoga; *Ahenda* JA 22 (KEFRI): Gombe-Kampala; *Ahenda* JA 20 (KEFRI): Pallisa-Mbale; *Ahenda* JA 23 (KEFRI): Bosolwe-Tororo; *C.V. Brasnett* s.n.(EA): Gombolola (Lake Kyoga); *P. Chandler* 832 (EA): Serere (Teso); *D. Dawkins* 415 (EA): Mengo; *W.J. Eggeling* s.n. (EA): Bugala-Sese Island (Lake Victoria); *Morris Godall* s.n. (EA): Lolui Island (Lake Victoria); *C.M. Harris* 219a (EA): Jinja; *A.B. Katende* K266 (EA): Teso-Mt. Abela; *A.B. Katende* 2193 (EA): Acholi-Ilamwo county; *A.B. Katende* 2346 (EA): East Mengo; *K.A. Lye* 4372 (EA): East Mengo; *Milchemore* 1278 (EA): Busoga; *A.S. Thomas* 4072 (EA): West Nile; *A.S. Thomas* 1222 (EA): Bugala-Sese Island (Lake Victoria); --- 1580 (EA): Moroto Mts.

TANZANIA: *Ahenda* JA 9 (KEFRI): Usagara-Mwanza; *Ahenda* JA 10 (KEFRI): Ibondo-Mwnaza; *Ahenda* JA 11 (KEFRI): Musoma; *Ahenda* JA 12 (KEFRI): Tarime; *Ahenda* JA 13 (KEFRI): Shirati; *Ahenda* JA 14 (KEFRI): Utegi; *Ahenda* JA 15 (KEFRI): Buhemba; *Ahenda* JA 16 (KEFRI): Shinyanga; *S. Azuma* 470 (EA): Kabogo Mts (Kigoma); *S. Azuma* 476 (EA): Kabogo Mts (Kigoma); *B.D. Burth* 3294 (EA): Shinyanga; *Carmichael* 835 (EA): Rubya forest reserve-Ukerewe Island (Lake Victoria); *T.H. Clutton-Brock* s.n. (EA): Kigoma; *T.H. Clutton-Brock* 433 (EA): Gombe (Kigoma); *E.A.H.* 13, 116 (EA): Musoma; *P.J. Greenway* 799 (EA): Muritikera; *P.J. Greenway & R.M. Polhill* 11431 (EA): Manyani (Dodoma); *J. Itani* s.n. (EA): Kigoma; *K. Kano* 253 (EA): Firabanga river (Kigoma); *Z.N. Kasika* 8056 (EA): Zanaki-Sukuma; *Kew* 336 (EA): Musoma; *J. Newbould* s.n. (EA): Geita-Maisamu Island (Lake Victoria); *S. Paulo* 53 (EA): Uluguru Mts (Morogoro); *J.R.A. Procter* s.n. (EA): Capri point (Mwanza); *Procter* 983 (EA): Bukoba; *Procter* 3478 (EA): Ngurdoto crater (Arusha); *V.V. Rauma* 255 (EA): Capri point (Mwanza); *W.A. Rogers* 1516 (EA): Biharamulo; *F.G. Smith* s.n. (EA): Butimba (Mwanza); *A. Suzuki* 105 (EA): Kasakati (Kigoma); *A. Suzuki* 121 (EA): Kasakati (Kigoma); *R.E.S. Tanner* (EA): Musoma; *TCB* 348 (EA): Kigoma; *Schleiben* 3148 (EA): Uluguru Mts (Morogoro); *S.R. Semsei* 3603 (EA): Lushoto Arboretum; *S.R. Semsei* 3994 (EA): Magamba (Lushoto); *Von Braun* 1053 (EA): Dodwe Derema road.

Other related specimens examined: *Mildred E. Mathias & Taylor* Al 51 (EA); *Peiper* 1053 (EA); *Reekmans* 1025 (EA): Burundi; *V.C. Gilbert* 5240 (EA): Tanzania; *Welwitsch* 5696 (EA): Angola.

2.6 Morphological and cytological characteristics of *V. keniensis* and *V. fischeri*

Besides examining the herbarium specimens, further studies were carried out to compare and contrast the four different populations of *V. keniensis* and *V. fischeri* in East Africa to determine whether the two species belong to different or the same taxon (species). The study involved the analysis of the morphological and cytological traits, mating systems and the genetic structure of their populations.

2.6.1 Morphological variation in *V. keniensis* and *V. fischeri* seed characteristics

In many genera fruits and seeds produce taxonomic criteria at different levels of hierarchy (Stace, 1980). The seed do differ inter-specifically, in certain cases it varies even at infraspecific level (e.g. *Spergula arvensis*) or even within an individual (e.g. *Spergularia media*) (Stace, 1980). Such quantitative characters tend to be more useful at lower levels of taxonomic hierarchy (Davis & Heywood, 1963). The morphology of *Vitex* seeds from the different populations was evaluated and compared.

Materials and methods

1. 100 trees were marked from each of the *V. keniensis* and *V. fischeri* populations;

Mt. Kenya, Nyambene hills, Mt. Elgon and Lake Victoria, totalling 400 trees from the four populations of the species.

2. At maturity, 10 fruits were collected from each of the sampled trees, processed (extracted and cleaned) and kept separately per mother tree.
3. The seeds from the trees of each *V. keniensis* and *V. fischeri* population were then bulked and 200 seeds randomly drawn from the seed-lot of each population.
4. Measurements on the length and width of the randomly drawn seeds were taken using vernier callipers and the weight of each was seed measured on an electronic weighing balance.

Results and discussions

The variability on seed characteristics of seeds from the different *Vitex* populations were evaluated based on their differences in seed length, width, shape (derived from length: width) and weight. Analyses of variance of the seed length, width, shape and weight were carried out using the SPSS computer program (Nouris, 1994) to investigate the differences among the *V. keniensis* and *V. fischeri* populations of Nyambene hills, Mt. Kenya, Mt. Elgon and Lake Victoria. Tukey-*hsd* tests at significance level of 0.05 in SPSS was used to separate the means.

The results of analyses of variance on the *Vitex* seed morphological traits showed significant differences at $p < 0.05$ level among the four *Vitex* populations (Table 2.1). The highest variation among the *Vitex* populations was recorded in seed shape (derived from the ratio of the seed length to width). Meanwhile the least variation was observed in seed length. The level of variations on seed weight and seed width between the studied *Vitex* populations were moderate.

Overall, the Mt. Kenya *Vitex* population had the longest mean seed length while Nyambene hills had the shortest (Table 2.2). There was no significant ($p < 0.05$) difference between Nyambene hills, Mt. Elgon and Lake Victoria populations in the means of the lengths of their seeds. The Lake Victoria population of the species produced the widest seed and Mt. Elgon, the narrowest. The mean seed width of Lake Victoria population did not differ ($p < 0.05$) significantly from those of Nyambene hills and Mt. Kenya. Lake Victoria *Vitex* produced lighter and more spherical seeds while Mt. Kenya *Vitex* seeds were the heaviest and most elongated in shape.

Conclusion

Most of the variation observed in the *V. keniensis* and *V. fischeri* seed morphological characteristics did not seem to follow any geographical or ecological pattern. No clear pattern of the distribution of the seed morphology was observed among the populations. Assuming the observed seed morphological variation is a reflection of the species' genetic make-up, then the random distribution of most seed characters could be a result of lack of difference between the *Vitex* populations; possibly a result of the common genetic background of the *Vitex* populations or because of their capacity to survive the different ecological conditions without conspicuous morphological change in their seeds.

Table 2.1. Analysis of variance of seed length (cm), seed width (cm), Seed shape (length : breadth) and seed weight (g) in the four *V. keniensis* and *V. fischeri* populations; Mt. Kenya, Nyambene hills, Mt. Elgon and Lake Victoria. (* denotes significance at the 5% level, ns denotes non-significance at 5% level).

Source of variation	DF	Length		Width		Shape		Weight	
		MS	F	MS	F	MS	F	MS	F
Population	3	0.49	8.62*	0.36	17.66	1.37	31.30*	0.59	14.35*
Replication	3	0.40	7.06*	0.17	8.51*	0.02	0.49ns	0.66	16.07*
Residual	793	0.06		0.02		0.04		0.04	

Table 2.2. Mean seed length, width, seed shape (length : width ratio) and weight of the *Vitex* seeds from the four different *Vitex* populations; Mt. Kenya, Nyambene hills, Mt. Elgon and Lake Victoria (Means denoted by the same letter are not significantly different at the 5% level of significance according to Tukey test).

Vitex Populations	Length (cm)	Width (cm)	Shape	Weight (g)
Mt. Kenya	1.3475 **b**	0.8570 **b**	1.5905 **b**	0.5535 **c**
Nyambene hills	1.2445 **a**	0.8655 **b**	1.4502 **a**	0.5299 **bc**
Mt. Elgon	1.2460 **a**	0.7890 **a**	1.5751 **b**	0.4888 **b**
Lake Victoria	1.2575 **a**	0.8875 **b**	1.4305 **a**	0.4292 **a**

However, it is also possible that most of the seed traits are selectively neutral and therefore do not respond to the forces of natural selection. That may make them neither adaptive nor sensitive to changes in the environmental conditions thus leading to the observed random distribution of seed morphological traits among the different populations. Despite the significant variation ($p < 0.05$) between *Vitex* populations on seed morphology, little link was observed in relation to the obvious different ecological conditions of the different habitats of the species. Therefore, basing on the seed morphology no consistent distinction can be made between the populations to support their separation into *V. keniensis* and *V. fischeri*. However, a further study was carried out on other morphological characteristics of the different populations of the species and are presented in the next sections.

2.6.2 Seedling leaf morphology of *V. keniensis* and *V. fischeri*

Materials and methods

1. In each of the four *V. keniensis* and *V. fischeri* populations 50 distantly distributed trees were randomly sampled.
2. One seedling was raised from each of the sampled trees making a total of 50 seedlings per *Vitex* population.
3. The 50 seedlings per *Vitex* population were raised in labelled pots under the same conditions in a glasshouse in 10 replicates of 5 seedlings.

4. At the age of three months when the seedlings started producing compound (palmate; 5-foliolate) leaves, the leaf length, width, and petiole length were measured using vernier callipers and the number of simple leaves, veins and teeth (serration) counted.

Results and discussions

Analyses of variance were carried out on the seedling leaf morphology using the SPSS computer program (Nouris, 1994) to investigate the differences among the four *Vitex* populations. Tukey's-hsd tests at significance level of 0.05 in SPSS was used to separate the means of the seedling leaf morphological traits.

The analyses of variance on leaf morphology of seedlings from the different populations of *Vitex* showed significant ($p < 0.05$) differences between them (Table 2.3). The seedlings were raised under similar environmental conditions in a glasshouse, therefore any differences among them should be a reflection of their taxonomic and genetic variation. The highest significant variation among the four *Vitex* populations was observed in seedling leaf length, width and number of veins. The least significant variation was observed in the number of teeth (serration). The within population variation in the seedling leaf morphology at $p < 0.05$ was insignificant except for the number of teeth (serration). That could be a result of similar genetic background and slow pace of population divergence.

Table 2.3. Analysis of Variance on Leaf length (cm), width (cm), petiole length (cm), number of simple leaves, veins and number of teeth of seedlings of *V. keniensis* and *V. fischeri* from Mt. Kenya, Nyambene hills, Mt. Elgon and Lake Victoria (* denotes significance at 5% level and ns non-significance at 5% level).

Source of variation	DF	Leaf length		Leaf width		Petiole length	
		MS	F	MS	F	MS	F
Population	3	21.80	12.38*	5.44	11.39*	0.73	4.12*
Pop.*Tree	12	1.22	0.70ns	0.48	1.01ns	0.24	1.34ns
Replication	9	2.38	1.37ns	0.57	1.20ns	0.89	4.98*
Residual	175	1.74		0.48		0.18	
Source of variation	DF	No. of teeth		No. of simple leaves		No. of veins	
		MS	F	MS	F	MS	F
Population	3	8.47	3.25*	5.77	5.79*	396.82	14.25*
Pop.*Tree	12	1.56	0.60*	0.85	0.86ns	37.04	1.33ns
Replication	9	5.58	2.14ns	3.14	3.16*	43.26	1.55ns
Residual	175	2.60		1.00		27.84	

Table 2.4. Means of length, width, petiole length, number of teeth, simple leaves and veins of *Vitex* seedlings from the four different *V. keniensis* and *V. fischeri* populations; Mt. Kenya, Nyambene hills, Mt. Elgon and Lake Victoria. (means denoted by the same letter are not significantly different at 5% significance level according to Tukey test).

Vitex Population	Leaf length, cm	Leaf width, cm	Petiole length, cm
Mt. Kenya	5.517**b**	3.140**b**	1.154**b**
Nyambene hills	4.491**a**	2.534**a**	0.970**ab**
Mt. Elgon	4.057**a**	2.375**a**	0.888**a**
L. Victoria	4.219**a**	2.711**a**	0.908**a**
Vitex population	No. of teeth	No. of simple leaves	No. of veins
Mt. Kenya	9.480**b**	4.100**ab**	26.940**c**
Nyambene hills	9.000**ab**	4.300**b**	33.820**a**
Mt. Elgon	9.460**ab**	3.820**a**	30.840**b**
L. Victoria	8.620**a**	3.520**a**	30.500**b**

On multiple range tests (Tukey-HSD test with significance level $p<0.05$), the four *Vitex* populations exhibited no clear pattern distinction (Table 2.4). Seedlings of Mt. Kenya and Nyambene hills *Vitex* differed significantly ($p < 0.05$) on most of the leaf morphological traits despite their geographical proximity and ecological similarity of their habitats. The same observation was made on the seedlings of Lake Victoria and Mt. Elgon. The pattern of distribution of most of the variation in the seedling leaf morphological traits were also inconsistent with the expected ecological gradient and geographical distances. The seedlings raised under the similar growth conditions were expected to exhibit their heritable (adaptive) traits. Therefore, the lack of distinct pattern of variation in seedling leaf morphology of the different *Vitex* populations seem to imply similarity between them.

2.6.3 Wood characteristics of *V. keniensis* and *V. fischeri*

Wood anatomy is of considerable taxonomic and phylogenetic significance as cited by Carlquist (1961), besides timber trade. There are a number of features of the xylem which show major trends of evolution in angiosperms. Distribution of vessels in transection is a taxonomically significant feature (Singh et al., 1994). The presence or absence of tylose and presence of visible pits in tylose also afford taxonomic criteria. Other features include vascular rays and storied wood (Stace, 1980). The study on the properties of wood of *Vitex* from the different populations was carried out to make comparisons between them. The results can also be used in the subsequent selection and genetic improvement of the species for commercial timber production.

Materials and methods

The characteristics of *V. keniensis* and *V. fischeri* wood from the different populations have been evaluated by analyzing samples from the four different populations of the species. That was an additional basis for assessing the distinction and similarity between the two "species".

1. 10 pieces of wood were collected from each of the *Vitex* populations. The wood samples were collected from mature trees of approximately the same age.
2. The specific gravity of each sample was measured as a ratio of the weight of a sample of wood to its volume.
3. Three major planes were used for describing the micro- and macro-features of the *Vitex* wood. The transverse, radial and tangential sections of the wood samples were studied. In the transverse section, planes were made perpendicular to the logs; radial sections, vertical planes were cut through the central axis of the logs parallel to the rays, tangential sections, parallel vertical planes but not through the longitudinal axis (Figure 2.4).
4. Wood hardiness in kilonewton and fibre length in millimeters (mm) of wood from the different *Vitex* populations were analyzed.

Results and discussions

The average wood density from the different *Vitex* populations varied between 0.42 and 0.51 g/cm^3. The mean values for Mt. Kenya, Nyambene hills, Mt. Elgon and Lake Victoria were 0.42, 0.45, 0.48 and 0.51 g/cm^3 respectively. The density of the sampled *Vitex* wood was moderate, falling between those of hard wood and soft wood. Soft woods growing in East Africa are ***Pinus radiata*** (0.33 g/cm^3), ***Pinus oocarpa*** (0.441 g/m^3) and *P. patula* (0.33 to 0.46 cm^3). Hardwood includes *Acacia senegal* (L.) Wild. (0.54 cm^3) and *Acacia nilotica* (L.) Del. (0.94 cm^3) (Giertz, 1995). The mean density of about 0.45g/cm^3 in *Vitex* would therefore be treated as medium in the classification by Wheeler et al. (1989). The difference between the different populations of *Vitex* in wood density ranged from 7 to 21%. The largest difference (21%) was observed between Mt. Kenya and Lake Victoria. The *Vitex* wood density seems to follow some consistent pattern in relation to the ecological and geographical ranges. However, at this stage, it is difficult to conclude whether the traits are heritable or not.

Transverse section (x100): The pores are solitary and the parenchyma vasi-centric. Some parenchyma were paratracheal or absent. The rays are predominantly multiserrate (2 to 4 cells wide) and the fibres septate. The fibres are thin to thick walled, growth rings indistinct and tylose present. Radial section (x100): Ray tissues are homogeneous composed wholly of procumbent cells. Tangential section (x100): The rays are 1 to 2 cells wide but predominantly uniseriate, fibres septate, one septum per fibre (Figure 2.4).

The mean radial hardiness of *Vitex* wood was between 1.87 and 2.62 kilonewton, while the tangential hardiness ranged from 1.94 to 3.20 kilonewton. The average

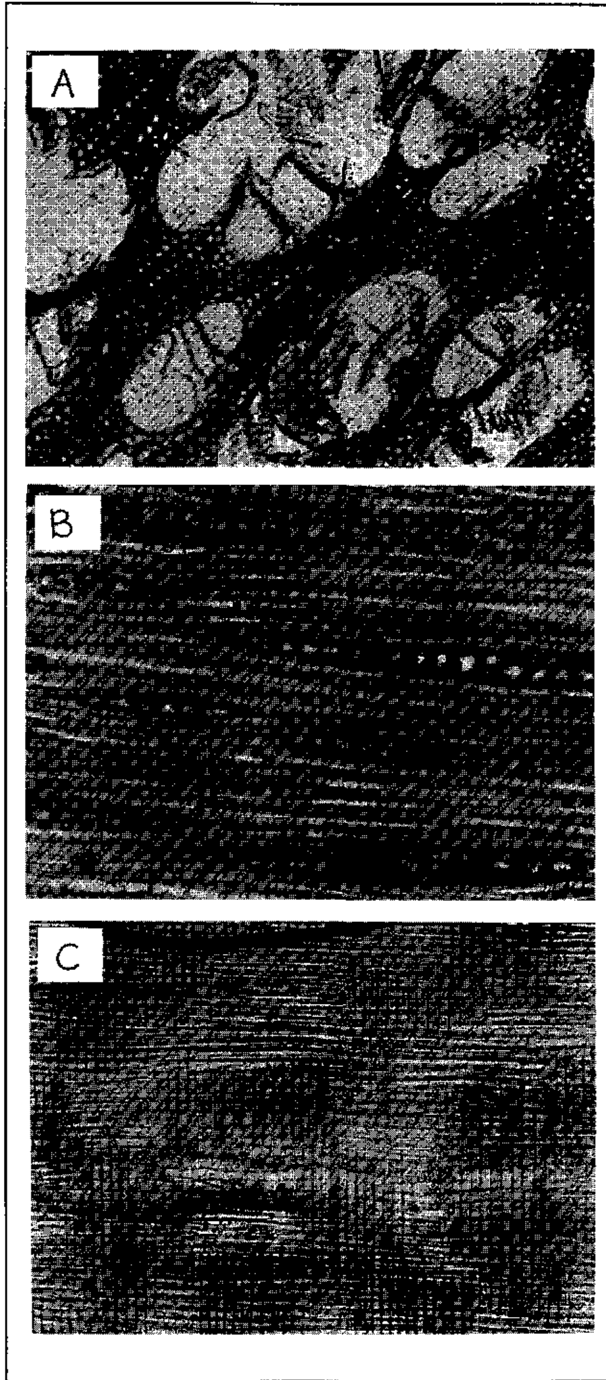


Figure 2.4. Morphological properties of wood of *V. keniensis*. a) Transverse section, b) Tangential section, c) Radial section (x100).

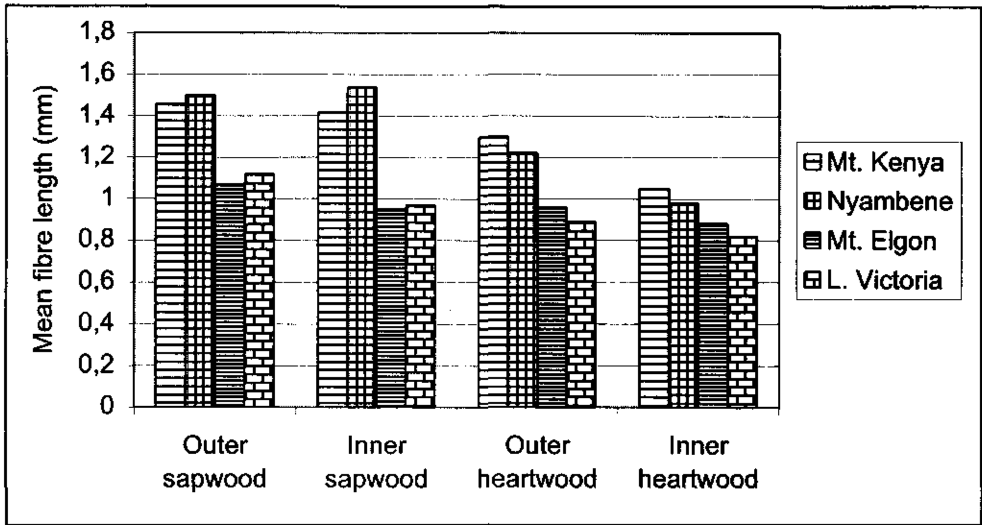


Figure 2.5. Mean fibre length of *V. keniensis* and *V. fischeri* wood.

hardiness of *Vitex* wood varied among the populations. The wood from Lake Victoria and Mt. Elgon had higher values of 2.62 and 2.5 kilonewton (radial hardiness) respectively. Lake Victoria had tangential hardiness of 2.54 and Mt. Elgon, 3.20 kilonewton. The mean hardiness of *Vitex* from Mt. Kenya was 1.87 (radial hardiness) and 1.97 kilonewton (tangential hardiness). Meanwhile, Nyambene hills had radial hardiness of 1.99 and tangential hardiness of 1.94 kilonewton. The properties of wood from Lake Victoria and Mt. Elgon seem to exhibit stronger physical properties than the timber of Mt. Kenya and Nyambene hills. The strong physical properties in wood are preferred in construction, therefore it could be one of the desirable traits for improvement breeding in forestry.

The heartwood of *Vitex* is dark brown in colour and the sapwood is light brown to yellow. The lighter colour of sap wood is a result of it being young, living and physiologically active. The heart wood is normally darker because of deposits of resins, tannin and other organic substances. They make the timber resistant to fungi and insects. The grains are straight especially in those from Nyambene and Mt. Kenya but slightly wavy and interlocked in those of Lake Victoria and Mt. Elgon. The texture is fine but relatively coarse among those from Lake Victoria and Mt. Elgon. Mt. Kenya *Vitex* had the longest fibres and Lake Victoria the shortest. The *Vitex* populations of Mt. Kenya and Nyambene hills had the longest mean fibre length (Figure 2.5). The wood from the different *Vitex* populations varied in their physical properties (density, hardiness and fibre length) but not in their micro- and macro-morphological characteristics.

There is a strong correlation between wood structure and macro-ecology of tree species (Soerianegara & Lemmens, 1993). Therefore, it is not expedient to separate

the different populations into taxonomic entities based on the wood density, hardiness and fibre length while the other morphological traits and genetic characteristics are similar. The variation in the physical properties of wood can also arise from the influence of the environment through phenotypic plasticity without necessarily involving of genetic change (Stuessy, 1990).

2.6.4 Cytological characteristics of *V. keniensis* and *V. fischeri*

Cytology in a broad sense deals with all aspects of cells, but in practice in taxonomic work the focus has been on chromosome number and their attributes (Lewis, 1969). Chromosomes play a special role as a source of comparative data because they contain genetic material responsible for maintaining biological integrity of species. Chromosome number as a taxonomic character is probably one of the most constant single features employed (Singh et al., 1994). Generally, with a few exceptions, the number of chromosomes in each cell of individuals of the same species is normally constant (Stace, 1980; Singh et al., 1994). The relative conservativeness renders the chromosome number an important and much used taxonomic character (Stace, 1980). Since chromosome numbers change in evolution through many diverse avenues, comparisons are normally valid only within certain narrow defined limits such as species within a genus (Stace, 1980).

The ploidy level effects a number of characteristics in plant life for instance, the morphology, physiology and even the mating systems (Stace, 1980). The distribution of cytotypes in species populations can therefore be used to distinguish them and make some conclusions on their taxonomic relationships. Studies on the cytological characteristics of *V. keniensis* and *V. fischeri* were carried out on the different populations of the species. The information on the distribution of the cytotypes was also essential for interpreting complex electrophoretic data generated from genetic (isozyme) analysis on the species' populations (Barrington, 1990; Haufler et al., 1990), in chapter 4.

Materials and methods

1. 10 mature trees were sampled from each of the four *Vitex* populations in shown in Figure 2.3.
2. Mature fruits were collected from the sampled trees and the seeds extracted, cleaned and germinated in vermiculite in labelled petri-dishes placed in an incubator set at 25°C.
3. Once the seeds germinated and produced radicles, 2 to 3cm of the meristematic root tips were cut off and immersed in 2 mM 8-hydroxyquinoline at room temperature for 4 to 7 hours.
4. The pre-treated root tips were fixed in freshly prepared acetic acid to alcohol (1:3 parts v/v glacial acetic acid to absolute alcohol), one to two hours.
5. The root tips were hydrolysed in a normal solution of hydrochloric acid in a water bath at 60°C for 8-12 minutes, then stained with Feulgen reagent in the

dark at room temperature for about one hour.

6. The stained root tips were then squashed in 2% acetocarmine solution and observed under a microscope.
7. 10 cells at metaphase stage with clearly visible chromosomes were counted in each slide and the number of chromosomes in each of them determined.

Results and discussions

The best samples of root-tips i.e. those with actively dividing cells were obtained from the fast growing seedlings.Therefore, the growth rates of the plants had direct influence on the possibility of getting cells with visible chromosomes at metaphase stage. The time of sample collection also had an influenced on the chances of getting good root-tips. The best root samples were harvested at noon when it was bright and warm, that is probably when the meristematic cells in the roots were undergoing rapid division. The roots collected in the morning or late in the evening had very low proportion of actively dividing cells. Root pruning and withholding watering of the seedlings for 24 hours before collecting root-tips also enhanced root growth.

However, the resultant chromosomes of *Vitex* were too tiny for their morphology to be distinguishable for detailed studies on the nature of their karyotypes. The chromosome counts from the randomly selected 10 trees of each of the four tree populations showed that the species had 96 somatic chromosomes (Figure 2.6). This figure was constant and applied to all the samples from the four different *Vitex* tree populations. Somatic chromosome numbers reported in the genus *Vitex* so far vary for 24 to 34. Some of the species in the genus have been reported to have more than one cytotypes; *V. agnus-castus* L. 2n=24 (Patermann, 1935) and 2n=32 (Sharma & Mukhopadhyay, 1963); *V. trifolia* L. 2n=26 (Sobti & Singh, 1961), 2n=32 (Suguira, 1936) and 2n=34 (Sharma & Mukhopadhyay, 1963); *V. negundo* L. 2n=24 (Malik & Ahmad, 1963), 2n=26 (Sobti & Singh, 1961) and 2n=34 (Sharma & Mukhopandyay, 1963). But only one cytotype has been reported on the following *Vitex* species; *V. cienkowskii* Kotschy et Peyr. 2n=32 (Mangenot & Mangenot, 1962), *V. fosteri* Wright 2n=32 (Mangenot & Mangenot, 1962), *V. grandifolia* Gürke 2n=32 (Mangenot & Mangenot, 1957), *V. micrantha* 2n=32 (Mangenot & Mangenot, 1957) and *V. rotundifolia* 2n=32 (Chuang et al., 1963). In the past, two basic chromosome numbers of x=6 and 8 have been reported for the genus *Vitex* (Darlington & Wylie, 1956). The chromosomes in the meiotic cells of *V. keniensis* and *V. fischeri* were too small to be observed so no conclusion could made on their basic chromosome number. Recently, isozyme and other molecular techniques have been used to determine ploidy level in plants (Crawford & Smith, 1984; Haufler et al., 1990; Harris & Ingram, 1992). Inferences on the nature and level of polyploidy in the species were therefore made from their isozyme phenotypes (chapter 4).

The *Vitex* species is therefore highly polyploid. The somatic chromosome number was the same in all the populations studied i.e. 2n=96. Polyploidy has long been recognised as a significant feature in many plant taxa and estimates vary, but 47 to 70% of all angiosperm species have polyploid origins (Masterson, 1994). Polyploids

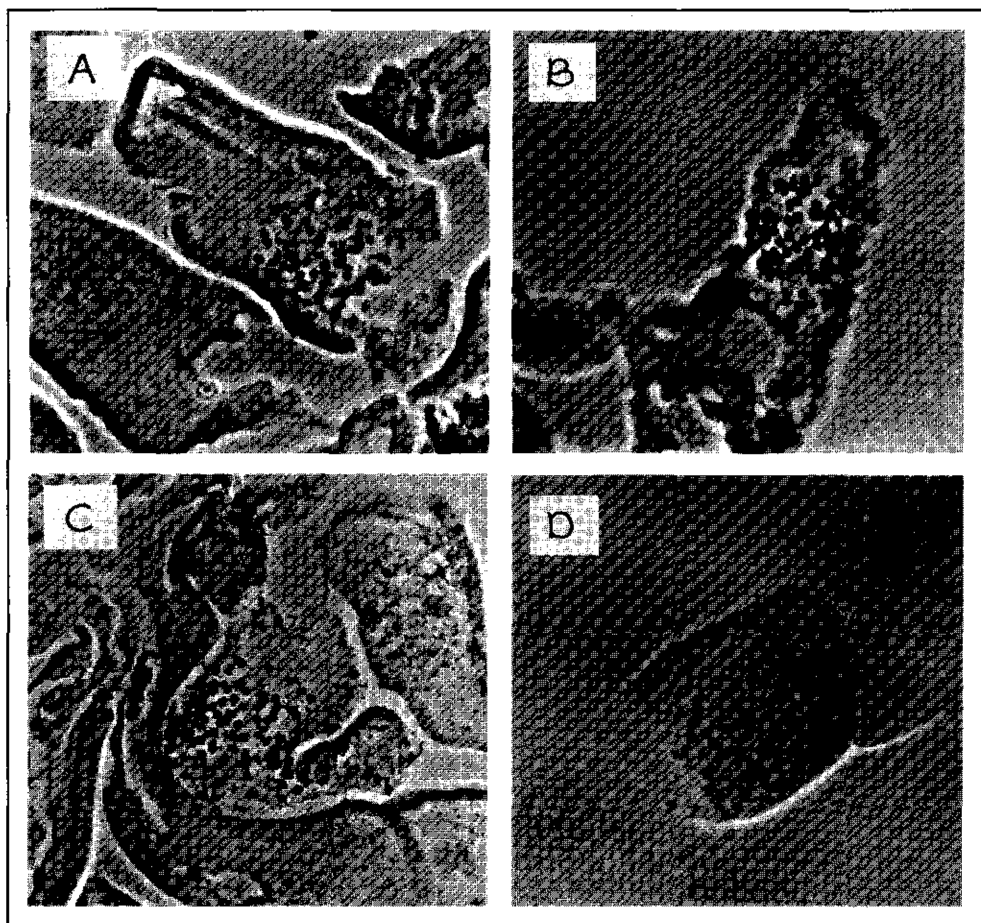


Figure 2.6. Somatic chromosomes of *V. keniensis* and *V. fischeri*. a) Mt. Kenya, b) Nyambene hills, c) Mt. Elgon, d) Lake Victoria (x 1000).

frequently have long ecological ranges different from their diploid progenitors (Ehrendorfer et al., 1996; Lewis, 1980). This attribute could be linked to the wide range of chromosomal combination possibilities in a polyploid. A number of studies have attempted to show the relationship of polyploidy to the environment. Past research has shown higher ecological tolerance in polyploid than the closely related diploids (Stebbins, 1957). Polyploidy therefore seem to confer additional ecological adaptability to many taxa making them capable of colonising a wide geographical range (Stuessy, 1990). Therefore, the observed high polyploidy in *Vitex* could partly explain the ability of the species to occur in the contrasting ecological zones without changing its morphological and genetic characteristics.

According to Stuessy (1990), variation in the number of chromosomes is more common in herbaceous than in woody dicots. Therefore many of the woody species

tend to exhibit a uniform chromosome number despite ecological diversity as in *Danthania sericea* (Fairbrothers & Quinn, 1970). The constant number of somatic chromosomes throughout the *Vitex* populations indicates that despite the variable ecological conditions, the species has retained one cytotype. The observation further underlines the similarity between *V. keniensis* and *V. fischeri*, casting doubt on their distinction. The close connection between chromosomes and reproductive isolation makes it logical to consider the difference in chromosome number between populations to be recognised as taxonomic at specific level. The chromosomal traits do reflect common ancestry rather than convergence (Stuessy, 1990), implying that the observed similarity in the cytotype of the *Vitex* populations might arose from their common origin especially considering the close similarity in their phenotypic traits as illustrated in the previous sections in this chapter.

2.7 Conclusion

The studies of the herbarium specimens, seed and seedling morphology, somatic chromosome number and wood properties of *Vitex* revealed more similarities than distinction between the populations from the different ecological areas. The patterns of variation in most of their morphological features were random, implying that *V. keniensis* and *V. fischeri* are not taxonomically different from each other.

In nature there are three possible ways through which such disjunct populations of species are formed (Lind & Morrison, 1974); they may originate separately in different areas simultaneously (multiple origin), they may be formed by long distance dispersal from one to another climatically favourable area, or they may occur through contraction or separation of a once continuous range following climatic change or geological events or human activities. The first postulation is unlikely especially under natural conditions. The second postulation may be ruled out because of the vast distances between the *Vitex* populations thus limiting the inter-population geneflow.

It is most probable that the *Vitex* populations were formed through the third postulation as a result of various geological and tectonic activities in East African. Biogeographical history study of East African vegetation indicated that during the early part of the Kainozoic Era East Africa had no high mountains and is believed to have consisted of a vast gently undulating plain (Lind & Morrison, 1974) probably consisting of similar vegetation cover. The present highlands are therefore a result of tectonic activities in the Miocene and Pleistocene which led to the formation of Rift Valley and volcanic mountains (Haughton, 1963; Lind & Morrison, 1974). It is therefore possible that most of the plant species were separated into different populations by the geological events and human activities that followed.

The biogeographical history could also explain the relict of highland species such as *Podocarpus latifolia* (Thunb.) Mirb., *Prunus africana* Mill., *Olea europea* L. ssp. *africana* (Mill.) P. Green and *Warburgia ugandensis* Sprague found in some low-lying

areas in the wet savanna around Lake Victoria at about 1000 to 1400 m above sea level. These species have been considered to be exclusively sub-montane or montane forest plants. The western shore of Lake Victoria in Maramagambo forest (Uganda) and Minziro forest (Tanzania) at about 1000 to 1200 m above sea level is dominated by *Podocarpus* species (Lind & Morrison, 1974). The natural occurrence of these species in this region is considered remarkable because this is far below their known natural altitudinal range. Meanwhile, in some parts of the wet savanna of Masai Mara, Migori and Kuria districts of Kenya *Vitex* do occur naturally in the same zone with *P. africana, O. europea* ssp. *africana* and *W. ugandensis* especially in some riverine areas where human activities are minimal. In Moroto mountains in Uganda also, the species grows in association with *O. europea* and *Teclea nobilis* Del. The distribution of *V. keniensis* and *V. fischeri* in the different geographical and ecological zones in East Africa can be explained by the biogeographical historical events which led to the formation of the Rift Valley and volcanic mountains separating the once continuous lowland vegetation stretching from West to East Africa.

The descent from common ancestry could therefore explain the morphological similarity observed in the populations of *V. keniensis* and *V. fischeri* occurring in the different geographical and even ecological regions of East Africa. In addition to common ancestry, the morphological similarity could have maintained by slow pace divergence between the isolated populations caused by the species' biological characteristics such as polyploidy, long life cycle (over 100 years) and phenotypic plasticity. These factors can slow down the rate of adaptive changes in geographically and ecologically isolated populations.

Alternatively, the difference in selection pressure between the regions may not be as much as it appears. Therefore the main morphological and physiological features of the species may be adequate for its populations to colonise the distinct ecological zones without the need for adaptive changes. The results of this study therefore indicates that the role of geographical and ecological separation in speciation was overestimated for the *Vitex* populations. The study has further indicated that geographical and ecological separation of populations of a species alone is inadequate for taxonomic separation unless supported by other factors, otherwise the ecophenes would end up in an endless species list.

The stability of *Vitex* over a wide ecological range could be an indication that so long as the morphological, physiological and genetic make-up of a species does not impose limitations on its survival, there would be less need for adaptive change thus divergence would be unnecessary or it proceeds at a slow pace. However, over time and as the natural environment changes and pressure from human activities increase, the species' populations might eventually undergo speciation events. But at the moment the observed morphological and cytological variations are not adequate for them to be placed in separate taxa. *V. keniensis* and *V. fischeri* should therefore be considered as a single species. This is also supported by the observations made by Dale & Greenway (1961).

V. fischeri Gürke in Engl. Bot. Jahrb. 18: 171 (1893).
Types: Tanzania: Mwanza district, near Lake Victoria, Kayenzi [Kahegi], *Fischer 476.* (B, all syntypes destroyed). Neotype: *van der Maesen & Ahenda*: 6203; Suba-Kuria (Lake Victoria basin). Paratypes: van der *Maesen & Ahenda*; 6204, 6205 (also Suba-Kuria).

Heterotypic synonyms: ***V. andongensis*** Bak. in Fl. Trop. Afr. 5: 329 (1900).
Type: Angola, Pungo Andongo, *Welwitsch 5696* (K, holo; BM, iso, not seen)

V. bequartii De Wild. in F.R. 13: 142 (1914).
Type: Zaire, Lubumbashi [Elisabethville], Bequaert 319 & Homble 202 (BR, hol.)

V. keniensis Turrill (syn. *V. balbi Chiov.*). in K.B. 1915: 47 (1915).
Type: N.E. & E. Mt. Kenya, probably Meru, D.K.S. Grant in F.D. 846 (K, syn.!, E.A., Isosyn.!).
This does not qualify as a *synonymus novus*, as in the past the species have been considered conspecific on and off.

V. payos (Lour.) Merr. var. *stipitata* Mold. in Phytologia 8: 72 (1961).
Type: Uganda, Mengo district, Kampala, E.H. Wilson 194 (UC, holo.!)

The name *V. fischeri* has priority over the well-known *V. keniensis* (Meru oak), so *V. keniensis* falls into synonymy, thus *V. fischeri* Gürke (syn. *V. keniensis* Turrill). The four populations should be considered as one species and be included in the proposed conservation or improvement breeding strategies. The subsequent chapters therefore treat *V. keniensis* and *V. fischeri* as one species; *V. fischeri* syn. *V. keniensis*. However, both names continues to be used in the text because further comparisons are being made on their mating systems and genetic structure, and for ease of understanding the subsequent chapters by those used to *V. keniensis* for so long a time.

3 Mating systems of *V. keniensis* and *V. fischeri*

3.1 Introduction

Mating systems in broad sense include all aspects of sex expression in plants which affect the relative genetic contribution to the next generation of individual within a species (Wyatt, 1983). The mating system of plants is one of the major aspects that influences their population structures, and hence their genetic diversity (Loveless & Hamrick, 1984; Hamrick, 1992). The ability of any organism to produce genetic variation for any trait including resistance to pathogens, is partially determined by its mating system; self-fertilisation yields little variation in progeny and out-crossing among divergent genomes creates greater genetic variability (Bell, 1983). Generally, high genetic variability have been observed within populations of many tree species indicating that the out-crossing is common in them (Hamrick & Loveless, 1988; Loveless, 1992; Bawa, 1992). Some species are intolerant to self-fertilisation and do counter it by developing either pre- or post-zygotic rejection of selfing (Lande & Shemske, 1985; Charlesworth & Charlesworth, 1987).

The mating system of a species (such as selfing rates) may vary along geographical or ecological gradients especially under stress (Dafni, 1992). Hence, the result from one population cannot always be applied to the whole species. Therefore in this study, the four *Vitex* populations were treated separately. Generally, plants exhibit five main classes of mating patterns, namely: predominantly self-fertilising, predominantly outcrossing, mixed selfing and outcrossing, apomixis (Richards, 1986; Brown, 1989; Brown et al., 1989). Within populations of selfing plants, variability is usually low but not depauperate, owing to limited outcrossing and segregation in the progeny of the few heterozygous maternal plants available (Allard, 1989; Shore, 1991).

Mating systems comprises of the mode of pollination, fertilisation, the method of reproduction and seed dispersal mechanism (Burley et al., 1986; Richards, 1986). It is widely used in agriculture and forestry to regulate and canalise the components of fecundity for selection purposes in cultivated plants. The knowledge of the sexual systems is an essential background for evaluation of the dependence of seed production on pollination rate and type, towards the understanding of the mechanisms of gene flow within and between populations. The information is crucial in breeding programmes where effective isolation of seed orchards and in developing seed collection strategies. Mating systems has a direct influence on genetic structure of a population and therefore is essential in sampling parental trees for breeding and gene conservation, designing multiplication populations such as seed orchards and to achieve the intended packaging of required genetic traits in progeny (Adams & Jolly, 1980; Ritland & El-Kassaby, 1985; Adams, 1992).

Mating systems i.e. the extent to which progenies are produced from self-fertilisation or outcrossing, vary considerably among plant species and among populations (Glover & Barrett, 1986). Therefore, the understanding of a species' mating system could also be of value to unravel its taxonomic complexities and evolutionary pathways (Stace, 1980). Certain reproductive traits such as dioecy and gynodioecy, have been interpreted as a way of avoiding self-fertilisation (Darwin, 1877; Faegri & van Pijl, 1979; Lloyd, 1975; Charlesworth & Charlesworth, 1979). Despite the benefits of outbreeding, selfing has evolved repeatedly from outcrossing taxa (Stebbins, 1957; Raven, 1976) possibly through reproductive assurance of selfing under conditions of low population density or pollinator uncertainty (Barrett, 1988).

Generally, the knowledge of the biology of tropical forests and, in particular the reproductive biology of their woody species is very limited (NRC, 1991). The reproductive syndrome of plants is an important facet of their adaptive responses. Like most of the tropical forest tree species, the mating system of *Vitex* has not been studied and is therefore unknown. This is a bottleneck to their conservation and improvement breeding programmes. However, different approaches have been used to gain an understanding of mating systems in plants and estimate parameters which describe mating systems. The most conventional method used is controlled pollination to assess self-compatibility and produce full-sibs (Ellis & Sedgeley, 1992; Perez-Nasser et al., 1993). I therefore carried out studies on floral morphology, phenological patterns and the mode of pollination to determine the mating systems of the different *Vitex* populations.

3.2 Floral morphology and phenology of *V. keniensis* and *V. fischeri*

Angiosperms have flowers of diverse size, shape and colour depending on the taxon. Flowers are functional organs whose structural complexities are presumably adapted to sexual reproduction. The flower structure, phenology and evolutionary ecology of pollination partnerships are so interwoven that systematists also rely on floral structure for identification and phylogenetic studies. The reproductive organ is considered a conservative character and is widely used in classification at different taxonomic levels (Stace, 1980).

The traits of flowers play an important role in controlling pollen flow within and between flowers, and within and between plants. The outcrossing and selfing levels of a given plant species shows a tight association between the mating system, the floral size, the temporary separation of anther dehiscence and stigma receptivity, and the spatial relationship of the stigma and anther (Cruden, 1977). The level of within and between population genetic variability and hence the evolutionary potential and niche width are reliant on floral traits and pollination strategies. Every conceivable facet of the variation of inflorescence, bracts, receptacle, calyx, corolla, stamens and ovules is valuable in plant classification at different taxonomic levels (Stace, 1980).

The temporal and spatial separation of the male and female functions of flowers in plants has traditionally been treated as a means to avoid self-pollination (Faegri & van der Pijl, 1979). Morphological analysis of a given flower and knowledge on its temporal pattern of development are essential and can be used for determining the species' mode of pollination. The male and the female functions of the flower may mature simultaneously (homogamy), the male (pollen) may be available before the female part (stigma) is receptive (protogyny) or the stigma may be receptive before the pollen grains mature (protandry). These have implications on pollination and the subsequent gene flow in a given population. The development of stigma and pollen grains can be checked in vivo or in vitro. Studies were therefore carried out on the flowers to determine their mode of pollination in populations of *Vitex* by monitoring phenological patterns of the different populations of the species, observing floral morphology, and trends in the maturation of both stigma and pollen grains.

Materials and methods

1. Ten trees were randomly sampled in each of the four *Vitex* populations.
2. 25 flower buds were selected from different parts of the crown and tagged with plastic identification markers in each tree, immediately before the flowering season as flower buds were forming.
3. The number of opened inflorescence buds were counted every week on each tagged individual tree once per week from January onwards in Mt. Kenya and Nyambene hills but from December in Mt. Elgon and Lake Victoria.
4. Finally, the number of fruits formed by the tagged flowers were also counted every week as they mature.
5. The morphology of the flowers was observed using magnifying lenses and comparison were made at different stages of development.

Results and discussions

The flowers are brightly coloured, scented and hermaphroditic, that is bearing both male and female parts (Figure 2.2). The stamens are slightly longer than the style, i.e. it is a thrum. The floral parts of the *Vitex* studied were similar in all the populations observed. The inflorescence is composed of flowers which vary in size from 6 to 8 mm long. The four calyx lobes are green. The corolla is bilabiate with 2 small, white lobes and one large mauve, lower lobe. The bright colour of the flowers and scent are intended to increase floral display to attract more pollinators to the inflorescence (Klinkhamer & de Jong, 1990) and hence increasing the chances of pollination and the possibility of fruit set. The flowers are scented at maturity and it is quite certain that the species is pollinated by bees (*Apis mellifera* L.). The bees trapped from the flowers using insect traps and observed under a microscope were found to have pollen grains of *Vitex* when examined. Other flower visitors included wasps and spiders but they did not have any pollen grains. They appeared to prey on the bees and other insects visiting the flowers. The local inhabitants reported that the species was popular in honey production and that honey from its flowers was of superior quality, fetching high prices in the market.

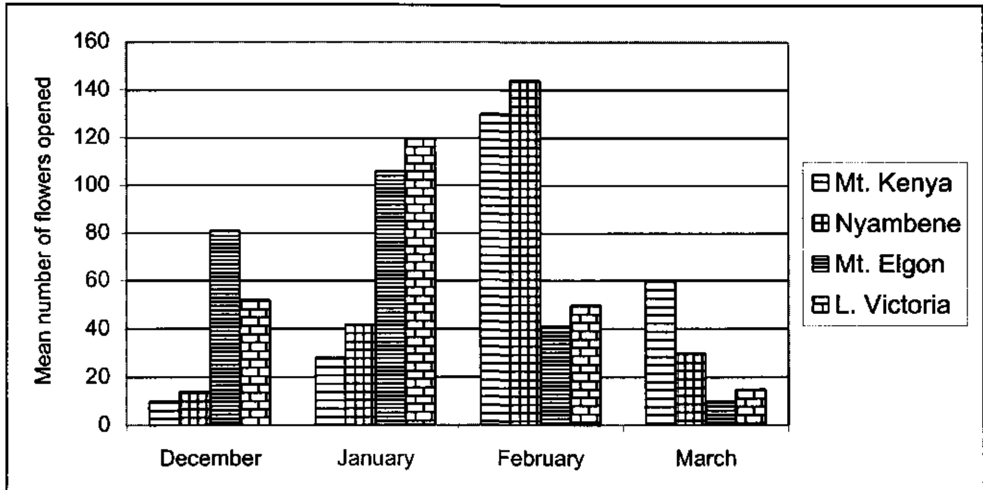


Figure 3.1. Flower phenology of populations *V. keniensis* and *V. fischeri.*

The androecium consists of 5 stamens, the anthers are club-shaped, slightly above the level of the fork-shaped stigma. The anthers bear sticky and yellow-orange pollen at maturity. The gynoecium consists of a white, superior ovary with a slender style and fork-shaped stigma. The ovary has four loculi, bearing up to four ovules. Even though the ovules are four in number, only one gets fertilised and produces only one viable seed while the rest (three) are normally aborted. The cause could be investigated further. However, this phenomenon has been attributed to zygotic competition in developing fruits (Bawa, 1990). It has also been explained by Mazer (1987) as a manifestation of sexual selection at post-fertilisation stage, a feature common in multi-ovuled species.

The *Vitex* trees are deciduous, shedding their leaves annually. The leaf fall coincides with fruit maturity. The shedding of leaves and mature fruits occur during the short rains between September and November. The shedding of leaves is normally followed by the formation of leaf buds, leaves and later flower buds in December (in Mt. Kenya and Nyambene hills) or in November (in Lake Victoria and Mt. Elgon). Flower longevity varied considerably between the *Vitex* populations. The flowers are ready for pollination between January and March in Mt. Kenya and Nyambene hills with the peak season at the end of February. But the floral longevity of each individual flower varied between 15 and 25 days, before wilting, drying and dropping of the corolla. In Lake Victoria and Mt. Elgon regions flowering occurs between December and February with the peak mid January (Figure 3.1 & 3.2). The floral longevity tended to be shorter in the Lake Victoria and Mt. Elgon *Vitex* populations (10 to 20 days). The duration of flower persistence on the plant after pollination appeared to depend on the prevailing climatic conditions (temperature and humidity). Similar observations were also made by Primack (1980). The flower longevity could contribute to the relatively prolonged duration of flower anthesis, increasing the chances of pollination.

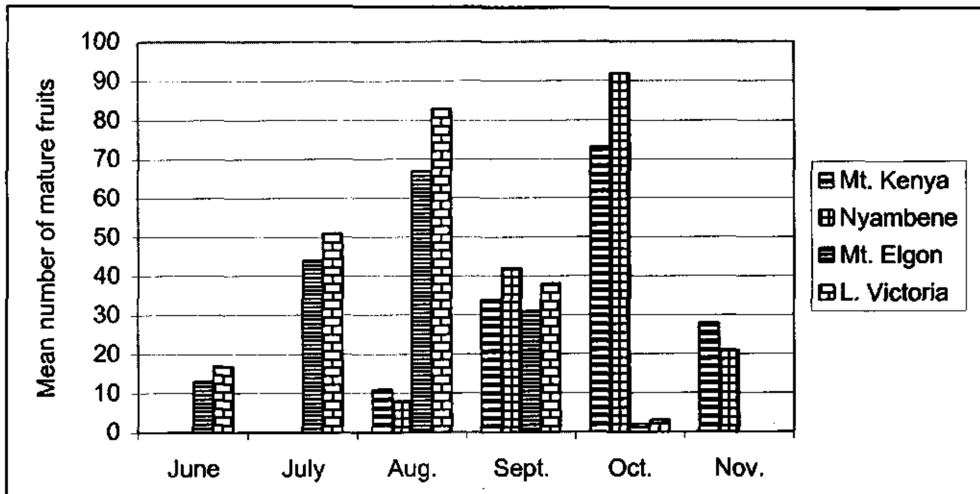


Figure 3.2. Fruit phenology of populations of *V. keniensis* and *V. fischeri.*

The nectar of the tree species is produced at the bottom of the flower and the production of nectar persists for about 8 to 10 days. The prolonged duration of nectar production may be intended to attract multiple visits from the pollinators mainly *Apis mellifera* L. (bees) thus increasing the chances of pollination. The stigma becomes sticky at the receptive stage which more or less coincides with pollen dehiscence, implying homogamy, an adaptation considered to promote selfing. Flowers are mostly shed and pollination complete before the onset of the long rains which start in early April. The rain dislodges the shrivelled corolla of the pollinated flowers leaving developing fruits. The late flowers are also damaged by the rains and the winds that accompany them.

In Mt. Kenya and Nyambene hills the fruits mature between September and November, while in Lake Victoria and Mt. Elgon they were ready earlier, that is from July to September. The ripe pulpy fruits fall to the forest floor together with dry leaves from the crowns. The fruits are mostly dispersed by hornbill birds, monkeys and even human beings who feed on the sweet pulp and discard the nuts containing seeds. The fruit and leaf fall is enhanced by the wet (rainy) and windy conditions during that period. The extracted seeds germinate readily and develop into trees especially in areas where the forest is opened up. The fruits that fall by gravity directly to the forest floor without having been depulped get attacked heavily by pests and fungi lowering the chances of germination. This interferes with the natural regeneration of the species in the forest. Most of these predators are attracted by the sweet (nutritious) pulp covering the nut. However, the predation by insects and fungi was less severe in the wet savanna populations of Lake Victoria and Mt. Elgon. That could be attributed to the sparse crown cover and thus, the exposure of the forest floor.

The morphology of flowers of the different *Vitex* populations showed no marked distinction between *V. fischeri* and *V. keniensis*. The phenological activities of the different populations were similar. However, the timing and the duration tended to follow the pattern of the environmental conditions. Therefore the long period between flowering and fruit maturity observed in *Vitex* of Mt. Kenya and Nyambene hills may be attributed to the cool environment caused by the high altitude and rainfall in the region. The hermaphrodity and homogamy of the flowers indicate the possibility of selfing in the plant species. The colourful corolla, sweet scent and sticky pollen grains is an indication that the species is zoophilous and is pollinated mainly by bees (*A. mellifera*). That was implied from the presence of pollen grains on the bees trapped from the species' flowers. There was also a conspicuous increase in the population of the insect when the *Vitex* populations are on flower. The fruits of the tree species are pulpy (drupes) and turn purple-black at maturity. In Mt. Kenya region the fruits are sold in markets for human consumption at a small scale, nevertheless contributing to the nutrition of the local population.

3.3 Reproductive biology of *V. keniensis* and *V. fischeri*

In the first attempts to execute controlled pollination from January to March 1996, most of the flowers aborted. That could be attributed to unusually dry weather conditions during that year. The experiments were repeated in 1997 and 1998. Prior to the pollination experiments to discern the species mating systems, the stage for optimum pollen viability and stigma receptivity were assessed in each of the *Vitex* populations. This is because pollen viability and stigma receptivity affect the success of pollination and the subsequent fertilisation process and seeding. The studies were also carried out to detect temporal separation of the sexual stages, if any.

3.3.1 Viability assessment of *V. keniensis* and *V. fischeri* pollen grains

Materials and methods

The viability of pollen grains of the species was tested at different stages of flower development from the onset of anthesis at the beginning of flowering season. Two methods of analysis were applied on the pollen viability assessment; staining by aniline blue and by tetrazolium salt (Dafni, 1992). The tests on pollen viability were crucial in ensuring that the pollen grains used in artificial pollination experiments were viable.

Five trees were marked in each of the four *Vitex* populations and 20 flowers of each sampled tree tagged at the onset of blooming. Pollen grains were collected using a fine brush and their viability was tested daily by staining, starting from just before anthesis for up to 20 days when the flowers wilted and the corolla and stamens are shed. The viability of pollen grains were tested by the two staining methods as a precaution against over- or under-estimation.

Aniline blue test on the viability of *Vitex* pollen grains

1. Samples of pollen grains taken at different stages of flower development were put on a glass slide and a drop of 1% aniline blue was added.
2. The pollen sample was covered with a glass cover slip.
3. The dye solution was replaced with water after 5 minutes of staining.
4. The pollen grains were put under a microscope at x40, the stained pollen counted, and the % of the stained pollen was considered as a measure of its viability.

Tetrazolium tests on viability of *Vitex* pollen grains. This was done by the method of Heslop-Harrison et al. (1984) and Stanley & Linkens (1974).

1. Samples of pollen grains were put in a drop of 0.5%, 2,3,5-triphenyl tetrazolium chloride in 35% sucrose solution and covered immediately to exclude oxygen, which can inhibit dye reduction.
2. The preparation was kept at 50°C in the dark for 2 hours.
3. The red-coloured pollen grains which were at the central area of the sample were counted under a microscope.
4. The proportion of the stained pollen was determined and converted into %, and taken as a measure of the pollen viability.

Table 3.1. Analysis of Variance on Pollen Viability of *Vitex* populations of Mt. Kenya, Nyambene hills, Mt. Elgon and L. Victoria (* denotes significance at $p < 5\%$ and ns non-significance).

Source of Variation	DF	MS	F
Staining Methods	1	5916.72	132.21 *
Vitex populations	3	344.34	7.69 *
Residual	3	44.75	

Table 3.2. Means of the viability of pollen grains of the *Vitex* populations from Nyambene hills, Mt. Kenya, Mt. Elgon and L. Victoria (means denoted by the same letter are not significantly different at $p < 5\%$ significance level according to Tukey Test).

Vitex Population	Mean staining % of pollen grains	
	Aniline blue	Tetrazolium salt
Mt. Kenya	28.810**ab**	18.381**ab**
Nyambene hills	24.476**a**	14.381**a**
Mt. Elgon	32.857**b**	18.286**ab**
Lake Victoria	31.619**b**	19.238**b**

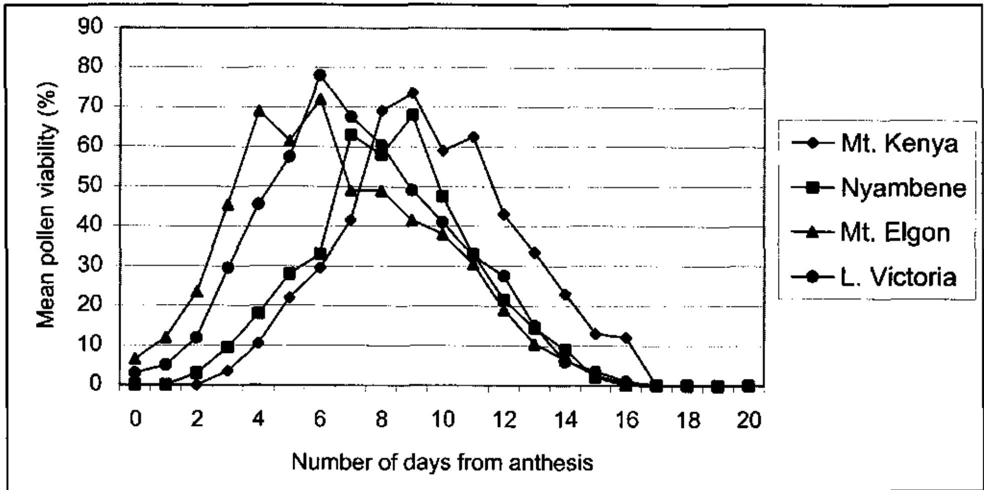


Figure 3.3. Trends in the mean pollen viability of *V. keniensis* and *V. fischeri* populations.

Results and discussions

The pollen staining methods differed significantly ($P < 0.05$) from each other. The pollen stainability by tetrazolium salt was lower than that of aniline blue. However, the difference was relatively constant in most samples. The observations indicate the difference in the sensitivity of the two methods in assessing pollen grain viability.

The two methods have their strengths and weaknesses; the advantages of using of aniline blue are that it is easy to handle and it allows large samples to be analyzed, but in some cases it may show low correlation to the real germination level *in vivo*; the use of tetrazolium tests is rapid and correlates fairly well with pollen viability and is also suitable for analysing large samples, but the set-back is that desiccated pollen grains in some instances may give false-positive results (Dafni, 1992). The use of the two pollen viability testing techniques was a cautionary measure against drawing inaccurate conclusions. The average of the two methods was therefore considered to reflect the estimates of the pollen viability level of pollen grains of *Vitex*. The four *Vitex* populations differed significantly ($p < 0.05$) in their pollen viability (Table 3.1).

Generally, from the mean pollen viability test results (Table 3.2) the pattern of variation was somewhat consistent with the ecological range of the *Vitex* populations. Nyambene hills did not differ ($P < 0.05$) significantly from Mt. Kenya. The same was observed between Mt. Elgon and Lake Victoria populations. Mt. Elgon *Vitex* had the highest pollen grain viability and they attained maturity much faster than the other populations (Figure 3.3). The slow pollen maturation and lower pollen viability in the Nyambene hills and Mt. Kenya could be attributed to the mild temperatures and wet conditions in the habitats of the two *Vitex* populations. Pollen grains from Lake

Victoria had a relatively high viability level but the loss in viability was quite rapid after the peak. This is probably because of the high temperatures and relatively high humidity from the Lake. The variation of the level of pollen viability and maturation between the different populations of *Vitex* is a possible indication of the role of environmental conditions. Similar observations have also been made by Johri & Vasil (1961), Stanley & Linskens (1974) and Stephenson et al. (1992) on the influence of the prevailing temperature and humidity on the competence of pollen.

Mt. Kenya and Nyambene hills *Vitex* reach optimum pollen maturity 9 days after anthesis while in Lake Victoria and Mt. Elgon it takes place 6 days after anthesis. In all cases, pollen grains lost their viability in about 17 days. Therefore, for higher vigour of *Vitex* pollen grains, it is necessary to collect them 9 days after anthesis in Mt. Kenya and Nyambene hills but 6 days after anthesis for Lake Victoria and Mt. Elgon. Further research is needed to determine the storage life of the pollen grains to enhance the species' *ex-situ* conservation and breeding programmes.

3.3.2 Evaluation of stigma receptivity of *V. keniensis* and *V. fischeri*

Stigma receptivity is a crucial stage in the maturation of the flower which may greatly influence the rate of self-pollination and pollination success at different stages in the flower life cycle (Galen et al., 1987). Therefore any success in breeding experiments or artificial pollination procedure should be accompanied by tests on timing and duration of stigma receptivity. Stigma receptivity can be determined experimentally by examining morphological changes such as colour and presence of exudate, determination of pollen germination or tube growth or fruit set after pollination at different times relative to the flower opening and by staining or testing for esterase presence (Dafni, 1992).

The assessment of stigma receptivity in *Vitex* was carried out *in-vivo* by artificial pollination of flowers at different stages of development. Flowers of sampled trees were emasculated, hand-pollinated at different stages of development (starting just before anthesis to wilting stage) and bagged to isolate the flowers from foreign pollen grains. That was expected to determine the optimum flower age in the different *Vitex* populations for the subsequent artificial-pollination experiments.

Materials and methods

1. Ten trees were randomly sampled in each of the four *Vitex* populations at the beginning of flowering season.
2. 20 flowers were marked and tagged on each sampled tree.
3. The flowers were then emasculated and hand-pollinated at an interval of one day (starting from onset of anthesis) with viability tested samples of pollen grains.
4. The number of fruits resulting from each day's treatment was considered an estimate of stigma receptivity.

Table 3.3. Analysis of variance on stigma receptivity of *V. keniensis* and *V. fischeri* (* denotes significance at 5% level).

Source of variation	DF	MS	F
Population	3	42.47	6.02*
Tree	9	2.68	0.38*
Residual	27	7.05	

Table 3.4. Means of the number of fruits formed by the *V. keniensis* and *V. fischeri* populations following hand-pollination.

Vitex Population	Mean number of fruits formed
Mt. Kenya	4.181b
Nyambene hills	4.490b
Mt. Elgon	3.814a
Lake Victoria	3.452a

Results and discussions

The number of fruits formed was assumed to indicate the level of stigma receptivity. The four populations differed ($P < 0.05$) significantly from one another in their stigma receptivity (Table 3.3). There was also a significant difference between trees in each of the populations.The observation could be attributed to the genetic make-up of the individual trees or micro-environmental variation.

The stigma receptivity level was higher in the flowers of Nyambene hills and Mt. Kenya populations and lower in Mt. Elgon and Lake Victoria populations (Table 3.4). This is contrary to their pollen viability levels. However, the stigmas of Lake Victoria and Mt. Elgon *Vitex* populations became receptive earlier, reaching the peak 6 days after anthesis, while the Nyambene hills and Mt. Kenya populations reach maximum stigma receptivity 10 days after anthesis (Figure 3.4). The rapid stigma development and decline in receptivity after the peak in the Lake Victoria and Mt. Elgon populations could be attributed to higher temperatures in the two regions. The delayed stigma maturation in Nyambene hills and Mt. Kenya *Vitex* populations may be a result of the lower temperatures and high humidity. The stigma receptivity in Lake Victoria and Mt. Elgon *Vitex* is critical, the graph assuming a conical shape which declines rapidly after reaching the peak. The Mt. Kenya and Nyambene hills *Vitex* populations were more prolific, producing large number of fruits and Lake Victoria had the least, thus exhibiting the lowest reproductive capacity. This suggests that in *Vitex*, stigma receptivity has a major role in successful pollination and seed production. Therefore, despite exhibiting slow pollen maturation and low pollen viability, Mt. Kenya and Nyambene hills *Vitex* produced the highest number of fruits. The observed significant differences within the *Vitex* populations in stigma receptivity was possibly caused by the differences in the ecological zones by the different populations of the species.

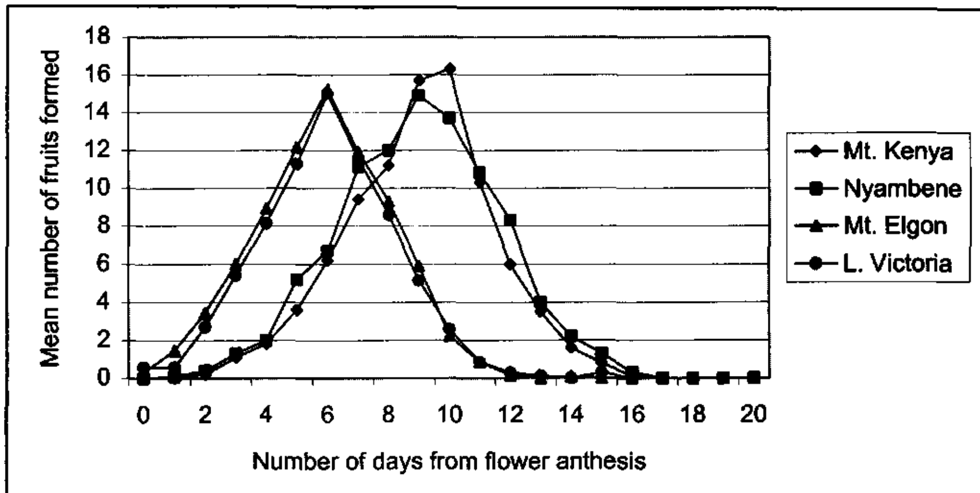


Figure 3.4. Trends in stigma receptivity in *V. keniensis* and *V. fischeri* populations.

3.3.3 Pollination mechanisms and self-compatibility in *V. keniensis* and *V. fischeri*

Materials and methods

Pollination experiments were carried out to investigate mating parameters of the *Vitex* populations. The methods of Dayanandan et al. (1990) was applied, but with a few modifications. I used numbered weather-resistant tags and exclusion bags made of perforated plastic material. Ten *Vitex* trees were sampled per population and 20 flowers were tagged for each treatment on each tree. The flowers were marked at anthesis stage and controlled pollination carried out 5 to 7 days later for Mt. Elgon and Lake Victoria populations but after 8 to 10 days in Mt. Kenya and Nyambene hills as recommended in the previous pollen viability and stigma receptivity assessments. A large isolation distance of at least 1 km was between the randomly sampled trees was maintained to avoid biparental inbreeding associated with outcrossing of closely spaced individuals and to ensure coverage of the species range.

Hand-pollination treatments on of the *Vitex* flowers

1. **NEO** Flowers non-emasculated and left open

The flowers were unmanipulated and acted as the control for comparison with the other treatments to detect the effects of emasculation and the species' reproductive capacity under natural conditions.

2. **NEB** Flowers non-emasculated and bagged

The flowers were unmanipulated but bagged to exclude foreign pollen sources. Fruit set in this treatment is an indication of apomixis or possibility of spontaneous self-pollination without pollinator.

3. **EB** Flowers emasculated and bagged

The anthers were removed by fine forceps. It is the best way to test for autonomous

apomixis (Richards, 1986). If seeds are produced without pollen, then pollen is not necessary and the seeds are apomictic. If no seeds are formed then either pollen grains are necessary or the flowers were so traumatized by the treatment that it aborted. The latter can be ruled out by comparison of seed set of the treatment with that of emasculated and crossed, and emasculated and selfed.

4. **NESB** Flowers non-emasculated, selfed and bagged
The flowers were unmanipulated but selfed and bagged. Comparison of fruit set with the other treatments gives an indication of the effects of emasculation on pollination.

5. **ESB** Flowers emasculated, selfed and bagged
The anthers were plucked out using fine forceps, the flowers selfed and bagged. The result from this treatment is to show the degree of self-compatibility or pseudogamy in the species. If seeds are set, then the species is either self-compatible or pseudogamous apomictic (Nybom, 1989; Campbell & Dickinson, 1990). However, lack of seed set is more difficult to interpret. It could also mean that the pollination technique was ineffective or the flowers were damaged by the treatment (emasculation), or that the flowers were self-incompatible. These possibilities can be tested by comparing the seed set from naturally open pollinated flowers (1) and emasculated and crossed treatment (7).

6. **NECB** Flowers non-emasculated, crossed and bagged
The flowers were unmanipulated, crossed and bagged to assess the effect of emasculation on fruit set, for comparison with other treatments.

7. **ECB** Flowers emasculated, crossed and bagged
The flowers had their anthers removed, crossed with a mixture of pollen from the different *Vitex* trees and bagged. The treatment was carried out to detect the degree of out-crossing in the species within the populations.

8. **EO** Flowers emasculated and left open.
The anthers were removed and the flowers left exposed to open pollination to assess if wind-pollination or cross-pollination by insects can occur.

Results and discussions

In the controlled pollination experiment, the different flower treatments varied significantly at $p < 0.05$. But there was no significant ($p < 0.05$) difference within and even between the *Vitex* populations (Table 3.5). This is a possible indication that the studied *Vitex* populations had a similar mode of pollination besides sharing similar floral morphology. The number of fruits formed varied from one treatment to another (Table 3.6).

The species produced fruits by both self- and cross-pollination. The summary of the assessments on the degree of each of the two modes of pollination are presented in the next section (Table 3.7). The mixed mating system in the species is evident from the formation of fruits by flowers that were emasculated, selfed and bagged produced fruits as well as the ones that were emasculated, crossed and bagged. The amount of fruits formed by flowers which were emasculated and bagged was negligible; an

indication of the need for pollen for fertilisation and possibly the absence of apomixis in the species. The few seeds formed in that treatment could have been from contamination of the flowers before the treatment. The tree species showed high seed production under the natural open pollination conditions, a characteristic commonly associated with self-compatible and self-pollinated plant species (Dafni, 1992). However, it is worth noting that bagging treatments do increase the temperature, thus can negatively affecting pollination of flowers.

Table 3.5. Analysis of variance of controlled pollination on *V. keniensis and V. fischeri* (* denotes significance at $p < 5\%$ level and **ns** non-significance).

Source of Variation	DF	MS	F
Treatment	7	726.81	166.77*
Population	3	4.18	0.96**ns**
Pop*Treat	21	1.72	0.39**ns**
Pop*Tree	27	3.93	0.90**ns**
Residual	261	4.36	

Table 3.6. Means of the number of fruits formed out of 20 flowers (per tree in each population) following different the hand-pollination treatments on *V. keniensis and V. fischeri* (Means denoted by the same letter are not significantly different at 5% significance level according to Tukey's Test).

Flower Treatment	Mean no. of fruits formed after hand pollination of 20 flowers per treatment.
NEO (Control)	14.425**e**
NEB	3.850**cd**
EB	0.050**a**
NESB	4.500**d**
ESB	4.125**cd**
NECB	4.100**cd**
ECB	2.950**bc**
EO	2.100**b**

The success in pollination and fruit formation in the non-emasculated treatments in the four *Vitex* populations showed a similar trend and decreased in the order of open, selfed, crossed and bagged treatments (Figure 3.5). The fruit set in non-emasculated and bagged treatments, suggested that pollination could occur in the species' flowers passively, without intervention of pollinators possibly by gravity or direct contact between the stigma and anther. In the flowers, the anthers are above the stigma so there is a possibility of the pollen being dislodged by gravity at maturity and deposited

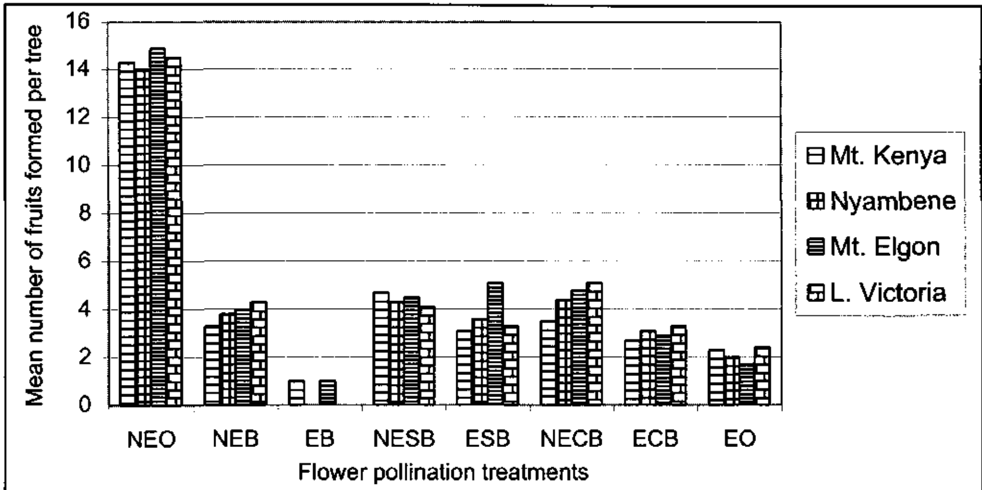


Figure 3.5. Effects of the different hand-pollination treatments on the production of fruits in the *V. keniensis* and *V. fischeri* populations.

on the stigma. Fruit-set from non-emasculated treatments was higher than the emasculated ones, indicating a possible negative influence of flower emasculation on fruiting through mechanical injury. Since most insect pollinators visit flowers for pollen grains, they tend to avoid emasculated flowers (Cruden et al., 1989; Young & Stanton, 1990; Donnelly, 1998). This can also lead to the low number of fruits among the flowers that were emasculated and left open. In some cases the stigma and style may function abnormally following emasculation thus leading to formation of lower numbers of fruits (Guo et al., 1990).

3.3.4 Estimates of the mating parameters of *V. keniensis* and *V. fischeri* populations

The data from the previous hand-pollination experiments were used for estimating the species' mating parameters, such as Self-incompatibility (ISI), Selfing rates (S) and Relative Reproductive Success (RRS).

Self-incompatibility in the *V. keniensis* and *V. fischeri* populations was estimated by the method of Zapata & Arroyo (1978). In this method, self-incompatibility is estimated as proportion of fruits formed by cross-pollination to those from self-pollination. According to the method, the values of the self-incompatibility reflect the following possibilities:

> 1 = self-compatible
$> 0.2 < 1$ = partially self-incompatible
< 0.2 = mostly self-incompatible
0 = completely self-incompatible

Based on the categorisation by Zapta & Arroyo (1978), the four *V. keniensis* and *V. fischeri* populations are partially self-incompatible (Table 3.7). However, in nature selfing rates may be controlled by other adaptive features such as dichogamy. If self-compatibility rates are genetically controlled as observed by Dafni (1992), then the similar levels of partial self-incompatibility in the different *Vitex* populations could imply their taxonomic or genetic similarity.

Table 3.7. The level of self-incompatibility in the *Vitex* populations.

Population	No. selfed fruits formed	No. crossed fruits formed	Self-incompatibility levels
Mt. Kenya	34	27	0.8
Nyambene hills	39	29	0.7
Mt. Elgon	52	28	0.5
Lake Victoria	40	33	0.8

According to Richards (1986) most polyploids tend to suffer from inability to reproduce sexually and establish, unless they are self-fertile. That may be one of the explanations for the observed partial self-incompatibility in *Vitex*. Self-compatibility has proved more successful in polyploids than in diploids, perhaps due to the greater capacity of polyploids for storing variability, and releasing it more slowly, when selfed (Richards, 1986). This phenomenon makes polyploids more capable of tolerating more selfing than in diploids.

However, in spite of self-compatibility being a desirable asset in a number improvement breeding strategies, in several instances selfing is associated with inbreeding depression. Therefore, the next section presents the evaluation of the vigour of the seeds (offspring) from self- and cross-pollination by assessing and comparing their germination levels.

3.3.5 Comparison of the germination vigour of seeds from cross- and self-pollination of *V. keniensis* and *V. fischeri*

The experiment was carried out as measure of vigour of offspring resulting from the different pollination treatments.

Materials and methods

1. Seeds from open-, cross- and self-pollination were processed, labelled and kept separate per population.
2. The seeds were sown in vermiculite in 4 replicates of 50, kept moist by watering and put in an incubator set at 25°C.
3. Their germination was recorded daily until no more seeds were germinating.
4. The results were summarised and expressed in %.

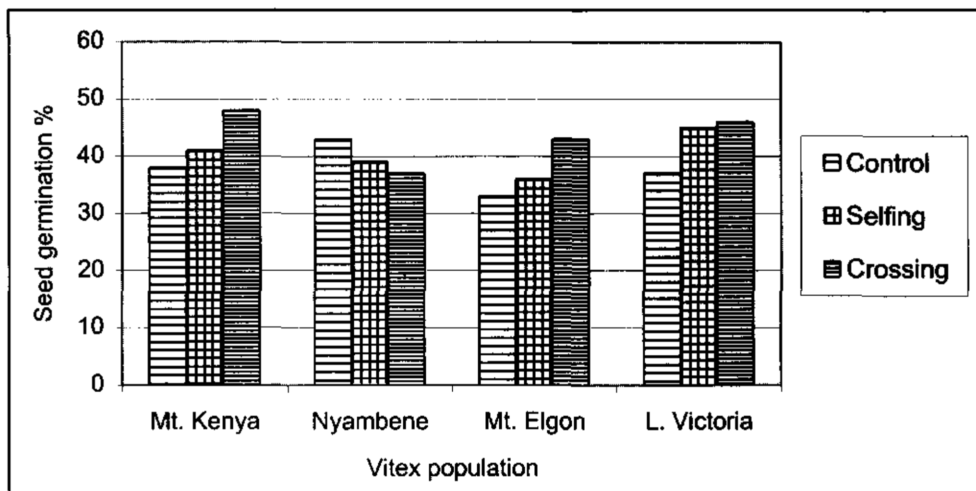


Figure 3.6. Germination vigour of *V. keniensis* and *V. fischeri* seeds from open-, self- and cross-pollination.

Results and discussions

The average germination of the species' seeds is normally ranging from 25 to 50% (Rhode, 1986) and it has been attributed to dormancy imposed by the hard nut on the seed. Therefore, the low germination recorded is normal for the tree species. However, in general seeds from cross-pollination had higher germination than those from open- and self-pollination except in Nyambene hills (Figure 3.6). Contrary to my expectation seeds from open pollination showed lower mean germination than those from self-pollination. The seeds from cross-pollination exhibited slightly higher mean germination than the other two modes of pollination. However, the difference in seed germination among the *Vitex* populations was marginal. The different *Vitex* populations did not differ much in the degree of loss in seed germination capacity among their selfed offsprings possibly indicating a low degree of inbreeding depression. The mild inbreeding depression could be due to the high polyploidy level of the species, $2n=96$. The phenomenon has also been observed by Richards (1986). Therefore, it seems the *Vitex* trees can also be selfed without the risks of loss in seed set and germination capacity. According to Curtis (1999), species exhibiting high, persistent inbreeding depression is unlikely to evolve towards increased self-fertility. Therefore, the self-compatibility in *Vitex* has possibly been maintained by the species' ability to tolerate or buffer inbreeding depression.

Further research is necessary to assess the effects of selfing on other desirable traits of the species. This is because although inbreeding depression was not detected in the study in seed set and germination, it is essential to sample indices of fitness throughout the life cycle of the progenies. Inbreeding depression may be detected at a later stage (e.g. Belaoussoff & Shore, 1995). In some cases inbreeding may not be detectable unless the plants are subjected to environmental stress (Eckert & Barrett, 1994).

Table 3.8. The degree of selfing rates (S) in *V. keniensis and V. fischeri* populations. The calculations were done according to Charlesworth & Charlesworth (1987).

Population	Fruits from cross-pollination	Fruits from open pollination	Fruits from self-pollination	Estimates of selfing rates
Mt. Kenya	27.00	143.00	34.00	16.57
Nyambene hills	29.00	140.00	39.00	11.10
Mt. Elgon	29.00	149.00	52.00	05.22
Lake Victoria	33.00	145.00	40.00	16.00

Estimates of selfing rates (S) in *V. keniensis* and *V. fischeri* were calculated according to the method by Charlesworth & Charlesworth (1987). Self-pollination rate is a criterion used to assess the fate of pollen on the stigma of the same flower or plant. A plant species may be self-compatible but exhibit low selfing rates due to timing events in the flowers, its structure and pollinator's behaviour. Therefore, selfing rates need to be evaluated besides the degree of self-compatibility.

The number of seeds from the different modes of pollination were compared according to Charlesworth & Charlesworth (1987) in estimating selfing rates in the various *Vitex* populations. Selfing rate is an important criterion in evaluating the fate of pollen on the stigma of the same flower of a plant. The method makes it possible to estimate the rate of selfing of a species in natural conditions (Dafni, 1992).

Vitex in Mt. Elgon showed the lowest selfing rates while those of Mt. Kenya and Lake Victoria the highest (Table 3.8). That implied that populations with high self-compatibility may not necessarily have high selfing rates. It supported the observation made by Dafni (1992) that a plant may be highly self-compatible but has low chances of self-pollination. So there may be a range of factors responsible for the low selfing rates observed in Mt. Elgon populations such as climatic conditions.

Estimates of Relative Reproductive Success (RRS) in the *Vitex* populations were done according to Stanton & Preston (1988). The estimates of relative reproductive success were as follows; Mt. Kenya had 0.179, Nyambene hills 0.175, Mt. Elgon 0,181 and Lake Victoria 0.186. These values are relatively low because out of the four ovules in the ovary of *Vitex* only one of them gets fertilised to form viable seed. The reason for this is not yet established but it could be a result of infra-fruit competition arising from resource limitation, thus influencing the final number of seeds per fruit and even the spacing of seeds in the fruit (Stanton & Preston, 1988). According to Stephenson et al. (1992) such selective abortion does improve the quality of the few seeds produced. The average relative reproductive success in *Vitex* is about 0.183. In nature, the species flowers and fruits profusely, that could be in response to the need to compensate for the low relative reproductive success.

3.3.6 Reproductive compatibility between the *V. keniensis* and *V. fischeri* populations and the vigour of the resultant seeds

The four *V. keniensis* and *V. fischeri* populations were crossed and the germination of the resultant seeds tested. The experiment was designed to determine the reproductive compatibility between the different *Vitex* populations and the vigour (germination) of the resultant seeds. The results were compared with the number of fruits formed from cross- and self-pollination. The study aimed at finding out the possibilities of outcrossing the different *Vitex* populations for possible improvement breeding work in the future and to detect if there is reproductive barrier between them.

Materials and methods

Ten trees in each of the four populations were selected and 10 flowers on each of them used per treatment. The flowers were emasculated and artificially pollinated by viability tested pollen from other populations (crossed) and bagged to avoid contamination from foreign pollen grains. The controls were marked and left to open pollination. The fruit development was monitored and harvested at maturity.

All the fruits obtained from the different treatments were extracted, the seeds processed, labelled and sown in vermiculite in four replicates. This is because the number of seeds obtained from the hand-pollination was not enough for four replicates of at least 25 seeds (ISTA, 1993). The seeds were sown in vermiculite and put in a germination cabinet set at 25°C and moistened with water. Germination was monitored and recorded daily until no more seeds were germinating and the mean germination % were calculated for each seed lot (Table 3.9).

Table 3.9. Number of fruits formed from open-pollination (*) and cross-pollination of the *V. keniensis* and *V. fischeri* populations and their respective seed germination percentage. A: Number of fruits formed; B: Seed Germination %.

Female / Male	Mt. Kenya		Nyambene		Mt. Elgon		L. Victoria	
	A	B	A	B	A	B	A	B
Mt. Kenya	72*	35%*	55	32%	48	14%	32	20%
Nyambene	53	26%	75*	39%*	50	19%	44	12%
Mt.Elgon	43	12%	34	11%	70*	24%*	56	17%
L.Victoria	26	9%	28	8%	39	13%	73*	47%*

Results and discussions

The results show that it is possible to cross the different *Vitex* populations and obtain viable seeds. More fruits and higher seed germination rates were recorded from the controls (open-pollination) (Table 3.9). The higher number of fruits from the control was probably because they were not subjected to mechanical damage by emasculation. According to Stuessy (1990) the more robust and fertile the hybrids, the closer will be

their genetic relationship. The observed germination of the out-crossed seeds is therefore a possible indication of close taxonomic or genetic relationship between the different *Vitex* populations. However, the long time scale involved in raising the tree species could not allow further assessment of offspring survival, performance and fertility upon maturation. However, it is necessary to monitor the performance of the progenies throughout a complete life cycle. That is because sometimes inbreeding depression may set in at a later stage of development or when they are faced with a stressful environment.

3.4 Conclusion

The similarity in the floral morphology between the *Vitex* populations could be a reflection of their common ancestry and lack of taxonomic distinction between them. They also exhibited similar sequence in their phenology although the timing and duration differed according to the ecological zones. That is a possible indication that the phenological pattern of the *Vitex* is influenced by the environmental conditions.

The differences in pollen viability and stigma receptive among the populations also tended to match the ecological and geographical distribution patterns. The phenomenon could be attributed to the influence of temperature and moisture on the pollen viability and longevity. The pollen viability is normally sensitive to environmental conditions especially temperature and moisture (Vogler et al., 1999). According to Vogler et al. (1999) also found that hot environments led to high pollen viability. Therefore the high pollen viability in Lake Victoria and Mt. Elgon *Vitex* could be a result of the relatively high temperatures in these regions. This is an indication that pollen viability in *Vitex* is affected by the prevailing environmental conditions.

The tree species has a mixed mating system. The partial self-incompatibility observed in the *Vitex* populations is possibly an adaptation to maintain fecundity even when pollinator availability is unpredictable. Pollinator-limited seed production is often presumed to favour evolution of self-compatible from self-incompatible ancestors (Wyatt, 1983, 1984; Dafni & Bernhardt, 1990; Weisler & Snow, 1992). Since *Vitex* is capable of producing seeds both by selfing and crossing. The level of selfing and crossing would depend on other factors such as population density and the availability of pollinator. Therefore, reduction in its population size and density is likely to promote selfing and inbreeding. Over time this can lead to reduction in genetic variation, inbreeding depression and reduced adaptability of its populations to environmental conditions.

Biparental inbreeding is also common where seed dispersal is limited. *Vitex* seeds are normally dispersed by monkeys, hornbill birds and to a limited extent humans. Continued exploitation of the forests might lead to destruction of the habitat and decline in the populations of seed dispersal agents. Over time, biparental inbreeding also induces the evolution of selfing in previously outcrossing species (Lloyd, 1979;

Uyenoyama, 1986). Outcrossing mating systems are common in most tropical tree species (Loveless, 1992; Bawa, 1992). The partial self-compatibility in *Vitex* could be arising from biparental breeding as a result of the decline in its population, seed dispersal agents and pollinators.

Selfing can evolve repeatedly in out-crossing taxa, and theory predicts that increases in the level of self-fertilisation occur in concert with changes in reproductive allocation and the magnitude of inbreeding depression (Parker et al., 1995). The results from fitness in consequence of selfing and crossing indicated some low degree of vigour loss in terms of seed production and germination levels. The apparent mild consequences of selfing, that is inbreeding depression in *Vitex*, could be attributed to its polyploidy and high heterozygosity. This concurs with the observation made by Lande & Schemske (1985) that diploids exhibit higher inbreeding depression than tetraploids (Richards, 1986). The phenomenon can enable *Vitex* to withstand the negative effects of inbreeding for some generations.

The possibility to cross the different *Vitex* populations is a reflection of genetic compatibility in a particular evolutionary line (Stuessy, 1985). This implies that the different *Vitex* populations have not yet undergone reproductive isolation. The high degree of reproductive compatibility between the *Vitex* populations is therefore a further indication of the close similarity between them in addition to their morphological traits. The inter-population reproductive compatibility means that desirable traits can easily be transferred from one population to another through a breeding programme.

4 The genetic structure of *V. keniensis* and *V. fischeri* populations

4.1 Introduction

In developing sampling strategies for conservation efforts or genetic improvement of tree species, it is useful to take into account overall geographic patterns of genetic differentiation. The geographical distribution of genotypes constitutes the spatial structure of a population revealing pockets of diversity (Boshier et al., 1995; Kuser et al. 1997). The genetic structure is influenced by and influences all aspects of species' population genetics such as mating system, seed and pollen dispersal, and natural selection (Epperson, 1992). The knowledge of the structure of genetic variation is vital in understanding the population genetics of a species as well as for the practical application of the knowledge in the sustained management of production forests (Finegan, 1992). It can also help us to understand the historical processes leading to the genetic diversity of a species (Sheely & Meagher, 1996). The value of data on genetic variation for conservation has gained increasing recognition (Falk & Holsinger, 1991). Large scale deforestation can lead to reduction in effective population size and genetic isolation of fragmented populations. Maintenance of the genetic diversity is considered crucial for long-term survival and evolutionary response of populations to changes in the environment (Hueneke, 1991) thus preserving its evolutionary potential. Taxonomy also plays a critical role in conservation since only formally named entities can be afforded legal protection (Avise, 1994). Taxonomic uniqueness has also been promoted as a factor leading to priority conservation status. Therefore, understanding genetic relationships of taxa may help define taxonomic entities and determine their conservation priorities (Stewart et al., 1996).

A common consequence of human interventions in plant populations is mostly fragmentation during exploitation, or clearing for other land uses. Depending on the scale and duration, these interventions could reduce genetic diversity. In recent years, information pertaining to patterns of genetic variation in forest tree taxa has increased (Hamrick & Godt, 1989; Hamrick et al., 1992). The upsurge can be attributed to several factors, among others, to the growing awareness of the role of forestry in biodiversity conservation (Williams, 1991), the need to evaluate genetic diversity before and after management interventions (e.g. Bawa & Krugman, 1991; Salvonainen & Karkkainen, 1992), the desire to understand genomic organisation for tree improvement programmes (Neale & Williams, 1991), and the availability of new and refined techniques for gathering this information (Cheliak & Rogers, 1988). Although there has always been the need to define patterns of forest tree populations for better exploitation and management, it is the current refined assay techniques that have revolutionised the investigations, reducing the time required for analyses and decision making.

The use of markers in plant population biology is increasing and the knowledge about their applications, choices of techniques and data analysis for molecular markers has become an important issue (Vekemans & Jacquemart, 1997). Molecular markers such as allozyme loci have been used in a wide array of applications in the field of plant biology such as population genetic structure, genetic diversity of natural populations and breeding systems. In the context of conservation management, molecular markers have been used gather genetic information on endangered plant species to aid sampling for ex-situ conservation (Schoen & Brown, 1995). Other application of molecular markers include phylogenetic analysis (Swofford et al. 1996), ecological genetics (Azzouzi et al., 1997), detection of hybridisation (Heinze, 1997) and cultivar identification (Hillis, 1996). The discovery that genes code for proteins, combined with the development of the techniques of protein electrophoresis, provided means of estimating genetic variation in natural populations (Crawford, 1990). These analyses have investigated various species, among them some forest tree species (e.g. Hamrick et al., 1992; Moran, 1992; Zimniak-Prybylska, 1995).

A number of other techniques have been developed to evaluate the genetic characteristics of plant populations (Crawford, 1990; Murphy et al., 1990; Neale & Williams, 1991). The choice of the technique depends on the aims of the study, the sensitivity and convenience of the technique, and availability of resources. Currently, these evaluations are implemented using methodologies including DNA (deoxyribonucleic acid) sequencing, gene mapping, restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPDs), and characterisation of seed storage proteins and specific proteins, including isozymes.

DNA sequencing is the most informative of all the molecular techniques, as it determines all the genetic variation (Crawford, 1990). However, the cost of DNA sequencing has limitations to its wider application. The use of DNA fragments, for example, RFLPs and RAPDs, have been developed as methods of intermediate cost (Neale & Williams, 1991; Krutovskii & Wagner, 1992). However, their costs are high compared to the isozymes, the use of radioisotopes (RFLPs), the limited number of probes (RFLPs), the long processing time (RFLPs) and the problems associated with dominant bands hence difficulty of scoring heterozygotes (RAPDs) make them less popular than isozymes (Neale & Williams, 1991). The isozyme technique is at the moment, therefore, one of the most cost-effective methodologies presently available for many purposes, and can be widely adopted for genetic analyses.

The distribution of genetic diversity of plant populations have been widely documented by means of allozyme markers for the last 20 years (Mahy & Neve, 1997). The studies on tropical forest tree species indicate that they maintain high levels of genetic diversity within populations, and are far less geographically differentiated than herbaceous plants (Hamrick et al., 1992). The ultimate goal of these investigations was to characterise *Vitex* populations in terms of their taxonomic status and genetic structure. Analyses of isozyme patterns of different taxa have proved to be of great value in establishing patterns of genetic variation, investigating evolutionary genetics,

and in the verification of crossings, hybridisation, introgression, ploidy and paternity (Adams, 1983; Murphy et al., 1990). Some of the characteristics that make isozyme markers appropriate for genetic studies were summarised by Brown & Allard (1970) and Brown et al. (1989).

Despite having been documented by many workers, gel electrophoresis has no universally ideal single system of assay, even within one species. A set of optimum conditions have to be established empirically, depending on the organism, tissue, enzyme system and, at times, the locus (Wendel & Weeden, 1989). However, whatever the source of variation is, polymorphism is useful as it reflects genetic control.

The principle of electrophoresis (isozymes) is based on the nature of amino acids. Amino acids have positive or negative charges but some are neutral, this leads to the differences in their mobility in an electric field. The variation in patterns is assumed to reflect amino acid substitution in an enzyme which leads to the difference in electrophoretic mobility on the supporting medium (Freifelder, 1976). Various supporting media such as paper, cellulose acetate, agarose, starch and polyacrylamide gels have been used (Feret & Bergmann, 1976; Freifelder, 1976; Wendel & Weeden, 1989; Murphy et al., 1990). Starch and polyacrylamide gels are widely employed (Freifelder, 1976). The electrophoretic data are gathered as isozymes and allozymes (Murphy et al., 1990), which are expressed as bands of different mobilities after staining. The bands reflect the allelic variation in the organism, they can be scored and interpreted to express genetic diversity (Gottlieb, 1981). To reduce loss of enzyme activities and protein denaturation, the tissue should be homogenized in a short time in an appropriate extraction buffer.

In this study, trees were sampled in the four *V. keniensis* and *V. fischeri* populations, the optimum electrophoretic assay conditions were determined by standardization of tissue homogenates, the buffer systems and staining requirements optimized with the aim of achieving comparable isozyme resolutions. The resultant isozyme profiles were used to elucidate the nature of polyploidy, segregation pattern, taxonomic status and genetic structure of the *Vitex* populations.

4.2 Analyses of the genetic structure of *V. keniensis* and *V. fischeri* populations

4.2.1 Sampling and isozyme analysis methods

The natural geographically isolated populations of *Vitex* in East Africa were identified and mapped (Figure 2.3). A hundred individual trees were randomly sampled and marked in each of the *Vitex* populations (Mt. Kenya, Nyambene hills, Mt. Elgon and Lake Victoria). A minimum distance of 250 m was maintained between the sampled trees with the aim of reducing the chance of assembling closely related individuals and to maximise the coverage or representation of the species' natural

population range. The appropriate sample size is still a debatable question in plant population analysis (Crawford, 1990; Bawa; 1992), but it is clear that the larger the sample size, the more accurate the estimated parameters.

Fifteen enzyme systems (Appendix 4.1) were first screened on *Vitex*. Then the optimum electrophoretic assay conditions of the best 8 were refined, which included standardisation of tissue homogenates, optimisation of buffer systems and staining requirements according to Wendel and Weeden (1989), with the aim of achieving comparable isozyme resolutions. Different tissues of *Vitex* such as seeds, seedlings (cotyledons and embryos), fresh leaves, roots and barks were tried out.

Homogenisation of samples

A complex extraction buffer of Tris-HCl (Wendel & Weeden, 1989) was used with some modification. The roles of buffer additives are explained by Kephart (1990). To minimise the variation arising from physiological and ontogenetic conditions, only materials at the same developmental stage were homogenised and used in the analyses. An extraction buffer (0.5 ml) was added to each sample and extraction performed in labelled microtubes over ice. Alternatively, microtubes with buffer were frozen at -4°C in a freezer prior to the addition of samples. The frozen buffer cooled the tissues and also helped in crushing before thawing. The plant materials (seeds, seedlings, leaves, bark and roots) were mechanically crushed with a tapered glass rod in the microtubes because grinding with chilled mortar and pestle was time-consuming and prone to contamination. Consequently, the use of a power homogeniser was inappropriate because of warming up. The glass rod was washed and rinsed with absorbent paper between samples to avoid contamination. The samples were loaded onto the gels and electrophoresis machine immediately. Delays or storage of the samples led to low enzyme activity and caused poor resolution. To avoid delays only homogenates enough for one day's work (i.e. 20 samples for 8 enzyme systems) were prepared. The homogenates were centrifuged at 1100 r.p.m. for 3 minutes at room temperatures (20-25°C). Otherwise longer centrifuging time led complete loss of activity of some enzymes such as ADH and ME and this could be attributed to warming of the homogenates in the process. The supernatant was decanted and a 10 μl used to load the gel. The 20 samples were loaded into the 20 wells of each gel which had been filled with the electrode (tank) buffer. Samples being denser than the buffer, they settled at the bottom of the wells.

The apparatus was then assembled and each tank run for 4 hours set at 500 volts (v) and 120 milliamperes (mA). Shorter running time led to lack of bands or poor separation of bands but longer duration allowed some bands to go overboard. The timing and constant voltage or current were maintained by a power supply (micro-computer electrophoretic power supply, model E 455). The temperature in the tank was maintained at 3°C by a multi-temperature thermostatic circulator (LLK Bromma 2219). The use of bromophenol blue to monitor mobility was discontinued because it coagulated some samples. The running time of 4 hours was found to be adequate for

most enzymes because it produced good band separation and allowed gel assembly, running, staining and recording within the same day. Therefore, the need for storing them overnight was eliminated, as storage had negative effects on enzymes like PGI and SDH. At the end other run, the gels were carefully removed and stained appropriately. Enzymes such as GOT, ADH, MR and DIA took shorter time to stain. Longer exposure of these enzymes to the stain led to over-staining and heavy streaking. The electrophoretic phenotypes were scored and recorded in specially designed zymographs. Furthermore, some of the gels were photographed.

Preparation of extraction buffer

Reagents	Quantity
ß-Mercaptoethanol	0.025 ml
EDTA	0.010 g
Potassium Chloride	0.019 g
Magnesium chloride	0.050 g
PVP 40 000	1.000 g
0.1M Tris-HCl	25.000 ml

Preparation of acrylamide gels for electrophoresis analyses

Stock solution:

1. Acrylamide stock solution: 63 ml of distilled water was added to acrylamide/bis-acrylamide stock in a bottle, shaken well, marked and kept in a fridge.
2. Ammonium persulphate solution was prepared by dissolving 50 mg ammonium persulphate in 10 ml distilled water. Ammonium persulphate was prepared and used when still fresh. Poor results were obtained when stored ammonium persulphate was used.

Preparation of gel buffer

The pH of the gel buffer was maintained at 8.4 by increasing (if low) with concentrated trizma base solution and reducing (if high) with dilute a (50%) hydrochloric acid solution.

Reagents	Quantity
Boric acid	0.09 ml of 5.56g/l
EDTA	0.0025 ml of 0.93g/l
Trizma base	0.09 ml of 10.89g/l

Preparation of acrylamide gels

Eight gels were prepared for each run. The acrylamide gels were prepared in the evening and used the following morning (day), allowing them time to form. The gels were prepared by mixing the solutions below:

Reagents	Quantity
Distilled water	48.00 ml
Gel buffer	224.00 ml
Acrylamide stock	88.00 ml
Ammonium persulphate	21.60 ml
Temed	0.72 ml

Preparation of tank buffers
For each tank 5 litres of the buffer was prepared. In 5 litres of distilled water, the following chemicals were added:

Reagents	Quantity
Boric acid	27.80 g
EDTA	4.65 g
Trizma base	54.45 g

A continuous buffer system of Tris-Borate-EDTA (Wendel & Weeden, 1989) was used. The pH was raised to 8.4 with trisma base (if low) and lowered with hydrochloric acid (if high). Polyacrylamide gels (16x18 cm) were prepared with a Hoeffer S.E. 600 apparatus for vertical electrophoresis. Using the accessory divider, four club gel sandwiches were put in each tank. Two tanks were used per run, with eight enzyme systems a total of twenty samples were screened per run (per day).

4.2.2 Staining procedures

The basic staining procedures for various enzymes for different taxa and tissues have been reviewed by Brewer & Singh (1970), Vallejos (1983) and Wendel & Weeden (1989). From these basic recipes, the optimum staining conditions was established for *Vitex* samples by manipulation of pH, substrate concentration and buffer ionic strength. Tris-HCl staining buffer was used throughout the study. The staining reagents used besides the enzyme specific substrates were; tetrazolium thiazolyl blue (MTT), diazonium salts (i.e. fast blue BB salt, fast blue RR salt), co-factors such as nicotinamide dinucleotide (NAD), ß-nicotinamide dinucleotide phosphate (ß-NADP), nicotinamide dinucleotide reduced (NADH), phenazine methosulfate (PMS), a catalyst for most assays with tetrazolium stains. Other than NADH, the frequently used reagents were retained refrigerated as aqueous solutions of 10 mg/ml and dispensed in 1 to 2 ml volumes. In order to mark the position of every sample during the staining procedure, a notch was cut at the top left hand corner of every gel next to the first specimen. A total of 15 enzymes were stained initially (Appendix 4.1), but only 8 showed consistently good resolution.

Results and discussions

The enzyme systems that exhibited superior resolution of banding patterns were ADH, DIA, GOT, ME, MR, 6-PGD, PGI and SDH (Table 4.1). They were then used in the studying genetic structure of the *Vitex* populations. The best isozyme polymorphisms were observed in cotyledons of germinating seeds (seedlings). That could be attributed to high metabolic rates and enzymatic activities in the developing seedlings. According to Smith (1981), cotyledons of seedlings are normally in an active state, mobilising resources for growth, thus they have higher levels of enzymes at that early stage of development. The use of germinating seeds also helped in avoiding dead or rotten seeds. The poor results from leaf samples could be attributed to the production of the photosynthetic substances associated with tannins, phenols and other inhibiting chemical by-products. Fresh leaves produce good bands with fewer enzymes (ADH, GOT, PGI and SDH) compared to seeds and germinating seeds. The activities were observed in leaves only when polyvinylpolypyrolidone (PVP 40 000) was included in the extraction buffer. That is likely to be a result of the ability of PVP 40 000 to render the inhibitors such as phenolic compounds in the samples ineffective. The barks and roots exhibited heavily streaked bands or no activity with the enzymes screened. It is possible that better results can be achieved from the remaining enzymes and tissues of *Vitex* if more screening is carried out, involving adjustment of extraction, staining and running conditions.

Table 4.1. Results from the trial screening of different enzymes in various *V. keniensis* and *V. fischeri* tissues. The staining protocol for each enzyme is presented in appendix 4.1.

Enzyme	Plant material				
	seeds	seedlings	leaves	roots	bark
ACP	*	*	**	*	**
ADH	+	++	++	*	*
DIA	+	++	**	*	**
α-EST	*	*	**	*	*
GOT	++	++	++	*	*
G-6-PD	*	*	*	*	*
GDH	*	+	*	*	*
LAP	*	*	*	*	*
MDH	*	*	**	**	*
ME	+	++	**	*	*
MR	+	++	**	*	*
6-PGD	+	++	*	*	*
PGI	+	++	++	*	*
PGM	*	+	*	*	*
SDH	+	++	++	*	*

* No reaction; ** Streaking;
+ Activity, faint bands; ++ Good, clear bands.

The analytical materials finally used in the study consisted of seeds from the 100 randomly selected trees in each of the four geographically isolated *Vitex* populations in Kenya, Uganda and Tanzania. Samples of mature fruits were collected from all over the crown, extracted by depulping, dried, cleaned and maintained separately for each of the selected trees. The processed seeds were maintained in cold storage at 4°C until they were required for isozyme analysis. The genetic variation within and between the *Vitex* populations was studied by sampling one seed from each of the 100 trees selected per population. Therefore a total of 400 trees were sampled in the four *Vitex* populations for the study.

4.2.3 Isozyme analyses on samples of germinating seeds of *V. keniensis* and *V. fischeri*

Materials and methods

1. The processed seeds from the 100 sampled trees per population were rinsed in distilled water to remove dust and empty, rotten seeds by flotation.
2. Single viable seeds from each of the sampled trees were sown in vermiculite in a polystyrene sheet in a glasshouse, making a total of 100 seed samples per population.
3. After 10 days, the radicle started emerging and the germinating seeds were removed, their cotyledons excised and digested in extraction buffer for isozyme analyses.

The 8 enzyme systems which gave good enzyme polymorphisms and the banding patterns that could be scored from the seedlings are listed in Table 4.2, while their staining schedules are summarised and presented in appendix 4.1. The staining was carried out in stainless steel trays with lids in an incubator to keep off light. This is because some of the staining reagents are photosensitive.

Table 4.2. Enzyme systems chosen and used in the analysis of *V. keniensis* and *V. fischeri* populations.

Enzyme	Abbreviation	Commission number
1. Alcohol dehydrogenase	ADH	1.1.1.1
2. Diaphorase	DIA	1.6.99.-
3. Gluterate oxaloacetate transaminase	GOT	2.6.1.1
4. Malic acid	ME	1.1.1.37
5. Manadione reductase	MR	1.6.99.-
6. 6-Phosphoglucose dehydrogenase	6-PGD	1.1.1.44
7. Phosphoglucose isomerase	PGI	5.3.1.9
8. Shikimate dehydrogenase	SDH	1.1.1.25

The staining intensity of the enzymes varied from one sample to another irrespective of the population. The response of different tissues to assay conditions was also reflected in the resolution of the enzymes. During tissue preparation, ME could not withstand longer centrifugation but MR could. When the tissues were stored at -4°C, MR, 6-PGD and PGI activities were easily lost while ME, ADH and GOT remained active and could be assayed the following day. The apparent differences in the activities of the enzymes could be reflecting the temperature ranges at which they function. The enzymes that were active in both leaves and seeds exhibited no noticeable differences in banding patterns between cotyledons of germinating seeds and fresh leaves.

Alcohol dehydrogenase (ADH)

The enzyme stained well with seedlings as well as fresh leaves but not with the root or bark samples. The bands from seed samples were relatively faint. The enzyme stained fast at incubation temperature of 37°C and was prone to over-staining when left to stain for long. However, slight over-staining was desirable for clear photographs and band intensity since the gel background was transparent. When rinsed with cold water the bands lasted for a longer period of time. ADH was less sensitive to storage of the digested tissues overnight and omission of any of the buffer additives (such as PVP) did not cause any noticeable difference in the resolution.

Diaphorase (DIA)

DIA exhibited activity in samples of seeds, seedlings, leaves and bark. However, leaf and bark samples streaked heavily making it somewhat difficult to evaluate electrophoretic phenotypes in some cases. The best, clear banding patterns were observed in seedling material while those of the seeds were good but faint. In an attempt to make the bands robust a little more than 0.2 mg of 2,6-dichlorophenol-indolephenol was added but they soon spread out into each other, making them a little difficult to score. The gels were incubated at 37°C and over-staining was avoided by limiting the staining time. The bands were clearer when the excess stain was rinsed off with cold water.

Gluterate Oxaloacetate (GOT)

GOT produced bands of good resolution with seeds, germinating seeds and leaves. It had activity with the roots and barks. Aspartic and α-ketoglutaric acid used in the assay of GOT have low solubility in the staining buffer, therefore the mixture had to be stirred and dissolved before the pH could be corrected to 7.25. The other staining reagents were added to the mixture when the gels were ready for staining. GOT showed stability and stained well even after overnight storage of extracts from the sampled plant tissues. The enzyme could be stained within a pH range of 7.25 to 8.0 but the bands were sharper at pH 7.25. Omission of any of the additives from the extraction buffer did not alter the resolution.

Malic Acid (ME)

ME displayed good activity and clear bands with seeds and germinating seeds but not with roots or bark. The leaves streaked heavily making it difficult to discern the bands. The intensity of staining of ME on samples of seeds and seedlings was reduced when 2-B-mercaptoethanol or PVP was omitted from the standard extraction buffer. ME took longer to stain and the optimum pH for staining buffer was between pH 7.0 and 8.0. Only one zone showed clear activity at pH 7.0, increasing to two in some populations at pH 8.0. ME was stable and did not lose activity when stored overnight.

Manadione reductase (MR)

The enzyme produced good clear bands with seeds and seedlings but streaked heavily with leaf samples. It displayed low activity after storage of tissue extracts overnight. No activity was observed in roots and bark. The effects of omitting some additives from the extraction buffer was not conspicuous. MR stained fast at 37°C and is also prone to over-staining. It streaked heavily when left to over-stain.

6-phosphogluconate dehydrogenase (6-PGD)

It produced no activity with leaves, roots or bark but seed samples exhibited faint bands. The best resolution was observed on samples of germinating seeds (seedlings). In the preparation of its staining reagent, 6-phosphogluconic acid was first dissolved in staining buffer and the pH corrected to 8.0. The enzyme exhibited stability but required longer staining time to allow slight over-staining for clear resolution. However, it mostly displayed one zone of activity but two in some rare cases.

Phosphoglucoisomerase (PGI)

PGI displayed good activity especially with seedlings, seeds and leaves. The activity on seeds was a little faint. It stained slowly, therefore it required longer in staining reagent. Good results were obtained when the staining process was allowed to continue over night. There was no activity in roots and bark extracts.

Shikimate dehydrogenase (SDH)

The activity and resolution data were obtained from extracts of seedlings, leaves and seeds. The bands from seed samples were faint but they could be scored. SDH was dissolved in staining buffer and pH corrected to 8.0 before mixing with the other reagents. The enzyme was stable but stained slowly thus needed longer staining period in an incubator at 37°C.

Conclusion

The observations made seem to be consistent with the general conclusion that the range of variables for good electrophoretic resolution are enormous, and the optimum

conditions can only be searched empirically. The variation that could be analyzed is represented by the isozyme patterns that account for the number of bands, their distribution and staining intensity, but the probability of experimental artifacts is also high until sufficient samples are assayed and analyzed genetically. The following chapter presents the genetic interpretation of the isozyme patterns observed in the gels run under the conditions which were optimised in the screening runs.

Following the above protocol the eight enzymes (ADH, DIA, 6-PGD, ME, MR, GOT, PGI and SDH) are suitable for genetic analysis on *Vitex*. The best tissues are from germinating seeds and to some extent fresh leaves (Table 4.1). The good results from germinating seeds could be attributed to high metabolic activity, resource mobilisation and thus high enzyme activities. Storage of tissue samples is not advisable as most of the enzyme systems lose activity when stored. However, failure of most of enzyme systems to produce bands with leaves of bark could be attributed to low enzyme activity or the presence of tannins, phenols and other inhibitors in those parts of the plant. The obstacle could be overcome by further research in inhibitor neutralising substances.

4.3 Electrophoretic analysis on polyploidy and allozyme polymorphism in *V. keniensis* and *V. fischeri*

The ploidy level of a given species can be obtained from chromosome counts, chromosome pairing pattern and morphological variation, but more recently, isozymes and other molecular techniques have become useful in determining ploidy levels (Harris & Ingram, 1992). This chapter presents an isozymic assessment of the level and nature of the polyploidy of the different the *V. keniensis* and *V. fischeri* populations in East Africa. The information is also valuable in interpretation of complex electrophoretic data that arise from increased ploidy level (Wolf et al., 1990; Barrington, 1990; Haufler et al., 1990).

The techniques and the results of chromosome counts are presented in chapter 2. The survey showed that all the *Vitex* populations studied had one cytotype of 96 somatic chromosomes. Despite the variability in the ecology of the zones occupied by the different *Vitex* populations, the species seem to have retained the same cytotype. However, the chromosomes were too tiny for the details of karyotypes to be studied. The analysis of allozymic pattern was used to reveal the segregation and inheritance pattern, and the nature of the polyploidy (auto-, allo- or segmental polyploid). The interpretation of allozyme data is directly affected by the species' ploidy level, and thus the basic understanding of the species' cytotype is necessary (Barrington, 1990; Haufler et al. 1990).

The application of enzyme phenotypes to ascertain ploidy level has been demonstrated for many plant species (Crawford & Smith, 1984; Haufler et al., 1990). In all these cases polyploids were shown to display isozyme multiplicity relative to diploids.

Through the allozyme data, the nature of the ploidy can be determined since multisomic inheritance is mostly associated with autopolyploids, while fixed heterozygosity is a character of allopolyploids (Waples, 1988; Krebs & Hancock, 1989; Cai & Chinnappa, 1989). The inheritance patterns of allozymes can also be used to identify the status of a species' cytotype (Odrzykoski & Gottlieb, 1984). This would allow the distinction between auto-, allo- and segmental polyploids. Electrophoretic characteristics associated with multisomic inheritance include the number of loci displayed, asymetrical banding patterns, fixed heterozygosity, and multiple alleles within the same locus (Barret & Shore, 1989; Wendel & Weeden, 1989; Kephart, 1990). Allopolyploids tend to exhibit fixed heterozygosity at many loci due to non-segregation of non-homologous chromosomes (Werth et al., 1985).

This chapter examines the patterns of inheritance in electrophoretic loci of *V. keniensis* and *V. fischeri*. The results were eventually applied in the scoring, analysis and interpretation of the genetic data from the 8 enzyme systems on the four *Vitex* populations, covering the geographical range of the species. Inferences are drawn about the possibility of higher level of ploidy, its nature and genetic polymorphism from the pattern of allozyme inheritance.

Materials and methods

1. The isozyme genotypes of five of the ten parent plants used in artificial pollination experiments (see chapter 3) were analyzed.
2. Seeds from emasculated, selfed and bagged flowers (treatments 3, 4 and 5 in the pollination experiments) enough to raise 40 progenies per selected tree in each population were sown in vermiculite in a germination cabinet at 25°C.
3. Young actively growing leaves were collected the seedlings at the age of 3 months.
4. The protocol of isozyme analysis is explained above. When more than one loci were observed for an enzyme, the most anodally migrating locus was designated as 1. The alleles were given letters with the most anodally migrating one designated as a, the second fastest b, and so on.
5. Four enzyme systems (ADH, GOT, PGI and 6-PGD) were tested using *Vitex* seedling leaf extracts. These were the only enzyme systems with relatively good resolution on *Vitex* leaf extracts.
6. A total of 800 progenies were analyzed covering the four *Vitex* populations. The banding patterns of the progenies were scored and compared with the parental phenotypes.
7. The segregation ratios of progenies were studied from electrophoretic phenotypes and were compared with the expected ratios derived from the genotypes of the parent plants.

Results and discussions

The enzyme electrophoresis resulted in a clear staining for the four enzymes encoded by 6 loci; ADH_1, ADH_2, GOT_2, PGI_1. PGI_2. and 6-PGD_2. Most of the plants exhibited these polymorphic loci. The bands migrated anodally for all the examined

enzymes. Several progenies exhibited heterozygous banding patterns at the different enzymes and were highly polymorphic. Some data on the segregation among progenies of the *Vitex* are presented in table 4.3. Most loci displayed three to four bands per locus for most enzymes and there was no evidence for fixed heterozygosity. They also exhibited unbalanced staining intensities in a number of enzyme loci, a possible indication for multiple doses of individual alleles. Genotypic interpretations of electrophoretic band patterns were inferred by assuming that banding intensity reflect allelic dosage. The segregation at the six enzyme loci was in close agreement with the genetic model of tetrasomic inheritance, codominant expression of alleles coding for enzymes and random chromosome segregation (Table 4.1).

These observations are evidence for tetrasomic inheritance in all the populations of *V. keniensis* and *V. fischeri*, enabling the species to maintain up to 3 to 4 alleles at a locus. However, complex banding patterns for ADH_2 consisting of up to five bands were also observed occasionally in some plants; these could be heterodimeric bands of intermediate mobility at times formed between the three allozymes (Soltis & Rieseberg, 1986). There was no evidence of fixed heterozygosity which is a predictable and consistently observed consequence of allopolyploidy (Gotlieb, 1981; Werth et al., 1985). In all the polymorphic loci, both homozygous, balanced and unbalanced heterozygous banding patterns were displayed in progenies. A number of electrophoretic investigations on allopolyploids have consistently revealed the presence of fixed heterozygosity (Soltis & Rieseberg, 1986) arising from lack of segregation. Therefore, the observed homozygosity, normal heterozygosity and complex banding (independent segregation) patterns in the progeny arrays is an evidence for autopolyploidy in the species. While, the maximum of four alleles per locus with occasional exceptions discussed above implies tetraploidy. The species may therefore be considered an autotetraploid. The results from *Vitex* is similar to those of obtained by Quiros (1982), Crawford & Smith (1984) from a number of taxa which showed that autotetraploid individuals carry up to four different alleles per locus.

Conclusion

The data from isozyme analysis on electrophoretic phenotypes and segregation in progeny arrays of the *Vitex* populations has provided additional insight into the nature of polyploidy in the plant species. The populations of *Vitex* exhibited autotetraploidy; as deduced from the 96 somatic chromosomes, enzyme multiplicity, the maintenance of up to three or four alleles at a locus and tetrasomic segregation pattern in the progenies (Table 4.3 & Figure 4.1). The independent (random) segregation of the four homologous chromosomes during the first meiotic division tends to lower the frequency of homozygous gametes (Krebs & Hancock, 1989) and that could explain the observed heterozygosity among the progeny of *Vitex*.

In general the tetrasomic inheritance results in a high proportion of heterozygous progeny as reflected in high levels of heterozygosity in autotetraploid populations (Li, 1955). It has been suggested that enzyme multiplicity affords a polyploid greater biochemical versatility (Barber, 1970; Manwell & Baker, 1970; Levin, 1983).

Table 4.3. An example of segregation patterns of some progeny genotypes of *Vitex*.

ADH_1: aabc x aabc		
Progeny	Expected	Observed
aaab	4.4	5
aaaa	1.1	2
aaac	4.4	2
aabc	11.1	13
aabb	4.4	5
abbc	4.4	4
aacc	4.4	4
abcc	4.4	3
bbcc	1.1	2
X^2		2.92ns

ADH_2: bccc x bccc		
Progeny	Expected	Observed
bbcc	10	12
cccc	10	9
bccc	20	19
X^2		0.55ns

GOT_2: aabc x aabc		
Progeny	Expected	Observed
aaac	4.4	3
aaab	4.4	6
aaaa	1.1	1
aabc	11.1	12
aabb	4.4	3
abbc	4.4	5
aacc	4.4	5
abcc	4.4	4
bbcc	1.1	1
X^2		1.7ns

6-PGD_2: bbcc x bbcc		
Progeny	Expected	Observed
bbbb	1.1	2
bbbc	8.9	11
bbcc	20.0	19
bccc	8.9	8
cccc	1.1	1
X^2		1.03ns

PGI_1: bbce x bbce		
Progeny	Expected	Observed
bbbc	4.4	6
bbce	11.1	11
bbbe	4.4	4
bbbb	1.1	2
bbcc	4.4	5
bcce	4.4	4
bbee	4.4	3
bcee	4.4	4
ccee	1.1	1
X^2		3.2ns

PGI_2: acef x acef		
Progeny	Expected	Observed
ceef	2.2	3
ccff	1.1	1
aacc	1.1	0
aace	2.2	2
aacf	2.2	3
acce	2.2	3
accf	2.2	2
ceff	2.2	1
acef	6.7	8
aaee	1.1	1
aaef	2.2	2
acee	2.2	1
eeff	1.1	0
aeef	2.2	4
aaaf	1.1	2
acff	2.2	3
aeff	2.2	2
ccee	1.1	0
ccef	2.2	2
X^2		2.82ns

Note: The example given is at each of the six loci in an array of progenies from one of the selfed trees. The statistically significant differences between the expected and the observed number of genotypes were analyzed by chi-square test, X^2 ($p < 0.05$ and $p < 0.10$). The difference between the expected and the observed number of progenies was insignificant in all the crosses (ns) both at $p<0.05$ and $p<0.10$.

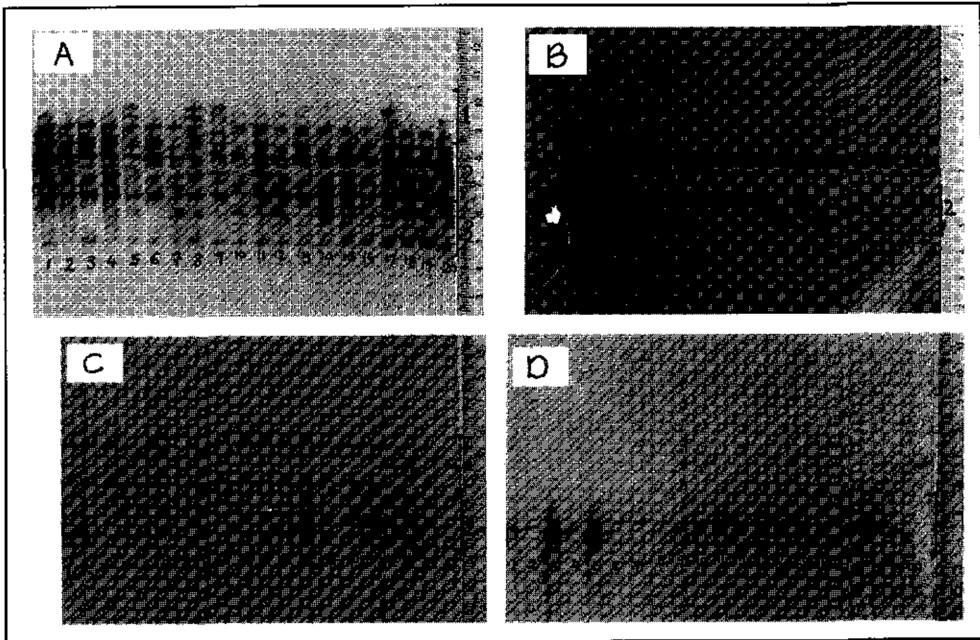


Figure 4.1. Photographs of polyacrylamide gels (PAGE) showing electrophoretic patterns and tetrasomic segregation in an array of 20 *Vitex* progenies from selfing. The numbers at the right of the photographs designate loci and alleles, respectively. Numbers at the bottom indicate individual samples. **A.** ADH (ADH_1 & ADH_2); homozygotes (one banded), normal heterozygotes, unbalanced heterozygotes and complex heterozygotes. Note the 5 band heterozygotes in ADH_2. **B.** GOT_2; homozygous (one band), normal heterozygous, unbalanced heterozygous. Three-banded patterns were also depicted. **C.** PGI_2; homozygous (one-band), normal heterozygous, unbalanced heterozygous, three-banded and complex patterns. **D.** 6-PGD_2; homozygous (one banded), normal heterozygous, unbalanced heterozygous and three band patterns.

According to Soltis & Rieseberg (1986) the multiple enzyme forms may minimise variation in substrate affinity, thus expanding the range over which physiological coordination of metabolic processes is possible. The observed heterozygosity and enzyme multiplicity may therefore be an adaptation by the species for greater genetic and biochemical versatility, a possible explanation for the wide ecological range of natural occurrence (i.e Savanna and montane rain forest) by the *Vitex* populations.

4.4 Electrophoretic phenotypes in *V. keniensis* and *V. fischeri* populations

In order to determine the electrophoretic (genetic) relationship within and among the four different *Vitex* populations, all the 8 enzymes assayed were scrutinised for variation in their banding patterns (Figures 4.2 & 4.3).

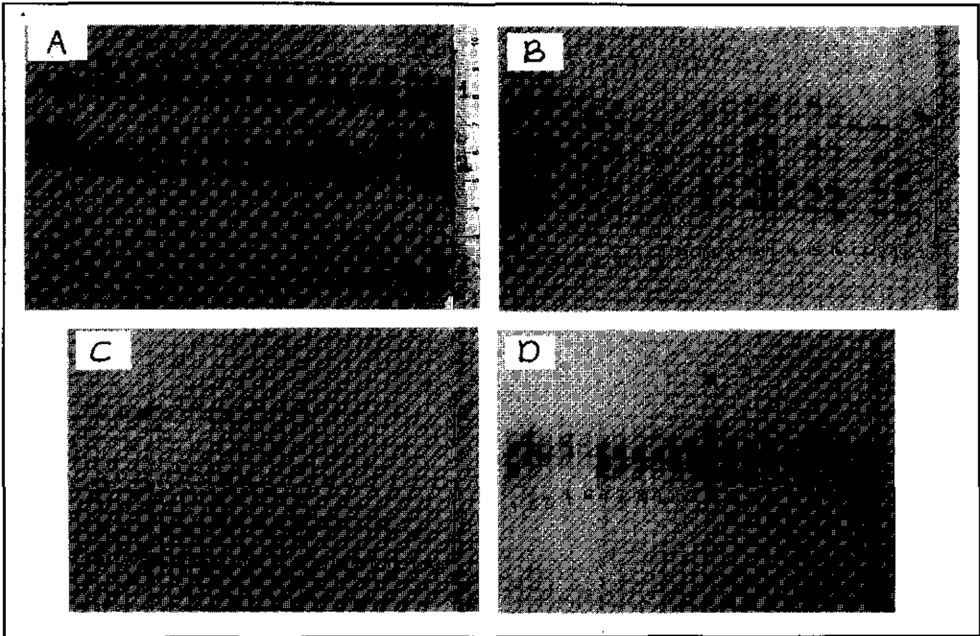


Figure 4.2. Electrophoretic phenotypes of *V. keniensis* and *V. fischeri* (isozyme loci): **A.** GOT_2 & GOT_3; **B.** ADH_1, ADH_2 & ADH_3; **C.** DIA_2; **D.** 6-PGD_2.

Twelve loci expressed banding patterns that could reflect the genetic structure of the study species' populations. The enzymes were designated according to the international nomenclature followed by arabic numbers, with the most anodal designated as the first locus. All the assayed enzymes migrated anodally, and the number and patterns of the bands which contributed to a locus were inferred from the sub-unit structure of the enzyme as described by Wendel & Weeden (1989), Kephart (1990), Crawford (1990) and Murphy et al. (1990). Alleles at a locus were specified by lower case letters following Cai and Chinnapa (1989) and Shore (1991). Where two loci migrated to the same position on a gel, the possible homoelogous alleles were assigned similar letters.

To confirm that the migration of allozymes at a locus was similar, samples from various populations were run adjacent to each other on the same gel and, in several combinations, for all the enzymes. Progenies (selfed) from parents of known genotypes were analyzed to reveal the species' allelic segregation (Table 4.3). The phenotypes were therefore scored into genotypes, assuming that the variation in banding patterns reflected allelic polymorphism, described as the occurrence together in the same habitat of two or more discontinuous forms of a species (trait or allele) in such proportions that the rarest of them cannot be maintained by recurrent mutation (Ford, 1976). The ratios of the various genotypes at every locus, for all polymorphic loci, to the total sample size were then expressed as genotypic frequencies. The populations were then entered into comparative analysis.

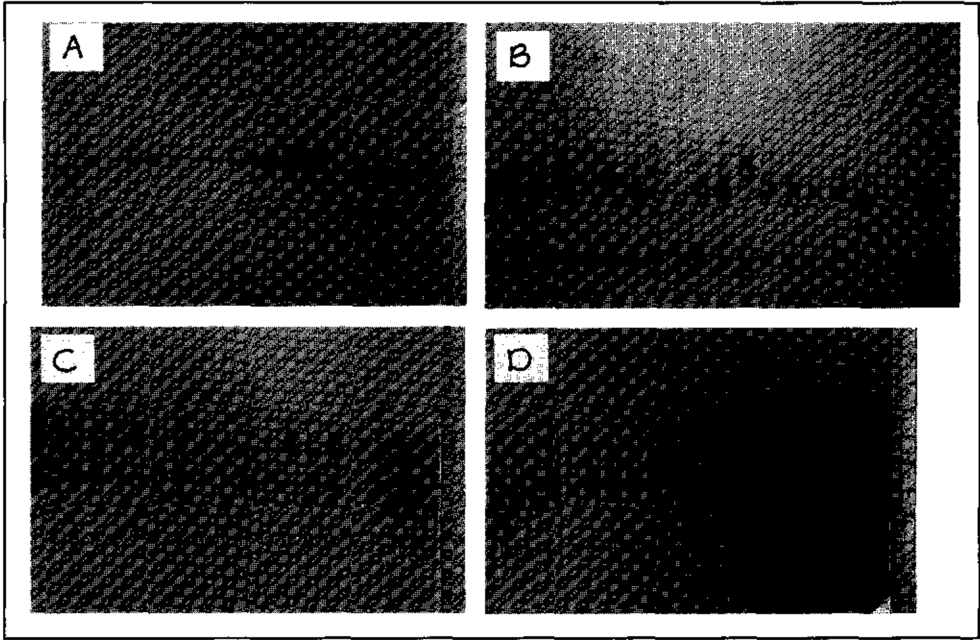


Figure 4.3. Electrophoretic phenotypes of *V. keniensis* and *V. fischeri*: **A.** SDH_1; **B.** ME_1; **C.** PGI_1 & PGI_2; **D.** MR_1.

The electrophoretic phenotypes were then scored into genotypes. The ratios of the various genotypes at every locus, for all polymorphic loci, to the total sample size was expressed as genotypic frequencies. The distinction in the allelic frequencies was compared with ecological conditions of each population. The 12 loci analyzed were GOT_2, GOT_3, Adh_1, ADH_2, ADH_3, DIA_2, MR_1, SDH_1, 6-PGD_2, PGI_2, PGI_3 and ME_1 (Table 4.2) for enzyme nomenclature and commission number. The observed electrophoretic patterns of the various enzymes and their interpretation are discussed below.

Gluterate Oxaloacetate (GOT)

The *Vitex* populations showed three zones of activity in GOT (Figure 4.2 A). However, the last two zones (loci) were consistent and clear for analysis. They were designated GOT_2 and GOT_3. The first locus (GOT_1) showed no variation between the trees and even populations. GOT_2 exhibited complex pattern, with some genotypes displaying a maximum of 4 alleles with varying degree of staining intensity in different samples. There were two alleles which could appear either as a pair or independently in the locus. That was noted in a number of alleles in the locus. The complex banding pattern observed in GOT_2 could have resulted from overlapping of two loci co-migrating on a gel. The presence of some homozygous genotypes assisted in maintaining the interpretation on the possibility that locus GOT_2 was a reflection of multiple alleles within the locus. GOT_2 and GOT_3 were predominantly expressed by

three alleles per locus with asymmetrical staining. The some of the samples exhibited four alleles per locus with asymmetrical staining intensities.

A secondary band activity cathodal to locus two was noticed in some samples but its genetic significance was not established because it was variable in intensity and was not consistent with any sub-unit structure. The observation was consistent with the activities that had been reported on *Stephanomeria exigua* (Gottlieb, 1973), which were referred to as 'ghost bands'. These bands could be a characteristics of some enzyme systems Kephart (1990) or they could be representing some silenced alleles. Their activities varied among individuals within the populations, possibly implying some inheritance pattern.

Alcohol dehydrogenase (ADH)

The enzyme exhibited three zones of activity in the samples of *Vitex*. The anodal locus was designated as ADH_1, the cathodal zone, ADH_3 and the middle ADH_2. The three zones of activities exhibited some variation within and between the populations. The pattern and number of bands varied considerably between the activity zones. Locus ADH_2 showed the highest number of bands. In some samples, as many as 8 bands were observed at ADH_2 in some samples. ADH_1 and ADH_3 displayed relatively fewer bands and less variation than the middle zone (ADH_2). This could be attributed to ADH being a dimeric enzyme. It is therefore expected to form intragenic heterodimers in heterozygous situation (Hart, 1969, 1970; Marshal et al., 1974; Brown et al., 1974; Roose & Gottlieb, 1980). According to Gottlieb (1982), the ability of two alleles to form heterodimers is a sign of structural similarity. Thus the likely reason for alleles a, b, c, d and e at ADH_2 to selectively form heterodimer bands could be based on their structural relationship (Figure 4.2 B).

Diaphorase (DIA)

DIA had 2 main zones of activity (loci). The anodal DIA_2 was clearer and exhibited polymorphism which was included in the study of the populations while DIA_1 was monomorphic in most samples. DIA_1 exhibited up to 4 bands but the majority of samples displayed 3 alleles (Figure 4.2 C). The enzyme is known to display considerable variation from one species to another in terms of the enzymes expressed, and quaternary structure. In reviews by Wendel & Weeden (1989) and Kephart (1990), the enzyme is presented as a monomer, a dimer or a tetramer. DIA is one of the reductase enzymes which are not substrate-specific (Wendel & Weeden, 1989), and thus may stain other reductase enzymes. That was not a common occurrence but in a few isolated cases some bands resemble MR. But parallel staining of DIA and MR on the same day assisted in eliminating the stray bands.

6-Phosphoglucose dehydrogenase (6-PGD)

6-PGD is one of the enzymes of the carbohydrate metabolism which is frequently

assayed in plant species. In *V. keniensis* and *V. fischeri*, 6-PGD displayed 2 loci. The anodal was designated 6-PGD_1 and the cathodal one 6-PGD_2. Of the two loci, 6-PGD_2 was the most consistent and polymorphic. 6-PGD_1 was rare and was mostly monoallelic whenever it occurred (Figure 4.2 D). For 6-PGD_2 up to 4 alleles were observed per locus but 3 alleles were more common. Some alleles stained darker, especially when the b allele was present. That could be expected since the enzyme is a dimer (Wendel & Weeden, 1989; Kephart, 1990; Murphy et al., 1990). In phenotypes with three bands, deep staining was also observed; this could be a reflection unbalanced heterozygosity. The staining intensity of four bands varied. That could be interpreted as dosage effect and further differentiated samples. 6-PGD_1 is usually (not always) located in cytosol, while 6-PGD_2 is a plastid allozyme (Oballa, 1993). The enzyme is highly conservative and is usually found as two isozymes, except in case of duplication (Gottlieb, 1981; 1982). The bands displayed asymmetrical staining and unbalanced heterozygosity (Figure 4.2 D).

Shikimate Dehydrogenase (SHD)

SDH_1 displayed one zone of activity with up to four bands, representing 4 alleles, designated from a to d. SDH is a monomer (Wendel & Weeden, 1989; Kephart, 1990), displaying single bands for homozygotes and, two to three bands for heterozygotes. The samples exhibited both homozygosity and heterozygosity (balanced and unbalanced). Some genotypes exhibited four alleles which migrated closely together while in others were the alleles were slightly separated but the pattern maintained. Genotypes with three bands were common in the four populations (Figure 4.3 A).

Malic Enzyme (ME)

ME is also known as MDPH (Murphy et al., 1990). It exhibited 2 enzyme activity zones (Figure 4.3 B). The anodal was designated ME_1 and the cathodal ME_2. ME_2 was mostly monomorphic and inconsitent in the samples. The phenotypes in ME_1 had variable number of bands ranging from one to four. The one-banded phenotypes were noted as homozygotes, and the remainder with two, three or four were considered heterozygotes. The three- to four-banded phenotypes were classified as balanced or unbalanced heterozygotes depending on the gene dosage effect and the patterns. Murphy et al. (1990) and Kephart (1990) described ME as a tetramer. However, the genotypes were a little difficult to score due to unclear sub-unit structure. The less clear banding patterns and sub-unit structure of the enzyme could be a possible expression of multiple bands, and variable dosage ratio which is only possible in polyploids (Oballa, 1993).

Phosphoglucoisomerase (PGI)

Two regions of activity, PGI_1 and PGI_2 were observed in the enzyme. Both exhibited up to six alleles per locus (Figure 4.3 C). The complex banding pattern could

be attributed to co-migration of the alleles. (Figure 4.3 C). The enzyme was highly polymorphic and displayed variation between the species' populations.

Generally, the polymorphism varied from one locus to another but the locus that displayed high number of alleles also showed high number of genotypes. For instance ADH_2 showed the highest number of alleles compared to other loci, meanwhile SDH_1 had the lowest number of alleles and polymorphic genotypes.

Manadione Reductase (MR)

MR exhibited one zone of activity zone, designated as MR_1. The activity zone had variable bands confirming the existence of homozygotes and heterozygotes (Figure 4.3 D). The enzyme has been reported to express tetrameric banding patterns by Bousquet et al. (1987) and Huenneke (1985). Heterozygotes expressed up to four bands and homozygotes one. Some samples displayed patterns with three intensely stained bands. This could have resulted from interaction factors when heterodimers co-migrate or individual variation in activity of the enzymes. However, it could also represent multiple alleles expressed by homologous chromosomes. The difficulty experienced was the sensitivity of the enzyme to assay conditions leading to some inconsistent variability.

4.5 Genetic diversity of *V. keniensis* and *V. fischeri* populations

The pattern of genetic diversity can be inferred from isozyme markers (Ledig, 1986). It provides an insight into historical biogeography of populations, revealing the past geographic structure and distribution (Ranker et al., 1994). Several investigations of the level of genetic diversity among congeneric species involving diploids and their polyploid counterparts have indicated that polyploids do exhibit higher heterozygosity than diploids (Schnabel et al., 1991). About 40% of plant species are known to be polyploids, and most of these are angiosperms (Stebbins, 1980). Evidence from cytological and isozyme patterns shows that *V. keniensis* and *V. fischeri* are polyploids (both having $2n=96$), thus raising the expectations of observing a high level of genetic variation.

Isozyme gene markers have been found to be stable and reliable indicators of genetic variation and population structure (Nei, 1972; 1973; 1978; Gottlieb, 1981; Adams, 1983; Hillis & Moritz, 1990) because of their direct link to genes. This Chapter addresses the genetic variability within and between the different *Vitex* populations. Twelve polymorphic isozyme loci of 400 samples from four populations were used to determine the variation of genetic parameters. The possible taxonomic distinction and evolutionary trend of the populations of the species are also illustrated using dendrograms. The application of the observed genetic variation in conservation strategies and breeding programmes are also discussed.

4.5.1 Statistical analysis on the electrophoretic data

The 8 enzymes examined resulted in the identification of 12 putative loci which were used the genetic analyses. The banding and segregation pattern confirmed autotetraploidy in the species. In addition to homozygotes, balanced and unbalanced heterozygotes were also evident. Such observations are normally a consequence of different allelic dosages (Soltis & Rieseberg, 1986; Ehrendorfer et al., 1996) which could have arisen from tetraploidy. The banding patterns (phenotypes) were scored into genotypes and further transformed into relative genotypic frequencies by counting individuals of similar genotypic categories and calculating the ratio to the total sample size. Appendix 4.2 the allelic frequencies at the 12 loci of the *Vitex* populations.

The parameters expressing genetic variation were calculated both for the species as a whole and individual populations (Table 4.4 & 4.5). The proportions of the polymorphic loci (P) and the mean number of alleles per locus (A) were calculated using the computer program BIOSYS-1 (Swofford & Selander, 1981). The allelic frequency, effective number of alleles and expected heterozygosities were calculated manually. Other genetic parameters were estimated following the formulae of Nei (1973, 1975) and Hedrick (1985). These included total gene diversity (H_T), expected gene heterozygosity (H_e), intrapopulation diversity (Hs), inter-population gene diversity (Dst), the coefficient of gene differentiation (G_{ST}), the coefficient of absolute gene differentiation (D_M) and the coefficient of intra-population gene diversity (R_{ST}). Nei's (1978) unbiased genetic identity (I) and genetic distance (D) were calculated using BIOSYS-1. The rooted Wagner Tree dendrogram was also plotted by BIOSYS-1.

4.5.2 The proportion of polymorphic loci (p)

The proportion of polymorphic loci (P) is one of the parameters used to determine genetic variation in natural populations using isozyme loci polymorphism. It is a statistic expressing the number of loci with two or more alleles. The parameter for each *Vitex* population was estimated following Hedrick (1985), where x is the number of loci polymorphic in the sample and m is the number of loci assayed, including monomorphic loci:

$$P = \frac{x}{m} \tag{1}$$

The average polymorphism for the species was then calculated as follows, where s is the number of populations of the species examined:

$$\overline{P} = \frac{\sum_{i=1}^{s} P}{s} \tag{2}$$

4.5.3 Estimates of genetic diversity of populations of *V. keniensis* and *V. fischeri*

The isozyme phenotypes were scored into genotypes and further transformed into genotypic frequencies by counting individuals with similar genotypes and calculating their ratio to the total sample. The observed mean heterozygosity (H_{OP}) in each population was calculated as follows:

$$H_{OP} = \frac{\sum_{i=1}^{m} \frac{\sum_{i=1}^{n} G}{N}}{m} \qquad (3)$$

where N is the sample size of a population, G is the number of observed heterozygous genotypes and m is the number of loci scored, including the monomorphic ones. The overall observed mean heterozygosity for the species (H_{OS}) was then estimated by averaging over the populations:

$$H_{OS} = \frac{\sum_{i=1}^{s} Hop_i}{s} \qquad (4)$$

where s is the number of populations, other terms are defined in formula 3.

The gene diversity of the species was calculated assuming autotetraploidy (presented in section 4.4) and that the populations were randomly mating i.e. Hardy-Weinberg equilibrium. In a homozygote of an autotetraploid, the four alleles are identical. Therefore for those isozyme loci with the four co-migrating alleles were considered homozygotes. The estimate of the expected heterozygosity was calculated using the formula of Hedrick (1985) for autotetraploids, as:

$$H_e = 1 - \sum_{i=1}^{n} p_i^4 \qquad (5)$$

where n is the number of alleles and pi^4 is the frequencies of the homozygotes.

The effective number of alleles (A_{ep}) in a population, which is the reciprocal of the frequency of the homozygotes, was calculated following Ayala and Kiger (1984), as (all terms are as defined above):

$$A_{ep} = \frac{1}{\sum_{i=1}^{n} p_i^4} \qquad (6)$$

The average expected heterozygosity (H_{ep}) of the populations was estimated as the average of the per locus heterozygosity estimates:

$$H_{ep} = \frac{\sum_{i=1}^{m} He_i}{m} \tag{7}$$

The genetic diversity within the species at each locus (H_{SP}) was calculated as (all terms are as defined above):

$$H_{sp} = \frac{\sum_{i=1}^{s} He_i}{s} \tag{8}$$

The overall average genetic diversity within the species' populations (H_{SS}) is then;

$$H_{ss} = \frac{\sum_{i=1}^{m} Hsp_i}{m} \tag{9}$$

The species' total genetic diversity, H_T at each locus is calculated as

$$H_T = \frac{\sum_{i=1}^{m} 1 - \sum_{i=1}^{n} r^{4_i}}{m} \tag{10}$$

where r is the weighted allele frequencies over s populations.

Inter-population genetic diversity (D_{ST}) is the absolute measurement of genetic differentiation among populations (Nei, 1973; 1987), and was calculated as follows:

$$D_{ST} = H_{ST} - H_S \tag{11}$$

The parameter above is a modification of Wright's F-statistic (Wright, 1965), adapted to allow analyses of loci with multiple alleles, because the original formula, [1-Fit=(1-Fis)(1-Fst)] could not handle a situation where more than two alleles are expressed at a locus (Nei, 1973). Fit, Fis and Fst are deviations of genotypes from the Hardy-Weinberg equilibrium, which is more complex when applied to polyploids (Oballa, 1993). The complexities such as overlapping alleles leading to either under- or over-estimations of genotypic frequencies in multi-allelic polyploids with homologous genes have been discussed by Asins & Carbonell (1987) and Waples (1988).

Nei (1973; 1975) defined the absolute measure of genetic differentiation (D_M) as the average minimum genetic distance between populations, and it is dependent on genetic diversities within populations. It excludes comparisons of populations with themselves and is appropriate for comparing genetic differentiation in different populations. It is estimated as (all terms are as defined above):

$$D_M = \frac{sD_{ST}}{s-1} \qquad \textbf{(12)}$$

The coefficient of genetic differentiation (G_{ST}) is an index expressing inter-population variation relative to total diversity, and was estimated as:

$$G_{ST} = \frac{D_{ST}}{H_T} \qquad \textbf{(13)}$$

The above estimate of G_{ST} is useful when comparing populations with similar breeding systems (Nei, 1973). It varies from 0 to 1 and is equivalent to Wright's Fst (gene differentiation among populations), as demonstrated by Nei (1973).

Coefficient of intra-population gene diversity (R_{ST}) is the index of genetic diversity due to absolute gene differentiation among populations (Nei, 1973). The coefficient of intra-population gene diversity was calculated from Dm (Nei, 1973), as (all terms are as defined above):

$$R_{ST} = \frac{D_M}{H_S} \qquad \textbf{(14)}$$

Total genetic diversity (H_T), intra-population genetic diversity (H_S), inter-population genetic diversity (D_{ST}), coefficient of genetic differentiation (G_{ST}), absolute coefficient of genetic differentiation (D_m) and coefficient of inter-population genetic diversity (R_{ST}). The allele frequencies of each of the populations at the 12 polymorphic loci (Appendix 4.2) are also estimated.

Results and discussions

The proportion of polymorphic loci is high (P=100%) but could be considered comparable to other tropical tree species reported; 95% for *Acacia tortilis* (Oln'gotie, 1992), 90% for *Faidherbia albida* (Joly et al., 1992), 98% for *Acacia karroo* (Oballa, 1993) and 66.7% for *Abronia macrocarpa* (Williamson & Werth, 1999). The high polymorphic value could be attributed to either the species' biological characteristics such as long life cycle, high ploidy and out-crossing level. However, it is worth mentioning that overestimation of polymorphism can also arise from deliberate exclusion of non-consistent, poorly resolved monomorphic loci from the analysis.

Table 4.4. Distribution of genetic diversity estimates for the four populations of *V. keniensis* and *V. fischeri*. Sample size (N), mean number of alleles per locus (A), effective number of alleles (A_{ep}), percentage polymorphic loci (P), observed heterozygosity (H_O) and expected heterozygosity (H_{ep}).

Population	N	A	Aep	P	Ho	Hep
Mt. Kenya	100	5.1	6.0	100	0.86	0.971
Nyambene	100	5.1	4.4	100	0.81	0.970
Mt. Elgon	100	5.1	6.4	100	0.87	0.971
L. Victoria	100	5.1	4.2	100	0.80	0.970
Species mean		5.1	5.3	100	0.84	0.970

Table 4.5. Genetic differentiation at 10 loci across the four *V. keniensis* and *V. fischeri* populations.

Locus	HT	H_S	D_{ST}	Gst	Dm	Rst
ADH_1	0.99	0.967	0.023	0.023	0.007	0.007
ADH_2	1.00	0.965	0.035	0.035	0.012	0.012
ADH_3	1.00	0.967	0.033	0.033	0.011	0.011
DIA_2	0.98	0.965	0.035	0.035	0.012	0.012
GOT_2	1.00	0.967	0.037	0.037	0.012	0.012
GOT_3	0.98	0.967	0.037	0.037	0.012	0.012
MR_1	0.99	0.963	0.037	0.037	0.012	0.012
ME_1	1.00	0.962	0.038	0.038	0.013	0.014
SDH_2	0.99	0.959	0.041	0.041	0.014	0.015
$6\text{-}PGD_1$	0.98	0.965	0.035	0.035	0.012	0.012
PGI_1	1.00	0.972	0.028	0.028	0.009	0.009
PGI_2	0.99	0.969	0.031	0.031	0.010	0.010
Species mean	0.99	0.965	0.035	0.035	0.012	0.012

The enzymes were polymorphic for three or more alleles per locus. The effective number of alleles per locus (A_{ep}) in the populations ranged from 4.2 to 6.0. While the mean number of alleles per locus (A) over the four populations was 5.1, this is similar in all the populations studied. Examples of reports on high average number of alleles per locus are *Pinus taeda* (3.89 to 4.78, Conkle, 1981) and *Maclura pomifera* (4.0, Schnabel et al., 1991). The high number of alleles per locus and polymorphisms in *Vitex* could also be attributed to the polyploidy. The constant number of alleles despite the variation in geographical and ecological conditions can also be a result of common origin, biological capacity of the populations to inhabit the different ecological zones without undergoing adaptive changes or the isozymes may be neutral to the forces of selection imposed by the environment, thus remaining unchanged over generations.

Generally, some angiosperms have higher levels of genetic diversity than those reported in conifers (Hamrick et al. 1981). The following are reports on the levels of the genetic diversity on some tropical angiosperms; *Acacia albida* 45% (Joly et al.,

1992) and *Acacia tortilis* (Oln'gotie, 1992), 73% for *Maclura pomifera* (Schnabel et al., 1991), 83% for *Acacia karroo* (Oballa, 1993) and 99% for *V. fischeri* (= *V. keniensis*) in this study.The high level of genetic diversity in *V. keniensis* and *V. fischeri* populations could be attributed to the polyploidy and the widespread pollen dispersal by bees, and seed dispersal by monkeys and hornbill birds within the populations. Most studies comparing genetic diversity in diploids and polyploids have also concluded that high variation is associated with high ploidy level (Shore, 1991). The attributes of polyploidy dominate the development and maintenance of genetic variation in a polyploid species (Arft & Ranker, 1998).

The observed heterozygosity (H_O) ranged from 80% in Lake Victoria to 87% in Mt. Elgon. The observed mean heterozygosity was 84% while the expected heterozygosity (H_e=97%) was higher than the observed heterozygosity. Estimates of other genetic diversity parameters are also presented in tables 4.4 & 4.5. The total genetic diversity of the populations based on the 12 loci (H_T) was 99% while the intra-population heterozygosity varied from (H_S) varied from 95.9% (SDH_2) to 97.2% (PGI_1) with the mean of 96.5%. This indicates that the bulk of the species' genetic diversity is retained within the populations and little is distributed between them.

The mean diversity among the populations (D_{ST}) was estimated as 0.035. This value is small, an indication that the *Vitex* populations are not significantly different from one another. The value of D_{ST} varied from 0.023 in ADH_1 to 0.041 in SDH_2. The proportion of between population heterozygosity to the total heterozygosity (G_{ST}) was 3.5%. The G_{ST} value is used to compare the ratio of the population component to the total diversity. This implies that 3.5% of the total genetic diversity was attributed to genetic differences among the populations and the remaining (96.5%) was maintained within the populations of the species. The low G_{ST} value indicated that the *Vitex* populations are very similar, this is surprising given the disjunct ecological and geographical distribution of its populations. However, this may be a further indication of taxonomic similarity between them as earlier presented in chapter 2.

High level of genetic diversity within the populations is mostly caused and maintained by an efficient gene flow and high fecundity (Hamrick et al., 1981; Loveless & Hamrick, 1984; Hamrick & Godt, 1989). Given the sedentary nature of trees, geneflow does occur through pollen and seed dispersal. This maintains and even increases the diversity of the genepool in a population. The high genetic diversity of the Vitex populations could be attributed to efficient gene flow through bee pollination and seed dispersal by monkeys and hornbill birds. This also suggests that although the tree species exhibits a mixed mating system, outcrossing seem to be predominant thus the maintenance of the high genetic diversity within its populations. According to Barrett & Shore (1989) observed that selfing plants tend to have more genetic variation between than within their populations. Hamrick & Godt (1989) also found that trees dispersed by ingestion of seeds by animals are genetically more variable within the population.

4.5.4 Genetic distance and identity of populations of *V. keniensis* and *V. fischeri*

The degree of similarity and dissimilarity between the different *Vitex* populations were estimated by Nei's genetic statistics (1972), modified Rogers genetic distance (Wright, 1978) and Prevosti genetic distance (1978). On Nei's genetic statistics (1978) the similarities between the populations were high ranging from 0.980 to 0.989. The highest genetic similarity was recorded between Nyambene hills and Lake Victoria. The mean genetic distance between the *Vitex* populations on Nei's genetic statistics was 0.984. The genetic distance based on Nei's statistics was between 0.011 and 0.020. The highest level genetic distance was observed between Mt. Elgon and Lake Victoria (Table 4.6). The analyses by Modified Rogers genetic distance (Wright, 1978) and Prevosti genetic distance (Wright, 1978) exhibited genetic distance patterns similar to those from Nei's genetic statistics (Table 4.7).

The pattern of variation in genetic identity and distance between the *Vitex* populations is random and does not follow the geographical distances, prevailing ecological conditions or the existing taxonomic delimitation. The highest divergence was observed between Mt. Elgon and Lake Victoria. This is in contrast to the fact that the two populations are geographically closer to each other, occupy relatively similar ecological zones and have been considered to be the same species (*V. fischeri*) different from the Nyambene hills and Mt. Kenya populations (*V. keniensis*) (Verdcourt, 1992; Beentje, 1994).

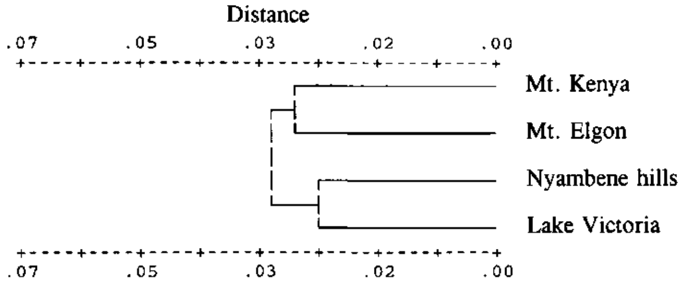
Table 4.6. Matrix of genetic similarity and distance coefficients. Below diagonal: Nei (1972) genetic identity (I); Above diagonal: Nei (1972) genetic distance (D) of *V. keniensis and V. fischeri* populations over 12 loci.

Vitex Population	1	2	3	4
1 Mt. Kenya	****	0.015	0.015	0.016
2 Nyambene hills	0.985	****	0.019	0.011
3 Mt. Elgon	0.985	0.981	****	0.020
4 Lake Victoria	0.984	0.989	0.980	****

Table 4.7. Matrix of genetic similarity (I) and distance (D) coefficients: Below diagonal: Modified Rogers distance (Wright, 1978): Above diagonal: Prevosti distance (Wright, 1978).

Population	1	2	3	4
1 Mt. Kenya	*****	.027	.028	.029
2 Nyambene hills	.023	*****	.032	.025
3 Mt. Elgon	.023	.026	*****	.035
4 Lake Victoria	.023	.020	.026	*****

Figure 4.4. UPGMA dendrogram of the four populations of *V. keniensis* and *V. fischeri*.



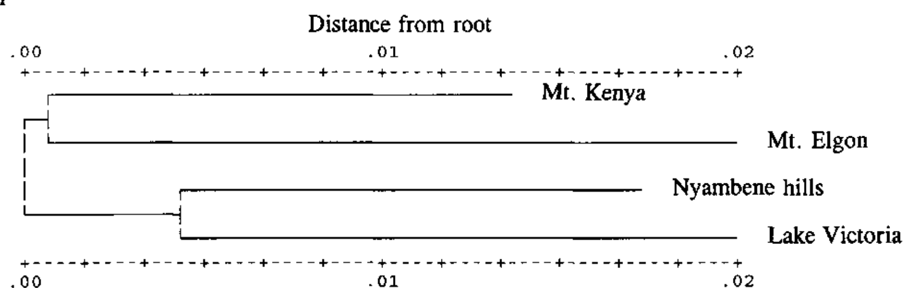
The current taxonomic separation of the four populations of *Vitex* into *V. keniensis* (Mt. Kenya and Nyambene hills) and *V. fischeri* (Mt. Elgon and Lake Victoria) also does not appear to be reflected in the genetic distances or in the morphological traits (Chapter 2) of the populations. The distribution of genetic similarity and distance among the four *Vitex* populations did not follow any consistent pattern, an indication of their being conspecific.

In a review of electrophoretic analysis and plant systematics, Gottlieb (1977) concluded that most conspecific populations have high mean genetic identities on Nei's genetic statistics (1972), usually above 0.90, while congeneric plant species have lower genetic identities varying from 0.5 to 0.6. The mean genetic identity of the four *Vitex* populations was 0.984 which is well above congeneric level of divergence. It is well within conspecific level. The mean genetic distance of 0.016 also seems to indicate that the populations of the species have not yet diverged to species level.

The dendrograms of the genetic relationships between the *Vitex* populations are illustrated in figures 4.4 and 4.5. Figure 4.4 is clustered by the unweighted pair group of arithmetic means (UPGMA) of Sneath and Sokal (1973), constructed using Prevosti genetic distance (Wright, 1978). The longest branch has a genetic distance of 0.035 and a cophenetic correlation of 0.0691. Figure 4.5 Wagner Tree (Farris, 1972) clustered by rooting the dendrogram at the midpoint of the longest path. The total length of the path is 0.058 and the cophenetic correlation is 1.00. It is also clustered from the genetic distance matrices (Table 4.7).

Both UPGMA and Wagner Tree dendrograms yielded branches of similar pattern. They grouped the four populations into two; Mt. Kenya and Mt. Elgon on one side, and Nyambene hills and Lake Victoria on the other (Figure 4.4 & 4.5). This also is in contrast to the prevalent taxonomic separation of the populations, geographical distances and the prevailing ecological conditions in their habitat. The *Vitex* populations exhibited high mean genetic identity and low mean genetic distances between them, an indication that they are remain conspecific despite the varying geographical and ecological range of distribution. This is also a further evidence that most of the variation exist within the populations rather than between them.

Figure 4.5. Rooted Wagner tree dendrogram of the four *V. keniensis* and *V. fischeri* populations.



4.6 Conclusion

The high genetic diversity in *Vitex* populations can be attributed to high level of outcrossing, pollen and seed dispersal within the populations by bees and monkeys and birds respectively, and by the apparent neutrality of the loci to natural selection. Outcrossing species tend to have high genetic diversity evident from increased polymorphism, allelic diversity and heterozygosity (Hooper & Haufler, 1997). Selfing species are more prone to lower levels of intra-population genetic diversity than outcrossing species, and in selfing species each population tends to be more differentiated from the others. According to Barrett & Shore (1989) and Crawford (1983) selfing plants tend to have more genetic variation between their populations than within them because different alleles are fixed during inbreeding. If these observations are correct then, although *Vitex* has a mixed mating system, out-crossing predominates. Therefore explaining the high intra-population gene diversity (H_S) and the low inter-population gene diversity (D_{ST}) in *Vitex* populations. The high heterozygosity in the populations of *Vitex* could also be attributed to the high polyploidy level (2n=96).

The low genetic variation between the disjunct populations of *Vitex* shows lack of divergence a possible indication that no transformation of genetic constitution has occurred. The divergence of populations mostly depends on the original genepool, time since separation, presence of natural barrier, ecological gradient (selection pressure) and physiological and phenotypic plasticity of the species. According to the history and biogeography of the vegetation of East Africa the region was composed of a uniform plain during the Kainozoic Era (Haughton, 1963; Lind & Morrison, 1974). That was changed by tectonic and volcanic activities that led to the formation of rift valleys and mountains during Miocene and Pleistocene periods (Lind & Morrison, 1974), thus separating the once continuous vegetation cover on the extensive plain. Therefore it is possible that the geographically isolated *Vitex* populations were once extensive and continuous, thus sharing a common ancestry. However, in spite of having been separated by the geological events, it seems they have not undergone sufficient drift for detectable morphological and genetic changes to warrant their taxonomic separation. The genetic relationship between the *Vitex* populations does not follow the current taxonomic separation and the level of genetic identity between them is well

above 0.90 (Table 4.6, Figures 4.4 & 4.5) implying that they are conspecific.

The study has shed some light on biogeographical history of the species by concluding that the *Vitex* populations are relicts of a once larger more widespread one. The lack of unique alleles or combination of alleles across loci also supports a once larger, more widespread distribution. The ability of the species' populations to maintain close morphological and genetic similarity could be attributed to factors driving the evolutionary process such as the species gene-pool, time, selection pressure, gene-flow and biological adaptability which include polyploidy and phenotypic plasticity. These features can allow a species occupy different habitats without the need to undergo genetic or conspicuous morphological change. It is also possible that the variation in the selection pressure between the different ecological zones inhabited by the *Vitex* populations may not be as wide as it appears, therefore allowing or sustaining the homogeneity among them. However, little is known about the nature and magnitude of selective factors acting across the *Vitex* populations.

According to Allphin et al. (1998), high levels of heterozygosity tend to buffer genotypes against environmental challenges. The phenomenon can allow species to inhabit a wide range of ecological zone and slow down the pace of genetic change in species' populations. Other reports indicate that polyploids have a wide range of geographical distribution relative to diploids (Soltis & Soltis, 1991). That could further explain the capacity of *Vitex* populations to occur in contrasting ecological zones. The long life span of the tree species (over 100 years) can also lead to time lag in transformation of demographic and genetic structure, slowing down the rate of population divergence. The high genetic identities and low genetic distances between the populations of *Vitex* populations therefore implies common ancestry and minimal or lack of divergence.

The high genetic variation within the *Vitex* populations, a possible sign of the absence of directional selection favouring particular genotypes, which can reduce heterozygosity. The species exhibited mixed mating system i.e. both out-crossing and selfing (Chapter 3), out-crossing appear to predominate thus maintaining the high heterozygosity in its populations. The high genetic variation within the populations seem to be maintained by efficient geneflow too within the populations through pollination and seed dispersal. It is therefore possible that the pollinators (bees) and seed dispersal agents (monkeys and hornbills) ensure extensive distribution of the genetic materials. The low inter-population variation between the *Vitex* populations concurs with the observation by Hamrick & Godt (1990) that geographic range has little influence on inter-population differentiation of species. It appears that so long as the morphological, physiological and genetic characteristics of an organism do not impose limitations to its survival in an environment, evolution or speciation would be a luxury, unfortunately nature operates only on necessities. That could explain the lack of synchrony between the pattern of genetic structure, the ecological gradient and geographical distances of the *Vitex* populations.

5. General conclusions and recommendations

In spite of the ecological and geographical different in their habitats, the morphological and genetic make-up of the *Vitex* populations appear similar. The studied traits of all the species' populations on the data from the available literature, herbarium specimens, morphology of freshly collected samples from the populations, cytology, mating systems and genetic structure revealed no reason for taxonomic separation of them into two species (*V. keniensis* and *V. fischeri*). Therefore *V. fischeri* and *V. keniensis* should be considered as one species. The name *V. fischeri* has priority and the well-known *V. keniensis* falls into synonymy. This supports the observation made by Dale & Greenway (1961) that *V. keniensis* and *V. fischeri* are not distinguishable. The resemblance in the morphological characteristics of the two species by different authors presented in appendix 2.1 leads to a similar conclusion.

The genetic identities between the *Vitex* populations (I= 0.984) were well within the range expected of conspecific populations (e.g. Crawford, 1983; Allphin et al. 1998). Therefore any efforts for improvement breeding and conservation on programmes on the famous Meru oak, any of the four populations would adequately represent the species' genepool. However, the variation in timber properties between the *Vitex* populations needs further research, although it is probable that the timber traits are more under environmental influence. The results of such work would contribute to the improvement of the quality of the timber produced by the species.

The *Vitex* populations exhibit a mixed mating system and produces seeds by selfing as well as through out-crossing. This could be an adaptation against the possibility of missing a pollinator or a mate. The seeds from self-pollination showed no marked loss in germination. This could be attributed to the high ploidy level and high heterozygosity of the species. The two phenomena are capable of cushioning inbreeding depression. However, many generations of inbreeding can lead to vigour loss and reduced adaptability to the prevailing environmental conditions. More research is necessary to investigate the vigour of the selfed offspring throughout a complete life cycle. Such an investigation was not possible at this stage because of the long life span of the tree species. The mixed mating system in the species allows both selfing and out-crossing. This may be considered advantageous in improvement breeding efforts, as it can allow exploitation of the species' heterozygosity and at the same time making it possible to benefit from the positive aspects of selfing such as the breeding of pure lines.

The tree species is pollinated mainly by bees (*A. mellifera*), therefore populations of these insects are vital for its survival. The current destruction of their habitats is likely to affect the pollinators adversely. Therefore, there is a need to preserve the species' natural habitat to maintain pollinator populations for enhanced geneflow in the

Vitex populations. Otherwise reduced population of pollinators can lead to increased selfing and possibly inbreeding depression over generations thus threatening the species' survival. Besides habitat preservation, the existing bee-keeping culture among the communities in the forest areas also need to be encouraged for the benefit of both the local population and the forest plant populations. The preservation of the habitat of the tree species is also be important protecting the populations of the seeds dispersal agents i.e monkeys and hornbill birds. They play an important role in facilitating geneflow through seed dispersal. They also play an essential role in promoting natural regeneration by pre-treatment of the seeds that pass through their digestive systems, enhancing germination.

The four *Vitex* populations had the same somatic chromosome number i.e. $2n=96$. Thus they are polyploids. Their electrophoretic phenotypes and progeny segregation patterns indicates that they are tetraploids. Meanwhile, their progeny arrays exhibits multiple alleles of homozygotes, balanced and unbalanced heterozygotes. The independent segregation patterns are normally associated with autopolyploids (Lewis, 1980). Therefore, the populations of *Vitex* are autotetraploids.

The level of total genetic diversity ($H_T=99\%$) estimated for *Vitex* was high. Reports on the total genetic diversity of other polyploid tropical tree species indicate that *Acacia karroo* has 88% (Oballa, 1993) and *Maclura pomifera* has 73% (Schnabel et al., 1991). From empirical evidence, it is possible to attribute the high genetic diversity in the *Vitex* populations to the presence of multiple alleles arising from the polyploidy. The genetic diversity may also maintained by the species' mixed mating systems and extensive geneflow through pollen and seed dispersal within the populations. The high morphological and genetic similarity between the populations could be attributed to the *Vitex* populations having a common origin, uniform selection pressure, long life span of the tree species and other biological features such as phenotypic plasticity, heterozygosity and polyploidy. Protein polymorphism is often correlated to fitness (Mitton, 1994), therefore the high total diversity could be a reflection of the species' ecological adaptability. The high level of genetic diversity in the *Vitex* populations reflect both outcrossing mode of reproduction and the potential longevity of the individual trees. The reasonably high levels of the existing genetic variation suggests that conservation efforts could still preserve most of the species diversity. However, it is worth noting as a caution that if the species is forced to resort to inbreeding over generations, genetic erosion is likely to set in, leading reduced genetic diversity and loss of vigour.

The maintenance of the genetic diversity is considered crucial for long term survival and evolutionary response of populations to changes in environment (Huenneke, 1991). Loss of genetic variation may reduce a population's ability to adapt to changing environmental conditions and lead to inbreeding depression. Despite the high genetic diversity in the *Vitex* populations, their natural range is under increasing pressure from human activities and it is becoming impossible for it to be maintained indefinitely. The on-going selective harvesting in the species natural populations can also lead to

dysgenic effects, resulting loss of the best genotypes. Therefore, a concerted effort towards *in-situ* and *ex-situ* conservation is necessary for preserving the stability of the species' genetic structure.

The information on the genetic structure and mating systems can be applied in the improvement breeding and raising of productive commercial plantations of the species to reduce the over-exploitation of the natural population. The species' natural populations could be protected and managed as gene banks. The observed inter-population reproductive compatibility in *Vitex* is advantageous because it can allow easy transfer of desirable inheritable traits from one population to another. However, the possibility of cross-pollination in the species implies that the designing of the seed orchards should ensure a separation distance from neighbouring unimproved individuals of the same species to avoid possible contamination through cross-pollination.

The variation between the *Vitex* populations was low (G_{ST}= 3.5%) implying lack of population divergence. This is in contrast to the variation in the ecological and geographical distances between the four disjucnt populations (Figure 2.3). The habitats of two populations (Mt. Kenya and Nyambene hills) may be described as montane rain forest, while Mt. Elgon and Lake Victoria occur in the wet wooded savanna. The Wagner Tree dendrogram divided *Vitex* populations into two main cluster groups, representing the level of isozyme divergence. However, judging from the small genetic distance between them, seeds collected from at least one of the populations would be enough to capture the majority of the genetic variation held within the species. Sampling intensity within the selected populations should be relatively high. This is because most of the species genetic diversity is retained within the populations. However, although there are no marked genetic differences between the populations, there is need to include the contrasting ecological zones in the conservation by including one from montane and the other from savanna forests as a precautionary measure, just in case some ecological-specific unique genetic traits were not detected by the isozyme technique.

Population genetic analyses of a threatened and highly exploited plant species such as Meru oak need a well-designed conservation programmes that would ensure preservation of maximum levels of natural genetic variation and local adaptation. Habitat fragmentation and loss of the species' natural populations is in the increase especially in the highly settled areas. This is bound to expose the species to decline in its genetic diversity and adaptability to the dynamic environment. Genetic erosion would also impose a limitation to the potentials of the species' improvement through breeding. The debate on the optimum sampling intensity for conservation of genetic diversity is on-going. In the absence of resource constraints, the more the sample size the higher the chances of preserving the species' genetic diversity. However, a minimum of one hundred genotypes sampled across the range of the chosen *Vitex* populations would be adequate for conserving its gene-pool. This is because the species is an autotetraploid with a maximum of four alleles per locus, therefore according to

the formulae of Waples (1988) the expected number of genotypic categories is a hundred. It is worth stressing that if resources allow assembling a higher number of genotypes would increase the chances of capturing more of the species' gene pool.

The distance between the sampled individuals is also critical and the optimum isolation distance is dictated by the neighbourhood size which depends on the extent of pollen and seed dispersal. An isolation distance of 300 m, being the flight distance of the pollinating bees (Boshier et al., 1995), between sampled trees would be recommended for *Vitex*. The seed dispersal range by monkeys and hornbill birds in these environments is still unknown. These need to be established for the purpose of improving the sampling strategy for the species' genepool.

Since most of selection by users is based on adaptability to site and expected end uses such as timber, fruits and fuelwood, more research is necessary on the desirable traits and their heritability for improvement purposes. The observed variation in wood properties need to be investigated further to exploit its potential. This would promote the production and utilisation of the species both at subsistence and commercial level.

Appendix 2.1. Comparison of *V. fischeri* and *V. keniensis* made by different authors.

Table A.1. Comparison of *V. fischeri* and *V. keniensis* by Dale & Greenway (1961). They concluded that collections away from Mt. Kenya are referable to as *V. fischeri* but it is doubtful if *V. keniensis* is distinguishable from that species.

Characteristics	*V. fischeri*	*V. keniensis*
Height	30 ft	100 ft with 50 ft clear bole and 6 ft diameter.
Bark	grey	thin, rough and slightly fissured
Slash	white	blaze cream yellow
Young branches	clothed with orange tawny tomentose	---
Leaves	5-foliolate	---
Petiole	2.5 to 5.5 inches long	---
Leaflets	entire, scabrous & puberulous above when young; scabrous & almost glabrous when mature, tomentose beneath, ovate-elliptic to elliptic or slightly obovate, 2.5 inches long, 1.25 to 2.5 inches broad; apex acuminate, base broadly cuneate to rounded, petiolule up to 0.5 inch long	---
Cymes	axillary, densely flowered peduncles 3.5 inches long	---
Flowers	pubescent, white mauve	---
Fruits	oblong-globose, 0.4 inches long, black when ripe, cupped in enlarged membraneous calyx.	---
Habitat	wet savanna; altitude 4000 to 6000 ft	east of Mt. Kenya; altitude 5000 to 6000 ft

Table A.2. Comparison of *V. fischeri* and *V. keniensis* by Verdcourt (1992). He observed that *V. keniensis* is usually synonymised with *V. fischeri* but there is no doubt that it is a distinct taxon worthy of specific rank.

Characteristics	*V. fischeri*	*V. keniensis*
Height	Savannah shrub or small to fairly large tree (1.8-) 4.5 - 9 or sometimes to 15(- 18) m with rounded crown.	Tree 12 - 30 m.
Diameter	---	1.8 - (2.3) m.
Clear bole	---	12 - 18 m.
Bark	grey to dark brown shallowly serrate and fissured.	very thin, rough and slightly fissured.
Slash	white to light brown with white sapwood	creamy yellow turning dirty green.
Stems & petiole	young branches clothed with velvety orange-brown hairs or yellow tomentose	beneath with more shaggy indumentum than *V. fischeri*
Leaves	scented, 5-foliolate	5-foliolate
Leaflets	oblong, ovate-elliptic to elliptic or slightly obovate elliptic, 5-19 cm. long, 3-10 cm. wide, shortly to long-acuminate at the apex, tip acute, cuneate to rounded at the base,entire scabrid-puberulous above but velvety woolly tomentose beneath with sparse to dense glands, venation evident, lateral veins rather numerous up to ± 15 veins.	obovate, 5.5 - 17 cm. long, 3.2 - 8.5 cm. wide, broadly rounded to obtusely acuminate at the apex, cuneate to rounded at the base, coriaceous, usually drying darker than *V. fischeri*, sparsely puberulous above, paler beneath and completely covered with soft ochraceous tomentum and glands.
Petioles	6.5-16.5 cm. long, petiolules 1-2 (3-8) cm. long.	13.5 - 17 cm. long, petiolules present or absent or intermediate 0.6-4 cm. long.
Cymes	axillary, extensive, many flowered, up to 11 (-15) cm. wide	ochraceous, tomentose, more lax than *V. fischeri*, axillary panicle up to 12 cm. long 24 cm. wide.
Peduncle	6-14 cm. long, pedicels 0-1 mm. long or that of central flower of cyme up to 12 mm. All axes woolly tomentose	6.3 - 13 cm. long, secondary branches to 4.5 cm. long, pedicels 1 - 4 mm. long, densely tomentose.
Bracts	0.3-2.2 cm. long, dark, velvety outside	0.5 - 1.0 cm. long, 1 - 4 mm. wide.

Calyx	tinged red, 3-4 mm. long, truncate or shallowly toothed, teeth up to 1 mm. long	densely ochraceous tomentose, more distinctly toothed than *V. fischeri.*
Tube	---	2 mm. long, lengthening to 5 - 6 mm. in fruit; teeth broadly triangular, 0.5 mm. long, lengthening to 3 mm.
Corolla	white with blue or mauve upper lips and lower cream or both blue; throat yellow; tube ±3 mm. long, the lip 3.5 mm. long and lower lobe 2.5 mm. long and wide.	white or white tinged blue mauve upper lips and with largest lobe mauve, tube curved, 5-6 mm. long, ochraceous tomentose above outside; limb 8 - 10 mm. wide, and largest lobe round or ovate, 3 - 5 mm. long, 3.5 mm. wide, entire, the others 2.5 - 3 mm. long 2 mm. wide, slightly emarginate, densely ochraceous tomentose outside.
Ovary	---	globose, with simple hairs and ovary
Style	---	filiform, 6 mm. long
Stigma	---	lobe-linear
Drupes	Black or purple green to black with green spots, oblong-globose, 1-1.3 mm long *sic*, 0.8-1.0 cm. wide, edible.	Black with white spots, obovoid, (1.1-) 2.2 - 2.4 cm. long, (0.8-) 1.3 - 1.5 cm. wide, shiny, glabrous, rounded at the apex, ± contracted at the base.
Mesocarp	calycine cup ±1 cm. wide.	fleshy, endocarp very woody, the putamen ±2 cm. long, 1.1 - 1.2 cm. wide, 6 - 7 mm. diameter at the base, obtusely 4-angled.
Habitat	Wooded grassland and thicket particularly on granite rocks, also rich bushland on termite mounds and *Bridelia*, *Maesopsis*, *Albizia* forest; 980-2080 m.	Evergreen forest, sometimes thicket particularly riverine; 1290-2100 m.

Table A.3. Comparison of *V. fischeri* and *V. keniensis* by H. Beentje (1994).

Characteristics	*V. fischeri*	*V. keniensis*
Height	3 - 15 m	12 - 35 m
Leaves	5-foliolate	5-foliolate
Leaflets	elliptic or slightly obovate, terminal leaflet 9 - 21 by 4 - 9 cm slightly sand papery or rarely glabrous above, densely pubescent beneath, apex shortly acuminate.	elliptic, base often unequal, terminal, apex short acuminate, terminal leaflet 9-21 by 3.5 - 10 cm; slightly sand papery above, densely pubescent beneath.
Flowers	white or pale mauve, with lower lip darker mauve in axillary dichasia 5 - 24 cm long, flowers 6 - 8 mm long.	white or purplish, with largest lobe dark mauve, in axillary dichasia 12 - 18 cm long; flowers 7 - 8 mm long.
Fruits	Purple to black, oblong-globose 8 - 12 mm long, edible.	black, ellipsoid, 13 - 16 mm long.
Habitat	wooded grassland rarely in forest margins.	moist evergreen forests.

Appendix 2.2. Climatic conditions in the natural range of *V. keniensis* and *V. fischeri* populations. Sources: Forest station ledgers, Jaetzold & Schmidt (1982), Langdale-Brown et al.(1964) and Lind & Morrison (1974).

Mt. Kenya Region. Altitude: 1300 - 2000 m.

Month		J	F	M	A	M	J	J	A	S	O	N	D	Annual
Temperature (°C)	Mean	21	22	22	23	22	21	20	20	21	22	22	21	21.4
	Max.	28	30	29	28	27	26	25	26	28	29	27	26	27.4
	Min.	13	14	16	17	16	15	14	14	14	15	16	15	14.9
Mean rainfall (mm)		75	63	117	444	369	99	75	81	69	231	252	132	2007
Mean potential evaporation (mm)		174	171	189	153	138	120	114	126	159	180	144	138	1806

Nyambene hills. Altitude: 1300 - 2200 m.

Month		J	F	M	A	M	J	J	A	S	O	N	D	Annual
Temperature (°C)	Mean	16	16	17	16	16	14	13	13	14	16	16	15	15.2
	Max.	22	23	22	21	20	19	17	17	20	21	20	21	20.3
	Min.	9	10	11	12	11	10	9	9	9	11	11	10	10.2
Mean rainfall (mm)		45	60	93	315	294	63	57	54	51	207	201	81	1521
Mean potential evaporation (mm)		120	123	138	105	102	87	78	84	114	129	96	102	1278

Mt. Elgon region. Altitude 1200 - 1500 m.

Month		J	F	M	A	M	J	J	A	S	O	N	D	Annual
Temperature (°C)	Mean	20	20	20	20	19	18	18	18	18	19	19	19	19.0
	Max.	26	27	27	26	25	24	23	22	25	25	25	25	25.0
	Min.	13	13	13	14	13	12	13	13	12	13	13	13	13.0
Mean rainfall (mm)		33	45	82	146	184	120	168	224	123	75	67	52	1419
Mean potential evaporation (mm)		165	154	164	135	115	105	102	108	123	136	129	152	1588

Lake Victoria region. Altitude 1000 - 1400 m.

Month		J	F	M	A	M	J	J	A	S	O	N	D	Annual
Temperature (°C)	Mean	23	23	23	23	23	22	22	22	23	23	23	22	22.7
	Max.	31	31	31	29	29	29	29	29	30	31	30	30	29.9
	Min.	14	15	16	16	16	15	15	14	14	15	15	14	14.9
Mean rainfall (mm)		72	101	120	187	130	81	78	89	71	66	108	73	1176
Mean potential evaporation (mm)		180	160	180	150	140	130	140	150	160	170	160	170	1890

Appendix 4.1. The enzymes screened on *V. keniensis* and *V. fischeri* and their respective recipes. They are named according to IUB (1984).* indicates the best enzymes finally used in the analysis.

1. Acid Phosphate (ACP, E.C. 3.1.3.2)

0.1 M Acetate buffer pH 4.8	50 ml
x-naphthylene acid phosphate	50 mg
Fast Garnet GBC	50 mg

***2. Alcohol Dehydrogenase (ADH, E.C. 1.1.1.1)**

0.1 M Tris-HCl pH 8.0	100 ml
95% Ethanol	1.0 ml
NAD	10 mg
MTT	10 mg
PMS	2 mg

***3. Diaphorase (DIA, E.C. 1.6.99.-)**

0.05 M Tris-HCl pH 8.0	100 ml
2,6 Dichlorophenol-indophenol	0.2 mg
NADH	10.0 mg
MTT	5.0 mg

4. α-Esterase (α-EST, E.C. 3.1.1.-)

0.1 M Tris-HCl pH 7.25	100 ml
α-Napthyl acetate	100 mg
Fast blue RR salt	100 mg

5. Glutamate dehydrogenase (GDH, E.C. 1.4.1.2)

0.1 M Tris-HCL pH 8.0	100 ml
L-Glutamic acid	1.0 g
NAD	1.0 ml/10 mg
MTT	1.0 ml/10 mg
PMS	0.5 ml/5 mg

6. Glucose-6-phosphate dehydrogenase (G-6-PD, E.C. 1.1.1.49)

0.1 M Tris-HCl pH 7.25	100 ml
D-Glucose-6-phosphate (disodium salt)	50 mg
NADP	1.5 ml/15 mg
MTT	1.5 ml/15 mg
PMS	0.3 ml/3 mg

***7. Gluterate Oxaloacetate Transaminase (GOT, E.C. 2.6.1.1)**

0.1 M Tris-HCl pH 8.35	100 ml
L-Aspartic acid	100 mg
α-Ketogluteric acid	33 mg
Pyridoxial-5-phosphate	4 mg
PVP Soluble	50 mg
Fast Blue BB Salt	100 mg

8. Leucine-amino peptidase (LAP, E.C. 3.4.11.1)

A 0.2 M Tris-HCl pH=7.5	50 ml
Malic acid	200 mg
Reduce pH to 6.0.	
Fast Garnet GBC	50 mg
(Stir and filter)	
B Distilled water	750 μl
Acetone	750 μl
L-leucyl-b-naphthylamide,	
HCl (endonuclease)	15 mg

Add A to B pour over the gel and incubate at 25°C.

9. Malate dehydrogenase (MDH, E.C. 1.1.1.37)

0.1 M Tris-HCl pH 8.0	100 ml
1.0 M DL-malic acid	3.0 ml
NAD	10 mg/1.0 ml
MTT	10 mg/1.0 ml
PMS	3 mg/0.3 ml

***10. Malic Acid (ME, E.C. 1.1.1.40)**

0.1 M Tris-HCl pH 8.0	100 ml
1.0 M DL-Malic acid pH 7.0	3.0 ml
NAD	10.0 mg
MTT	10.0 mg
PMS	3.0 mg
MgCl2	100 mg

***11. Manadione reductase (MR, E.C. 1.6.99.-)**

0.1 Tris-HCl pH 7.25	100 ml
Manadione (sodium bisulphate)	20 mg
NADH	10 mg
MTT	20 mg

***12. 6-Phosphogluconate Dehydrogenase (6-PGD, E.C. 1.1.1.44)**

0.1 M Tris-HCl pH 7.5	100 ml
1.0 M Magnesium chloride	1 ml
NADP	30 mg
MTT	40 mg
PMS	8 mg

***13. Phosphoglucoisomerase (PGI, E.C. 5.3.1.9)**

A 0.05 M Tris-HCl, pH 8.0	50 ml
NAD	10 mg
Fructose-6-phosphate, Na salt	20 mg
Glucose-6-phosphate dehydrogenase	20 units
B MTT	20 mg
PMS	8 mg

Add A to B, pour over gel and incubate.

14. Phosphoglucomutase (PGM, E.C. 5.4.2.2)

A 0.1M Tris-HCl, pH 7.5	100 ml
1M MgCl2	1 ml
Glucose-1-phospahte, sodium salt	150 mg
Glucose-6-phosGlucose-6-phosphate dehydrogenase	20 µl
B MTT	20 mg
NADP	20 mg
PMS	8 mg

***15. Shikimate Dehydrogenase (SDH, E.C. 1.1.1.25)**

0.1 M Tris-HCl pH 8.0	100 ml
Shikimic acid	50 mg
NADP	15 mg
MTT	15 mg
PMS	4 mg

Appendix 4.2. Genetic variability in each of the *V. keniensis* and *V. fischeri* populations at the different polymorphic loci derived from allelic frequencies.

Population: Mt. Kenya

Alleles	Locus											
	ADH_1	ADH_2	ADH_3	DIA_2	GOT_2	GOT_3	MR_1	ME_1	SDH_2	$6PGD_2$	PGI_1	PGI_2
A	.080	.105	.095	.095	.090	.075	.080	.110	.070	.075	.080	.085
B	.085	.115	.090	.090	.080	.075	.090	.110	.100	.110	.075	.070
C	.080	.080	.085	.100	.095	.100	.100	.085	.105	.100	.085	.080
D	.085	.055	.070	.090	.085	.090	.115	.060	.125	.090	.070	.095
E	.070	.045	.055	.020	.050	.060	.015	.030	.000	.025	.055	.050
F	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.025	.015
He	.973	.969	.973	.969	.972	.972	.967	.969	.963	.968	.977	.975

Population: Nyambene hills

Alleles	Locus											
	ADH_1	ADH_2	ADH_3	DIA_2	GOT_2	GOT_3	MR_1	ME_1	SDH_2	$6PGD_2$	PGI_1	PGI_2
A	.970	.080	.085	.090	.080	.070	.080	.100	.080	.080	.090	.070
B	.100	.085	.075	.090	.070	.100	.085	.110	.075	.100	.075	.085
C	.075	.125	.095	.100	.105	.095	.100	.090	.115	.090	.080	.110
D	.085	.050	.080	.085	.090	.080	.120	.080	.130	.095	.105	.065
E	.070	.045	.070	.035	.055	.055	.015	.020	.000	.040	.045	.065
F	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.005	.005
He	.972	.971	.972	.970	.971	.972	.967	.968	.963	.970	.972	.972

Population: Mt. Elgon

Alleles	Locus											
	ADH_1	ADH_2	ADH_3	DIA_2	GOT_2	GOT_3	MR_1	ME_1	SDH_2	$6PGD_2$	PGI_1	PGI_2
A	.080	.095	.095	.080	.095	.050	.095	.100	.085	.070	.085	.080
B	.065	.090	.080	.105	.080	.100	.090	.125	.115	.110	.080	.070
C	.075	.110	.090	.085	.100	.095	.095	.105	.105	.090	.075	.085
D	.095	.065	.085	.075	.080	.080	.080	.055	.095	.105	.080	.085
E	.085	.040	.050	.055	.045	.070	.045	.015	.000	.025	.050	.060
F	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.025	.030
He	.972	.970	.972	.972	.971	.972	.970	.965	.964	.968	.976	.975

Population: Lake Victoria

Alleles	Locus											
	ADH_1	ADH_2	ADH_3	DIA_2	GOT_2	GOT_3	MR_1	ME_1	SDH_2	$6PGD_2$	PGI_1	PGI_2
A	.060	.090	.095	.105	.090	.080	.090	.090	.090	.070	.075	.075
B	.080	.095	.065	.080	.055	.075	.075	.135	.085	.115	.085	.090
C	.105	.095	.080	.105	.105	.105	.110	.095	.095	.090	.075	.105
D	.085	.055	.105	.080	.095	.080	.100	.060	.130	.080	.110	.065
E	.070	.065	.060	.030	.055	.055	.025	.020	.000	.040	.045	.050
F	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.010	.015
He	.972	.971	.971	.969	.971	.972	.968	.965	.964	.971	.972	.973

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