MAURITIUS RESEARCH COUNCIL

THE PHYLOGENETIC RELATIONSHIPS AND PHARMACOLOGICAL PROPERTIES OF THE ENDEMIC EBONY TREES OF THE MASCARENES

Final Report

Year 2004

MAURITIUS RESEARCH COUNCIL

Address:

Level 6, Ebène Heights,
34, Cybercity,
Ebène 72201,
Mauritius.

Telephone: (230) 465 1235
Fax: (230) 465 1239
Email: mrc@intnet.mu
Website: www.mrc.org.mu

This report is based on work supported by the Mauritius Research Council under award number MRC/RUN-0020. Any opinions, findings, recommendations and conclusions expressed herein are the author's and do not necessarily reflect those of the Council.

APPENDIX D
(Form 1050)

PI Name and Address

Dr G. Khittoo
Professor T. Bahorun
University of Mauritius
Reduit
Mauritius

MAURITIUS RESEARCH COUNCIL

FINAL REPORT

PART I- PROJECT IDENTIFICATION INFORMATION

1. Type the name of the MRC Scheme under which grant is made Unsolicited Research Grant Scheme
2. Award Dates From: 2001 To: 2004
3. Organisation and Address: University of Mauritius, Reduit
4. Award Number: MRC/RUN-0020
5. Project Title: The phylogenetic relationships and pharmacological properties of the endemic ebony trees of the Mascarenes

Mauritius Research Council

Table of Contents

Contents **Page**

Contents **Page**

List of Figures

List of Tables and Plates

Acknowledgements

We would like to extend our warmest thanks to all people and institutions that helped us in this project on the ebony trees. We feel we owe these magnificent species this small tribute after their almost complete demise through sheer lack of vision and appreciation.

Indeed, with the help of our friends, we were able to unveil some fascinating aspects of a genus which once covered the island in all its splendour.

We particularly thank:

- The MRC for their patience and financial support.
- The University of Mauritius for providing the necessary infrastructure.
- The Forestry Service which at the inception of the project was under the direction of the late and much regretted Mr S. Paupiah. He encouraged all his staff to participate in our project and to provide us with whatever materials and help we needed. For these we thank, Mr A. Khurun, R. Ramjaun, R. Rutty, S. Poonoosamy and their colleagues.
- The National Park & Conservation Services especially Mr S. Gopal and Mr M. Allet for the sharing of their field experience.
- Mr Vikash Tatayah from the Mauritian Wildlife Foundation for helping us locate some of the rare species.
- Professor Sterling Keeley from the University of Hawaii for opening her lab for molecular work and for her constructive criticisms.
- Dr Armand Seguin and his team from the Forest Genomics, Laurentian Forestry Centre Quebec, Canada for helping in the analysis of the DNA sequences.
- Our research assistants and students for their technical help.
- Dr Shadila Venkatasamy for her input in all aspects of the project from the design to the analysis of the results.
- To others who helped us but who are not listed here please accept our gratitude.

Summary

Studies on the ecology, morphology and molecular phylogeny were undertaken with a view to understand the colonisation patterns and evolution of the *Diospyros* species in Mauritius. On another front, phytochemical screening and antibacterial properties of plant extracts from the *Diospyros* species were carried out to investigate the pharmacological properties of these species.

The distribution of the *Diospyros* species and populations were examined with respect to their presence in the different altitudinal ranges, humidity zones, soil and forest types. Analysis of the results obtained showed that except for *D. egrettarum* which is restricted to the east coast of Mauritius, the other *Diospyros* species have a relatively broad distribution. However, the abundance of these species indicated that some of them have a marked preference for humid to super humid habitats while others seem to favour sub humid to dry regions. Phenological observations revealed that *Diospyros* species, which share neighbouring habitats, tend to have staggered flowering periods that act as reproductive barriers among these species.

Although the Mascarene *Diospyros* species are all dioecious, female flowers still retain a number of staminodes suggesting that they have the potential of developing functional stamens while the male flowers have only male reproductive organs. Examination of the flowers of the local *Diospyros* species indicated the occurrence of leaky dioecy in one species namely, *D. egrettarum*. Furthermore, these leaky dioecious plants produced fruits with viable seeds. A male-biased sex ratio was noted in the species *D. egrettarum*, *D. leucomelas, D. melanida*, *D. revaughanii* and *D. tessellaria*. Given that in all *Diospyros* species, the germination of *Diospyros* seeds is relatively low and the sex ratio in *Diospyros* populations is male-biased, germination of the seeds which first landed in Mauritius would have led to more adult male than female plants. Therefore, leaky dioecy could have come to the rescue of a solitary male *Diospyros* plant whose survival would have depended on its ability to bear fruits with viable seeds.

In an attempt to find molecular markers to distinguish between female and male *Diospyros* plants, the genomic DNA of male and female *D*. *egrettarum* plants was assessed by Random amplified polymorphic DNA (RAPD), using fifty random decamer primers. The RAPD profiles generated were highly monomorphic. Out of the three polymorphic bands for the female samples tested only the one generated by the primer OPC02 consistently appeared in all the

female samples and was hence considered as a potential sex-associated RAPD band for *D. egrettarum*

Phylogenetic trees based on 35 morphological characters and the Internal Transcribed Spacer (ITS) regions of the ribosomal DNA were reconstructed for the Mascarene *Diospyros* species. The morphology and molecular trees had limited agreement on the relative positions of the major clades. It would seem that parallel or convergent evolution of some morphological traits obscured the actual phylogenetic relationships of some species and their relative positions could not be determined in the morphology based strict consensus tree. On the other hand, the molecular data generated well resolved phylogenetic trees that have provided additional information on the colonisation patterns of the *Diospyros* species in Mauritius. The general trend outlined by the phylogenetic analysis showed that closely related species shared neighbouring habitats. Morphological and molecular data sets also suggest that Mauritius could have acted as a centre for the dispersal of *Diospyros* species in the Mascarenes as both *D. borbonica* (Reunion) and *D. diversifolia* (Rodrigues) are nested within the Mauritian *Diospyros* species.

The Mauritian endemic *Diospyros* species were screened for their phytochemical contents, polyphenolic contents and their antibacterial properties. Preliminary phytochemical screening showed the presence of reducing sugars, tannins, cardiac glycosides and terpenoids in all the eleven species. Steroids, saponins and anthraquinones were present in all species except for *D.pterocalyx* and *D.egrettarum*. However, alkaloids were present in *D.neraudii* and *D.revaughanii* only. *D.eggretarum* contained the smallest amount of polyphenolics compounds whereas *D.melanida* had the highest amount of polyphenols. Bacterial strains *B.cereus, B.spizizenii, S.aureus, S.epidermidis, P.mirabilis, K.pneumoniae, A.baumanii, E.coli and P.aeruginosa* were sensitive to the *Diospyros* extracts while *S.aureus, P.aeruginosa* and *A.baumanii* were resistant to positive controls but were sensitive to the plant extracts. These properties can be attributed to the high presence of polyphenols, flavonoids, tannins, saponins, anthraquinones and especially terpenoids.

1.0 Introduction

The Mascarene archipelago which is found in the south west Indian Ocean consists of the islands of Mauritius, Reunion and Rodrigues. An estimated 70% of the Mascarene flora is thought to have originated from Madagascar and Africa (Cadet, 1977). While some of these species seem to closely resemble their Malagasy and African relatives, most of them have evolved to give rise to genera and species endemic to the Mascarenes (Cadet, 1984). Several vegetation surveys carried out over the years, (Vaughan and Wiehe, 1937; Strahm, 1994; Page and D'Argent, 1997) have indicated that Mauritius harbours a diverse flora comprising of at least 900 native species and ten endemic genera. The most recent survey estimates that less than 2% of the original Mauritian endemic plants survive in forest reserves and on mountain slopes (Page and D'Argent, 1997).

Prior to Dutch settlers arriving in Mauritius in 1598, *Diospyros* (Ebenaceae), commonly known as the ebony were the dominant species of the native Mauritian forest (Pitot, 1905). However, during the 17th century, the *Diospyros* species especially *D. tessellaria* were overexploited for their excellent timber quality and black wood (Brouard, 1963). This resulted in a drastic reduction in the population sizes of these endemic plants. Over recent years, the *Diospyros* populations have been under severe threat due to increased demand for land space mainly for agriculture and urbanisation. These species are now reduced to pockets of individuals located mostly in reserves and inaccessible areas. For the past several years, twelve endemic species have been identified in Mauritius. They are, *D. angulata* Poir, *D. boutoniana* A.DC, *D. chrysophyllos* Poir, *D. egrettarum* I.B.K Richardson, *D. hemiteles* I.B.K Richardson, *D. leucomelas* Poir, *D. melanida* Poir, *D. neraudii* A.DC, *D. nodosa* Poir, *D. pterocalyx* Bojer, *D. revaughanii* I.B.K Richardson and *D. tessellaria* Poir. Unfortunately, in 2000, the last known individual of *D. angulata* went extinct lowering the number of endemic *Diospyros* species to eleven (pers observation).

The Ebenaceae, a widespread family of woody dicot trees and shrubs occur mainly in tropical and subtropical regions. This family is divided into three main genera, namely *Diospyros*, *Euclea* and *Tetraclis* (Brummit, 1992). *Diospyros* species seem to be more prevalent in regions of Asia, Africa and Central to South America while the genera *Euclea* and *Tetraclis* have been found to occur only in Madagascar, Eastern and Southern Africa (Cronquist, 1981; Ng, 1986). The genus *Diospyros* represented by more than 350 species is the most important one both numerically and economically (Mallavadhani *et al.* 1998).

Interestingly, this genus is the only representative of the Ebenaceae family in the Mascarene Islands.

Another important aspect of the *Diospyros* is the medicinal uses of various *Diospyros* species. Some 300 organic chemicals have been isolated and identified so far (Mallavadhani et al, 1998). The uniqueness of the genus is the elaboration of a large number of pentacyclic triterpenes and juglone based 1,4-naphthoquinone metabolites. These metabolites can be used as chemical markers for taxonomic studies and have antibacterial (Alake LB, 1994; Adeniyi et al, 2000), antiviral (Vlietinck et al, 1995), antimalarial (Likhitwitayawuid et al, 1999), antihypertensive (Kameda et al, 1987), antihelmintic (Maki et al, 1983), antiinflammatory (Recio et al, 1995), antifungal (Vu et al, 1994), antihaemorrhagic activities (Martz, 1992), antitumouric (Kapadia et al, 1997) among others. The potential for pharmaceutical exploitation of *Diospyros* is impressive.

Unfortunately there is a paucity of information concerning the biology and pharmaceutical potential of the Mauritian *Diospyros* species. Furthermore, given the grim survival outlook of these *Diospyros* species, there is an urgency to initiate projects that would lead to a better understanding of the biology of endemic plants with a view to enhance their survival.

In this study, we have attempted to understand important aspects of the biology of the *Diospyros* species and investigated some of their pharmaceutical properties with the hope that our findings will foster scientific interest vital for the conservation of these once dominant endemic species.

2.0 Distribution of *Diospyros* species

2.1 Background

Historical records of the approximate distribution of *Diospyros* in Mauritius date back to 1598 when the Dutch Admiral, Van Warwijck first landed in Mauritius. He reported the island to be literally submerged in a dense forest largely dominated by *Diospyros* species and identified a number of *Diospyros* on the coast that produced good quality timber (Pitot, 1905). From 1598 to 1710 the Dutch exploited all the accessible coastal forests exporting the good quality ebonies to Holland. During their stay in Mauritius, the succession of Dutch governors reported that the *Diospyros* species were found in great numbers on the eastern and some northern plains and coasts. The western part of the island was also described as supporting a large number of ebony plants while the regions around Port-Louis and part of the northern regions were said to be dominated by a palm savanna. Four distinct *Diospyros* species found in the east and north east areas were described by the Dutch Governor Lamotius. During the French occupation a report by Alexander Dalrymple indicated that the ebony trees were common everywhere (Rouillard and Guého, 1999). Furthermore, the notes kept by Dutch, French and British residents of the island, reveal that the *Diospyros* species were present in abundance from the coast line to the mountain tops (Pitot, 1905).

Nowadays, after a legacy of indiscriminate wood cutting and agricultural clearing, the situation is completely different with only pockets of small populations of *Diospyros* restricted to the mountain slopes and generally inaccessible areas. Consequently, little is known on the natural distribution of the *Diospyros* species as historical records only give a general sketch of the original native forest. Habitat diversity, elevation, rainfall, soil types as well as biological factors such as phenology and physiological adaptations have been suggested to influence species richness, distribution and dispersal (Verheyen *et al*, 1999; Aldasoro, 2004). It has also been suggested that biogeographic analyses provide a useful platform that draws on the above aspects to document patterns of diversity and analyse the role of environmental factors in shaping them (Crisci *et al*, 2003).

2.2 Materials and Methods

2.2.1 Data Collection

Herbarium specimens found at the Mauritius Sugar Industry Research Institute (MSIRI) were revisited to establish baseline information on the locations of the *Diospyros* species in Mauritius. Twelve extant species were identified: *D. angulata*, *D. boutoniana*, *D. chrysophyllos*, *D. egrettarum*, *D. hemiteles*, *D. leucomelas*, *D. melanida*, *D. neraudii*, *D. nodosa*, *D. pterocalyx*, *D. revaughanii* and *D. tessellaria*. However, in 2000, the only female tree representing *D. angulata* went extinct (personal observation). Additional information on field locations was obtained from the Forestry Department and the National Parks and Conservation Services. Whenever possible locations of populations were established using Global Positioning System (GPS) measurements.

The *Diospyros* species were reported from 173 different locations around Mauritius. Some of the major locations of the *Diospyros* species are indicated in figure 2.1. To understand their relationships to habitat and distribution, seven forest types based on the forest classification of Vaughan & Wiehe (1937) and Page & D'Argent (1997) were identified along with one marsh type and species distributions recorded within them. In addition, a variety of soil types as described by Parish and Feillafe (1965) were also found to be important in describing the distribution of the *Diospyros* species.

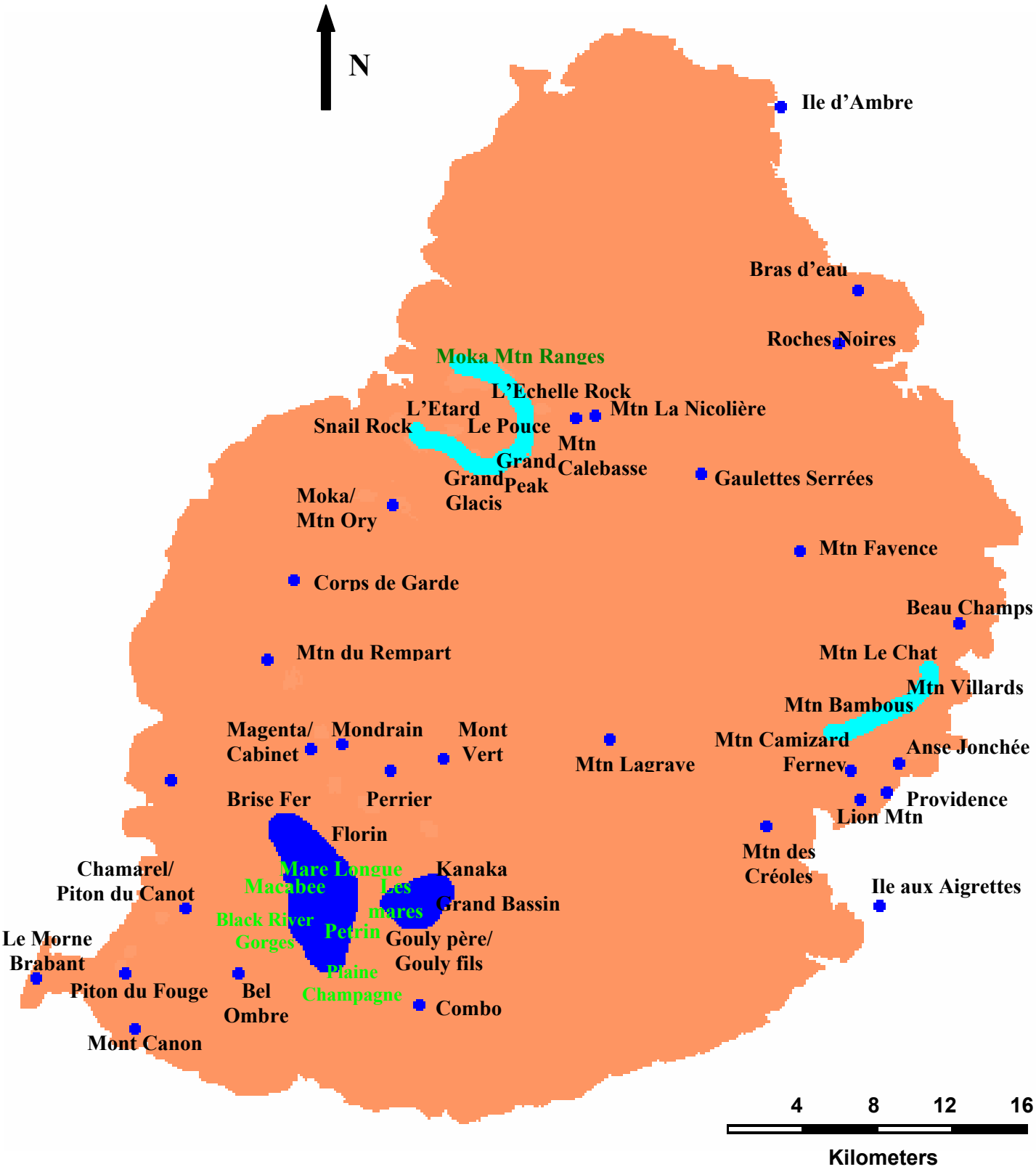


Figure 2.1 : Locations of the main populations of the *Diospyros* species in Mauritius
The regions where populations of the *Diospyros* species are encountered are indicated in this map.

2.2.2 Data Analysis

The software SPSS 13.0 was used to generate figures showing the abundance of the *Diospyros* species, their distribution with respect to mean annual rainfall and elevation above sea levels (Padya, 1984; 1989) altitudinal ranges of the *Diospyros* species, number and species of *Diospyros* encountered at different altitudinal zones, soil and forest types. The raw data are shown in appendix 2.1.

Using the GPS measurements of the *Diospyros* populations, a map showing their distribution was constructed with the software DIVA GIS (Hijmans *et al*, 2001; Jarvis *et al*, 2003; Serrato *et al*, 2004). This software was also utilised to analyse the data obtained in order to produce a map of the island indicating species richness in cells of $0.03^{o}X0.03^{o}$. Based on the distribution of the *Diospyros* populations, the software enabled calculation of the number of species present in each of the $0.03^{o}X0.03^{o}$ grids.

Two other maps with grids of $0.03^{o}X0.03^{o}$ were generated to grade the diversity of *Diospyros* species and populations in the different types of forest and soil. The Brillouin index which reflects the number of species and their abundance was used to calculate the diversity index. This was carried out to show the distribution of the soil and forest types that support *Diospyros* species in Mauritius and to assess their diversity.

It should be noted that regions where only a few isolated trees (without any forest structure) of *Diospyros* occur have not been included in the map where the forest diversity index is shown.

2.2.3 Flowering and Fruiting Periods

It is important to note that during the flowering periods, all mature females of the same species flower at the same time while the male plants produce flowers over a longer period of time.

The flowering and fruiting phenology of each of the *Diospyros* species were observed and recorded over a period of four years (see tables 2.1 and 2.2 for an average). Both male and female flowers were considered when in full bloom for the flowering period while fruit production was measured at maturity. Full bloom is considered when most of the branches are covered with flowers (5mm to 35mm in diameter).

2.2 Results

2.2.1 Distribution of the *Diospyros* Species in Mauritius

Each of the known populations of the twelve endemic *Diospyros* species is shown in figure 2.2. Only one species, *D. egrettarum* is restricted to the eastern coastal regions. Species such as *D. neraudii*, *D. nodosa* and *D. pterocalyx* showed a marked preference for upland habitats even though some isolated trees have been found on the mountains of the lowland regions. Areas where the *Diospyros* species are absent represent the land under anthropogenic use, where all the indigenous vegetations have been wiped out.

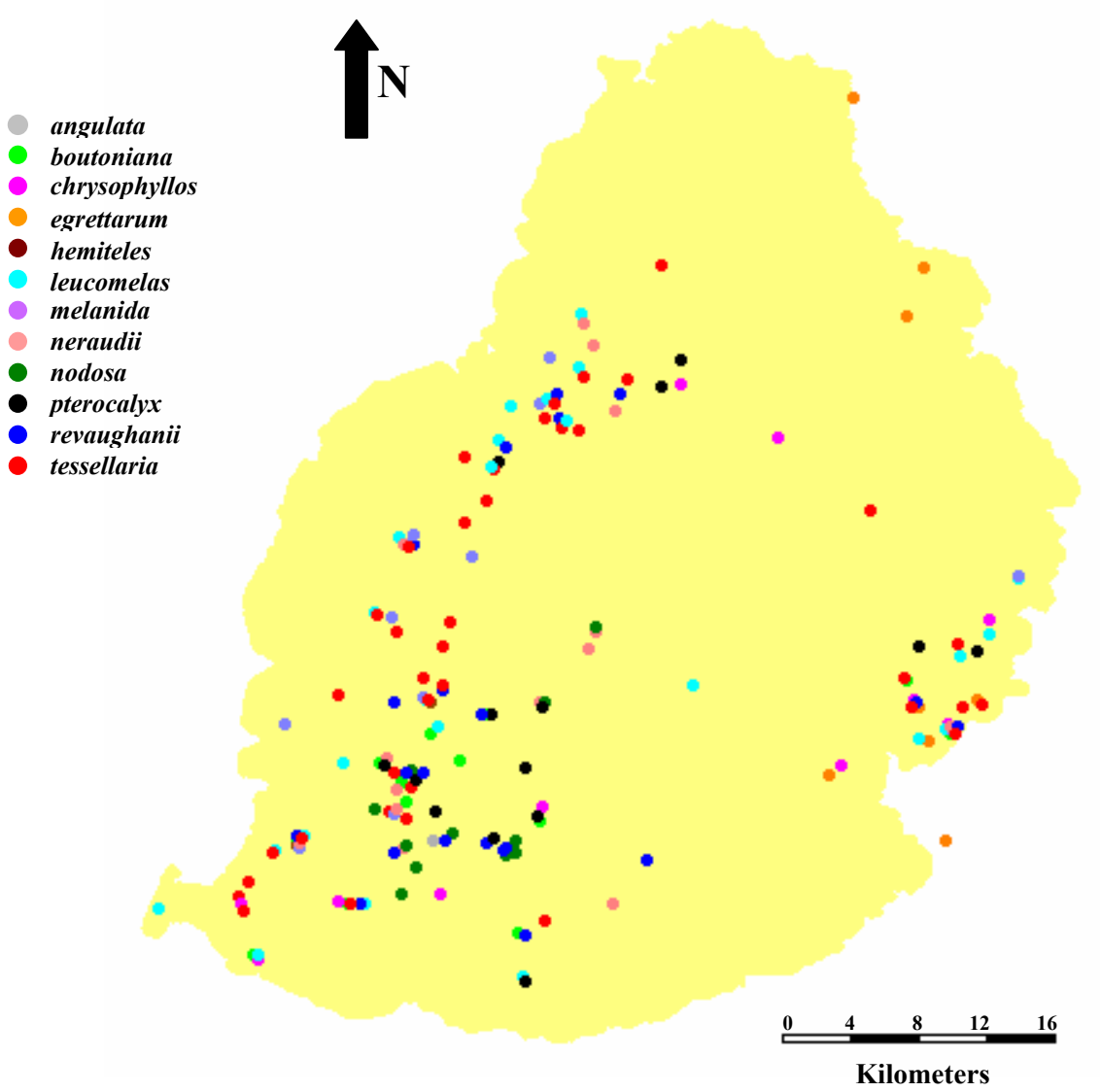


Figure 2.2 : Distribution of *Diospyros* species over Mauritius
This figure shows the recorded populations of all the *Diospyros* species.

Figure 2.3 complements the information in figure 2.2 and identifies species richness in the different geographical regions. For instance, it becomes clear that species richness is highest in two areas (in red). These are the humid region in the south west and a sub humid region in the east of the island. Six different *Diopsyros* species were present in some super humid and humid locations with progressively fewer in the other regions. Areas with 1-2 species indicate degraded regions that range from humid to dry climate.

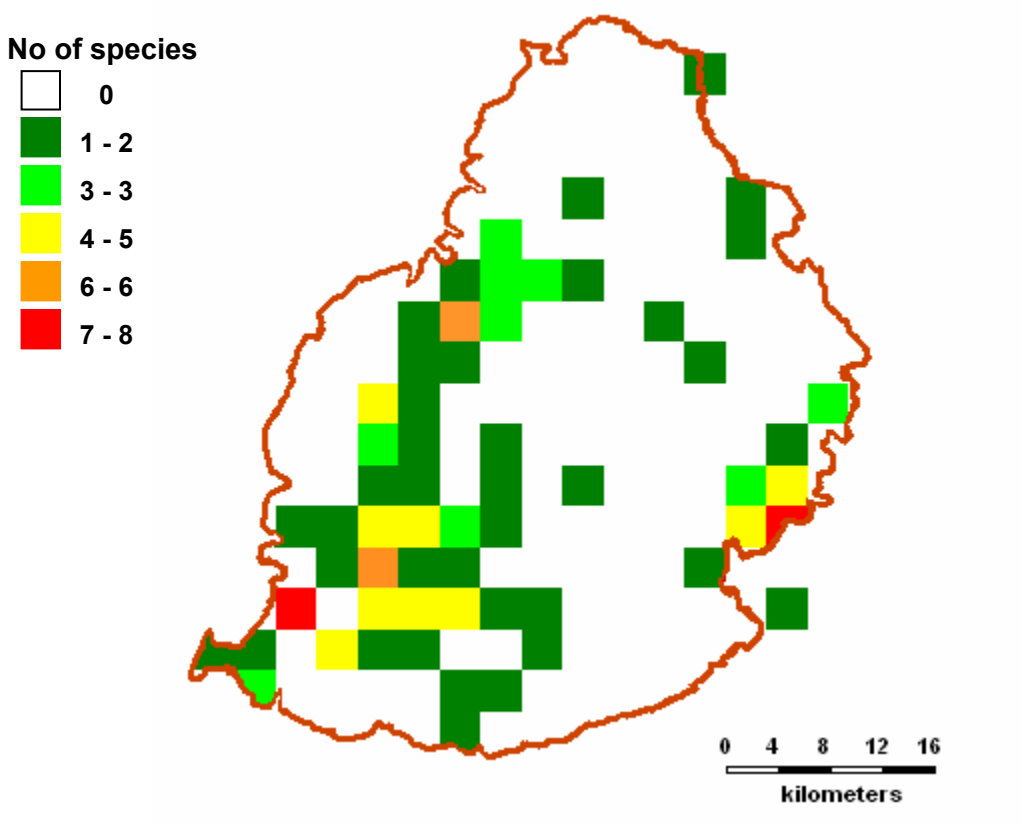


Figure 2.3 : Map showing *Diospyros* species richness in grids 0.03°X 0.03°
In this figure, grids have been superimposed on the various locations to indicate the number of *Diospyros* present in the different regions of Mauritius.

A rank-abundance chart shown in figure 2.4 illustrates the overall status of the *Diospyros* species. Most species are endangered and the majority of the populations consist of only a few individuals. The rarest species, *Diospyros hemiteles* is critically endangered with only one reproductively viable population in Chamarel and one female individual inhabiting the Cabinet state land. *Diospyros egrettarum* can be encountered in five locations on the east coast only.

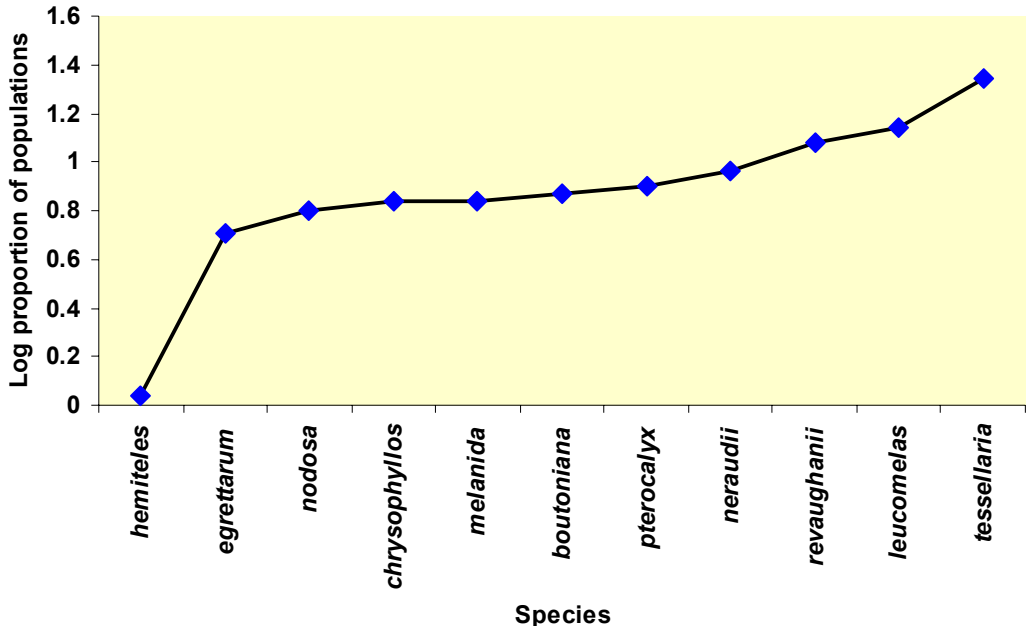


Figure 2.4 : Rank-abundance chart
This chart indicates the abundance of each of the eleven extant *Diospyros* species.

Diospyros tessellaria seems to be the most successful species followed by *D. leucomelas* and *D. revaughanii* as these species have multiple populations and individuals. The altitudinal zones occupied by the *Diospyros* species are shown in figure 2.5 and the number of species at each altitudinal range is also shown in figure 2.6. The species with the broadest elevational ranges are *D. tessellaria* followed by *D. leucomelas* and *D. revaughanii*. Altitude data indicate that most of the *Diospyros* species exist in a relatively broad altitudinal range. *Diospyros hemiteles* is an exception as it has been found only at mid altitude elevations while populations of *D. nodosa* are found only at high elevations on the central plateau. A peak in the number of *Diospyros* species and populations was observed in the mid altitude areas.

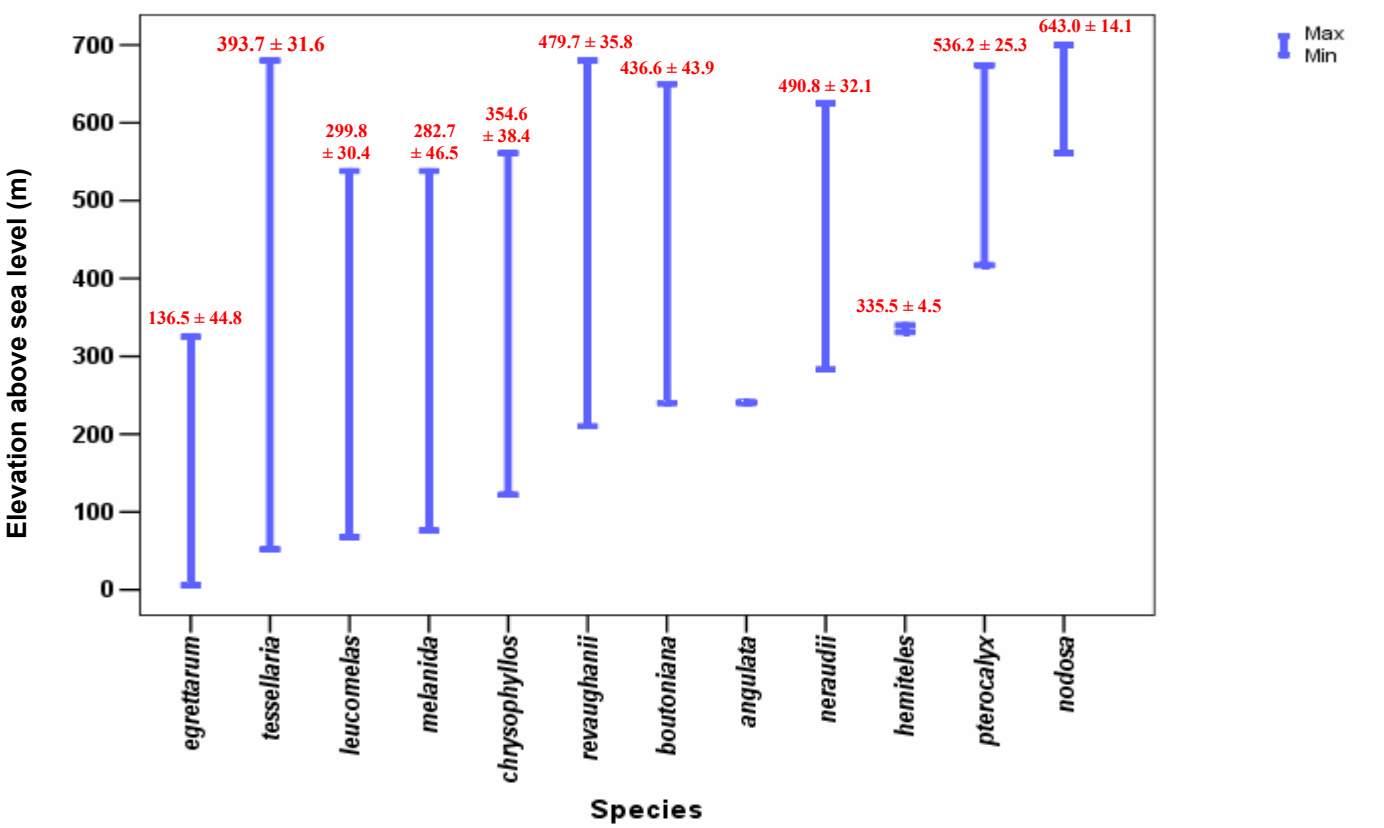


Figure 2.5 : Altitudinal ranges of the *Diospyros* species
The altitudinal ranges occupied by the twelve *Diospyros* species are shown. Standard error values are given in red.

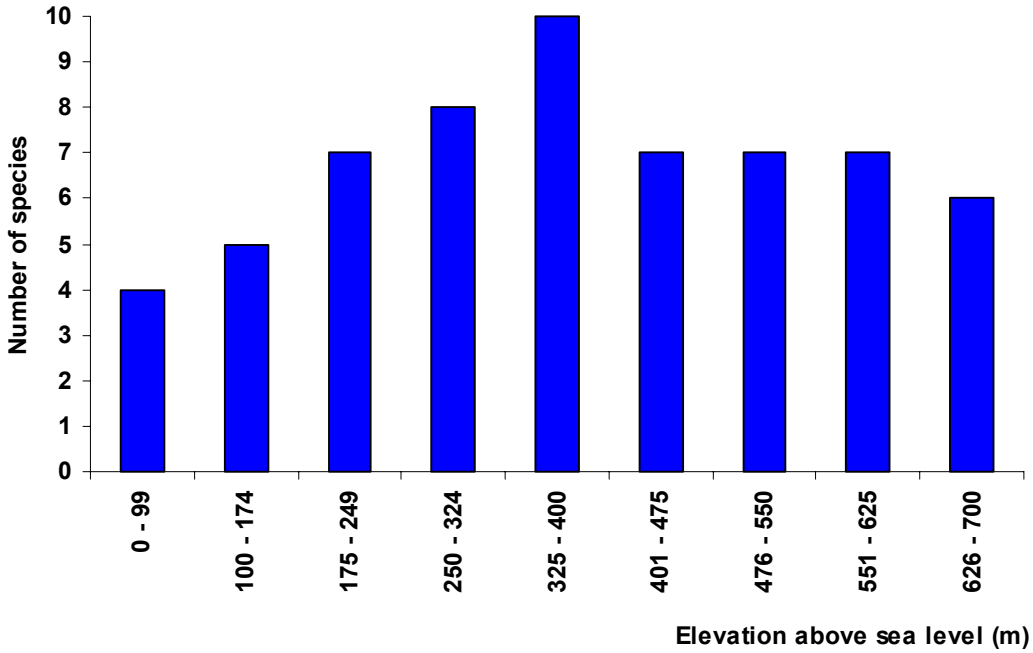


Figure 2.6 : Frequency of the *Diospyros* at different altitudinal ranges
The number of *Diospyros* species present in the different altitudinal ranges are indicated.

In the low altitude habitats (0-99m), *D. egrettarum*, *D. leucomelas*, *D. melanida* and *D. tessellaria* were found in the dry and dry evergreen forest on the lithosols, latosolic reddish prairie, mountain slope complexes, dark magnesium clays and low humic latosols. In the range of 100-174m, *D. boutoniana*, *D.chrysophyllos*, *D. leucomelas*, *D. melanida* and *D. tessellaria* were encountered in the dry evergreen forests located on the mountain slope complexes, lithosols and dark Magnesium clays. At an elevation of 175-249m, *D. chrysophyllos*, *D. egrettarum*, *D. leucomelas*, *D. melanida*, *D. revaughanii*, *D. tessellaria* and the now extinct individual of *D. angulata* were growing in dry evergreen and transitional forests that are situated on the dark Magnesium clays, mountain slope complexes, humic latosols and lithosols. The species *D. boutoniana*, *D.chrysophyllos*, *D. egrettarum*, *D. leucomelas*, *D. melanida*, *D. neraudii*, *D. revaughanii* and *D. tessellaria* were recorded at the altitudes of 250-324m in the dry evergreen and transitional forests found on the mountain slope complexes, humic latosols, latosolic brown forest, low humic latosols and lithosols. The highest number of species was recorded between 325m and 400m in dry evergreen, transitional and *Sideroxylon* formation/ridge forests covering the mountain slope complexes in addition to the lithosols and to a lesser extent the low humic latosols, humic latosols and latosolic brown forest soils. These ten species are *D. boutoniana*, *D. chrysophyllos*, *D. egrettarum*, *D. hemiteles*, *D. leucomelas*, *D. melanida*, *D. neraudii*, *D. pterocalyx*, *D. revaughanii* and *D. tessellaria*. In the range of 401m to 475m, *D. chrysophyllos*, *D. leucomelas*, *D. melanida*, *D. neraudii*, *D. pterocalyx*, *D. revaughanii* and *D. tessellaria* were growing in dry evergreen, transitional and *Sideroxylon* formation/ridge forests that have developed on mountain slope complexes, dark magnesium clays, lithosols, low humic latosols, latosolic brown forest soils. *Diospyros boutoniana*, *D. leucomelas*, *D. melanida*, *D. neraudii*, *D. pterocalyx*, *D. revaughanii* and *D. tessellaria* were found between 476m and 550m in dry evergreen, transitional and *Sideroxylon* formation/ridge forests on the soil types mountain slope complexes, lithosols, latosolic brown forest, latosolic reddish prairie, low humic latosols and dark Magnesium clays. At 551-625m, *D. boutoniana*, *D. chrysophyllos*, *D. neraudii*, *D. nodosa*, *D. pterocalyx*, *D. revaughanii* and *D. tessellaria* were observed in the cloud, mossy rainforest, climax montane forest growing on the humic ferruginous soil, the *Sideroxylon* formation/ridge developed on the latosolic brown forest, the dry evergreen and transitional forests located on the mountain complexes, lithosols and the marshes situated on the ground water laterites. Six species, *D. boutoniana*, *D. neraudii*, *D. nodosa*, D. pterocalyx, *D. revaughanii* and *D. tessellaria* were observed in the range 626-700m. These species occurred in the climax montane rainforest and the mossy rainforests located on humic

ferruginous latosols in addition to the marshes found on the hydromorphic soil and the ground water laterites. Except for the marsh community of Florin, all the other marsh forests are found in this altitudinal range. In Figure 2.7, the populations of the *Diospyros* species have been plotted with respect to the amount of annual rainfall received and their elevations above sea level. The highest number of *Diospyros* species and populations were found between 200m and 500m above sea level in regions receiving 1500mm to 2000mm of rain annually. Most of the mountain slopes are found in this altitudinal and humidity range. A smaller cluster of *Diospyros* populations were observed in areas with altitudes of 550m to 600m with a mean annual rainfall of 3500mm to 4500mm.

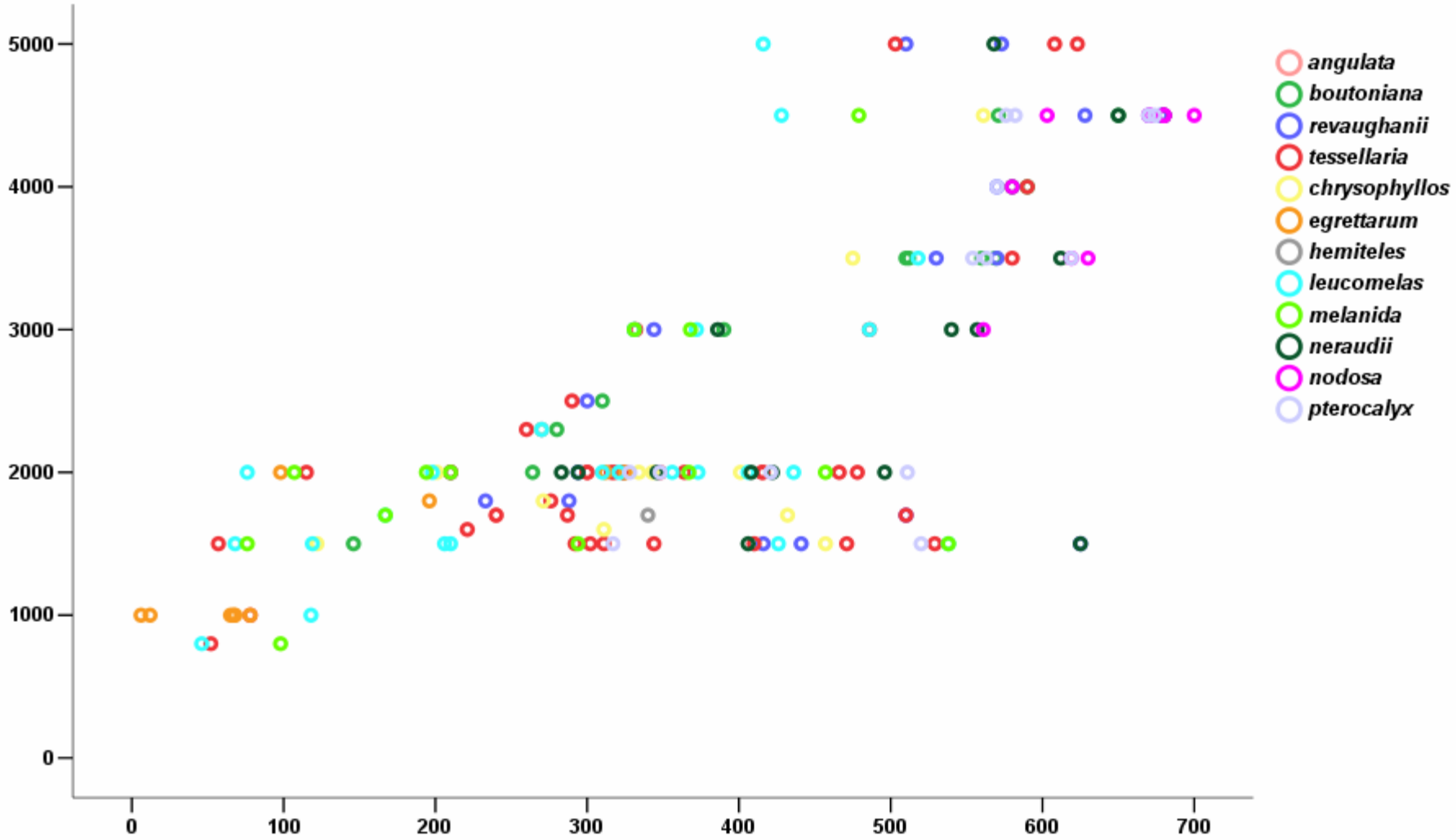


Figure 2.7 : Scatter plot of rainfall and altitude with respect to the populations of the *Diospyros* species
The markers of the scatter plot represent the *Diospyros* populations and the above figure indicates the altitudinal and humidity ranges preferred by the *Diospyros* species.

2.2.2 Diversity of *Diospyros* Species in Different Forest Types

Figure 2.8 shows the diversity of *Diospyros* species in the different types of forests encountered in Mauritius.

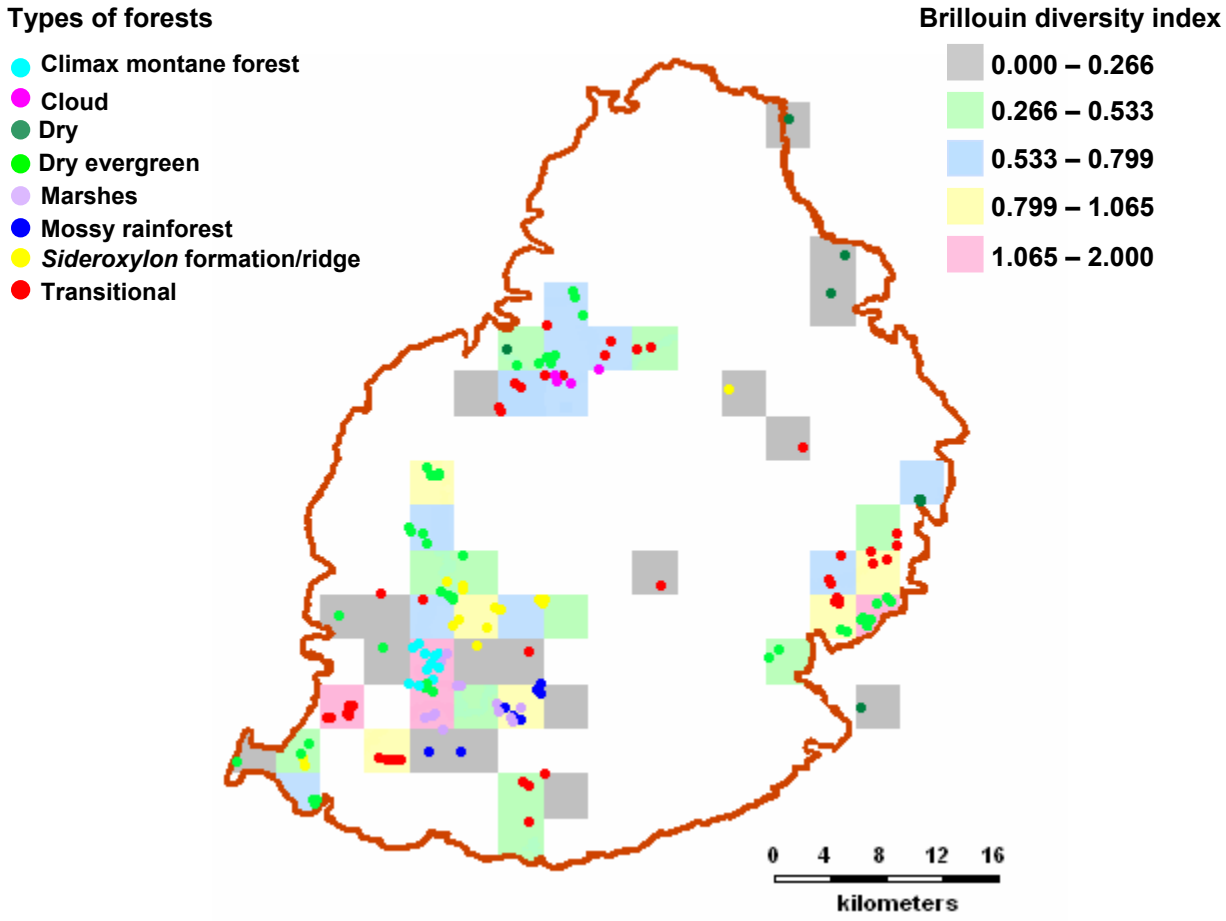


Figure 2.8 : Diversity of *Diospyros* species in the different types of forests
The different types of forest are superimposed in grids of 0.03° X 0.03° whose colours reflect the Brillouin indices for the diversity of *Diospyros* in these locations.

The highest diversity index (pink grids) was observed in the transitional forests of the Chamarel region, encompassing the climax montane forests of Macabée, the dry evergreen forest of Black River Gorges, the marsh forest of Plaine champagne and the dry evergreen forests of Providence, Anse Jonchée in the east.

The second highest diversity index (yellow grids) was observed in the following six grids:

1. Transitional forest of Bel ombre.
2. Mossy rainforest of Kanaka crater and Grand Bassin, the marsh forest of Les Mares, Gouly fils and Gouly Père.

3. *Sideroxylon* formation/ridge forest of Tamarin falls/reservoir and Perrier.
4. Dry evergreen forest of Montagne Corps de Garde.
5. Transitional forest of Ferney and the dry evergreen forest of Lion Mountain in the east.
6. Transitional forest of Mountains Bambous and Villards.

The third highest diversity index (blue grids) includes:

1. Dry evergreen forest at Mont Canon in the south and Montagne du Rempart in the west.
2. Transitional, dry evergreen and cloud forests on the Moka mountain range.
3. Transitional forests on Montagne Ory and Guibies peak.
4. Dry forest at Beau Champs and transitional forest at Ferney in the east.
5. Transitional forest and dry evergreen forest at Grosse Roche and Cabinet/Magenta.
6. *Sideroxylon* formation/ridge forest at Perrier and Mondrain Nature Reserve.
7. Marsh forest of Pétrin and Plaine Champagne.

The fourth highest diversity index (green grids) was measured in the

1. Dry evergreen and sideroxylon/formation ridge forests on mountains in the south-west and in the centre.
2. Dry evergreen forests on the mountain peaks near Port-Louis and Lion Mountain in the east.
3. Transitional forests on the mountain slopes of La Nicolière in the north, montagnes Bambous and Villards in the east, at Combo and two mountain peaks in the south.

The lowest diversity index (grey grids) was observed mostly on the dry and some dry evergreen forests in the west, transitional forests in the west, centre and east where there are some villages and mossy rainforest in the centre.

The highest number of species and populations are located in the transitional and dry evergreen forests. The transitional forests are found on the humid mountains and hills while the dry evergreen forests are encountered on mountains, hills, gorges and valleys in the dry to sub humid regions. The climax montane rainforests and the mossy rainforests which represent the relatively good forest canopy of the super humid regions have significantly fewer species and populations as they cover only a small area of the central plateau. The *Sideroxylon* formation/ridge forests which are found in the sub humid to humid regions support both upland and lowland species and are the most favoured habitats of the *Diospyros* species after the dry evergreen and transitional forests. Only four species, *D. neraudii*, *D. nodosa*, *D, pterocalyx* and *D. revaughanii* occur in the marsh forest. The cloud forests are found on the few high (from 568 to 608m) mountain tops and harbour a few trees of *D. neraudii*, *D. revaughanii* and *D. tessellaria*. The dry forests are coastal and are found

mainly on the east coast. Three species, *Diospyros egrettarum*, *D. leucomelas* and *D. melanida* can be found in this type of forest with populations of *D. egrettarum* being the most abundant. Species of the dry forest are not present in the marsh forest and vice versa.

2.2.3 Diversity of *Diospyros* Species in Different Soil Types

The diversity indices of figure 2.9 are slightly different from those of figure 2.8 because the isolated trees which cannot be classified in any forest types have been excluded from figure 2.9. However, these *Diospyros* trees have been mapped in figure 2.9, thus increasing the diversity indices in some areas.

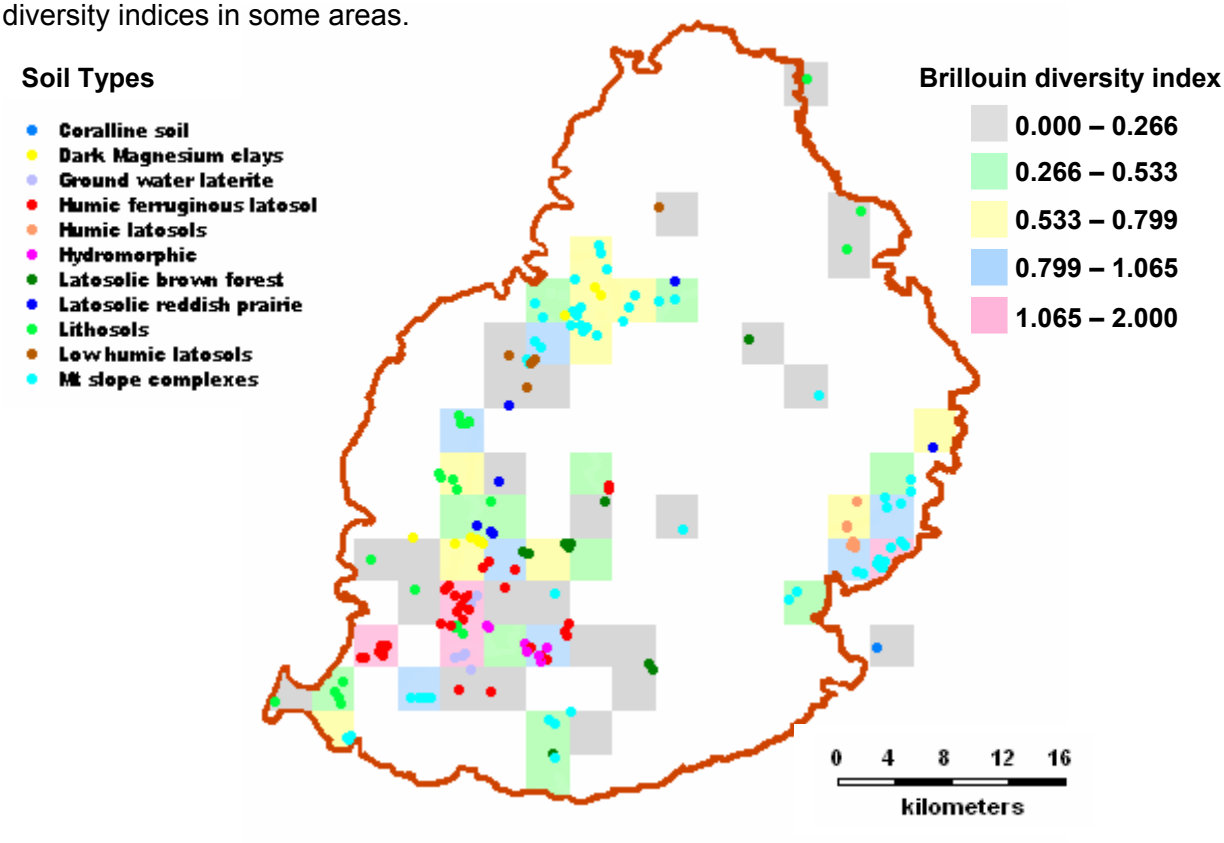


Figure 2.9: Diversity of *Diospyros* species in the different types of soil
The different types of soil are superimposed in grids of 0.03° X 0.03° whose colours reflect the Brillouin indices for the diversity *Diospyros* in these locations.

According to figure 2.9, the highest diversity of *Diospyros* species and populations was found in the following areas (pink grids):

1. Chamarel area (humic ferruginous latosols).
2. regions of Black River gorges (lithosols), Plaine champagne (ground water laterites) and Macabée (humic ferruginous latosols).

3. area encompassing Providence and Lion Mountain (mountain slope complexes) in the east of Mauritius.

The second highest diversity index (blue grids) was found in the following regions:

1. Moka/Ebene region (low humic latosols) and the mountain slopes near Moka (mountain slope complexes).
2. Perrier (latosolic brown forest) and the Tamarin falls, Plaine sophie, Mare aux Vacoas (Humic ferruginous latosols) areas.
3. Mondrain area (latosolic reddish prairie) and the cabinet state land/Magenta valley (dark Magnesium clays).
4. Brise fer, Mare longue and Macabée plateau (humic ferruginous latosols).
5. Kanaka crater (humic ferruginous latosols) and Gouly père/Gouly fils (hydromorphic).
6. Corps de Garde mountain and the region around (lithosol).
7. Mountain slopes in the east (Mountain slope complexes).

The third highest diversity index (yellow grids) was recorded on the:

1. Mountain slope complexes in the north, west and south of the island.
2. Humic latosols in the eastern regions.
3. Lithosols, dark magnesium clays and humic ferruginous latosols in the west.
4. Hydromorphic soils in the centre.

The fourth highest diversity index (green grids) represents mainly:

1. Some mountain slope complexes in the north, east and south of the island.
2. Lithosols in the west and centre.
3. Latosolic reddish prairie in the centre and north.
4. Latosolic brown forest in the centre and south.
5. Humic ferruginous latosols in the centre of Mauritius.

The lowest diversity index (grey grids) was found on the mountain peaks, the two islets in the east of Mauritius and in the inhabited areas where a few indigenous trees still persist. These areas occur in all the types of soil.

The populations of the *Diospyros* species occurring on the latosolic brown forest are few although some individuals of eight different *Diospyros* species have been found in these soils. The lithosols are the relatively rich but dry soils found on the steeper slopes of the mountains, gorges and the rocky lands. Only populations of multistemmed *D. egrettarum* have been encountered in the dry, hot and very degraded habitats on the rocky lands. The latosolic reddish prairie represents the fertile and well drained soils found in the sub humid and drier regions. These soils are either under agriculture or town/villages. Only a few

Diospyros individuals are left in the patches of degraded forests that have been spared. The humic latosols occur in the sub humid regions close to the humic ferruginous latosols or the latosolic brown forest soils. These soils do not cover large areas and are mostly under anthropogenic use except for a few fragmented forests.

The dark magnesium clays have developed only in a few regions located in the Western part of the island. Most of these locations have been wiped out of their indigenous cover and only some *Diospyros* species can be found near Port-Louis (the smallest district harbouring the capital of Mauritius) and the valley of Magenta/Cabinet state land where access is restricted and endemic trees are no longer felled. The low humic latosols can be found in the sub humid regions where agricultural cultures, towns and villages have been established. Consequently, except for a few plants that have somehow been left unharmed, the other *Diospyros* trees have all been cut down. The ground water laterites and the hydromorphic soils are marshy lands where the trees are dwarfs. Only some *D. neraudii*, *D. nodosa*, *D. pterocalyx* and *D. revaughanii* are adapted to water logged soils. On the other extreme, the shallow and poor coralline soil of Ile aux Aigrettes supports around fifty different indigenous plants including some 400 individuals of *D. egrettarum*.

2.2.4 Flowering and Fruiting Phenology

Tables 2.1 and 2.2 show the flowering and fruiting periods of the *Diospyros* species studied. The flowering ranges shown in table 2.2 reflect the time period when both female and male flowers were produced at the same time. Some species like *D. egrettarum*, *D. leucomelas*, *D. melanida*, *D. revaughanii* and *D. tessellaria* flower for longer periods. Species that are found in close proximity such as *D. boutoniana*, *D. neraudii*, *D. pterocalyx*, *D. revaughanii* on the upland regions and *D. leucomelas*, *D. melanida*, *D. hemiteles* on mid altitude habitats produce flowers in a staggered way. As flowering phenology was recorded only on one female plant of *D. angulata*, the true flowering period of this species cannot be determined.

It should be noted that in most of the species, small bursts of male flower production were observed two to three times a year even when the female flowers were not present in the populations. These erratic flowering episodes of the male trees are not included in the table.

Table 2.1 : Flowering phenology of the *Diospyros* species

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
D. angulata												
D. boutoniana												
D. chrysophyllos												
D. egrettarum												
D. hemiteles												
D. leucomelas												
D. melanida												
D. neraudii												
D. nodosa												
D. pterocalyx												
D. revaughanii												
D. tessellaria												

Table 2.2 : Fruiting phenology of the *Diospyros* species

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
D. angulata												
D. boutoniana												
D. chrysophyllos												
D. egrettarum												
D. hemiteles												
D. leucomelas												
D. melanida												
D. neraudii												
D. nodosa												
D. pterocalyx												
D. revaughanii												
D. tessellaria												

Observations during the flowering periods especially among the *Diospyros* populations on Bras d'Eau, Brise fer, Chamarel, Ile aux Aigrettes, Magenta/Cabinet and Perrier Nature Reserve, indicated that the flowers of the *Diospyros* species were visited by insects ranging from honey bees and other hymenopteran insects, dipteran flies, coleopteran beetles and small butterflies and moths (Lepidoptera). Small coleopteran beetles (approximately 0.5cm in length) were observed in the male and female flowers of all species of *Diospyros*. The flowers of *D. tessellaria* were observed to exert a more powerful attraction on all the above insects compared to the other *Diospyros* species. Fruiting followed flowering phenology in a

predictable sequence. Fruit development takes approximately two weeks. For populations of *D. tessellaria* which occur at all altitudinal ranges, flowering and fruiting occur over a period of 4 months. Other species like *D. egrettarum*, *D. leucomelas, D. melanida* and *D. revaughanii* produce fruits over a period of 3 months. The last group is made up of *D. boutoniana, D. chrysophyllos, D. hemiteles, D. neraudii* , *D. nodosa* and *D. pterocalyx* and they bear fruits over a period of 2 months.

3.0 Morphology and Phylogeny

3.1 Background

Morphological studies of the Mascarene *Diospyros* species were carried out as early as 1804 (Richardson, 1980), and these were identified and classified into 14 distinct species, twelve of which were found to be endemic to Mauritius, one to Reunion and another one to Rodrigues. Although the Mascarene *Diospyros* species have been collected for herbaria, no information is available on their colonisation patterns or adaptive radiation. As these species are endangered, there is an urgency to understand the biology of the remaining *Diospyros* species. Findings are likely to be invaluable for conservation and reforestation.

The purpose of this study was to analyse selected morphological characters with a view to establish the phylogenetic relationships within the Mascarene *Diospyros* species.

3.2 Materials and Methods

For each species, thirty-five stable morphological characters were selected based on their presence in all the species and their potential for phylogenetic informativeness (table 3.1). In the case of *D. angulata* measurements were taken from plant materials we collected in 2000 while other characters were obtained from herbarium (Mauritius Sugar Industry Research Institute- MSIRI) specimens as this species is now believed to be extinct in the wild. Data, which could not be obtained from live specimens of *D. borbonica* (Reunion), were complemented with measurements from herbarium (MSIRI) specimens. Measurements for *D. kaki*, that is a native of Asia has been used as outgroup as adult plants of this species are found in Mauritius with flowers and fruits which were readily available at the time of study.

The morphological characters (12 vegetative and 23 reproductive) were scored and a data matrix was constructed for each of the fourteen species (Matrix I). This morphological data matrix contained two taxa that were polymorphic for one character and 3% of missing data.

Matrix I

D.kaki	1111222110011020002?01?0121?2110202
D.angulata	2000340110220??????11??0?1?2132211
D.boutoniana	200034010022031212101112011221320 11
D.borbonica	1020230200010102121110120112122221 1
D.chrysophyllos	0021222200010210100111100001112221 1
D.diversifolia	1020000110011002130010020122112221 1
D.egrettarum	1130230011100121121201111211011021 1
D.hemiteles	1021112210011003230210130311110121 1
D.leucomelas	1130230011100122121201121211021021 1
D.melanida	1032121210011003120210230311112221 1
D.neraudii	0010110200011202111110020122112111 1
D.nodosa	(0,1)1111112000112011111101101221120111
D.pterocalyx	0111001210011001111110010122110111 0
D.revaughanii	(0,1)1302220111000211211011112121211011
D.tessellaria	2001220200010010001111000000100120 2

Table 3.1: Morphological characters and character states used in the maximum parsimony analysis of the Mascarene *Diospyros* species

Morphological characters	Character States				
	0	**1**	**2**	**3**	**4**
Tree height (m)	2-6	7-12	13-20		
No of tree trunk	single	multiple			
Bark colour	Black	Nearly black	Greyish black	Grey	
Leaf:					
Colour	Dark green	Green	Greyish Green		
Length (cm)	3-6	7-10	11-16	20-30	
Width (cm)	1-3	4-6	7-9	10-12	13-15
Shape	Elliptic	Oblong	Oval		
Base	Cordate	Cuneate	More or less rounded		
Tip	Acute	Obtuse			
Texture	Sub-leathery	Leathery			
Petiole thickness (mm)	20-25	30-35	40-45		
Petiole length (mm)	1.5-1.9	2-10	11-20		
Male flowers:					
Clusters	Aggregate	Solitary			
Calyx length (mm)	5-6	7-8	9-10	10-11	
Calyx shape	Cupuliform	Ovoid	Cupuliform-cylindrical		
Corolla diameter (mm)	5-9	10-15	16-22	25-35	

Morphological characters	Character States				
	0	**1**	**2**	**3**	**4**
No of corollar lobes	4	5-6	7-8		
No of stamens	9-15	16-26	27-40	60-70	
Anther length (mm)	3.0-4.9	5.0-7.0			
Filament length (mm)	1	2	3	4	
Female flowers:					
Clusters	Aggregate	Solitary			
Calyx shape	cupuliform	More or less spherical			
Calyx diameter (mm)	5-6	7-8	9-10		
Corolla diameter (mm)	7-9	10-12	15-22	25-35	
No of stigmas	4-6	7-8			
Staminodes	2-10	11-19	20-24	25-40	
Surface of calyx	Dense hairs	Sparse hairs	Absence of hairs		
Flower fragrance	Very fragrant	Fragrant	Very slightly fragrant		
Fruit:					
Length	2.5-3.0	3.1-4.0	4.1-5.0		
No of Calyx lobes	4	5	6-7		
Calyx height (mm)	3.0-4.0	10-15	16-18	20-25	
Shape	ovoid	ellipsoid	spherical		
Outer surface	Very sticky	Sticky	Not sticky		
Texture when mature	Soft	Hard			
Wings on calyx	Very pronounced	Less pronounced	No wings		

All characters were treated as independent, unordered and of equal weight. The phylogenetic analysis was performed with PAUP 4.0b. The most parsimonious trees were found using a heuristic search with 100 random stepwise additions and MULTREES option. Branch lengths for the trees were calculated using the ACCTRAN (accelerated transformation optimisation) option in PAUP. A strict consensus tree was then constructed from the most parsimonious trees obtained. Bootstrap analyses (100) using simple stepwise additions were conducted to examine the relative level of support for individual clades of the cladograms (Felsenstein, 1985).

3.3 Results

Cladistic analysis of the morphological data generated five most parsimonious trees, with a length of 125, a consistency index (CI) of 0.57, a homoplasy index (HI) of 0.43, a retention index (RI) of 0.63 and a rescaled consistency index (RC) of 0.36. One of the five trees was arbitrarily selected and shown with the synapomorphies along the branches in figure 3.1.

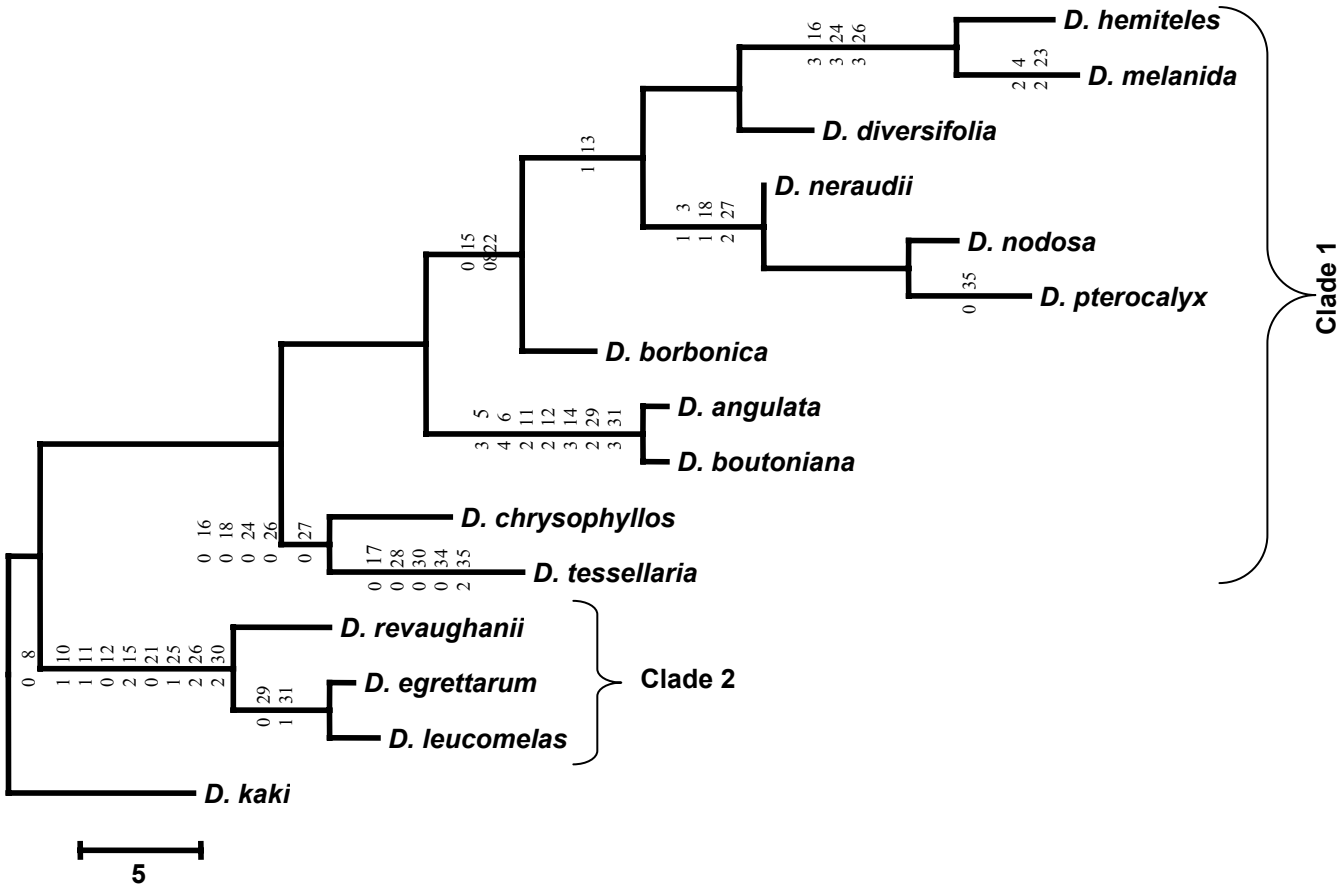


Figure 3.1: One of the five cladograms generated from the maximum parsimony analysis of 35 equally weighted morphological characters (CI= 0.57, HI= 0.43, RI= 0.63, RC= 0.36). The synapomorphies and their character states are indicated above and below the branches respectively.

The fourteen *Diospyros* species are divided into two major clades. Clade 1 consists of *D. chrysophyllos*, *D. tessellaria*, *D. angulata*, *D. boutoniana*, *D. borbonica*, *D. diversifolia*, *D. neraudii*, *D. nodosa*, *D. pterocalyx*, *D. hemiteles* and *D. melanida*. Clade 2 is made up of *D. revaughanii*, *D. egrettarum* and *D. leucomelas*. The relative positions of the species in clade 1 vary slightly among the five trees while the order of clade 2 species is identical for all the cladograms. In three of the cladograms, *D. chrysophyllos* and *D. tessellaria* are grouped as

sister species in a subclade which collapsed in the other two trees. *Diospyros angulata* and *D. boutoniana* are represented as sister species in all five trees and their relative positions only differ in one cladogram. The order of the minor clade consisting of *D. neraudii*, *D. nodosa*, *D. pterocalyx* are similar in all the cladograms and are shown to be among the last species to have evolved. *Diospyros hemiteles* and *D. melanida* are also indicated as recent species except for one cladogram where both sister species are shown to have appeared just before *D. chrysophyllos* and *D. tessellaria*. *Diospyros borbonica* in Reunion Island and *D. diversifolia* in Rodrigues Island seem to have emerged after the lineage consisting of *D. angulata and D. boutoniana* in all five trees. Figure 3.1 also provides some information on the accumulated morphological changes for each of the fourteen Mascarene *Diospyros* species.

Clade 2 is supported by cordate leaf base, leathery leaf texture, intermediate petiole thickness, shortest petiole length, cupuliform-cylindrical male flower calyx shape, aggregate female flower cluster, 7-8 stigmas, 20-24 staminodes and 6-7 fruit calyx lobes (characters 8, 10, 11, 12, 15, 21, 25, 26, 30 respectively). Within clade 2, the species *D. egrettarum*, *D. leucomelas* can be grouped together by the shortest fruit length and a calyx height of 10-15mm (characters 29 and 31 respectively).

Within clade 1, the node leading to *D. angulata* and *D. boutoniana* is supported by the broadest leaf, thickest petiole and longest leaf, petiole, flower calyx, fruit, fruit calyx (characters 6, 11, 5, 12, 14, 29, 31). The clade consisting of *D. neraudii*, *D. nodosa*, *D. pterocalyx* is typified by nearly black bark and 16-26 stamens (characters 3, 18) while the fruit calyx of *D. pterocalyx* is characterised by very pronounced wings (character 35). The species *D. chrysophyllos* and *D. tessellaria* share a number of similarities namely, 9-15 stamens, the smallest corolla diameter in both male and female flowers, 2-10 staminodes in the female flowers, the presence of dense hairs on the surface of the flower calyx (characters 18, 16, 24, 26 and 27). *Diospyros tessellaria* is however quite distinct and can be separated from the other species by having the smallest number of corolla lobes and fruit calyx lobes (characters 17, 30), very fragrant flowers, fleshy fruits and no wings on the fruit calyx (characters 28, 34, 35). *Diospyros chrysophyllos*, *D. tessellaria*, *D. boutoniana* have an ovoid shape male calyx (character 15) while *D. tessellaria*, *D. boutoniana* and *D. angulata* have characteristic black barks (character 1). *Diospyros diversifolia*, *D. hemiteles*, *D. melanida*, *D. neraudii*, *D. nodosa* and *D. pterocalyx* all have a solitary male flower cluster (character 13) while only the species *D. neraudii*, *D. nodosa* and *D. pterocalyx* are characterised by the absence of hairs on the surface of the flower calyces (character 27). *Diospyros borbonica* together with *D. diversifolia*, *D. hemiteles*, *D. melanida*, *D. neraudii*, *D. nodosa* and *D.*

pterocalyx have cupuliform male and female flower calices in common (characters 15, 22). *Diospyros hemiteles* and *D. melanida* typically have the largest male and female flower corolla (characters 16, 24) and 20-24 staminodes in the female flowers (character 26). *Diospyros melanida* can be distinguished by its greyish green leaves and largest female flower calyx diameter (characters 4, 23). *Diospyros hemiteles* on the other hand exhibit the highest number of corolla lobes (character 17).

Figure 3.2 represents a strict consensus tree with bootstrap values of more than 50%. Bootstrap estimates were relatively good for some basal nodes but low bootstrap values for the other nodes and the ambiguity in the exact order of some species resulted in the polytomies observed in the consensus tree.

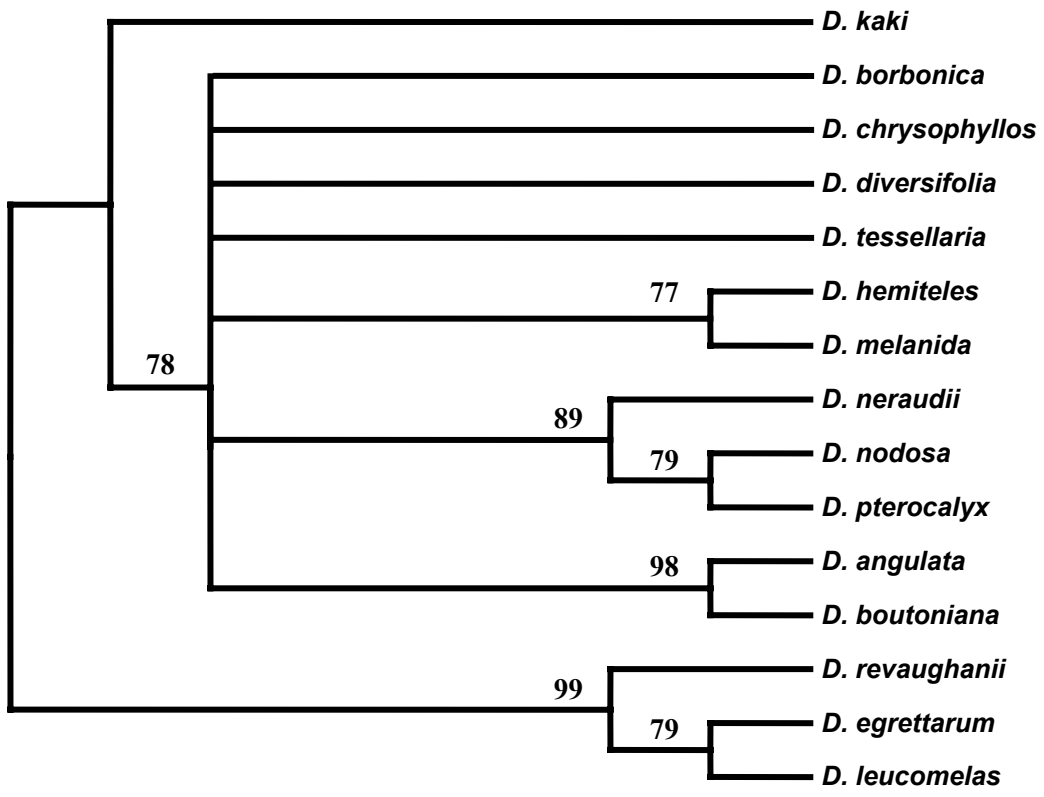


Figure 3.2: Strict consensus tree of the five most parsimonious trees obtained. The figures above the branches are the bootstrap values.

According to figure 3.2, there is strong support that the species *D. revaughanii*, *D. egrettarum*, *D. leucomelas* are among the most ancient and are morphologically different from the rest of the Mascarene species. Moreover, the upland species *D. neraudii*, *D. nodosa*

and *D. pterocalyx* are also shown to be closely related. The consensus tree also suggests that *D. hemiteles* and *D. melanida* which grow in the same habitat can be considered as sister species while *D. angulata* and *D. boutoniana* seem to share many similarities. However, *D. chrysophyllos, D. tessellaria, D. borbonica*, and *D. diversifolia* could not be placed in any exact order.

4.0 Leaky Dioecy in Diospyros egrettarum

4.1 Background

Oceanic islands have provided unique environments for the study of dioecy which refers to the existence of separate male and female individuals in natural plant populations (Bawa, 1982; Baker and Cox, 1984; Sakai *et al.,* 1995a). For instance, compared o the 6% of flowering plant species which are known to exhibit dioecy worldwide (Renner and Ricklefs, 1995), the estimated dioecious flora are believed to be 14.7% for Hawaii (Sakai *et al.,* 1995b), 12 to 13% for New Zealand (Godley, 1979) and 9% for Juan Fernandez Islands (Anderson *et al.,* 2000). In Mauritius little is known on the occurrence of leaky dioecy in the endemic dioecious plants. Nevertheless, an early study conducted by Baker in 1877 indicated that 11% of the Mauritian endemic plants were dioecious while De Cordemoy (1895) observed only 4% of dioecy in the endemic flora of Reunion Island. More recently, studies in Reunion Island (Humeau *et al.,* 1999) revealed that the percentage of dioecy ranges from 15 to 20%. New studies are therefore necessary to establish a more exact level of dioecy in the Mauritian endemic plants for comparison with other oceanic islands and a better understanding of the evolutionary biology of the Mauritian endemic plants. In this present study, the occurrence of leaky dioecy among the *Diospyros* species of Mauritius is examined and the viability of seeds produced by these leaky dioecious plants is investigated.

4.2 Materials and Methods

4.2.1 Study species

We examined the flowers of all the eleven species of *Diospyros* during their respective flowering periods and only *D. egrettarum* was found to exhibit leaky dioecy. *Diospyros egrettarum* is a 5-6m multistemmed tree that can be observed on the East coast of Mauritius only. Two main populations can be encountered, one on Ile aux Aigrettes (an islet, 900 m off the South-East coast of Mauritius) and the other at Bras d'Eau on the North-East coast of

Mauritius. Both male and female trees have grey barks, dark green leaves and produce numerous small insect pollinated white flowers, approximately 2cm in diameter. Male flowers are produced in the leaf axils in groups of 6 to 10 while female flowers are produced in groups of 5 to 7. During the flowering period of *D. egrettarum* the total number of male and female trees was counted in the two populations and their sex ratios calculated. The number of male and female plants was also recorded for the major populations of *D. leucomelas*, *D. melanida*, *D. revaughanii* and *D. tessellaria* during their respective flowering periods. Sex ratios were also calculated for these species. A chi square test was performed to test departures from the 1:1 sex ratio.

4.2.2 Pollination trial

During the flowering period of *D. egrettarum* usually from July to September, a pollination experiment was designed to study leaky dioecy in *D. egrettarum*. Eight individuals from a total of 388 plants were found to produce both staminate and pistillate stems on the same tree (Table 4.1). Two treatments were carried out on these hermaphrodite individuals to test the sexual functionality of these morphological female flowers. Fifteen female flower buds from each female stem were selected and bagged with mosquito netting material prior to the pollination experiment.

Table 4.1. Number of shoots observed on the leaky dioecious individuals and the number of female flowers treated

Individual number	No of shoots	Total number of female flowers bagged
1	1 shoot producing female flowers 1 shoot producing male flowers	15
2	2 shoot producing female flowers 1 shoot producing male flowers	30
3	3 shoot producing female flowers 1 shoot producing male flowers	45
4	2 shoot producing female flowers 2 shoot producing male flowers	30
5	1 shoot producing female flowers 7 shoot producing male flowers	15
6	3 shoot producing female flowers 2 shoot producing male flowers	45
7	2 shoot producing female flowers 1 shoot producing male flowers	30
8	2 shoot producing female flowers 5 shoot producing male flowers	30

Three days after bagging the flower buds, the following treatments were carried out:

Treatment A (hand self pollination)
Five of the fully opened female flowers from each female stem were hand pollinated with pollen obtained from male flowers found on the same plant.

Treatment B (hand cross pollination)
Another set of five fully opened female flowers from each female stem were pollinated with pollen obtained from male trees other than the tree from which the female flowers were selected.
All artificially pollinated female flowers (treatments A and B) were re-bagged with mosquito netting material.

Treatment C (apomixy test)
As a control, the five remaining flowers from each female stem were left bagged with the mosquito netting material and no artificial pollination was carried out on those flowers.

After three weeks, the bags were removed and the number of successful pollination trials was noted for each female shoot. The positions of the immature fruits resulting from the pollination experiment were carefully marked. One week after removing the bags, the number of fruits formed was noted and after another three weeks, the number of fruit abortions was recorded. The number of mature fruits obtained for each treatment was noted three months after carrying out the hand pollination. The results obtained for initial fruit set, number of fruits aborted and number of mature fruits produced in treatment A and B were compared using a G test of independence (Sokal and Rohlf, 1997).

4.2.2.1 Fruit set and germination trial

Two samples comprising of one hundred seeds each obtained from treatment A and treatment B were sown separately to determine the germination success of the seeds produced by the leaky dioecious trees. Concurrently, one hundred seeds of *D. egrettarum* collected from other female plants not exhibiting leaky dioecy were also germinated as control.

4.3 Results

4.3.1 Sex ratio

Only five *Diospyros* species were found to occur in groups of more than 25 individuals in a close proximity. The rest of the *Diospyros* species were found to be comprised of a few individuals trees distributed sparsely over a large area making it difficult to establish a reliable sex ratio that would reflect the natural distribution. Examination of the population structures of *D. egrettarum*, *D. leucomelas*, *D. melanida*, *D. revaughanii* and *D. tessellaria* have revealed that there exists a male- biased ratio (table 4.2) in the five species. The ratio of female to male individuals in populations of *D. egrettarum*, *D. leucomelas* and *D. melanida* was found to be approximately 1:3 while the sex ratio of *D. revaughanii* and *D. tessellaria* was found to be 1 female: 2males. Table 4.2 also shows the altitudes of the populations that have been surveyed for the above five *Diospyros* species.

Table 4.2. Ratio of female:male trees in some *Diospyros* species

Species	***egrettarum***				***leucomelas***		***melanida***		***revaughanii***		***tessellaria***	
Location	**B. d'eau**		**IAA**		**Magenta**		**Magenta**		**Petrin**		**B. fer**	
Altitude (m)	50		13		210		210		580		612	
Sex	F	M	F	M	F	M	F	M	F	M	F	M
No. of trees	28	91	71	198	8	26	7	22	22	43	28	59
Ratio	1:3.3		1:2.8		1:3.3		1:3.1		1:2		1:2.1	
χ^2	33.353		59.959		9.529		7.759		6.785		11.046	
	(p=0.000)		(p=0.000)		(p=0.002)		(p=0.005)		(p=0.009)		(p=0.001)	

B. d'eau= Bras d'eau; IAA= Ile aux Aigrettes; B.fer= Brise Fer; F= female; M=male.

4.3.2 Leaky dioecy

Ten species of the eleven known *Diospyros* were found to exhibit strict dioecy. In only one species, we observed leaky dioecy which amounts to 2% of the trees studied. These leaky dioecious plants were subjected to pollination trials. Table 3 shows the number of flowers treated and the results obtained for treatments A and B. No fruit formation was noted when the female flower buds were bagged in the control experiment. From the data obtained, the initial fruit formation was found to be 59.2% in treatment A and 54.6% in treatment B. However, abortion of the immature fruits reached 24.6% in treatment A and 25.2% in treatment B. Consequently, the pollination experiment of treatment A resulted in the production of 43.8% mature fruits while treatment B generated 40.8% mature fruits.

Germination success of the *D. egrettarum* seeds was recorded as 28% for seeds from treatment A, 36% for seeds from treatment B and 32% for seeds collected from other female *D. egrettarum* trees not exhibiting leaky dioecy. No significant differences were found in the number of initial fruit set (G=2.392, p=0.935), abortions (G=3.027, p=0.883) or the production of mature fruits (G=2.668, p=0.914) between treatment A and treatment B.

5.0 Sex Determination using RAPD

5.1 Background

Gender determination based on RAPD markers has been successful in *Silene latifolia* (Mulcahy et al. 1992), *Pistacia vera* (Hormaza *et al*. 1994), *Atriplex garettii* (Ruas et al. 1998), rattan (*Calamus simplicifolius*, Yang *et al*. 2005), kiwi plant (*Actinidia chinensis,* Xiao *et al*., 2003), black pepper (*Piper longum,* Manoj *et al*. 2005) and pointed gourd (*Trichosanthes dioica* Roxb. Singh *et al*. 2002). The usual procedure for identification of sex-linked RAPD markers in dioecious and/or hermaphrodite plants involves screening of bulked sample of DNA extracted from male and female plants (plates 5.1 and 5.2).

Plate 5.1: Male flowers of *D. egrettarum*

Plate 5.2: Female flower of *D. egrettarum*

5.2 Materials and Methods

5.2.1 Plant Samples

The leaf materials used in this study were collected randomly from male and female *Diospyros egrettarum* plants from Ile aux Aigrettes. Prior to genomic DNA extraction, the leaf materials were rinsed with distilled water and sterilised in 70% alcohol followed by washing in 5% bleach water. The leaf materials were further rinsed in 70% alcohol and finally rinsed in distilled autoclaved water.

5.2.2 DNA extraction

About two grams of leaf tissue were ground into a fine powder using liquid nitrogen in a mortar. Total genomic DNA was extracted using the DNeasy® Plant Mini kit (QIAGEN, GmbH, Hilden, Germany) following the manufacturer's protocol. DNA quality was evaluated on a 1.0% agarose gel stained with ethidium bromide (Sambrook et al, 1998). Quantity was measured using GeneQuant *pro* RNA/DNA Calculator (Biochrom Ltd, Cambridge, England).

5.2.3 DNA amplification

Optimisation of PCR conditions and thermal cycling parameters were performed in preliminary experiments. Optimised PCR amplification mixtures contained 2.5 mM Mg^{2+}, 200µM of each dNTP (Roche Diagnostics GmbH, Mannheim, Germany), 2.5 pmol decamer primer (Operon Technologies, Alameda, California, USA), 0.5 units HotstarTaq™ DNA polymerase (QIAGEN GmbH, Germany), 50 ng of template DNA, 1x PCR buffer, 250 ng BSA(Life Technologies, UK) and 1x Q solution all in a final volume of 25 µl. RAPD reactions were performed in a PTC 220 DNA Engine Dyad (MJ Research. Inc.,Waltham , MA, USA). The thermal cycling program used was: 94^0C /15 mins ; 3 cycles of 94^0C /25 seconds 35^0C /25 seconds and 72^0C/ 2 mins followed by 40 cycles of 94^0C / 25 seconds, 37^0C / 25 seconds and 72^0C/ 2 mins. Following a final extension of 72^0C/ 7 mins, reactions were ended with an indefinite hold at 4^0 C.

Negative control containing the amplification mixture without template DNA was also included to check the reproducibility and accuracy of PCR reactions. A 100 base pair ladder (DNA molecular weight marker XIV, Roche Diagnostics, Germany) was loaded on the gel for calculations of fragment size. Amplified DNA fragments were mixed with 10x loading buffer (20%w/v Ficoll 400, 0.1M EDTA, 1.0% SDS, 0.25% w/v Bromophenol blue) and separated on 1.8% (w/v) agarose gels (Agarose MP, Roche Diagnostics GmbH, Germany) in 1x TAE

buffer. Gels were run for 2 hrs at 100V, stained with ethidium bromide (0.4 µg ml^{-1}) for 45 mins, washed in distilled water for 1.0 hr and photographed on a UV transilluminator.

5.2.4 Primer screening

A total of fifty RAPD primers (Operon Technology Inc.; OPC 01-20, OPL 01-20 and OPAJ 01-10) was screened with DNA samples from twenty-five female and twenty-male individuals. Amplifications were repeated and consistent markers were included in the analysis.

5.3 Results

5.3.1 DNA extraction

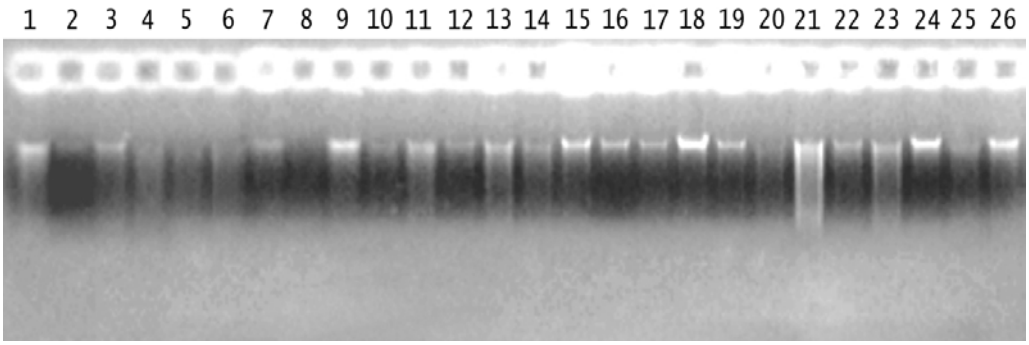


Figure 5.1. Agarose gel electrophoresis profile of genomic DNA extracted from female *D. egrettarum* plants using the DNeasy® Plant Mini kit

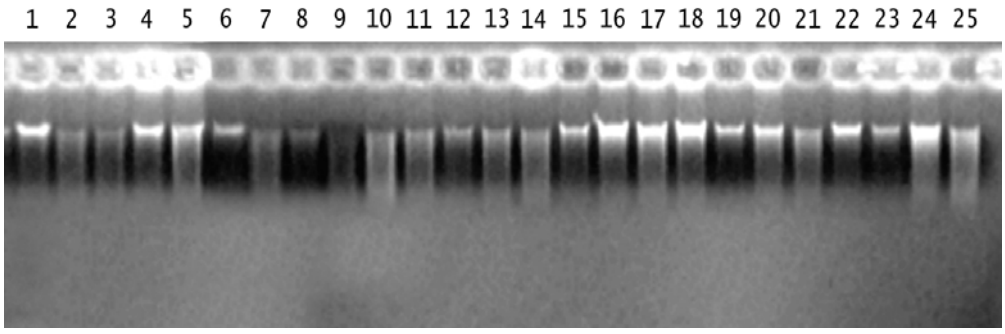


Figure 5.2. Agarose gel electrophoresis profile of genomic DNA extracted from male *D. egrettarum* plants using the DNeasy® Plant Mini kit

5.3.2 RAPD Analysis

RAPD profiles of twenty-five male and twenty-five female *D. egrettarum* individuals were generated with fifty different 10-mer primers of arbitrary sequences from Operon kits and compared. Monomorphism and polymorphism in the RAPD patterns were assessed by counting the number of RAPD bands generated. Monomorphic bands are the same sized amplified bands present in all the males and females while polymorphic bands are the amplified bands present only in either the females or the males. Some of the results are shown in figures 5.3 to 5.5. Although the RAPD profiles of the female and male samples showed some differences in band patterns (for instance in OPC02, figure 5.3), we were not able to these results could not satisfactorily reproduced. This indeed is a weakness of the RAPD method.

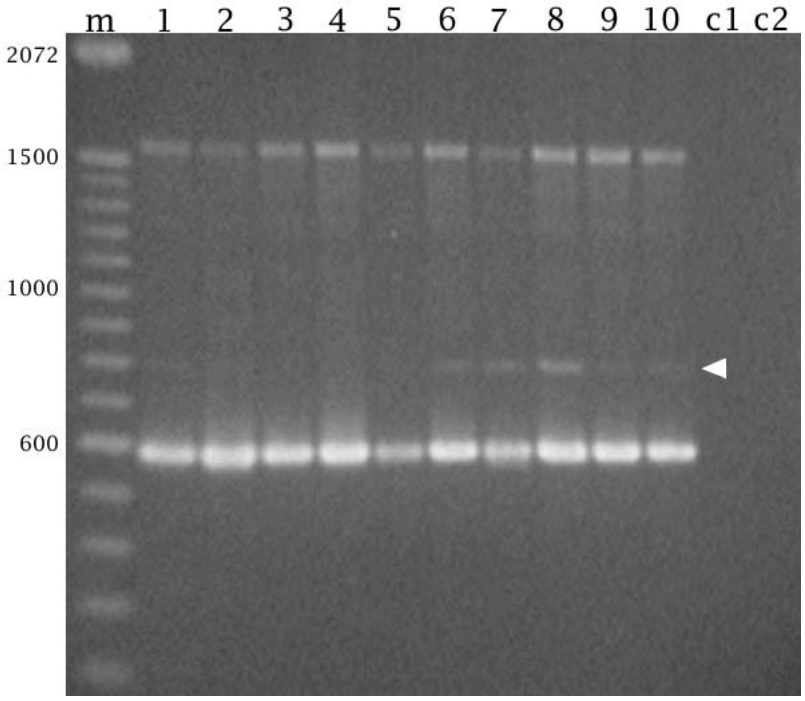


Figure 5.3. RAPD profile generated from 5 male and 5 female *Diospyros egrettarum* plants with primer OPC02. Lane m: marker (100bp ladder); lanes 1 through 5: RAPD products from DNA of male plants; lanes 6 through 10: RAPD products from DNA of female plants; lane c1: control without primer; lane c2: control without DNA sample. Arrow head indicates the sex-specific RAPD band for female *D. egrettarum* plant

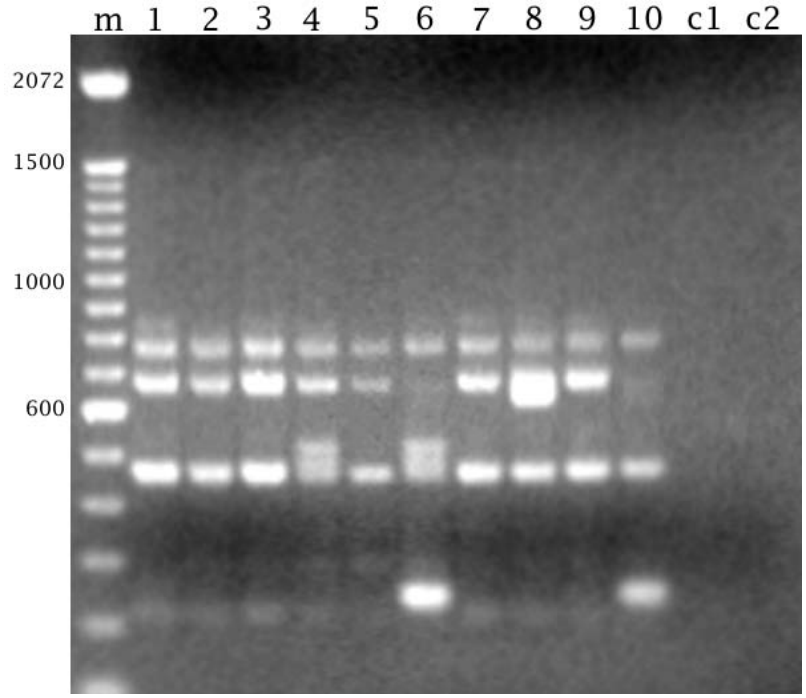


Figure 5.4. RAPD profile generated from 5 male and 5 female *Diospyros egrettarum* plants with primer OPC04. Lane m: marker (100bp ladder); lanes 1 through 5 – RAPD products from DNA of male plants; lanes 6 through 10 – RAPD product from DNA of female plants; lane c1: control without primer; lane c2: control without DNA sample

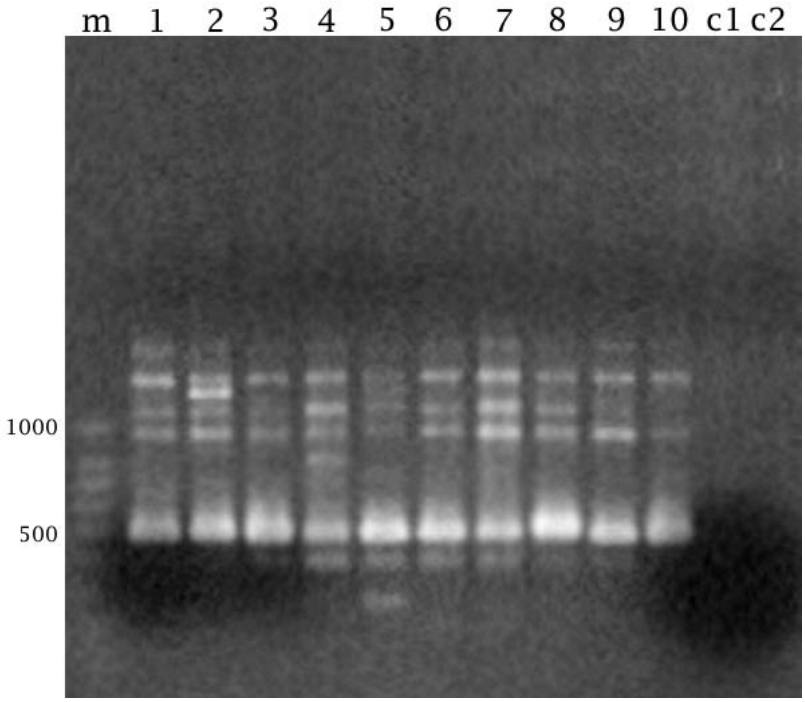


Figure 5.5. RAPD profile generated from 5 male and 5 female *Diospyros egrettarum* plants with primer OPC11. Lane m: marker (100bp); lanes 1 through 5: RAPD products from DNA of male plants; lanes 6 through 10: RAPD products from DNA of female plants; lane c1: control without primer; lane c2: control without DNA sample.

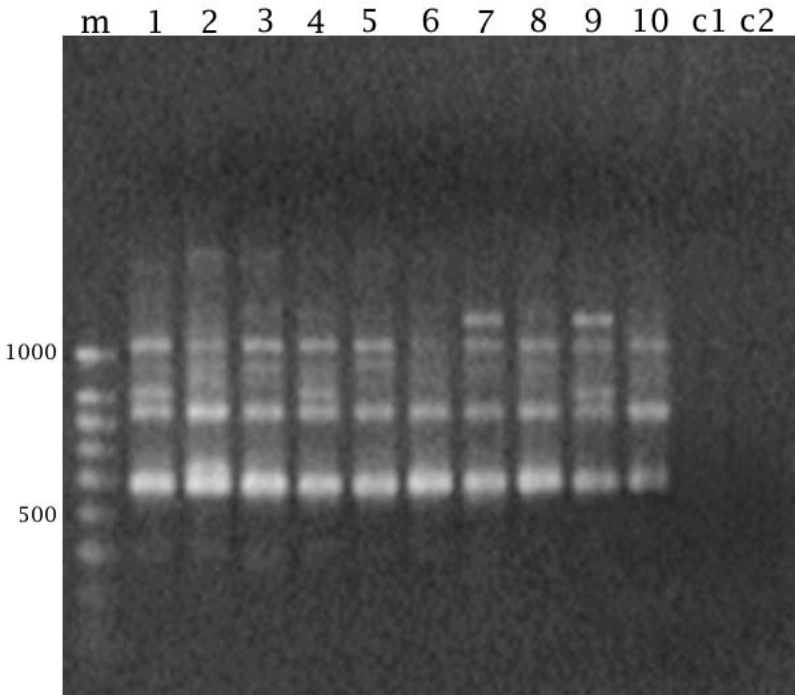


Figure 5.6. RAPD profile generated from 5 male and 5 female *Diospyros egrettarum* plants with primer OPC13. Lane m: marker (100bp); lanes 1 through 5: RAPD products from DNA of male plants; lanes 6 through 10: RAPD products from DNA of female plants; lane c1: control without primer, lane c2: control with DNA sample

6.0 Molecular Phylogeny

6.1 Background

With the advent of powerful techniques in molecular biology and bioinformatics, more and more systematists are utilising DNA-based data for inference of plant phylogeny. Molecular data have been more successful in determining the origin, monophyly and evolution of a number of endemic plants on islands such as the Canary (Francisco-Ortega, 1996; Struwe *et al*, 1998), Hawaii (Wagner and Funk, 1995; Kim *et al*, 1998), Galapagos (Schilling *et al*, 1994), Juan Fernandez (Sang *et al*, 1994), Macaronesia (Francisco-Ortega *et al*, 1997) and the Caribbean (Negrón-Ortiz *et al*, 2003). The choice of an appropriate DNA region for comparative sequencing is also an important factor as this will determine the amount of informative characters generated. To compensate for the limitations of cp DNA as well as to obtain additional and independent estimates of phylogeny, nuclear rDNA has been widely

adopted as a tool in systematics. Hence, at lower taxonomic levels, internal and external intergenic spacers are commonly employed (Alvarez and Wendel, 2003). Ribosomal genes exist in tandem arrays composed of hundreds to thousands of copies (Baldwin *et al*, 1995). This high copy number facilitates amplification of rDNA by PCR based strategies. Furthermore, the repetitive structure of these arrays promotes a process of homogenisation fuelled by concerted evolution that may result in a single predominant sequence across all copies. Although rDNA has been very useful to promote understanding of plant systematics, there have been potential difficulties related to the presence of multiple copies per array and multiple arrays per genome. For instance, in the absence of complete concerted evolution, sequence variants can arise and when maintained, yield multiple distantly related rDNA types within individuals (Bailey *et al*, 2003). These phylogenetic distant sequences may be preferentially amplified over functional rDNA loci because of differences in genomic copy number or primer affinity (Wagner *et al*, 1994; Chase *et al*, 2003). This results in the masking of the complexity of the nuclear rDNA content by failing to detect sequence polymorphism. This drawback could in fact preferentially reveal paralogous rDNA sequences in different taxa or accessions. Nevetheless, several authors have maintained that the Internal transcribed spacer (ITS) region of the 18S – 26S nuclear ribosomal DNA remains a strong tool to resolve phylogenetic relationships at the generic and species level in plants (Kim and Jansen, 1994; Baldwin *et al*, 1995; Soltis *et al*, 1998; Koch *et al*, 2003). The popularity of the ITS regions as a valuable source of information for phylogenetic reconstructions include, its small size (~ 700bp in angiosperm), ease of sequence alignment and the presence of highly conserved flanking sequences which is ideal for designing primers. For these reasons we have used the ITS region in this study to

(a) Determine the phylogenetic relationships among the *Diospyros* species endemic to the Mascarenes and
(b) Assess the degree of congruence between morphological and molecular data sets.

6.2 Materials and Methods

6.2.1 Plant Samples

Fresh tender leaf materials were collected from individual plants found in the major populations of the *Diospyros* species in Mauritius and Rodrigues. Plant material, from three individuals of *D. borbonica* was generously provided the Jardin Botanique des Mascareignes

of Reunion Island. During a training funded by the International Plant Genetic Resource Institute (IPGRI) to Denmark, DNA was isolated from fresh leaves of *D. mespiliformis* (endemic to Mozambique) and *D. whyteana* (endemic to South-Africa). These two species were used as outgroups for our phylogenetic reconstruction.

6.2.2 DNA Extraction

Except for *D. angulata* (only one known individual), DNA was screened from five different individuals of each *Diospyros* species. All the DNA extractions were carried out using the DNeasy Plant minikit (QIAGEN). The quality and purity of the DNA obtained were assessed by the optical density measurements of GeneQuant II DNA/RNA (Pharmacia). The yield of the genomic DNA was also visually gauged by loading them of 1.5% agarose gels.

6.2.3 Polymerase Chain Reaction (PCR) and DNA Sequencing

The primers used to amplify and sequence the entire ITS region were based on the sequences of White *et al* (1990) as they have been extensively used in other angiosperms studies (Baldwin, 1992). These include the forward primer ITS5 (5' GGAAGTAAAAGTCGTAACAAGG3') and the reverse primer ITS4 (5' TCCTCCGCTTATTGATATGC3'). The concentrations of Magnesium chloride, BSA, dNTPs of the reaction mixture and the temperature of the cycle reactions were varied with a view to improve the amplification of the ITS region. Therefore, several optimisations of the PCR were required before appropriate conditions were reached for the satisfactory yield of bands with expected sizes. The following reaction mix was used:

Reagents	**1 reaction (µl)**
10X Qiagen buffer	2
Q solution	4
Magnesium chloride	0.5
dNTPs (10 mM)	0.2
Primer ITS5 (10pmol/ µl)	0.8
Primer ITS4 (10pmol/ µl)	0.8
BSA	0.5
Hotstar Taq polymerase	0.1
MilliQ water	10.1
Total volume	19

19µl of the PCR mix was added to each PCR tube followed by 1µl of diluted genomic DNA (approx. 50ng/µl). The PCR cycles finally adopted was as follows:

95^{o}C for 15mins; (94^{o}C for 30s;58^{o}C for 1min;72^{o}C for 2min) X 4 cycles; (94^{o}C for 30s;56^{o}C for 1min;72^{o}C for 2min) X 26 cycles; 72^{o}C for 7mins; 4^{o}C for ever

5µl of the resulting PCR products were loaded on 1.5% agarose gels to assess the success of the amplification reactions. A 100bp ladder (SIGMA) was also loaded (5µl) on the agarose gels to determine the weight of the products obtained. The remaining 15µl of the successful PCR products were then loaded on 1.5% agarose gels with the Genechoice 100bp ladder (PSG Scientific) as marker to estimate their yield (in ng) based on the intensity of the bands produced by the 100bp ladder as shown in figure 6.5. The gels were stained with Ethidium bromide (EtBr) and placed on UV transilluminator. The required bands were excised and purified using the QIAquick gel extraction kit (QIAGEN) under conditions specified by the manufacturer.

The purified PCR products were then used in a sequencing reaction:

Reaction mix	**1 Reaction (µl)**
Terminator Ready Reaction mix	2
2.5 X sequencing buffer	6
ITS5 or ITS4 primer (10pmol/µl)	0.32
MilliQ water	11

19µl of the reaction mix and 1µl of the PCR product were added to each 0.2µl tube and the following amplification reaction was performed:

94^{o}C for 2mins; (96^{o}C for 10s;50^{o}C for 5mins;60^{o}C for 4mins) X 25 cycles; 4^{o}C for ever

The resulting products were run on the automated sequencer ABI Prism 310 (Perkin Elmer) to generate the sequences.

6.2.4 Sequence Alignment and Analysis

All sequences used were represented by forward and reverse directions. The reverse complementation of the reverse sequences were determined using the software CHROMA (Goodstadt and Pointing, 2001) and the overlap regions of the forward and reverse sequences for each *Diospyros* species were found with the help of the software BioEdit version 7.05 (Hall, 2004). Complete sequences of the ITS region were thus found for all the *Diospyros* species. Each of these raw sequences were then compared to published sequences using the mega blast option in the NCBI server to find out if our results could be related to the sequences of the ITS region of other angiosperms.

The *Diospyros* sequences were aligned using CLUSTAL W provided in BioEdit. However, a final manual alignment was carried out to fine tune the concatenation of the sequences. Pairwise distances were calculated across all sites as the Kimura 2-parameter (Kimura, 1980) using PAUP 4.0b 10 (Swofford, 2001).

The sequences were then analysed using the heuristic maximum parsimony (MP) searches in PAUP 4.0b10 with *D. philipensis* as outgroup. The data were treated as unordered and of equal weight. The search strategy consisted of 1000 replicates of random taxon addition with the options, TBR branch swapping and MULTREES. The relative support for the branches was determined using 100 bootstrap replicates.

The trees generated by PAUP4.0b were viewed in the software TREEVIEW (Page, 1996) and TreeExplorer version 2.12 (Tamura, 1999).

6.3 Results

The yield of DNA from the Plant DNEASY minikit was relatively good and ranged from 142.7 µg/ml to 198.8 µg/ml while the purity was 98% to 99%. A yellowish brown coloration was obtained with the DNA of *D. angulata*, *D. boutoniana* and *D. borbonica* and an extra step of cleaning with 98% ethanol had to be used on the column to remove any trace of colour. *Diospyros revaughanii*, *D. pterocalyx* and *D. nodosa* produced more white precipitated proteins and the lysate obtained from these species were always clear.

Typical migration patterns of the DNA obtained in 1.5% agarose gels that have been stained with EtBr and viewed under UV are shown in figure 6.1.

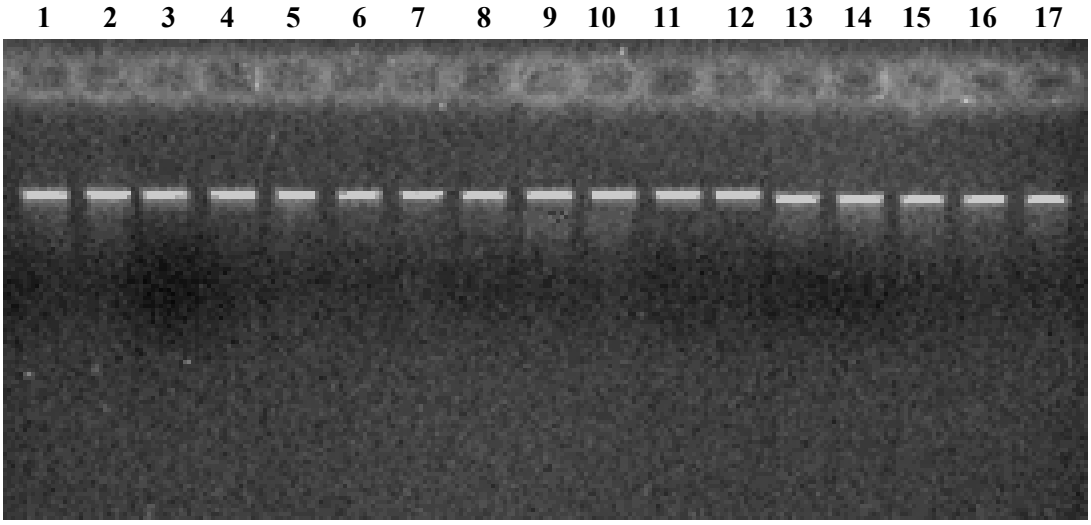


Figure 6.1: A 1.5% agarose gel showing the migration pattern of the genomic DNA of *Diospyros*
Lane 1: *D. angulata* **Lane 2: *D. boutoniana*** **Lane 3: *D. chrysophyllos*** **Lane 4: *D. egrettarum***
Lane 5: *D. hemiteles* **Lane 6: *D. leucomelas*** **Lane 7: *D. melanida*** **Lane 8: *D. neraudii***
Lane 9: *D. nodosa* **Lane 10: *D. pterocalyx*** **Lane 11: *D. revaughanii*** **Lane 12: *D. tessellaria***
Lane 13: *D. borbonica* **Lane 14: *D. diversifolia*** **Lane 15: *D. mespiliformis*** **Lane 16: *D. whyteana***
Lane 17: *D. angulata* (dried leaves)

6.3.1 Amplification of the ITS Region

Figures 6.2 and 6.3 show typical results obtained when DNA of the *Diospyros* species were used as template to amplify the ITS region.

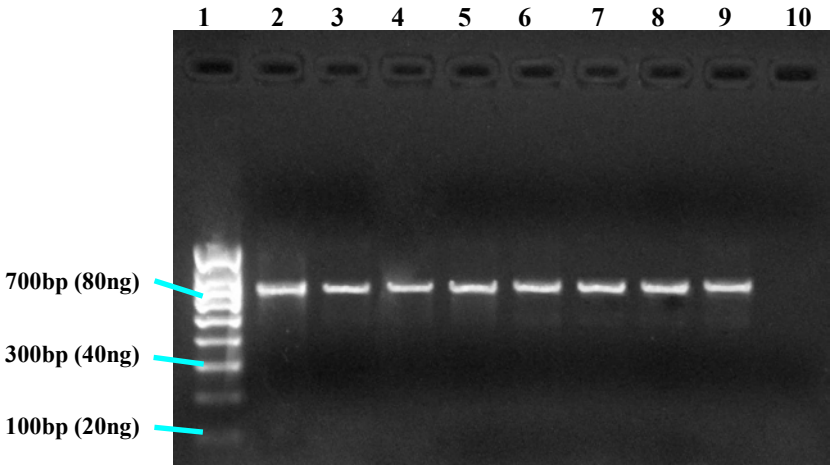


Figure 6.2: A 1.5% agarose gel showing migration of the PCR products under optimised conditions
Lane 1: 100bp ladder Lane 2: *D. angulata* Lane 3: *D. boutoniana* Lane 4: *D. chrysophyllos*
Lane 5: *D. egrettarum* Lane 6: *D. hemiteles* Lane 7: *D. leucomelas* Lane 8: *D. melanida*
Lane 9: *D. neraudii* Lane 10: -ve control

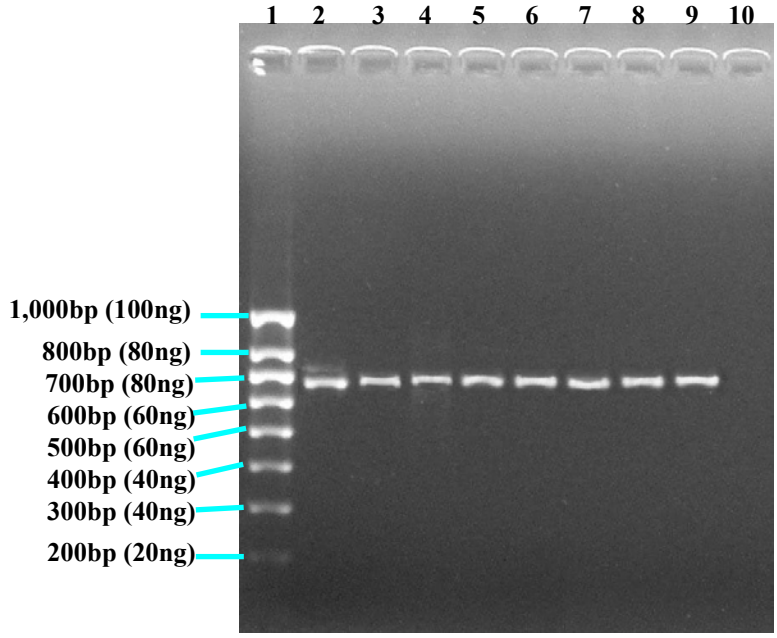


Figure 6.3: A 1.5% agarose gel showing migration of the PCR products under optimised conditions
Lane 1: 100bp ladder Lane 2: *D. nodosa* Lane 3: *D. pterocalyx* Lane 4: *D. revaughanii*
Lane 5: *D. tessellaria* Lane 6: *D. borbonica* Lane 7: D. *diversifolia* Lane 8: *D. mespiliformis*
Lane 9: *D. whyteana* Lane 10: -ve control

6.3.2 Sequences of the ITS Region

When the mega blast option of the NCBI server was used to test our sequences, our *Diospyros* were found to have more pairwise similarities with the *Diospyros* species from South Asia and some of those species have been included in our phylogenetic analysis.

6.3.4 Phylogenetic Analysis

In the maximum parsimony analysis, the total number of molecular characters was 776 with 251 constant characters, 133 variable characters that were parsimony uninformative and 392 parsimony informative characters. The phylogenetic analysis using PAUP 4.0b10 generated two most parsimonious trees with a tree length (TL) of 1140, a consistency index (CI) of 0.5904, a homoplasy index (HI) of 0.4096, a CI excluding uninformative characters of 0.5037, an HI excluding uninformative characters of 0.4963, a retention index (RI) of 0.4823 and a rescaled consistency index (RC) of 0.2847. The two equally parsimonious trees differed only in the relative positions of *D. rhodocalyx* and *D. rhombifolia* which are native to south Asia. One of the trees with branch lengths is shown in figure 6.4. The lengths of the branches indicate the genetic changes that accumulated in each species with time.

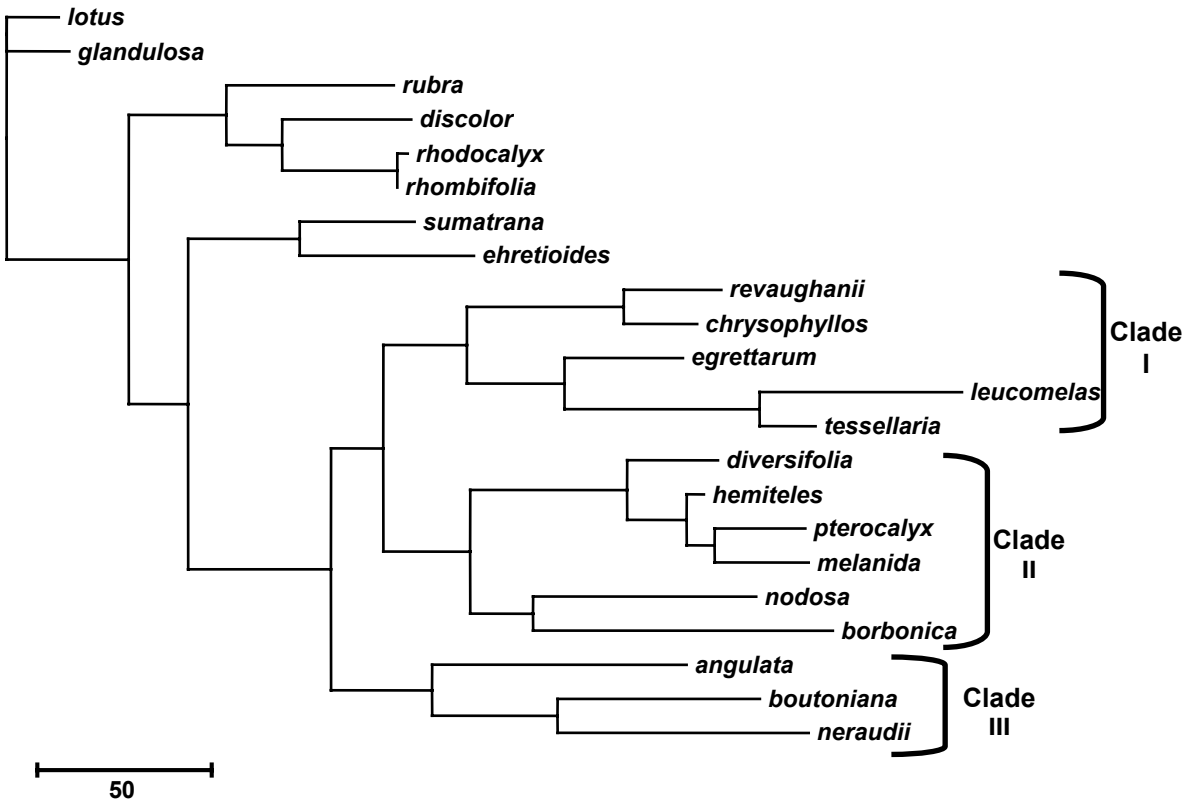


Figure 6.4: One of the three most parsimonious trees generated from the maximum parsimony analysis of the ITS region using PAUP 4.0b. TL = 1140, CI = 0.5904, HI = 0.4096, CI excluding uninformative characters = 0.5037, HI excluding uninformative characters = 0.4963, RI = 0.4823 and RC = 0.2847.

Three principal clades were identified among the Mascarene *Diospyros* as indicated in figure 6.4. Clade I consisted of the sister species *D. revaughanii* and *D. chrysophyllos* as a subgroup while *D. leucomelas, D.tessellaria* and *D. egrettarum* formed the other subclade.

Clade II comprised of *D. borbonica* (endemic to Reunion Island) and *D. nodosa* in a subclade as well as *D. diversifolia* (endemic to Rodrigues Island), *D. hemiteles*, *D. pterocalyx* and *D. melanida* in another subclade. The species *D. angulata, D. botoniana* and *D. neraudii* made up clade III which was basal to the other *Diospyros* species.

The ITS sequences of the Mascarene *Diospyros* were found to share more genetic similarities with the South Asian *Diospyros* (obtained from the NCBI server) and these species were included in our phylogenetic analyses in an attempt to probe into the ancestry of the Mascarene *Diospyros*.

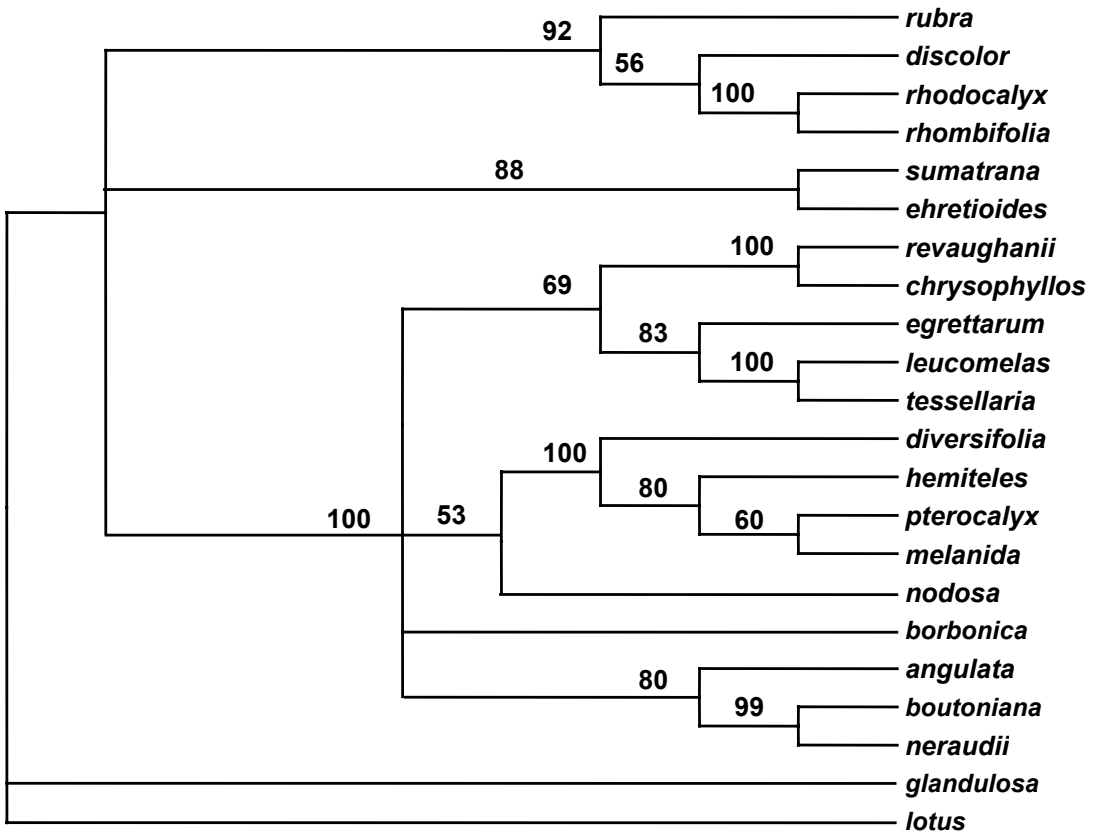


Figure 6.5: Strict consensus tree of the two equally parsimonious trees based on phylogenetically informative nucleotide substitutions from the ITS region. Bootstrap values >50% are indicated above the branches.

The strict consensus tree with bootstrap values higher than 50 is shown in figure 6.5. Good support for the branches are indicated by bootstrap values of greater than 75, moderate support designated by values between 65 to 75 while less than 65 is considered as weak. Only the branch leading to *D. borbonica* collapsed as the exact positions of this species within this clade could not be resolved. The other branches were supported by relatively high bootstrap values as shown in figure 6.5.

7.0 Discussion

7.1 Distribution of *Diospyros* species in Mauritius

Mauritius has more *Diospyros* species compared to Reunion or Rodrigues Islands which have only one endemic *Diospyros* species each. In the Mascarenes, age has been the major factor in determining the total number of endemic species which is estimated to be 311 in Mauritius (8mya), 189 in Reunion (3 mya) and 47 in Rodrigues (1.5 mya) as reported by Strahm (1994).

The data shown in table 2.1 reveal that *Diospyros* species, which are found to share the same habitats and often occur in close physical proximity, tend to have staggered flowering periods. This staggering of flower production may act as a reproductive barrier in preventing cross fertilisation among the *Diospyros* species given that pollination of most of these species is carried out mainly by the generalist pollinators such as honey bees, dipteran flies, coleopteran beetles and small butterflies and moths (personal observation). The situation of *D. tessellaria* is interesting in that although there is overlapping of its flowering period with the other *Diospyros* species growing close by, no hybrids have been reported.

Only *D. tessellaria* was observed to produce fleshy fruits while the other *Diospyros* species bear fruits which have a hard exocarp and hardly any flesh. The wide geographic range of *D. tessellaria* can be attributed to the effective dispersal of its seeds by bats, which relish the soft fleshy semi-sweet fruits (Nyhagen, 2001). Three endemic bats, namely, *Pteropus niger*, *P. subniger* and *P. rodricensis* are known to have existed in Mauritius with the two later species being now extinct (Cheke and Dahl, 1981). This wide dispersal and the ability of *D. tessellaria* to grow in different habitats has enabled this species to become the most prevalent *Diospyros* species on the island even though it was the most sought after for its hard black core (Rouillard and Gueho, 1999). The fruits of the other *Diospyros* species fall under the mother plant when they are still hard but become softer once on the ground. The now extinct giant tortoises and the ground birds could have then played a role in dispersing

the seeds from their origins. The fact that eleven of the twelve *Diospyros* species endemic to Mauritius produce relatively big non fleshy fruits has hindered their dispersal by birds. Consequently, gene flow through movement of seeds was limited to where the ground herbivores could go while micro-geographic isolation could have also helped in the adaptive radiation of the *Diospyros* species in Mauritius. This limited seed dispersal of most of the *Diospyros* species could have induced local speciation as the various mountain ranges and peaks located in Mauritius have acted as natural barriers for the movement of the seeds. Indeed, variations in topology and climatic contrasts have been shown to often provide over short distances a wide array of niches for immigrants to adaptively radiate (Wagner *et al*, 1990; Givnish, 1997).

The picture that seem to emerge from the spatial distribution of *Diospyros* indicates that despite the fact that the *Diospyros* species are now a mere fraction of their original numbers, they are found in nearly all the available native habitats in Mauritius. Moreover, the current distribution of the *Diospyros* species seems to have depended on rainfall regime irrespective of topography. In the surveys carried out in this study, most of *Diospyros* species were observed to be part of the highest canopies in their respective forest types and are consequently important in the development and establishment of lower stratal species. Furthermore, other surveys have shown that the regions with good *Diospyros* species richness are also rich in the other endemic species (Page and D'Argent, 1997). Therefore, *Diospyros* species may perhaps be viewed as a bioindicator species reflecting the status of the Mauritian remnant forests.

7.2 Morphology and phylogeny

The Mascarene *Diospyros* species are all dioecious and they can easily be differentiated from each other by their leaves and tree stature. The leaf colour and structures of the *Diospyros* species in Mauritius often vary regardless of the type of habitats. However, *D. diversifolia* has the smallest and narrowest leaves which could be an adaptation to arid areas. Indeed, this species can be encountered only in Rodrigues which is drier and warmer than Mauritius or Reunion. It is sometimes difficult to distinguish among the flowers and fruits of some species as in the case of *D. egrettarum* and *D. leucomelas* even though they inhabit different geographical altitudes. The leaves of both species also share many similarities except for the red midrib of the leaves of *D. leucomelas*. A few studies shave shown that reddish coloration in leaves could act as a deterrent to herbivory (Hansen, 2004). These authors have argued that the herbivores would regard leaves with reddish patterns as toxic

and would therefore stay away from those plants. Leaves with reddish venations can be observed in quite a few species indigenous to Mauritius (*Tarenna borbonica*, *Gastonia mauritiana*, *Casinne orientalis*, *Tambourissa* sp to name a few) but this phenomenon is mostly encountered in the juvenile stage. The colorations then disappear to produce uniform green leaves as the plants mature and gain in height. Interestingly, in the case of *D. leucomelas*, the red mid becomes more prominent on the underside of the leaves in the adult stage. This could well be an added protection against herbivory given the multistemmed tree habit of *D. leucomelas*, with leaves only a short distance from the ground.

The Mascarene *Diospyros* species bear small white flowers except for *D. tessellaria* which sometimes produce flowers that have a pink tinge. Our data have also indicated that there are no dramatic variations in the flower morphology among the different species while male and female flowers of the same species appear almost identical. Studies have shown that female flowers which cannot produce pollen to attract the insect pollinators bear close resemblance to the male flowers in order to be pollinated by deceit (Renner and Feil, 1993; Le Corff et al, 1998). Some non-discriminating insects will therefore visit both male and female flowers thereby pollinating the female flowers with pollen from the male flowers. The female flowers of *Diospyros* also have staminodes which increase their resemblance to male flowers. Moreover, male flowers are produced earlier and in more frequent cycles than the female flowers which are generated only once a year. This strategy could have evolved to ensure that rewarding male flowers are available earlier and for longer periods to the pollinators so as to make these insects less discriminating when the rewardless female flowers are produced. Furthermore, the close similitude among the flowers of *Diospyros* species would suggest that selection pressures to attract generalist pollinators have favoured parallel evolution or convergence in floral characters.

Our phylogenetic analysis suggests that the clade *D. revaughanii, D. egrettarum, D. leucomelas* could have been the first pioneering species of Mauritius. *Diospyros revaughanii* has colonised areas ranging from low altitude habitats to marshy lands on the central plateau. This species has also shown a certain phenotypic plasticity in that it occurs either as a tree in low altitude regions or as a shrub in the upland marshy areas. Given that its fruits have a sweet-scented smell and are sticky, they may have been picked or stuck to birds thus dispersing the seeds over broader distances. *Diospyros egrettarum* is the only true coastal species with fragmented populations occurring only on the eastern lowland regions and on a 25 hectares islet (Ile aux Aigrettes) off the South-East coast of Mauritius. It is interesting to note that *D. leucomelas* and *D. egrettarum* which exhibit a high degree of morphological

similarity are both located in close proximity in the remnant forests on the east coast of Mauritius. However, most of the populations of *D. leucomelas* are found in mid altitude areas. On the other hand, *D. chrysophyllos* which exhibits the closest morphological resemblance to *D. tessellaria,* have been encountered in a few low to high altitude regions as isolated individuals. *Diospyros tessellaria* is the most widely distributed species indicating adaptation to most of the ecological conditions of Mauritius in that it has been able to establish viable populations in regions of low, mid and high altitudes. This broad distribution and significant population size is certainly linked to the fact that the fruits of *D. tessellaria* are fragrant and fleshy enough to be eaten and dispersed by the endemic bat, *Pteropus niger*. It should be noted that *D. tessellaria* is the only *Diospyros* species in the Mascarenes that bear fleshy and fragrant mature fruits while the rest of the Mascarene species produce fruits that remain hard even when they are mature.

Diospyros boutoniana and *D. angulata* are the two species that have the broadest leaves in this genus. Although, *D. angulata* and *D. boutoniana* have been found to be morphologically quite close, they do not share the same ecological habitats. *Diospyros boutoniana* occur mostly in upland forests with only a few individuals inhabiting low and mid altitude areas while the only plant representing *D. angulata* was located in a mid altitude region. However, as *D. angulata* was down to one single female individual, it is difficult to ascertain its real geographical distribution. *Diospyros borbonica* which is endemic to Reunion Island has been reported to occur only in the South-East coast of this island (Bosser *at al*, 1976 ongoing) while *Diospyros diversifolia* is endemic to Rodrigues Island and can be found as very fragmented populations in several locations. Given that Mauritius is the oldest Mascarene island (8M years old) and based on the phylogenetic analysis, it is tempting to speculate that the *Diospyros* group evolved and speciated in Mauritius until at some point in time one species moved to the coastal region of Reunion Island (3M years old) to give rise to *D. borbonica*. This event may have been followed by another migration to produce *D. diversifolia* in Rodrigues Island (1.5M years old). The species *D. hemiteles* and *D. melanida* which appear to be closely related (figures 3.1 and 3.2) are known to occur only in mid altitude regions. Despite the fact that the group *D. leucomelas*, *D. tessellaria*, *D. hemiteles* and *D. melanida* all occur in very close proximity in mid altitude habitats, they have a staggered flowering period so that no two species flower at the same time. It is therefore not surprising that hybrids have never been observed among these species, which sometimes are only a few meters apart. The high altitude species most likely emerged to colonise the humid habitats on the central plateau of Mauritius. *Diospyros pterocalyx, D. neraudii* and *D.*

nodosa all occupy the same niches in the upland forests of Mauritius. Like the above mid altitude species, they have developed reproductive barriers so as to remain as distinct species.

In essence, the phylogenetic trees obtained from morphological data support the notion that colonisation of the *Diospyros* group most probably started from the coastal areas and then with speciation and adaptive radiation, the species moved to the mid altitude regions and then finally the upland species arose to colonise the humid habitats. The seeds of *D. tessellaria* on the other hand were most probably dispersed by the *Pteropus niger* (and other endemic bats, now extinct) some distance away from the mother plant, explaining the wide distribution of *D. tessellaria* over the whole island. Unfortunately, little is known on the eating habits of the now extinct herbivores species like the flightless Dodo (*Raphus cucullatus*), the red rail (*Aphanapteryx bonasia*) and giant land tortoises (*Cylindraspis inepta*, *Cylindraspis triserrata*), which could have contributed in the dispersal of *Diospyros* seeds to different niches.

Homoplasy in some character traits from both vegetative and reproductive parts of the *Diospyros* species has created ambiguities in the relative positions of a few species in the phylogenetic tree.

7.3 Leaky dioecy in *D. egrettarum*

7.3.1 Sex ratio

Several observations have shown that sex ratios in dioecious species are not always 1:1 (Bram and Quinn 2000). For instance, male-biased sex ratios and female-biased sex ratios (Crawford and Balfour, 1983; Ornduff, 1985; Sakai and Weller, 1991; Houle and Duchesne, 1999) have been well documented. However, Correia and Diaz Barradas (2000) found no significant differences between the number of male and female plants of *Pistacia lentiscus* growing in abandoned old agricultural areas where the soil was fairly rich in nutrients. Furthermore, male-biased sex ratios have also been recorded irrespective of environmental factors, habitat quality and population density (Espirito-Santo *et al.,* 2003). In our study, a male-biased sex ratio (1 female:3 males) was found in the two populations of *D. egrettarum* surviving on a low nutrient soil and dry condition. In the populations of *D. leucomelas* and *D. melanida* which share a mid altitude habitat (where the growing conditions are more favourable compared to that of *D. egrettarum*), a similar male-biased sex ratio (1 female:3 males) was also observed for both species. In the species, *D. revaughanii* and *D. tessellaria*

which both inhabit a high altitude, rich and moist region, the number of male plants was also higher than the number of female plants (1female: 2males). It would seem that the *Diospyros* species endemic to Mauritius are generally male-biased with a less pronounced difference at higher altitudes. Unfortunately, the other *Diospyros* species occur as a few isolated individuals and their sex ratios cannot be ascertained. It is noteworthy that many male plants of *D. egrettarum*, *D. leucomelas*, *D. melanida*, *D. revaughanii* and *D. tessellaria* were seen to produce flowers more frequently and even in periods where the female plants of these species were not flowering. In fact, in the case of *D. egrettarum* some of the male plants produced flowers even at the time when all the female individuals were bearing fruits. In order to understand this behaviour we should bear in mind that the reproductive investment of male plants is significantly less than female plants which have to produce flowers, fruits, seeds (Roucheleau and Houle, 2001). It has also been shown that given the higher cost of reproduction, female plants can exhibit a decline in survival rate, frequency of flowering or variation in reproductive effort (Antos and Allen, 1999; Nicotra, 1999). Consequently, the more numerous male individuals with their frequent flowering periods could well be an attempt to extend the availability of pollen so as to give a chance to the female plants to cope with environmental demands that sometimes could make their flowering behaviour somewhat erratic.

7.3.2 Leaky dioecy

Our results show that each individual leaky *D. egrettarum* tree has the ability to generate fertile male and female flowers and produce viable seeds. Moreover, there were no significant difference among the germination rates of seeds from treatment A, treatment B and the control indicating that the production of seeds through leaky dioecy is as efficient as in strict dioecy.

Diosyros egrettarum is the only *Diospyros* species that can be encountered in low altitude habitats that are closest to the sea and the two populations studied have been subjected to immense pressures from woodcutters, poor soil quality and drought conditions for several years. Our results concur with the work carried out by Humeau *et al.,* (1999) on the *Dombeya* endemic to Reunion Island. They showed that the *Dombeya* species, which exhibited leaky dioecy, occur in highly isolated relict populations at lower altitudes forests where human disturbance via habitat destruction and fragmentation was greatest. On the other hand, the species of *Dombeya* occurring in large populations in mid to high altitude cloud forest were found to exhibit strict dioecy. Humeau *et al.,* therefore suggested that leaky dioecy usually

occurs at low altitude and /or in small threatened, fragmented populations. A similar trend seems to exist in the Mauritian endemic *Diospyros* species as only *D. egrettarum* can be found at the low altitude and marginal areas. However, it is important to note that in the case of *Dombeya*, the male and female flowers retained (to different degrees) morphologically well developed opposite sex organs (Humeau *et al.,* 1999) leading to cryptic dioecy whereas in *D. egrettarum*, the same plant was observed to produce distinct male and female flowers on separate stems. This sort of leaky dioecy has been noted in *Freycinetia scandens* in which the same plant produced staminate and pistillate spikes at the same time (Cox and Webster in Baker and Cox, 1984). At this point in time, the mechanism of leaky dioecy is not clearly understood although one would expect a hormonal connection. Indeed, Baker and Cox (1984) suggested that a mere alteration of the hormone system in a plant could produce separate staminate and pistillate flowers even on the same shoot. Yin and Quinn (1995) proposed a model where the hormone, gibberellin has both male and female receptors thereby regulating sex expression. They suggested that when the concentration of gibberellin was higher that the sensitivity level of the male receptor, maleness would be induced. Alternatively, the gibberellin concentration should be lower than the sensitivity level of the female receptor for the female sex organs to develop. This sort of hormonal regulation needs to be investigated in the leaky individuals of *D. egrettarum*.

Diospyros egrettarum is one of the rare *Diospyros* species that are multistemmed (from the base), the others being, *D. leucomelas*, *D. revaughanii*, *D. nodosa* and *D. pterocalyx*. The number of stems produced by *D. egrettarum* is however superior to the other multistemmed *Diospyros* species. The fact that *D. egrettarum* is the only *Diospyros* species that can be found close to the sea on the East coast is puzzling. The strong South-East trade winds that dominate the East of Mauritius could have enabled fruits of a *Diospyros* species to land on the Eastern coast. Interestingly, the largest population of *D. egrettarum* and six of the leaky dioecious individuals studied can be encountered on Ile aux Aigrettes, located 900m off the South-East coast of Mauritius. Given that in all *Diospyros* species, the germination of *Diospyros* seeds is relatively low (a maximum of 40% - pers. observation) and the sex ratio in *Diospyros* populations is male-biased (especially in dry and lower altitude regions), it is conceivable that germination of the seeds which first landed in Mauritius eventually led to more adult male plants. Contrary to female flowers of *Diospyros* which still have a number of staminodes and consequently, have the potential of developing functional stamens again, the male flowers have only male reproductive organs.

Therefore, leaky dioecy could well come to the rescue of a solitary male *Diospyros* plant whose survival would depend on its ability to bear fruits with viable seeds. This mechanism might have been an important factor in the colonisation and survival of the pioneer species of *Diospyros* on the island of Mauritius as it would have ensured the establishment of a population from one single leaky dioecious individual.

7.4 Sex determination using RAPD

Random Amplified Polymorphic DNA (RAPD) has been found to be effective for the identification of sex-linked molecular markers in several dioecious plant species such as Papaya (*Carica papaya* L.), hemp (*Cannabis sativa*), Black pepper (*Piper longum* L.*)*, kiwifruit (*Actinidia* species) nutmeg (*Myristica fragrans*) and rattan (*Calamus simplicifolius*). In addition, in plants such as, *Piper longum* L., (Manoj *et al*. 2005) more than one sex-specific markers have even been found. Nevertheless, Hormaza *et al*. (1994) hypothesized that in the case of *Pistacia*, low frequency of sex-linked bands indicated that the DNA segments involved in sex determination were very small and probably represented a single gene or very few genes. With the fifty decamers (Operon Technologies, Alameda, California, USA) tested in *Diospyros egrettarum*, the majority of RAPD reactions resulted in highly monomorphic banding patterns of amplified bands. These monomorphic RAPD profiles demonstrated the high genomic similarity between males and females of this species. Only primer OPC02 produced an approximate 800bp fragment that was consistently present in most of 25 female plants to a varying intensity but absent in the 25 male plants investigated. The determinants of sexual phenotype in plants are diverse, ranging from sex chromosomes in *Marchantia polymorpha* and white campion (*Silene latifolia*) to hormonal regulation in maize (*Zea mays*) and cucumber (*Cucumis sativa*) (Tanurdzic and Banks, 2004). In *Diospyros* species, the female-associated RAPD band generated by decamer OPC02 may either represent amplified DNA from a sex chromosome or it may simply represent DNA polymorphisms (e.g. point mutations). Moreover, the failure to obtain reproducible clear cut differences between the RAPD profiles of male and female *D. egrettarum* could be due the complexity of the mechanism involved in sex determination of this dioecious species.

In addition, it is possible that the sex of *Diospyros egrettarum* is controlled by regions of the genome not sampled by the set of primers used in the present study. Screening a larger number of decamers may be required to find a more tightly sex-linked amplification product. Also, the larger the genome is, the more random primers could be needed to find a sex-

specific RAPD marker (Yang *et al*., 2005). Indeed, Hormaza *et al*. (1994) and Manoj *et al*. (2005) stressed on the necessity to screen large numbers RAPD primers in order to identify sex-linked markers. Shibu *et al*. tested 80 primers for the RAPD analysis of nutmeg (*Myristica fragrans* Houtt.) while Manoj *et al*. (2005) used 80 primers to generate RAPD profiles in *Piper longum*. Other research groups used even higher numbers of decamers in trying to discriminate between sexes in dioecious plants. Ruas *et al*. (1998) who reported a male-linked RAPD marker in *Atriplex garrettii* tested 158 primers; Alstrom-Rapaport *et al*. (1998) screened 380 decamers to generate RAPD products from *Salix viminalis* L while Harvey *et al*., (1997) screened 500 primers before identifying two sex-linked markers in *Actinidia*; Gunter *et al*. (2003) screened approximately 1000 RAPD primers before finding female-linked genetic markers in *Salix viminalis* L. and Yang *et al*. (2005) tested 1040 random primers in rattan before identifying an approximate 500 bp male-specific RAPD fragment. On the other hand, other research groups screened only small numbers of primers in their sex determination studies: Mandolino *et al*. (1999) tested 20 primers before identifying the $OPA8_{400}$ marker they reported in *Cannabis*. Their work is in agreement with the one performed by Sakamoto *et al*. in 1995, who tested only 15 decamers, two of which generated two male-linked markers of 500 and 730 bp each.

7.5 Molecular Phylogeny

Although, there was limited agreement between morphology-based and molecular-based trees, the major groups remained almost the same with changes in the relative positions of the *Diospyros* species within the clades. Divergence between mophological (venkatasamy et al, 2005) and molecular phylogenetic trees can be largely attributed to the convergent and parallel evolution of the morphological characters of the *Diospyros* species. Moreover, both morphological and molecular data indicated that *D. borbonica* (endemic to Reunion) and *D. diversifolia* (endemic to Rodrigues) originated from Mauritius.

Nevertheless, compared to the morphology-based analysis, the molecular data on the *Diospyros* species generated better resolved phylogenetic trees that provided more accurate information on their evolution. Indeed, based on the strict consensus tree which also included South Asian *Diospyros* species and the high bootstrap values generated for the molecular-based phylogenetic tree, it would seem very likely that the Mascarene *Diospyros* species are monophyletic. Interestingly, in the strict consensus tree, the basal branches of the Mascarene *Diospyros* collapsed into polytomies revealing that molecular data could not determine which species first colonised Mauritius. This result suggests a number of missing

links exist. At this point in time and given the history of massive destruction of the *Diospyros* species, one cannot exclude the fact that some ancestors of the extant *Diospyros* species were pushed to extinction.

The phylogenetic relationships of the *Diospyros* species as reconstructed by the analysis of ITS sequence largely concur with the results on the biogeography presented in chapter 2. For instance, molecular data showed that *D. revaughanii*, *D, chrysophyllos*, *D. egrettarum*, *D. leucomelas* and *D. tessellaria* which all occur on the eastern coastal areas of Mauritius are close relatives. *Diospyros egrettarum* is confined to that region whereas *D. leucomelas* has moved to a number of mid altitude habitats and mountain slopes. On the other hand, *D. chrysophyllos*, *D. revaughanii* and *D. tessellaria* have expanded their ranges and are found from dry to super humid areas. They are also close neighbours in a number of habitats.

The other two species that have acquired the ability to adapt to other altitudinal zones are *D. boutoniana* and *D. neraudii* which exist as viable populations in regions of low, mid and high altitudes. Their sister species, *D. angulata* is now extinct and used to occur as a single female plant in a mid altitude habitat (pers observation). Unfortunately, no data is available on the original distribution of *D. angulata*.

The species, *D. hemiteles*, *D. pterocalyx*, *D. melanida* and *D. nodosa* are clustered in the same clade and it is interesting to note that the latter three species bear fruits with pronounced wings on the calyces. This morphological character can also be observed in *D. borbonica* which is also a member of this clade. The only two species of this clade that exhibit unwinged fruit calyces are *D. diversifolia* and *D. hemiteles* (Venkatasamy *et al*, 2005). *Diospyros pterocalyx* and *D. nodosa* share super humid habitats and also thrive in water logged soils. On the other hand, *D. hemiteles* which prefers mid altitude regions does not occur in the same habitats as it's sister species *D. pterocalyx*. Furthermore, the flowers and fruits of *D. hemiteles* resemble those of *D. melanida* and in fact, the morphology-based phylogeny grouped these two species in the same minor clade. Interestingly, *D. melanida* and *D. hemiteles* share the same ecological niche in the Magenta valley and they have probably evolved the same type of reproductive system to survive. As *D. hemiteles* is represented by only a few individuals, we do not have enough information on its biogeography to establish its relationship with *D. pterocalyx* and *D. nodosa*.

Based on both the morphological and molecular phylogenetic trees obtained, it is clear that some species that are closely related also share the same habitats. In a study on *Calochortus*, Patterson and Givnish (2003) found out that the closely related species shared neighbouring habitats and they also develop parallel adaptive radiation to survive in their

immediate environment. Rieseberg *et al* (1996) used a cpDNA phylogeny on *Helianthus*, to show that neighbouring species were often close relatives.

Both morphological and molecular data sets seem to confirm that Mauritius acted as a centre of dispersal for the *Diospyros* species in the Mascarenes as *D. borbonica* (Reunion) and *D. diversifolia* (Rodrigues) are nested within the Mauritian *Diospyros* clades. It would appear that a single colonisation event from Mauritius allowed the evolution of *Diospyros* into *D. borbonica* in Reunion while another separate dispersal event gave rise to *D. diversifolia* in Rodigues. Dispersal of the *Diospyros* seeds to Rodrigues would have involved a movement against the persistent south east trade winds and ocean currents. This would suggest that large birds could have been responsible for the long distance dispersal of seeds to Rodrigues found 550km to the north east of Mauritius. In the case of Reunion Island which is located 150km to the south west of Mauritius, *Diospyros* seeds could have been dispersed both by large birds or ocean currents. Dispersal of seeds from Mauritius to Reunion or Rodrigues could also be the result of cyclones with gusts reaching frequently above 150km/hr and which sometimes sweep the Mascarene Islands.

Except for *D. tessellaria* which bear fleshy, edible fruits (Nyhagen, 2001), the *Diospyros* species under study have no obvious morphological adaptation for dispersal. This characteristic could explain that *D. tessellaria* has the broadest distribution over Mauritius and has successfully colonised a range of habitats. It is also noteworthy that the fruits of *D. revaughanii* and *D. boutoniana* produce sticky exudates which have the ability to help the fruits to stick to the feathers of birds thus promoting their dispersion over longer distances. The weak long distance dispersability of the *Diospyros* seeds across the ocean can perhaps account for the single colonisation event from Mauritius to Reunion or Rodrigues. Within Mauritius however, the *Diospyros* species have been able to colonise a relatively wide range of habitats as discussed in Chapter 2.

It is noteworthy that the results obtained in the biogeography study (chapter 2), indicated that the different types of soil had practically no effect on the distribution of *Diospyros* species. However, the distribution of lavas from the old, intermediate and early volcanic series might shed some light on the colonisation and evolution of the *Diospyros* species as reconstructed by molecular data. Indeed, Juan (2000) reported that in an oceanic island environment, lava flows followed by local extinctions and subsequent recolonisations are important factors for vicariance events and consequently speciation within islands. Although Mauritius dates back to some 8Ma, the old series lava flows that caused the emergence of Mauritius lasted from 7.6 to 5Ma (Sadul, 1995). These eruptions gave rise to the mountain ranges (lithosols and

mountain slope complexes) and the dark Magnesium clays partially (Parish and Feillafé, 1965). Therefore colonisation of Mauritius could only have started around 5Ma. Interestingly, the eastern part of Mauritius shown to have one of the highest species richness and Brillouin indices (Chapter 2) has developed on the lavas of this older volcanic series. Indeed, populations of most of the *Diospyros* species namely, *D. boutoniana*, *D. chrysophyllos*, *D. egrettarum*, *D. leucomelas*, *D. melanida*, *D. neraudii*, *D. pterocalyx*, *D. revaughanii* and *D. tessellaria* occur in this region. Therefore, it can be hypothesised that this geologically oldest region has provided the *Diospyros* species a safe haven before their propagules have been dispersed in various areas of the island.

The results in chapter 4 showed that *D. egrettarum* exhibits leaky dioecy and it was argued that this type of reproductive plasticity could well be an adaptation to colonise new environment if a single male plant happen to be the result of long distance dispersal. Molecular data could not establish whether *D. egrettarum* was among the earliest colonists as the basal branches of the phylogenetic tree had no good bootstrap support.

The lower slopes of the mountain ranges (mountain slope complexes) have developed between 3.5 to 5Ma and occur between the intermediate/late lavas of the younger volcanic series and the older volcanic series. The super humid regions were formed by the early lavas of the younger volcanic series and turned into a number of soil types between 3.5 and 1.7Ma. The areas under the latosolic brown forest and latosolic reddish prairie developed only between 0.7Ma to 0.02Ma. Assuming that there has been a single or very few long distance dispersal events of *Diospyros* in Mauritius, it would seem that a first introduction event most likely occurred on the old series lavas soil of the east coast some 5Ma. The fruits of the early colonists were then dispersed to some extent to other niches. The upland species colonised the super humid regions only some 1.7 Ma. Continuous lava flows between 5Ma to 0.02Ma years ago have acted as barriers to gene flow, probably causing local extinction in some populations and favouring the formation of new species. The dry to sub humid regions under the latosolic reddish prairie soils and the humid to super humid locations developed on the latosolic brown forest soils were the last areas to be colonised by the *Diospyros* species.

The results obtained from the morphology and molecular studies of the *Diospyros* species in Mauritius seem to indicate that the Mascarene *Diospyros* species are most probably monophyletic and the evolution of this genus could have started on the geologically oldest regions of the east coast. Dispersal of the fruits by relatively large birds (which are now extinct) and cyclonic winds have helped in the colonisation of new habitats. The lava flows

and mountain ranges have probably acted as natural barriers thereby inducing local speciation. By the time all volcanic activities of Mauritius completely ceased after some 0.02Ma, a number of distinct *Diospyros* species must have already been thriving. Dispersal of the fruits by extinct endemic tortoises or birds such as the dodo and adaptation of these species to all possible environments during subsequent years resulted in their presence from the coast line to the mountain tops as described by the Dutch in 1598 (Pitot, 1905).

Part II

8.0 Pharmacological Properties of the endemic Diospyros species

8.1 Background

Many scientists, including Farnsworth (1991) and Verpoorte (2000) have alerted the scientific community to the fact that only a small percentage (1-10%) of plant species (some 500 000) have been studied for their biochemical and pharmacological properties. Despite its fragile ecosystem, Mauritius still offers a diverse and rich flora with potentially useful chemical compounds that await discovery. In view of the dwindling size of the Mauritian native forests, there is some urgency to investigate the pharmacological potential of local plants.

8.2 Materials and Methods

8.2.1 Extraction

Plant materials were collected from the eleven *Diospyros* species and samples (50 g) were thoroughly washed with running water, gently dabbed and then weighed. They were then homogenised in a warring blender with 70 % acetone (2 x 300 ml) and were left overnight at 4ºC. On the next day, the mixture was filtered. The filtrates were collected whereas the residue was left to macerate (24 hrs) in 100 % of methanol (2 x 300 ml). The filtrates were concentrated *under vacuo* at 37°C. The filtrates were pooled together and were washed with dichloromethane. The extracts were then lyophilised.

8.2.2 Qualitative Phytochemical Analysis

Extracts from the *Diospyros* species were screened for phytochemical constituents including, alkaloids, tannins, saponins, reducing sugars (glycosides), steroids, terpenoids, cardiac tonics/glycosides and anthraquinones. All the phytochemical tests were done in 6 replicates.

8.2.2.1 Methods for phytochemical screening

1. Alkaloids (Harborne, 1973;Trease and Evans, 1989): 250 µl of aqueous extracts was added to 1.5 ml of 10 % of 0.1 M of HCL. The mixture was warmed for some minutes. They were left to cool for a few minutes then a few drops of Mayer's solution were added to the samples. A white to yellow precipitate indicated the presence of alkaloids.

2. Reducing sugars: 250 µl of aqueous extracts was added to 2 ml of distilled water followed by 500 µl of Fehling's reagent. The mixture was boiled for a few minutes. A red precipitate indicated the presence of glycosides.

3. Tannins (Trease and Evans, 1989): 250 µl volumes of aqueous extracts were mixed with 2.5 ml of distilled water. The mixtures were shaken using a vortex and a few drops of 10 % ferric chloride were added. A dark blue/dark green precipitate showed the presence of tannins.

4. Saponins (Wall *et al*, 1954): 250 µl volumes of aqueous extracts were added to 2 ml aliquots of distilled water. The tubes were vigorously shaken for 2 minutes. The formation of frothing which persisted after 5 minutes when warmed indicated the presence of saponins.

5. Keller-Kiliani's test (Sofowora, 1996): 0.5g quantities of extracts were added to 5 ml volumes of glacial acetic acid. Then, 250 µl volumes of the mixture were added to 1 ml of glacial acetic acid followed by a drop of 10 % ferric chloride. Finally, a few drops of concentrated sulphuric acid were carefully added. A positive result was recorded when a brown ring formed at the interphase.

6. Lieberman's test (Kolawole *et al*, 2007): 250 µl volumes of extracts were mixed with 250 µl volumes of distilled water. Then 500 µl aliquots of glacial acetic acid were added followed by 500 µl of chloroform. The mixtures were mixed and 500 µl of concentrated sulphuric acid was carefully added to each of them. A reddish colour at the interphase showed that steroids were present.

7. Terpenoids (Kolawole *et al*, 2007): 250 µl of samples were added to 750 µl of distilled water and 500 µl volumes of 0.05 % of 2,4 dinitrophenlyhydrazine were added to them. An orange yellow colour indicated the presence of terpenoids.

8. Anthraquinones test /Bornträger's test (Onwukaeme *et al,* 2007): 250 µl of extracts were added to 1 ml volumes of chloroform followed by 1 ml of 20 % of ammonia. The mixtures were gently agitated. A pink red colour showed the presence of anthraquinones derivatives.

8.2.3 Quantitative determination of phenolics

8.2.3.1 Determination of Total phenols (Singleton et al, 1965)

125 µl of samples were added to aliquots of 1.75 ml of distilled water followed by 125 µl of 50 % Folin-Ciocalteu reagent (Merck). After three minutes, 500 µl volumes of 20 % sodium carbonate were added and the test tubes were well shaken. They were then incubated at 40 ºC in a water bath for 40 minutes. After incubation, the tubes were left to cool in the dark. A blue coloration obtained indicated the presence of phenols and the absorbance of the samples was measured at 685 nm. The results were expressed in µg of gallic acid per 25 mg fresh weight of plant materials.

8.2.3.2 Determination of Proanthocyanidin Content (Porter *et al,* 1986)

Volumes of 125 µl of methanolic plant extracts were each added to 1.5 ml of 95% solution of n-butanol- HCl (95:5 v/v) in tightly sealed test tubes. Aliquots of 100 µl of 2 % $NH_4Fe(SO_4)_2.12H_2O$ in 2 M HCl were then added. The test tubes were shaken and their absorbance was measured at 550 nm. The samples were then incubated at 95º C for 40 minutes. A pink red coloration indicated the presence of proanthocyanidins and the absorbance was read again at 550 nm. The results were expressed in mg of Cyanidin chloride per 25 mg fresh weight of plant materials.

8.2.3.3 Determination of Flavonoids Content (Lamaison et al, 1990)

The flavonoids content was estimated by mixing 1.5 ml of methanolic extracts to an equal volume of 2 % methanolic aluminium trichloride ($AlCl_3.6H_2O$). The mixtures were left for 10 minutes at room temperature. The absorbance was read at 440 nm and the results were expressed in µg of Quercetin per 25 mg fresh weight of plant materials.

8.2.4 Antibacterial assay

Samples were prepared at a concentration of 0.1g/ml by dissolving the lyophilised extracts in 10 % of dimethyl sulphoxide (DMSO, Merck). The disc diffusion method was used for the antibacterial assay. Volumes of 100 µl of a 1:100 dilution of an overnight culture of each bacterial strain (0.5 Mc Farland or 10^8- 10^9 CFU /ml) were spread onto Müller Hinton Agar (MHA). Sterile filter paper discs of 6 mm diameter were placed onto the MHA and were gently pressed. The discs were impregnated with the *Diospyros* extracts (0.1g/ml) and were allowed to diffuse for 20 minutes. Penicillin G discs (10µg), ampicillin(10µg), tetracycline (20 µg), chloramphenicol discs (30 µg) and gentamicin (10 µg) were used as positive controls whereas 10 % of DMSO was used as negative control. The plates were incubated for 24/48

hrs at 35 ºC. After incubation the plates were examined and the diameters of the zone of inhibition (mm) were measured and recorded. All tests were done in triplicates.

8.2.5 Preliminary antimalarial and antitumour assays

The barks, leaves and roots of the *Diospyros* species were harvested and extracts were made as in section 8.2.1. The alkaloid extracts were made using 100 g of lyophilized plant material.

8.2.5.1 In vitro antimalarial assay

The antimalarial activity of plant extracts was assessed against a chloroquine susceptible strain of *Plasmodium falciparum* available at the Royal Danish School of Pharmacy, University of Copenhagen. The *P. falciparum* strain was cultivated in human red blood cells (O+), diluted to 2% hematocrit in RPS10 (RPMI 1640 medium supplemented with 25 mM HEPES and 25 mM NaHCO3 and complemented with 10% human A+ serum and 5% Neomycin). Aqueous extracts were dissolved in ultrapure water while methanol and alkaloid extracts were dissolved in DMSO These solutions were filtered through Millipore sterile filters (mesh 0.22µm, Millipore) and diluted 1/20 in RPS10 to obtain the tests stock solutions. Three concentrations namely, 100, 50 and 25µg/ml were tested in triplicate cultures. The negative control was red blood cells without *Plasmodium* and positive control consisted of infected blood cells culture not treated with plants extract. The antimalarial assays were performed in 96-well tissue culture plates. The cultures were incubated at 37°C for 48 h in an atmosphere of 2% O2, 5%CO2 and 93% N2. Twenty four hours before termination of incubation, 20µl of L-[2,3,4,5,6-3H] phenylalanine (37ml:ml) were added into each well. The effect of the extracts in the cultures were monitored by the measurement of L-[2,3,4,5,6-3H]phenylalanine incorporation into the parasite nucleic acids. The cultures were performed in triplicates.

The antimalarial activity of plant extracts was expressed by the inhibitory concentrations 50 (IC50), representing the concentration of drug that induced a 50% decrease compared to the positive control culture referred as 100% parasitaemia. Activity of an extract was evaluated according to Rasaonaivo *et al*, 1999 (very active if IC50 < 5µg/ml, active 5µg/ml < IC50 < 50µg/ml, weakly active 50µg/ml < IC50 <100µg and inactive IC50 > 100 µg/ml).

8.2.5.2 Antitumour Assay

Most of the lyophilised plant samples were found to be strongly cytotoxic at 25µg/ml. A cell free and a cell based mitogen activated protein kinase (MAPK) assay were used to evaluate the inhibitory activities of the plant extracts according to the manufacturers' protocol (Stratagene, Promega).

8.3 Results

8.3.1 Phytochemical screening and polyphenol analysis

Phytochemical screening enabled us to highlight different classes of compounds in the Mauritan *Diospyros* extracts (Table 8.1). Reducing sugars, tannins, cardiac glycosides and terpenoids were found to be present in all the eleven species. Steroids, saponins and anthraquinones were also found to be present in nearly all species. However, alkaloids were present in two species (*D.neraudii* and *D.revaughanii*) only. Polyphenolic analysis showed that Mauritian *Diospyros* contain a relatively high concentration of total phenols including proanthocyanidins and flavonoids (Graph 8.1) and complex polyphenolic profiles (HPLC traces in appendix). *D.eggretarum* contained the lowest amount of phenolics compounds (total phenol: 321.1±1.78 µg/25mg FW; proanthocyanidins: 0.09 mg/25mg FW and flavonoids: 31.13±0.5 µg/25mg FW) whereas *D.melanida* had the highest amount of polyphenols with total phenolic content 1535±32.65 µg/25mg FW, proanthocyanidins 0.67±0.02mg/25mg FW and flavonoids 462.6±2.15µg/25 mg FW.

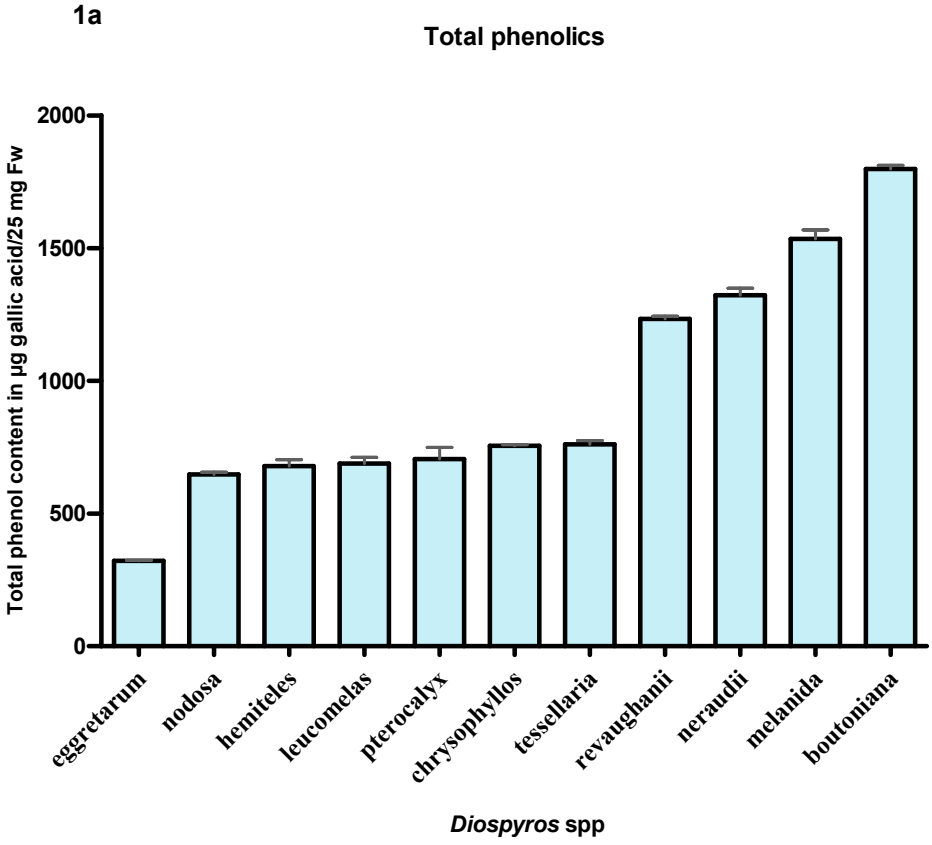
Table 8.1: Phytochemical screening of *Diospyros* species

Samples	Saponins	Reducing sugars	Alkaloids	Phenols	Cardiac glycosides	Steroids	Anthra-quinones	Terpenoids
D. nodosa	+	++	-	++(bg)	++/+	++	+++	++
D. pterocalyx	~	+	-	++ (bg)	+	+	~	+
D.boutoniana	++	+++	-	+++ (bg)	++/+	++	+++	+++
D.melanida	++	+ ++	-	++(bg)	+++	+++	+++	++/+
D.tessellaria	++	+	~	++	++	++	++	++
D.chrysophyllos	++	++	~	++ (bg)	++	++	++	++
D.neraudii	++	+++	++	+++ (bg)	+++	++	++/+	++/+
D.leucomelas	++	++	-	++ (bg)	+++	++	+++	++
D.egrettarum	++	++	~	+ (b)	+	~	~	+
D.revaughanii	+	+++	+	++	++	++	++/+	++
D.hemiteles	+	+	-	++	++/+	+	+	+

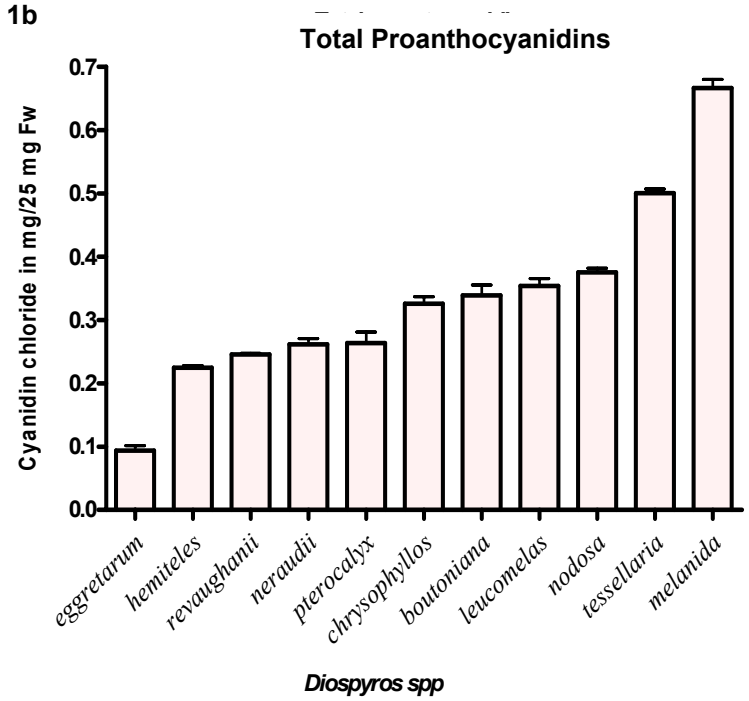
Key: ~: trace; -: not detected; (b): blue precipitate; **(**bg**)**: both blue and green precipitate

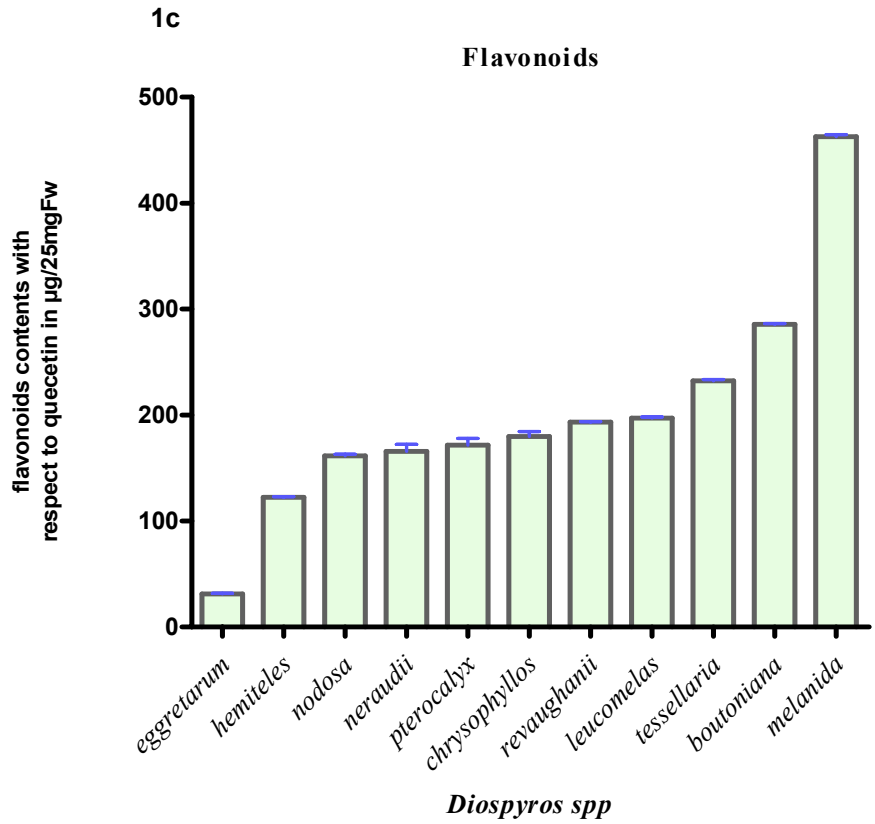
+++/++/+: Evaluation of results according to Malec *et al*. (2003).

1a


Total phenolics
Total phenol content in µg gallic acid/25 mg Fw
2000
1500
1000
500
0
eggretarum
nodosa
hemiteles
leucomelas
pterocalyx
chrysophyllos
tessellaria
revaughanii
neraudii
melanida
boutoniana
Diospyros spp

1b


Total Proanthocyanidins
Cyanidin chloride in mg/25 m g Fw
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
eggretarum
hemiteles
revaughanii
neraudii
pterocalyx
chrysophyllos
boutoniana
leucomelas
nodosa
tessellaria
melanida
Diospyros spp



Figures 8.1a, 8.1b and 8.1c : Amount of total phenol, proanthocyanidins and flavonoids in 11 species of *Diospyros*/25 mg FW

8.3.2 Antibacterial Assay

The antibacterial activities of the *Diospyros spp.* are shown in Table 8.2. The extracts showed different degree of bactericidal properties as both gram-negative and gram-positive bacteria were susceptible to them. The zones of inhibition obtained from these extracts were pronounced compared to positive controls (tetracycline, ampicillin, gentamicin and chloramphenicol) in certain bacteria. *D.egrettarum* and *D.hemiteles* showed the lowest antimicrobial activity compared to the rest of the species. *P.mirabilis* and *S.aureus* were resistant to extracts of *D.pterocalyx* and *D.tessellaria* respectively whereas all the bacterial strains were sensitive to the *Diospyros* extracts. It is noteworthy to point out that some *S.aureus, P.aeruginosa* and *A.baumanii* were resistant to antibiotics used (positive control) but were sensitive to most of the extracts.

Table 8.2: Antibacterial activities of Mauritian *Diospyros* species

	Test Organisms / Zone of inhibition (mm)								
Sample extracts	*B.cereus (ATCC 10876)*	*B.spizizenii (ATCC 6633)*	*Staphylococcus areus (ATCC 33592)*	*Staphylococcus epidermidis (ATCC 12228)*	*Proteus mirabilis (ATCC 7002)*	*Pseudomonas aeruginosa (ATCC 9027)*	*E.coli (ATCC0876)*	*Klebsiella pneumoniae (ATCC 4352)*	*Acinetobacter baumanii (ATCC 19 606)*
D.revaughanii	16	15	15	14	12.5	12	12	13	11
D. pterocalyx	14	13	13	12	-	11	11	15	10
D. nodosa	14	14	15	11	11	13	10	13	10
D.tessellaria	13	14	-	12	12	13	14	14	15
D.boutoniana	17	14	16	14	15	15	15	15	13
D.melanida	17	15	16	13	12	15	15	16	13
D.neraudii	15	15	14	14	12	12	15	14	12
D.leucomelas	10	14	17	10	11	11	13	14	14
D.chrysophyllos	15	14	12	8	12	11	14	13	14
D.egrettarum	12	-	10	-	-	10	0	-	12
D.hemiteles	12	-	12	-	-	10	0	-	10
Tetracycline (20 µg)	20	27	10	-	-	10	20	20	15
Ampicillin (10µg)	-	26	-	19	17	-	18	10	0
Gentamicin (10 µg)	16	27	-	30	17	15	20	20	0
Chloramphenicol (30µg)	10	28	-	32	15	-	25	20	0
Penicillin (10µg)	-	18	-	12	-	-	0	0	0
DMSO	-	-	-	-	-	-	-	-	-

8.3.3 Antimalarial and antitumour assays

Only the methanol extract of the root and bark of *D. egrettarum* showed a weak activity (IC_{50}=51.5±1.12 for root and 56.8±2.01 for bark). All the other samples were found to have no significant antimalarial activity.

In the cell-free MAPK, the plant extracts (25µg/ml) showed strong inhibitory activities as determined by a reduction (≥ 50%) of ^{32}P into the model peptide used to measure MAPK. Prompted by these activities, the three most potent extracts (*D. chrysophyllos, D. egrettarum* and *D. melanida*) were further assayed in a cell-based MAPK. The treated cells were then lysed and cellular proteins analysed by electrophesis and the gel treated with an antibody directed against the phosphorylated form of the MAPK substrate. Because of the strong cytotoxic effect of the plant extracts, only very low concentrations (< 5µg/ml) could be used. This reduced dose of plant extracts did not produce any reduction in phosphorylation of the MAPK protein substrate. At this point it is not clear whether the reduction in the MAPK activity observed earlier (cell-free MAPK) is a non specific reaction with the protein rather than enzyme inhibition by a competitive mechanism. Perhaps, different methods of extraction (especially for *D. chrysophyllos, D. egrettarum* and *D. melanida*) need to be explored for the MAPK assay.

8.4 Discussion

The bactericidal activity of *Diospyros* showed that both Gram positive and Gram negative bacterial strains were sensitive to almost all the eleven extracts. It is important to note that *S.aureus, A.baumanii* and *P.aeruginosa* were resistant to the commercial antibiotics but were sensitive to the plants extracts. Consequently, there is a great potential that these species possess molecules, which have characteristics that could be useful in the search for new antibacterial agents. The antibacterial properties of the plant extracts could perhaps be associated with the high amount of polyphenols (Figures 8.1a, 8.1b and 8.1c). Indeed, *D.boutoniana, D.melanida, D.neraudii* and *D. revaughanii* were among the species with high phenolic contents (1233 ±10.4 – 1798±13.7 µg/25 mg FW) and all the nine bacteria used in the assays were sensitive to those extracts. However, *D.egrettarum* and *D.hemiteles*, which showed the lowest amount of polyphenols, inhibited the growth of only 4 bacterial strains. This strengthens the notion that polyphenols constituents could be responsible for *Diospyros* antibacterial characteristics as have been widely reported (Bruneton, 1995 and Trease and Evans, 1989) and flavonoids (Iinuma *et al*. 1994; Alcaráz *et al*, 2000; Martini *et al*, 2004 and

Johann *et al*, 2007). However, antibacterial properties can also be linked to the presence of natural bioactive constituents such as: tannins (Izo *et al*, 1995 and Onuwukaeme *et al*, 2007), saponins (Tschesche, 1970) and anthraquinones (Akinyemi *et al*, 2005) or terpenoids including naphthoquinones (diospyrin; isodiospyrin and diosquinone) show active antibacterial activity in this genus (Sheherbanovskill *et al*, 1972; Alake, 1994; Khan *et al*, 1999; Adeniyi *et al*, 2000 and Gu *et al*, 2004). It is important to note that more and more molecules are being discovered in this genus which shows promising results against different pathogens. Amyrins and ursolic acid inhibits both Gram (+) and Gram (-) bacteria, where *P.syringae* were the most sensitive (Mallavadhani *et al*, 2004). Kaneshiro *et al*, 2000 have reported that atovaquone and diospyrin-based drugs was effective against plasmodium and *Pneumocytis carinii* organisms. Napthoquinones and triterpenes showed ichthyotoxic (Higa *et al*, 2002), antimicrobial (Khan *et al*, 1999 and Gu *et al*, 2004) and antitumor (Gu *et al*, 2004; Kuo *et al*, 1997). Diospyrin had also been isolated from *Diospyros montana* Roxb, an Indian species which possessed significant anti-tumour and anti-leishmanial activities (Hazra *et al*, 1984 and Yardley *et al*, 1996). Diospyrin and its derivatives were also reported to exhibit antimycobacterial (Lall *et al*, 2003) and antiparasitic properties (Hazra *et al*, 1994 & 1995). Furthermore, diospyrodin from *D.nigra* showed antibacterial activity (Dinda *et al*, 2006).

Interestingly, Adeniyi *et al* (1996) showed that extracts of other members of the *Diospyros* genus namely *D.mespilformis* inhibited the growth of *S.aureus* and *P.aeruginosa*. We confirmed that all the extracts of Mauritian *Diospyros* also inhibited *S.aureus* (except *D.tessellaria*), *P.aeruginosa* and *A.baumanii*. In the light of the results obtained so far, further studies need to be carried out in order to determine which compounds can be ascribed to the antibacterial properties of the Mauritian *Diospyros* species.

Although modest antimalarial and antumour activities have been detected in some of the Diospyros species, alternative extraction procedures that would diminish cell cytotoxity need to be applied. We would then be in a better position to reevaluate antimalarial and antitumour properties of the local *Diospyros* species.

9.0 References

1. Adeniyi BA, Fong HH, Pezzuto Jm, Luyengi L, Odelola HA. 2000. Antibacterial activity of diospyrin, isodiospyrin and bisisodiospyrin from the root of *Diospyros piscatoria*. ***Phytotherapy Research***. 14:112-7.
2. Adeniyi BA, Odelola HA, Oso BA. 1996. Antimicrobial potentials of *Diospyros mespiliformis* (Ebenaceae). ***African Journal of Medical Science****.* 25:221-224.
3. Akinyemi KO, Oluwa OK, Omomigbehin OE. 2006. Antimicrobial activity of crude extracts of three medicinal plants used in South- West Nigerian folk medicine on some food borne bacterial pathogens. ***African Journal of Traditional, Complementary and Alternative Medicines****.* 3:13-22.
4. Alake LB. 1994. Antibacterial activity of diosquinone isolated from *Diospyros tricolor*. ***Planta Medica***.60:477.
5. Alcaráz LE, Blanco SE, Puig ON, Tomás F, Ferretti FH. 2000. Antibacterial activity of Flavonoids against methicillin-resistant *Staphylococcus aureus* strains. ***Journal of theoretical biology***. 205,231-240.
6. Aldasoro JJ, Cabezas F, Aedo C. 2004. Diversity and distribution of ferns in Sub-Saharan Africa, Madagascar and some islands of the South Atlantic. ***Journal of Biogeography***. 31: 1579-1604.
7. Alstrom-Rapaport C, Lascoux M, Wang YC, Roberts G, Tuskan GA. 1998. Identification of a RAPD marker linked to sex determination in the basket willow (*Salix viminalis* L.) ***The Journal of Heredity***. 89: 44-49.
8. Alvarez I, Wendel JF. 2003. Ribosomal ITS sequences and plant phylogenetic inference. ***Molecular Phylogentics and Evolution***. 29:417-434.
9. Anderson GJ, Bernardello G, Lopez P, Stuessy TF, Crawford DJ. 2000**.** Dioecy and wind pollination in *Pernettya rigida* (Ericaceae) of the Juan Fernandez Islands. ***Botanical Journal of the Linnean Society*.** 132:121-141.
10. Antos JA, Allen GA. 1999. Patterns of reproductive effort in male and female shrubs of *Oemleria cerasiformis*: a 6-year study. ***Journal of Ecology***. 87:77-84.
11. Baker HG, Cox PA. 1984. Further thoughts on dioecism and islands. ***Annals of the Missouri Botanical Garden****.* 71:244-253.
12. Baker HG, Cox PA. 1984. Further thoughts on dioecism and islands. ***Annals of the Missouri Botanical Garden***. 71:244-253.

13. Baker HG. 1877**.** Flora of Mauritius and the Seychelles. Reeve L, London.
14. Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. ***Annals of the Missouri Botanical Garden***. 82:247-277.
15. Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. ***Annals of the Missouri Botanical Garden***. 82:247-277.
16. Baldwin BG, Sanderson MJ. 1998. Age and rate of diversification of the Hawaiian silversword alliance (Compositae). ***Proceedings of the National Academy of Sciences, USA.*** 95:9402-9406.
17. Ballard HE Jr, Systma JJ. 2000. Evolution and biogeography of the woody Hawaiian violet (*Viola*, Violaceae): arctic origins, herbaceous ancestry and bird dispersal. ***Evolution.*** 54:1521-1532.
18. Barber JC, Francisco-Ortega J, Santhos-Guerra, Marrero A, Jansen RK. 2000. Evolution of endemic *Sideritis* (Lamiaceae) in Macaronesia: insights from a chloroplast DNA restriction site analysis. ***Systematic Botany***. **25**:633-647.
19. Bawa KS. 1980**.** Evolution of dioecy in flowering plants. ***Annual Review of Ecology & Systematics***. 11:15-40.
20. Böhle UR, Hilger HH, Martin WF. 1996. Island colonization and evolution of the insular woody habit in *Echium* L (Boraginaceae). ***Proceedings of the National academy of Sciences, USA.*** 93:11740-11745.
21. Bosser J, Cadet TH, Gueho J, Julien HR, Marais W. 1976 ongoing. Flore des Mascareignes, La Reunion, Maurice et Rodrigues. MSIRI, ORSTROM, RBG Kew, Port-Louis, Mauritius.
22. Bram MR, Quinn JA. 2000. Sex expression, se-specific traits, and the effects of salinity on growth and reproduction of *Amaranthus cannabinus* (Amaranthaceae), a dioecious annual. ***American Journal of Botany***. 87:1609-1618.
23. Brummitt RK. 1992. Vascular plant families and genera. Royal Botanic Gardens, Kew, Richmond, Surrey, UK.
24. Bruneton J. 1995. Pharmacognosy, Phytochemistry and Medicinal Plants. Intercept Ltd London, New York. 2nd Edn. p. 386.
25. Cadet TH. 1977. La végétation de l'Ile de la Réunion : Etude phytoécologique et phytosociologique. Ph.D thesis, University of Aix-Marseille III.

26. Cadet TH. 1984. Fleurs et plantes de la Reunion et de l'ile Maurice. Les Editions du pacifique. Singapore.
27. Cheke AS, Dahl JF. 1981. The status of bats in the Western Oceanic Islands with special reference to Pteopus. ***Mammalia***. 45:205-238.
28. Correia O, Diaz Barradas MC. 2000. Ecophysiological differences between male and female plants of *Pistacia lentiscus* L. ***Plant Ecology***. 149:131-142.
29. Crawford RMM, Balfour J. 1983. Female predominant sex ratios and physiological differentiation in arctic willows. ***Journal of Ecology***. 71:149-160
30. Crisci JV, Katinas L, Posadas P. 2003. Historical Biogeography, An Introduction. Harvard University Press.
31. Cronquist A. 1981. An integrated system of classification of flowering plants. Columbia University Press, New York.
32. De Cordemoy EJ. 1895. Flore de l'Ile de la Reunion. P.Klincksiet, Paris.
33. Dinda B, Bhattacharya A, De UC, Arima S, Takayanagi H, Harigaya Y. 2006. Antimicrobial *C*-Glucoside from Aerial Parts of *Diospyros nigra.* Chemical & pharmaceutical bulletin**.** 54:679-681.
34. Esperito-Santo MM, Madeira BG, Neves FS, Faria MI, Fagundes M, Wilson-Fernandes G. 2003. Sexual differences in reproductive phenology and their consequences for the demography of *Baccharis dracunculifolia* (Asteraceae), a dioecious tropical shrub. ***Annals of Botany***. 91:13-19.
35. Farnsworth NR, Soejarto DD. 1991. Global importance of medicinal plants. In: Akerele O, Heywood V, Synge, H. (Eds). Conservation of Medicinal Plants. Cambridge University Press, Cambridge.
36. Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. ***Evolution***. 39:783-791.
37. Francisco-Ortega J, Crawford DJ, Santos-Guerra A, Jansen RK. 1997. Origin and evolution of *Argyranthemum* (Astercaceae:Anthemideae) in Macaronesia. In Givnish TJ, Systma KJ (eds). Molecular evolution and adaptive radiation. Cambridge University Press, Cambridge, UK. Pp 407-432.
38. Francisco-Ortega J, Fuertes-Aguilar J, Kim SC, Santhos-Guerra A, Crawford DJ, Jansen RK. 2002. Phylogeny of the Macaronesian endemic *Crambe* section *Dendocrambe* (Brassicaceae) based on internal transcribed spacer sequences of nuclear ribosomal DNA. ***American Journal of Botany***. 89:1984-1990.

39. Francisco-Ortega J, Jansen RK, Santos-Guerra A. 1996. Chloroplast DNA evidence of colonization, adaptive radiation and hybridization in the evolution of the Macaronesian flora. ***Proceedings of the National Academy of Sciences, USA***. 93:4085-4090.
40. Francisco-Ortega J, Jansen RK, Santos-Guerra A. 1996. Chloroplast DNA evidence of colonization, adaptive radiation and hybridization in the evolution of the Macaronesian flora. ***Proceedings of the National Academy of Sciences, USA***. 93:4085-4090.
41. Givnish TJ. 1997. Adaptive radiation and molecular systematics: issues and approaches. In Givnish TJ and Systma KJ (eds). Molecular evolution and adaptive radiation. Cambridge University Press. Cambridge. Pp 1-54.
42. Godley EJ. 1979. Flower biology in New Zealand. ***New Zealand Journal of Botany*** 17:441-466.
43. Goodstadt L, Ponting C.P. 2001. CHROMA: Consensus-based colouring of multiple alignments for publication. ***Bioinformatics***. 17:845-846.
44. Gu JQ, Graf TN, Lee D, Chai HB, Mi Q, Kardono LBS, Setyowati FM, Ismail R, Riswan S, Farnsworth NR, Cordell GA. 2004. ***Journal of Natural Products***. 67:1156-161.
45. Gunter LE, Kopp RF, McCord RP, Tuskan GA. 2003. Analysis of sex-linked, sequencecharacterized amplified region markers in *Salix eriocephala*. Canadian Journal Of Forest Research-Revue Canadienne De Recherche Forestiere. 33:1785-1790.
46. Hall T. 2004. BioEdit version 7.05.
47. Hansen I, Brimer L, Mølgaard P. 2004. Herbivore-deterring secondary compounds in heterophyllous woody species of the Mascarene Islands. ***Perspectives in Plant Ecology, Evolution and Systematics***. 6:187-203.
48. Harborne JB. 1983. Phytochemical Method: A guide to Modern Techniques of Plants Analysis. 2nd Edn. Chapman and Hall New York.
49. Harvey CF, Fraser LG, Gill GP. 1997. Sex determination in *actinidia*. ***Acta Horticulturae*** (ISHS). 444: 85-88
50. Hasegawa M, Kishino H, Yano T. 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. ***Journal of Molecular Evolution***. 22: 160-174.
51. Hazra B, Ghosh R, Banerjee A, Kirby GC, Warhurst DC, Phillipson JD. 1995. *In vitro* antiplasmodial effects of diospyrin, a plant-derived naphthoquinoid, and a novel series of derivatives. ***Phytotheraphy Research***. 9**:**72–74.

52. Hazra B, Pal S, Ghosh R, Banerjee A. 1994. Studies on changes in tumour-inhibitory activities through structural modification of a diospyrin derivative. ***Medical Science Research***. 22**:**621-623.

53. Hazra B, Sur P, Roy DK, Sur B, Banerjee A. 1984. Biological activity of diospyrin towards Ehrlich ascites carcinoma in Swiss A mice. ***Planta Medica***. 51:295-7.

54. Hijmans RJ, Cruz M, Rojas M, Guarino L. 2001. DIVA-GIS, Version 1.4. A geographic information system for the management and analysis of genetic resources data. Manual. International Potato Center, Lima, Peru.

55. Hormaza JI, Dollo L, Polito VS. 1994. Identification of a RAPD marker linked to sex determination in *Pistacia vera* using bulked segregant analysis. ***Theoretical and Applied Genetics***. 89: 9-13.

56. Houle G, Duchesne M. 1999. The spatial pattern of a *Juniperus communis* var *depressa* population on a continental dune in subarctic Quebec, Canada. ***Canadian Journal of Forest Research***. 29:446-450.

57. Humeau I, Pailler T, Thompson JD. 1999. Cryptic dioecy and leaky dioecy in endemic species of *Dombeya* (Sterculiaceae) on La Reunion. ***American Journal of Botany*** 86:1437-1447.

58. Humeau I, Pailler T, Thompson JD. 1999. Cryptic dioecy and leaky dioecy in endemic species of *Dombeya* (Sterculiaceae) on La Reunion. ***American Journal of Botany***. 86:1437-1447.

59. Iinuma M, Tsuchiya H, Sato M, Yokoyama J, Ohyama M, Ohkawa Y, Tanaka T, Fujuwara S, Fujii T. 1994. Flavanones with potent antibacterial activity against methicillin-resistant *Staphylococcus aureus*. ***The Journal of Pharmacy and Pharmacology***. 46: 892-895.

60. Izo AA, Cario GD, Briscards D. 1995. Biological screening of Italian medicinal plants for antibacterial activity. ***Phytotherapy Research***. 9: 281-286.

61. Jarvis A, Ferguson ME, Willaims DE, Guarino L, Jones PG, Stalker HT, Valls JFM, Pittman RN, Simpson CE, Bramel P. 2003. Biogeography of wild *Arachis*: Assessing conservation status and setting future priorities. ***Crop Science***. 43: 1100-1108.

62. Johann S, Lopes De Oliveira V, Moacir G, Pizzolatti MG, Schripsema J, Braz-Filho R, Branco A, Smânia A Jr. 2007. Antimicrobial activity of wax and hexane extracts from Citrus spp. Peels. *Memórias do Instituto Oswaldo Cruz, Rio de Janeiro*. 102: 681-685.

63. Juan C, Emerson BC, Oromi P, Hewitt GM. 2000. Colonisation and diversification: towards a phylogeographic synthesis for the Canary Islands. ***Trends in Ecology and Evolution***.15:104-109.
64. Kameda K, Takaku T, Okuda H, Kimura Y, Okuda T, Hatano T, Agata I, Arichi S. 1987. Inhibitory effects of various flavonoids isolated from leaves of persimmon on angiotensin-converting enzyme activity. ***Journal of Natural Produce***. 50:680-3.
65. Kapadia GJ, Balasubramanian V, Tokuda H, Konoshima T, Takasaki M, Koyama J, Tagahaya K, Nishino H. 1997. Anti-tumour promoting effects of naphthoquinone derivatives on short term Epstein-Barr early antigen activation assay and in mouse skin carcinogenesis. ***Cancer Letters***. 113:47-53.
66. Khan MR, Timi D. 1999. Constituents of *Diospyros lolin*, *D. maritima* and *D. novoguinensis*. ***Fitoterapia***. 70:194-196.
67. Kim HG, Keeley SC, Vroom PS, Jansen RK. 1998. Molecular evidence for an African origin of the Hawaiian endemic *Hesperomannia* (Astercaceae). ***Proceedings of the National Academy of Sciences, USA***. 95:15440-15445.
68. Kim KJ, Jansen RK. 1994. Comparison of phylogenetic hypotheses among different data sets in dwarf dandelions (*Krigia*): additional information from internal transcribed spacer sequences of nuclear ribosomal DNA. ***Plant Systematics and Evolution***. 190:157-185.
69. Kim SC, Crawford DJ, Francisco-Ortega J, Santos-Guerra A. 1996. A common origin for woody *Sonchus* and five related Macaronesian islands: molecular evidence for extensive radiation. ***Proceedings of the National Academy of Sciences, USA***. 93:7743-7748.
70. Kim SC, Crawford DJ, Francisco-Ortega J, Santos-Guerra A. 1996. A common origin for woody *Sonchus* and five related Macaronesian islands: molecular evidence for extensive radiation. ***Proceedings of the National Academy of Sciences, USA***. 93:7743-7748.
71. Koch MA, Dobes C, Mitchell-Olds T. 2003. Multiple hybrid formation in natural populations: concerted evolution of the Internal Transcribed Spacer of the Nuclear Ribosomal DNA (ITS) in North American *Arabis divaricarpa* (Brassicaceae). ***Molecular Biology Evolution***. 20:338-350.
72. Kolawole OM, Oguntoye SO, Agbede O, Olayemi AB. 2007. Studies on the efficacy of *Bridelia ferruginea* Benth. Bark extract in reducing the coliform load and BOD of domestic wastewater. ***Bulletin of the chemical society of Ethiopia***. Vol 21, No 2.
73. Kuo YH, Chang CI, Li SY, Chou CJ, Chen CF, Kuo YH, Lee KH. 1997. Cytotoxic Constituents from the Stems of Diospyros Maritima. ***Planta Medica***. 63:363-365.

74. Lamaison JLC, Carnet A. 1990. Teneurs en principaux flavonoids des fleurs de *Cratageus monogyna* Jacq et de Cratageus laevigata (Poiret DC) en function da la vegetation. ***Pharmaceutica acta Helvetiae***. 65:315-320.

75. Le Corff J, Ågren J, Schemske DW. 1998. Floral display, pollinator discrimination, and female reproductive success in two monoecious *Begonia* species. ***Ecology***. 79:1610-1619.

76. Likhitwitayawuid K, Dej-adisai S, Jongbunprasert V, Krungkrai J. 1999. Antimalarials from *Stephania venosa*, *Prismatomeris sessiliflora*, *Diospyros montana* and *Murraya siamensis*. ***Planta Medica***. 65:754-6.

77. Maki J, Kondo A, Yanagisawa T. 1983. Effects of alcoholic extract from Ma-Klua (*Diospyros mollis*) on adults and larvae of the dwarf tapeworm, *Hymenolepis nana* in mice and on the infectivity of the eggs. ***Parasitology***. 87:103-11.

78. Mallavadhani UV, Panda AK, Rao YR. 1998. Pharmacology and chemotaxonomy of *Diospyros*. ***Phytochemistry***. 49:901-51.

79. Mandolino G, Carboni A, Forapani S, Faeti V, Ramalli P. 1999. Identification of DNA markers linked to the male sex in dioecious hemp (*Cannabis sativa* L.). ***Theoretical and Applied Genetics***. 98:86-92.

80. Manoj P, Banerjee NS, Ravichandran P. 2005. Development of sex-associated SCAR markers in *Piper longum* L. ***Plant Genetic Resources Newsletter***. 141:44-50.

81. Martini ND, Katereke DRP, Eloff JN. 2004. Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (Combretaceae). ***Journal of Ethnopharmacology***. 93:207-212.

82. Martz W. 1992. Plants with a reputation against snakebite. ***Toxicon***. 30:1131-42.

83. Moore MJ, Francisco-Ortega J, Santos-Guerra A, Jansen RK. 2002. Chloroplast DNA evidence for the roles of island colonization and extinction in *Tolpis* (Asteraceae: Lactuceae). ***American Journal of Botany***. 89:518-526.

84. Mort ME, Soltis DE, Soltis PS, Francisco-Ortega J, Santos-Guerra A. 2002. Phylogenetics and evolution of the Macaronesian clade of Crassulaceae inferred from nuclear and chloroplast sequence data. ***Systematic Botany***. 27:271-288.

85. Mulcahy D L, Weeden NF, Kesseli R, Carroll SB. 1992. DNA probes for the Y-chromosome of *Silene latifolia*, a dioecious angiosperm. Sexual plant reproduction. 5:86-88.

86. Negrón-Ortiz V and Watson LE. 2003. Hypotheses for the colonization of the Caribbean Basin by two genera of the Rubiaceae. ***Systematic Botany***. 28:442-451.
87. Ng FSP. 1986. Tree flora of Malaya: a manual for foresters. Longman Group Ltd. London.
88. Nicotra AB. 1999. Reproductive allocation and the long-term costs of reproduction in *Siparuna grandiflora*, a dioecious neotropical shrub. ***Journal of Ecology***. 87:138-149.
89. Nyhagen FD. 2001. Foraging ecology of the Mauritian flying fox *Pteropus niger* (Megachiroptera). MSc thesis. University of Aarhus.
90. Onwukaeme DN, Ikuegbvweha TB, Asonye CC. 2007. Evaluation of Phytochemical constituents, antibacterial activities and effect of exudates of *Pycanthus Angolensis* Weld Warb (Myristicaceace) on corneal ulcers in rabbits. ***Tropical Journal of Pharmaceutical Research***. 6:725-730.
91. Ornduff R. 1985. Male-biased sex ratios in the cycad *Macrozamia riedlei* (Zamiaceae). ***Bulletin of the Torrey Botanical Club***. 112:393-397.
92. Padya BM. 1984. Climate of Mauritius. 2nd Edition. Government printer. Mauritius.
93. Padya BM. 1989. Weather and climate of Mauritius. Geography of Mauritius Series. Mahatma Gandhi Institute.
94. Page WS, D'Argent G. 1997. A vegetation survey of Mauritius (Indian Ocean) to identify priority rainforest areas for conservation management. Unpublished.
95. Parish DH, Feillafé SM. 1965. Notes on the 1:100 000 soil map of Mauritius. Mauritius Sugar Industry Research Institute. Mauritius.
96. Parish DH, Feillafe SM. 1965. Notes on the 1:100,000 soil map of Mauritius. Mauritius Sugar Industry Research Institute. Occasional paper No 22.
97. Pitot A. 1905. T'Eylandt Mauritius. Esquisses historiques, 1598-1710. Port-Louis. Mauritius.
98. Pitot A. 1905. T'Eylandt Mauritius. Esquisses historiques, 1598-1710. Port-Louis. Mauritius.
99. Porter LJ, Hrstich LM, Chan BC. 1986. The conservation of proanthocyanidins and prodelphinidins to cyanidins and delphinidins. ***Phytochemistry***. 25:225-230.
100. Rasoanaivo P, Ratsimamanga-Urverg S, Ramanitrhasimbola D, Rafatro H, Rakoto-Ratsimamanga A. 1992. Criblage d'extraits de plantes de Madagascar pour recherche d'activité antipaludique et d'effet potentialisateur de la chloroquine. ***Journal of Ethnopharmacology***. 64 :117-126.

101. Recio MC, Giner RM, Manez S, Gueho J, Julien HR, Hostettmann K, Rios JL. 1995. Investigations on the steroidal anti-inflammatory activity of triterpenoids from *Diospyros leucomelas*. ***Planta Medica***. 61: 9-12.

102. Renner S, Ricklefs R. 1995. Dioecy and its correlates in the flowering plants. ***American Journal of Botany***. 82:596-606.

103. Richardson IBK. 1980. Notes on *Diospyros* (Ebenaceae) in the Mascarene Islands. ***Kew Bulletin***. 34:723-735.

104. Rieseberg LH, Whitton J, Linder CR. 1996. Molecular marker incongruence in plant hybrid zones and phylogenetic trees. ***Acta Botanica of the Netherlanda***. 45:243-262.

105. Rocheleau AF, Houle G. 2001. Different cost of reproduction for the males and females of the rare dioecious shrub *Corema conradii* (Empetraceae). ***American Journal of Botany***. 88:659-666.

106. Rouillard G, Guého J. 1999. Les plantes et leur histoire à l'Ile Maurice. MSM Limited. Mauritius.

107. Ruas CF, Fairbanks DJ, Evans RP, Stutz HC, Andersen WF, Ruas PM. 1998. Male-Specific DNA in the Dioecious Species *Atriplex garrettii* (Chenopodiaceae). ***American Journal of Botany***. 85:162-167

108. Sadul P. 1995. Mauritius, a geomorphological analysis. Mahatma Gandhi Institute, Mauritius.

109. Sakai AK, Wagner WL, Ferguson DM, Herbst DR. 1995a. Origins of dioecy in the Hawaiian flora. ***Ecology***. 76:2517-2529.

110. Sakai AK, Wagner WL, Ferguson DM, Herbst DR. 1995b. Biogeographical and ecological correlates of dioecy in the Hawaiian flora. ***Ecology***. 76:2530-2543.

111. Sakai AK, Weller SG. 1991. Ecological aspects of sex expression in subdioecious *Schiedea globosa* (Caryophyllaceae). ***American Journal of Botany***. 78:1280-1288.

112. Sakamoto K, Shimomura K, Komeda Y, Kamada H, Satoh S. 1995. A male-associated DNA sequence in a dioecious plant, *Cannabis sativa* L. ***Plant & cell physiology***. 36:1549-1554

113. Sang T, Crawford DJ, Kim SC, Stuessy TF. 1994. Radiation of the endemic genus *Dendroseris* (Asteraceae) on the Juan Fernandez Islands: Evidence from sequences of the ITS regions of nuclear ribosomal DNA. ***American Journal of Botany***. 81:1494-1501.

114. Sang T, Crawford DJ, Stuessy TF. 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal

DNA: Implications for biogeography and concerted evolution. ***Proceedings of the National Academy of Sciences USA***. 92:6813-6817.

115. Schilling EE, Panero JL, Eliasson UH. 1994. Evidence from chloroplast DNA restriction site analysis on the relationships of *Scalesia* (Asteraceae:Heliantheae). ***American Journal of Botany***. 81:248-254.

116. Serrato A, Ibarra-Manriquez G, Oyama K. 2004. Biogeography and conservation of the genus Ficus (Moraceae) in Mexico. ***Journal of Biogeography***. 31:475-485.

117. Sheherbanovskill LR, Nilov GI. 1972. *Fitontsidy Mater Sovesch, 6th Edition,* 104-115.

118. Singh M, Kumar S, Singh AK, Ram D, Kalloo G. 2002. Female sex-associated RAPD marker in pointed gourd (*Trichosanthes dioica* Roxb.) ***Current Science***. 82:131-132.

119. Singleton VL, Rossi JA. 1965. Colorimetry of total phenolics with phosphomolybdic-photosynthetic acid reagents. ***American Journal of Enology and Viticulture***. 16:144-153.

120. Sofowora A. 1982. Medicinal Plants and Traditional Medicine in Africa. John Wiley and Sons Ltd. Chichester, pp 142-145.

121. Sokal RR, Rohlf FJ. 1997. Biometry. W H Freeman & Co. San Francisco. USA.

122. Soltis DE, Soltis PS, Bothel KD. 1990. Chloroplast DNA evidence for. the origins of the monotypic *Bensoniella* and *Conimitella* (Saxifragaceae). ***Systematic Botany***. 15: 349–362.

123. Strahm W. 1994. Regional overview: Indian Ocean Islands. In WWF, IUCN: Centres of plant diversity. A guide and strategy for their conservation. IUCN Publication Unit. Cambridge. UK.

124. Struwe L, Thiv M, Kadereit JW, Pepper ASR, Motley TJ, White PJ, Rova JHE, Potgieter K, Albert VA. 1998. *Saccifolium* (Saccifoliaceae), an endemic of Sierra de la Neblina on the Brazilian-Venezuelan border, is related to a temperate alpine lineage of Gentianaceae. ***Harvard Papers in Botany***. 3:199-214.

125. Swofford DL. 2001. PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b8. Sunderland, MA. USA. Sinauer Press.

126. Tamura K. 1999. TreeExplorer version 2.12. http://evolgen.biol.metro-u.ac.jp/TE/TE_man.html.

127. Tanurdzic M, Banks JA. 2004. Sex-Determining Mechanisms in Land Plants. ***The Plant Cell***. 16:S61-S71.

128. Trease GE, Evans WC. 1989. Pharmacognosy. Bailliere Tindall. London. UK

129. Tschesche R. 1970. Advances in the chemistry of antibiotics substances from higher plants: Pharmacognosy and phyto-chemistry. In *Proceeding of the 1st International Congress, Murich, 1970*. Edited by: Wagner H, Horharmmer L. Springer-Verlag, Berlin Heidelberg, New York; 274-289.

130. Vaughan RE, Wiehe PO. 1937**.** Studies on the vegetation of Mauritius I. A preliminary survey of the plant communities. ***Journal of Ecology*.** 25:289-343.

131. Verheyen K, Bossuyt B, Hermy M, Tack G. 1999. The land use history (1278-1990) of a mixed hardwood forest in Western Belgium and its relationship with chemical soil characteristics. ***Journal of Biogeography***. 26: 1115-1128.

132. Verpoorte R, Alfermann AW 2000. Metabolic Engineering of Plant Secondary Metabolism. Kluwer Academic Publishers, Dortrecht, The Netherlands.

133. Vlietinck AJ, Van Hoof L, Totte J, Lasure A, Vanden Berghe D, Rwangabo PC, Mvukiyumwami J. 1995. Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. *Journal of Ethnopharmacology*. 46:31-47.

134. Vu L, Huynh QK. 1994. Isolation and characterisation of a 27-Kda antifungal protein from the fruits of *Diospyros texana*. ***Biochemical and biophysical research communication***s. 202:666-72.

135. Wagner WL, Funk VA. 1995. Hawaiian biogeography: evolution as a hot spot archipelago. Smithsonian Institution Press. Washington.

136. Wagner WL, Herbst DR, Sohmer SH. 1990. Manual of the flowering plants of Hawaii. University of Hawaii Press and Bishop Museum Press (Special publication 83). Honolulu. Hawaii. USA.

137. Wall JM, Krider MM, Krewson CF, Eddy CR, Willaman JJ, Corell DS, Gentry HS. 1954. Steroidal sapogenins VII. Survey of plants for steroidal sapogenins and other constituents. ***Journal of the American Pharmacists* Association**. 63.1D7.

138. Xiao X, Zhang L, Li S, Testolin R, Cipriani G . 2003. Molecular Markers For Early Sex Determination In *Actinidia.* ***Acta Horticulturae*** 610: 533-538,

139. Yang H, Gan S, Yin H, Xu H. 2005. Identification of Random Amplified Polymorphic DNA Markers Linked to Sex Determination in *Calamus simplicifolius* C. F. Wei. ***Journal of Integrative Plant Biology***. 47: 1249-1253.

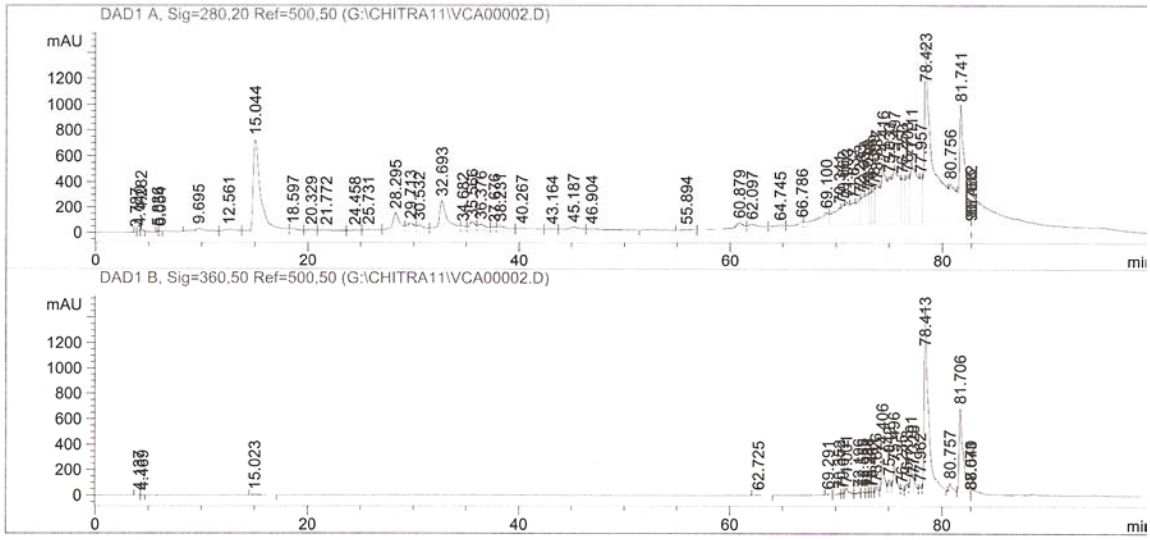
140. Yardley V, Snowdon D, Croft S, Hazra B. 1996. Antitrypanosomal activity of an indigenous plant product, diospyrin, and its synthetic derivatives. ***Phytotherapy Research***. 10:559-62.

141. Yin T, Quinn JA. 1995. Tests of a mechanistic model of one hormone regulating both sexes in *Buchloe dactyloides* (Poaceae). ***American Journal of Botanical***. 82:745-751.

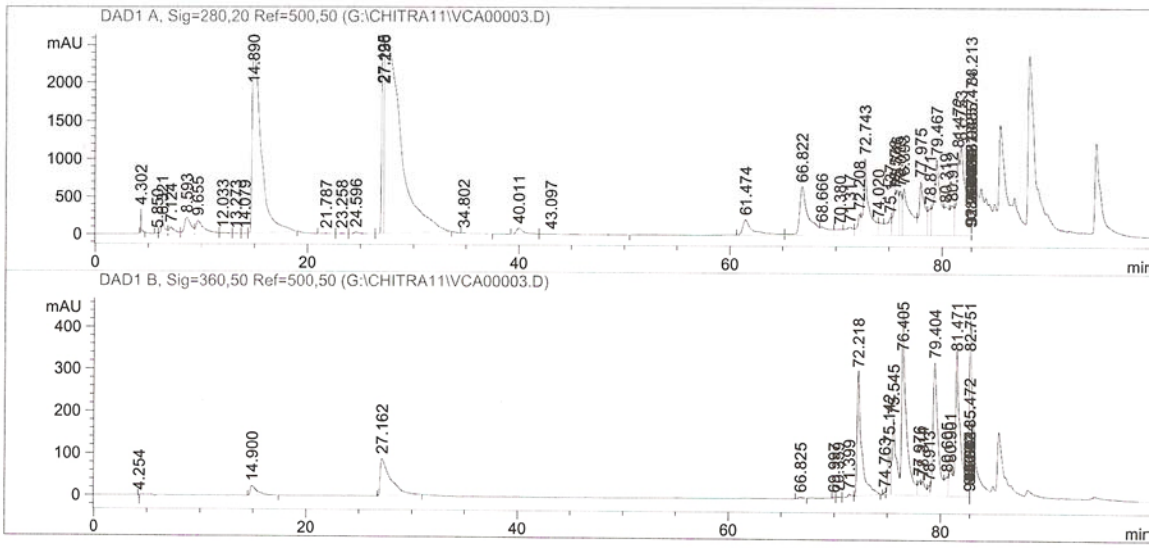
Appendix A

HPLC Profiles

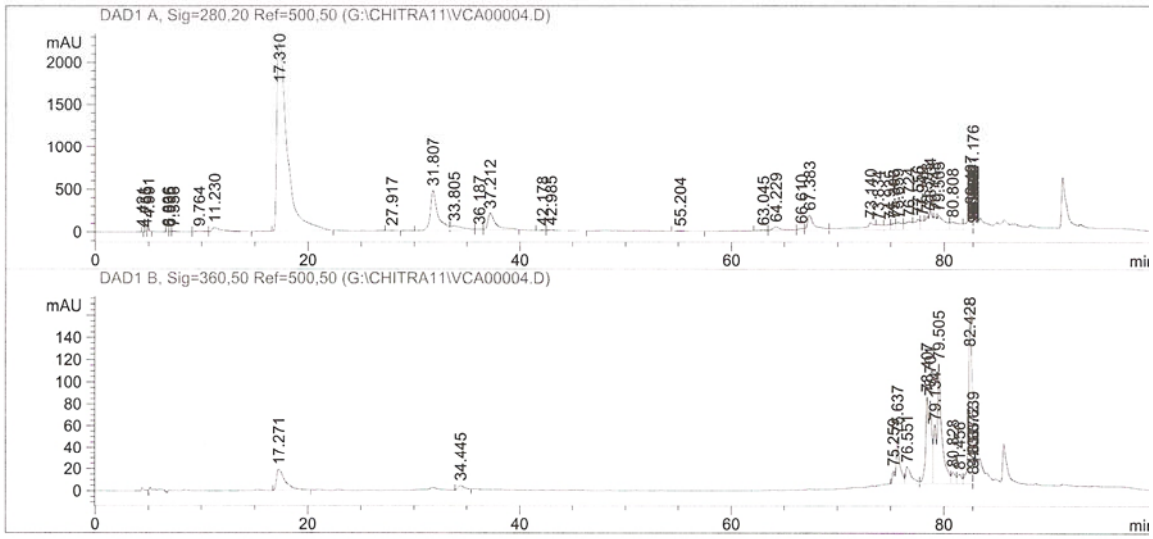
D.melanida



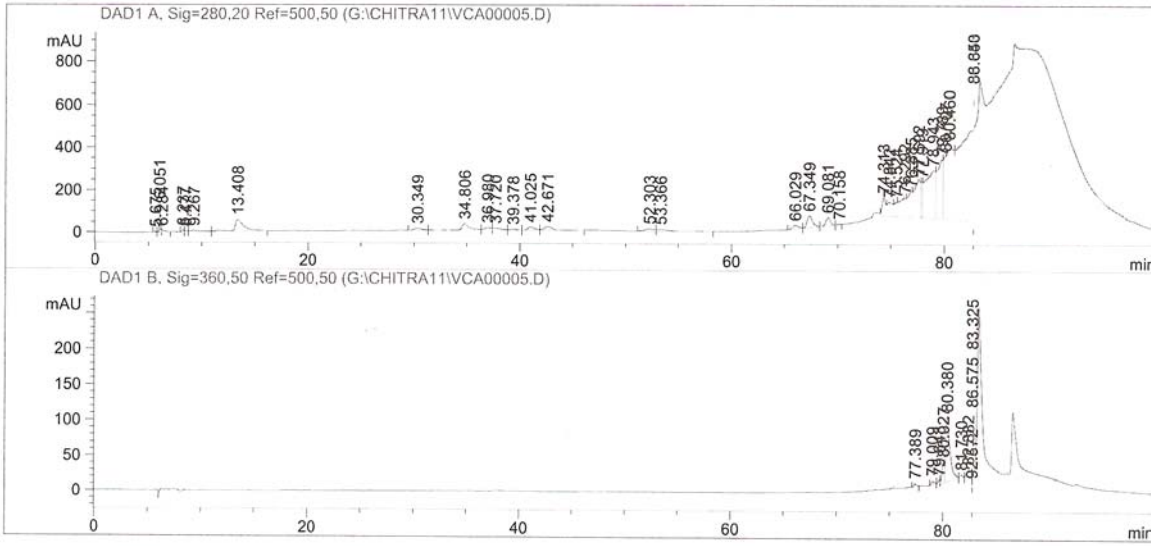
D.pterocalyx



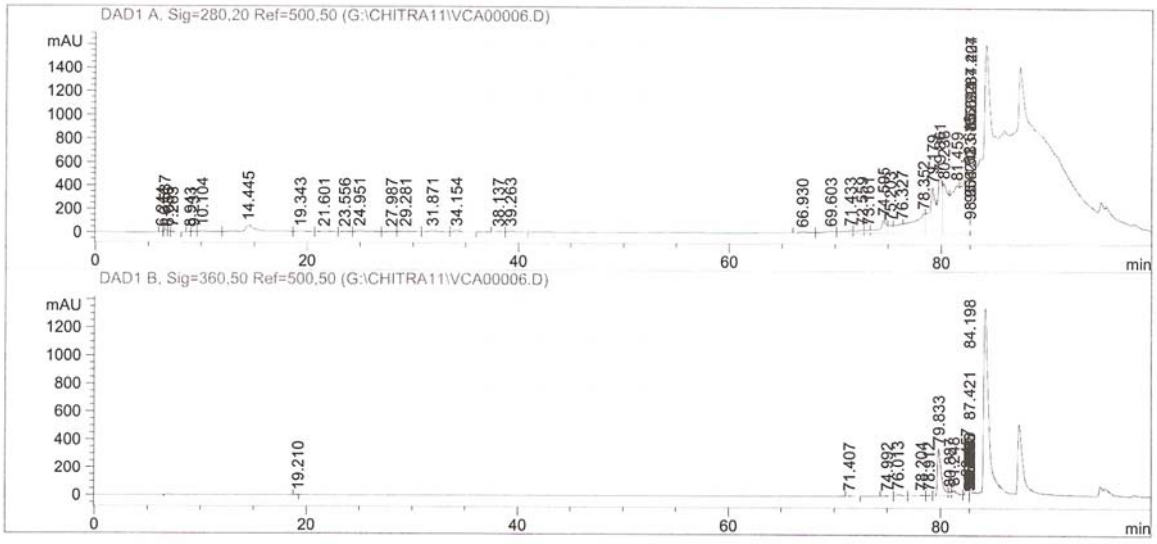
D.hemiteles



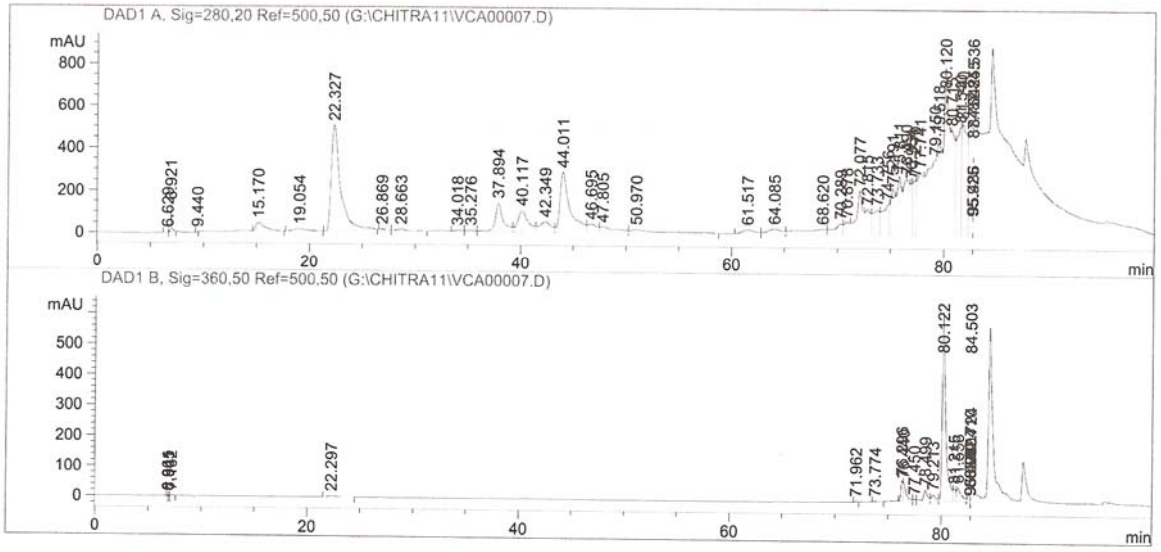
D.revaughanii



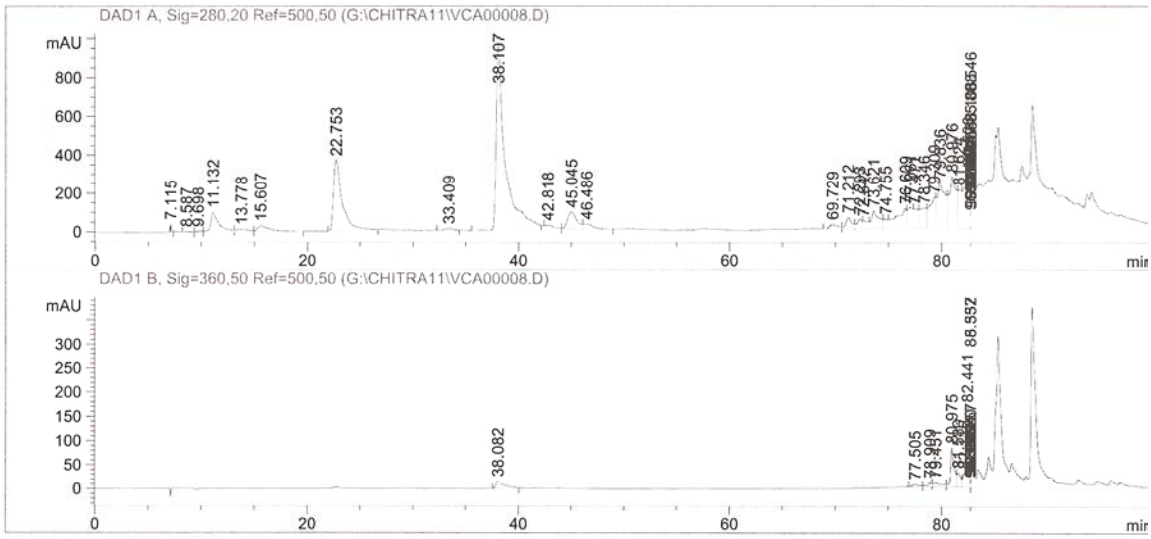
D.neraudii



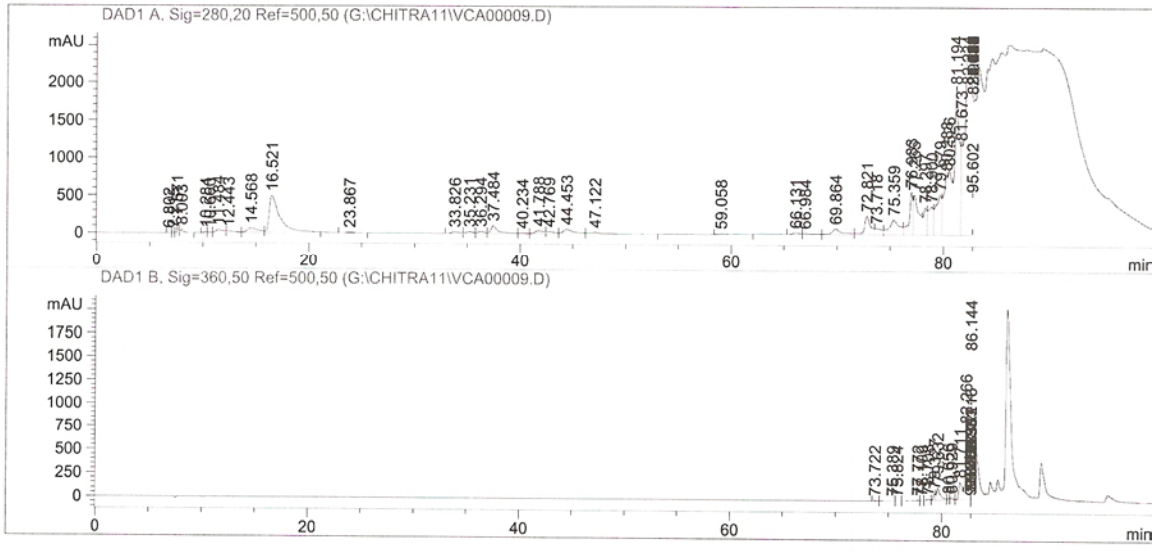
D.leucomelas



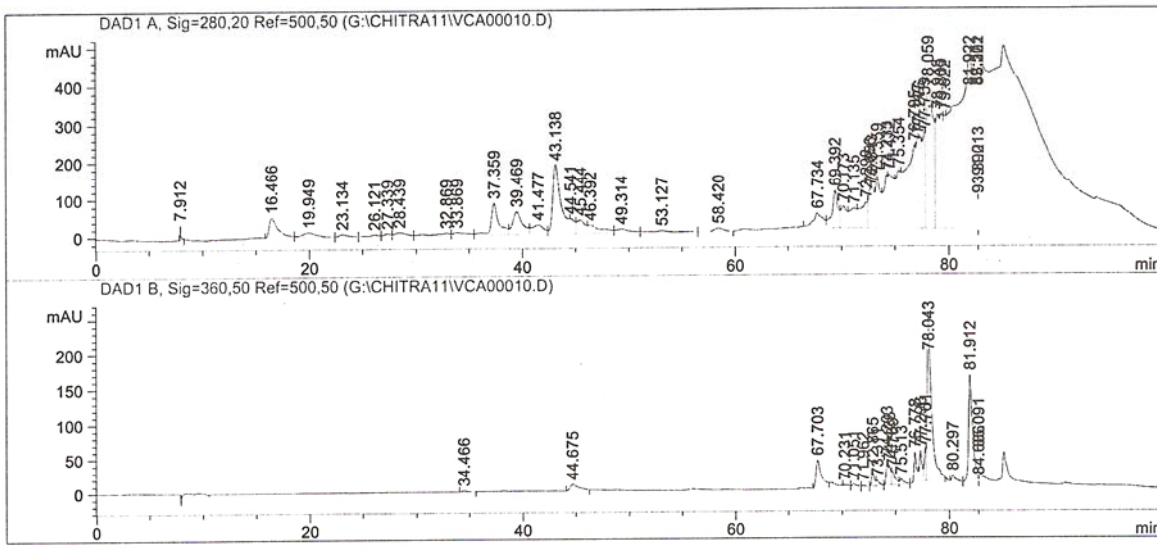
D.nodosa



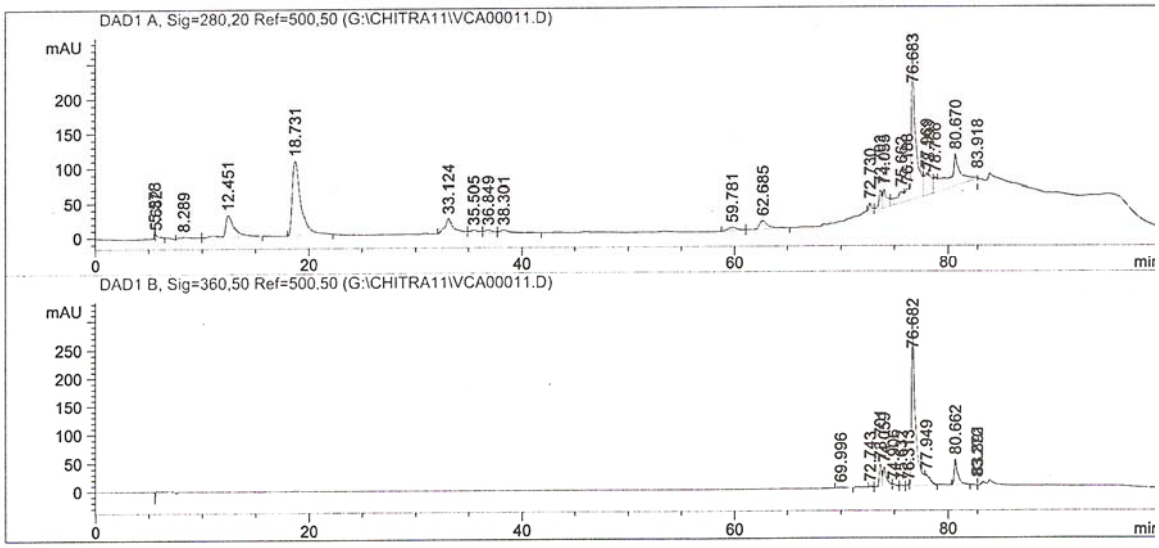
D.boutoniana



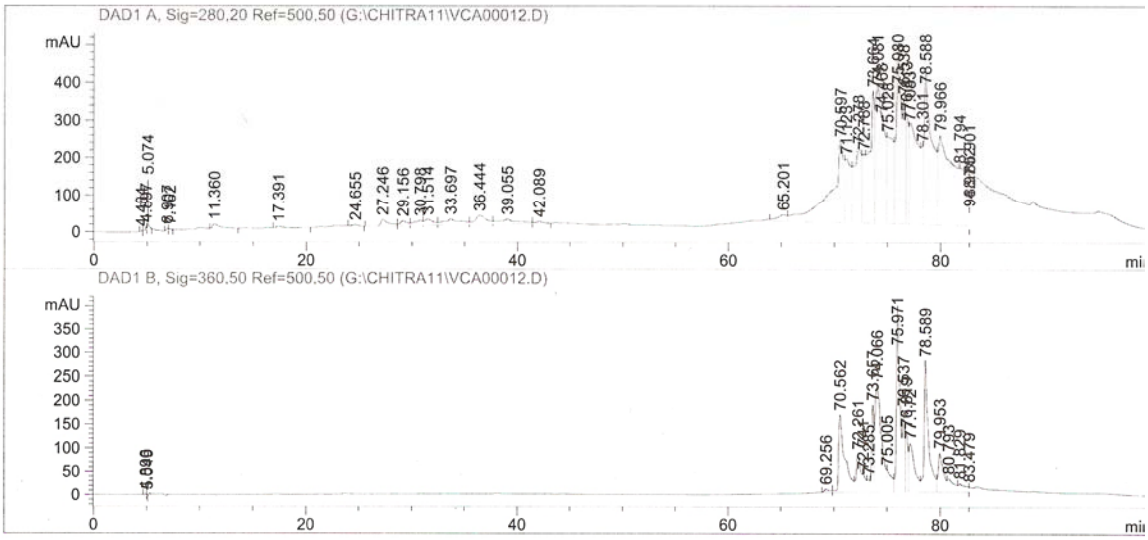
D.chrysophyllos



D.eggrettarum



D.tessellaria



Appendix B

Reprints

Plant Ecol (2007) 189:139–146
DOI 10.1007/s11258-006-9171-y

ORIGINAL PAPER

Leaky dioecy in *Diospyros* (Ebenaceae) endemic to the Island of Mauritius

Shadila Venkatasamy · Govindranathsing Khittoo · Sterling Keeley

Received: 12 August 2005 / Accepted: 28 May 2006 / Published online: 18 January 2007

Abstract Dioecy, a rather rare phenomenon in the plant kingdom seems to be more prevalent on oceanic islands. The high incidence of dioecy on these islands could result from dioecious colonists among which a small percentage show leaky dioecy, which is an ability to self-fertilise. In this study, we report the occurrence of leaky dioecy in one of the 11 extant *Diospyros* species endemic to Mauritius. Female flowers on the leaky dioecious plants were artificially pollinated and bagged. Populations of *D. egrettarum*, *D. leucomelas*, *D. melanida*, *D. revaughanii*, *D. tessellaria* were all male-biased with a ratio of at least 2:1. Leaky dioecy occurred only in one *Diospyros* species, *D. egrettarum* where hermaphrodite plants represented 2% of the populations studied. Seeds collected from them had the same germination rate (approximately 40%) as the ones obtained from strictly unisexual female plants of *D. egrettarum*. The fact that leaky dioecy led to the production of fertile seeds opens the possibility that a single pioneer *Diospyros* plant could have played a major role in the establishment of reproductively viable populations in Mauritius.

Keywords Ebony · Dioecy · Mascarene archipelago · Mauritius · Sex ratio

S. Venkatasamy
Faculty of Agriculture, University of Mauritius, Reduit, Mauritius

G. Khittoo (✉)
Faculty of Science, University of Mauritius, Reduit, Mauritius
e-mail: gkhittoo@uom.ac.mu

S. Keeley
Department of Botany, University of Hawaii, Manoa, Hawaii

Introduction

Oceanic islands have provided unique environments for the study of dioecy, the existence of separate male and female individuals in natural plant populations (Bawa 1982; Baker and Cox 1984; Sakai et al. 1995a). Although 6% of flowering plant species are known to exhibit dioecy worldwide (Renner and Ricklefs 1995), the estimated frequency of dioecy is 14.7% in the Hawaiian flora (Sakai et al. 1995a), 12%–13% in the New Zealand flora (Godley 1979) and 9% in the Juan Fernandez Islands flora (Anderson et al. 2000).

In the classic explanation of the high incidence of dioecy in island floras, the original colonists of isolated islands were assumed to be hermaphrodites or monoecious species and their dioecious descendants then developed outcrossing breeding systems autochthonously (Baker 1967). Bawa's studies (1982) of the Hawaiian flora showed that

genera which originally were presumed to have evolved dioecy autochthonously in Hawaii already existed as dioecious genera outside the Hawaiian Islands. Furthermore, Sakai et al. (1995a) proposed that the high incidence of dioecy in the Hawaiian flora was the result of both dimorphic colonists as well as evolution of dimorphism in Hawaii. Similar observations have also been reported by Godley (1979) for the New Zealand flora. Several ecological correlations have been proposed to explain the ability of dioecious plants to colonise oceanic islands. For instance, the association of dioecy with fleshy fruit may reflect dispersal of these fruits to remote islands by birds (Bawa 1980; Fox 1985). Dioecy is often linked with woody perennials which increase the opportunity for individuals of opposite gender to become reproductive simultaneously due to their longer life-span (Baker and Cox 1984; Thompson and Brunet 1990). Dioecious species could enhance their survival on remote islands by the production of multi-propagule units instead of single seeds (Wickens 1979; Baker and Cox 1984). Movement of multiseeded fruits to remote islands would thus ensure that staminate and pistillate trees grew in close proximity both in time and space. However, Sakai et al. (1995b) expressed concern that several ecological traits associated with dioecious species were based only on a small number of colonists and that care was necessary in their interpretation.

Other survival mechanisms that enable a single individual of a dioecious species to colonise oceanic islands include apomixis (that is the clonal reproduction by seeds) and hermaphroditism (Baker 1955; Richards 1990; Baker and Cox 1984). The presence of occasional opposite sex or hermaphrodite flowers on the same individual of a dioecious species is referred as leaky dioecy. Several cases of leaky dioecy have been reported on oceanic islands, the most well-known examples being, *Coprosma* in the Juan Fernandez Islands, *Sanctambrosia* in the Desventuras Islands (see Baker and Cox 1984), *Fragaria*, *Plantago* in Hawaii (Carlquist 1974) *Cotula*, *Clematis*, *Pittosporum*, *Dodonea*, *Alectryon*, *Anisotome*, *Astelia* in New Zealand (Godley 1979) and *Dombeya* in Reunion Island (Humeau et al. 1999) situated in the Indian Ocean, 150 km to the South West of Mauritius. These two islands are volcanic and form part of the Mascarene archipelago. Mauritius emerged some 8 million years ago (McDougall and Chamalaun 1969) while Reunion is believed to be 3 million years old (McDougall 1971).

Nevertheless, little is known on the occurrence of leaky dioecy in the endemic dioecious plants of Mauritius. The main reason for this paucity of data on dioecy and leaky dioecy is that the indigenous forest has been largely decimated by early settlers and less than 2% of the original forest remains (Page and d'Argent 1997). An early study conducted by Baker in 1877 indicated that 11% of the Mauritian endemic plants were dioecious while De Cordemoy (1895) observed only 4% of dioecy in the endemic flora of Reunion Island. More recently, studies in Reunion Island revealed that the percentage of dioecy ranges from 15% to 20% on that island and highlighted the presence of leaky dioecy in the endemic *Dombeya* species (Humeau et al. 1999). New studies are therefore necessary to establish a more exact level of dioecy and leaky dioecy in the Mauritian endemic plants for comparison with other oceanic islands and for a better understanding of the evolutionary biology of the Mauritian endemic plants.

In this study, we investigated the occurrence of leaky dioecy in the *Diospyros* species endemic to Mauritius. Historical records indicate that prior to exploitation by the Dutch settlers (in the late 16th and early 17th century) the Mauritian forest was dominated by *Diospyros* species which spanned from the lowlands to the central plateau and on the mountains (Pitot 1905). Twelve endemic *Diospyros* species had been identified in Mauritius but in 2000, the last known individual of *D. angulata* Poir went extinct lowering the number of endemic *Diospyros* species to 11. These species are now reduced to pockets of individuals that are scattered over various ecological habitats ranging from low (0–199 m), mid (200–499 m) to high altitudes (500–800 m). At low elevations, the *Diospyros* species survive in nutrient poor soils and marginal lands that receive less than 1,500 mm of rain annually. There is a gradual increase in annual rainfall and soil quality from mid (2,000–3,000 mm of rain) to high altitude (3,500–5,000 mm of rain) regions (Padya 1989).

The objectives of this study were to determine whether (1) the 11 extant endemic *Diospyros* species exhibited strict or leaky dioecy (2) the sex ratio of the species showing leaky dioecy was different from the other species located at different altitudinal ranges (3) apomixis occurred in the female trees of the species with hermaphroditic individuals and (4) the seeds from strict female and hermaphrodite trees were viable.

Materials and methods

Study species

We examined the flowers of all the 11 species of *Diospyros* during their respective flowering periods. However, some of the *Diospyros* species consist of a few isolated individuals. For instance flowers were collected from accessible trees of *D. chrysophyllos* Poir (17 trees), *D. hemiteles* I.B.K Richardson (14 trees), *D. neraudii* A.D.C (18 trees), *D. nodosa* Poir (20 trees) and *D. pterocalyx* Bojer (12 trees). For the rest of the species with larger populations (*D. boutoniana* A.D.C, *D. egrettarum* I.B.K Richardson, *D. leucomelas* Poir, *D. melanida* Poir, *D. revaughanii* I.B.K Richardson and *D. tessellaria* Poir), from 29 to over 200 individuals were examined. After the above observations, the main species for the remaining part of this study was *D. egrettarum*. This species is a 5–6 m multistemmed tree that grows on the East coast of Mauritius only. Two main populations can be encountered, one on Ile aux Aigrettes (an islet, 900 m off the South-East coast of Mauritius) and the other at Bras d'Eau on the North-East coast of Mauritius. Both male and female trees have grey barks, dark green leaves and produce numerous small insect pollinated white flowers, approximately 2 cm in diameter. Male flowers are produced in the leaf axils in groups of 6–10 while female flowers are produced in groups of 5–7.

Sex ratio

During the flowering period of *D. egrettarum* the total number of male and female trees was counted in the two populations and their sex ratios calculated. The number of male and female plants was also recorded for the major populations of *D. leucomelas*, *D. melanida*, *D. revaughanii* and *D. tessellaria* during their respective flowering periods. Sex ratios were also calculated for these species. A chi-square test was performed to test departures from the 1:1 sex ratio. The height (m) above sea level of the *Diospyros* populations were recorded as their habitats range from low to mid and high altitudinal zones.

Pollination trials

During the flowering period of *D. egrettarum* usually from June to August, a pollination experiment was designed to study leaky dioecy in *D. egrettarum*. Eight individuals from a total of 388 plants produced both staminate and pistillate stems on the same tree (Table 2). For two consecutive years, three treatments were carried out on these hermaphrodite individuals to test the sexual functionality of these morphological female flowers. Fifteen female flower buds from each female stem were selected and bagged with mosquito netting material prior to the pollination experiment. Three days after bagging the flower buds, the following treatments were carried out.

Self-pollination

Five of the fully opened female flowers from each female stem were hand pollinated using pollen from male flowers found on the same plant.

Outcross pollination

Another set of five fully opened female flowers from each female stem were pollinated with pollen from male trees other than the tree from which the female flowers were selected.

All artificially pollinated female flowers (selfed and outcrossed) were re-bagged with mosquito netting material.

Apomixy test (control)

As a control, the five remaining flowers from each female stem were left bagged with the mosquito

netting material and no artificial pollination was carried out on those flowers.

Field observation

After 3 weeks, the bags were removed and the number of successful pollination trials was noted for each female shoot. The positions of the immature fruits resulting from the pollination experiment were carefully marked. One week after removing the bags, the number of fruits formed was noted and after another 3 weeks, the number of fruit abortions was recorded. The number of mature fruits obtained for each treatment was noted 3 months after carrying out the hand pollination.

The results obtained for initial fruit set, number of fruits aborted and number of mature fruits produced in the self and outcross pollination trials were compared using a G test of independence (Sokal and Rohlf 1995).

Fruit set and germination trial

The fruits obtained from the self-pollination were shelled and the seeds obtained from the eight plants were bulked. That step was also carried out with the fruits obtained from the outcross pollination. The seeds obtained from selfed and outcrossed trials were kept in two different bags.

Five replicates of a sample of 50 seeds obtained from the self-pollination were sown in five different trays and the same trial was applied to seeds from the outcross pollination. This experiment was done to determine the germination success of the seeds produced by the leaky dioecious trees.

Concurrently, 250 seeds of *D. egrettarum* collected from other female plants not exhibiting leaky dioecy were also germinated as control. The same experiment was repeated with the seeds obtained in year 2.

Results

Occurrence of leaky dioecy

Out of the 388 *D. egrettarum* trees studied, leaky dioecy was observed only in 8 individuals (representing a total of 2%).

Sex ratio

Only five *Diospyros* species occurred in groups of more than 25 individuals in very close proximity (an average of 2–8 m apart). The rest of the *Diospyros* species comprised of individual trees distributed over a large area making it difficult to establish a reliable sex ratio that would reflect the natural distribution. Examination of the population structures of *D. egrettarum*, *D. leucomelas*, *D. melanida*, *D. revaughanii* and *D. tessellaria* have revealed that there exists a significant male bias in the five species (Table 1). The ratio of female to male individuals in populations of *D. egrettarum*, *D. leucomelas* and *D. melanida* was approximately 1:3 while the sex ratio of *D. revaughanii* and *D. tessellaria* was 1 female: 2 males. Table 1 also shows Chi-square tests for

Table 1 Ratio of female:male trees in some *Diospyros* species

Species	*egrettarum*				*leucomelas*		*melanida*		*revaughanii*		*tessellaria*	
Location	B. d'eau		IAA		Magenta		Magenta		Pétrin		B. fer	
Altitude (m)	50		13		210		210		580		612	
Sex	F	M	F	M	F	M	F	M	F	M	F	M
No. of trees	28	91	71	198	8	26	7	22	22	43	28	59
Ratio	1:3.3		1:2.8		1:3.3		1:3.1		1:2		1:2.1	
χ^2	33.353		59.959		9.529		7.759		6.785		11.046	
	(P = 0.000)		(P = 0.000)		(P = 0.002)		(P = 0.005)		(P = 0.009)		(P = 0.001)	

B. d'eau = Bras d'eau; IAA = Ile aux Aigrettes; B.fer = Brise Fer; F = female; M = male. χ^2: test for departures from the 1:1 sex ratio

Table 2 Initial fruit set, abortions and final fruit set for the pollination experiment carried out on the eight leaky dioecious individuals of *D. egrettarum*

	Pollination treatment	Number of	Plant no.								%
			1	2	3	4	5	6	7	8	
		Female shoots	1	2	3	2	1	3	2	2	
		Male shoots	1	1	1	2	7	2	1	5	
		Female flowers bagged	15	30	45	30	15	45	30	30	
Year 1	Self	Initial fruit set	8	14	30	18	11	23	17	21	59.2
		Abortions	3	3	6	4	3	5	2	9	24.6
		Mature fruits	5	9	24	14	8	18	15	12	43.8
	Outcross	Initial fruit set	10	11	28	13	8	28	16	17	54.6
		Abortions	6	3	5	2	4	2	2	7	23.7
		Mature fruits	4	9	23	11	4	24	14	10	41.3
Year 2	Self	Initial fruit set	12	21	39	16	9	37	12	19	68.8
		Abortions	5	7	6	2	1	8	3	4	21.8
		Mature fruits	7	14	33	14	7	29	9	15	53.3
	Outcross	Initial fruit set	9	18	35	22	10	36	24	21	72.9
		Abortions	1	2	4	2	1	6	4	3	13.1
		Mature fruits	8	16	31	20	9	30	20	18	63.3

deviations from a 1:1 sex ratio and the altitudes of the populations that were surveyed for the above five *Diospyros* species.

Pollination trials: selfed vs. outcrossed

Ten species of the 11 known *Diospyros* exhibited strict dioecy. In only one species, *D. egrettarum* we observed leaky dioecy which amounted to 2% of the trees of this species studied. These leaky dioecious plants were subjected to pollination trials. Table 2 shows the number of flowers treated and the results obtained for self and outcross pollination trials. There was no fruit formation when the female flower buds were bagged in the control experiment showing the absence of apomixy in *D. egrettarum*.

From the total number of flowers pollinated in year 1, an average of 59.2% gave rise to the formation of fruits. Out of these, an average of 24.6% fruit abortions were observed resulting in the production of 43.8% mature fruits (Table 2). In contrast, the outcross pollination had an initial fruit formation of 54.6%, an abortion of 23.7% and yielded 41.3% mature fruits. Self and outcross pollinations did not differ in the number of initial fruit set ($G = 2.392$, $P = 0.935$), abortions ($G = 3.027$, $P = 0.883$) or the production of mature fruits ($G = 2.668$, $P = 0.914$) in year 1. Furthermore, the same experiment carried out the following year also did not show any significant variation in the initial fruit set ($G = 5.685$, $P = 0.577$), abortions ($G = 3.544$, $P = 0.831$) and mature fruits ($G = 4.004$, $P = 0.779$) between selfed and outcrossed. The overall results obtained in years 1 and 2 were not significantly different ($P > 0.05$).

Germination success of the *D. egrettarum* seeds was 28.8% for seeds from the self-pollination, 36.4% for seeds from the outcross pollination and

Table 3 Germination success of seeds obtained from the pollination trials carried out on the leaky dioecious individuals of *D. egrettarum*

	Treatment	Number of seedlings germinated from replicate					% germination
		1	2	3	4	5	
Year 1	Self	21	8	17	12	14	28.8
	Outcross	17	11	24	21	18	36.0
	Control	23	19	21	7	13	33.2
Year 2	Self	6	28	16	34	12	38.4
	Outcross	28	16	13	31	15	41.2
	Control	14	8	22	19	23	34.4

33.2% for seeds collected from other female *D. egrettarum* trees not exhibiting leaky dioecy (control). The percentage of seedlings reached 38.4% and 41.2% for self and outcross pollination respectively when seeds obtained from the experiment of year 2 were sown. In the control of year 2, the seed germination obtained was 34.4% (Table 3).

Discussion

Male-biased sex ratio

Several observations have shown that sex ratios in dioecious species are not always 1:1 (Bram and Quinn 2000). For instance, male-biased sex ratios and female-biased sex ratios (Crawford and Balfour 1983; Ornduff 1985; Sakai and Weller 1991; Houle and Duchesne 1999) have been well documented. It is often observed that sex ratios are skewed towards maleness in many dioecious species (Esperito-Santo et al. 2003) especially in more stressful habitats (Rocheleau and Houle 2001). However, Correia and Diaz Barradas (2000) found no significant differences between the number of male and female plants of *Pistacia lentiscus* growing in abandoned old agricultural areas where the soil was fairly rich in nutrients. In our study, a male-biased sex ratio (1 female:3 males) was found in the two populations of *D. egrettarum* surviving on a low nutrient soil and dry condition. In the populations of *D. leucomelas* and *D. melanida* which share a mid altitude habitat (where the growing conditions are more favourable compared to that of *D. egrettarum*), a similar male-biased sex ratio (1 female:3 males) was also observed for both species. In the species, *D. revaughanii* and *D. tessellaria* which both inhabit a high altitude, the number of male plants was also higher than the number of female plants (1 female:2 males). It would seem that the *Diospyros* species endemic to Mauritius are generally male biased with a less pronounced difference at higher altitudes where the soil is relatively richer and the environment more humid. Similar results have been obtained in a number of studies where it was shown that males were more abundant than females in regions with lower soil moisture (Bierzychudek and Eckhart 1988; Sakai and Weller 1991; Dawson and Ehleringer 1993). In dioecious species males only produces pollen while females have to bear flowers as well as fruits. Consequently, female plants need additional resource allocation for reproduction (Antos and Allen 1994; Jonasson et al. 1997; Hogan et al. 1998). Moreover, in Mauritius, the *Diospyros* populations studied are located in areas where the land is not exploited for agriculture due to the poorer quality of the soil, a factor known to favour male bias (Bram and Quinn 2000).

It is noteworthy that many male plants of *D. egrettarum*, *D. leucomelas*, *D. melanida*, *D. revaughanii* and *D. tessellaria* were seen to produce flowers more frequently and even in periods where the female plants of these species were not flowering. In fact, in the case of *D. egrettarum* some of the male plants produced flowers even at the time when all the female individuals were bearing fruits. This pattern can perhaps be explained by the fact that the reproductive investment of male plants is significantly less than female plants which have to produce flowers, fruits and seeds (Roucheleau and Houle 2001). It has also been shown that given the higher cost of reproduction, female plants can exhibit a decline in survival rate, frequency of flowering or variation in reproductive effort (Antos and Allen 1999; Nicotra 1999).

Leaky dioecy

Our results show that each individual leaky *D. egrettarum* tree has the ability to generate fertile male and female flowers and produce viable seeds. Moreover, there were no significant differences among the germination rates of seeds from the self, outcross and control trials ($P > 0.05$) indicating that there is no evidence for inbreeding depression at this stage.

Diosyros egrettarum is the only *Diospyros* species that can be encountered in low altitude habitats that are closest to the sea and the two populations studied have been subjected to immense pressures from woodcutters, poor soil quality and drought conditions for several years. Our results concur with the work carried out by Humeau et al. (1999) on the *Dombeya* endemic

to Reunion Island. They showed that the *Dombeya* species, which exhibited leaky dioecy, occur in highly isolated relict populations at lower altitude forests where human disturbance via habitat destruction and fragmentation was greatest. On the other hand, the species of *Dombeya* occurring in large populations in mid to high altitude cloud forest were found to exhibit strict dioecy. Humeau et al. (1999), therefore suggested that leaky dioecy usually occurs at low altitude and /or in small threatened, fragmented populations. A similar trend seems to exist in the Mauritian endemic *Diospyros* species as only *D. egrettarum* can be found at the low altitude and marginal areas. However, it is important to note that in the case of *Dombeya*, the male and female flowers retained (to different degrees) morphologically well-developed opposite sex organs (Humeau et al. 1999) leading to cryptic dioecy whereas in *D. egrettarum*, the same plant was observed to produce distinct male and female flowers on separate stems. This sort of leaky dioecy has been noted in *Freycinetia scandens* in which the same plant produced staminate and pistillate spikes at the same time (Cox and Webster in Baker and Cox 1984).

The fact that *D. egrettarum* is the only *Diospyros* species that can be found close to the sea on the east coast supports the notion that the strong south east trade winds that dominate the east of Mauritius could have favoured the landing of fruits of a *Diospyros* species on the eastern coast. Indeed, the largest population of *D. egrettarum* and six of the hermaphrodite individuals studied were encountered on Ile aux Aigrettes, located 900 m off the south east coast of Mauritius. In all *Diospyros* species, the germination of seeds is relatively low (a maximum of 40%—pers. observation) and the sex ratio in *Diospyros* populations is male-biased (especially in dry and lower altitude regions). In case the germination of *Diospyros* seeds which first landed in Mauritius gave rise to only male plants, a plastic morph like leaky dioecy would have enabled these male individuals to bear fruits with viable seeds. This mechanism might have been an important factor in the colonisation and survival of the pioneer species of *Diospyros* on the island of Mauritius as it would have ensured the establishment of a population from one single leaky dioecious individual. Interestingly, phylogenetic studies based on morphology suggest that colonisation of the *Diospyros* group most probably started from the coastal areas and that the oldest clade consisting of *D. egrettarum*, *D. leucomelas* and *D. revaughanii* were the first pioneering species of Mauritius (Venkatasamy et al. 2006). It is noteworthy that *D. egrettarum* which exhibits leaky dioecy is the only true coastal species of Mauritius. Based on these observations, one possible scenario is that leaky dioecy which was important for the colonist species subsequently disappeared during speciation and adaptive radiation to favour strict dioecy among *Diospyros* species. These new species migrated from the coastal areas to more humid habitats at higher altitudes and have maintained strict dioecy. Interestingly, when the *Diospyros* species occur in close proximity, they tend to have staggered flowering periods which act as a form of reproductive barrier (Venkatasamy et al. 2006). However, the phylogenetic relationships based on morphology cannot exclude the possibility that more than one colonisation event from other *Diospyros* species could have occurred.

Acknowledgements The authors thank the Mauritius Research Council (MRC) for financial support and the Tertiary Education Commission (TEC) for the scholarship awarded to S. Venkatasamy. We also thank the two anonymous reviewers for their constructive comments.

References

Anderson GJ, Bernardello G, Lopez P, Stuessy TF, Crawford DJ (2000) Dioecy and wind pollination in *Pernettya rigida* (Ericaceae) of the Juan Fernandez Islands. Bot J Linn Soc 132:121–141

Antos JA, Allen GA (1994) Biomass allocation among reproductive structures in the dioecious shrub *Oemleria cerasiformis* – a functional interpretation. J Ecol 82:21–29

Antos JA, Allen GA (1999) Patterns of reproductive effort in male and female shrubs of *Oemleria cerasiformis*: a 6-year study. J Ecol 87:77–84

Baker HG (1877) Flora of Mauritius and the Seychelles. Reeve L, London

Baker HG (1955) Self-incompatibility and establishment after "long-distance" dispersal. Evolution 9:347–348

Baker HG (1967) Support for Baker's law—as a rule. Evolution 21:853–856

Baker HG, Cox PA (1984) Further thoughts on dioecism and islands. Ann Miss Bot Garden 71:244–253

Bawa KS (1980) Evolution of dioecy in flowering plants. Annu Rev Ecol Syst 11:15–40
Bawa KS (1982) Outcrossing and the incidence of dioecism in island floras. Am Nat 119:866–871
Bierzychudek P, Eckhart V (1988) Spatial segregation of the sexes of dioecious plants. Am Nat 132:34–43
Bram MR, Quinn JA (2000) Sex expression, se-specific traits, and the effects of salinity on growth and reproduction of *Amaranthus cannabinus* (Amaranthaceae), a dioecious annual. Am J Bot 87:1609–1618
Carlquist S (1974) Island biology. Columbia University Press, New York, USA
De Cordemoy EJ (1895) Flore de l'Ile de la Reunion. P.Klincksiet, Paris
Correia O, Diaz Barradas MC (2000) Ecophysiological differences between male and female plants of *Pistacia lentiscus* L. Plant Ecol 149:131–142
Crawford RMM, Balfour J (1983) Female predominant sex ratios and physiological differentiation in arctic willows. J Ecol 71:149–160
Dawson TE, Ehleringer JR (1993) Gender-specific physiology, carbon isotope discrimination and habitat distribution in boxelder, *Acer negundo*. Ecology 74:798–815
Esperito-Santo MM, Madeira BG, Neves FS, Faria MI, Fagundes M, Wilson-Fernandes G (2003) Sexual differences in reproductive phenology and their consequences for the demography of *Baccharis dracunculifolia* (Asteraceae), a dioecious tropical shrub. Ann Bot 91:13–19
Fox JF (1985) Incidence of dioecy in relation to growth form, pollination and dispersal. Oecologia 67:244–249
Godley EJ (1979) Flower biology in New Zealand. N Z J Bot 17:441–466
Hogan KP, Garcia MB, Cheeseman JM, Loveless MD (1998) Inflorescence photosynthesis and investment in reproduction in the dioecious species *Aciphylla glaucescens* (Apiaceae). N Z J Bot 36:653–660
Houle G, Duchesne M (1999) The spatial pattern of a *Juniperus communis* var *depressa* population on a continental dune in subarctic Quebec, Canada. Can J For Res 29:446–450
Humeau I, Pailler T, Thompson JD (1999) Cryptic dioecy and leaky dioecy in endemic species of *Dombeya* (Sterculiaceae) on La Reunion. Am J Bot 86:1437–1447
Jonasson S, Medrano H, Flexas J (1997) Variation in leaf longevity of *Pistacia lenticus* and its relationship to sex and drought stress inferred from leaf $\delta^{13}C$. Funct Ecol 11:282–289
McDougall I (1971) The geochronology and evolution of the young volcanic island of Reunion, Indian Ocean. Geochim Cosmochim Acta 35:261–288
McDougall I, Chamalaun FH (1969) Isotopic dating and geomagnetic polarity studies on volcanic rocks from Mauritius, Indian Ocean. Geol Soc Am Bull 80:1419–1442
Nicotra AB (1999) Reproductive allocation and the long-term costs of reproduction in *Siparuna grandiflora*, a dioecious neotropical shrub. J Ecol 87:138–149
Ornduff R (1985) Male-biased sex ratios in the cycad *Macrozamia riedlei* (Zamiaceae). Bull Torrey Bot Club 112:393–397
Page WS, D'Agent G (1997) A vegetation survey of Mauritius to identify priority rainforest areas for conservation management. Final Report
Padya BM (1989) Weather and climate of Mauritius. Geography of Mauritius Series, Mahatma Gandhi Institute, Mauritius
Pitot A (1905) T'Eylandt Mauritius. Esquisses historiques, 1598–1710. Port-Louis, Mauritius
Renner S, Ricklefs R (1995) Dioecy and its correlates in the flowering plants. Am J Bot 82:596–606
Richards AJ (1990) Studies in *Garcinia*, dioecious tropical forest trees: agamospermy. Bot J Linn Soc 103:233–250
Rocheleau AF, Houle G (2001) Different cost of reproduction for the males and females of the rare dioecious shrub *Corema conradii* (Empetraceae). Am J Bot 88:659–666
Sakai AK, Weller SG (1991) Ecological aspects of sex expression in subdioecious *Schiedea globosa* (Caryophyllaceae). Am J Bot 78:1280–1288
Sakai AK, Wagner WL, Ferguson DM, Herbst DR (1995a) Origins of dioecy in the Hawaiian flora. Ecology 76:2517–2529
Sakai AK, Wagner WL, Ferguson DM, Herbst DR (1995b) Biogeographical and ecological correlates of dioecy in the Hawaiian flora. Ecology 76:2530–2543
Sokal RR, Rohif FJ (1995) Biometry. Freeman, New York
Thompson JD, Brunet J (1990) Hypotheses for the evolution of dioecy in seed plants. Trends Ecol Evol 5:11–16
Venkatasamy S, Khittoo G, Nowbuth P, Vencatasamy DR (2006) Phylogenetic relationships based on morphology among the *Diospyros* (Ebenaceae) species endemic to the Mascarene Islands. Bot J Linn Soc 150:307–313
Wickens GE (1979) Speculations on seed dispersal and the flora of the Aldrabra archipelago. Phil Trans Roy Soc, Lond Ser B 286:85–97

Botanical Journal of the Linnean Society, 2006, **150**, 307–313

Phylogenetic relationships based on morphology among the *Diospyros* (Ebenaceae) species endemic to the Mascarene Islands

SHADILA VENKATASAMY,[1] GOVINDRANATHSING KHITTOO,[2]* PRAKASH NOWBUTH[2] and DASS ROY VENCATASAMY[1]

[1]*Faculty of Agriculture, University of Mauritius, Reduit, Mauritius*
[2]*Faculty of Science, University of Mauritius, Reduit, Mauritius*

Received February 2005; accepted for publication August 2005

The *Diospyros* (Ebenaceae) species which colonized the Mascarene Islands, namely Mauritius, Reunion and Rodrigues, have been decimated over the years by human settlements. Of the 14 endemic species that have been described and collected for herbaria, *Diospyros angulata* is now believed to be extinct in Mauritius. The phylogenetic relationships of the 14 *Diospyros* species were determined using maximum parsimony analysis of 35 morphological characters. This analysis separated the Mascarene *Diospyros* into two major clades, with *D. revaughanii*, *D. egrettarum* and *D. leucomelas* grouped in the same strongly supported most basal clade while the rest of the species formed the other major clade. High bootstrap values were obtained for the sister species *D. angulata* and *D. boutonania*, and the clade clustering the upland species *D. neraudii*, *D. nodosa* and *D. pterocalyx*. There was also relatively strong support for the clade comprising *D. hemiteles* and *D. melanida*, which are located in mid altitude regions. These results indicate that *Diospyros* species most probably colonized the coastal areas of Mauritius and then moved to mid altitude habitats before finally reaching the upland regions. There are also strong indications that *D. borbonica* and *D. diversifolia*, endemic to Reunion and Rodrigues, respectively, resulted from migrations from Mauritius. © 2006 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2006, **150**, 000–000.

ADDITIONAL KEYWORDS: Mauritius – Reunion – Rodrigues – systematics.

INTRODUCTION

The Ebenaceae, a widespread family of woody dicot trees and shrubs, occurs mainly in tropical and subtropical regions. This family is divided into three main genera, namely *Diospyros*, *Euclea* and *Tetraclis* (Brummit, 1992). *Diospyros* species seem to be more prevalent in regions of Asia, Africa and Central to South America, while the genera *Euclea* and *Tetraclis* have been found to occur only in Madagascar, Eastern and Southern Africa (Cronquist, 1981; Ng, 1986). The genus *Diospyros*, represented by more than 350 species, is the most important both numerically and economically (Mallavadhani, Panda & Rao, 1998). Interestingly, this genus is the only representative of the Ebenaceae family in the Mascarene Islands namely, Mauritius, Reunion and Rodrigues. These islands are believed to be the results of massive submarine volcanic eruptions and are located approximately 700–900 km to the east of Madagascar in the Indian Ocean. Mauritius, the oldest of these islands, emerged some 8 million years ago (McDougall & Chamalaun, 1969), followed by Reunion which has an estimated 3 million years of existence (McDougall, 1971); the latest island to appear is the 1.5 million-year-old Rodrigues (McDougall, Upton & Wadsworth, 1965). Mauritius, situated at longitude 57°30′E and latitude 20°20′S, is some 150 km to the north-east of Reunion and 574 km to the south-west of Rodrigues. An estimated 70% of the Mascarene flora is thought to have originated from Madagascar and Africa (Cadet, 1977). While some of these species seem to closely resemble their Malagasy and African relatives, most of them have evolved to give rise to genera and species endemic to the Mascarenes (Cadet, 1984). Several vegetation surveys carried out over the years (Vaughan & Wiehe, 1937; Strahm, 1994; Page & D'Argent, 1997) have indicated that Mauritius harbours a diverse flora

*Corresponding author. E-mail: gkhittoo@uom.ac.mu

comprising at least 900 native species and ten endemic genera. The most recent survey estimates that less than 2% of the original Mauritian endemic plants survive in forest reserves and on mountain slopes (Page & D'Argent, 1997).

Prior to the arrival of Dutch settlers in 1598 *Diospyros*, commonly known as the ebony, were the dominant species of the native Mauritian forest (Pitot, 1905). However, during the 17th century, the *Diospyros* species, especially *D. tessellaria*, were overexploited for their excellent timber quality and black wood (Brouard, 1963). This resulted in a drastic reduction in the population sizes of these endemic plants. Over recent years, the *Diospyros* populations have been under severe threat due to increased demand for land space mainly for agriculture and urbanization. These species are now reduced to pockets of individuals located mostly in reserves and inaccessible areas. For the past several years, 12 endemic species have been identified in Mauritius: *D. angulata* Poir, *D. boutoniana* A.DC, *D. chrysophyllos* Poir, *D. egrettarum* I.B.K. Richardson, *D. hemiteles* I.B.K. Richardson, *D. leucomelas* Poir, *D. melanida* Poir, *D. neraudii* A.DC, *D. nodosa* Poir, *D. pterocalyx* Bojer, *D. revaughanii* I.B.K. Richardson and *D. tessellaria* Poir. Unfortunately, in 2000 the last known individual of *D. angulata* went extinct, lowering the number of endemic *Diospyros* species to 11 (pers. observation).

Morphological studies of the Mascarene *Diospyros* species were carried out as early as 1804 (Richardson, 1980), and these were identified and classified into 14 distinct species, 12 of which were found to be endemic to Mauritius, one to Reunion and another to Rodrigues. Although the Mascarene *Diospyros* species have been collected for herbaria, no information is available on their colonization patterns or adaptive radiation. As these species are endangered, there is an urgency to understand the biology of the remaining *Diospyros* species. Findings are likely to be invaluable for conservation and reforestation. Furthermore, the proven good quality of the timber of the *Diospyros* species, together with potential medicinal properties (Mallavadhani *et al*., 1998), strengthens the need to study these species at different levels.

The purpose of this study was to analyse selected morphological characters with a view to establishing the phylogenetic relationships within the Mascarene *Diospyros* species.

MATERIAL AND METHODS

For each species, 35 stable morphological characters were selected based on their presence in all the species and their potential for phylogenetic informativeness (Table 1). In the case of *D. angulata* measurements were taken from plant materials we collected in 2000, while other characters were obtained from herbarium (Mauritius Sugar Industry Research Institute: MSIRI) specimens as this species is now believed to be extinct in the wild. Data, which could not be obtained from live specimens of *D. borbonica* (Reunion), were complemented with measurements from herbarium (MSIRI) specimens. Measurements for *D. kaki*, which is a native of Asia, have been used as outgroup, as adult plants of this species are found in Mauritius with flowers and fruits that were readily available at the time of study.

The morphological characters (12 vegetative and 23 reproductive) were scored and a data matrix was constructed for each of the 14 species (Appendix I). This morphological data matrix contained two taxa that were polymorphic for one character and 3% of missing data.

All characters were treated as independent, unordered and of equal weight. The phylogenetic analysis was performed with PAUP 4.0b. The most parsimonious trees were found using a heuristic search with 100 random stepwise additions and MULTREES option. Branch lengths for the trees were calculated using the ACCTRAN (accelerated transformation optimization) option in PAUP. A strict consensus tree was then constructed from the most parsimonious trees obtained. Bootstrap analyses (100) using simple stepwise additions were conducted to examine the relative level of support for individual clades of the cladograms (Felsenstein, 1985).

RESULTS

Cladistic analysis of the morphological data generated five most parsimonious trees with a length of 125, a consistency index (CI) of 0.57, a homoplasy index (HI) of 0.43, a retention index (RI) of 0.63 and a rescaled consistency index (RC) of 0.36. One of the five trees was arbitrarily selected and shown with the synapomorphies along the branches in Figure 1.

The 14 *Diospyros* species are divided into two major clades. Clade 1 consists of *D. chrysophyllos*, *D. tessellaria*, *D. angulata*, *D. boutoniana*, *D. borbonica*, *D. diversifolia*, *D. neraudii*, *D. nodosa*, *D. pterocalyx*, *D. hemiteles* and *D. melanida*. Clade 2 is made up of *D. revaughanii*, *D. egrettarum* and *D. leucomelas*. The relative positions of the species in clade 1 vary slightly among the five trees, while the order of clade 2 species is identical for all the cladograms. In three of the cladograms, *D. chrysophyllos* and *D. tessellaria* are grouped as sister species in a subclade which collapsed in the other two trees. *Diospyros angulata* and *D. boutoniana* are represented as sister species in all five trees and their relative positions only differ in one cladogram. The order of the minor clade consisting of *D. neraudii*, *D. nodosa* and *D. pterocalyx* is similar in

Table 1. Morphological characters and character states used in the maximum parsimony analysis of the Mascarene *Diospyros* species

Morphological characters	Character states				
	0	1	2	3	4
Tree height (m)	2–6	7–12	13–20		
No. of tree trunk	Single	Multiple			
Bark colour	Black	Nearly black	Greyish black	Grey	
Leaf					
Colour	Dark green	Green	Greyish green		
Length (cm)	3–6	7–10	11–16	20–30	
Width (cm)	1–3	4–6	7–9	10–12	13–15
Shape	Elliptic	Oblong	Oval		
Base	Cordate	Cuneate	More or less rounded		
Tip	Acute	Obtuse			
Texture	Sub-leathery	Leathery			
Petiole thickness (mm)	20–25	30–35	40–45		
Petiole length (mm)	1.5–1.9	2–10	11–20		
Male flowers:					
Clusters	Aggregate	Solitary			
Calyx length (mm)	5–6	7–8	9–10	10–11	
Calyx shape	Cupuliform	Ovoid	Cupuliform-cylindrical		
Corolla diameter (mm)	5–9	10–15	16–22	25–35	
No. of corollar lobes	4	5–6	7–8		
No. of stamens	9–15	16–26	27–40	60–70	
Anther length (mm)	3.0–4.9	5.0–7.0			
Filament length (mm)	1	2	3	4	
Female flowers:					
Clusters	Aggregate	Solitary			
Calyx shape	cupuliform	More or less spherical			
Calyx diameter (mm)	5–6	7–8	9–10		
Corolla diameter (mm)	7–9	10–12	15–22	25–35	
No. of stigmas	4–6	7–8			
Staminodes	2–10	11–19	20–24	25–40	
Surface of calyx	Dense hairs	Sparse hairs	Absence of hairs		
Flower fragrance	Very fragrant	Fragrant	Very slightly fragrant		
Fruit:					
Length	2.5–3.0	3.1–4.0	4.1–5.0		
No. of Calyx lobes	4	5	6–7		
Calyx height (mm)	3.0–4.0	10–15	16–18	20–25	
Shape	ovoid	ellipsoid	spherical		
Outer surface	Very sticky	Sticky	Not sticky		
Texture when mature	Soft	Hard			
Wings on calyx	Very pronounced	Less pronounced	No wings		

all the cladograms and these species are shown to be among the last to have evolved. *Diospyros hemiteles* and *D. melanida* are also indicated as recent species except for one cladogram, where both sister species are shown to have appeared just before *D. chrysophyllos* and *D. tessellaria*. *Diospyros borbonica* in Reunion Island and *D. diversifolia* in Rodrigues Island seem to have emerged after the lineage consisting of *D. angulata* and *D. boutoniana* in all five trees. Figure 1 also provides some information on the accumulated morphological changes for each of the 14 Mascarene *Diospyros* species.

Clade 2 is supported by a cordate leaf base, leathery leaf texture, intermediate petiole thickness, shortest petiole length, cupuliform-cylindrical male flower calyx shape, aggregate female flower cluster, 7–8

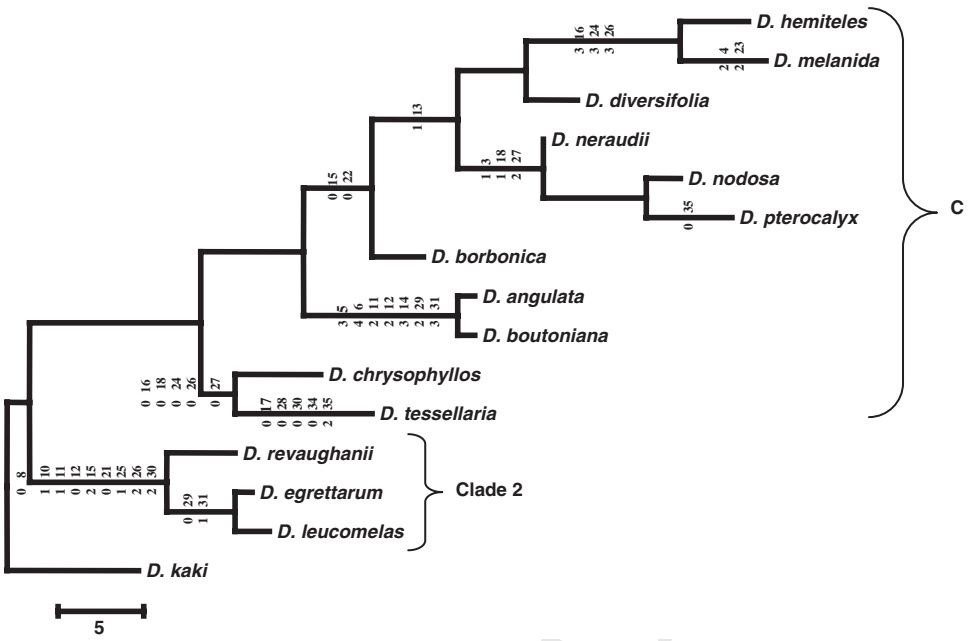


Figure 1. One of the five cladograms generated from the maximum parsimony analysis of 35 equally weighted morphological characters (CI = 0.57, HI = 0.43, RI = 0.63, RC = 0.36). The synapomorphies and their character states are indicated above and below the branches, respectively.

stigmas, 20–24 staminodes and 6–7 fruit calyx lobes (characters 8, 10, 11, 12, 15, 21, 25, 26 and 30, respectively). Within clade 2, the species *D. egrettarum* and *D. leucomelas* can be grouped together by the shortest fruit length and a calyx height of 10–15 mm (characters 29 and 31, respectively).

Within clade 1, the node leading to *D. angulata* and *D. boutoniana* is supported by the broadest leaf, thickest petiole, and longest leaf, petiole, flower calyx, fruit and fruit calyx (characters 6, 11, 5, 12, 14, 29 and 31). The clade consisting of *D. neraudii*, *D. nodosa* and *D. pterocalyx* is typified by nearly black bark and 16–26 stamens (characters 3 and 18), while the fruit calyx of *D. pterocalyx* is characterized by very pronounced wings (character 35). The species *D. chrysophyllos* and *D. tessellaria* share a number of similarities, namely, 9–15 stamens, the smallest corolla diameter in both male and female flowers, 2–10 staminodes in the female flowers and the presence of dense hairs on the surface of the flower calyx (characters 18, 16, 24, 26 and 27). *Diospyros tessellaria* is, however, quite distinct and can be separated from the other species by having the smallest number of corolla lobes and fruit calyx lobes (characters 17 and 30), very fragrant flowers, fleshy fruits and no wings on the fruit calyx (characters 28, 34 and 35). *Diospyros chrysophyllos*, *D. tessellaria* and *D. boutoniana* have an ovoid male calyx (character 15), while *D. tessellaria*, *D. boutoniana* and *D. angulata* have characteristic black barks (character 1). *Diospyros diversifolia*, *D. hemiteles*, *D. melanida*, *D. neraudii*, *D. nodosa* and *D. pterocalyx* all have a solitary male flower cluster (character 13), while only the species *D. neraudii*, *D. nodosa* and *D. pterocalyx* are characterized by the absence of hairs on the surface of the flower calyces (character 27). *Diospyros borbonica*, together with *D. diversifolia*, *D. hemiteles*, *D. melanida*, *D. neraudii*, *D. nodosa* and *D. pterocalyx*, have cupuliform male and female flower calices in common (characters 15 and 22). *Diospyros hemiteles* and *D. melanida* typically have the largest male and female flower corolla (characters 16 and 24) and 20–24 staminodes in the female flowers (character 26). *Diospyros melanida* can be distinguished by its greyish green leaves and largest female flower calyx diameter (characters 4 and 23). *Diospyros hemiteles*, on the other hand, exhibits the highest number of corolla lobes (character 17).

Figure 2 represents a strict consensus tree with bootstrap values of more than 50%. Bootstrap estimates were relatively good for some basal nodes, but low bootstrap values for the other nodes and the ambiguity in the exact order of some species resulted in the polytomies observed in the consensus tree.

According to Figure 2, there is strong support that the species *D. revaughanii*, *D. egrettarum* and *D. leucomelas* are among the most ancient and are morphologically different from the rest of the Mascarene species. Moreover, the upland species

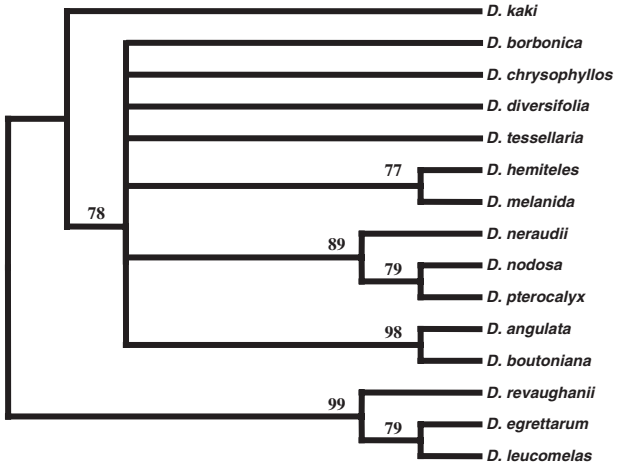


Figure 2. Strict consensus tree of the five most parsimonious trees obtained. The figures above the branches are the bootstrap values.

D. neraudii, *D. nodosa* and *D. pterocalyx* are shown to be closely related. The consensus tree also suggests that *D. hemiteles* and *D. melanida*, which grow in the same habitat, can be considered as sister species while *D. angulata* and *D. boutoniana* seem to share many similarities. However, *D. chrysophyllos*, *D. tessellaria*, *D. borbonica* and *D. diversifolia* could not be placed in any exact order.

DISCUSSION

The Mascarene *Diospyros* species are all dioecious and can easily be differentiated from each other by their leaves and tree stature. The leaf colour and structures of the *Diospyros* species in Mauritius often vary regardless of the types of habitat. However, *D. diversifolia* has the smallest and narrowest leaves, which could be an adaptation to arid areas. Indeed, this species can be encountered only in Rodrigues, which is drier and warmer than Mauritius or Reunion. It is sometimes difficult to distinguish among the flowers and fruits of some species, as in the case of *D. egrettarum* and *D. leucomelas*, even though they inhabit different geographical altitudes. The leaves of both species also share many similarities except for the red midrib of the leaves of *D. leucomelas*. A few studies have shown that reddish colouration in leaves could act as a deterrent to herbivory (Hansen, Brimer & Mølgaard, 2004). Hansen *et al.* have argued that herbivores would regard leaves with reddish patterns as toxic and would therefore stay away from those plants. Leaves with reddish venations can be observed in quite a few species indigenous to Mauritius (*Tarenna borbonica*, *Gastonia mauritiana*, *Casinne orientalis*, *Tambourissa* sp. to name a few) but this phenomenon is mostly encountered in the juvenile stage. The colourations then disappear to produce uniform green leaves as the plants mature and gain height. Interestingly, in the case of *D. leucomelas*, the red mid becomes more prominent on the underside of the leaves in the adult stage. This could well be an added protection against herbivory, given the multistemmed tree habit of *D. leucomelas* with leaves only a short distance from the ground.

The Mascarene *Diospyros* species bear small white flowers, except for *D. tessellaria* that sometimes produces flowers that have a pink tinge. Our data have indicated that there are no dramatic variations in flower morphology among the different species, while male and female flowers of the same species appear almost identical. Studies have shown that female flowers, which cannot produce pollen to attract insect pollinators, bear a close resemblance to the male flowers in order to be pollinated by deceit (Renner & Feil, 1993; Le Corff, Ågren & Schemske, 1998). Some nondiscriminating insects will therefore visit both male and female flowers, thereby pollinating the female flowers with pollen from the male flowers. The female flowers of *Diospyros* also have staminodes which increase their resemblance to male flowers. Moreover, male flowers are produced earlier and in more frequent cycles than the female flowers that are generated only once a year. This strategy could have evolved to ensure that rewarding male flowers are available to the pollinators earlier and for longer periods, so as to make these insects less discriminating when the rewardless female flowers are produced. Furthermore, the close similitude among the flowers of *Diospyros* species would suggest that selection pressures to attract generalist pollinators have favoured parallel or convergent evolution in floral characters.

Our phylogenetic analysis suggests that the clade *D. revaughanii*, *D. egrettarum* and *D. leucomelas* could have been the first pioneering species of Mauritius. *Diospyros revaughanii* has colonized areas ranging from low altitude habitats to marshy lands on the central plateau. This species has also shown a certain phenotypic plasticity in that it occurs either as a tree in low altitude regions or as a shrub in the upland marshy areas. As its fruits are sweet scented and sticky, they may have been picked by or stuck to birds, thus dispersing the seeds over broader distances. *Diospyros egrettarum* is the only true coastal species, with fragmented populations occurring only on the eastern lowland regions and on a 25 ha islet (Ile aux Aigrettes) off the south-east coast of Mauritius. It is noteworthy that we have recorded the occurrence of leaky dioecy in individuals from two populations of the multistemmed *D. egrettarum*. Indeed, the same plant was observed to produce distinct male and female flowers on separate stems. Furthermore, these leaky

dioecious plants generated seeds that had a germination rate of approximately 30% (unpublished data). Therefore, the mechanism of leaky dioecy could have been a strategy to ensure that a single pioneer *Diospyros* plant had the ability to generate fertile seeds and establish a reproductively viable population. It is interesting to note that *D. leucomelas* and *D. egrettarum*, which exhibit a high degree of morphological similarity, are both located in close proximity in the remnant forests on the east coast of Mauritius. However, most of the populations of *D. leucomelas* are found in mid altitude areas. On the other hand, *D. chrysophyllos*, which exhibits the closest morphological resemblance to *D. tessellaria*, has been encountered in a few low to high altitude regions as isolated individuals. *Diospyros tessellaria* is the most widely distributed species, indicating adaptation to most of the ecological conditions of Mauritius in that it has been able to establish viable populations in regions of low, mid and high altitude. This broad distribution and significant population size is certainly linked to the fact that the fruits of *D. tessellaria* are fragrant and fleshy enough to be eaten and dispersed by the endemic bat, *Pteropus niger*. It should be noted that *D. tessellaria* is the only *Diospyros* species in the Mascarenes that bears fleshy and fragrant mature fruits, while the rest of the Mascarene species produce fruits that remain hard even when they are mature.

Diospyros boutoniana and *D. angulata* are the two species that have the broadest leaves in this genus. Although *D. angulata* and *D. boutoniana* have been found to be morphologically quite close, they do not share the same ecological habitats. *Diospyros boutoniana* occurs mostly in upland forests with only a few individuals inhabiting low and mid altitude areas, while the only plant representing *D. angulata* was located in a mid altitude region. However, as *D. angulata* was down to one single female individual, it is difficult to ascertain its real geographical distribution. *Diospyros borbonica*, which is endemic to Reunion Island, has been reported to occur only on the south-east coast of this island (Bosser *et al*., 1976 ongoing), while *D. diversifolia* is endemic to Rodrigues Island and can be found as very fragmented populations in several locations. Given that Mauritius is the oldest Mascarene island (8 million years), and based on the phylogenetic analysis, it is tempting to speculate that the *Diospyros* group evolved and speciated in Mauritius until at some point in time, one species moved to the coastal region of Reunion Island (3 million years old) to give rise to *D. borbonica*. This event may have been followed by another migration to produce *D. diversifolia* in Rodrigues Island (1.5 million years old). The species *D. hemiteles* and *D. melanida*, which appear to be closely related (Figs 1, 2), are known to occur only in mid altitude regions. Despite the fact that the group *D. leucomelas*, *D. tessellaria*, *D. hemiteles* and *D. melanida* all occur in very close proximity in mid altitude habitats, they have a staggered flowering period so that no two species flower at the same time. It is therefore not surprising that hybrids have never been observed among these species, which are sometimes only a few metres apart. The high altitude species most likely emerged to colonize the humid habitats on the central plateau of Mauritius. *Diospyros pterocalyx*, *D. neraudii* and *D. nodosa* all occupy the same niche in the upland forests of Mauritius. Like the above mid altitude species, they have developed reproductive barriers so as to remain as distinct species.

In essence, the phylogenetic trees obtained from morphological data support the notion that colonization of the *Diospyros* group most probably started in the coastal areas. Then, with speciation and adaptive radiation, the species moved to the mid altitude regions and finally, the upland species arose to colonize the humid habitats. The seeds of *D. tessellaria*, on the other hand, were most probably dispersed by the *Pteropus niger* (and other endemic bats, now extinct) some distance away from the mother plant, explaining the wide distribution of *D. tessellaria* over the whole island. Unfortunately, little is known of the eating habits of the now extinct herbivore species such as the flightless dodo (*Raphus cucullatus*), the red rail (*Aphanapteryx bonasia*) and giant land tortoises (*Cylindraspis inepta*, *Cylindraspis triserrata*), which could have contributed to the dispersal of *Diospyros* seeds to different niches.

Homoplasy in some character traits from both vegetative and reproductive parts of *Diospyros* species has created ambiguities in the relative positions of a few species in the phylogenetic tree. It is hoped that future molecular analyses will clarify the effects of parallel or convergent evolution, and provide evidence on whether or not the shared derived characters arose independently in the different *Diospyros* species as adaptations to a common environment.

ACKNOWLEDGEMENTS

The authors wish to thank the Mauritius Research Council for financial support. The contributions of officers from the Forestry Department and the National Parks & Conservation Services are gratefully acknowledged. We are also thankful to the Tertiary Education Commission for the scholarships awarded to PN and SV.

REFERENCES

Bosser J, Cadet TH, Gueho J, Julien HR, Marais W. 1976. *Flore des Mascareignes, La Reunion, Maurice et Rodrigues*

[ongoing]. RBG Kew, Port-Louis, Mauritius: MSIRI, ORSTROM.

Brouard NR. 1963. *A history of woods and forests in Mauritius.* Port-Louis. Mauritius: Government Printers.

Brummitt RK. 1992. *Vascular plant families and genera.* Kew, UK: Royal Botanic Gardens.

Cadet TH. 1977. La végétation de l'Ile de la Réunion: Etude phytoécologique et phytosociologique. PhD Thesis, University of Aix-Marseille III.

Cadet TH. 1984. *Fleurs et plantes de la Reunion et de l'ile Maurice.* Singapore: Les Editions du pacifique.

Cronquist A. 1981. *An integrated system of classification of flowering plants*. New York: Columbia University Press.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39:** 783–791.

Hansen I, Brimer L, Mølgaard P. 2004. Herbivore-deterring secondary compounds in heterophyllous woody species of the Mascarene Islands. *Perspectives in Plant Ecology, Evolution and Systematics* **6:** 187–203.

Le Corff J, Ågren J, Schemske DW. 1998. Floral display, pollinator discrimination, and female reproductive success in two monoecious *Begonia* species. *Ecology* **79:** 1610–1619.

Mallavadhani UV, Panda AK, Rao YR. 1998. Pharmacology and chemotaxonomy of *Diospyros*. *Phytochemistry* **49:** 901–951.

McDougall I. 1971. The geochronology and evolution of the young volcanic island of Reunion, Indian Ocean. *Geochimica et Cosmochimica Acta* **35:** 261–288.

McDougall I, Chamalaun FH. 1969. Isotopic dating and geomagnetic polarity studies on volcanic rocks from Mauritius, Indian Ocean. *Geological Society of America Bulletin* **80:** 1419–1442.

McDougall I, Upton BGJ, Wadsworth WJ. 1965. A geological reconnaissance of Rodriguez Island, Indian Ocean. *Nature* **206:** 26–27.

Ng FSP. 1986. *Tree flora of Malaya: a manual for foresters*. London: Longman Group Ltd.

Page WS, D'Argent G. 1997. A vegetation survey of Mauritius (Indian Ocean) to identify priority rainforest areas for conservation management. Unpublished.

Pitot A. 1905. *T'Eylandt Mauritius. Esquisses historiques, 1598–1710.* Port-Louis, Mauritius.

Renner SS, Feil JP. 1993. Pollinators of tropical dioecious angiosperms. *American Journal of Botany* **80:** 1100–1107.

Richardson IBK. 1980. Notes on *Diospyros* (Ebenaceae) in the Mascarene Islands. *Kew Bulletin* **34:** 723–735.

Strahm W. 1994. Regional overview: Indian Ocean Islands. In: *WWF, IUCN: Centres of plant diversity. A guide and strategy for their conservation.* Cambridge, UK: IUCN Publication Unit.

Vaughan RE, Wiehe PO. 1937. Studies on the vegetation of Mauritius I. A preliminary survey of the plant communities. *Journal of Ecology* **25:** 289–343.

APPENDIX 1

5

D. kaki	111122211001102000??01?0121?2110202
D. angulata	2000340110220???????11??0?1?2132211
D. boutoniana	2000340100220312121011201122132011
D. borbonica	1020230200010102121110120112122221 1
D. chrysophyllos	0021222200010210100111000011122211
D. diversifolia	1020000110011002130010020122112221 1
D. egrettarum	1130230011100121121201111211011021 1
D. hemiteles	1021112210011003230210130311110121 1
D. leucomelas	1130230011100122121201121211021021 1
D. melanida	1032121210011003120210230311112221 1
D. neraudii	0010110200011202111110020122112111 1
D. nodosa	(0,1)1111112000112011111101101221120111
D. pterocalyx	0111001210011001111110010122110111 0
D. revaughanii	(0,1)1302220111000211211011112121211011
D. tessellaria	2001220200010010001111000000100120 2

MARKED PROOF

Please correct and return this set

Please use the proof correction marks shown below for all alterations and corrections. If you wish to return your proof by fax you should ensure that all amendments are written clearly in dark ink and are made well within the page margins.

Instruction to printer	*Textual mark*	*Marginal mark*
Leave unchanged	··· under matter to remain	Stet
Insert in text the matter indicated in the margin		New matter followed by
Delete	through matter to be deleted	
Delete and close up	through matter to be deleted	
Substitute character or substitute part of one or more word(s)	/ through letter or through word	New letter or new word
Change to italics	— under matter to be changed	
Change to capitals	≡ under matter to be changed	
Change to small capitals	= under matter to be changed	
Change to bold type	~ under matter to be changed	
Change to bold italic	≈ under matter to be changed	
Change to lower case	Encircle matter to be changed	
Change italic to upright type	(As above)	
Insert 'superior' character	/ through character or where required	under character e.g.
Insert 'inferior' character	(As above)	over character e.g.
Insert full stop	(As above)	
Insert comma	(As above)	
Insert single quotation marks	(As above)	and/or
Insert double quotation marks	(As above)	and/or
Insert hyphen	(As above)	
Start new paragraph		
No new paragraph		
Transpose		
Close up	linking letters	
Insert space between letters	between letters affected	
Insert space between words	between words affected	
Reduce space between letters	between letters affected	
Reduce space between words	between words affected	