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GENETIC AND DEMOGRAPHIC PROCESSES UNDERLYING REPRODUCTION IN *LODOICEA MALDIVICA,* THE LARGEST-SEEDED PLANT IN THE WORLD

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SUMMARY

The ancient islands of the Seychelles archipelago are a biodiversity hotspot, hosting many endemic species. One of these is the iconic, endangered coco de mer palm, *Lodoicea maldivica* (J. F. Gmel.) Pers., which is famous for producing the largest seeds in the plant kingdom. Today, the majority of these seeds are sold to tourists as souvenirs, with the consequence that remaining fragments of coco de mer forest are no longer regenerating.

Seed and pollen dispersal are important processes influencing the reproductive success and genetic structure of all plant populations. Habitat fragmentation can disrupt these processes, leading to deleterious genetic effects such as increased drift, elevated inbreeding, reduced genetic diversity and intensification of genetic structure. Despite its ecological and economic significance, surprisingly little is known about the genetic and ecological processes acting within natural populations of *Lodoicea*, and about how these are affected by habitat fragmentation. In this study, I used a range of molecular and ecological approaches to investigate how environmental and genetic factors affect the genetic structure of *Lodoicea* populations, sex ratios, and overall reproductive success. I also investigated impacts of habitat fragmentation on this system.

My results show that the huge seeds of *Lodoicea* are dispersal-limited, which results in a strongly developed fine-scale spatial genetic structure (FSGS). Thus, despite relatively high genetic diversity, inbreeding was also high, and potential mates within close proximity were, on average, closely related. Similar genetic patterns were observed across all remaining sub-populations, irrespective of the degree of fragmentation of the habitat or growth stage of the plants. This suggests that recent habitat disturbance has either had no negative genetic effects on the species, or that the disturbances occurred too recently for genetic changes to become apparent.

Paternity analyses performed on offspring showed that around 80% of all pollen flow occurred over distances of < 80 m. However, I also found evidence of occasional pollen dispersal over much longer distances, which is probably important for maintaining genetic connectivity across the landscape. I postulate that the restricted pollen flow contributes to the high levels of FSGS,

and drives the high levels of bi-parental inbreeding. Shorter average pollen dispersal distances were recorded in the more degraded population on Curieuse, suggesting that long-distance pollen flow occurs less often among isolated forest fragments.

A detailed study of persistent female inflorescences revealed large variation in reproductive output among individuals over a seven-year period. Fecundity was limited by soil nutrient concentrations and by pollen availability, albeit at different phases of the reproductive cycle. Many trees, especially in open areas, bore abnormal fruits that failed to produce viable seeds. One possible cause for this is pollen limitation. Indeed, historical evidence indicates that the average fecundity of female trees has declined in recent years, which presumably reflects the reduced density of male trees in the modern landscape.

Previous research has shown that most *Lodoicea* populations have unbalanced sex ratios. However, the reason for this - whether different numbers of male and female seeds are produced or mortality rates of established plants are unequal – was not previously known. I developed two new male sex-specific markers, which allowed me to investigate sex ratios in phenotypically similar male and female non-mature plants. My results show approximately balanced sex ratios in all sub-populations, with no detectable effects of the environment upon this ratio. It is likely that higher female susceptibility to environmental stress and subsequent earlier death results in male-biased ratios in some populations, though patterns vary among private and protected *Lodoicea* populations. I conclude that the reasons for the biased adult sex ratios are probably complex and may in part reflect human activities.

Lodoicea is both an ecological keystone species and a flagship species for conservation. This thesis demonstrates the wide range of ecological processes that interact to shape its genetic and demographic structure. It shows how recent habitat disturbance can alter patterns of pollen dispersal, which can reduce a species' overall fecundity*.* The results contribute to a growing body of literature highlighting the importance of understanding how ecological trade-offs in plants can shape a species' evolution, and how habitat disturbance can alter natural dynamics. I conclude that to properly manage species of conservation concern it is important to consider the genetic responses of fragmentation in a wider ecological context.

ZUSAMMENFASSUNG

Die erdgeschichtlich sehr alten Inseln des Archipels der Seychellen sind ein Biodiversitätshotspot, welcher unzählige endemische Arten beheimatet. Eine dieser Arten ist die einzigartige, jedoch gefährdete Coco de Mer Palme, *Lodoicea maldivica* (J. F. Gmel.) Pers., welche bekannt dafür ist, die grössten Samen des gesamten Pflanzenreiches zu produzieren. Heutzutage werden die meisten dieser Samen als Souvenirs an Touristen verkauft, was dazu führt, dass sich die wenigen, noch erhaltenen Fragmente der Coco de Mer Wälder nicht mehr verjüngen.

Samen- und Pollenausbreitung sind wichtige Prozesse für den reproduktiven Erfolg und die genetische Struktur aller Pflanzenpopulationen. Habitat-Fragmentierung kann diese Prozesse stören und zu schädlichen genetischen Effekten führen wie Gendrift (zufällige Änderung der Allelfrequenzen innerhalb einer Population) oder einer die Zunahme der genetischen Struktur. Trotz ihrer ökologischen und ökonomischen Bedeutung ist über die genetischen und ökologischen Prozesse erstaunlich wenig bekannt, welche die natürlichen Populationen von *Lodoicea* beeinflussen sowie dieselben durch Habitat-Fragmentierung beeinflusst werden. In dieser Studie habe ich mit einer Reihe molekularer und ökologischer Ansätze untersucht, wie ökologische und genetische Faktoren die genetische Struktur, Geschlechterverhältnisse sowie den reproduktiven Erfolg von *Lodoicea*-Populationen beeinflussen. Zusätzlich habe ich die Einflüsse der Habitat-Fragmentierung auf dieses System untersucht.

Meine Resultate zeigen, dass die riesigen Samen von *Lodoicea* in ihrer Verbreitung eingeschränkt sind, was in einer starken kleinräumigen genetischen Struktur (fine-scale spatial genetic structure, FSGS) resultiert. Trotz einer relativ grossen genetischen Vielfalt zeigte sich eine starke Inzucht und, dass nah-stehende, potentielle Partner im Schnitt eng verwandt waren. Ähnliche genetische Muster wurden in allen verbleibenden Subpopulationen gefunden, unabhängig von der Fragmentierung des Habitats oder dem Wachstumsstadium der Pflanzen. Das legt nahe, dass Störungen in jüngster Zeit entweder keine negativen genetischen Effekte auf die Art hatten oder, dass die Störungen vor zu kurzer Zeit geschahen, so dass sich die genetischen Veränderungen noch nicht nachweisen lassen.

Vaterschaftstests an Nachkommen zeigten, dass ungefähr 80% des gesamten Pollenflusses über Distanzen von mehr als 80m stattfanden. Dennoch fand ich Evidenz für gelegentliche Pollenausbreitung über viel grössere Distanzen, was vermutlich wichtig ist, um die genetische Verbindung der Populationen über die Landschaft zu erhalten. Ich postuliere, dass der eingeschränkte Pollenfluss zu dem hohen Ausmass an FSGS beiträgt und die starke Inzucht vorantreibt. Im Schnitt wurden kürzere Pollenausbreitungsdistanzen in der stärker degradierten Population auf der Insel Curieuse gefunden, was darauf hindeutet, dass Pollenflüsse über weite Distanzen weniger oft in isolierten Waldfragmenten vorkommt.

Eine detaillierte Untersuchung von lange bestehenden weiblichen Blütenständen über 7 Jahre, zeigte grosse Variationen bei der Anzahl überlebensfähige Nachkommen zwischen den Individuen. Die Fruchtbarkeit wurde eingeschränkt durch spezifische Konzentrationen von Bodennährstoffen sowie durch die Pollenverfügbarkeit, wobei diese Einflüsse in verschiedenen Phasen im Reproduktionszyklus variierten. Viele Bäume, speziell in offenen Gebieten, trugen abnormale Früchte, welche keine überlebensfähigen Samen produzierten. Ein möglicher Grund dafür ist ein Mangel an verfügbarem Pollen. In der Tat zeigen historische Daten, dass die mittlere Fruchtbarkeit weiblicher Bäume in den letzten Jahren zurückgegangen ist, was vermutlich durch die reduzierte Dichte männlicher Bäume in der modernen Landschaft induziert wird.

Frühere Untersuchungen haben gezeigt, dass die meisten *Lodoicea*-Populationen unausgewogene Geschlechtsverhältnisse haben. Die Gründe dafür - ob eine unterschiedliche Anzahl männlicher und weiblicher Samen produziert wurde oder die Sterblichkeitsrate der entwickelten Pflanzen verschieden war - waren nicht bekannt. Ich habe zwei neue geschlechtsspezifische Marker für männliche Pflanzen entwickelt, was mir erlaubte, die Geschlechtsverhältnisse von nicht-adulten Pflanzen zu bestimmen, die trotz unterschiedlicher Geschlechter phänotypisch nicht unterscheidbar sind. Meine Resultate zeigen annähernd ausgeglichene Geschlechtsverhältnisse in allen Subpopulationen, mit keinen nachweisbaren Effekten der Umwelt auf das Verhältnis. Es ist wahrscheinlich, dass eine stärkere weibliche Empfindlichkeit auf Umweltbelastungen und eine daraus folgende frühere Sterblichkeit zu dem unausgewogenen Verhältnis, mit einem Überschuss von männlichen Pflanzen, in einigen Populationen führt. Die Muster variieren jedoch stark zwischen ungeschützten (meist in Privatbesitz) und geschützten Populationen von *Lodoicea*. Daraus folgere ich, dass die Gründe für die unausgewogenen Geschlechtsverhältnisse adulter Pflanzen vermutlich verschiedenste Einflüsse beinhalten wobei einige davon menschliche Aktivitäten mit einschliessen.

Lodoicea ist gleichzeitig eine Schlüssel- und eine Flaggschiff-Art und somit sehr wichtig für den Naturschutz. Diese Arbeit zeigt die breite Palette ökologischer Prozesse, welche gegenseitig interagieren und so die genetische und demographische Struktur von *Lodoicea* formen. Sie zeigt auf, wie jüngste Störungen des Lebensraumes die Muster der Pollenausbreitung und damit die Fruchtbarkeit der Art verändern können. Die Resultate tragen zu einem wachsenden Kenntnisstand bei, der aufzeigt, wie wichtig es ist zu wissen, wie ökologische Zielkonflikte in Pflanzen die Evolution der Art beeinflussen und wie Störungen des Lebensraumes natürliche Dynamiken verändern können. Ich schliesse aus den Resultaten, dass es für ein gutes Management von schutzwürdigen Arten zwingend ist, die genetischen Reaktionen von Fragmentierung in einem breiteren ökologischen Kontext zu betrachten.

CHAPTER 1

General introduction

The coco de mer *Lodoicea maldivica* is an endemic palm that occurs only on two small islands in the Seychelles archipelago. This extraordinary species is surrounded by myth and legend; it was once thought that the trees grew beneath the sea and the nuts fell upwards, and it was also said that male trees wandered around at night to find females. Despite recent advances in our understanding of the natural history and ecology of this species, there are still many aspects of its demography, genetics and reproduction that we have very little information on. In this thesis I aim to advance our understanding of the ecological and evolutionary processes that occur in *Lodoicea,* and in dioecious plants in degraded island ecosystems, in general.

I begin, however, by presenting the theoretical ideas behind the high extinction rates in island endemics, and how genetic processes on islands might differ from on the mainland. I then introduce the study system of the Seychelles, the study species *Lodoicea maldivica*, and finally summarise the main aims of the thesis.

GENETIC PROCESSES AND REPRODUCTION IN ISLAND PLANTS

Islands are ideal places for the evolution of unique species, due to their isolation from continental species (Grant 1998). Recently formed oceanic islands, especially those volcanic in origin, or those that were previously submerged, provide many opportunities for evolutionary diversification and adaptive radiation (Losos & Ricklefs 2009). For this reason, islands have been of great interest to biologists since the time of Darwin and Wallace. Levels of endemism of plants are 9.5 times higher on islands compared to continental regions, which often results in extremely high levels of biodiversity (Kier *et al.* 2009).

Due to drastic global environmental change predominantly caused by human activities, we are currently in the midst of the sixth mass extinction. Loss of species is estimated to be 100 to 1000 times faster than the normal background level (Rockström *et al.* 2009). In this current climate, species-rich islands are important reservoirs of biodiversity, but rates of extinction are disproportionately higher on islands (Diamond 1984; Frankham 1998), with island endemics being especially at risk (McDowall 1969). Recent animal extinctions have been concentrated in larger-bodied fauna, and the same trend is apparent in plants, with extinct large palms being over-represented in the fossil records on many islands around the world (Prebble & Dowe 2008). A synergy of processes can act to eventually cause extinctions (Brook *et al.* 2008), and may include habitat loss, climate change or other drivers. It has been suggested that invasive rats may have driven the extinction of palm species by predating on seeds or damaging plants (Meyer & Butaud 2009). Understanding the processes that drive reproduction and population extinction of endemic plants is key to ensuring resilient island systems. Differences in the genetics and mating systems of island versus mainland plant populations are thought to be especially important (Barrett 1996).

The isolation and small size of most island populations can make them more sensitive to stochastic environmental, demographic, or genetic changes such as drift (Barrett *et al.* 1996). The higher risk of small populations experiencing reduced genetic diversity (Ellstrand & Elam 1993) may increase with population isolation (Shapcott 2000). Island populations may also be predisposed to higher levels of inbreeding depression than mainland populations, particularly if the populations experienced genetic bottlenecks at their foundation (Kirkpatrick & Jarne 2000). For example, juvenile cohorts of the conifer *Araucaria nemorosa* on New Caledonia showed elevated inbreeding and loss of rare alleles as a response to habitat destruction (Kettle *et al.* 2007). It is commonly thought that dioecy evolved to promote outcrossing (Baker 1959; Lloyd 1972; Ross & Weir 1976), which could explain the especially high incidence of dioecy on islands (Thomson & Barrett 1981). It has been proposed that island populations that are naturally fragmented are less sensitive to habitat fragmentation than formally widespread species, although the mating system probably plays an important role (Finger *et al.* 2012).

Seed and pollen dispersal are the key processes shaping the genetic structure of populations. For example, recent anthropogenic barriers to biotic seed and pollen dispersal on the tropical island of Trinidad resulted in spatial and temporal genetic differentiation, and high levels of nearest-neighbour mating in the dioecious palm *Mauritia flexuosa* (Federman *et al.* 2014). On the other hand, inbreeding and genetic diversity erosion were avoided in another fragmented island palm *Phoenix canariensis* by effective long-distance wind dispersal of pollen (Saro *et al.* 2014).

It has been observed that many island organisms evolve a loss of dispersal ability (Carlquist 1966; Cody & Overton 1996), often caused by the loss of features or traits that aid in gamete dispersal (Hughes *et al.* 1994). This is probably a consequence of selection for short-distance dispersal, which has an obvious advantage on small islands, since fewer gametes or propagules are lost by being transported out to sea. Genetic processes such as increased inbreeding and reduced gene dispersal may act to reduce the overall fecundity of island plants, for example by reducing germination rates (Carlquist 1974), or indirectly through the extinction of animal pollinators or seed dispersal vectors (Anderson *et al.* 2011).

THE SEYCHELLES ARCHIPELAGO

Human history on the islands

The islands of the Seychelles archipelago were first colonised by the French between 1742–1770 (Stoddart 1984), but the earliest written records date back to 1502 (Galvano, in Hakluyt 1862). Between 1800 and 1900, deforestation and exploitation of species increased drastically and agricultural practices gradually intensified, after the British took control of the islands. The need to protect the forests was first mentioned by Malavois in 1787 (Fauvel 1909). This was reinforced in 1881 by General Charles George Gordon (Gordon of Khatoum), who regarded the forest on Praslin as the ‘Garden of Eden’ (McAteer 2000). From around 1900–1970, most agricultural practices decreased (except for the production of cinnamon) and reforestation of alien species started to become the trend. The post-colonial era coincided with the opening of the airport in 1972 on the largest granitic island of Mahé, and tourism became central to the Seychelles economy (detailed descriptions in Küffer 2006).

Biogeography

The Seychelles are an ancient archipelago that formed from the breakup of Gondwanaland. About 130 million years ago the Seychelles, Madagascar and India broke off together from the African continent. This was followed by the separation of Seychelles and India from Madagascar ~85 million years ago, and then by the separation of Seychelles from the Indian subcontinent ~65 million years ago (Briggs 2003). The ~115 islands of the Seychelles lie in the Indian Ocean, 1000 km northwest of Madagascar and 1700 km east of Kenya.

If the Seychelles were never completely submerged during their long-isolation, it is likely that some of the original species could have persisted. Although some Seychelles species have affinities with African and Asian species (Stoddart 1984), the long isolation of the granitic islands has allowed for the evolution many very distinct species. Six main habitat types can be found across the islands (Küffer 2006): beach fringe and coastal vegetation (including mangroves), lowland forest, mid-altitude forest, inselberg (rocky outcrop) vegetation, montane forest, and perhaps the most striking of them all, palm forest. These are composed of different proportions of six endemic species - *Deckenia nobilis* (palmis)*, Nephrosperma vanhoutteanum* (latannyen milpat)*, Phoenicophorium borsigianum* (latannyen fey)*, Roscheria melanochaetes* (latannyen oban)*, Verschaffeltia splendida* (latannyen lat) and *Lodoicea maldivica* (coco de mer). On the islands of Praslin and Curieuse, the dominant species was *Lodoicea maldivica*.

LODOICEA MALDIVICA

Biology and life-history

Lodoicea maldivica (Arecaceae) belongs to a monotypic genus within the tribe Borasseae (Uhl & Dransfield 1987). This species occurs in a natural state only on Praslin (37.4 km^2) and Curieuse (3.6 km^2), though historically it also grew on a few neighbouring islets. It is generally confined to steep slopes and valleys, and can be found growing on a range of soil types (Uhl & Dransfield 1987). This 'island giant' (Whittaker 1998) is the dominant canopy tree where it grows in dense forest, and adult males grow to around 30 m tall, though historically the palms reached heights of perhaps more than 50 m (Ward 1866). The palm is long-lived and it has been suggested that it may live for up to 350 years, though data on this topic are sparse (Savage & Ashton 1983). The dioecious mating system of the species is in itself uncommon (Renner 2014), and the extent of sexual dimorphism observed in the male and female flowers is remarkable. The females produce woody inflorescences 1–2 m long, which bear between 1 and 13 large flowers (at ~5 cm in diameter, the largest of any palm). Male trees produce equally large, leathery catkins that bear at any time between 50 and 170 individual spirally arranged, sweet-smelling flowers, producing abundant pollen. The pollination mechanism remains uncertain but it has been hypothesised that wind (Edwards *et al.* 2002), flying insects (Blackmore *et al.* 2012), endemic geckos (Fischer *et al.* 2008) or a combination of vectors (Galen *et al.* 1985) could be important. Seeds are reported to take around seven years to fully mature on the tree (Corner 1966; Blackmore *et al.* 2012), and due to their huge size (up to 18 kg), they are only dispersed by gravity.

Female flower

Male catkin

Germination can take anything between eight weeks (own data) to a few years. An extended cotyledonary axis buries the embryo below the soil, then extends horizontally until the seedling establishes at a suitable spot, at some distance from the seed (Thistleton-Dyer 1910; Tomlinson 1990; Edwards *et al.* 2002). The species has an extended juvenile phase, during which time there is extensive underground development of the stem-base (Bailey 1942). A tough, bowl-shaped structure, up to one metre in diameter, is formed, along with a root structure that grows through the bowl's perforations to anchor the palm to the ground. During the seedling and juvenile phases, the greatly elongated petioles grow up to 10 m tall, allowing the enormous leaf blades (up to 10 m^2 in area) to reach into the canopy (Edwards *et al.* 2002). The immature stage is characterised by the development of a visible trunk, and each successive new leaf is associated with an extension of the trunk length by around 12.2 cm. In closed forest, *Lodoicea* start producing flowers when the trunk height reaches around 3.5 m. How long plants take to reach sexual maturity remains uncertain, though anecdotal evidence suggests that it may take several decades under forest conditions.

Lodoicea is a keystone species, which structures the ecosystem in which it occurs. Animal species that are either associated with or confined to *Lodoicea* forest include five endemic gecko species, the Seychelles black parrot, the Seychelles bulbul, a newly described species of sooglossid frog, and the Seychelles white slug (Beaver & Chong 1992; Noble *et al.* 2011; Reuleaux *et al.* 2013). The palm is also an ecosystem engineer. Its leaves form an efficient funnel that directs large volumes of rainwater (and with it, organic matter) down the trunk, and thereby enhances nutrient and moisture conditions around its base (Edwards *et al.* 2015).

Seed with cotyledonary rope

Lodoicea bowl

Management/Conservation

The huge seeds (nuts) produced by *Lodoicea* have been treasured for centuries, since they were first found washed up on the shores of India and the Maldives (where it was originally assumed the seeds came from, hence the species name *L. maldivica;* Baker 1942). After the true source was discovered in 1768, the history of exploitation of the species started, and the demand for seeds has been increasing ever since (Savage & Ashton 1983). The vulnerability of the species was recognised, and the species is now protected by the 'Coco-de-Mer (Management) Decree' of 1978 (revised in 1994) and the 'Breadfruit and other Trees Act' (Laws of Seychelles 1991). *Lodoicea* has been listed as 'Endangered' on the IUCN Red List (Fleischer-Dogley *et al.* 2011a), and trade is controlled by the Convention on International Trade in Endangered Species (CITES).

Historical texts report that *Lodoicea* was once widespread, and dominant across the islands on which it grew (Brayer du Barre 1773, quoted in Fauvel 1909; Ward 1866). However, due to sustained harvesting of seeds and leaves, logging for timber, and numerous serious fires (Bailey 1942; Fischer & Fleischer-Dogley 2008), *Lodoicea* populations have declined by over 30% over three generations, and it now occupies less than 100 km^2 in total (Fleischer-Dogley *et al.* 2011a). Only three substantial populations remain, which together contain almost three quarters of extant individuals: in Vallée de Mai (VdM)/Fond Peper (FP; 55°44' E, 4°19' S) and Fond Ferdinand (55°45' E, 4°21' S) in the south of Praslin, and on Curieuse (55°43' E, 4°16' S) (Fleischer-Dogley *et al.* 2011b). These populations lie within the Praslin and Curieuse National Parks, and are protected by the Seychelles National Parks Authority (SNPA; Curieuse), the

Ravin de Fond Ferdinand Nature Reserve, and the Seychelles Islands Foundation (SIF; VdM/FP). In 1983 the VdM was declared a UNESCO World Heritage Site, and it is now a popular tourist destination (Kaiser-Bunbury *et al.* 2014). The entry fees and profits from the sale of seeds are used by SIF to manage both the VdM and the Aldabra Atoll World Heritage Site. It is increasingly recognised that harvesting levels are unsustainable, and precautionary levels were recently proposed to ensure the long-term viability of the species (Rist *et al.* 2010). To encourage the natural regeneration of *Lodoicea,* an incentive-driven stewardship scheme was recently initiated in the VdM (Kaiser-Bunbury *et al.* 2014), but in most other populations regeneration is virtually absent. Most areas of the remaining forest are far from natural in structure, having been subjected to selective logging and the translocation and removal of seeds, as well as the introduction of invasive alien species.

Several large-scale attempts have been made to replant *Lodoicea* across Praslin and Curieuse, although some were largely unsuccessful (details compiled in Fleischer-Dogley 2006). This may partly be because little is known about natural patterns of reproduction or the genetic processes at work in undisturbed forest.

Small cluster of *Lodoicea*

Juvenile

THESIS OUTLINE

In this thesis I use a range of molecular and ecological approaches to investigate the demographic and genetic structure of *Lodoicea maldivica* stands and their patterns of regeneration:

(i) Neutral genetic markers (microsatellites) are used to investigate aspects of the ecology and mating system of *Lodoicea,* processes that are otherwise difficult to explore in species with long generation times.
(ii) Genetic relationships between female trees and established offspring are used to investigate seed dispersal patterns.
(iii) Paternity analysis and spatially explicit mating models are used to explore pollen dispersal patterns.
(iv) Morphological evidence from the large woody female inflorescences is used to investigate past reproductive success of female trees.
(v) A Next Generation Sequencing approach is used to investigate the sex determination system in *Lodoicea*, and study the sex ratios across ontological stages.

In addition to the introduction, this thesis contains four empirical papers (Chapters 2–5) and a general discussion (Chapter 6).

Chapter 2: Keeping it in the family: genetic implications of limited seed dispersal for the dioecious palm *Lodoicea maldivica*, the largest-seeded plant in the world. The impact of realised seed dispersal patterns on the fine-scale spatial genetic structure of *Lodoicea* sub-populations is explored. Genetic diversity and inbreeding levels are compared across sub-populations (exhibiting varying degrees of disturbance) and age cohorts (the oldest of which probably established before large-scale habitat disturbance).

Chapter 3: Limited pollen dispersal in natural populations of *Lodoicea maldivica* (J. F. Gmel.) Pers. Little is currently known about pollen dispersal in *Lodoicea,* although a range of possible pollination vectors have been proposed. Paternity analysis and spatially explicit mating models are used to explore pollen dispersal patterns and the mating system of the palm.

Chapter 4: Tracing coco de mer's reproductive history: pollen and nutrient limitation reduce fecundity. The availability of nutrient resources and pollen are important factors affecting plant fecundity. The significance of soil nutrient conditions, isolation of females from males, genetic diversity and vegetation type are related to flower and seed production in *Lodoicea.*

Chapter 5: Identification of sex-linked markers for coco de mer (*Lodoicea maldivica)* and their application in exploring sex ratios of non-mature plants. Most adult populations of the dioecious *Lodoicea* display biased sex ratios. Sex-linked markers were developed for the species using a ddRAD approach. These markers were then used to explore sex ratios across life stages in non-mature plants.

Chapter 6: General discussion. I place the key results of my thesis in the context of a range of evolutionary and ecological processes in plants. Specifically, reproductive success and trade-offs in dioecious species are discussed. The relevance of my findings will be useful for advancing our knowledge of the genetic and reproductive sensitivity of plants to habitat change, and in guiding the conservation of *Lodoicea*.

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CHAPTER 2

Keeping it in the family: genetic implications of limited seed dispersal for the dioecious palm *Lodoicea maldivica*, the largest-seeded plant in the world

with Christopher N. Kaiser-Bunbury, Peter J. Edwards, Frauke Fleischer-Dogley and Chris J. Kettle

ABSTRACT

Patterns of seed dispersal strongly influence the fine-scale spatial genetic structure (FSGS) of plant populations. The endangered palm *Lodoicea maldivica* presents an extreme case of limited dispersal as the huge fruits (c. 20 kg) can only disperse by gravity. We investigated patterns of realised seed dispersal and FSGS in populations of this extraordinary palm. We sampled 1252 individual adults and regenerating offspring from across the species' natural range in the Seychelles, and genotyped these at 12 microsatellite loci. The average dispersal distance was very short (8.7 ± 0.7 m), with the largest distances occurring on steep slopes. FSGS was intense, especially in younger cohorts. Levels of inbreeding were high, contrary to what might be expected in a dioecious species, and most pairs of male and female trees ≤ 10 m apart were closely related. However, levels of genetic diversity were relatively high and similar among sub-populations, despite variation in habitat disturbance and fragmentation. We conclude that the potential fitness costs associated with limited dispersal, and especially FSGS and inbreeding, are compensated by the benefits of maternal resource provisioning of progeny. These factors have an important influence upon the demography of this monodominant flagship species. Our results suggest that genetic factors are unlikely to compromise the reproductive success of this species.

INTRODUCTION

Dispersal allows offspring to move away from their parents, thereby facilitating gene flow, reducing sibling competition and avoiding inbreeding. In plants, most long-distance gene dispersal, especially in wind-pollinated species, occurs through the transport of haploid pollen (Petit *et al.* 2005), which moves only paternal genes. In contrast, seed dispersal - which is the principal means by which progeny establish at some distance from related individuals - moves both maternal and paternal genes. Both processes influence the degree to which related individuals are aggregated, and therefore the fine-scale spatial genetic structure (FSGS) of a population (Hardy *et al.* 2006). Limited dispersal of either seeds or pollen may result in an increased intensity of FSGS (Ennos 1994; Hardy *et al.* 2006; Seidler & Plotkin 2006) and greater genetic 'isolation-by-distance' (Wright 1943). Conversely, any aspect of a plant's breeding system that promotes outbreeding might tend to reduce the intensity of FSGS. An extreme case is dioecy, which is usually regarded as a strategy to avoid inbreeding, especially in small populations (Darwin 1877; Baker & Cox 1984), and which occurs more frequently on tropical islands than among flowering plants generally (12–27% versus 4%; Yampolsky & Yampolsky 1922; Bawa 1980).

In general, the intensity of FSGS appears be more affected by seed dispersal than by pollen dispersal, especially in large, long-lived plant species (Vekemans & Hardy 2004; Torimaru *et al.* 2007; Grivet *et al.* 2009). However, any disruption to dispersal – whether of seeds or pollen – can be expected to intensify the FSGS of a population. Several studies have demonstrated that habitat disturbance or fragmentation can disrupt seed (Cramer *et al.* 2007) and pollen dispersal (Ismail *et al*. 2012), leading to elevated inbreeding and reduced genetic diversity. However, in some systems long-distance pollen flow remains sufficient to counteract these negative effects (Dick *et al.* 2007; Ismail *et al*. 2014). In addition to these dispersal-related effects, several other factors can also influence FSGS, including local adaptation in heterogeneous habitats (Galen *et al.* 1991; Parisod and Christin 2008), recent founder events in pioneer tree species (Silvestrini *et al.* 2015), distance and density-dependent mortality (Choo *et al.* 2012), and the spread of sterility-causing mutations (De Cauwer *et al.* 2010).

The coco de mer palm *Lodoicea maldivica* (J.F.Gmel.) Pers. (Arecaceae) is a dioecious plant endemic on two small islands in the Seychelles. Female trees bear the largest flowers of any palm and the heaviest seeds in the plant kingdom. Geckos are thought to be the main pollination agents, though wind dispersal may also be important (Edwards *et al.* 2002; Blackmore *et al.* 2012). In contrast, the seeds, which are enclosed in a massive fruit weighing around 20 kg (Edwards *et al.* 2002, maximum of 45 kg; Tomlinson 2006), lack any mechanism for dispersal apart from gravity, so that seedlings usually establish in dense clusters around female trees. Although this leads to intense sibling competition, the leaves of the adult trees function as huge funnels, channelling water and organic material to the base of the tree and thereby improving nutrient and moisture conditions for young plants (Edwards *et al.* 2015).

Lodoicea provides a remarkable opportunity to study the genetic consequences of extremely limited seed dispersal across the entire range of a dioecious species in a fragmented habitat. We used microsatellites to investigate patterns of FSGS and genetic variation in adults and offspring of *Lodoicea* from a range of sites covering the entire species' range. We hypothesised that restricted seed dispersal leads to highly developed FSGS, while dioecy mitigates high levels of inbreeding, at least in the older cohorts which pre-date fragmentation. Specifically we addressed the following questions: (1) How far do seeds disperse? (2) Does topography influence patterns of realised seed dispersal? (3) Does limited seed dispersal result in intense FSGS across different sub-populations, cohorts and sexes? (4) How has recent habitat fragmentation affected patterns of inbreeding and genetic diversity? We discuss the genetic implications of limited seed dispersal and intense FSGS observed in *Lodoicea*, in the broader ecological and evolutionary context of inbreeding avoidance.

MATERIALS AND METHODS

Study area and study species

The Seychelles archipelago is exceptional among tropical islands in that it was uninhabited until comparatively recently, when Europeans colonised the islands in the 18th century (Lionnet 1976). Historical records suggest that *Lodoicea,* in the tribe Borasseae (Dransfield *et al.* 2008), was once the most abundant species across the islands of Praslin and Curieuse (Malavois 1787, quoted in Fauvel 1909), where it formed dense, monodominant forest (Edwards *et al.* 2015). Following settlement, and over the past 150 years especially, populations on both islands were drastically reduced, as timber, leaves and nuts were exploited (Fischer & Fleischer-Dogley 2008), and habitat cleared for cultivation and development (Bailey 1942). Extensive, semi-continuous stands now remain only in the southern part of Praslin, and especially in Vallée de Mai World Heritage Site. On Curieuse the species occurs mainly as isolated individuals or in small clusters. More recent pressures on *Lodoicea* include the spread of invasive alien species (Fischer & Fleischer-Dogley 2008) and the unsustainable harvesting of nuts. Indeed, there is now almost no natural regeneration except in protected areas. Nonetheless, because the trees may live for up to 350 years (Savage & Ashton 1983), many of the larger adults may have established before major human disturbance and have a genetic structure reflecting processes operating under natural conditions. All sub-populations on Praslin are male-biased (Vallée de Mai (VdM) and

Fond Peper (FP): 0.57; Fond Ferdinand (FF): 0.51), while Curieuse (CU) is female-biased (0.54; Fleischer-Dogley *et al*. 2011).

Sample collection

We investigated the FSGS of *Lodoicea* at three locations (hereafter referred to as sub-populations) on the island of Praslin–VdM, FP and FF, and one on the island of Curieuse (Fig. 1). Each sub-population on Praslin is characterised by continuous semi-natural vegetation, although the forest on Curieuse is patchier. Within each of the four sub-populations, we selected four clusters of *Lodoicea* plants that appeared to represent a naturally regenerating patch, and included plants of different age cohorts, ranging from seedlings to adult trees. We avoided areas where *Lodoicea* might have been planted, and instead selected areas with minimal signs of human disturbance. Within each of the 16 clusters selected (mean area ± SD: 904.4 ± 800.9 m^2), the locations of all adult trees were geo-referenced (Garmin 60CSx), and the positions of all young plants were mapped by taking bearings and measuring distances taken from a central female tree. Distances between next nearest cluster ranged from 13–360 m. GPS locations of all males within a radius of 80–120 m of each cluster were also recorded. We classified individuals into age classes using the following criteria: *seedlings* had two or fewer leaves; *juveniles* were trunkless plants with more than two leaves; *immatures* were individuals with trunks but without flowers; and *adults* had reached sexual maturity (from perhaps age 25–50 years), as indicated by the presence of male or female flowers. '*Offspring*' refers generally to all non-mature plants. Sampling of leaves for genetic analysis was carried out on all individuals in clusters, and was extended in the downhill direction until all potential offspring were sampled (likely over-sampling, to ensure any long distance seed dispersal events were identified), and uphill until all potential mothers were sampled. Samples were also collected from male trees within a radius of 80–120 m, and dried and stored in silica gel. This resulted in a total of 1252 samples (sub-population mean *N* ± SE: 313 ± 58.8) (see Table 1 for sample sizes of each group).

Genotyping

Total genomic DNA was extracted from the leaf tissue using the DNeasy® 96 Plant Kit (Qiagen). Twelve nuclear microsatellite markers were designed for *Lodoicea* at ecogenics GmbH (Balgach, Switzerland), using Roche 454 sequencing. For details of the microsatellite loci and PCR conditions see Morgan *et al.* (2016). Fragment length was analysed using the internal size marker LIZ 500 HD in an ABI3730 capillary sequencer (Applied Biosystems), and scored with GeneMarker 2.6.0 (Holland & Parson 2011).

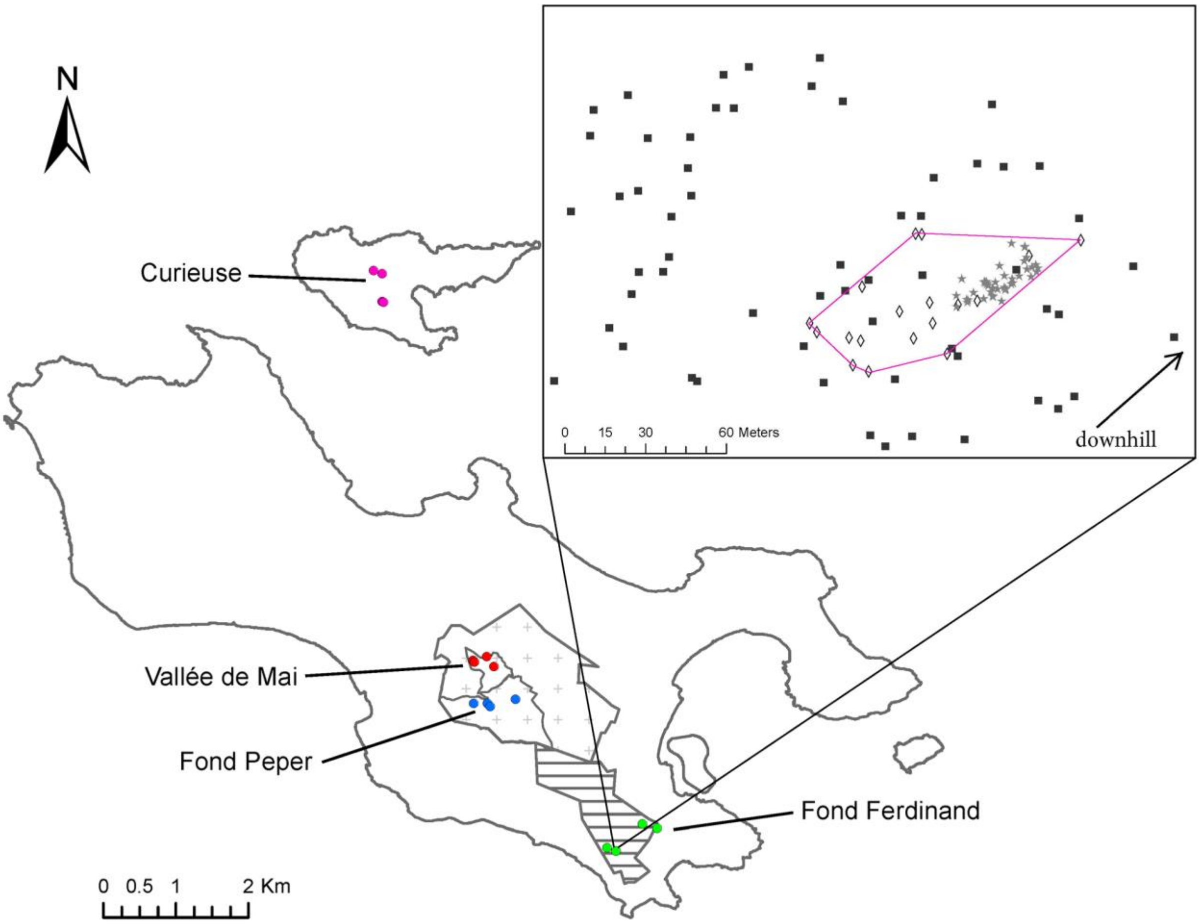

Fig. 1. Locations of the sites of sampled *Lodoicea maldivica* on Praslin and Curieuse islands. Centres of the clusters are shown by coloured circles (red = Vallée de Mai; blue = Fond Peper; green = Fond Ferdinand; pink = Curieuse). The crossed area indicates Praslin National Park, and within it, Vallée de Mai and Fond Peper boundaries, and the lined area indicates Fond Ferdinand. Inserted map shows the Fond Ferdinand 2 cluster with boundary and some of the surrounding males (black squares = males; diamonds = females; grey stars = offspring).

Genetic analysis

Genetic diversity, inbreeding and differentiation

GenAlEx 6.5 (Peakall & Smouse 2006) was used to calculate deviations from Hardy-Weinberg equilibrium (HWE), the mean number of alleles (N_a), the number of private alleles (P_A) and the observed (H_O) and unbiased expected (uH_E) heterozygosities. To control for differences in sample size among groups, we also calculated mean private rarefied allelic richness over loci (Π_S) using HP-RARE 1.1 (Kalinowski 2005). The presence of null alleles and allelic dropout were tested in Micro-Checker 2.2.3 (van Oosterhout *et al*. 2004).

Inbreeding coefficients (F_{IS}), allelic richness (A_R; i.e. the number of alleles per locus, corrected for differences in sample size) and pairwise levels of differentiation (F_{ST}) were estimated in FSTAT 2.9.3.2 (Goudet 1995) using 1,000 permutations. Linkage disequilibrium was tested using the log likelihood ratio statistic (G-test) in GENEPOP 4.2 (Raymond & Rousset 1995) using 10,000 iterations. We performed these analyses for each sub-population using data for all adults and offspring combined, and for each age cohort (including males surrounding clusters). In addition, we analysed each cohort separately, but considering only plants within the clusters (so as to have the same sample area for each cohort). We also analysed topographical position of the trees. For this we selected and pooled the uppermost and lowermost ten individuals from each slope considered to be steep enough (slope > 25%) to permit occasional long-distance seed dispersal (number of clusters: FP N = 3; FF N = 4; CU N = 2). Analyses were carried out on the sex of adult trees using only individuals from within the clusters, excluding the surrounding males.

Quantifying realised seed dispersal distances

To determine the most likely mother tree of each individual within a cluster we used the delta maximum-likelihood exclusion analysis in CERVUS 3.0 (Marshall *et al*. 1998; Kalinowski *et al*. 2007). Females from within the cluster were included as candidate mothers. This analysis simulated 10,000 offspring, with the minimum number of typed loci set at 9, the proportion of mistyped loci set at 1%, with 96.1% of the loci typed, and the significance threshold for maternity assignments set at 95%. We calculated mean dispersal distances for (i) all offspring, (ii) for offspring in each sub-population, and (iii) for offspring in clusters on relatively flat and sloped terrain (slopes < 25% and >25%, respectively). Strictly, our data do not record dispersal distances but the distances between mother plants and surviving offspring, though any resulting bias due to differential survival of offspring probably has only a small effect upon estimated dispersal distances.

Estimating FSGS across populations and life stages

To investigate FSGS, we used the software SPAGeDi 1.4c (Hardy & Vekemans 2002) and GeneAlex (Peakall & Smouse 2006). We calculated correlations of pairwise relatedness (r) (Peakall *et al*. 2003) and multilocus kinship coefficients (F; Loiselle *et al*. 1995) using the pairwise spatial distances of the plants. Separate analyses were performed for: (i) all plants in each sub-population, (ii) for offspring and adults separately, and (iii) for male and female plants (adults from within clusters) separately. For the analysis of cohorts and sexes, the reference sample was the whole dataset. The distance classes varied among but not within analyses (see Table 4, Figs. S1, S2), and were specified to ensure there were sufficient pairs in each class (minimum numbers: sub-population = 341; adults vs. offspring = 172; sexes = 104). To compare the intensity of FSGS - among populations, between adults and offspring, and between sexes - we used the *Sp*-statistic $Sp = -b_F/(1 - F_1)$, where F_1 is the mean kinship coefficient between the pairs of individuals within the first distance class, and b_F is the mean regression slope of the regression of kinship over the natural log of the distance (Vekemans & Hardy 2004).

An auto-correlational heterogeneity test (as described by Smouse *et al.* 2008) was carried out in GeneAlex (Peakall & Smouse 2006) to test for differences in FSGS among sub-populations, age cohorts and sexes in adult trees. We calculated the squared paired-sample *t*-test statistic (t^2) to test for differences in relatedness within each of the distance classes among the sub-populations. We also carried out a test of correlogram-level heterogeneity (Ω-test) among sub-populations, between adults and offspring, and between males and females, taking 1% as the level for a significant effect (Banks & Peakall 2012). Sequential Bonferroni corrections (Holm 1979) were applied to all multiple tests. To evaluate the relatedness of potential father and mother adult trees we plotted kinship (F) between male and female pairs from all samples, at several distance classes.

RESULTS

Genetic diversity, inbreeding and differentiation

In the complete sample of 1252 *Lodoicea maldivica* plants, we recorded a total of 158 alleles at 12 microsatellite loci, with a mean of 13 alleles per locus (range 5–21). Over all loci, the inbreeding coefficient (F_{IS}) was 0.272. All loci deviated significantly from Hardy-Weinberg Equilibrium (HWE), reflecting an excess of homozygotes. After sequential Bonferroni correction, no significant linkage was detected between loci pairs.

Sub-populations, sexes, age cohorts and topographical position

No significant variation was detected across groups for any measure of genetic diversity (N_a, A_R, H_o or uH_e; Table 1). However, mean private rarefied allelic richness over loci was significantly higher at CU (Π_s = 1.47 ± 0.47) than at VdM (Π_s = 0.29 ± 0.09, U = 26.0, $P \leq$

0.01) and FP ($\Pi_s = 0.24 \pm 0.12$, $U = 32.0$, $P \leq 0.05$). Allelic richness increased with cohort age from seedlings to adults, although the differences were not significant (Table 1).

Table 1. Genetic diversity and inbreeding coefficient summary statistics for *Lodoicea maldivica* at the four sub-populations, Vallée de Mai (VdM), Fond Peper (FP), Fond Ferdinand (FF) and Curieuse (CU), for the adult and offspring cohorts (and within offspring: seedlings, juveniles and immatures), for males and females (from within the cluster boundaries), and for the tops and bottoms of slopes. N, sample size; N_a, mean number of alleles and its SE; A_R allelic richness and its SE; P_A, number of private alleles; Π_s, mean rarefied number of private alleles over loci plus SE; H_o, observed heterozygosity with SE; uH_E, unbiased expected heterozygosity with SE; F_{IS}, inbreeding coefficient and significance of deviation from zero.

		N_a	A_R	P_A	Π_s	H_o	uH_E	F_{IS}
Sub-population	***N* 1252**							
VdM	482	10.58 (1.42)	9.57 (1.33)	5	0.29 (0.09)	0.489 (0.056)	0.667 (0.054)	0.268***
FP	293	10.00 (1.31)	9.53 (1.26)	1	0.24 (0.12)	0.530 (0.045)	0.675 (0.047)	0.216***
FF	265	9.67 (1.39)	9.34 (1.32)	5	0.48 (0.14)	0.498 (0.053)	0.672 (0.050)	0.260***
CU	212	10.58 (1.25)	10.39 (1.23)	17	1.47 (0.47)	0.490 (0.053)	0.714 (0.033)	0.314***
Cohort (2 groups)	***N* 1252**							
Adult	759	12.75 (1.53)	11.99 (1.50)	25	-	0.504 (0.053)	0.690 (0.048)	0.269***
Offspring	493	11.08 (1.37)	10.98 (1.38)	5	-	0.495 (0.050)	0.681 (0.049)	0.274***
Cohort (4 groups)	***N* 1252**							
Adult	759	12.75 (1.53)	6.03 (0.69)	25	-	0.504 (0.053)	0.690 (0.048)	0.269***
Immature	25	7.50 (1.08)	5.94 (0.79)	1	-	0.450 (0.068)	0.667 (0.054)	0.344***
Juvenile	454	10.67 (1.39)	5.84 (0.68)	4	-	0.497 (0.050)	0.682 (0.049)	0.271***
Seedling	14	5.75 (0.64)	5.48 (0.60)	0	-	0.512 (0.058)	0.672 (0.050)	0.246***
Sex	***N* 178**							
Male	78	8.42 (1.15)	8.39 (1.14)	6	1.31 (0.36)	0.472 (0.046)	0.666 (0.051)	0.293***
Female	100	9.42 (1.22)	9.14 (1.17)	18	0.56 (0.19)	0.496 (0.060)	0.699 (0.049)	0.291***
Elevation	***N* 180**							
Top	90	9.00 (1.28)	8.84 (1.24)	8	0.75 (0.33)	0.477 (0.049)	0.681 (0.047)	0.300***
Bottom	90	9.33 (1.36)	9.13 (1.30)	12	1.03 (0.26)	0.503 (0.051)	0.683 (0.049)	0.265***

*** $P \leq 0.001$.

The inbreeding coefficients (F_{IS}) for each sub-population, age cohort and sex were significantly greater than zero. F_{IS} values were similar among sub-populations and age cohorts, and did not differ according to sex or topography (Table 1). There were also no differences in genetic diversity or inbreeding among age cohorts, when considering only individuals from within cluster boundaries (Table S1).

At the sub-population level, all pairwise F_{ST} values differed significantly from each other at the adjusted 5% level (which was 0.83%; Table 2). Genetic differentiation across sub-populations was low at $F_{ST} = 0.018 \pm 0.004$. The greatest differentiation was found between the sub-population on CU and those on Praslin, while sub-populations FP and FF - although not the closest geographically (Fig. 1) - were the most similar. Genetic differentiation among sub-populations was greater for offspring than for adults ($F_{ST} = 0.027 \pm 0.006$ vs. 0.015 ± 0.003).

Table 2. Pairwise F_{ST} values and standard errors across *Lodoicea maldivica*'s range in the Seychelles, among adults only, offspring only and overall. **P* values below the adjusted level of 0.0083 at the 5% level.

	Pairwise F_{ST}				
F_{ST}		VdM	FP	FF	CU
Overall	VdM	0			
0.018 (0.004)	FP	0.014*	0		
	FF	0.010*	0.009*	0	
	CU	0.030*	0.024*	0.029*	0
Adults	VdM	0			
0.015 (0.003)	FP	0.009*	0		
	FF	0.009*	0.008*	0	
	CU	0.025*	0.018*	0.028*	0
Offspring	VdM	0			
0.027 (0.006)	FP	0.026*	0		
	FF	0.015*	0.017*	0	
	CU	0.043*	0.037*	0.036*	0

Realised seed dispersal

Seed dispersal distances were estimated using the maternity analyses for offspring within clusters (Fig. 2). Across all clusters we assigned 56.5 ± 5.3% (mean ± SE; *N* = 267) of offspring to the most likely mother tree within the site. However, the assignment rate increased to 78.0 ± 3.1% by excluding offspring at the extremities of the clusters (these individuals were sampled to capture possible long-distance dispersal events, and the seeds could have come from trees outside the sample area), as well as two FF sites with very low assignment rates. The rates varied across clusters from 93.3% at VdM2 to 7.3% at FF1 (Table S2). Overall, 50.6% of assigned offspring were growing less than 5 m from the mother tree, while the largest detected realised dispersal distance was 77.4 m (mean ± SE across all sub-populations: 8.7 ± 0.7 m). Realised seed dispersal was larger in FF (17.0 ± 2.6 m) than in the other sub-populations (CU: 5.6 ± 1.2 m; FP: 7.5 ± 1.0 m; VdM: 7.8 ± 0.8 m; $\chi^2 = 32.241$, df = 3, pairwise tests all $P \leq 0.001$), and was also larger on strongly sloping compared to level terrain (11.3 ± 1.2 m vs. 6.5 ± 0.6 m; $U = 7{,}499.0$, df = 1, $P \leq 0.05$).

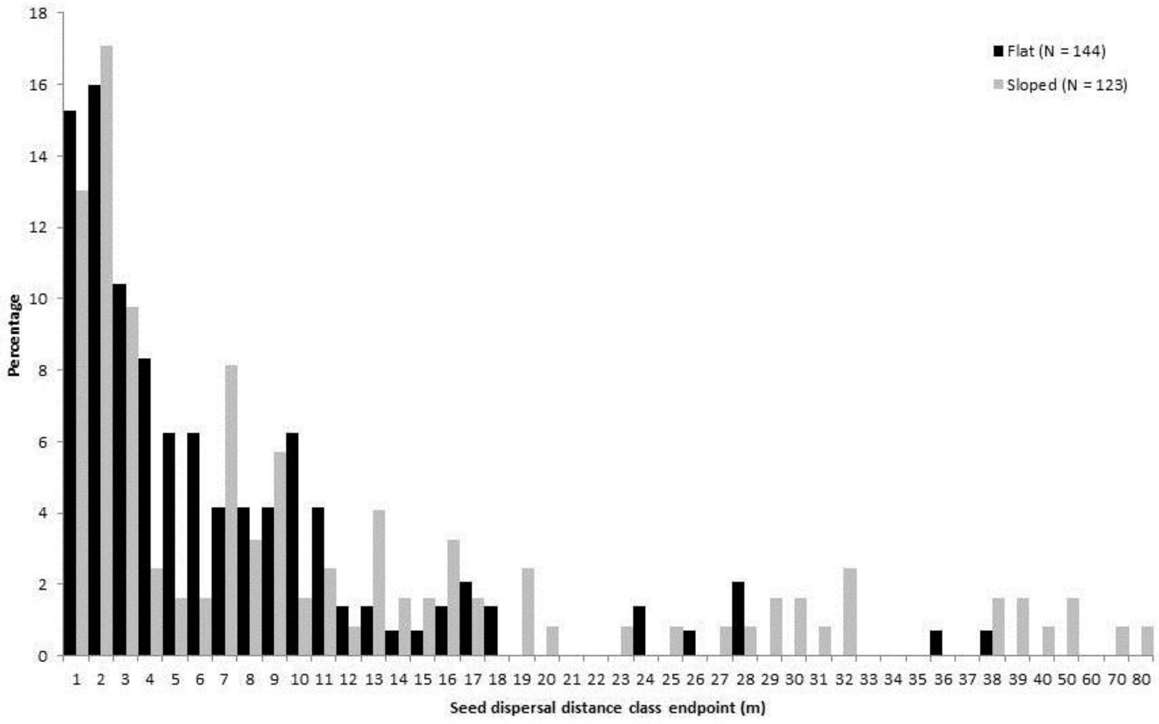

Fig. 2. Frequency distribution of realised seed dispersal distances in *Lodoicea maldivica*. Flat = seven clusters from relatively flat ground (slope < 25%), sloped = nine clusters from relatively steep slopes (slope > 25%). Obtained using maternity analysis assignments at the 95% confidence level. Candidate mothers were restricted to the same cluster as the offspring.

Evaluating patterns of FSGS

Sub-populations

FSGS was most intense at FP (*Sp* = 0.017 ± 0.002), and least intense at FF (*Sp* = 0.010 ± 0.002; Fig. 3, Table 3). Both Loiselle's *F* in SPAGeDI and the spatial autocorrelation *r* in GenAlEx indicated similar relatedness levels between tree pairs in all sub-populations (Table 3). At the shortest distance class (≤ 10 m), *F* was highest in FP (F_1 = 0.098 ± 0.010) and lowest in FF (F_1 = 0.041 ± 0.006), a pattern supported by *r* (Table 3). Using the multi-class test criterion (*Ω*) to test for heterogeneity of the multilag correlograms, FSGS differed significantly between all sub-population pairs, with the strongest difference being between FP and CU (*Ω* = 100.796, *P* ≤ 0.001). The largest single difference was between FP and FF at the 10 m class (t^2= 92.028, *P* ≤ 0.001; Table 4), a pattern consistent with the *Sp*-statistic.

Adult and offspring cohorts

FSGS was more strongly developed in offspring cohorts (*Sp* = 0.018 ± 0.002) than in adults (*Sp* = 0.009 ± 0.001) (Fig. S1, Table S3). The degree of kinship differed significantly between adult and offspring cohorts for nine of 21 distance classes (Table S4), with both *F* and *r* being higher in the offspring than in the adults for the ≤ 10 m distance class (offspring: F_1 = 0.093 ± 0.009, r_1 = 0.134; adults: F_1 = 0.066 ± 0.006, r_1 = 0.098). We also investigated the intensity of FSGS within adult and offspring cohorts separately in the various sub-populations. Overall, intensity was greatest for FP offspring (*Sp* = 0.027 ± 0.003), and least for FP adults (*Sp* = 0.006 ± 0.001) (Table S5).

TABLE 3. Summary table of fine-scale spatial genetic structure of *Lodoicea maldivica* across the four sub-populations, using 13 distance classes: 10, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400, 500 and 1000 m. Including: sample size (*N*); F_1, mean pairwise kinship coefficient and its SE among individuals within the first distance class (0-10 m); r_1, the spatial autocorrelation coefficient *r* at the first distance class; $Dist_F$, the distance class (m) up to which *F* significantly deviates from zero; $Dist_r$, the distance class (m) up to which *r* significantly deviates from zero; b_{Ld}, the slope of kinship (*F*) over the natural logarithm of the geographic distance between pairs, and its SE; *Ω*, multi-class test criterion for null hypothesis *r* = 0; *Sp*, the intensity of fine-scale spatial genetic structure with associated SE. Significant values at *P* ≤ 0.001 are displayed in bold.

Sub-population	*N*	F_1	r_1	$Dist_F$	$Dist_r$	b_{Ld}	*Ω*	*Sp*
VdM	482	**0.067** (0.007)	**0.100**	30	150	-0.011 (0.0016)	**156.211**	0.012 (0.002)
FP	293	**0.098** (0.010)	**0.151**	100	100	-0.015 (0.0016)	**163.756**	0.017 (0.002)
FF	265	**0.041** (0.006)	**0.063**	100	100	-0.009 (0.0015)	**145.763**	0.010 (0.002)
CU	212	**0.076** (0.015)	**0.112**	30	30	-0.017 (0.0032)	**113.318**	0.014 (0.003)

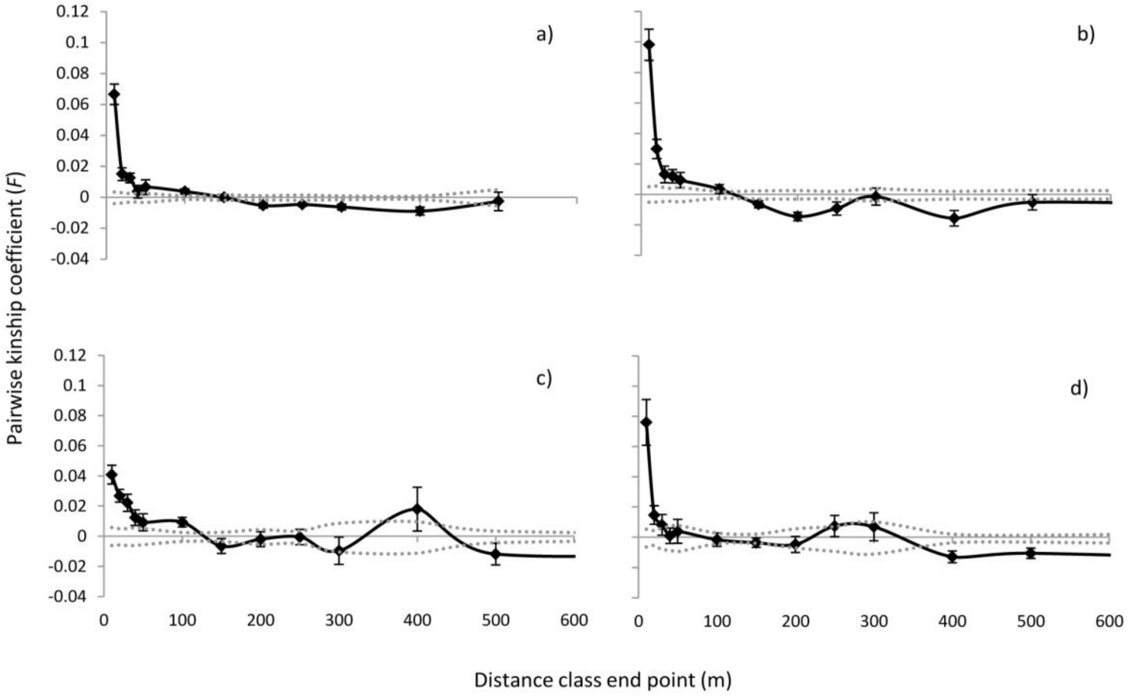

Fig. 3. Fine-scale spatial genetic structure for all individuals of *Lodoicea maldivica* from each of the four sub-populations. **a)** Vallée de Mai, **b)** Fond Peper, **c)** Fond Ferdinand and **d)** Curieuse. Solid lines represent mean *F* values (Loiselle *et al.* 1995). Dotted lines represent bootstrapped upper and lower 95% confidence intervals. Error bars denote 1SE.

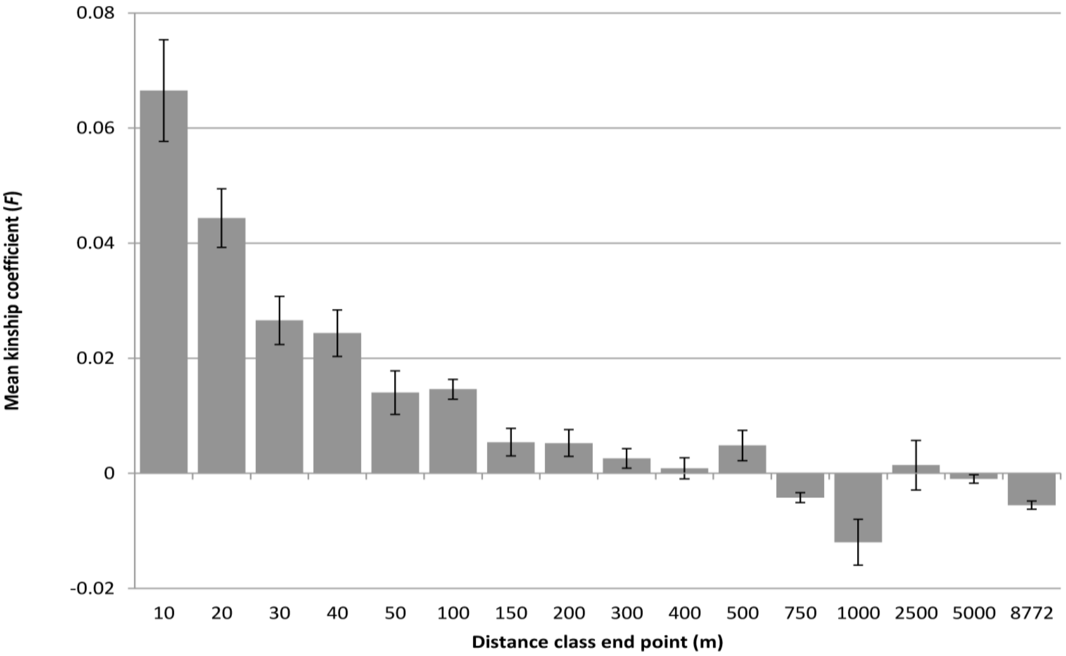

Fig. 4. Mean kinship coefficient (*F* ± 1SE) among male and female *Lodoicea maldivica* pairs at various distance classes.

Table 4. Single-class (t^2), and multi-class (Ω) test criterion and associated *P* values for heterogeneity tests of spatial genetic structure using paired comparisons between the four sub-populations of *Lodoicea maldivica. N*, number of pairs of individuals within each distance class. Significant values, after a sequential Bonferroni correction starting at the recommended probability of $P \leq 0.01$, are displayed in bold.

Distance class	1	2	3	4	5	6	7	8	9	10	11	12	13	Ω-test
intervals (m)	10	20	30	40	50	100	150	200	250	300	400	500	1000	criterion
N VdM	2468	3697	4508	4660	4630	19370	10979	13461	10831	17377	21241	1259	0	
N FP	1453	1417	1676	1978	1594	5721	3598	3994	4871	1940	4583	3454	5916	
N FF	1010	1302	1157	1169	1255	3905	3195	1611	1732	440	296	2151	14971	
N CU	1131	1521	754	642	553	2602	3285	903	627	341	3782	5247	978	
Population pairs														
VdM v. FP t^2	**51.251**	**8.485**	0.611	3.560	0.038	1.023	**13.528**	**13.337**	6.195	**10.315**	**11.205**	0.001	1.143	**99.301**
P	0.001	0.006	0.416	0.058	0.842	0.337	0.001	0.001	0.013	0.002	0.001	0.974	0.263	0.001
VdM v. FF t^2	**20.601**	3.255	1.624	0.968	0.690	2.969	**9.160**	5.244	2.534	0.456	**13.456**	2.920	0.836	**77.867**
P	0.001	0.071	0.205	0.328	0.400	0.081	0.001	0.018	0.110	0.521	0.001	0.089	0.869	0.001
VdM v. CU t^2	2.339	0.067	3.812	2.789	0.185	2.305	3.216	0.279	8.150	11.604	0.042	4.983	**2.866**	**66.670**
P	0.132	0.802	0.052	0.095	0.652	0.131	0.060	0.609	0.005	0.004	0.851	0.020	0.006	0.001
FP v. FF t^2	**92.028**	0.682	3.157	0.227	0.349	6.682	0.144	**22.544**	**11.534**	4.810	**21.580**	5.289	1.560	**91.620**
P	0.001	0.421	0.076	0.620	0.557	0.010	0.725	0.001	0.001	0.029	0.001	0.027	0.203	0.001
FP v. CU t^2	**20.046**	6.849	1.976	**7.803**	0.276	0.973	1.637	6.293	**16.882**	3.586	6.331	**10.984**	2.827	**100.796**
P	0.001	0.009	0.154	0.006	0.598	0.311	0.204	0.012	0.001	0.046	0.004	0.002	0.093	0.001
FF v. CU t^2	**28.324**	3.588	**7.524**	5.112	0.820	**9.309**	0.810	1.179	3.158	**10.045**	**14.288**	0.297	5.500	**95.241**
P	0.001	0.061	0.008	0.025	0.356	0.003	0.359	0.284	0.069	0.002	0.001	0.598	0.019	0.001

Male and female adults

Male and female *Lodoicea* within the clusters showed a similar FSGS (*Sp* = 0.012 ± 0.003 vs. *Sp* = 0.012 ± 0.002) (Fig. S2, Table S6), though the distance over which females were significantly more related than by chance was slightly greater than for males ($Dist_F$ = 30 m vs. 15 m, respectively).

The relatedness (*F*) between male and female adult pairs varied according to distance class (χ^2 = 375.90, df = 15, $P < 0.0001$; Fig. 4). Mean kinship values between male/female pairs were highest at distances of less than 10 m (*F* = 0.066 ± 0.009), and remained significantly higher than expected from spatially random pairs up to a distance of 500 m.

DISCUSSION

The results presented here demonstrate that natural populations of *Lodoicea maldivica* exhibit highly developed fine-scale spatial genetic structure (FSGS). This finding is consistent with our initial prediction, based upon the absence of any mechanism for seed dispersal and the apparently limited pollen dispersal (preliminary evidence suggests that geckos are the principal dispersal agents). However, previous molecular studies of *Lodoicea* using AFLP markers found relatively high levels of genetic diversity, and remarkably little differentiation among populations across its natural range (Fleischer-Dogley *et al.* 2011). In this discussion, we attempt to clarify the patterns of genetic variation in this species, and with the additional knowledge on patterns of relatedness and inbreeding across cohorts, to resolve this apparent paradox.

Patterns of seed dispersal and fine-scale spatial genetic structure

As expected, our data revealed very limited seed dispersal, with more than half of all offspring establishing within 5 m of mother plants. Dispersal distances were greatest at the steeply sloping Fond Ferdinand site, where the clusters of offspring occurred in elongated ellipses downslope of the mother trees. This site also had the least developed FSGS. Clusters FF1 and FF3 were situated on dry slopes in areas that had previously been subjected to fire, and the lower maternity assignment rates in these areas was probably because many of the mother trees had been killed. Of the unassigned seedlings, 11% had genotypes that were incompatible with the candidate mother’s genotype by only one allele, but may not have been assigned due to the pair possessing common alleles.

FSGS was more developed in offspring than in adults, presumably because of thinning of seedling and juvenile cohorts over time (Hamrick *et al*. 1993; Zhou & Chen 2010). Adult *Lodoicea* are rarely observed growing in close proximity to other adults, while under natural conditions seedlings are clumped around female trees. This could result in small-scale founder effects, with a few individuals contributing to the colonisation of a cluster, thus amplifying the FSGS. If selection favours those progeny least related to the nearest adult tree, FSGS would decline as the plants mature, a process that has been documented for other palm species (Choo *et al.* 2012). Alternatively, the observed decline might reflect a disruption in pollen-mediated gene flow due to recent habitat fragmentation. Our data do not enable us to distinguish between these two processes.

Within all sub-populations, pairs of individuals within 10 m from each other had an average kinship coefficient value of $F = 0.07$. To put this into perspective, an F value between 0.125 and 0.031 suggests that the most recent common ancestor of these individuals is between one or two generations (equivalent to first or second cousins). We also found that relatedness between male and female adult trees increased with proximity, and that pairs separated by less than 10 m also had a mean F value of 0.07. The high relatedness over short geographical distances, coupled with the very high inbreeding coefficients at all life stages (see the ‘Patterns of genetic diversity and inbreeding’ section below), is consistent with the idea that mating between spatially proximate pairs is common in *Lodoicea,* with little evidence for selection for outbred pollen during fertilisation. Studying contemporary pollen dispersal in *Lodoicea* will be important in resolving this.

Patterns of genetic diversity and inbreeding

Are there differences in genetic diversity and differentiation among sub-populations and cohorts?

We expected the larger, more intact sub-populations on Praslin to be genetically more diverse than the very fragmented sub-population on Curieuse (Pither *et al*. 2003). We also expected to see greater genetic diversity in adult trees at the bottoms of slopes, and a decline in genetic diversity from the adults to the seedling, as has been observed in other fragmented species (Vranckx *et al.* 2012). Our results confirmed none of these effects; the most we detected were non-significant trends towards higher genetic diversity in adults. The only significant difference was a greater mean rarefied number of private alleles on Curieuse, which could reflect restricted gene flow between the two islands, followed by genetic drift (Slatkin 1985).

High levels of genetic diversity in endangered species may indicate either historically large populations or continuing gene flow among apparently separate populations (Shapcott *et al*. 2007). The levels of genetic diversity found here are similar to, or higher than, those found in other rare or endangered tropical palm species (Shapcott *et al*. 2007; Shapcott *et al.*, 2012; Nazareno & dos Reis 2014), lending support to the idea of historically large and continuous populations of *Lodoicea* (Fauvel 1915).

Although outcrossing rates and levels of genetic diversity are typically high in tropical trees, their populations tend to be more differentiated than those of temperate species, possibly as a result of higher inbreeding levels (reviewed in Dick *et al.* 2008). In *Lodoicea*, low but significant genetic differentiation was observed among all sub-population pairs, with the greatest differences being between Curieuse and Praslin. This suggests that the channel between the islands, albeit a relatively recent result of sea level rise around 8000 years ago, is a barrier to gene dispersal (see also Fleischer-Dogley *et al.* 2011). Another historical factor to consider is that the offspring established under conditions of greater fragmentation and disturbance than did the adults, which could explain the higher genetic differentiation in the offspring than the adults (Browne *et al.* 2015). Historically, the Fond Peper and Fond Ferdinand *Lodoicea* on Praslin formed one continuous tract of forest, and even today the two areas are still partially connected, which presumably accounts for the low genetic differentiation between these sub-populations.

Is inbreeding higher in the more isolated sub-populations and in the younger cohorts?

Inbreeding was unexpectedly high in all sub-populations and, contrary to our initial hypothesis, was not higher in the smaller fragmented sub-population on Curieuse, nor in the younger, post-fragmentation age cohorts (Kettle *et al.* 2007; Finger *et al.* 2012). Several factors, not mutually exclusive, can produce a deficit of heterozygotes including inbreeding, null alleles and the Wahlund effect (Wahlund 1928). Of these, null alleles can be excluded because the pattern of homozygote excess is consistent across loci (with only one locus, Lm6026, showing a frequency slightly over $P = 0.20$), which would be unlikely for null alleles (Dakin & Avise 2004). We can also exclude the Wahlund effect, since this would require significant barriers to gene flow that evidently do not exist on the two islands.

In general, theory predicts that a decrease in population size will result in an increase in inbreeding (Oostermeijer *et al.* 2003), as has been observed in another long-lived dioecious plant in a fragmented habitat (Dubreuil *et al*. 2010). Dioecy is thought to have evolved as a strategy to overcome inbreeding depression, though it has clearly been insufficient to prevent high inbreeding in *Lodoicea*. Indeed, the evolution of a very large seed has resulted in a breeding system characterised by high levels of inbreeding. This raises the question of how *Lodoicea* avoids the deleterious effects of inbreeding depression? One possibility is that deleterious recessive alleles have been purged from this long-lived species over generations for thousands of years. This is consistent with the ancient origins of the Seychelles archipelago (Baker & Miller 1963). Infrequent long-distance pollen or seed dispersal (e.g. downhill) may be sufficient to prevent inbreeding depression at the population level. However, without genotype data from pollen and seeds it is difficult to relate pollination success and survival rates of offspring to the genetic relatedness of mother and father pairs.

Placing the genetic implications of limited seed dispersal in an ecological context

Monodominant tropical forests such as those of *Lodoicea* appear to be restricted to regions that have been stable for very long periods (Hart *et al.* 1989). Indeed, it has been argued that *Lodoicea*, with its huge seed, could only have evolved under very stable conditions (Edwards *et al*. 2015). If pollen dispersal is frequently over short distances, the intense FSGS and relatively high relatedness among male and female individuals in close proximity would give rise to the high inbreeding coefficients we observe. The consistently high levels of inbreeding and similar levels of genetic diversity among life stages and study sites, regardless of different levels of habitat degradation, suggest that mating between related individuals has long been the strategy of reproduction in *Lodoicea*. The advantages of maternal resource provisioning for progeny via the interception and funnelling of nutritious material to the tree's base (Edwards *et al.* 2015), thus appear to outweigh the potentially negative costs of inbreeding.

Increased forest fragmentation, reduced system stability, disruption of pollinators, and sustained harvesting of seeds may lead to an elevated frequency of inbreeding, which is unlikely to result in fitness costs for *Lodoicea*. More important may be how contemporary patterns of gene flow vary among sites, and the implications this could have for sex ratios in future generations (Stehlik & Barrett 2005). What is more, preserving the local pollinator communities will be more important for conserving patterns of genetic diversity *per se*. These results highlight that for species with extremely limited seed dispersal and intense FSGS, fragmentation genetics needs to be placed in the wider ecological context.

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SUPPLEMENTARY MATERIAL

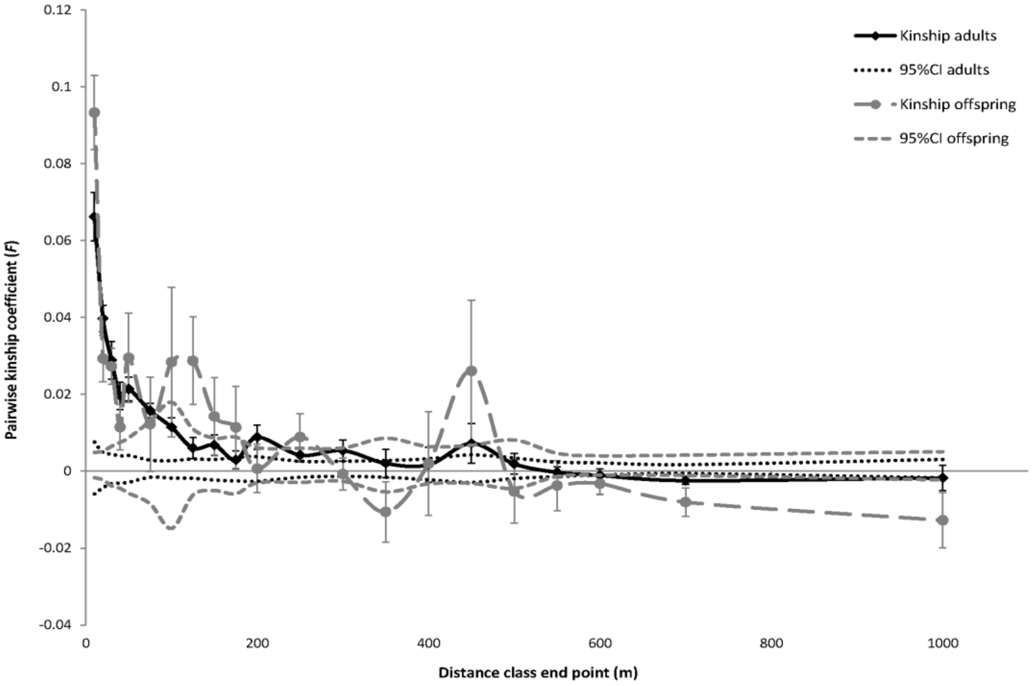

Fig. S1. Fine-scale spatial genetic structure for adult and offspring cohorts of *Lodoicea maldivica*. Mean *F* values (Loiselle *et al.* 1995) plotted at 21 distance classes: 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700 and 1000 m. Error bars denote 1SE.

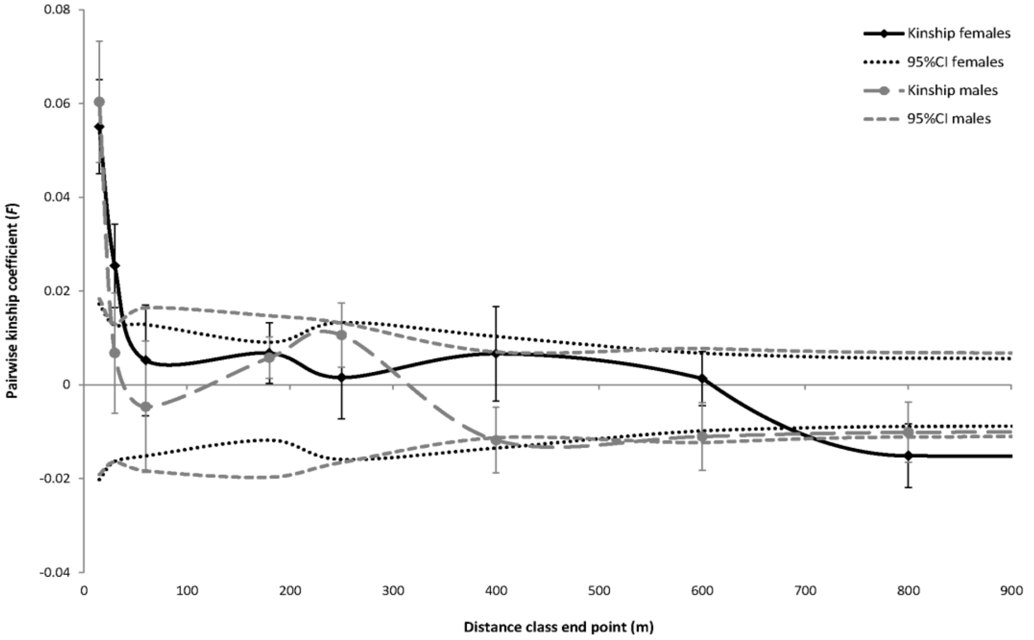

Fig. S2. Fine-scale spatial genetic structure for male and female adult cohorts of *Lodoicea maldivica.* Mean *F* values plotted at 8 distance classes: 15, 30, 60, 180, 250, 400, 600 and 800 m. Error bars denote 1SE.

Table S1. Genetic diversity and inbreeding coefficient summary statistics for *Lodoicea maldivica* within adult and offspring cohorts. Individuals are from within the cluster boundaries (and within offspring: seedlings, juveniles and immatures). N, sample size; N_a, mean number of alleles and its SE; A_R, allelic richness and its SE; P_A, number of private alleles; Π_s, mean rarefied number of private alleles over loci plus SE; H_o, observed heterozygosity with SE; uH_e, unbiased expected heterozygosity with SE; F_{IS}, inbreeding coefficient and significance of deviation from zero.

		N_a	A_R	P_A	Π_s	H_o	uH_e	F_{IS}
Cohort (2 groups)	***N* 671**							
Adult	178	9.92 (1.34)	9.85 (1.32)	6	0.70 (0.17)	0.485 (0.053)	0.686 (0.050)	0.293***
Offspring	493	11.08 (1.37)	10.20 (1.38)	20	1.05 (0.22)	0.495 (0.050)	0.681 (0.049)	0.274***
Cohort (4 groups)	***N* 671**							
Adult	178	9.92 (1.28)	5.93 (0.66)	6	0.45 (0.09)	0.485 (0.053)	0.686 (0.050)	0.293***
Immature	25	7.50 (1.08)	5.94 (0.79)	4	0.61 (0.19)	0.450 (0.068)	0.681 (0.055)	0.344***
Juvenile	454	10.67 (1.39)	5.84 (0.68)	16	0.55 (0.12)	0.497 (0.050)	0.682 (0.049)	0.271***
Seedling	14	5.75 (0.64)	5.48 (0.60)	0	0.35 (0.15)	0.512 (0.058)	0.672 (0.050)	0.246***

*** $P \leq 0.001$.

Table S2. Maternity assignment rates at the 95% confidence level of *Lodoicea maldivica* offspring within each of the 16 clusters. Core offspring excludes those offspring at the left and right extremities of the clusters (Vallée de Mai (VdM), Fond Peper (FP), Fond Ferdinand (FF) and Curieuse (CU)).

Cluster	Assignment rate (%)	
	All offspring	Core offspring
VdM1	64.4	90.6
VdM2	73.7	93.3
VdM3	68.9	72.1
VdM4	45.5	71.4
FP1	88.2	88.2
FP2	37.1	75.0
FP3	54.5	91.7
FP4	70.5	79.5
FF1	5.6	7.3
FF2	61.5	80.0
FF3	20.0	22.2
FF4	68.4	72.2
CU1	66.7	66.7
CU2	70.6	80.0
CU3	57.1	75.0
CU4	45.9	50.0

Table S3. Summary table of fine-scale spatial genetic structure of adult and offspring cohorts of *Lodoicea maldivica*. Twenty-one distance classes used: 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700 and 1000 m. Including: sample size (*N*); F_1, mean pairwise kinship coefficient and its SE among individuals within the first distance class (0-10 m); r_1, the spatial autocorrelation coefficient *r* at the first distance class; $Dist_F$, the distance class (m) up to which *F* significantly deviates from zero; $Dist_r$, the distance class (m) up to which *r* significantly deviates from zero; b_{Ld}, the slope of kinship (*F*) over the natural logarithm of the geographic distance between pairs, and its SE; *Ω*, multi-class test criterion for null hypothesis $r = 0$; *Sp*, the intensity of fine-scale spatial genetic structure with associated SE. Significant values at $P \leq 0.001$ are displayed in bold.

Cohort	*N*	F_1	r_1	$Dist_F$	$Dist_r$	b_{Ld}	*Ω*	*Sp*
Adults	755	**0.066** (0.006)	**0.099**	300	150	-0.009 (0.001)	**209.478**	0.009 (0.001)
Offspring	489	**0.093** (0.009)	**0.134**	50	175	-0.016 (0.002)	**253.296**	0.018 (0.002)

Table S4. Single-class (t_2), and multi-class (Ω) test criterion and associated P values for heterogeneity tests of spatial genetic structure using paired comparisons between adult and offspring cohorts of *Lodoicea maldivica*. N, number of pairs of individuals within each distance class. Significant values, after a sequential Bonferroni correction starting at the recommended probability of $P < 0.01$, are displayed in bold.

Distance class	1	2	3	4	5	6	7	8	9	10	11	12
intervals (m)	10	20	30	40	50	75	100	125	150	175	200	250
N adults	778	1744	2520	2909	3119	8178	7497	6136	4748	4379	4003	7558
N offspring	3628	3125	1686	1235	719	416	172	643	931	819	2101	2257
Cohort pairs												
adults v. offspring t^2	**19.866**	**9.466**	0.377	1.470	**7.970**	0.016	1.383	5.632	1.969	**16.274**	1.341	**7.554**
P	0.001	0.004	0.533	0.229	0.004	0.895	0.222	0.017	0.157	0.001	0.240	0.005
Distance Class	13	14	15	16	17	18	19	20	21	22		Ω-test
Intervals (m)	300	350	400	450	500	550	600	700	1000	more		criterion
N adults	8487	8196	5416	3007	5019	10177	14824	24037	6424	145479		
N offspring	2659	916	2048	1734	996	4812	7933	7040	3343	70103		
Cohort pairs												
adults v. offspring t^2	1.362	3.114	1.964	**10.459**	5.578	**10.089**	2.424	**14.158**	**52.855**			**157.929**
P	0.239	0.075	0.155	0.001	0.016	0.004	0.115	0.002	0.001			0.001

Table S5. Summary table of fine-scale spatial genetic structure of adults and offspring cohorts within each of the four sub-populations (Vallée de Mai (VdM), Fond Peper (FP), Fond Ferdinand (FF) and Curieuse (CU)) of *Lodoicea maldivica*. Eight distance classes used: 10, 20, 100, 200, 300, 400, 700 and 1000 m. Including: sample size (*N*); F_1, mean pairwise kinship coefficient and its SE among individuals within the first distance class (0-15 m); r_1, the spatial autocorrelation coefficient *r* at the first distance class; $Dist_F$, the distance class (m) up to which *F* significantly deviates from zero; $Dist_r$, the distance class (m) up to which *r* significantly deviates from zero; b_{Ld}, the slope of kinship (*F*) over the natural logarithm of the geographic distance between pairs, and its SE; Ω, multi-class test criterion for null hypothesis $r = 0$; *Sp*, the intensity of fine-scale spatial genetic structure with associated SE. Significant values at $P \leq 0.001$ are displayed in bold; not significant $P \leq 0.01$.

Group	*N*	F_1	r_1	$Dist_F$	$Dist_r$	b_{Ld}	Ω	*Sp*
VdM adults	339	**0.055** (0.006)	**0.086**	100	100	-0.011 (0.002)	**88.270**	0.011 (0.002)
VdM offspring	143	**0.065** (0.006)	**0.099**	10	10	-0.016 (0.002)	**69.751**	0.017 (0.003)
FP adults	158	**0.040** (0.009)	**0.068**	100	100	-0.006 (0.001)	**74.774**	0.006 (0.001)
FP offspring	135	**0.098** (0.012)	**0.152**	100	100	-0.025 (0.003)	**90.192**	0.027 (0.003)
FF adults	143	**0.031** (0.014)	0.042	100	100	-0.009 (0.002)	**71.830**	0.009 (0.002)
FF offspring	122	**0.037** (0.007)	**0.061**	100	100	-0.010 (0.002)	**76.147**	0.010 (0.002)
CU adults	119	**0.076** (0.017)	**0.115**	100	20	-0.008 (0.002)	**67.657**	0.009 (0.002)
CU offspring	93	**0.065** (0.008)	**0.115**	10	20	-0.016 (0.002)	**62.669**	0.017 (0.003)

Table S6. Summary table of fine-scale spatial genetic structure of female and male *Lodoicea maldivica*. Eight distance classes used: 15, 30, 60, 180, 250, 400, 600 and 800 m. Including: sample size (*N*); F_1, mean pairwise kinship coefficient and its SE among individuals within the first distance class (0-15 m); r_1, the spatial autocorrelation coefficient *r* at the first distance class; $Dist_F$, the distance class (m) up to which *F* significantly deviates from zero; $Dist_r$, the distance class (m) up to which *r* significantly deviates from zero; b_{Ld}, the slope of kinship (*F*) over the natural logarithm of the geographic distance between pairs, and its SE; Ω, multi-class test criterion for null hypothesis $r = 0$; *Sp*, the intensity of fine-scale spatial genetic structure with associated SE. Significant values at $P \leq 0.001$ are displayed in bold.

Sex	*N*	F_1	r_1	$Dist_F$	Dist_r	b_{Ld}	Ω	*Sp*
Male	78	**0.060** (0.013)	**0.091**	15	15	-0.011 (0.003)	**47.900**	0.012 (0.003)
Female	100	**0.055** (0.010)	**0.074**	30	30	-0.011 (0.002)	**56.703**	0.012 (0.002)

CHAPTER 3

Limited pollen dispersal in natural populations of *Lodoicea maldivica* (J. F. Gmel.) Pers.

with Peter J. Edwards, Christopher N. Kaiser-Bunbury, Frauke Fleischer-Dogley and Chris J. Kettle

ABSTRACT

Pollen dispersal is important for maintaining genetic connectivity between plant populations, and may be critical for rare species, which would otherwise suffer from inbreeding and reduced genetic diversity. *Lodoicea maldivica* is an ecologically and economically important palm native to two islands of the Seychelles archipelago. Little is currently known about pollen dispersal in this species, though endemic geckos are thought to be important vectors. We used genetic analyses based upon 12 hypervariable microsatellite loci to quantify pollen flow and dispersal in four natural sub-populations. We assigned 44% of offspring to male trees growing within the sample area (< 80 m radius). However, a further 26% of fathers were estimated to have died in the period between pollination and sampling, bringing the proportion of fathers within 80 m to around 70%. 57% of this short distance pollen flow occurred within distances up to 30 m, and the average pollen dispersal distance detected within sub-populations was 73 m. These results indicate that pollen dispersal on Praslin is mostly over rather short distances, but that long-distance dispersal does occur occasionally and is probably important to prevent inbreeding. However, long-distance pollen flow is probably insufficient to maintain gene flow between the isolated *Lodoicea* forest fragments on Curieuse. We discuss our results in the context of management of this endangered species. Our results are consistent with a growing body of evidence demonstrating reduced genetic connectivity as a consequence of habitat fragmentation.

INTRODUCTION

Plants can disperse their genes in two ways: through haploid pollen dispersal and diploid seed dispersal. Pollen dispersal is usually the principal means by which genes are spread across a landscape (Ennos 1994), and is essential for maintaining genetic connectivity, particularly in fragmented or naturally low-density populations (Petit & Hampe 2006; Dick *et al.* 2008). In many ecosystems, pollen dispersal kernels (i.e. the probability density function of pollen dispersal to any position relative to the father plant) typically follow a leptokurtic distribution, with the majority of matings occurring between close neighbours (Levin & Kerster 1974). This can result in high rates of correlated paternity (i.e. the probability that two progeny drawn at random from the same mother share the same father). The shape of the tail of leptokurtic distributions is often used to inform researchers about the relative contribution of long-distance dispersal events as a metric of the effective neighbourhood size (Austerlitz *et al.* 2004), although the complementary characterisation of ecological factors affecting pollen dispersal will enhance our understanding of gene flow. The relative proportion of short and long distance dispersal influences the genetic structure of a population, and especially the intensity of its fine-scale spatial genetic structure (FSGS).

Anthropogenic disturbance often leads to formerly continuous habitats becoming fragmented, or to important pollen vectors becoming scarce. These changes reduce the mating opportunities for plants (Jump & Peñuelas 2006), and may lead to increased genetic drift, elevated inbreeding (Wright 1946; Chybicki & Burczyk 2009), reduced genetic diversity (Young *et al.* 1996; Aguilar *et al.* 2006, 2008), and an intensification of FSGS (Loveless & Hamrick 1984; Smouse & Sork 2004). In some species, however, these effects are small because long-distance pollen dispersal, for example by wind, is a significant process (Dick *et al.* 2003; Lowe 2005; Sork & Smouse 2006). For example, pollen flow over distances of several kilometers has been detected in some wind-pollinated tropical trees (Ashley 2010). However, in tree species that normally experience high genetic connectivity across the landscape through long-distance pollen-flow, an elevated frequency of short-distance pollen dispersal and an increase in inbreeding may be observed when adult densities are low (Ismail *et al.* 2012).

The Seychelles coco de mer *Lodoicea maldivica* (Arecaceae) is a remarkable dioecious palm that produces the world's largest seeds (Chapter 4) that can only disperse by gravity. The short average seed dispersal distances result in highly clustered growth patterns, in which neigbouring individuals are usually closely related and often share the same mother (Chapter 2). This pattern of seed dispersal is reflected in a highly developed FSGS (Chapter 2). In contrast, almost nothing is known about pollen dispersal, though we assume that in the absence of human disturbance long-distance dispersal was sufficient to prevent excessive inbreeding and maintain a uniformly high level of genetic diversity across the species' natural range (Chapter 2; Fleischer-Dogley *et al*. 2011). However, much of *Lodoicea*'s natural habitat has been destroyed through deforestation and fires, leaving the population on Curieuse particularly fragmented, which has probably altered the balance between short- and long-distance dispersal.

Our objectives were to investigate realised pollen dispersal patterns and the mating system in *Lodoicea* across its natural range, using a variety of methods based on microsatellite markers. These included paternity analysis to assess short-distance pollen flow (Jones *et al.* 2010), and indirect spatially explicit mating models (introduced by Adam & Birkes 1991; Chybicki & Burczyk 2010) and TWOGENER analyses (Smouse *et al.* 2001) to detect long-distance pollen flow. Specifically, we expected to find differences in pollen dispersal patterns across sub-populations on Praslin and Curieuse, where landscape context and levels of fragmentation vary. We discuss the evidence for disruption to the pollen dispersal system in *Lodoicea* and its management implications for this endangered species. In addition we discuss our findings in the wider context of how forest fragmentation affects the reproductive ecology of palms and more generally of forest trees.

MATERIALS AND METHODS

Study species and sites

The granitic islands of the Seychelles form part of a tectonic plate fragment that separated from India during the break-up of Gondwanaland some 70 million years ago (Baker & Miller 1963). During this long period of isolation, a unique flora has evolved, including the remarkable *Lodoicea*. Prior to colonisation of the Seychelles archipelago in the 18th century (Lionnet 1976), *Lodoicea* grew densely across the whole of Praslin and Curieuse. Since then, populations have been decimated due to high demand for its timber and nuts, and to vegetation clearance for habitation and agriculture. Today, only a few semi-connected sub-populations remain on the islands of Praslin (Vallée de Mai, Fond Peper and Fond Ferdinand) and Curieuse. Scattered clumps and individuals also persist at several other locations across Praslin, mainly in steep valleys. Human-mediated seed movement and removal may have altered the spatial genetic structure of *Lodoicea* across much of its range. We therefore focused our sampling in the most natural areas, and chose sites ("clusters") showing natural regeneration and minimal signs of human interference. All these sites consisted of dense patches of individuals ranging from seedlings to trunked adults.

Where this giant borassoid palm (Dransfield *et al.* 2008) grows in closed forest, it is the dominant canopy tree. Males reach heights of up to 30 m, but emergents growing to over 50 m tall were reported in the past (Ward 1866). Individuals in closed forest start flowering when the trunk reaches about 3.5–4 m (own data; Savage & Ashton 1983), and male trees vary widely in height. *Lodoicea* produces striking dimorphic inflorescences and flowers. Male trees bear up to four huge 0.9–1.8 m long catkins (Corner 1966) containing clusters (cincinni) of 50 to 170 fragrant yellow flowers that emerge sequentially from pit-like structures and last for less than one day. Female trees also produce large inflorescences, which bear between 1 to 13 flowers (at ~5 cm diameter, the largest of any palm; Chapter 4). Although flowering females can be observed at any time of year, the flowers on individual trees are only receptive for a short period soon after a new inflorescence has emerged (Blackmore *et al.* 2012). The relative importance of different pollen vectors remains uncertain, and may vary among sites; geckos are probably important (Fischer & Fleischer-Dogley 2008; Kaiser-Bunbury *et al.,* unpubl. data), but other biotic (trigonid bees, flies, slugs; Blackmore *et al.* 2012) or abiotic vectors (wind or rain; Edwards *et al.* 2002) may also play a role.

The massive fruit of *Lodoicea* is reported to take around 7 years to develop (Corner 1966; Blackmore *et al*. 2012). Like many palms *Lodoicea* has a trunkless juvenile phase during which there is considerable development below-ground of the stem-base, which forms a massive saucer-shaped structure up to 100 cm across, and of the associated root system. During the juvenile phase, leaves with enormously elongated petioles are produced from the buried stem apex, to form a huge basal rosette. In the following immature phase, leaves are produced at regular intervals from the developing trunk, each leaf being associated with an increase in trunk length of approximately 12.2 cm. Little precise information is available on how long plants under natural conditions take to develop, though anecdotal evidence indicates that the period between germination and reaching maturity probably lasts several decades. In the case of seedlings, for example germination usually takes about four years, and subsequent leaves are produced at a rate of one every three years.

Sample collection, genotyping and genetic diversity

In a previous study we genotyped plants from four clusters (mean area ± SD: 904.4 ± 800.9 m^2) within each of four sub-populations (Vallée de Mai (VdM), Fond Peper (FP), Fond Ferdinand (FF) and Curieuse (CU); Fig. 1A) using 12 nuclear microsatellite loci (Morgan *et al.* 2016). These clusters were situated within extensive forest on Praslin, and within forest fragments on Curieuse. Fragment length was analysed using the LIZ 500 HD internal size marker with an ABI3730 capillary sequencer (Applied Biosystems, Zug, Switzerland) and scored with GeneMarker 2.6.0 (Holland and Parson 2011). Within the 16 clusters, we included all male and female adults and young *Lodoicea* plants, and we also sampled all male trees within a radius of 80–120 m (Table 1) around each cluster (see Fig. 2 for sampling strategy). We recorded the spatial co-ordinates of each adult tree using GPS (Garmin 60CSx), and measured the height of the trunk from ground level to just beneath the lowest leaf using a laser range-finder (TruPulse 360). The precise positions of young plants were determined using bearings and distances taken from a central female (Chapter 2). Young plants were classified as seedlings (plants with only two leaves), small juveniles (plants with basal rosettes), large juveniles, and immature plants (trunk present). Juveniles were categorised according to the girth of the largest petiole: small and large juveniles had petiole girths below and above the median, respectively, for each sub-population. Distances between cluster edges within sub-populations ranged from 13–360 m, and as such, the sampling of males in radii around clusters overlapped in some cases. Sampled males surrounding clusters within sub-populations were separated by between 354–8043 m and were treated as separate entities for pollen dispersal and immigration analyses.

Genetic diversity and inbreeding analyses for all sampled individuals were conducted following the protocols detailed in Morgan *et al.* (2016) and Chapter 2. In brief, deviations from Hardy-Weinberg equilibrium (HWE), observed (H_o) and unbiased expected heterozygosities (uH_E), mean number of alleles (N_a) and the number of private alleles (Π_s) were calculated using GenAlEx 6.5 (Peakall & Smouse 2006). Inbreeding coefficients (F_{IS}) and allelic richness (A_R) were calculated using FSTAT 2.9.3.2 (Goudet 1995), and the presence of null alleles and allelic dropout were tested in Micro-Checker 2.2.3 (van Oosterhout *et al.* 2004). Analyses were carried out for each sub-population and overall.

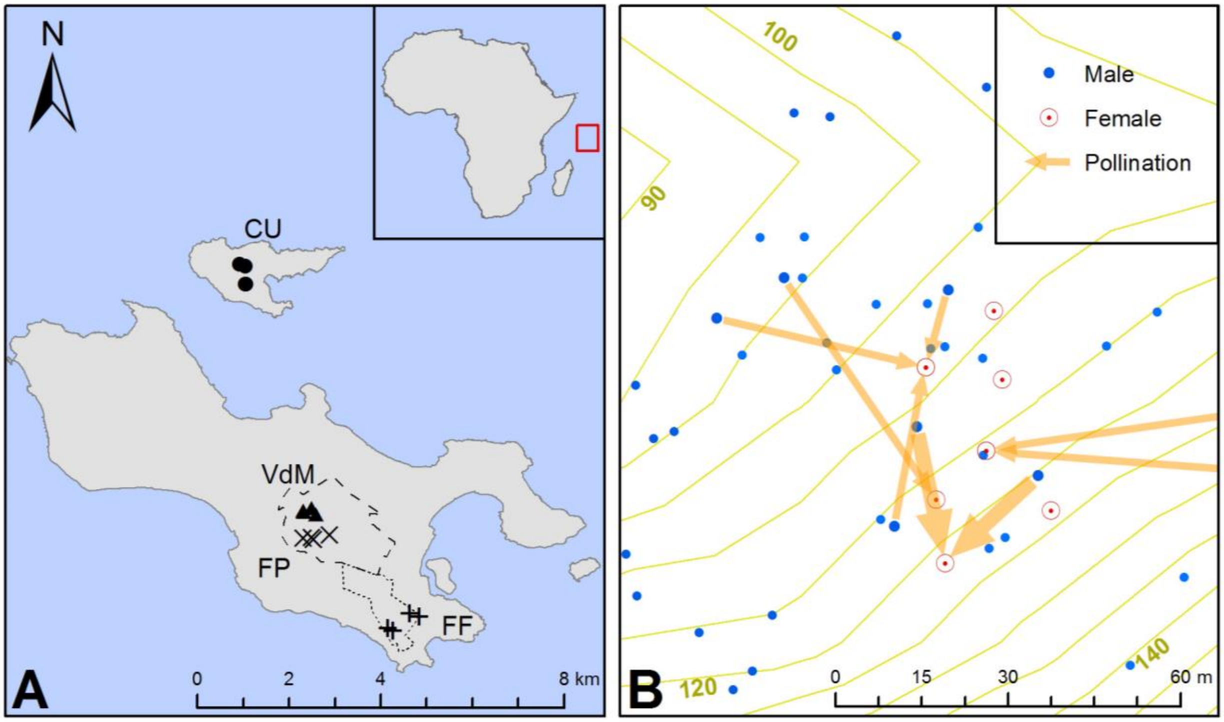

Fig. 1. Locations of sampled *Lodoicea maldivica* on the Seychelles, for pollen flow analysis. **A.** Centres of clusters on Praslin, indicated by: triangles = Vallée de Mai (VdM), 55°44'11''E, 4°19'43''S; × = Fond Peper (FP), 55°44'17''E, 4°20'01''S; **+** = Fond Ferdinand (FF), 55°203 45'39''E, 4°21'02''S, and circles = Curieuse (CU), 55°43'25''E, 4°16'45''S). The dashed and dotted lines indicate Praslin National Park and Ravin de Fond Ferdinand Nature Reserve, respectively. **B.** Network of realised pollination events detected between males and females in the paternity analysis for the FP4 cluster. Width of arrows equivalent to frequency of detected events. Some pollen immigration events from outside of the cluster are shown.

Pollen dispersal patterns and mating system

Direct estimates

We directly evaluated pollen flow distances using paternity analyses of all offspring with known mother trees (sample sizes in Table 1). Maternity assignments had previously been used to determine the probable mothers of all offspring (Chapter 2). For the paternity analysis we used CERVUS 3.0 program (Marshall *et al.* 1998; Kalinowski *et al.* 2007), which is based on the maximum-likelihood exclusion method (Meagher & Thompson 1986). Paternity was determined by the Δ-estimated statistic, calculated using 10,000 simulations, 0.01 error rate at the loci and 0.961 loci typed. We simulated 0.05 candidate fathers that are related to offspring at 0.008 (equivalent to third cousins), as this was close to the average estimated relatedness level among pairs of adults separated by 150 m (Chapter 2). All adult males sampled within sub-populations were considered as potential fathers of offspring (assuming 0.75 pollen donors sampled). The strict confidence level of 95% for the trio assignment (mother-father-offspring) was used. Combined exclusion probabilities for the first and second parents were 0.9961 and 0.9999, respectively.

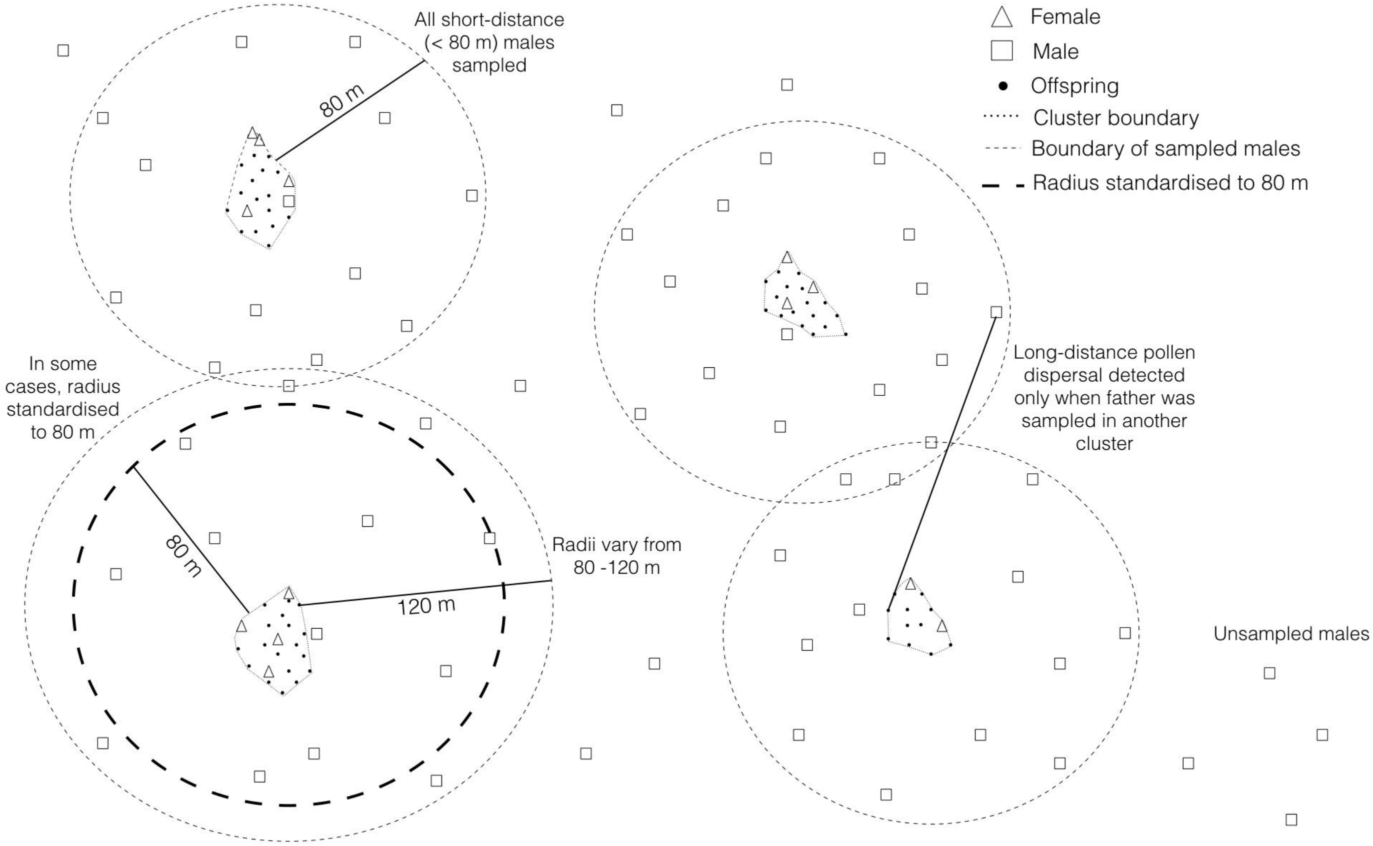

Fig. 2. Diagram of *Lodoicea maldivica* sampling scheme. Four clusters within one sub-population are shown (not to scale).

Realised pollen dispersal distances (*d*) for each offspring were calculated as the Euclidian distances between the assigned mother and father trees. The pollen flow distances were grouped into 10 m classes up to 100 m, and then 50 m classes up to 650 m, and the percentage of the total detected pollination events was calculated within each distance class. As the sampled radii of males, and distances between clusters varied across sub-populations, the possibility of detecting long-distance pollen flow was more likely in some sub-populations than others. Therefore, we also calculated the proportion of total mating events occurring within a distance of < 80 m, as this was the minimum sampling radius shared by all sub-populations. Unassigned offspring were assumed to have been sired by a male > 80 m from the mother tree. We used Kolmogorov-Smirnov tests in SPSS 21.0 (SPSS, Armonk, NY, USA) to determine whether the observed distribution of pollen flow distances departed significantly from the null hypothesis of random mating (i.e. whether the dispersal kernel was 'flat'). We did this by comparing the frequency distributions of realised pollination distances and the pairwise distances of all mother trees and sampled males. We estimated the pollen immigration rate (m_p) for each sub-population as the number of offspring unassigned to a father, divided by the total number of offspring (Smouse & Sork 2004). The genetic relatedness among assigned parental pairs was calculated using the kinship coefficient (F; Loiselle *et al.* 1995) in SPAGeDI 1.4c (Hardy & Vekemans 2002). Differences between pollen flow distances and kinship coefficients across sub-populations were compared with Mann-Whitney or two-tailed independent sample t-tests using SPSS 21.0. The numbers of males contributing to reproduction were also calculated.

The analysis of pollen dispersal was complicated by the very slow development of *Lodoicea* (seeds take seven years to mature and offspring may take several decades to mature), which meant that many of the fathers had died by the time we sampled their offspring. Monitoring studies by the Seychelles Island Foundation have found that trunk height increases by an average of 0.146 m per year, and therefore provides a means of determining the age of a tree. By analysing the frequency distribution of trunk heights of male trees in our sample, we were therefore able to estimate their mean reproductive lifespan, and hence the proportion of fathers that must have died during a given period.

Indirect estimates

Mating models that do not require complete parentage assignments can be used to infer the dispersal kernel, and pollen immigration rates and dispersal distances. Thus, they provide complementary information to the direct estimates of pollen dispersal obtained by the paternity analyses (Smouse & Sork 2004). We used the KINDIST (Robledo-Arnuncio *et al.* 2006) and TWOGENER (Smouse *et al.* 2001) methods in POLDISP 1.0c (Robledo-Arnuncio *et al.* 2007) to analyse the mating system and obtain alternative estimates of pollen dispersal. We used KINDIST to estimate correlated paternity rates (r_p) within and among the maternal samples of each sub-population, a measure that is expected to decay with distance. TWOGENER provided us with a second estimate of average pollen dispersal distances (δ) for sub-populations. We tested the normal and exponential (Austerlitz & Smouse 2002) dispersal functions to estimate pollen dispersal distances (δ) using the spatial locations of surrounding males for at least 80 m and fixed effective male densities (d_e) estimated from field observations. We estimated the effective densities within the sampled areas (Table 2) to be one tenth (Austerlitz *et al.* 2004) of the male densities we observed. The effective number of fathers per mother tree (N_{ep}) was calculated as N_{ep} = ½ ϕ_{ft}, where ϕ_{ft} is the intra-class correlation measure, estimated through an AMOVA procedure (Excoffier *et al.* 1992). TWOGENER requires complete maternal genotypes with no missing data, and a minimum of two assigned offspring per maternal family. As a result, the total number of mother trees was reduced from 61 to 21.

Finally, we fit within-sub-population pollen dispersal kernels using a spatially explicit mating model, or NEIGHBORHOOD model (Adams & Birkes 1991; Burczyk *et al.* 2002) with the software NM+ (Chybicki & Burczyk 2010). We fixed the selfing rate at zero, and the neighbourhood parameter to 'infinite' to treat the total sampled adult sub-population as the neighbourhood, and jointly estimated genotyping error rates. As NM+ assumes monooecy, we included a trait that gave values to femaleness and maleness, and fixed the selection gradients to reflect the fact that males produce no seeds and females produce no pollen. We tested initial values for all parameters by adding parameters sequentially, and applied the exponential-power pollen dispersal kernel (Clark 1998). We also used this method to estimate average pollen dispersal distances and pollen immigration rates.

RESULTS

Genetic diversity

In the sample of 1252 *Lodoicea* from four sub-populations, we detected between 5 and 21 alleles for each of the 12 individual microsatellite loci, representing 158 alleles in total. All loci deviated significantly from HWE, denoting an excess of homozygotes. The putative presence of null alleles was detected in 11 loci, although inbreeding was deemed the most likely explanation. There was no evidence for large allelic dropout. No significant variation in any of the measures of genetic diversity (N_a, A_R, H_o, uH_E or Π_s) could be detected among the four sub-populations. Inbreeding coefficients were significantly greater than zero, but did not differ among sub-populations (see Morgan *et al.* 2016 and Chapter 2 for further details).

Table 1. Characteristics of pollen dispersal in Lodoicea maldivica across the four sub-populations, using CERVUS. Including: radius, minimum radius of males surrounding offspring; numbers of mothers and offspring; numbers of fathers contributing to paternity, paternity assignment rate; d, mean pollen dispersal distance, and median in brackets; proportion of mating events at distances ≤ 80 m, and number in brackets; m_p, pollen immigration rate; F, mean pairwise kinship coefficient of all mates, with standard error; F < 80 m; mean pairwise kinship coefficient of mates less than 80 m apart, with standard error.

Sub-population	Radius (m)	Mothers (N)	Offspring (N)	Fathers (N)	Assignment rate	d (m)	Mating events ≤ 80 m (N)	m_p	F (SE)	F < 80m (SE)
Overall	90	61	269	93	0.517	72.5 (24.8)	0.439 (118)	0.483	0.076 (0.010)	0.074 (0.011)
VdM	80	23	90	36	0.567	65.3 (33.3)	0.467 (42)	0.433	0.088 (0.017)	0.087 (0.018)
FP	105	13	84	23	0.429	54.1 (23.6)	0.393 (33)	0.571	0.028 (0.018)	0.031 (0.019)
FF	80	14	42	14	0.500	127.1 (33.6)	0.357 (15)	0.500	0.121 (0.018)	0.134 (0.021)
CU	95	11	52	20	0.596	68.7 (19.0)	0.519 (27)	0.404	0.081 (0.021)	0.070 (0.02)

Pollen flow and mating systems

Direct estimates

About half (0.517) of the total sample of 269 offspring could be assigned with > 95% confidence to a male tree within the sub-populations, with assignment rates ranging from 0.429 (in FP) to 0.596 (in CU) for the individual sub-populations (Tables 1 and S1). The assignment rates varied considerably among cohorts in all sub-populations, declining from 0.667 for seedlings to 0.20 for immature trees.

Less than half (0.44) of all mating events could be assigned to a father within an 80 m range, with proportions ranging from 0.36 to 0.52 across sub-populations (see Fig. 1B for pollination events within the FP4 cluster). Over all offspring to which we could assign a parent-pair, we only detected a small proportion of pollination events at distances > 80 m (range across sub-populations 0.08–0.29). Mean realised pollen dispersal distance was greater at FF (d = 127 ± 34 m) than at CU (d = 69 ± 19 m, U = 212.0, $P \leq 0.05$), but was similar among all other sub-population pairs (Table 1; d among clusters, Table S1), although there was a trend for greater dispersal distances at FF compared to FP (d = 54 ± 24 m, U = 264.5, P = 0.06). The peak in the frequency of realised pollination dispersal was found in the 10 m distance class overall for sub-populations (Fig. 3, but see Fig. S1 for sub-populations separately). Kolmogorov-Smirnov tests showed that the observed and potential frequency distributions of pollen flow distances and distances between potential parent trees were significantly different overall and for each sub-population (all $P \leq 0.001$; Fig. 4), indicating that the success of mating was not a function of the spatial distribution.

Across all sub-populations, assigned mother-father pairs separated by up to 80 m had an average relatedness of F = 0.076. The kinship was higher at VdM (F = 0.088 ± 0.017) than at FP (F = 0.028 ± 0.018, U = 690.0, $P \leq 0.05$), and also higher at FF (F = 0.121 ± 0.018) than at FP (t_{55} = -3.391, $P \leq 0.001$). The kinship coefficient only decreased slightly when we included paternity matches at distances greater than 80 m (Table 1). Ninety-three males contributed paternity to the 61 mother trees.

Correcting for tree mortality

Figure 5 shows the frequency distribution of trunk heights for all male trees sampled on Praslin (but see Fig. S2 for male heights on Curieuse). These data clearly show the impact of the trade

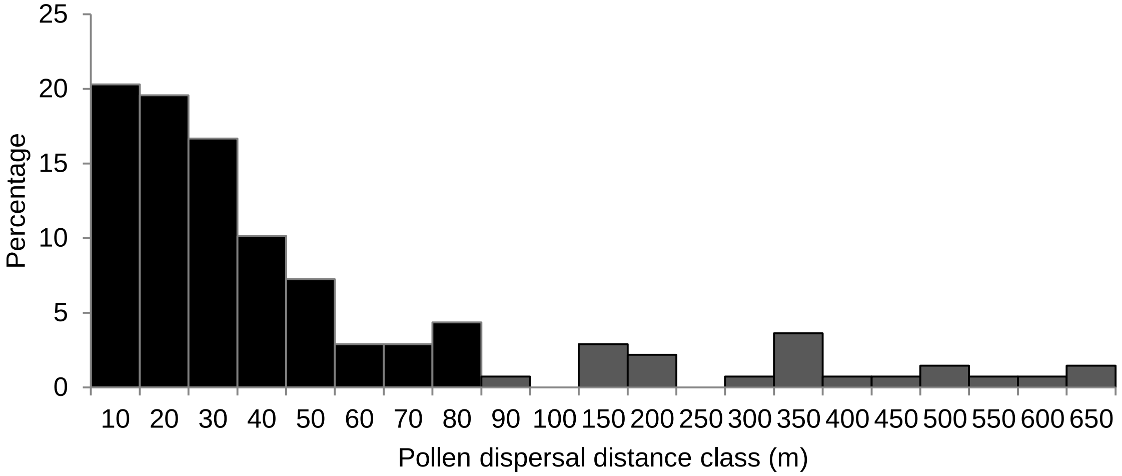

Fig. 3. Frequency distribution of detected realised pollen dispersal distances in *Lodoicea maldivica.* Paternity analysis assignments at the 95% trio-confidence level. Candidate fathers were restricted to same sub-population as the offspring. Black bars indicate the minimum distance at which all adult males were sampled around the 16 clusters; grey bars also include males surrounding other clusters within the sub-population.

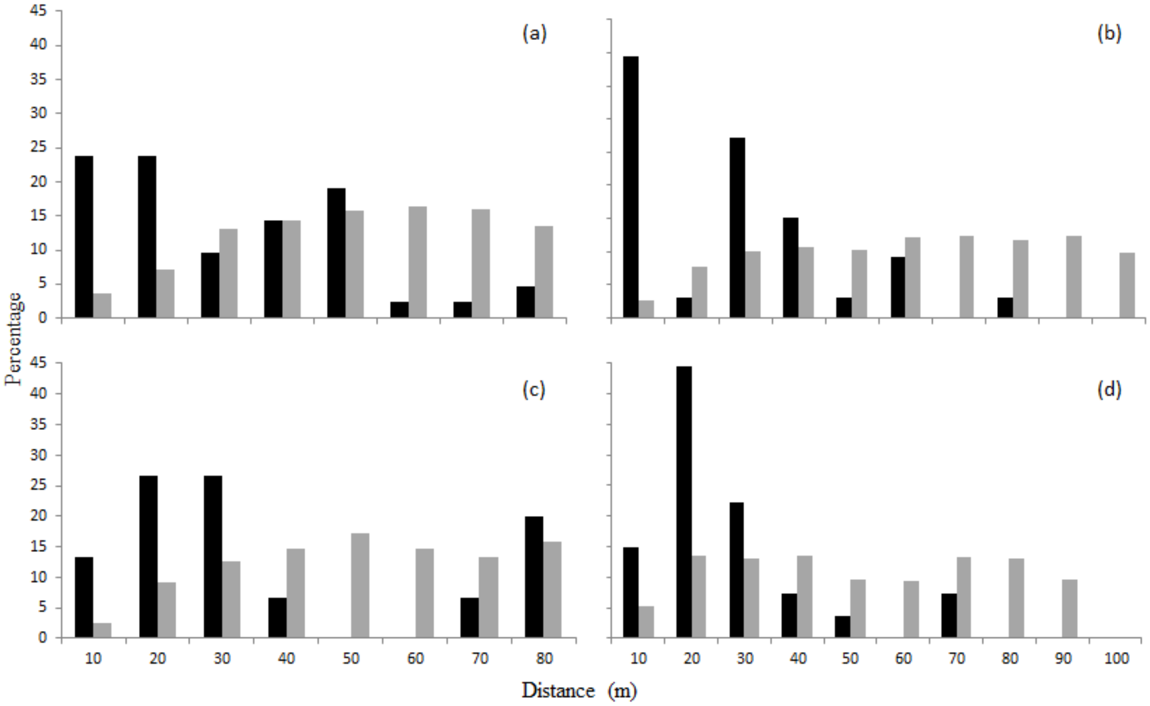

Fig. 4. Frequency distribution of observed realised pollination distances for *Lodoicea maldivica* offspring in each sub-population (black bars) and inter-tree distances of all adult males relative to maternal trees (grey bars). **(a)** Vallée de Mai, **(b)** Fond Peper, **(c)** Fond Ferdinand and **(d)** Curieuse. Distances restricted to minimum radius size of sampled males around offspring clusters.

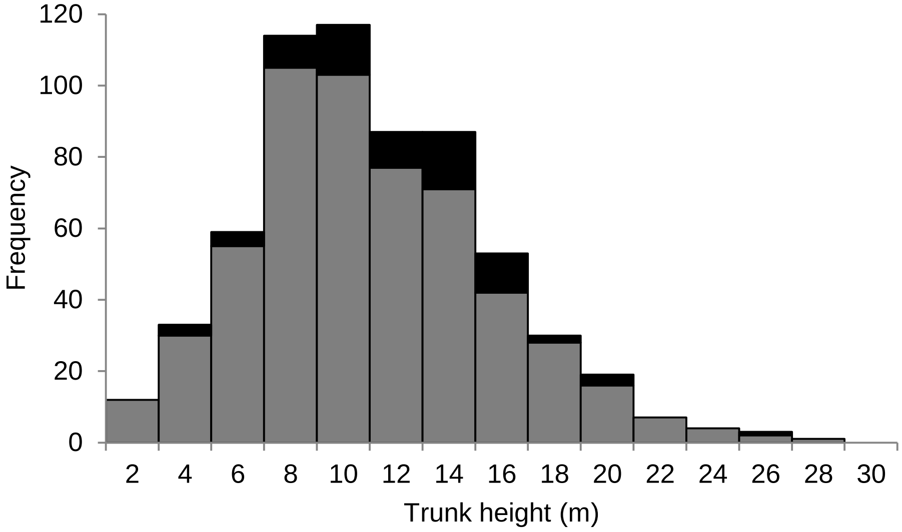

Fig. 5. Frequency distribution of male *Lodoicea* trunk heights in the Vallée de Mai, Fond Peper and Fond Ferdinand sub-populations on Praslin. Grey and black bars indicate all sampled male trees, and males that fathered at least one offspring, respectively.

in nuts upon the structure of the forest, since there are very few young trees present (trunk height < 6 m). The data also show that numbers of male trees decline linearly with increasing height above a height of about 6 m, with very few trees reaching more than 20 m. From this linear decline, and assuming an annual height increment of 0.146 m, we estimated that the mean reproductive lifespan of all male trees was 59 years, which corresponds to an average annual mortality of 1.69% per year of the trees originally present. However, only around 10% of these trees were recorded as fathers, and many of the 'non-fathers' were probably very small and unproductive, which may over-estimate the mortality rate of fathers. Considering only those trees recorded as fathers, we obtained a mean reproductive life span of 85 years, which is equivalent to an annual mortality of 0.118% per year. We used this value to calculate the proportion of missing fathers.

Based upon monitoring data from the Seychelles Island Foundation, we estimate that the mean age of seedlings was 16 years since pollination (i.e. seven years as a seed plus nine years as a seedling), and would therefore expect 18.8% of fathers within the sample area to have died. Thus, the fathers of the seedling cohort can be accounted for as 66.7% assigned and 15.3% dead (i.e. 18.8% of 0.667), which leaves a further 18.0% of fathers that lay outside the sample area. Applying the same value of 18% to all cohorts, we find that the proportions of fathers that died were 25% for young juveniles, 37% for old juveniles and 62% for immature plants, with a value of 31% for the entire sample. These data also allow us to estimate the mean ages of the three cohorts as 19, 31, and 52 years, respectively, and that of all offspring as 25 years.

Indirect estimates

The correlation of paternity within maternal families (i.e. the proportion of full-sibs) ranged from moderate to high degrees (sub-population averages ranged from r_p = 0.097 in FF to r_p = 0.517 in FP). Family-level estimates varied widely from -0.377 to 1.894 (effectively null to unity; Table S2). KINDIST is based on the decay of correlated paternity among maternal sibship pairs with distance; according to isolation by distance theory (Robledo-Amuncio *et al.* 2007). However, in our study sub-population correlations did not decrease with distance (all Spearman's rank $P >$ 0.05), therefore we carried out no further analyses with the program.

Using the TWOGENER approach, levels of global differentiation among the pollen pools fertilising mother palms ranged from relatively low to high (FF: ϕ_{ft} = 0.014; VU: ϕ_{ft} = 0.082; FP: ϕ_{ft} = 0.085; VdM: ϕ_{ft} = 0.144). The models that gave the lowest error values were the 'normal' model for VdM and FP, the 'exponential' model for CU, and both fit equally well for FF. Using the results from the best fitting models, we estimated short average pollen dispersal distances (VdM 21.9 m, FP 33.1 m, FF 80.3 and 92.6 m, and CU 46 m; Table 2). These equate to effective numbers of pollen donors per mother tree (N_{ep}) ranging from 4.4 in VdM (where adult male density was highest) to 34.5 in FF (where male density was lowest). However, it must be noted that the TWOGENER analysis was based on low numbers of maternal families, particularly for FF.

Average pollen distances estimated using the exponential-power model in NM+ were similar to those estimated using CERVUS for VdM (53.8 ± 43.3 m) and FP (55.7 ± 32.8 m), but lower for CU (22.2 ± 2.7 m) (Table 3). The model would not converge for FF. Pollen immigration rates for VdM, FP and CU were 0.535 (± 0.063), 0.689 (± 0.051) and 0.461 (±0.066), respectively, and the order from highest to lowest immigration among sub-populations was in agreement with the order given by the CERVUS analysis. The shape of the pollen dispersal kernels for VdM and FP indicated fat-tailed distributions (*bp* = 0.31 ± 0.15 and 0.52 ± 0.29), suggesting intensive dispersal at short distances, with the probability of long-distance dispersal decelerating slowly. However, placed in the context of this system, which experiences intensive short-distance pollen dispersal, the 'long-distance' pollen dispersal distances estimated are not likely to be relatively far. Conversely, the Curieuse dispersal kernel had a thinner tail (bp = 2.44 ± 1.07), suggesting infrequent long-distance pollen dispersal events (Austerlitz *et al.* 2004).

Table 2. Characteristics of pollen dispersal for *Lodoicea maldivica* sub-populations, estimated with TWOGENER. Numbers of maternal families and offspring, ϕ_{ft} differentiation parameter between the pollen clouds of the sample of females and N_{ep} effective number of pollen donors per mother tree are shown. Normal and exponential dispersal parameters were tested with fixed male densities estimates (d_e), to estimate mean pollen dispersal distances (δ). Model error values are given.

Sub-population	Mothers (*N*)	Offspring (*N*)	ϕ_{ft}	N_{ep}	Dispersal function	d_e (males/m^2)	δ (m)	Error
VdM	7	35	0.114	4.4	Normal	0.001105	21.9	0.153
					Exponential	0.001105	25.1	0.154
FP	4	43	0.085	5.9	Normal	0.000325	33.1	0.154
					Exponential	0.000325	37.5	0.156
FF	3	14	0.014	34.5	Normal	0.000224	80.3	0.013
					Exponential	0.000224	92.6	0.013
CU	7	41	0.082	6.1	Normal	0.000333	14.7	0.384
					Exponential	0.000333	46.0	0.350

Table 3. Dispersal parameters for sub-populations of *Lodoicea maldivica* estimated with the exponential-power model in NM+. Including: *mp*, pollen immigration rate; *dp*, mean estimated pollen dispersal distance; and *bp*, shape of the pollen dispersal kernel. Standard deviations are given in brackets. Parameters not estimated are represented by '–'.

Sub-population	*mp*	*dp* (m)	*Bp*
VM	0.535 (0.063)	53.8 (43.3)	0.31 (0.15)
FP	0.689 (0.051)	55.7 (32.8)	0.52 (0.29)
FF	–	–	–
CU	0.461 (0.066)	22.2 (2.7)	2.44 (1.07)

DISCUSSION

This is the first genetic study to look at pollen flow patterns and the mating system in natural sub-populations of *Lodoicea maldivica.* Overall, pollen dispersal was relatively short-distance, with more than half (57%) of all detected pollen flow occurring at distances up to 30 m, and rarely occurring over more than 400 m (6%). Our study provides evidence that fragmentation influences pollen dispersal. The mating system in natural *Lodoicea* sub-populations departed from random, with closer males significantly more likely to father offspring than would be expected by chance. Overall, a high proportion of mating events were found to occur among related individuals.

Evidence for short-distance pollen dispersal

Within relatively small sampling areas we were able to assign around half (52%) of all progeny to a father tree. In addition, we estimated that around 31% of fathers had died and could therefore not be sampled. Across all samples we detected an overall short-distance pollen dispersal of 72.5 m. Paternity analyses and the spatially explicit mating models in NM+ (which can estimate pollen dispersal distances from outside of sampled areas; Chybicki & Burczyk 2010), yielded similarly short pollen dispersal distances at Vallée de Mai (65 vs. 54 m) and Fond Peper (54 vs. 56 m), though the mean distance estimated by CERVUS was lower than that estimated by NM+ for Curieuse (65 vs. 22 m). This indicates that the collection of leaf samples of larger numbers of male *Lodoicea* in paternity analyses is unlikely to be worth the substantial effort involved due to the high density and height of trees in natural sub-populations.

Pollen dispersal distances were greatest at Fond Ferdinand (127 m; although estimates were not calculated using NM+); the site with the lowest observed male tree density. The greatest pollen dispersal distances detected at Fond Ferdinand, along with the greatest realised seed dispersal distances detected in a previous study, contribute to FF having the least intense fine-scale spatial genetic structure of the four sub-populations (Chapter 2). The within-subpopulation dispersal distances detected here are similar to those estimated in other tropical palms (81 m, Ramos *et al.* 2016; 71 m, Saro *et al.* 2014), and it has also been shown that pollen dispersal rarely exceeds 300 m in dense forests (Degen & Sebbenn 2014). Using directly estimated pollen flow distances and the proportions of unassigned progeny, we showed that almost half (44%) of all pollen flow occurred with 80 m distances. This is less than the 70% of pollen flow that occurred within 75 m distances in the Canarian palm *Phonenix canariensis* (Saro *et al.* 2014). We estimated lower effective pollen dispersal distances with TWOGENER than with CERVUS and NM+, though our study system and sampling strategy was not well-suited to the TWOGENER method. Larger sample sizes of offspring produced by individual females would be necessary to increase the numbers of maternal families included in our analyses. However, natural regeneration is now severely limited in natural *Lodoicea* populations, making it difficult to find large “family-clusters”. KINDIST is known to yield more accurate estimates of the pollen dispersal distribution than TWOGENER (Robledo-Arnuncio *et al.* 2007), but we were not able to use the method, possibly due to low sample sizes of mothers that were assigned to more than two offspring, or due to a limited range in pollen flow distances. We only fitted the exponential-power dispersal function in NM+, but other functions could be trialed to find the most appropriate model for the data.

Considering detected pollen flow events at all distances, and allowing for trees that died, 18% of all pollen was estimated to have originated from outside of the sampled trees within sub-populations. It is difficult to make comparisons on pollen immigration rate because it is so influenced by the sampling design and area sampled. However, pollen immigration rates were lower than for other non-isolated tropical trees (49% in *Symphonia globulifera*, Carneiro *et al.* 2009; 61% in *Theobroma cacao*, Silva *et al.* 2011), although sampling areas in these studies were much larger. Indirect methods can provide us with a better idea of pollen flow outside of sampled areas, but the direct and indirect methods in the *Lodoicea* sub-populations yielded similar results. This corroborates the idea that the majority of pollen flow occurs over distances not much greater than our sampling distances, and thus, the paternity analysis captured the majority of pollen flow events. Only slightly higher pollen immigration rates were detected with NM+ than with CERVUS, and this could also be due to fairly recent forest discontinuities (e.g. fire breaks and areas damaged by fire) being at the edges of some of the clusters in this study. This is likely to have reduced pollen immigration into the sampled area, leading to parentage underestimating long-distance pollen flow (Ottewell *et al.* 2012).

Indications of potential pollination vectors of *Lodoicea maldivica*

Dense areas of *Lodoicea* forest host a larger diversity of animal species, including some geckos (Gerlach & Ineich 2006; Noble *et al.* 2011), which are thought to be important in *Lodoicea* pollination. A number of theories have been proposed about *Lodoicea* pollination. These include: wind (Savage & Ashton 1983), wind and insects (Good 1951), wind and rain (Edwards *et al.* 2002), animals such as geckos (Corner 1966; Fischer & Fleischer-Dogley 2008), flying insect vectors (Blackmore *et al.* 2012), or a combination of biotic and abiotic vectors (Fleischer-Dogley *et al.* 2011). The most likely combination, based on the results presented in this study, is infrequent long-distance dispersal by wind, and more frequent short-distance dispersal, possibly by animal vectors. The floral characteristics of *Lodoicea* such as the bright colour of male flowers and the sweet scents of the male pollen and female nectar certainly suggest that they evolved to attract animal pollinators. With Kolmogorov-Smirnov tests, we showed that random mating did not occur within the sub-populations, but closer males were much more likely to sire offspring than would be expected by chance, as has been observed in other studies (Meagher 1986; Saro *et al.* 2014). This also supports the idea that most pollination is effected over short-distances, possibly by relatively sedentary or territorial animal dispersers, or by wind-dispersal, particularly below the canopy where air movement is likely to be more limited. Long-distance wind-dispersal of the pollen of tall canopy males is likely to play an important role in maintaining gene flow, particularly in the dense VdM and FP sub-populations. This is reflected by the fatter tail of the dispersal curve estimated for the VdM and FP sub-populations, and in general, wind-pollination in trees tends to result in fat-tailed dispersal kernels (Austerlitz *et al.* 2004; Pluess *et al.* 2009; Klein *et al.* 2006; Saro *et al.* 2014). As larger floral displays usually attract more pollinators (Makino & Sakai 2007), the lower pollen immigration rates on Curieuse could also be explained by a lower abundance of animals transferring pollen long distances.

NM+ can also be used to identify ecological correlates of siring success and it would be interesting to relate this to phenotypic traits such as male tree height. This could provide us with further insights into modes of pollen dispersal in *Lodoicea,* and whether, for example, longer-distance pollen dispersal is more prevalent in taller canopy males, while short-distance biotic dispersal of pollen is more prevalent in shorter males that may be more accessible to animals such as geckos that are known to cross between trees via overlapping canopies.

Evidence that fragmentation affects pollen dispersal

We detected the highest pollen immigration rates in FP, and the lowest in CU (using both direct and indirect methods). Curieuse had the greatest proportion of mating events that took place over distances of less than 80 m, the lowest estimated pollen dispersal distance estimated with NM+, and was the only sub-population predicted to have a thin-tailed pollen dispersal kernel, indicating that long-distance pollen dispersal events are very uncommon. In an earlier study (Chapter 2) we found that realised seed dispersal was also most limited on Curieuse, and mean private rarefied allelic richness over loci was higher than at VdM and FP, though the mean pollen dispersal distance estimated with CERVUS did not support Curieuse as being the sub-population with the most limited gene flow. Curieuse is the most fragmented sub-population of the four, with large clusters of *Lodoicea* plants being separated by up to 200 m or more of bare ground or low layers of vegetation, in particular the invasive coco plum (*Chrysobalanus icaco*) shrub. Although long distance pollen flow (possibly wind-mediated) is relatively infrequent, it likely plays an important evolutionary role in contributing towards the relatively low genetic differentiation among sub-populations, and high genetic diversity levels. However the results show that the majority of realised pollen dispersal events are over relatively short distances (94% of detected events < 400 m) and this is consistent with the high levels of bi-parental inbreeding observed in adult sub-populations, and high levels of inbreeding overall (Chapter 2). Dispersal dynamics are likely to change in disturbed landscapes (Dick *et al.* 2013), therefore it is plausible that pollen flow patterns on Curieuse differ from those in the VdM and FP, and to a large extent, FF, which are characterised by dense, continuous *Lodoicea* forest.

***Lodoicea* mating system**

We previously showed that mating between highly related, spatially proximate pairs was likely to be common in natural *Lodoicea* systems (Chapter 2), and our results from this study support the hypothesis of high biparental inbreeding rates. A high correlation of paternity was estimated in some females, which can result from repeated matings with near neighbours, though overall, a large range was observed in the number of males contributing paternity to each female. This is consistent with another dioecious palm species *Phonenix canariensis* (Saro *et al.* 2014). Estimated effective numbers of fathers per mother tree for VdM, FP and CU (N_{ep} = 4.4, 5.9 and 6.1, respectively), are within a similar range to the values obtained for other palm species (N_{ep} = 5.8 for *P. canariensis;* Saro *et al.* 2014, and N_{ep} = 5.4 for *Oenocarpus bataua*; Ottewell *et al*. 2012).

Conclusions

We used a combination of methods to provide us with complementary information on pollen dispersal dynamics across the species distribution. Because of the very slow development of young *Lodoicea* in their natural habitat, our analysis had to take account of tree mortality during the period between pollination and sampling. We demonstrated frequent pollen dispersal at short distances across all sub-populations of *Lodoicea*. On Praslin, where forests are less disturbed, a larger proportion of pollen flow events were long-distance, whereas on the highly fragmented Curieuse, few long-distance pollination events were estimated. Pollen dispersal did not follow a model of isolation-by-distance, but mating occurred at shorter distances than would be expected. Our results suggest that *Lodoicea* demonstrates mixed modes of pollen dispersal, with a combination of abiotic and biotic vectors. Although long-distance dispersal by wind or flying insects is relatively infrequent, it is likely to be important over evolutionary timescales in maintaining genetic connectivity within and possibly among sub-populations. The frequent short-distance pollen dispersal observed in all sub-populations is likely to be natural and not a recent artifact of habitat disturbance. Combining pollen dispersal data with data on putative pollen vector (especially gecko) movement patterns would provide novel insights to *Lodoicea* pollination dynamics.

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SUPPLEMENTARY MATERIAL

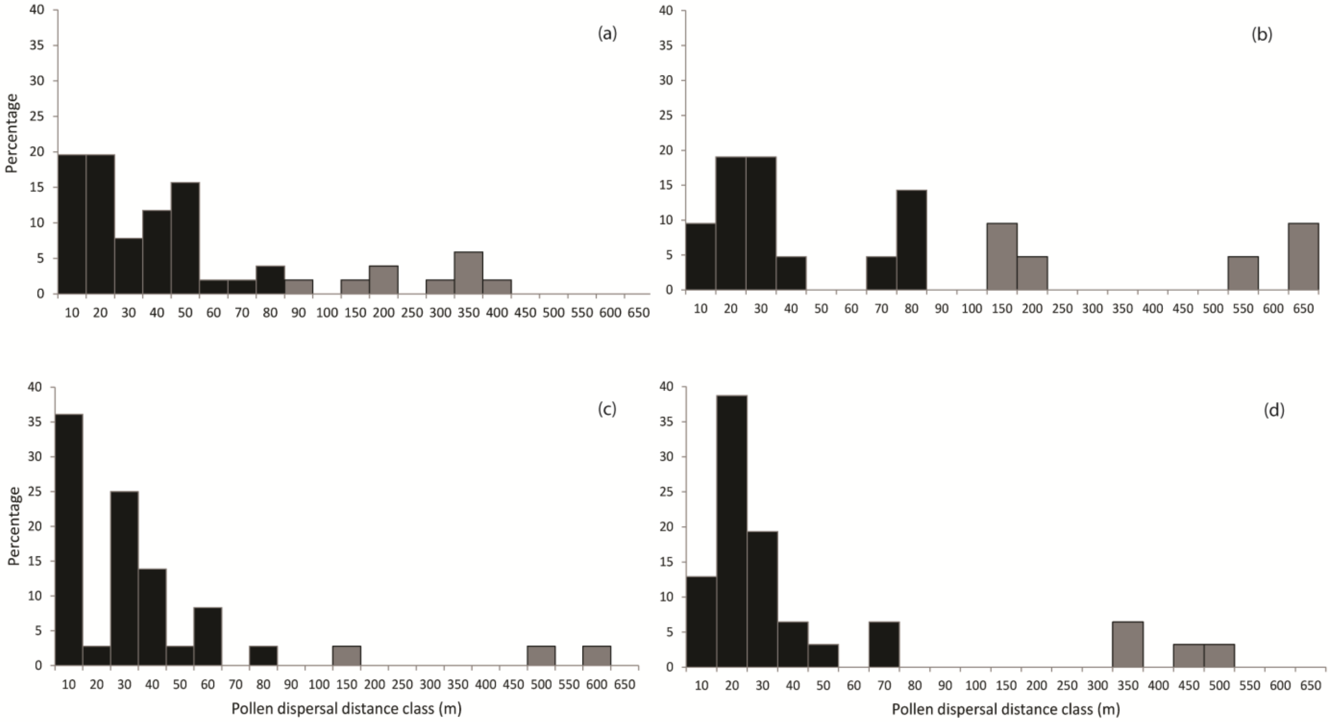

Fig. S1. Frequency distribution of detected realised pollen dispersal distances in *Lodoicea maldivica* sub-populations. **(a)** Vallée de Mai, **(b)** Fond Peper, **(c)** Fond Ferdinand and **(d)** Curieuse. Paternity analysis assignments at the 95% trio-confidence level. Candidate fathers were restricted to same sub-population as the offspring. Black bars indicate the minimum distance at which all adult males were sampled around clusters; grey bars also include males surrounding other clusters within the sub-population.

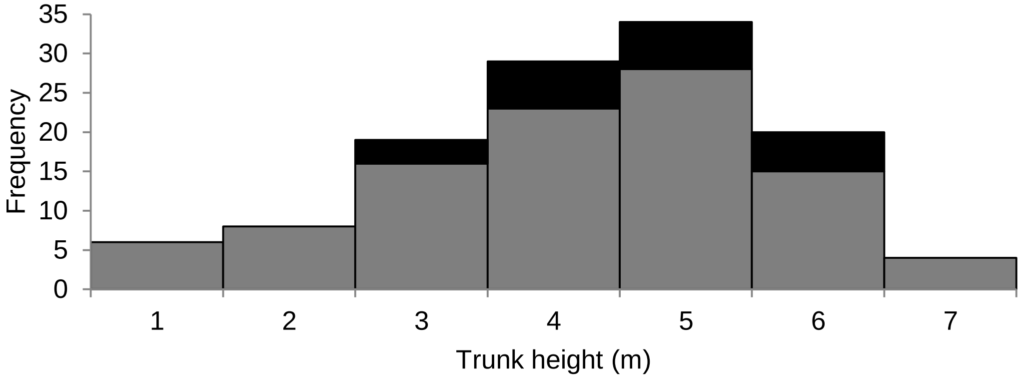

Fig. S2. Frequency distribution of male *Lodoicea* trunk heights on Curieuse. Grey and black bars indicate all sampled male trees, and males that fathered at least one offspring, respectively.

Table S1. Characteristics of pollen dispersal in *Lodoicea maldivica* across the 16 clusters, using CERVUS. Including: radius; minimum radius of males surrounding offspring; numbers of mothers and offspring; numbers of fathers contributing to paternity; paternity assignment rate; *d*, mean pollen dispersal distance, and median in brackets; proportion of mating events at distances ≤ 80 m, and number in brackets; m_p, pollen immigration rate; *F,* mean pairwise kinship coefficient of all mates, with standard error; *F* <80 m; mean pairwise kinship coefficient of mates less than 80 m apart, with standard error.

Cluster	Radius (m)	Mothers (*N*)	Offspring (*N*)	Fathers (*N*)	Assignment rate	*d* (m)	Mating events ≤ 80 m (*N*)	m_p	*F* (SE)	*F* <80m (SE)
VdM1	80	5	30	10	0.567	27.8 (14.1)	0.533 (16)	0.433	0.091 (0.037)	0.09 (0.039)
VdM2	80	3	15	6	0.533	60.7 (46.3)	0.467 (7)	0.467	0.091 (0.036)	0.110 (0.036)
VdM3	80	11	31	12	0.581	88.2 (19.2)	0.452 (14)	0.419	0.087 (0.021)	0.076 (0.025)
VdM4	80	4	15	8	0.533	97.8 (49.9)	0.333 (5)	0.467	0.077 (0.050)	0.067 (0.045)
FP1	100	1	30	9	0.400	72.2 (26.1)	0.367 (11)	0.600	0.003 (0.034)	0.003 (0.038)
FP2	100	4	11	4	0.455	34.8 (31.6)	0.455 (5)	0.545	0.050 (0.067)	0.050 (0.067)
FP3	120	2	12	4	0.750	13.3 (8.4)	0.750 (9)	0.250	0.102 (0.012)	0.102 (0.012)
FP4	100	6	31	8	0.323	78.9 (26.0)	0.258 (8)	0.677	-0.021 (0.028)	-0.021 (0.032)
FF1	80	2	3	1	0.333	607.0 (607.0)	0	0.667	-0.074 (0)	–
FF2	80	8	24	7	0.500	128.8 (26.0)	0.333 (8)	0.500	0.107 (0.020)	0.098 (0.027)
FF3	80	1	2	0	0	–	0	1.000	–	–
FF4	80	3	13	7	0.615	64.7 (66.5)	0.538 (7)	0.385	0.167 (0.024)	0.176 (0.025)
CU1	80	1	12	4	0.500	92.2 (51.7)	0.417 (5)	0.500	0.173 (0.052)	0.166 (0.063)
CU2	80	1	12	5	0.667	17.2 (11.3)	0.667 (8)	0.333	0.119 (0.012)	0.119 (0.012)
CU3	100	3	12	6	0.583	59.7 (20.5)	0.500 (6)	0.417	0.037 (0.023)	0.038 (0.028)
CU4	120	6	16	7	0.625	102.1 (17.5)	0.500 (8)	0.375	0.025 (0.046)	-0.004 (0.042)

Table S2. Within-sibship correlated paternity estimates for *Lodoicea maldivica* mother trees within each sub-population. r_p = correlated paternity.

Sub-population	Mother	Offspring (*N*)	r_p
VdM	315	8	0.198
	317	5	0.438
	347	13	0.125
	398	2	-0.150
	406	3	0.117
	408	2	0.378
	434	2	0.071
	Mean	5	0.168
FP	1	30	0.017
	38	4	0.173
	77	2	1.894
	83	7	-0.018
	Mean	10.75	0.517
FF	643	3	0.222
	644	7	0.039
	1261	4	0.030
	Mean	4.67	0.097
CU	471	12	0.088
	488	12	0.106
	712	5	0.764
	787	3	0.164
	799	2	-0.377
	819	5	-0.055
	828	2	0.117
	Mean	5.86	0.115

CHAPTER 4

Tracing coco de mer's reproductive history: pollen and nutrient limitation reduce fecundity

with Christopher N. Kaiser-Bunbury, Peter J. Edwards, Frauke Fleischer-Dogley and Chris J. Kettle

ABSTRACT

The availability of nutrient resources and the quality and quantity of pollen are important factors affecting flower and fruit production. The Seychelles endemic palm coco de mer (*Lodoicea maldivica)* produces the largest seeds in the plant kingdom (average 8.5 kg), and the nutrient costs of reproduction are high. A detailed study of persistent female inflorescences enabled us to track the reproductive history of individuals over seven years, and to investigate the factors determining reproductive success. We recorded large variation in reproductive output among individuals, with inflorescence and flower production being related to available soil nitrogen and potassium, and fruit production being limited primarily by pollen availability. Habitat degradation led to reduced fruit set and higher fruit abortion rate, which substantially reduced overall fruit set. We also documented the largest variation in seed size in any species (16.3-fold range in fresh weight, N = 2415). We discuss the implications of our results for the sustainable management of this iconic palm species and more broadly, the factors that shape reproductive output in threatened plants.

INTRODUCTION

The reproductive output of plants is influenced by a number of biotic and abiotic factors acting at different stages of the lifecycle (Henry *et al.* 2004; Kim & Donohue 2011), and understanding these factors is important from an evolutionary perspective, and for conservation management. According to theory, the numbers of seeds a plant can produce is governed by the availability of resources such as nutrients, light and water, which are all essential for producing flowers and fruits (Bateman 1948). Rather than merely responding passively to nutrient availability, there is evidence that plants can control to some degree their investment in reproduction (Lloyd 1980) by selectively abscising damaged flowers, developing fruits and genetically inferior seeds (Janzen 1977). In this way reproduction can be adjusted to match the resources available for the maturation of the offspring (Stephenson 1981). Although resource availability strongly influences reproductive output (especially flower production; Winn 1991), other factors including trade-offs between nutrient- and pollen-limitation and pollen attraction effort, genetic load and photosynthetic costs (Haig & Westoby *et al.*1988; Helenurm & Schall 1996; Ne'eman *et al.* 2006) may also be important.

In many plant communities, seed set is limited by the quantity and quality of available pollen (Aizen & Harder 2007; Fernández *et al.* 2012; Wang *et al.* 2013). For example, fertilisation may be reduced if much of the pollen delivered to a flower is incompatible because it is related too closely or too distantly to the ovule (Bertin 1982). A reduction in seed set is particularly likely to occur in low-density, fragmented plant populations where levels of inbreeding are high (Liu & Koptur 2003; Severns 2003). Habitat fragmentation may also result in a decline in pollinator abundance and species richness (Steffan-Dewenter & Tscharntke 1999). These effects, in turn, can cause a disruption of plant-pollinator interactions, which can often result in reduced pollinator visitation rates, reduced gene flow and thus a reduced seed set (Ågren 1996; Aguilar & Galetto 2004; reviewed in Knight 2005). It has also been shown that the amount of outcrossing provided by pollinators can limit seed size, as well as seed number (Galen *et al.* 1985). The role of genetic effects in flower and seed production, such as heterozygosity, which have been shown to correlate with growth rates (Breed *et al.* 2012) and survival (del Castillo *et al.* 2011) in some plants, is largely unknown. Heterozygous individuals may cope better under stressful environmental circumstances (Badyaev 2005), but understanding whether there is a direct link to reproductive success requires further study.

Another factor that may affect reproductive success is the size and quality of seeds produced. Large seeds are known to confer fitness benefits in shady habitats and on nutrient-poor soils (Westoby 1992; Vaughton & Ramsey 1998) because they develop into large seedlings, better able to survive under these conditions than the seedlings of small-seeded species (Wulff 1986b; Moegenburg 1996). On the other hand, smaller seeds can be produced in larger numbers, and are more readily dispersed (Harper *et al.* 1970). Seed size is thought to be under strong stabilising selection, varying much less than vegetative structures such as leaves (Harper *et al.* 1970). Although this appears to be true for most species, considerable variation has been observed both within and among species (Thompson 1984). Factors shown to affect seed mass include resource constraints (Wulff

1986a; Vaughton & Ramsey 1998), seed number (Wolf 1986), pollen availability (Wolf 1986), and a combination of these factors (Galen *et al.* 1985).

The species with by far the largest seeds in the plant kingdom is the Seychelles coco de mer *Lodoicea maldivica* (J. F. Gmel.) Pers. (Arecaceae), which produces seeds weighing as much as 18 kg fresh weight (Fig. 1a, c, g). A survey in the UNESCO World Heritage Site at the Vallée de Mai (VdM), the finest remaining stand of *Lodoicea* in the Seychelles, found that the mean number of developing fruits per female was 7.03, though with considerable variation among trees (Edwards *et al*. 2002). Given that it takes about seven years for the fruits to mature (comments in Corner 1966; Blackmore *et al*. 2012; Anders Lindström pers. obs. from Nong Nooch Botanical Garden, Thailand), and most fruits contain only one seed, this represents an unusually low reproductive output of around one seed per female per year. *Lodoicea* is ecologically well-adapted to the poor environmental conditions on the islands, such as low light levels for the first 50 to 100 years of life and low nutrient levels. In such a nutrient poor environment, even such a low reproductive output represents a considerable cost in terms of nutrients, accounting for some 88% of a female tree's aboveground phosphorus (P) budget (Edwards *et al*. 2015). Investing so much in reproduction is only possible through an adaptation of *Lodoicea* foliage that forms a funnel, allowing nutrient-rich organic matter to be washed down to the base of the trunk, thereby maximising the tree's nutrient supply. *Lodoicea* evolved in isolation on the Seychelles for around 70 million years in the absence of major disturbances (Baker & Miller 1963), and thus, likely represents an 'evolutionary end-point'. We therefore expect main drivers for reproductive success in *Lodoicea* to be well-established.

The extraordinary biological features of *Lodoicea* are the reason for its substantial contribution to the Seychelles economy. Around 40% of all tourists visiting the Seychelles pay an entrance fee for the VdM primarily to see the *Lodoicea* palm forest ecosystem, and considerable additional revenue is generated from the sale of seeds (nuts) (Seychelles Islands Foundation, 2009 unpubl. report), where the main demand comes from tourists. As well as being ecologically and economically important, the palm has high cultural significance and is considered a national icon. *Lodoicea* seeds, leaves and timber were used traditionally for making Seychellois crafts, and the palms are a central part of folklore on the islands. Despite the high importance of this keystone species, it is severely exploited for its seeds. It has also been proposed that *Lodoicea* has a reduced reproductive performance where it grows solitarily or in small groups in less fertile degraded shrubland (Fig. 1i), as opposed to forest habitat (Fig. 1h) (Edwards *et al.* 2015). Despite recent advances in our understanding, we still lack a detailed knowledge of what influences the reproductive success of this palm.

The aim of this study was to investigate variation in flower and fruit production in *Lodoicea* in its natural habitat, and to determine the main factors influencing this variation. Specifically, we asked whether soil nutrient and pollen (as measured by the distance from and abundance of male trees) availability and the genetic variation of female trees influenced flower and fruit production. Abnormal fruits that failed to mature were also included in our study as they contributed a notable proportion of fruit produced in the *Lodoicea* forest. In addition to exploring variation in numbers of flowers and fruits, we present data on the large variation in seed size in *Lodoicea.* We discuss the implications of our results for the future sustainable management of *Lodoicea* and drivers of plant reproduction in general.

Fig. 1. Photographs of *Lodoicea maldivica* on Praslin. **(a)** Female bearing a large fruit set. The most recently produced fruits can be observed on the uppermost inflorescences, and successively more mature fruits can be seen on inflorescences hanging lower down on the palm. **(b)** Dissected abnormal fruit. **(c)** Dissected fruit with seed inside. **(d)** Female bearing fruits and abnormal fruits. **(e)** Receptive female flower. **(f)** Gecko (*Ailuronyx trachygaster*) feeding on the nectar of a male inflorescence. **(g)** Seed**. (h)** Closed forest in Vallée de Mai. **(i)** Degraded shrubland at Cherie Mon. Two adult males can be observed amongst the shrub.

MATERIALS AND METHODS

Study area

Lodoicea maldivica is endemic on the islands of Praslin (37.4 km^2) and Curieuse (3.5 km^2) in the Republic of Seychelles. Until the 19th century, dense monospecific stands of *Lodoicea* covered much of the islands (Fauvel 1915). Today little-disturbed *Lodoicea* forest remains only in protected areas (VdM and Fond Peper within Praslin National Park, and Fond Ferdinand) in the south of Praslin (Fig. 2). On Curieuse and elsewhere on Praslin, the species persists as small clusters and isolated individuals (Polunin and Procter 1973), with poor natural regeneration. *Lodoicea* kernel is CITES-listed and protected from illegal exploitation (Kaiser-Bunbury *et al*. 2014), though many nuts are poached due to their high black-market value (Rist *et al.* 2010).

Fieldwork was conducted at six sites across Praslin (VdM, Fond Peper, Fond Ferdinand, Cherie Mon, Zimbabwe and Anse Boudin (Fig. 2), consisting of two main habitat types: a) dense, closed *Lodoicea* forest and b) degraded open shrubland/mixed forest. The highly weathered granitic soils on the island are infertile and deficient in nitrogen (N), P, potassium (K), calcium and magnesium (Dobrovol'sky 1986), particularly so in the rocky valleys, where the surface soil is minimal. Praslin has a tropical humid climate, with a mean daily rainfall of 10.6 ± 1.1 mm and 17.1 ± 1.2 mm in the dry and wet seasons, respectively (Edwards *et al.* 2015).

Method for assessing female flower and fruit production

In closed forest on Praslin, trees reach sexual maturity when the trunk is about 4 m tall (Savage & Ashton 1983), though in open areas this may happen when the trunk is shorter. The male trees bear one to four long-lived (3–4 months) cylindrical inflorescences up to 90 cm long that produce spirally arranged clusters of flowers (Fig. 1f). Female plants bear large lignified inflorescences that are produced at the stem apex in the axils of emerging leaves (Fig. 1a). As each inflorescence grows in length, up to 13 flowers are produced sequentially over a period of three to four weeks (CKB pers. obs) (Fig. 1e). During the first five to six months the fertilised ovules expand rapidly to reach the final size in the form of large, bilobed seeds within a thick husk, and then slowly mature over six to seven years. Most fruits contain a single seed, but some (9.2%) contain two seeds, and very few (0.03%) contain three (N = 307, own data, trees on Praslin and Curieuse). Strictly, the husk represents the epicarp and mesocarp of the fruit, which surrounds all carpels, while a separate endocarp surrounds the locule of each carpel (in cases where there is more than one) to form a hard woody 'pyrene'

(Uhl & Dransfield 1987). Thus the structure loosely referred to here as the ‘seed’ or nut is in botanical terms the true seed plus the surrounding pyrene (i.e. maternal tissue). The mature seeds vary greatly in size and shape. Unfertilised ovules become lignified and persist as prominent, hemispherical lumps on the inflorescence. Some fruits fail to develop normally, being narrow and elongated in shape (Fig. 1b, d), and are shed before reaching maturity. The reasons for this abnormal development are not known. Inflorescences are shed soon after the last fruit has matured.

The inflorescences of female trees provide a record of the tree’s reproductive history over seven years (see Discussion), from which it is possible to determine how many flowers were produced, how many ovules were fertilised, and how many of these developed normally. The successful production of a mature fruit can be recognised from the distinctive bowl-shaped scar surrounded by lignified perianth parts that is left on the oldest inflorescences. A similar scar in a younger inflorescence (i.e. one with still maturing fruits) can be interpreted as the loss of an immature fruit. The order of inflorescence production can be observed by their position on the tree, with older inflorescences hanging down close to the trunk.

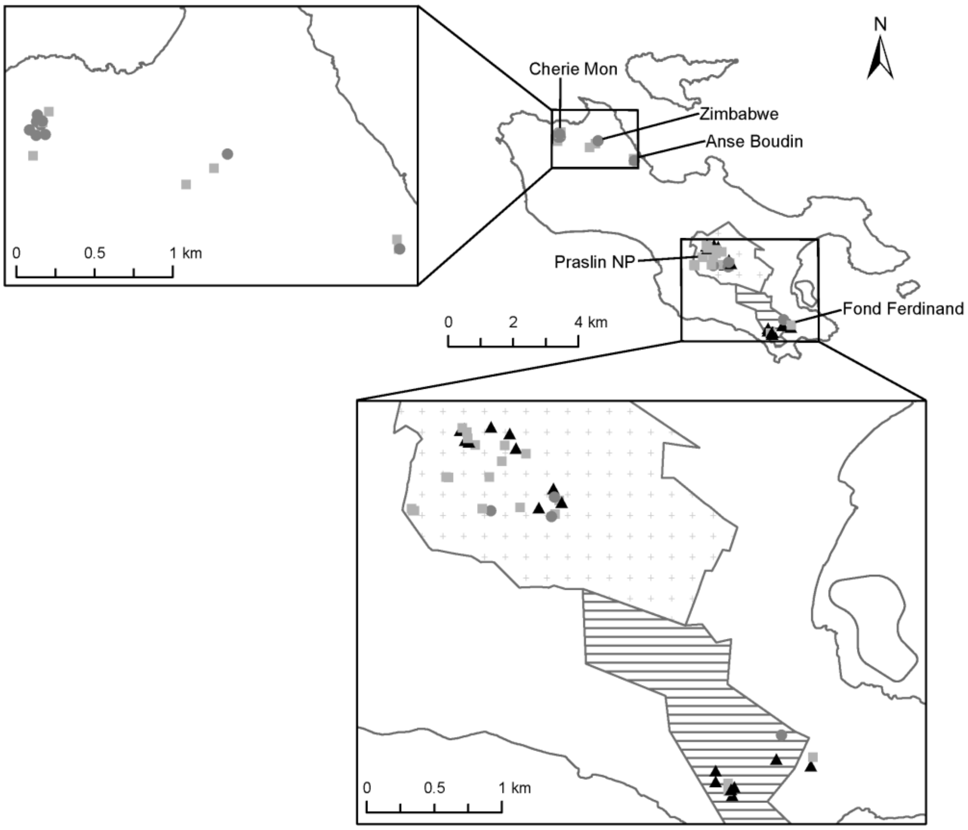

Fig. 2. Locations of the sites of sampled female *Lodoicea maldivica* on Praslin. Black triangles are individuals that had six or more fruits; dark grey circles are those with no fruits; light grey squares are all others. The crossed area indicates Praslin National Park, and the lined area Fond Ferdinand.

Field survey

We used the method described above to study the reproductive output of 57 female *Lodoicea* trees, chosen to represent varying degrees of isolation from male trees. To achieve a balanced representation of female trees along a gradient of distance to the nearest male, females were randomly selected within distance classes. Thirty-nine of the trees were in more-or-less closed forest, while 18 were in degraded shrubland.

For each tree, we examined all inflorescences, recording the numbers of undeveloped ovules, developing fruits, abnormal fruits, and successfully shed mature fruits. We then aggregated these data to obtain ‘all flowers’ (the sum of unfertilised flowers, and normal and abnormal fruits), abnormal fruits, and ‘all fruits’ (the sum of all developing fruits plus any mature fruits that had been shed). ‘Fruit set’ was calculated as the proportion of flowers that developed into fruits.

For each tree we also recorded the distance to the nearest male *Lodoicea*, and the total number of males within a 10 m radius. Ten metres was a feasible distance to count males, and a previous study showed that male and female pairs within 10 m from each other are significantly related (Chapter 2). Distances from females to the nearest male ranged from 0.4 to 159 m, and the numbers of males within a 10 m radius ranged from zero to nine. *Lodoicea* have a long lifespan (possibly around 350 years, Savage & Ashton 1983), thus it is unlikely that adult male densities would have changed during the seven year reproductive period covered in this study. The number of flowering catkins per male (recorded between May and July 2014) ranged from zero to four (mean ± SD: 0.67 ± 0.03; N = 320). We obtained genotypes for all females in this study, using 12 microsatellite loci developed by Morgan *et al.* (2016). These genotypes were used to calculate the individual standardised multilocus heterozygosity (MLH; following Slate *et al.* 2004) to test the relationship between genetic variability and reproductive success.

Soil nutrient status around female trees

Available P and K, and pH

Samples of soil at 10 cm depth were collected at distances of 0.5 and 1 m downhill from each female tree in April to May 2014. Means of both measurements were used for determining available P and K concentrations and pH (Edwards *et al.* 2015). Sites with insufficient soil were excluded from analyses. The samples were passed through a 2 mm sieve, air-dried and extracted in a solution of ammonium acetate and EDTA (1:10; FAL *et al.* 1996). The extracts were then analysed using inductively coupled plasma atomic emission spectroscopy (Vista-MPX CCD Simultaneous ICP-OES; Varian). Each ICP-OS run included sample blanks and an external reference sample. Soil pH was determined in a 1:2.5 soil to distilled water solution using a portable pH meter (Microprocessor pH 95 Meter, WTW, Weilheim, Germany).

Available N

The availability of nitrogen (N) was measured by placing small mesh bags containing 2.0 g (dry weight) ion-exchange resin (Amberlite IRN-150, H+ & OH- form; Sigma-Aldrich Logistik GmbH, Schnelldorf, Switzerland) in the soil (IER; Lundell 1989). The 5 x 5 cm bags were made from fine nylon mesh (60 µM mesh width, Sefar Nitex 03-60/35; Sefar AG, Thal, Switzerland). Prior to use, the bags were shaken for 2 h with 2 M KCl, rinsed with distilled water, and then kept moist until use. The resin bags were installed by cutting an oblique slot in the soil to a depth of 5 cm, inserting the bag, and gently pressing back the soil. The bags were incubated in the field for ~ 30 days, also at distances of 0.5 and 1 m downhill from females. Mean daily rainfall during the time of the experiment was 8.2 ± 2.2 ml/day (within the normal range for the time of year). Upon collection the bags were rinsed with distilled water to remove surface soil, and subsequently air-dried. In the laboratory, the resin was extracted for 2 h in 30 ml 2 M KCl (Keeney & Nelson 1982). The extract was filtered through Whatman no. 45 filter paper and analysed using colorimetric assays for NH_4^+ (adapted from Mulvaney 1996) and NO_3^- (plus NO_2^-; Doane & Horwáth 2003) (see Appendix 1 for detailed methods).

Statistical analysis

Individual correlations were conducted to test the relationships of each variable with numbers of inflorescences, flowers and fruits, and fruit set, and to help select variables to include in subsequent modelling. We used five different General Linear Models (GLMs) and three functions (indicated below as ‘package::function()’) in the RStudio environment v. 0.98.1102 (RStudio Team 2015). Co-linearity of variables was tested using usdm::vifstep() (Naimi 2015) by calculating the variance inflation factors (VIFs). All variables had VIF values well below the recommended threshold value of 10 (max. VIF = 2.08), indicating no collinearity problems.

Inflorescence and flower production: To test the influence of soil nutrients and pH, MLH and vegetation type on the production of inflorescences and flowers, we separately modelled inflorescence and flower number as a function of the main effects N, P, K, pH, MLH and vegetation type (dense closed forest or degraded shrubland), along with the following two-way interactions in the full model: N × P, N × K, P × K, P × pH, K × pH, and vegetation type × MLH. Number of inflorescences was analysed using a GLM assuming a Poisson distribution and log link, with the function stats::glm() (R Core Team 2014). Flower number was analysed using a GLM assuming a negative binomial distribution and a log link, correcting for over-dispersion with the function MASS::glm.nb() (Venables & Ripley 2002).

Fruit production: To analyse the effects of pollen availability, MLH and vegetation type on fruit production, we used a model that included the main effects distance to the nearest male, number of males within a 10 m radius, MLH and vegetation type, as well as the two-way interactions: distance to the nearest male × vegetation type, distance to the nearest male × MLH, number of males within 10m × vegetation type, number of males within 10m × MLH and vegetation type × MLH. As the fruit set data was zero-inflated, we first ran a binary model (i.e. fruit-setting probability), modelling the occurrence of successes (fruits > 0) and failures (fruits = 0), followed by a 'proportional' model (i.e. fruit set size) on non-zero data. For the binary model we used a GLM with a binomial distribution (across all populations, and within closed forest separately). The proportion data (from all populations) was analysed using a quasi-binomial distribution to account for over-dispersion. We used the cbind() function (Becker *et al.* 1988) to combine fruits and non-fruits into one response variable; a method ideally suited to analyse unbalanced data. Both GLMs used a logit link, and the function stats::glm() (R Core Team 2014). Between 2009 and 2013, freshly fallen seeds from the VdM and Fond Peper were weighed (*N* = 2416), and their lengths and diameters measured (*N* = 2368; Seychelles Islands Foundation, unpubl. data).

Abnormal fruit production: To study the effects of nutrients, pollen availability, MLH and vegetation type on the occurrence of abnormal fruits, we modelled the abnormal fruit as a function of N, P, K, distance to the nearest male, number of males within 10m, vegetation type and MLH, and the two-way interactions: N × P, N × K, P × K, N × vegetation type, K × vegetation type, P × vegetation type, distance to the nearest male × MLH, number of males within 10m × MLH and vegetation type × MLH. The data on abnormal fruits was modelled using the occurrence of successes (abnormal fruits > 0) and failures (abnormal fruits = 0) using the stats::glm() function, assuming a quasi-binomial distribution with a logit link.

Model selection: We applied a backward stepwise model selection for all GLMs to obtain minimum adequate models. For the Poisson, negative binomial and binomial models, we excluded variables using Akaike's second order information criterion (AICc; for smaller sample sizes). The removal criteria was ΔAICc ≤ 2 compared to the reduced model, to ensure parsimonious model selection and avoid over-fitting (Arnold 2010). Main effects were only removed when higher-order effects were removed first. For the quasi-binomial models we used the function stats::drop1() (R Core Team 2014) to exclude terms using *P*-values from *F* tests (based on analysis of deviance tests). Significance level for model selection was $\alpha = 0.05$.

RESULTS

Inflorescences and flowers

Female trees within closed forest and degraded shrubland produced a similar number of inflorescences per tree (range 1–15, $t_{55} = 0.309$, $P = 0.76$), and flowers per inflorescence (range 2.4–9.1, $t_{55} = 0.591$, $P = 0.56$, Table 1). Similarly, the total number of female flowers per tree (i.e. all flowers produced over the last seven years, range 6–123) did not differ between vegetation types ($W = 352$, $P = 0.99$, Table 1). Numbers of inflorescences and flowers were both positively associated with available soil N and K, but not with P and pH (Tables 2a, b; see also Table 3). Soil nutrients and pH were highly variable but were similar in forest and degraded shrubland (Wilcoxon all $P > 0.05$).

Table 1. Variation in reproductive output of female *Lodoicea maldivica* included in this study. Reported are the means (± SD). Fruit set is defined as proportion of flowers that developed into fruits.

Reproductive output	Closed forest (*N* = 39)	Degraded shrubland (*N* = 18)	Overall (*N* = 57)
No. inflorescences	6.97 (3.00)	6.78 (2.56)	6.91 (2.85)
No. flowers (all)/ inflorescence	5.26 (1.77)	4.95 (0.04)	5.16 (1.66)
No. flowers (all)/ tree	39.62 (28.14)	35.72 (21.00)	38.39 (25.97)
No. undeveloped ovules/tree	31.92 (24.99)	31.06 (17.68)	31.65 (22.78)
No. fruits (all)/ tree	6.18 (7.27)	0.72 (1.02)	4.46 (6.54)
No. developing fruits/tree	5.62 (6.80)	0.61 (1.04)	4.04 (6.10)
No. fallen immature fruits/tree	0.36 (1.14)	0.00 (0.00)	0.25 (0.95)
No. fallen mature fruits/tree	0.15 (0.43)	0.06 (0.24)	0.12 (0.38)
Fruit set	0.21 (0.19)	0.03 (0.04)	0.16 (0.18)
No. abnormal fruits/tree	1.54 (4.53)	4.00 (6.37)	2.32 (5.25)

Table 2. Final GLM models.

	Estimate	SE	z value	Pr(>|z|)
a) Response: inflorescence number				
Intercept	1.6789	0.0988	16.998	<2e-16 ***
N	0.0121	0.0077	1.577	0.1148 .
K	0.0012	0.0005	2.396	0.0166 *
Null deviance: 58.065 on 50 df Residual deviance: 49.966 on 48 df				
b) Response: flower number				
Intercept	3.2506	0.1483	21.924	<2e-16 ***
N	0.0209	0.0125	1.672	0.0944 .
K	0.0018	0.0008	2.173	0.0298 *
Null deviance: 61.526 on 50 df Residual deviance: 53.194 on 48 df				
c) Response: Presence of fruit(s) (all populations)				
Intercept	2.1691	0.5278	4.110	3.96e-05 ***
Degraded shrubland	-2.1691	0.7076	-3.065	0.00218 **
Null deviance: 61.210 on 56 df Residual deviance: 50.746 on 55 df				
d) Response: Presence of fruit(s) (closed forest)				
Intercept	2.1691	0.5278	4.11	3.96e-05 ***
Null deviance: 25.793 on 38 df Residual deviance: 25.793 on 37 df				
e) Response: Fruit set when fruit(s) present (all populations)				
Intercept	-0.64972	0.22576	-2.878	0.00639 **
Distance to nearest male	-0.0690	0.0182	-3.781	0.00051 ***
Degraded shrubland	-2.1272	0.8579	-2.596	0.01312 *
Distance to nearest male × degraded shrubland	0.0624	0.0214	2.920	0.00573 **
Null deviance: 273.27 on 43 df Residual deviance: 134.99 on 40 df				
f) Response: Presence of abnormal fruit(s)				
Intercept	-2.2305	0.4968	-4.490	3.69e-05 ***
Distance to nearest male	0.0507	0.0143	3.549	8e-04 ***
Null deviance: 71.097 on 56 df Residual deviance: 48.910 on 55 df				

*** P ≤ 0.001, ** P ≤ 0.01, * P ≤ 0.05, . P < 0.01.

Table 3. Variables tested in this study for each female *Lodoicea maldivica*, including resin adsorption rates for nitrogen (N; NH_4^+ and NO_3^- combined), available soil phosphorus (P) and potassium (K), and soil pH (all measurements combined from 0.5 and 1 m sampling distances from females). Also measured were the distance to the nearest male and number of males within 10 m from the female, and the standardised multilocus heterozygosity (MLH) of the female. Spearman's *rho* correlation coefficients (except inflorescence number against soil pH and MLH, which were tested with Pearson's correlations), and significance levels are given. Correlations remaining significant after sequential Bonferonni corrections for each response variable are in bold. One outlying female that produced 43 fruits was excluded from all correlations.

Variable	*N*	Mean (SD)	Range	Correlation coefficient			
				Inflorescence no.	Flower no.	Fruit no.	% fruit set
Available N (µg N/g/day)	56	4.90 (6.31)	0.46–28.79	0.317*	**0.381****	0.002	-0.090
Soil P (µg P/g dry soil)	52	3.72 (3.20)	0.31–14.99	0.315*	0.196	0.164	0.126
Soil K (µg K/g dry soil)	52	129.35 (97.92)	31.00–509.05	0.370**	0.235	0.241	0.166
Soil pH	52	4.93 (0.43)	3.76–6.34	0.267	0.158	0.094	0.069
Distance to nearest male (m)	57	28.06 (34.87)	0.4–159	0.066	0.159	**-0.483*****	**-0.529******
No. males ≤ 10m	57	0.93 (1.69)	0–9	-0.143	-0.240	**0.465*****	**0.533******
MLH	57	0.768 (0.243)	0.360–1.321	-0.073	-0.041	0.020	-0.023

**** P ≤ 0.0001, *** P ≤ 0.001, ** P ≤ 0.01, * P ≤ 0.05.

Fruits

Few inflorescences (4%) showed scars indicating that a mature fruit had been shed, confirming that inflorescences generally fall soon after the last fruit has been shed (Table 1). The number of fruits per tree ranged from zero to 43, yet the frequency distribution was highly skewed (median = 2). Thirteen trees (22.8%) produced no fruits and 17 (29.8%) produced six or more (these six accounting for 75% of all fruits recorded; Fig. 3).

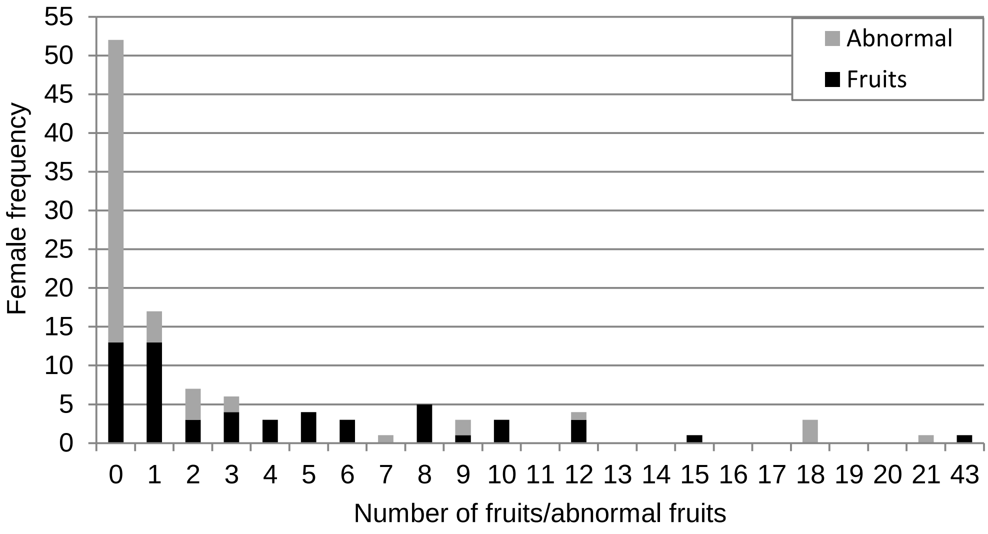

Fig. 3. Frequency histogram showing numbers of fruits and abnormal fruits produced by individual female *Lodoicea maldivica* trees. Each female is represented twice: once each for the numbers of fruits and abnormal fruits.

Trees in closed forest produced > 8 times as many fruits as trees in degraded shrubland (Table 1, $W = 607$, $P < 0.0001$), and the presence of fruits was dependent upon the tree growing in closed forest (Table 2c). As only seven trees had fruits in degraded shrubland, we did not try to explain their presence. All tested variables were unsuitable to explain the presence of fruiting/non-fruiting trees in closed forest, where 87% of trees produced fruits over a seven-year period (Table 2d). Fruit set, however, was dependent on distance to the nearest male and vegetation type. As the distance to the nearest male increased, fruit set decreased, this pattern being more marked in closed forest than in degraded shrubland (Table 2e).

The number of flowers was independent of the number of fruits produced by individual fruit-bearing females (outlier excluded, adj $R^2 = -0.02$, $P = 0.66$). Across all trees, and assuming a seven-year maturation period for fruit, the mean rate of production in closed forest was 0.88 fruits/year compared to 0.10 fruits/year in degraded shrubland (overall 0.67 fruits/year; fruit-bearing trees only, 0.98 vs. 0.21 fruits/year). Individual correlation analyses showed that the number of males within a 10 m radius of the female, as well as the distance to the nearest male significantly influenced fruit number and fruit set (Table 3).

Abnormal fruits

Over half of all fruits were of the abnormal, elongated type (51.2%) (Fig 3). The number of abnormal fruits was higher in degraded shrubland than in closed forest (range 0–21, 61% (11/18) vs. 18% (7/39) females with abnormal fruit, *W* = 201.5, *P* = 0.002, Table 1). Within closed forest, abnormal fruits were highly aggregated on certain females, with most trees having either only normal or only abnormal fruits (Fisher's two-tailed exact test, *P* = 0.006; Table 4). However, no significant aggregation of abnormal fruits was found in degraded shrubland (Fisher's two-tailed exact test, degraded shrubland: *P* = 1; Table 4). The probability of bearing abnormal fruits increased with distance from the nearest male (Table 2f).

Seed size and mass

Seeds varied greatly in size. Although we did not measure the size of seeds in our sample, seeds collected over a four-year period showed a 16.3-fold range in fresh weight, from 1.04 to 18 kg (mean ± SD: 8.50 ± 2.39 kg; *N* = 2415, Fig. 4). Seed length and diameter (*N* = 2368) ranged from 17 to 48 cm (mean ± SD: 29.57 ± 3.85 cm) and 12.2 to 40.6 cm (mean ± SD: 28.28 ± 3.87 cm), respectively (Supplementary Material, Figs. S1, S2).

Table 4. Contingency table of female *Lodoicea maldivica* with fruits and abnormal fruits in closed forest, degraded shrubland and overall populations. Total numbers for each category are given in brackets. Fisher's two-tailed exact probabilities are shown.

Closed forest	Fruits		
Abnormal fruits	+	-	Total
+	42.86% (3)	57.14% (4)	100%
-	93.75% (30)	6.25% (2)	100%
Total	84.62% (33)	15.38% (6)	(39)
2-tailed exact test *P* = 0.006 **			
Degraded shrubland			
Abnormal fruits			
+	54.55% (6)	45.45% (5)	100%
-	42.86% (3)	57.14% (4)	100%
Total	50.00% (9)	50.00% (9)	(18)
2-tailed exact test *P* = 1 ns			

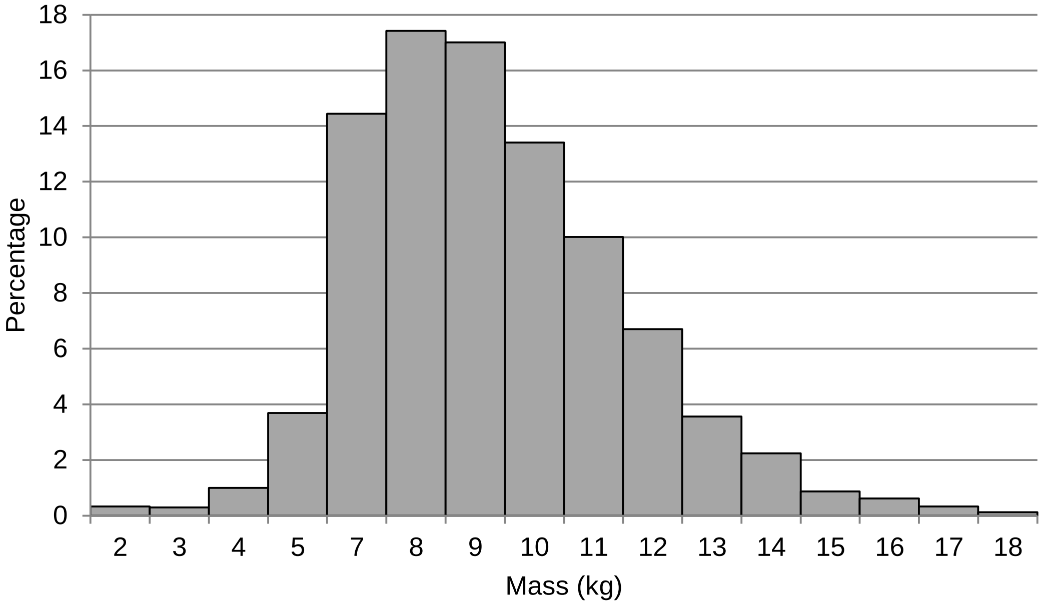

Fig. 4. Frequency distribution of the fresh weights (kg) of *Lodoicea maldivica* seeds (*N* = 2416).

DISCUSSION

This study reveals that pollen and nutrient limitation affect reproductive success of *Lodoicea* at different phases of the reproductive cycle, as suggested by previous studies (Galen *et al.* 1985; Haig & Westoby 1988). On the nutrient-poor soils of Praslin, the production of female flowers is limited by the availabilities of both N and K. In contrast, both vegetation type and pollen availability are important factors limiting fruit production. Large numbers of abnormal, non-viable fruit substantially reduces reproductive output in *Lodoicea*, and are most likely the result of seed abortion early on in the fruit's development, possibly as a consequence of pollen incompatibility or unavailability. Female reproductive output varies widely in the numbers of flowers and fruits, and in the sizes of seeds produced.

Assessing female reproductive success

Various lines of evidence suggest that inflorescences persist for around seven years. First, we found very few older inflorescences bearing scars of fallen mature fruits, indicating that inflorescences are shed when the fruits have matured. Second, our method yields a mean rate of mature fruit production of 0.88 fruits/year in closed forest, which is similar to that estimated from a survey of all trees in the VdM (1.00 fruit per tree, Edwards *et al*. 2002), and to that estimated by the productivity of female trees (0.8 fruit per year between 1995 and

2000, Fleischer-Dogley 2006). Finally, the value corresponds well with results from a study of the rate of leaf production, which found that trunked immature *Lodoicea* in the VdM produced between 0.62 and 2.18 leaves per year (mean value 1.2 leaves per year, $N = 15$; Edwards *et al.* 2015). Since one inflorescence is produced per leaf, we would expect trees to bear somewhere between 4 and 15 inflorescences, with a mean of about 8, which corresponds fairly closely to what we observed.

A possible limitation of this assumption would be if it did not apply for inflorescences bearing only unfertilised ovules or a mixture of unfertilised ovules and abnormal fruits. We might expect these inflorescences to be shed sooner, and our analysis would then overestimate the proportion of ovules producing mature fruits. There is, however, some evidence to suggest that such an effect, if it exists, is probably small; our results show that just over half (54.5%) of individuals with fruit-bearing inflorescences also had older inflorescences with only unfertilised flowers, indicating that even these unfertilised inflorescences may persist for many years. We therefore base our analysis on the assumption that all inflorescences live for the same time span of approximately seven years, although further research is needed to determine a more accurate estimate.

Does soil nutrient availability affect flower production in *Lodoicea*?

Both N and K are important for flower production in *Lodoicea* on Praslin. Based on high nutrient reabsorption before leaves are shed, there are indications that both N and P are important limiting nutrients for *Lodoicea* (Edwards *et al.* 2015), though the significance of K as a limiting factor was previously unknown. The importance of nutrients for flower production has been demonstrated in other species (Winn 1991; Ne'eman *et al.* 2006).

Does pollen availability influence fruit production?

Fruit set was strongly limited by the degree of isolation of females from males, particularly in closed forest (Table 2e), where there was huge variation in fruit set (Table 1). The presence of fruits was also reliant upon the female growing in closed forest (Table 2c). Our individual correlations also showed the number of males within 10 m to be important for fruit production; however, our best-fitting GLM models selected the most parsimonious final variables and could have excluded other relevant variables. A shorter male-female distance facilitates greater pollen transfer by either wind or biotic pollinators, and although the *Lodoicea* pollen dispersal mechanism is uncertain, it is likely that endemic geckos play an important role. Two potential candidate gecko species, the day gecko (*Phelsuma sundbergi*) and the giant bronze gecko (*Ailuronyx trachygaster*), are specialised on *Lodoicea* forest, and rarely found or absent from degraded shrub vegetation (Noble *et al.* 2011; Gerlach & Ineich 2006). The smaller numbers of pollinators, along with lower densities of male trees (Ågren 1996) may contribute towards the reduction of pollen quantity and/or quality in pollination, and hence the reduced fruit set in degraded shrubland (Aguilar & Galetto 2004). Additionally, the dioecious nature of *Lodoicea* may predispose the species to pollen limitation (Wilson & Harder 2003).

Factors leading to the production of abnormal fruits

Abnormal fruits could be found on over half of all *Lodoicea* surveyed. Abnormal fruits were highly aggregated on certain individuals, often resulting in little or no normal fruit. Below, we discuss several non-mutually exclusive hypotheses that could explain their presence.

Ovules could be aborted during an early stage of development in order to reduce the plant's investment into fruits, as a response to inadequate resources (Lloyd 1980; Stephenson 1981; Verdú & García-Fayos 1998). Some trees consistently aborted seeds, which would have required resources to have been low over several years, even in dense forest. Aborted fruits were more common in degraded shrubland, despite nutrient levels being similar in both habitat types; indicating soil nutrients are unlikely to play an important role. One limitation of our method is that we only measured soil nutrients once, although some temporal heterogeneity in soil nutrient status and pollen availability over the years is likely (Gibson 1986).

An alternative explanation is that abnormal fruits reflect a genetic effect, whereby some genotypes are either more susceptible to parthenocarpy (i.e. the development of unfertilised fruit, which may or may not have been pollinated or otherwise stimulated to grow) (Simmonds 1952; Gorguet 2008) or abnormal meiosis (Varoquaux *et al.* 2000). However, abnormal fruits are more common in isolated trees, and there is no evidence to suggest these plants are genetically different. Pests or disease could also cause fruit abortion. Stenospermocarpy (i.e. the abortion of fruit after fertilisation of the ovule) has been associated with fungal infections (Berry 1960), but there was no evidence of diseases affecting fruits of *Lodoicea* in our study. Rats are a fairly common pest on Praslin, and bite marks have been observed on female flowers and fruits, but there is no evidence to suggest that rats are more common in open habitat, nor that they would non-uniformly affect specific trees.

The absence of pollination and the subsequent abortion of unfertilised ovules is a more plausible explanation for abnormal fruits. Abnormal fruits were more likely to occur with increasing isolation distances of females to males (Table 2f), although certain trees in dense forest appear to be affected over many years. Historically, *Lodoicea* forest was very dense and pollinator species were more abundant. Now, particularly in highly fragmented habitats, biotic pollinators need to move greater distances between male and female trees.

Another credible idea is that pollen received by females was too closely-related, or otherwise incompatible. Indeed, Hameed (2016) recently showed the importance of the condition and source of *Lodoicea* pollen through hand-pollination experiments. In *Lodoicea,* genes are frequently transferred short distances among male and female pairs (Chapters 2 & 3); therefore it is unlikely that inbreeding results in abortion of fruits, in the absence of any other interacting external factors. However, if nutrients were limiting, the maturation of the most fit, or genetically diverse ovules could be prioritised over the less fit ovules (Janzen 1977; Stephenson 1981; Helenurm & Schall 1996). Unfortunately, the compatibility of pollen grains reaching a particular female flower is virtually impossible to assess (de Nettancourt 1977). In this study we also show that a shorter distance to the nearest male results in a significantly greater fruit set (Table 2e), which runs counter to this hypothesis. It is possible, however, that some females consistently aborting fruits regularly received pollen from an incompatible male, regardless of the isolation distance.

At the Nong Nooch Botanical Garden in Thailand it was found that abnormal fruits only formed after pollination (Anders Lindström, pers. obs.), which would rule out parthenocarpy as a cause. However, stenospermocarpy remains a possibility, or some other environmental (Gustafson 1937; Verdú & García-Fayos 1998) or hormonal effect (Mesejo *et al.* 2014), or even a combination of these factors. Whatever the cause, abnormal fruit production is certainly a major factor in reducing reproductive success in *Lodoicea*, and further field studies are needed to ascertain the cause and its consequences for fitness.

Fruit mass and size variation

Lodoicea exhibits great plasticity in seed size (Fig. 4, S1, S2), exceeding that of any other palm (Moegenburg 1996) or plant species documented (Thompson 1984). As there are higher costs to producing larger seeds, seed mass is likely to be negatively related to local environmental nutrient availability (Vaughton & Ramsey 1998).

Does anthropogenic habitat disturbance influence female reproductive success?

Anthropogenic disturbance appears to have a limited effect on production of inflorescences and flowers in *Lodoicea.* Similar levels of nutrients were found within 1 m of mother trees in both natural closed forest and degraded shrubland, resulting in the production of similar numbers of inflorescences and flowers. It should be noted, however, that some sites in degraded shrubland could not be sampled due to the rockiness and lack of soil depth, resulting in the exclusion of the lowest nutrient samples, and thus overestimation of the mean available nutrients. Soil nutrient levels are also unknown for distances further than 1 m from females, that do not benefit from the effective water and nutrient funnelling mechanism of *Lodoicea* (Edwards *et al.* 2015).

Conversely, pollen limitation in *Lodoicea,* resulted in significantly lower fruit production overall, and possibly higher abnormal fruit production in degraded shrubland. This is likely to be a consequence of anthropogenic forest degradation. Most male trees produce copious amounts of pollen throughout the year, making it unlikely that pollen limitation is common in closed forest where there is a high density of both male trees and pollinators (Edwards *et al.* 2015). Furthermore, hand-pollinated flowers result in an almost 100% fruit set and very often, in two or three seeds per fruit (FF-D, pers. comm.), supporting this idea. Indeed, it appears that average fruit production in 1866 was higher than it is today (around four or five per inflorescence, max. 11; Ward 1866). Many evolutionary theories predict that plants evolve to reduce pollen limitation, either by the attraction of pollinators (Haig & Westoby 1988), the reduced reliance on pollinators (Lloyd 1974) or the evolution of sexual reproductive traits (e.g. monoecy). However, *Lodoicea* populations were severely reduced and fragmented only very recently (Lionnet 1976) - within just a single generation of this slow-growing species -, so evolutionary responses to change in habitat conditions would not be possible.

Fragmentation caused by forest clearance and fires not only reduced the numbers and densities of reproductive adults, but highly fragmented populations are also less attractive to pollinators (Sih & Baltus 1987), some of which are endemic to *Lodoicea* forests. The over-harvesting of seeds also severely limits natural regeneration. Although we did not study trees that showed signs of poaching, it cannot be ruled out that some fruits had been removed from trees, especially in the unprotected, degraded habitat where the threat was higher.

Management recommendations

Our results suggest that the systematic collection, removal and translocation of *Lodoicea* seeds should be avoided. Although fruit poaching is still a huge threat to this ecologically, economically and culturally important species, as many regenerating seeds as possible should be left in the forest to facilitate *Lodoicea* forest natural regeneration, particularly in protected areas. Females in closed forest produced many more fruits than those in degraded shrub, where abnormal fruits were more common. Therefore, management should first aim to maintain the quality of, and to restore closed *Lodoicea* forest as opposed to sparsely planting across larger, degraded areas (as was previously carried out on Curieuse Island in the early 2000s). The conservation and promotion of local pollinator communities will also be crucial for reducing future pollen limitations and increasing fruit production.

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SUPPLEMENTARY MATERIAL

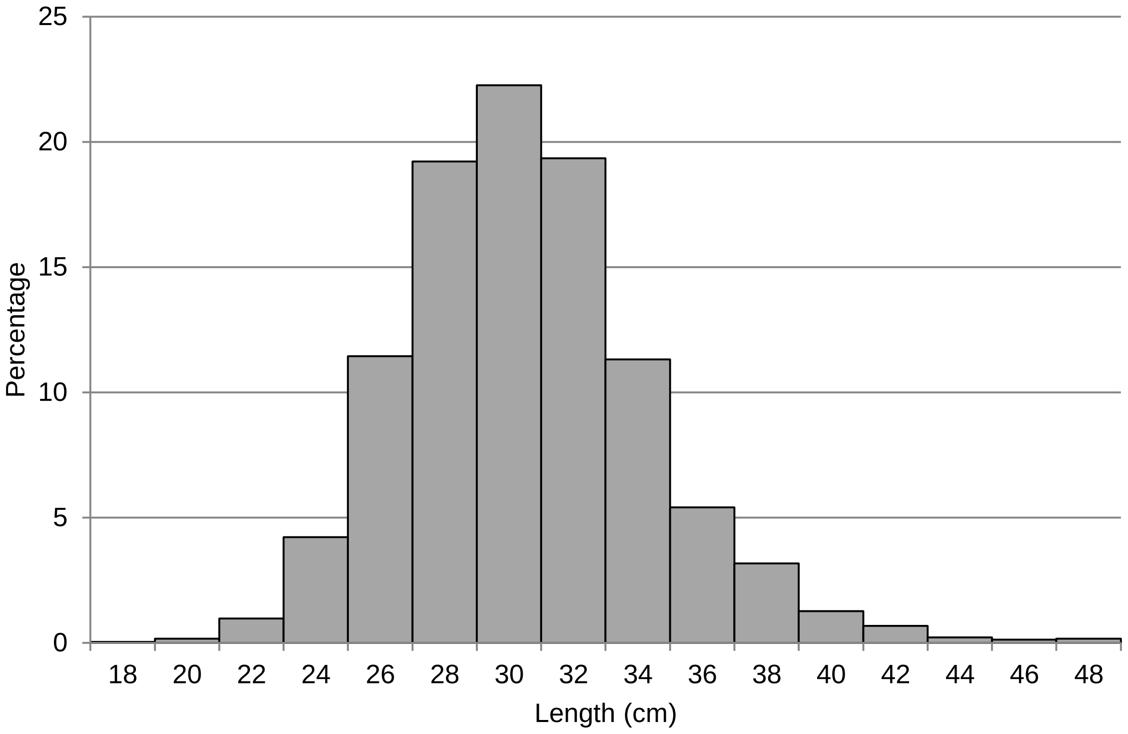

Fig. S1 Frequency distribution of the lengths (cm) of *Lodoicea maldivica* seeds (*N* = 2368).

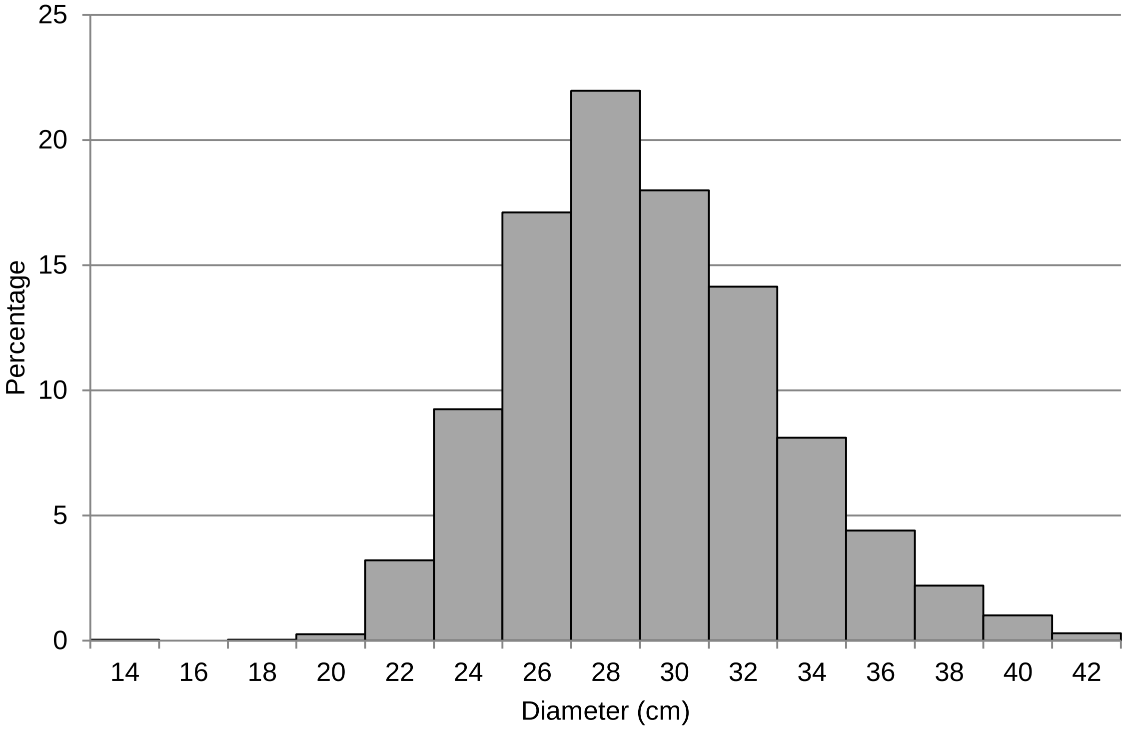

Fig. S2 Frequency distribution of the diameters (cm) of *Lodoicea maldivica* seeds (*N* = 2368).

APPENDIX

Appendix 1. Preparation of reagents.

NH_4^+ salicylate reagent

The salicylate reagent solution to assay for NH_4^+ was prepared by adding 0.05 g sodium nitroprusside, 13 g sodium salicylate, 10 g sodium citrate and 10 g sodium tartrate to 100 ml dH_2O. The hypochlorite reagent was made by dissolving 6 g sodium hydroxide in 100 ml dH_2O and 2 ml sodium hypochlorite. 200 µl each reagent were added to 800 µl sample in cuvets. After 60 min, absorbance was determined at 650 nm using a V-1200 Spectrophotometer (VWR International GmbH, Dietikon, Switzerland), and readings compared to standard solutions (0-3 ppm; VWR International GmbH). Sample filtrates were diluted with 2 M KCl to give values in the linear range of absorbency.

NO_3^- vanadium reagent

The vanadium reagent to assay for NO_3^- was prepared by dissolving 0.5 g vanadium (III) chloride, 0.2 g sulfanilamide and 0.01 g N-(1-naphthyl)ethylenediamine dihydrochloride in 200 ml 0.5 M HCl. 1000 µl reagent was added to 45 µl sample in cuvets. Absorbencies were read at 540 nm after 6 h, and regressed against standard solutions (0-30 ppm; VWR International GmbH). Sample filtrates were also diluted when necessary. N adsorption rates were calculated (µg N/g resin/day; hereafter referred to as N), using the means of the 0.5 and 1 m sampling distances from the females.

CHAPTER 5

Identification of sex-linked markers for coco de mer (*Lodoicea maldivica)* and their application in exploring sex ratios of non-mature plants

with Peter J. Edwards, Christopher N. Kaiser-Bunbury, Mathias Scharmann, Frauke Fleischer-Dogley and Chris J. Kettle

ABSTRACT

Globally about 6% of all flowering plants are dioecious, meaning that each individual is functionally either male or female. *Lodoicea maldivica* is a long-lived dioecious palm in which immature plants are phenotypically similar while adults are sexually dimorphic. Most adult populations in the Seychelles exhibit biased sex ratios; though it is unknown whether this is a result of different numbers of the two sexes being produced or subsequent differences in mortality. In this study we applied a ddRAD approach to identify sex-linked markers in *Lodoicea,* enabling us to reliably determine the gender of non-mature individuals. We screened 589 non-mature individuals at these markers to explore sex ratios across life stages in natural *Lodoicea* sub-populations. Each of the two sex-specific markers resulted in the amplification of a male-specific band (Lm123977 at 405 bp and Lm435135 at 130 bp), suggesting that *Lodoicea* has a mammal-like sex determination system (XX female and XY male). We estimate the physical size of a potential contiguous Y-chromosomal region to be very short, at between 7 and 470 kb. Our study of four sub-populations of *Lodoicea* on the islands of Praslin and Curieuse revealed that the two sexes were produced in approximately equal numbers, and that there was no significant deviation from a 1:1 ratio before the adult stage. The reasons for the biased adult ratios are probably complex and vary among sites. Levels of genetic diversity and inbreeding were similar

between male and female *Lodoicea* in all sub-populations and cohorts. We discuss the results in the context of the life history, ecology and management of *Lodoicea*.

INTRODUCTION

Around 6% of all flowering plants are dioecious, meaning that each individual is functionally either male or female (Renner 2014). Dioecy occurs in many families of flowering plants, and even within genera of otherwise monoecious species, indicating that the condition has evolved independently hundreds or thousands of times (Charlesworth 2002; Janousek & Mrackova 2010; Renner 2014) but in evolutionary terms is usually short-lived (Lewis 1942). Given this scattered distribution, it is not surprising that the genetic mechanisms underlying dioecy vary widely. The commonest is probably the XY system, in which an X chromosome pairs with a Y chromosome bearing the genes associated with maleness. Degeneration of the Y chromosome results in the suppression of recombination between (at least part of) the sex chromosomes, ensuring that gametes receiving a Y chromosome are male. In some cases, the Y chromosome is different in appearance from the X chromosome (usually smaller, although see Ming & Moore 2007), and the sex chromosomes are said to be heteromorphic. Other evolutionary pathways for plant sex chromosomes include the ZW system whereby the female is heterogametic, and multiple sex chromosome systems where sex is determined by X/autosome ratios (e.g. in *Rumex* and *Humulus*; Parker & Clark 1991).

In theory, both the XY and ZW systems of sex determination should produce equal numbers of male and female progeny. However, biased sex ratios are not uncommon, and may arise for a number of ecological, genetic and physiological reasons. One of these is the intensity of pollination, with high intensities favouring female pollen (Stehlik & Barrett 2005, Stehlik *et al.* 2008). This effect arises because the accumulation of deleterious mutations in Y chromosomes (as a result of low crossing over rates) reduces the vigour of ‘male’ pollen tubes, so that the successful pollen is more likely to be female (Muller 1964). Similar effects may also occur when a diverse pollen pool is available (Stehlik & Barrett 2005; Stehlik *et al.* 2008); the so-called process of “certation” (Correns 1922). Male tree density (Pickup & Barrett 2013), relative distances of seed and pollen dispersal (de Jong *et al.* 2002) and environmental resources (Adams 2011; Vandepitte *et al.* 2010) can also affect sex ratios, as well as genetic factors such as inbreeding levels (Bailey & McCauley 2005; Barrett & Case 2006) and sex chromosome segregation distorters (Taylor & Ingvarsson 2003; Meiklejohn & Tao 2010).

The evolutionary significance of dioecy has long perplexed scientists (Darwin 1876). A dioecious system has the disadvantage that only half of the individuals in a population can produce seeds, which reduces overall reproductive output and potentially restricts the population's capacity to spread into new habitats. On the other hand, dioecy does ensure cross-fertilisation, though this benefit is only realised when compatible pollen is successfully transferred from a male to a female tree (Bawa & Opler 1975; Givnish 1982). If population densities are low (Arista & Talavera 1996) or sex ratios biased (Stehlik *et al.* 2008), the opportunities for pollination may be reduced, which can lead to reduced effective population sizes and increased genetic drift (Reed & Frankham 2003). These effects in turn may result in decreases in genetic diversity (Vandepitte *et al.* 2010), increases in mating between related individuals, or population divergence (Jump & Peñuelas 2006).

Many long-lived dioecious plants including a number of palms (Arecaceae) are economically or ecologically important (Henderson *et al.* 1995). For species bearing harvestable fruits, such as the palms *Borassus flabellifer* and *Phoenix dactylifera,* female plants are clearly of more value than males. In such cases, it would be more efficient if a plant's sex could be identified at an early stage, thereby avoiding the need to grow large numbers of unproductive male plants. However, determining the gender of young dioecious plants from their vegetative traits is rarely possible (Lloyd & Webb 1977; Delph *et al.* 1996), though traditional karyotype studies can be applied to determine the sex of species with heteromorphic sex chromosomes (Westergaard 1958). Recently, new methods using genetic markers have also been developed, though some of these are inconvenient, poorly reproducible, time-consuming to develop and use, or costly (reviewed in Milewicz & Sawicki 2013).

This paper concerns the dioecious coco de mer palm, *Lodoicea maldivica* (J. F. Gmel.) Pers., which is an endemic palm of two small islands in the Seychelles, and a species of both economic and conservation importance. *Lodoicea* belongs to the tribe Borasseae (Arecaceae) (Dransfield *et al.* 2008), which includes several palm species with a Gondwanaland distribution, and in which dioecy is clearly an old-established trait. Having a method to determine the sex of non-mature *Lodoicea* would be useful to support the sustainable management of the species and to advance our understanding of its reproductive ecology. *Lodoicea* is a large, long-lived palm (~350 years, Savage & Ashton 1983; or older, Polunin & Procter 1973) that takes at least 20 years to reach reproductive maturity (Edwards *et al.* 2002). Female trees of this extraordinary species produce the largest seeds (mean 8.5 kg; Chapter 4) in the plant kingdom. Thanks to their unusual form, these seeds (nuts) are highly sought after as souvenirs, and fetch a price of

€190–450 each (Rist *et al*. 2010). Together, the sale of seeds and fees paid by visitors to the Vallée de Mai UNESCO World Heritage Site (Seychelles Islands Foundation 2009 unpubl. report) make a significant contribution to the Seychelles economy.

Previous studies have shown that adult populations of *Lodoicea* often exhibit biased sex ratios, but it is unclear whether this bias is due to unequal numbers of males and females being 'born' or to differential rates of mortality subsequently (Fleischer-Dogley 2006). In this study we present a new molecular method for identifying the sex of immature *Lodoicea.* We then apply this method as part of a study of sex ratios in sub-populations of *Lodoicea*, and investigate how these ratios change with age. We also explore whether levels of genetic diversity and inbreeding differ among the sexes, possibly as a result of the distribution of individuals or biased sex ratios (Nosrati *et al.* 2012). Ultimately this study will contribute important insights into the growing knowledge base on genetic and environmental factors influencing sex determination in dioecious plants.

MATERIALS AND METHODS

Study area and species

Lodoicea maldivica is a remarkable palm endemic to the Seychelles (lat. 4°S, long. 55°E). Prior to the discovery of Praslin in 1743 (Bailey 1942), *Lodoicea,* of the tribe Borasseae (Dransfield *et al.* 2008), grew in dense monodominant stands that covered both Praslin (37 km^2) and Curieuse (2.73 km^2) (Brayer du Barre 1773, quoted in Fauvel 1909). However, due to exploitation for timber and the increasing demand from tourists for nuts, as well as several serious fires, the *Lodoicea* forests have recently become severely degraded, and there is now very little natural regeneration (Rist *et al.* 2010). The species is classified as endangered (Fleischer-Dogley *et al.* 2011) and the only remaining large populations are in the southern part of Praslin (where areas are still partially connected) and on Curieuse. In the north of Praslin and some areas on Curieuse, the species persists mainly as isolated individuals or in small patches, in scrubby habitat. The reproductive structures of the two sexes are very distinct (Fig. 1). Females produce inflorescences that bear between one and thirteen (Chapter 4) of the largest flowers of any palm (~ 5 cm diameter), on a zigzag rachilla (Fig. 1a.). Male palms bear large catkins, 1.2–1.8 m long (Corner 1966) and 8–10 cm wide, consisting of 60-70 spirally-arranged fragrant, yellow flowers embedded in leathery bracts (Fig. 1b).

Fig. 1. *Lodoicea maldivica* inflorescences. **(a)** Female with fruits and unfertilised flowers. **(b)** Male catkin.

Discovery of *Lodoicea* sex-linked markers

Sequencing and bioinformatics

We identified potential sex-linked genetic markers using the approach of Scharmann *et al.* (in prep.). Genomic DNA was extracted from the leaf tissue of 20 male and 20 female adult *Lodoicea*, following an optimised version (Morgan *et al.* 2016) of the DNeasy® 96 Plant Kit (Qiagen, Hombrechtikon, Switzerland) manufacturer's protocol. The genomes of the 40 individuals were sequenced using a ddRAD protocol (Peterson *et al.* 2012), with the restriction enzymes ecoRI and TaqI. The library with 40 individuals was sequenced in a single Illumina HiSeq 2500 lane for 136-bases single-end reads. Raw reads were de-multiplexed and quality-filtered (the entire read was discarded if quality dropped below Phred 20 in any window of 15% read length) using process_radtags.pl from the Stacks pipeline (Catchen *et al*. 2013). Reads were mapped (following a customised dDocent-like pipeline (Puritz *et al.* 2014)) against six different *de novo* reference assemblies, in order to reduce the chance of false positives caused by arbitrary choice of the assembly method. Three alternative single-end references, using the reads of all 40 individuals, were assembled with identity cutoffs 0.8, 0.9, and 0.95 in clustering steps (vsearch, https://github.com/torognes/vsearch) and cd-hit-est (Li & Godzik 2006). Additionally, three alternative paired-end references were assembled (rainbow, Chong *et al.* 2012) using the reads of one male and one female sequenced on the MiSeq (identical library protocol); this was done with reads trimmed to 146 bases for identity cutoffs 0.9 and 0.95 (cd-hit), and also with reads trimmed to 140 bases for identity cutoff 0.95. Single-end reads from the 40 individuals were mapped to each reference and formed the basis of the sex-specificity scan. Each individual was represented by an average of 2.9 million filtered reads (min. 370,000, max. 4.9 million), of which between 59–89% (depending on the reference), could be mapped using bwa-mem (Li 2013), at quality ≥ 1. Presence/absence statistics (samtools, Li *et al.* 2009) for all individuals and reference contigs were then subjected to the permutation procedure of Scharmann *et al.* (in prep.). This algorithm separates real biological genomic presence/absence from stochasticity, yielding a list of contigs that, with high confidence, occur in one of the two sexes exclusively (with bootstrap support). We also observed a divergence in counts of male and female-specific candidates with increasing stringency, which is characteristic for organisms with sex chromosomes (Scharmann *et al*. in prep.).

For all six *Lodoicea* reference assemblies we observed a statistical signature characteristic of a male-heterogametic system (Y-chromosome), although the Y-specific chromosomal region appeared to be extremely small. Consequently, we identified male-specific contigs suitable for a molecular sexing assay. From each reference assembly, we retained all contigs that received ≥ 50% bootstrap support for male specificity at stringency level ≥ 3. We enhanced the sequence length of these contigs by blasting for 100% identical and full-length aligned matches in the unassembled (but quality filtered) 150-bases paired-end reads from the MiSeq run. The non-redundant candidate male-specific contigs (cd-hit-est at similarity 1.00) were ranked according to the cumulative support they received (sum of the passed stringency levels over all alternative reference assemblies), and the top 15 were used for primer design. Detailed results for each scan can be found in Appendices 1–3 (read and mapping statistics per individual, candidate contig figures, list of contigs with > 50% bootstrap support).

Molecular sexing assay

Male sex-linked nuclear markers were designed for *Lodoicea* using Primer3web 4.0.0 (Koressaar & Remm 2007; Untergasser *et al.* 2012). We tested 11 potentially promising primer pairs with PCRs using eight males and eight females, and the most reliable two pairs (Table 1) that amplified only in males, were optimised. PCR amplifications used forward primers labeled with M13 tails (5′-TGTAAAACGACGGCCAGT-3′) at the 5′ ends (Schuelke 2000). Amplifications were performed at an 11-µl final volume of 1X PCR Buffer (colorless Flexi GoTaq PCR buffer), 0.2 mM DNTPs, 1.5 mM $MgCl_2$, 0.025 U/µL *Taq* Polymerase (all Promega Corporation, Zurich, Switzerland), 0.18 µg/µL bovine serum albumin (BioConcept, Allschwil, Switzerland), 1 µL DNA, 0.08 µl forward primers with M13 tails, 0.32 µl reverse primers and 0.32 µl M13-primer universal tails labeled with either FAM (Lm123977) or ATTO565 (Lm435135) (Microsynth, Balgach, Switzerland). Touchdown PCRs were carried out on a Bio-Rad Dyad Cycler (Bio-Rad Laboratories, Hercules, California, USA) with the following conditions: 94°C for 5 min; 5X (denaturation 94°C/30 s, starting annealing temperature 62.5°C (Lm123977) or 60.5°C (Lm435135)/45 s, decreasing by 1°C/cycle, extension 72°C/30 s); 20X (denaturation 94°C/30 s, annealing 57.5°C (Lm123977) or 56.5°C (Lm_435135)/45 s, extension 72°C/30 s); 8X (denaturation 94°C/30 s, annealing 53°C/45 s, extension 72°C/30 s); final extension 72°C/10 min and subsequent storage at 10°C.

Table 1. Characteristics of the two male sex-linked loci in *Lodoicea maldivica*[a].

Locus	GenBank accession no.	Primer sequences (5’-3’)	Size (bp)[b]
Lm123977		F: GCCGGACCAACAAAATGTG	405
		R: CATTTACGATCCACACCAAAAGT	
Lm435135		F: TTCAAATATCAGCTTCACAAGTATTTT	130
		R: TTTCCAATCACTTTAGAAGACACG	

[a]Values based on samples collected from eight males in the Vallée de Mai and Fond Peper, Praslin.

[b]Allele sizes include M13 tail (5′-TGTAAAACGACGGCCAGT-3′) attached to the forward primer (as described by Shuelke 2000).

For a subset of eight samples of each sex, band sizes for each marker were quantified via fragment analysis. PCR product (3 µl) from each primer pair were combined, and added to 10 µl HIDI formamide and 0.15 µl GeneScan 500 LIZ Size Standard (Applied Biosystems). Samples were denatured for 3 min at 92°C then run on an ABI 3730xl automatic capillary sequencer (Applied Biosystems, Waltham, Massachusetts, USA). Electropherograms were scored with GeneMarker 2.6.0 (SoftGenetics, State College, Pennsylvania, USA). Marker Lm123977 produced a strong peak (~ 9000 RFUs) at 405 bp only in the males, and another small peak (~

400 RFUs) at 81 bp that amplified in males and females. Marker Lm435135 produced a peak (~ 8000 RFUs) in the males at 130 bp and a smaller peak (~ 300 RFUs) in both sexes (Table 1).

Gel electrophoresis using a 1.6% agarose gel with a 100-bp ladder (both Promega) was used to assess the accuracy of the sex-linked markers (Fig. S1). For this, we selected a subset of known female and male adults from across the geographic range. The adult samples (DNA extracted as above) were screened with the two sex-linked markers, along with negative controls and a positive control for each DNA sample (primer pair Lm2407 (Morgan *et al.* 2016)). Females did not amplify 100% of the time (*N* = 95 for both primers), and males amplified 99% of the time (Lm123977 *N* = 105; Lm435135 *N* = 98). The same male sample failed to amplify with both primer pairs. DNA samples of all *Lodoicea* offspring (see ‘sample collection’ below) were screened using the two sex-linked markers, and positive and negative controls. Offspring sex assignments were determined with gel electrophoresis, and these were used for all future analyses.

Sex ratios in sub-populations of *Lodoicea maldivica*

Sample collection and genotyping

Leaf samples from across the whole of *Lodoicea*’s natural range (Fig. 2) were collected for DNA extraction. Plants were classified according to their growth stage: ‘adults’ were reproductive individuals producing flowers, ‘immature’ were non-reproductive individuals with a trunk, ‘juveniles’ possessed more than two leaves, ‘seedlings’ had one or two leaves and ‘young seedlings’ were plants that had been weighed and measured as seeds in 2013, having been extracted from freshly-fallen fruits, and allowed to germinate close to where they were found. We use the term ‘offspring’ to refer to all non-mature plants.

We sampled offspring from four naturally regenerating dense clusters of individuals within each of the four main sub-populations: on Praslin (Vallée de Mai (VdM), Fond Peper (FP) and Fond Ferdinand (FF)), and on Curieuse (CU) (*N* = 493; the sex of only one individual could not be reliably determined). Additionally, we sampled offspring from locations in the northern part of Praslin (Cherie Mon and Zimbabwe) and other areas outside of clusters in VdM (*N* = 48). We also sampled the bayonets (first leaf spikes) or first leaves of young seedlings in VdM and FP (*N* = 49) (total offspring *N* = 589), as well as leaf tissue from potential mother (*N* = 100) and father (*N* = 659) trees in the four sub-populations.

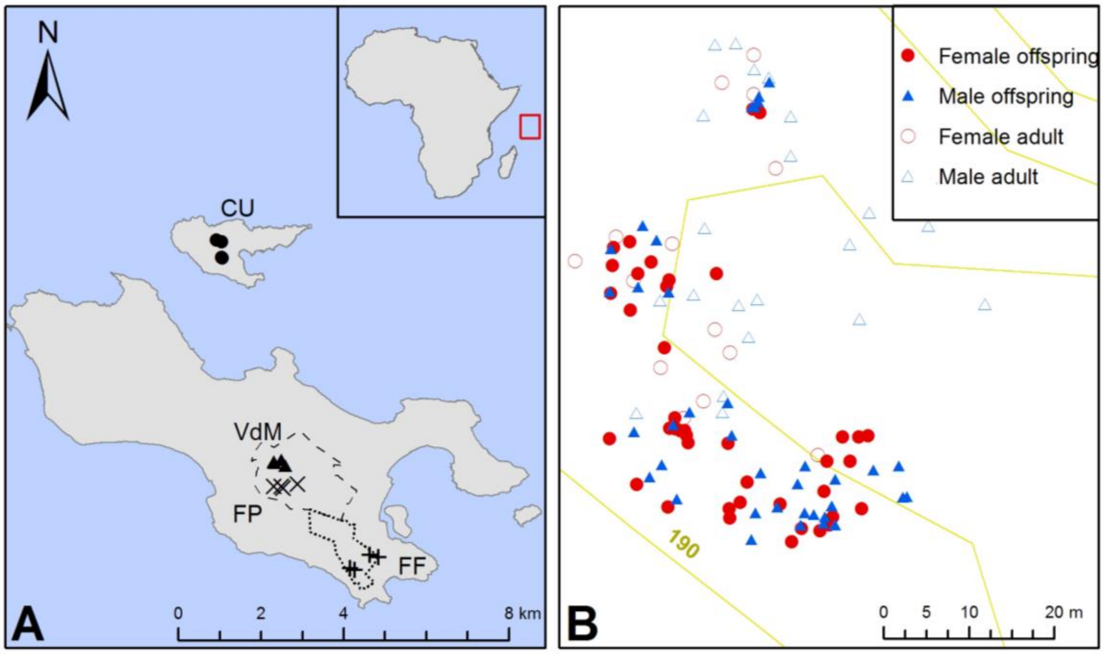

Fig. 2. Locations of sampled *Lodoicea maldivica* on the Seychelles. **A.** Centres of clusters on Praslin, indicated by: triangles = Vallée de Mai (VdM), 55°44'11"E, 4°19'43"S; **×** = Fond Peper (FP), 55°44'17"E, 4°20'01"S; **+** = Fond Ferdinand (FF), 55°203 45'39"E, 4°21'02"S, and circles = Curieuse (CU), 55°43'25"E, 4°16'45"S). The dashed and dotted lines indicate Praslin National Park and Ravin de Fond Ferdinand Nature Reserve, respectively. **B.** Distribution of male and female offspring and adults in the VdM 3 and 4 clusters.

DNA was extracted using the DNeasy 96 Plant Kit (QIAGEN, Hombrechtikon, Switzerland). All offspring and adults were genotyped using 12 polymorphic microsatellite loci (Morgan *et al.* 2016). Genotypes of offspring within clusters, and adults were available from Chapter 2, but offspring outside of clusters and young seedlings were genotyped for this study (see Appendix 2 for allele frequencies of samples additional to those included in Chapter 2). Fragment analysis was carried out in an ABI3730 capillary sequencer (Applied Biosystems) using the LIZ 500 HD size marker, and scored using GeneMarker 2.6.0 (Holland & Parson 2011).

Exploring possible causes of biased sex ratios in Lodoicea

A census of adult *Lodoicea* conducted in 2004 found that sex ratios in most populations in the native range were biased (data from Fleischer-Dogley 2006, presented in Table 2). We tested the significance of the observed sex ratio biases using the exact binomial test (two-sided) with the function stats::binom.test v. 3.1.2 (R Core Team 2014) in the RStudio environment v. 0.98.1102 (RStudio Team 2015). This function tests whether the observed proportion of females differs from the expected proportion (0.5), with a confidence level of 95%. We also tested the effects of a number of ecological and genetic factors (see below) on the sex ratios of the offspring, in the same way (for female and male counts and total sample sizes for each group, see Tables 2 and S1).

Do offspring sex ratios vary among sub-populations or cohorts? We analysed whether the proportion of female offspring differed from the expected 0.5 within each of the four sub-populations, each of the four clusters within each sub-population, each of the age cohorts (young seedlings, seedlings, juveniles and immature plants), and overall.

Does pollen flow distance or pollen heterogeneity influence offspring sex ratios? We analysed the effect of pollen flow distance on the sex of the offspring. Parentage analysis was carried out using maximum-likelihood maternity and paternity assignment using CERVUS 3.0 (Marshall *et al.* 1998; Kalinowski *et al.* 2007), based on the multilocus genotypes of the offspring and adult *Lodoicea*. The putative mother trees were first identified with 95% confidence, and the parameters used in Chapter 2. When a mother could be assigned, a father was then assigned to the offspring using a lower confidence level of 80% (a trio-assignment is more robust than a pair assignment). Offspring were grouped according to the distance between the two parent trees (10, 20, 30, 40, 50, 100, 300 and 650 m). Minimum and maximum detected pollen flow distances were 4.2 and 637.5 m, respectively.

Subsequent analysis was based on the assumption that offspring with an assigned mother but no father, had been fathered by a tree outside the sampling area. The minimum radius of sampled males around a cluster was 80 m. We then re-grouped the offspring according to short- (≤ 80 m), and long-distance (> 80 m) pollination events. Long-distance pollination events included offspring with assigned parents > 80 m apart, plus those for which no father could be assigned.

As well as pollen flow distances, we also tested directly the effect of parental relatedness on the sex of the offspring produced. The pairwise kinship coefficient (*F*; Loiselle *et al*. 1995) was calculated for each parent-pair (SPAGeDI 1.4c; Hardy & Vekemans 2002), and the offspring grouped according to parental relatedness less than or greater than the overall median level (*F* ≤ 0.0654 or > 0.0654).

Do individual female plants produce offspring with biased sex ratios? We tested whether the proportions of female offspring produced by individual female palms deviated from the expected level. We used maternity assignments to match offspring to mother trees, to test sex production at the finest scale.

Does male plant density influence offspring sex ratios? As estimates of pollen availability, we recorded the distance of the mother tree to the nearest adult male, and the number of males within a 10 m radius of the mother. The correlation between each of these measurements and the proportion of female offspring assigned to each mother tree (number of offspring assigned to each mother ranged from one to 30, mean = 4.6), was tested using a Pearson's correlation with the R package stats::cor.test (R Core Team 2014).

Can seed size predict its sex? We tested the relationship between seed size (fresh weight and length) and the sex of the 49 seeds planted in 2013, using a Pearson's correlation.

Does genetic variation vary among gender? We explored whether there was selection for more outbred and genetically diverse female than male offspring. We compared among the sexes across all individuals sampled, within four distinct age cohorts (see description above), and within the juvenile age cohorts in each of the four *Lodoicea* sub-populations. The number of alleles per locus (N_A), the number of private alleles (P_A), and observed (H_O) and unbiased expected (uH_E) heterozygosities were estimated using GenAlEx 6.5 (Peakall & Smouse 2006). Mean private rarefied allelic richness over loci (Π_s) was calculated using HP-RARE 1.1 (Kalinowski 2005), to account for sample size differences. Allelic richness, adjusted for sample size (A_R), and inbreeding coefficients (F_{IS}) using 10,000 permutations were estimated in FSTAT 2.9.3.2 (Goudet 1995).

Does the sex ratio influence levels of genetic variation? We tested whether there was a relationship between the degree of sex ratio bias and the genetic diversity (N_a, A_R, P_A, $\boldsymbol{\Pi}_s$, H_o and uH_E) and inbreeding of offspring within each of the 16 clusters.

Table 2. *Lodoicea maldivica* sex ratios on Praslin and Curieuse. Female, male and total counts, observed proportion of females (significant values in bold), probability that the observed sex ratios deviate from the expected 1:1 (with a confidence level of 95%) are given. Categories include adult populations (complete census data from Fleischer-Dogley 2006), total offspring, and offspring: from the four main sub-populations, within each of the age cohorts (across all populations), produced from short- (≤ 80 m) and long-distance (> 80 m) pollination events, with relatively low and high relatedness (*F*) values of the assigned parents, and offspring assigned to two individual females that produced an excess of females.

Category	**Female**	**Male**	**Total**	**Prop. female**	***P* (2-tailed)**
Adults: population					
Vallée de Mai	623	818	1441	**0.432**	<0.0001
Praslin NP	653	428	1081	**0.604**	<0.0001
Praslin–private land	905	1031	1936	**0.467**	0.0045
Fond Ferdinand	675	705	1380	0.489	0.44
Curieuse	948	802	1750	**0.542**	0.0005
All offspring	309	280	589	0.525	0.25
Offspring: sub-population					
Vallée de Mai	112	110	222	0.505	0.95
Fond Peper	77	66	143	0.538	0.40
Fond Ferdinand	64	59	123	0.520	0.72
Curieuse	53	40	93	0.570	0.21
Cohort					
Young seedling	23	26	49	0.469	0.78
Seedling	28	26	54	0.519	0.89
Juvenile	247	213	460	0.537	0.12
Immature	11	15	26	0.423	0.56
Offspring: pollen flow distance (m)					
< 80	67	61	128	0.523	0.66
> 80	73	63	136	0.537	0.44
Parent kinship (*F*)					
≤ 0.0654	41	43	84	0.488	0.91
> 0.0654	44	39	83	0.530	0.66
Offspring from individual females					
Female 1 - Vallée de Mai	11	2	13	**0.846**	0.022
Female 2 - Curieuse	10	2	12	**0.833**	0.039

F = kinship coefficient ; Loiselle *et al.* (1995)

RESULTS

Lodoicea sex-linked genomic region

The sex-specific markers identified in this study allowed us to reliably determine the gender of non-mature *Lodoicea* plants. Each of the two markers resulted in the amplification of a male-specific band (Lm123977 at 405 bp and Lm435135 at 130 bp). This indicates that *Lodoicea* has a mammal-like sex determination system (XX female and XY male). Although no information on the genome size and karyotype of *Lodoicea* is available, we can make predictions using estimates from some of its closest relatives in the tribe Borasseae (Baker *et al.* 2009). These suggest that *Lodoicea* has a very large genome, with a moderate number of chromosomes (*Bismarckia nobilis*: 1n = 36, 1C = 2 Gb; *Borassus flabellifer*: 1n = 36, 1C = 8.4 Gb; *Latania lontaroides*: 1n = 28, 1C = 3.4 Gb, Bennett & Leitch 2012). Using the upper and lower values of these as bounds for the genome size of *Lodoicea*, together with the proportion of male-specific contigs at stringency level 10 (0.001– 0.0056%), and assuming that contigs are equally (randomly) spaced along the genome, our results suggest that *Lodoicea* does not have a sex chromosome heteromorphic system. We estimate the physical size of a potential contiguous Y-chromosomal region to be 7–470 kb long. This region (an estimated 0.01% of the whole genome size) would cover less than 1% of the length of a single chromosome, assuming 28– 36 chromosomes of similar size.

Application of markers to *Lodoicea*

Sex ratios of adults and offspring

All adult sub-populations except Fond Ferdinand showed highly biased sex ratios: Praslin National Park (which includes Fond Peper plus the wider area) and Curieuse had strong female biases (0.604 and 0.542; binomial test $P < 0.0001$ and $P = 0.0005$, respectively), whereas Vallée de Mai and private land on Praslin had significant male biases (0.432 and 0.467; binomial test $P < 0.0001$ and $P = 0.0045$, respectively).

In contrast, there was a slight trend towards a female bias in most offspring groups, but no significant deviations from 1:1 sex ratios were found overall (0.525; Table 2) or within any of the four sub-populations (female ratio range 0.505–0.570; Table 2) or 16 clusters (female ratio range 0.368–0.649; Table S1) (binomial test all $P > 0.05$). Equal sex ratios were also observed in all offspring age cohorts, from young seedlings to immature trees (female ratios between 0.423–

0.537, binomial test all $P > 0.05$). However, two female palms, one in VdM and one on CU, produced offspring with a significant female-bias (11/13 and 10/12 females, respectively; binomial test both $P < 0.05$; Table 2). Nothing unusual could be observed about these particular trees or their surroundings.

Effects of pollen flow distance and relatedness of parent-pairs

The distances between pairs of assigned mother and father trees of female offspring ranged from 4–638 m (mean 83 m), and for male offspring 1–604 m (mean 93 m). Binomial tests indicated that the ratios of females did not differ from the expected 0.5 in response to different realised pollination distances (Tables 2, S1; all $P > 0.05$). The range in kinship (*F,* Loiselle *et al.* 1995) of parent-pairs of female offspring ranged -0.1555–0.424, and in males -0.2121–0.3864, but the level of relatedness of the parent trees had no effect on the sex of the resultant offspring (binomial test both $P > 0.05$).

Effect of male tree density

The distances between mother trees and the nearest adult male trees in this study ranged from 0.9–18.6 m. The number of males within 10 m of the mother tree ranged from 0–8. Pearson's correlations were calculated to analyse the relationships between the proportion of female offspring produced by mother trees and distance to the nearest male tree, and number of males within a 10 m radius. However, we found no relationships ($r_{56} = 0.252$, $P = 0.06$ for distance; $r_{56} = -0.177$, $P = 0.18$ for number of males; Fig. 3).

Seed size in predicting sex

Female seeds ranged in fresh weight from 6–12 kg, with a mean and SE of 8.9 ± 0.4 kg ($N = 23$). Male seeds ranged in weight from 3–13 kg, with a mean and SE of 8.4 ± 0.5 kg ($N = 26$; Fig. 4a). The average lengths of female and male seeds were 30.8 (± 0.5) cm and 30.3 (± 0.7) cm, respectively, and again with a larger range in males than females (Fig. 4b). We found no relationship between the mass or length of seeds and the sex of the plant (Pearson's correlation, both $P > 0.05$).

Genetic diversity and inbreeding

Levels of genetic diversity (N_a, A_R, P_A, Π_s, H_o and uH_E) did not differ between female and male offspring, whether for the entire sample, within cohorts, or between male and female juveniles within the sub-populations. Inbreeding levels (F_{IS}) were significantly greater than zero in all groups, but levels did not differ between males and females within groups (Table 3). Values of Pearson correlation coefficient calculated between female bias within offspring clusters and various measures of genetic diversity (N_a, A_R, P_A, Π_s, H_o and uH_E) and F_{IS} levels were all non-significant (P > 0.05, Fig. S2).

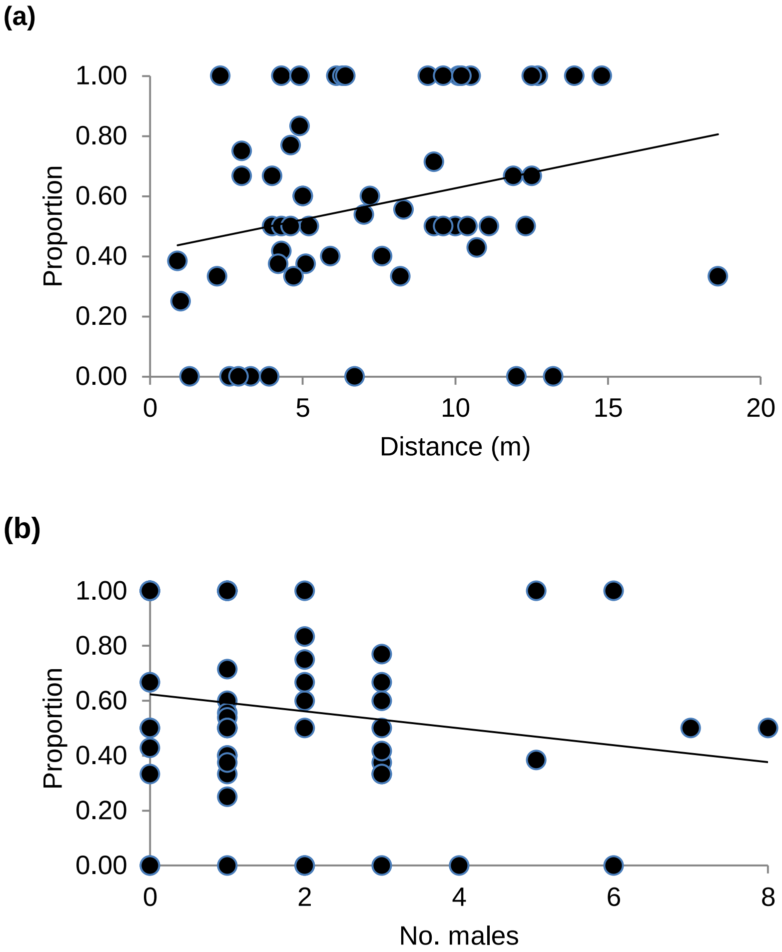

Fig. 3. Proportion of female offspring produced by each *Lodoicea maldivica* mother tree (*N* = 58), in relation to **(a)** the isolation distance of their mother tree to the nearest male *Lodoicea* (regression line of the non-significant relationship; *r* = 0.252, *P* = 0.06); and **(b)** the number of male *Lodoicea* within a 10 m radius of their mother tree (regression line of the non-significant relationship; *r* = -0.177, *P* = 0.18). Established offspring were assigned to mother trees using maternity analyses. Total offspring sample size *N* = 266. Number of offspring assigned to each mother tree ranged from one to 30.

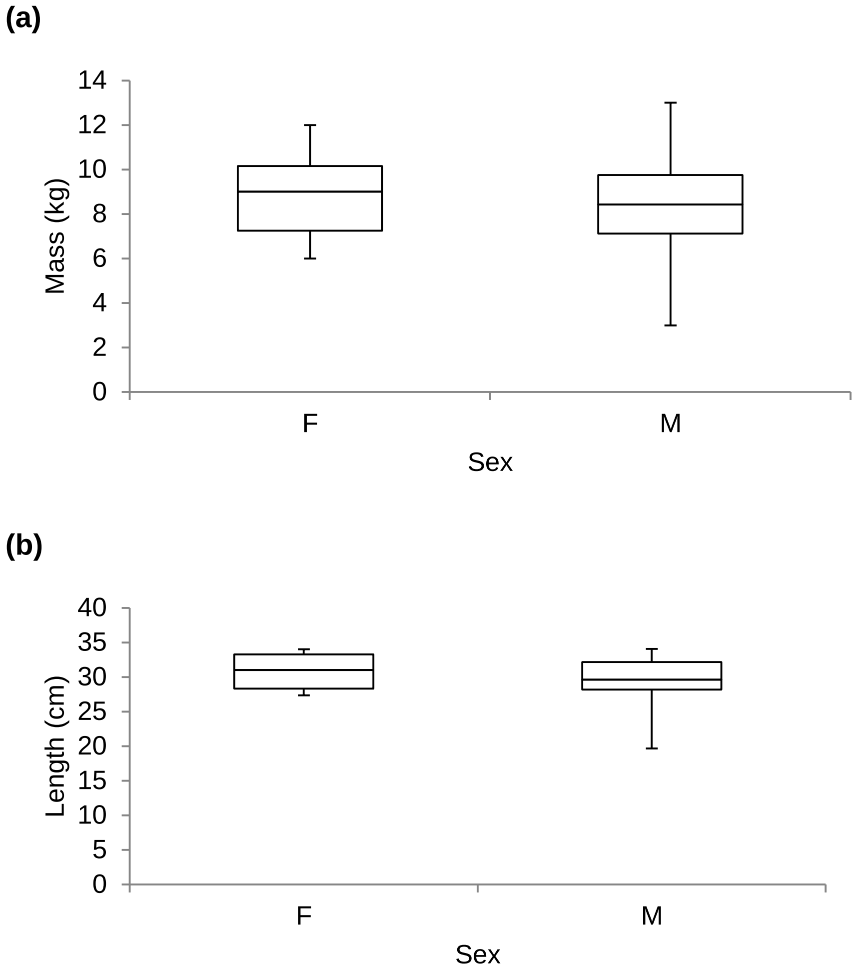

Fig. 4. Boxplots showing **(a)** masses (kg) and **(b)** lengths (cm), of female and male *Lodoicea maldivica* seeds (*N* = 49). First quartiles, medians and third quartiles indicated by boxes. Whiskers extend to minimum and maximum values. Seeds were weighed and measured in 2013 and sexed with two sex-linked markers (Lm123977 and Lm435135) using DNA extracted from the first bayonet or leaf, in 2015.

Table 3. Genetic diversity and inbreeding coefficient summary statistics for female and male *Lodoicea maldivica* offspring overall (Vallée de Mai, Fond Peper, Fond Ferdinand, Cherie Mon and Zimbabwe on Praslin, and Curieuse), offspring within age cohorts, and juveniles within the main sub-populations. Samples were collected across Praslin and Curieuse. N, sample size; N_a, mean number of alleles and its SE; A_R allelic richness and its SE; P_A, number of private alleles; Π_s, mean rarefied number of private alleles over loci plus SE; H_o, observed heterozygosity with SE; uH_E, unbiased expected heterozygosity with SE; F_{IS}, inbreeding coefficient and significance of deviation from zero.

		N_a	A_R	P_A	Π_s	H_o	uH_E	F_{IS}
All offspring	***N* 589**							
F	309	11.08 (1.45)	10.85 (1.44)	12	0.88 (0.24)	0.494 (0.051)	0.683 (0.049)	0.276***
M	280	11.08 (1.58)	11.01 (1.57)	12	1.03 (0.28)	0.503 (0.049)	0.684 (0.049)	0.264***
Cohorts	***N* 589**							
Immature F	11	5.42 (0.73)	5.21 (0.69)	1	0.25 (0.16)	0.415 (0.090)	0.684 (0.065)	0.407***
Immature M	15	6.25 (0.80)	5.28 (0.69)	1	0.26 (0.10)	0.493 (0.058)	0.678 (0.054)	0.281***
Juvenile F	249	10.42 (1.46)	5.38 (0.60)	6	0.22 (0.05)	0.494 (0.052)	0.682 (0.049)	0.276***
Juvenile M	215	10.25 (1.50)	5.50 (0.63)	6	0.27 (0.07)	0.500 (0.049)	0.684 (0.049)	0.269***
Seedling F	21	7.00 (0.81)	5.44 (0.60)	3	0.27 (0.06)	0.538 (0.058)	0.676 (0.054)	0.208***
Seedling M	24	7.25 (0.91)	5.57 (0.59)	0	0.27 (0.12)	0.525 (0.060)	0.686 (0.055)	0.240***
Seed F	30	7.25 (0.88)	5.25 (0.62)	1	0.13 (0.04)	0.496 (0.048)	0.678 (0.056)	0.272***
Seed M	28	7.00 (0.94)	5.10 (0.54)	5	0.27 (0.10)	0.512 (0.061)	0.663 (0.049)	0.232***
Juveniles within sub-populations	***N* 453**							
VdM F	66	7.67 (1.00)	6.79 (0.85)	1	0.06 (0.04)	0.485 (0.055)	0.650 (0.056)	0.256***
VdM M	62	7.42 (1.08)	6.78 (0.95)	1	0.09 (0.05)	0.471 (0.057)	0.647 (0.060)	0.273***
FP F	70	7.83 (1.10)	6.79 (0.93)	1	0.08 (0.04)	0.529 (0.054)	0.654 (0.050)	0.193***
FP M	57	7.58 (1.10)	6.77 (0.92)	1	0.12 (0.05)	0.553 (0.047)	0.657 (0.053)	0.160***
FF F	58	7.83 (1.16)	6.96 (0.96)	1	0.11 (0.05)	0.493 (0.054)	0.675 (0.051)	0.271***
FF M	53	8.17 (1.25)	7.27 (1.04)	1	0.21 (0.10)	0.488 (0.054)	0.689 (0.048)	0.295***
CU F	50	7.58 (0.84)	6.81 (0.72)	2	0.17 (0.10)	0.448 (0.065)	0.680 (0.035)	0.344***
CU M	37	6.83 (0.67)	6.64 (0.64)	2	0.18 (0.12)	0.494 (0.055)	0.691 (0.037)	0.288***

Note: F = female; M = male; VdM = Vallée de Mai ; FP = Fond Peper ; FF = Fond Ferdinand; CU = Curieuse.

*** $P \leq 0.001$

DISCUSSION

Using the two molecular markers, we were able to determine accurately the sex of young plants of *Lodoicea maldivica* from across the whole of the native range on Praslin and Curieuse. Although the sex ratios of most adult populations were strongly biased, we detected no such biases in the offspring. Also, we found no effects of the environment on sex ratios of non-mature plants. This strongly suggests that the two sexes of *Lodoicea* are produced in approximately equal numbers, as in the date palm, which is itself evidence for a genetic basis to sex determination (Daher *et al.* 2010). Deviations from a 1:1 ratio do not occur before adult stage, and the reasons for these later deviations are likely to be complex and to differ among sites. The use of these markers, along with pollination experiments in which quantities and diversity of pollen is carefully controlled could yield further insights into certation and sex determination processes in this species.

Lodoicea sex-linked markers

Although sex-determining genomic regions and pairs of homomorphic X/Y chromosomes have been found in the date palms (*Phoenix dactylifera*; Cherif *et al.* 2013, 2016; Mathew *et al.* 2014; Maryam 2016), we present here the first sex-specific molecular markers for a palm species that allows the determination of sexually immature males. Our results suggest that the Y-chromosomal region is exceptionally small, and the X- and Y-chromosomes of *Lodoicea* are therefore likely to be homomorphic (heteromorphic chromosomes are much less common in plants, though they do occur in genera including *Silene, Rumex* and *Cannabis* (Parker 1990; Westergaard 1958)). Further studies on the *Lodoicea* karyotype would be useful as a starting point for mapping and sequencing the sex-linked genes.

Besides *Lodoicea*, all other palms in the tribe Borasseae are strictly dioecious (Dransfield *et al.* 2008), though nothing is known about the sex determination systems. Cherif *et al.* (2016) showed that sex-related loci in the genus *Phoenix* are highly conserved and shared amongst species. This makes it likely that the Y-chromosomal system of *Lodoicea* is ancestral and also shared among the other members of the tribe. If so, the molecular sexing of *Lodoicea* might be applicable to other species, including the economically important *Borassus flabellifer*.

Lodoicea sex ratios

Sex ratios across populations and life stages

Most adult *Lodoicea maldivica* populations exhibit a significant bias in the male to female sex ratio. Until now there was no way to determine the sex of juveniles or if a biased sex ratio occurred early in the establishment of individual plants, or if male and female offspring are produced in unequal proportions. Our results detected slight tendencies towards a female bias in non-mature individuals from most clusters (11/16; Table S1), from sub-populations (3/4; Table 2), and when females grew further from the nearest male, but none was significant. Equal sex ratios were also found in all age cohorts from seedlings to trunked immature trees. In dioecious species, offspring sex ratios should typically be balanced due to negative frequency-dependent selection, providing both sexes are equally as costly to produce (Fisher 1930). This indicates that the production of the sexes in *Lodoicea* is balanced, though there may be small fluctuations due to the species' long generation time (Savage & Ashton 1983) and low reproductive output (Chapter 4). Sex biases due to differential mortality may begin to accumulate in younger cohorts but only become significant at the adult stage. Unfortunately, our data do not allow us to explore exactly when these biases become significant, though this is apparently not at the earliest reproductive stages.

Evidence for effects of pollination conditions and male density

For some dioecious species, it has been shown that the sex ratio of progeny is affected by the density of male individuals in the neighbourhood. One reason is that higher pollination intensities result in greater pollen tube competition, which may favour female pollen, which is likely to be more competitive and faster growing than male pollen (Stehlik & Barrett 2005, Stehlik *et al.* 2008). Conversely, a low pollen load would indicate there are few males reproducing (Taylor *et al.* 1999; Field *et al.* 2013), so in this case, the over-production of male offspring would be more beneficial. However, our results revealed no effects of pollen quantity upon sex ratios in *Lodoicea,* whether in terms of numbers of male trees or distance to the nearest male.

Various studies have shown that the degree of inbreeding can also influence sex determination in dioecious species (Bailey & McCauley 2005; Barrett & Case 2006). For example, a female bias may develop when both pollen and seed dispersal are limited, to increase the inbred population's relative contribution to the next generation. On the other hand, when seed dispersal is much more limited than pollen dispersal, which appears to be the case for *Lodoicea*, a male bias would reduce the chance of related individuals being close together and competing for the same resources (de Jong *et al.* 2002). *Lodoicea* across all sub-populations are relatively highly inbred with short-distance seed dispersal (Chapter 2) and relatively short pollen dispersal (Chapter 3), but this did not result in significant biases in female production.

Other environmental processes determining sex

The sex of some species is largely regulated by the environment. For example in the ‘temporally dioecious’ (Cruden & Hermann-Parker 1977) oil palm (*Elaeis guineensis*), water deficit, higher elevations and higher latitudes result in the production of more male flowers (Adams 2011), and defoliation-related stress results in higher female flower abortion (Corley 1973). Vandepitte *et al.* (2010) demonstrated that male-biased ratios in *Mercurialis perennis,* were associated with increased light penetration and greater growth of males. Water stress and population densities have also been found to affect sex ratio bias in other species (Lovett Doust *et al.* 1987). The degree of fragmentation differed between the two vegetation types on Praslin: dense closed forest and highly degraded shrubland. It is likely that environmental conditions (e.g. light, rainfall and nutrient levels) also differed, though we did not measure specific environmental conditions locally, and our sample size was small for *Lodoicea* growing in heavily degraded areas.

Sex ratios have also been shown to vary depending on the number of seeds produced within fruits. *Borassus flabellifer* demonstrated a female-bias in two-seeded fruits, but equal ratios in one- and three-seeded fruits and overall (George & Karun 2011). Only a small proportion of *Lodoicea* fruits produce two seeds (9.2%) and an even smaller proportion three seeds (0.03%; Chapter 4), so it would not be feasible to test this.

Seed size and sex relationship

We found no evidence that male and female *Lodoicea* seeds in this study were sexually dimorphic, as has been observed in *Rumex nivalis* (Stehlik & Barrett 2005). It could be hypothesised that differences in seed provisioning by maternal parents could occur, for example, to provide female seeds with a larger nutrient reserve to offset the higher metabolic load they will have later in life. In our study larger ranges in both length and mass were observed in male seeds, and a larger sample size could clarify any potential patterns not identified here. From a management perspective, the random harvesting and replanting of seeds should not inadvertently be altering sex ratios in the regenerating young cohorts.

Evidence for effects of sex on genetic diversity

Levels of genetic diversity and inbreeding were similar in male and female *Lodoicea* in all sub-populations and cohorts, although other studies have shown differences among the sexes (Nosrati *et al*. 2012; Heikrujam *et al.* 2015). The higher costs associated with reproduction in females of some species (Lloyd & Webb 1977; Cipollini & Stiles 1991; Obeso 2002), has been found to result in selection for outbred female progeny. We were unable to detect such an effect, possibly due to the high offspring survival rates in the species. The narrow range in the degree of female bias across clusters (0.368–0.649), together with the similarly high levels of genetic diversity across compared groups, might account for the lack of relationship between genetic diversity and sex bias. In a study of *Mercurialis perennis*, Vandepitte *et al. (*2010) found a significant relationship between genetic variation and sex ratio, but the ranges in both genetic variation and female ratio (0–0.85) were much larger than in our study.

Biased adult sex ratios

The sex ratio biases in adult populations were inconsistent in that Vallée de Mai and Praslin private land had more males, while Fond Ferdinand and Praslin National Park had more females. It could be argued that the VdM population has the most natural structure because it has been under protection longer than any other site in the Seychelles. In a 1983 study of *Lodoicea* in the VdM, Savage and Ashton recorded similar numbers of male and female palms entering maturity, although females started flowering at shorter heights. Numbers of females dropped off more rapidly than males at successively taller height classes; male palms reached up to 28 m tall, but the tallest female encountered was 18 m (Savage & Ashton 1983). This could be interpreted as the earlier death of female trees (Allen & Antos 1993; Purrington & Schmitt 1995).

In general, female-, rather than male-biased sex ratios are less common in dioecious species (Lloyd 1974), possibly due to higher susceptibility of females to environmental stress (Meagher 1981), or a trade-off between reproduction and survival (Wang *et al.* 2013). For example, heavy female *Lodoicea* crowns bearing many fruits are thought to be more vulnerable to wind-damage than male crowns (Savage & Ashton 1983). Another factor that could act simultaneously with earlier female death is delayed female reproductive maturity (Lyons *et al.* 1995). It is possible that females grow more slowly, and remain as immature trees for a longer period than males. This would lead to a positive association between the proportion of males and immature plants

(Lloyd 1973; Allen & Antos 1993). Identification of the sex of a larger sample of immature trees might elucidate the sex ratio patterns at this crucial development stage.

Male plants in dioecious species encounter less local resource competition due to longer distance pollen- than seed-dispersal (Taylor 1994). The limited seed dispersal distances and highly clustered natural growth patterns in *Lodoicea* (Chapter 2) result in high competitive stress on females that compete with their own progeny. It is therefore not surprising that the dense VdM population, where competition should be greater, has a male bias, whereas the more fragmented Curieuse has a female-bias. A female bias is expected when pollen and seed dispersal are both severely limited (de Jong 2002), and it has been shown that both seed and pollen dispersal are both short-distance, and more restricted on Curieuse than any sub-population on Praslin (Chapters 2 & 3).

Almost equal sex ratios were found in other dioecious tropical palm species, including *Chamaedorea tepejilote* (Oyama 1990) and *C. pinnatifrons* (Ataroff & Schwarzkopf 1992; as *C. bartlingiana*), *Mauritia flexuosa* (Urrego Giraldo 1987) and *Phytelephas seemannii* (Bernal 1998). The patterns of adult sex ratios across Praslin and Curieuse may not be entirely natural, but largely manipulated by humans. Unprotected sites such as state-owned lands on Praslin may have been subjected to higher levels of selective felling of one sex over the other: either felling of females for their superior higher-density wood (Edwards *et al.* 2002), or the preservation of females because of their capacity to produce economically and culturally important nuts. Human-mediated changes in natural adult sex ratios and increased habitat fragmentation may alter patterns of gene exchange, and reduce the contribution of individual males. This in turn may lead to an increase in mating between related individuals in future generations (Dubreuil *et al*. 2010).

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SUPPLEMENTARY MATERIAL

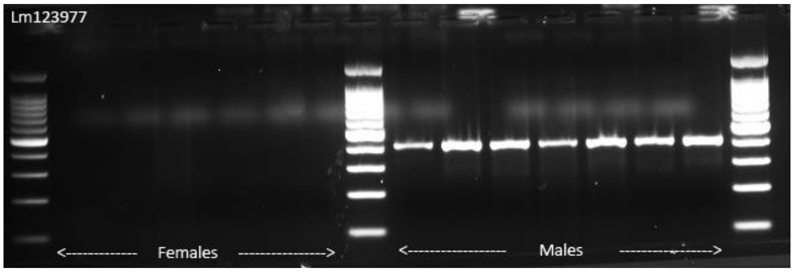


Fig. S1. Digital photograph of agarose gel after electrophoresis of female and male *Lodoicea maldivica* PCR products, amplified with the Lm123977 marker. DNA was stained with florescent GelRed™ (Biotium, Fremont, California, USA). 100 bp ladders shown.

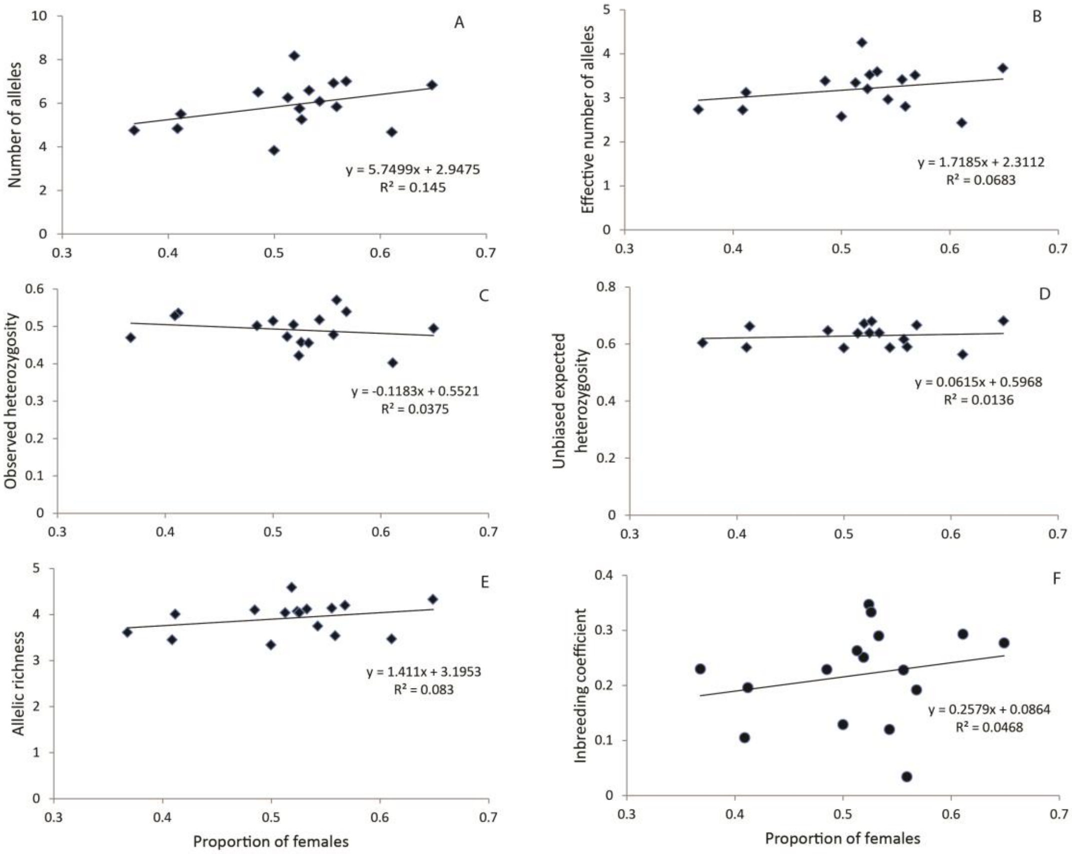


Fig. S2. Relationships between female ratio and genetic diversity and inbreeding in 16 *Lodoicea maldivica* clusters on Praslin and Curieuse (Pearson's correlations all not significant; $P > 0.05$). A: number of alleles; B: effective number of alleles; C: observed heterozygosity; D: unbiased expected heterozygosity; E: allelic richness; F: inbreeding coefficient (F_{IS}).

Table S1. *Lodoicea maldivica* sex ratios. Female, male and total counts, observed proportion of females, probability that the observed sex ratios deviate from the expected 1:1 (with a confidence level of 95%). Categories include: offspring from each of the sampled clusters within sub-populations on Praslin and Curieuse (offspring sampled for Chapter 2), and offspring grouped according to the pollen flow distance (using only assigned offspring, 80% confidence).

Category	**Female**	**Male**	**Total**	**Prop. female**	***P* (2-tailed)**
Cluster					
VdM1	24	21	45	0.533	0.77
VdM2	7	12	19	0.368	0.36
VdM3	25	20	45	0.556	0.55
VdM4	16	17	33	0.485	1.00
FP1	19	15	34	0.559	0.61
FP2	19	16	35	0.543	0.74
FP3	9	13	22	0.409	0.52
FP4	25	19	44	0.568	0.45
FF1	28	26	54	0.519	0.89
FF2	20	19	39	0.513	1.00
FF3	5	5	10	0.500	1.00
FF4	10	9	19	0.526	1.00
CU 1	11	7	18	0.611	0.48
CU 2	7	10	17	0.412	0.63
CU3	11	10	21	0.524	1.00
CU4	24	13	37	0.649	0.10
Praslin - other	28	24	52	0.538	0.68
Pollen flow distance (m)					
10	13	8	21	0.619	0.38
20	15	14	29	0.517	1.00
30	13	13	26	0.500	1.00
40	9	8	18	0.500	1.00
50	4	7	11	0.364	0.55
100	12	7	19	0.632	0.36
300	5	10	15	0.333	0.30
650	9	10	19	0.474	1.00

Note: VdM = Vallée de Mai ; FP = Fond Peper ; FF = Fond Ferdinand ; CU = Curieuse.

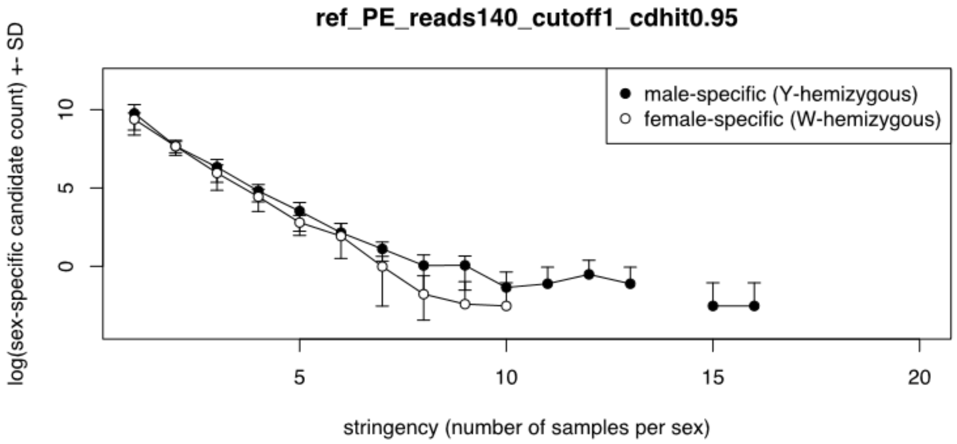
APPENDIX

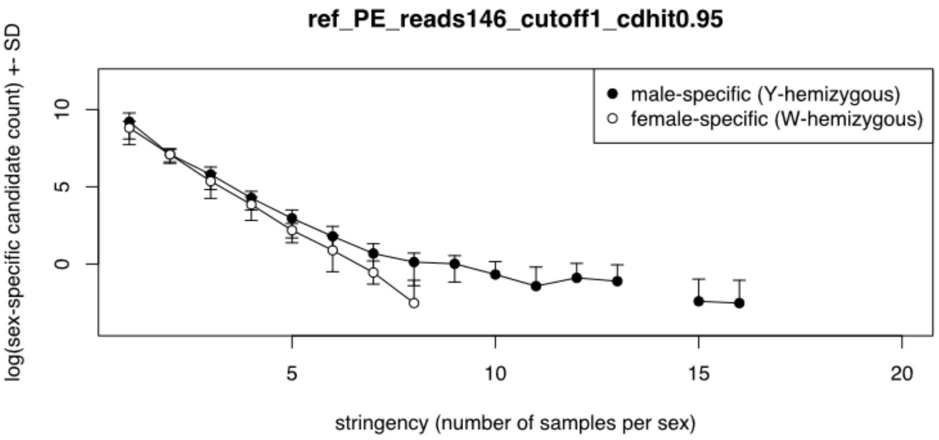
Appendix 1. Read and mapping statistics per individual (20 females and 20 males) for six alternative *de novo* reference assemblies.

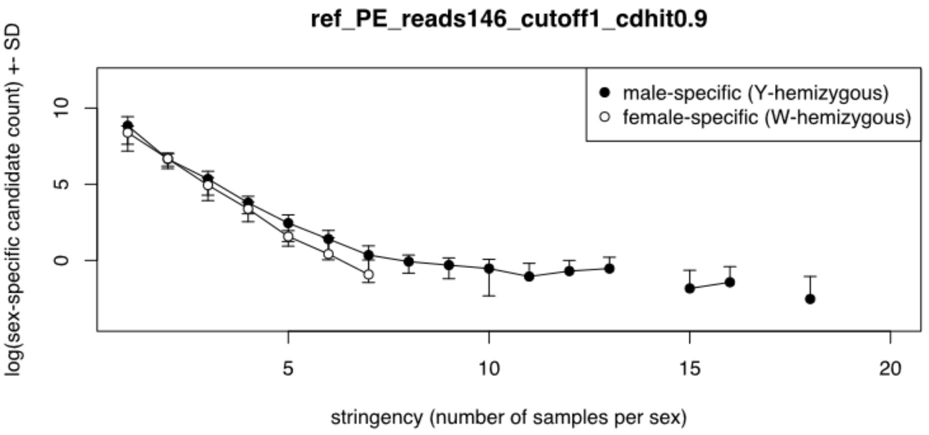
		Reference assembly					
Sample	Filtered read count	1	2	3	4	5	6
F1	2476602	1742452	1496518	1495208	1458899	2205327	2017362
F2	2447404	1714600	1461449	1461593	1462391	2182864	2008812
F3	3356752	2349691	2024555	2026204	1966710	2981769	2728661
F4	3082747	2155978	1846900	1845225	1849114	2744832	2522469
F5	3099330	2166579	1855537	1856907	1852730	2763265	2541487
F6	2038683	1431341	1227881	1228583	1217966	1815709	1673603
F7	3797833	2681836	2296112	2291038	2266891	3381520	3104403
F8	2429562	1713557	1468308	1468107	1442304	2165950	1982977
F9	370922	261164	223435	222737	218816	331906	302866
F10	2128985	1504849	1283097	1279195	1252163	1895301	1730624
F11	2677562	1875109	1601643	1601582	1605167	2385779	2198647
F12	4476654	3130071	2690421	2692295	2648144	3991590	3660343
F13	1121131	799081	681067	678030	662583	997499	912886
F14	3224552	2242422	1918703	1920681	1944280	2873491	2651101
F15	3201770	2264541	1931254	1925185	1909285	2854465	2622567
F16	3170988	2214569	1898622	1898829	1912693	2820464	2600877
F17	3151360	2201010	1882981	1886120	1860503	2809773	2573482
F18	3434662	2448960	2087232	2071563	2071370	3063713	2799830
F19	1485113	1040139	885385	885349	895960	1324080	1223132
F20	1574339	1112673	946271	940365	966312	1406887	1296666
M1	1433183	1007389	859724	858869	860424	1282351	1179070
M2	3296751	2299743	1967688	1970369	1975195	2937894	2706949
M3	3763510	2630527	2252847	2251743	2250702	3357997	3091159
M4	3427846	2430514	2071636	2059117	2088943	3054998	2798565
M5	3602321	2583782	2197253	2176128	2193295	3219623	2943434
M6	2972387	2110124	1802108	1791234	1810769	2645235	2426092
M7	4039043	2852565	2445599	2442026	2390544	3601943	3298448
M8	3512477	2496022	2147697	2141435	2043700	3121800	2840489
M9	4818225	3395486	2915937	2909838	2854129	4303317	3928164
M10	4625543	3268403	2793387	2788401	2765073	4126078	3778205
M11	3700688	2586735	2215075	2215720	2197924	3304534	3035541
M12	2348873	1678660	1435642	1420499	1420308	2079968	1906279
M13	4899753	3438167	2954408	2946673	2896255	4352419	3974563
M14	3100316	2163107	1857557	1856842	1826552	2752332	2519368
M15	2135044	1501993	1285420	1283108	1264989	1901541	1742187
M16	4392027	3090940	2653131	2649018	2579345	3901876	3569682
M17	2198362	1547935	1328625	1328302	1293266	1956195	1788918
M18	1009794	708611	607062	606179	601489	899634	828023
M19	1999191	1407799	1211054	1207497	1185128	1776555	1629230
M20	1981524	1389555	1191109	1191270	1173101	1764300	1619041

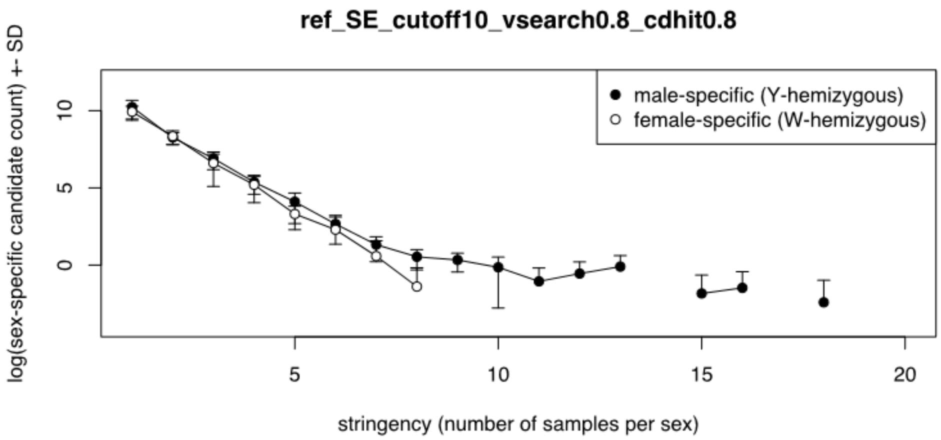
Note: Reference assemblies 1: paired-end reads, 140 bp, identity cutoff 1, cdhit 0.95, 160107 contigs; 2: paired-end reads, 146 bp, identity cutoff 1, cdhit 0.95, 97732 contigs; 3: paired-end reads, 146 bp, identity cutoff 1, cdhit 0.9, 72492 contigs; 4: single-ended, identity cutoff 10, vsearch 0.8, cdhit 0.8, 183243 contigs; 5: single-ended, identity cutoff 10, vsearch 0.95, cdhit 0.95, 526691 contigs; 6: single-ended, identity cutoff 10, vsearch 0.9, cdhit 0.9, 288991 contigs.

Appendix 2. Candidate sex-specific contigs for each reference assembly. Error bars indicate bootstrap support for male- and female-specific contigs.





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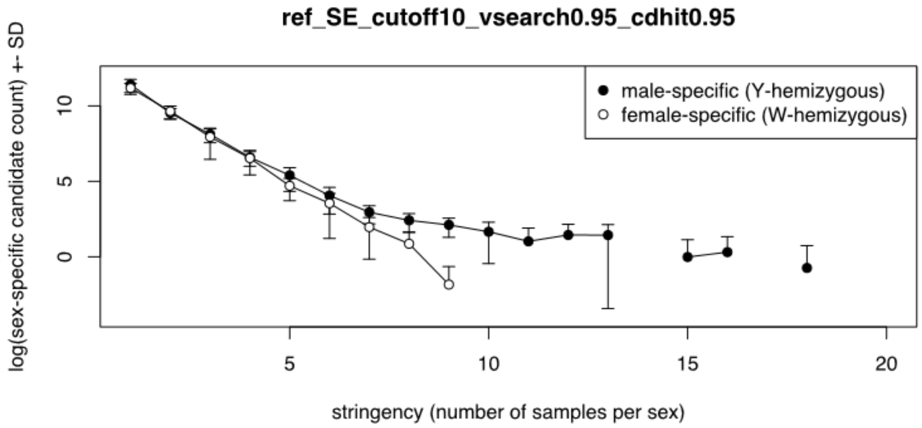
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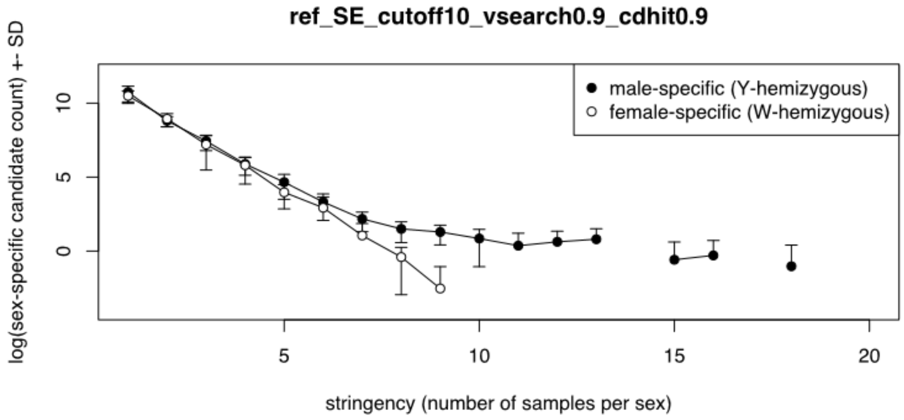
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Appendix 3. Non-redundant male-specific contigs suitable for a molecular sexing assay. 'N' represents an unknown number of bases in paired-end contigs.

```
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Appendix 4. Genetic properties of 12 microsatellite markers in *Lodoicea maldivica* offspring. Offspring outside clusters were seedlings, juveniles and immature trees sampled in Cherie Mon, Zimbabwe, Vallée de Mai or Fond Ferdinand. Young seedlings grew from seeds left to regenerate in 2013. *N*, sample size; N_a, number of alleles; uH_E, unbiased expected heterozygosity; H_O, observed heterozygosity; *HWE*, Hardy-Weinberg Equilibrium.

	Offspring outside clusters *(N = 48)*				Young seedlings (*N* = 49)			
Locus	N_a	H_O	uH_E	*HWE*[a]	N_a	H_O	uH_E	*HWE*[a]
Lm4716	3	0.617	0.502	3.262 ns	5	0.542	0.578	8.551 ns
Lm2630	14	0.689	0.868	95.163 ns	13	0.422	0.812	277.124***
Lm8853	4	0.375	0.576	43.639***	4	0.375	0.508	7.561 ns
Lm5648	12	0.729	0.851	96.736**	9	0.809	0.837	122.064***
Lm6782	15	0.370	0.703	239.636***	12	0.489	0.795	133.116***
Lm1153	14	0.587	0.841	151.860***	11	0.556	0.815	115.056***
Lm4293	5	0.186	0.360	42.878***	6	0.217	0.387	72.077***
Lm1750	5	0.660	0.703	14.785 ns	5	0.612	0.659	8.119 ns
Lm2407	7	0.354	0.483	30.703 ns	5	0.347	0.322	1.909 ns
Lm6026	9	0.479	0.798	177.922***	8	0.375	0.709	107.784***
Lm0144	8	0.500	0.739	34.741 ns	6	0.449	0.651	0.010*
Lm2071	12	0.771	0.856	66.680 ns	12	0.857	0.856	57.087 ns

[a] Deviations from *HWE* using χ^2 tests: *$P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; ns = not significant.

CHAPTER 6

General discussion

Amongst oceanic islands, the Seychelles are unusual for their great age, with the consequence that they support many species that have evolved *in situ* over millions of years. It was not until 1742 that the islands were colonised, and so many of these species still survive. However, especially over the past ~150 years, plant populations across many of the islands have been subjected to deforestation for timber and agriculture (Stoddart 1984). Large areas of *Lodoicea maldivica* forest on Praslin and Curieuse were cleared, and none of the remaining forest is completely undisturbed. The chapters in this thesis investigate different aspects of the demographic and genetic structure of *Lodoicea,* as well as considering how habitat disturbance has disrupted reproductive processes.

Palms (Arecaceae) are some of the most economically and ecologically important plants in the world (Henderson *et al.* 1995), but many species, including *Lodoicea,* are threatened by deforestation and unsustainable harvesting of fruits. As well as being a conservation priority, the unusual features of *Lodoicea* provide a unique opportunity to explore a range of important ecological and evolutionary processes. In aspects such as the huge seed with no mechanism for dispersal it is very distinct from other systems, and represents an extreme "evolutionary endpoint". In this thesis I applied a range of genetic and ecological techniques to look at various aspects of the demographic and genetic structure of this species, which is both a keystone species in the ecosystem where it occurs and a flagship species for conservation. After describing my key results below, I will place them in the context of a range of evolutionary and ecological processes, and discuss their relevance for our understanding of plant survival in fragmented and degraded habitats.

KEY RESULTS

In **Chapter 2,** I used maternity analyses to investigate seed dispersal distances in naturally regenerating areas of *Lodoicea.* The evolution of such a large seed has resulted in extremely limited seed dispersal and an intense fine-scale spatial genetic structure (FSGS). This FSGS could be observed in all remaining large sub-populations, and in all age cohorts, including adult trees that probably established before human settlement on the islands. Sub-populations were characterised by high levels of inbreeding, and pairs of potential mates within close proximity were, on average, closely related. On the other hand, relatively high levels of genetic diversity were maintained among the sub-populations, which varied in their degree of disturbance. This presented something of a paradox because dioecy is thought to have evolved to promote outbreeding. My findings suggest that mating between close relatives is a natural feature of the *Lodoicea* mating system*,* and I discuss the trade-offs between the potential negative costs of inbreeding versus the advantages of maternal resource provisioning for offspring (Edwards *et al.* 2015).

To get a more complete picture of gene-flow patterns, I investigated pollen dispersal in **Chapter 3**. I used a range of direct and indirect methods, and found that pollen flow and immigration rates were also limited, with around 80% of pollen flow occurring at distances of < 80 m. This limited pollen dispersal contributes to the intense FSGS and high inbreeding previously observed. My results suggested mixed pollination vectors for *Lodoicea.* The majority of pollen was dispersed over short-distances, possibly by geckos, but long-distance dispersal by wind probably maintains genetic connectivity over longer time-scales. My results suggest that long-distance pollen flow is less frequent among the isolated fragments of forest on Curieuse, where the natural habitat has been severely disturbed.

Investigating the factors influencing female fecundity in **Chapter 4**, I found that both soil nutrients and pollen availability were important limiting factors at different stages of reproduction. By studying persistent inflorescences on female trees, I recorded large variation in the numbers of flowers and fruits produced. Once again, I found evidence for detrimental effects of habitat degradation, specifically in relation to a lower fruit set and higher production of abnormal fruit in more isolated female trees.

In **Chapter 5,** I explored a further aspect of the demography of *Lodoicea*, namely in the patterns of sex ratios of non-mature plants. To do this I developed two novel male sex-linked markers using a Next Generation Sequencing approach. I used these markers to identify the sex of phenotypically similar male and female progeny, and explored a range of ecological factors that could result in the biased sex ratios that were observed in adult populations. I found no significant deviations from a 1:1 ratio before the adult stage, and concluded that the reasons for the unequal adult sex ratios are probably complex. Females may be susceptible to earlier mortality, but human activities also probably play a significant role in shaping population structure.

MAJOR DRIVERS OF REPRODUCTIVE SUCCESS IN DIOECIOUS TREES, AND SUSCEPTIBILITY TO HABITAT CHANGE

The details of how organisms reproduce vary greatly, covering a wide spectrum of sexual and asexual processes. Although sexual reproduction halves an individual's genetic contribution to the next generation in comparison to asexual reproduction (Maynard Smith 1978), it provides a vital means of maintaining genetic variation, which allows species to adapt to novel or changing environments. The sexually polymorphic system of dioecy, although uncommon among flowering plants (Renner 2014), has one major advantage over other breeding systems in that outcrossing is assured (Darwin 1876; Carlquist 1966; Lloyd 1975). It has been proposed that the fitness benefits of obligatory cross-fertilisation must outweigh the associated negative ecological constraints (Thomson & Barrett 1981). It is generally thought that the absence of self-fertilisation in dioecious plants reduces their colonising ability ('Baker's Law'; Baker 1955, 1967), but it has recently been shown that dioecious trees are significantly over-represented in young successional areas (Réjou-Méchain & Cheptou 2015). This suggests that dioecious trees may indeed have a competitive advantage over trees with other breeding systems, and this may be attributed to a higher average fitness of outcrossed seeds, especially in stressful environments (Barrett *et al.* 2001; Réjou-Méchain & Cheptou 2015). Dioecy presumably evolved in *Lodoicea* before the Seychelles and Indian land-masses separated, given that its closest relatives in the tribe Borasseae are also dioecious (Baker *et al.* 2009). The dioecious nature of *Lodoicea* probably gave it an initial competitive advantage over other tree species, which helped facilitate its dominance on the newly formed islands.

It has been shown that dioecious species are over-represented on other remote tropical islands, in comparison to the mainland (La Réunion, cited in Humeau *et al.* 1999; Hawaii, Bawa 1980; New Caledonia, Schlessman *et al.* 2014), although this pattern does not hold true everywhere (Barrett *et al.* 1996). Island species (usually characterised by having small geographic ranges and population sizes) are disproportionately susceptible to extinctions (Frankham 1998), due to random factors such as genetic drift and natural environmental disturbances. In addition to natural events, humans can exacerbate the threat of extinction through deforestation and the introduction of alien invasive species, pests and diseases, and it is therefore not surprising that the human impact index - a measure of current threat - is significantly higher on islands than on the mainland (Kier *et al.* 2009).

Long before extinction occurs, however, effects of disturbance may be detectable as elevated bi-parental inbreeding, and reduced genetic diversity and connectivity, especially in predominantly outbreeding plants (Ng *et al.* 2006). A species' response to habitat change may depend largely on the mating system (Aguilar *et al.* 2006; Honnay & Jacquemyn 2007; Ng *et al.* 2006). Dioecious trees may be particularly sensitive, as imbalances in sex ratios caused by habitat disturbances (Yu & Lu 2011) can reduce effective population sizes and intensify the other genetic effects of fragmentation. However, in my study I found no evidence of higher inbreeding rates, lower genetic diversity levels or significant biases in the sex ratios amongst offspring that established post-fragmentation, nor did I detect lower genetic variation on Curieuse. Possible reasons are that the response to fragmentation has been delayed in this long-lived species (Kramer *et al.* 2008), or because long-distance gene flow was sufficient to maintain genetic variation and connectivity (Aguilar *et al.* 2008).

Plant reproductive success depends on a number of ecological and genetic processes acting at three key stages: pollination, fertilisation and seed maturation (Lyons *et al.* 1989). Pollination depends on the successful transfer of pollen to female stigmas by biotic or abiotic pollination vectors, and reproduction in dioecious species is strongly dependent on the efficient transport of pollen (Bawa 1980; Givnish 1982). Habitat fragmentation can result in the disruption of pollen flow, thereby reducing reproductive success and ultimately, the population viability (Aguilar *et al.* 2006; Newman *et al.* 2013).

I showed in Chapter 4, that different stages of reproduction in *Lodoicea* were limited by both soil nutrients and distance to the nearest male, as has been observed in other plant species (nutrient availability: Bloom *et al.* 1985; Winn 1991, pollen availability: Wang *et al.* 2013). Nutrient availability has also been shown to affect the population structure of another palm species, where dense clumps of immature *Borassus aethiopum* plants can be found restricted to nutrient-rich patches (Barot *et al.* 2005). Nutrient availability may be particularly important for reproduction in dioecious species, as changing soil nutrient conditions can affect plant sex ratios (Yu & Lu 2011). Males and females can differentially allocate resources to reproduction when nutrients are limiting (Teitel *et al.* 2015), which can also indirectly alter reproductive sex ratios.

I demonstrated significant pollen limitation in *Lodoicea*, which resulted in a lower seedset in isolated trees. As male trees typically produce vast amounts of pollen year-round, we would not expect reproduction in dense, monotypic *Lodoicea* forests to be limited by the pollen supply. The most likely biotic pollinators of *Lodoicea* have been shown to be less common, or absent in degraded forest (Gerlach & Ineich, 2006; Noble *et al.,* 2011), and this probably results not only in lower pollen immigration rates and pollen flow (Chapter 3) but also in lower female fecundity (Chapter 4). Negative genetic consequences of fragmentation are likely to take several generations to manifest themselves (Kettle *et al.* 2007), and I was unable to detect such genetic changes in *Lodoicea.* However, the reductions in female fecundity and very limited natural regeneration are likely to represent the early stages of genetic fragmentation, of which the consequences may become apparent in successive generations.

Overall, the evidence suggests that dioecious species are particularly vulnerable to habitat change. Although tree species may be partially buffered against short-term changes by their long life-spans, and the potential for long-distance pollen dispersal, dioecious species are likely to experience more immediate reductions in fecundity if pollination is disrupted. *Lodoicea* is an ecosystem engineer and is very efficient at modifying local nutrient conditions (Edwards *et al.* 2015), giving its seedlings a competitive advantage over those of other species. However, a sufficient number of seeds must be left to germinate in the forests, and the promotion of local pollinator communities would help maintain the stability of the *Lodoicea* ecosystems. Support from practitioners and the local community will be crucial in attaining this goal, and will rely upon effective communication among scientists, policy-makers and the public (Kaiser-Bunbury *et al.* 2014).

RECONCILING ECOLOGICAL VERSUS EVOLUTIONARY TRADE-OFFS IN DIOECIOUS SPECIES

Trade-offs are a fundamental aspect of the evolution of organisms, and occur when one trait cannot increase without being accompanied by a decrease in another (Garland 2014). For example, the evolution of plant herbivore defence may entail changes in the resources available for growth, storage and reproduction. Another example of a trade-off is between producing many small seeds and few large seeds, referred to as the seed size/number trade-off (SSNT) (Smith & Fretwell 1974, but see Venable 1992). A larger number of seeds ultimately increases reproductive output (Leishman *et al.* 2000), and smaller seeds are more readily dispersed and may facilitate colonisation into new habitats (Harper *et al.* 1970). On the other hand, species with large seeds with a large nutrient reserve are likely to be competitively superior to species with small seeds (Leishman *et al.* 2000), especially in shady, dry or nutrient-poor habitats (Leishman & Westoby 1994; Vaughton & Ramsey 1998).

At the extreme end of the SSNT spectrum is *Lodoicea maldivica.* The annual seed production is extremely low, with an average of around one seed per year (Chapter 4), the lowest documented for any tropical tree. However, the few seeds that are produced are the largest in the plant kingdom (Janzen 1977; Thompson 1984; Michaels *et al.* 1988). The huge plasticity in seed size, exceeding that of any other palm species (Moegenburg 1996), is likely to be a response to available nutrients resources (Vaughton & Ramsey 1998), although size could have a hereditary component (Wulff 1986). The large nutrient reserve of a *Lodoicea* seed is known to support the growing seedling for up to four years, and as a result, offspring have a very high survival rate (100% of the 493 offspring sampled in 2013 were still alive in 2015). Although estimates of species-specific mortality are scarce in the literature, it appears that the survival rate of *Lodoicea* offspring not only exceeds that of any palm (Ataroff & Schwarzkopf 1992; Wright & Duber 2001) but also of any tropical tree species documented (Augspurger 1984; Primack 1985; Alvarez-Buylla & Martinez-Ramos 1992) (although varying resource availability must be taken into account). This provides a huge competitive advantage against the species of smaller-seeded plants, particularly in the dense closed-canopy of natural *Lodoicea* forests.

In fact, many aspects of *Lodoicea's* ecology contribute towards the maintenance of its dominance on the two islands, and the competitive exclusion of other species. Tropical rainforests include some of the most species-rich ecosystems of the world, though exceptional cases exist, such as that of *Lodoicea,* of forests dominated by a single species. These forests share many similar evolutionary and ecological features (Hart *et al.* 1989; discussed in Peh *et al.* 2011; and the dioecious canopy palm *Mauritia flexosa* in the Amazon, Holm *et al.* 2008), including large seeds capable of establishing in deep leaf litter, poor seed dispersal (discussed in relation to *Lodoicea* in Edwards *et al.* 2015), and shade-tolerant seedlings capable of regenerating under a closed canopy.

In this thesis, I provide further indications of how *Lodoicea* has managed to succeed in excluding other species and thus maintaining dominance. In Chapter 4, I measured low levels of nutrients in the soils on Praslin. Although *Lodoicea* has evolved novel mechanisms to cope with low nutrients, other competing species are likely to be less efficient at acquiring nutrients (Hart *et al.* 1989; Torti *et al.* 2001). In Chapter 2, I showed just how restricted is seed dispersal in *Lodoicea.* Given the highly gregarious establishment of seedlings beneath the mother trees, the species can only expand its range very slowly (Connell & Lowman 1989). A very long period of undisturbed conditions must therefore have been an essential factor in *Lodoicea's* historical dominance across the whole of Praslin and Curieuse (Fauvel 1909).

I put forward the hypothesis that a significant contribution towards *Lodoicea*'s success as a species involves another trade-off. Limited seed dispersal results in highly clustered growth patterns (Chapter 2), whereby siblings are in high competition with each other and with their mother. The species experiences limited pollen dispersal and, as a consequence, bi-parental inbreeding and rates of correlated paternity are moderate to high (Chapter 3); this, in turn, means that seedlings are often in competition with full-sibs that share closely related parents. I propose that the potentially negative costs of inbreeding are traded-off by maternal resource provisioning for progeny. *Lodoicea* demonstrates an effective funneling system of the leaves, whereby organic matter, including nutrient-rich pollen, is washed down to the base of the trunk with rainwater (Edwards *et al.* 2015). Offspring growing close to the mother tree benefit from improved nutrient and moisture conditions, which may give them a high competitive advantage in their early growth.

Plants in nutrient-poor soils normally internally allocate resources more efficiently than other species (Aerts 1996; Hiremath *et al.* 2002), particularly in older forest stands (Waring *et al.* 2015). A variety of strategies exist to increase the efficiency of nutrient uptake and recycling. For example, nutrients may be resorbed from senescing leaves (Milla *et al.* 2005; also in *Lodoicea*, Edwards *et al.* 2015), or gained via interactions with mycorrhizal fungi (Perez-Moreno & Read 2001), which incidentally can be sex-specific due to differing investments in reproduction by the sexes (Vega-Frutis *et al.* 2013; Vega-Frutis *et al.* 2015). Other trees are known to filter high volumes of water down their trunks (Herwitz 1986; Germer *et al.* 2010), but we do not know of any species that is able to filter and recollect nutrients as efficiently as *Lodoicea* (Edwards *et al.* 2015).

Despite high bi-parental inbreeding, *Lodoicea* appears to avoid inbreeding depression and maintains relatively high levels of genetic diversity. Deleterious recessive alleles can be purged from species (Byers & Waller 1999), and it is possible that this has been a continuing process in *Lodoicea* over many thousands of generations. This is consistent with the Seychelles' ancient origins (Baker & Miller 1963), and *Lodoicea's* closest relatives being the Asian Borassus palms (Baker *et al.* 2009).

Trade-offs give species the flexibility to adapt to changing environmental conditions. If we are able to understand how changes in certain conditions (e.g. resources or population densities) might affect the demography or reproduction of a species, we will be better equipped to manage and conserve important flagship species. This will be especially important as we progress further into the current biodiversity crisis we are facing. The research conducted for this thesis gives important insights to how the SSNT can allow species to attain and maintain dominance in an ecosystem.

FUTURE RESEARCH DIRECTIONS

- **Direct impact of human-mediated forest change**

  The research in Chapters 2 and 3 focused on the most natural areas of *Lodoicea* remaining across Praslin and Curieuse. These areas were characterised by dense clusters of individuals ranging from seedlings to adults. Although the majority of seeds are now removed from populations, I deemed it unlikely that seeds were planted in the study sites, thus the resultant patterns of established individuals I studied were “natural”. This contrasts with many areas which have clearly been managed, where the forest resembles a plantation of widely spaced individuals. The genetic structure of these manipulated areas is likely to be very different from the areas I studied. Other genetic and ecological processes such as pollen dispersal are also likely to differ because reduced *Lodoicea* densities could alter biotic pollinator or wind movement through the forest. On the other hand, human-mediated movement of seeds around the forest could result in lower inbreeding levels and a reduced FSGS at the local scale. The current stable system appears to have developed over millions of years, and it is unknown whether continued manipulation could result in inbreeding or outbreeding depression. Investigating the FSGS in these contrasting areas would give us a better indication of the impacts of past manipulation of populations.

- **Inbreeding depression in *Lodoicea***

  Theory suggests that such high levels of bi-parental inbreeding should reduce offspring fitness (Waser & Price 1991; Robertson & Ulappa 2004; Hirao 2010). Organisms tend to accumulate deleterious mutations, which in the long run may reduce fitness. These harmful mutations can be eliminated from inbred populations by selection (Barrett & Charlesworth 1991), a process that is most effective under particular circumstances (discussed in detail in Byers & Waller (1999)). Purging of negative alleles may be more likely in species with (a) short generation times (i.e. more opportunities for recombination), (b) high reproductive output (so there can be strong selection against less optimal genotypes), (c) small population sizes (Nei 1968; Hedrick 2002), (d) a mating system with higher rates of selfing (Husband & Schemske 1996), or (e) species that have gone through bottlenecks (Kirkpatrick & Jarne 2000). Some of these conditions do not apply for *Lodoicea*: it has a long generation time, low reproductive output (Chapter 4) and generally low levels of outbreeding (Chapter 3). Although *Lodoicea* populations have been vastly reduced and are fragmented to varying degrees, the species historically had a much larger range across the two islands, and the vegetation was almost monospecific (Fauvel 1915). However, the effects of continued disturbance to the system on the genetics of the species are largely unknown, but may become apparent in future generations.

High survival and slow growth rates in *Lodoicea* make it difficult to observe fitness differences in established offspring, but the data could indirectly allow us to make inferences about inbreeding depression. The data in Chapter 2 identified a reduced FSGS in adult compared to offspring cohorts, and in Chapter 3 I estimated high levels of bi-parental inbreeding using parentage analysis with highly variable microsatellite markers. Frequency histograms of kinship levels in each age cohort might reveal the earlier death of the most inbred individuals at each stage. Inbreeding depression can first become apparent at seed maturation or germination (Hirao 2010). In Chapter 4 I documented the significant reduction in female fecundity as a result of abnormal fruit production but I was unable to ascertain the cause. Hand-pollination experiments involving parent trees of varying levels of kinship could identify whether bi-parental mating results in higher rates of seed abortion or reduced seed germination rates (I was unable to genotype mature seeds that failed to germinate). At the same time, outbreeding depression, or the reduction in fitness of offspring resulting from mating between too-distantly related parents, could also be investigated. The outcome of these experiments could directly be used for more successful hand-pollination of *Lodoicea* in botanical gardens or natural populations.

PRACTICAL MANAGEMENT RECOMMENDATIONS

The main aim of my thesis was to understand the natural genetic and ecological processes that occur in *Lodoicea maldivica,* and to understand the effects of habitat degradation. Using my results, I am able to recommend specific management suggestions for the conservation of the species.

- Ultimately, *Lodoicea maldivica* has no future as a species if natural regeneration is prevented from occurring. The increasing demand from tourists for nuts is putting an immense pressure on the species. Although the high price of the nuts provides a valuable income for the Seychelles economy, and in some instances for generating money to conduct other conservation work, this needs to be carefully balanced. The systematic collection, removal and translocation of seeds should be avoided. Regeneration schemes that ensure a proportion of nuts remain in the forest, such as that carried out by Seychelles Islands Foundation, should be adopted by all organisations managing *Lodoicea* populations.
- When seeds are left to regenerate in the forest they should be placed as near to their mother tree as possible, so the natural population structure is not disrupted. Seeds grown close to the base of an adult *Lodoicea* will also benefit from the rainwater and nutrients filtered down its trunk. However, it is recognised that the risk of the nut being poached has to be minimised, so the strategic placing of nuts is crucial.
- Fruit production is most limited by pollen availability, particularly in more fragmented areas. The preservation and promotion of local pollinator communities (such as endemic gecko species) will be important in ensuring that future pollen limitations do not result in reduced fruit production.
- Alternatively, if hand-pollination were to be used as a tool to increase reproductive output of female trees, pollen should be taken from a male at ~600 m from the female (the furthest detected pollen flow distance). *Lodoicea* has fairly high natural bi-parental inbreeding levels, and it is unknown whether outbreeding depression from taking too-distant pollen would occur in the progeny of a female.
- Protection of natural habitats and increased security measures would help ensure the continued survival of the species. As a priority, closed, natural *Lodoicea* forest should be maintained and restored, and if possible, extended, as opposed to planting seeds in highly degraded areas. Trees in dense forest produce more normal fruits and fewer abnormal fruits, and the higher abundance of pollinators possibly contributes to the higher reproductive output.

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APPENDIX A

Development of 12 polymorphic microsatellite loci for the endangered Seychelles palm *Lodoicea maldivica* (Arecaceae)

with Kirsti Määttänen, Christopher N. Kaiser-Bunbury, Andres Buser, Frauke Fleischer-Dogley and Chris J. Kettle

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ABSTRACT

- ***Premise of the study:*** The evolutionarily and ecologically distinct coco de mer palm *Lodoicea maldivica* (Arecaceae) is endemic to two islands in the Seychelles. Before colonization of the islands by man, the endangered palm formed large monodominant stands, but its natural range is now restricted to four main populations and several patches of isolated individuals. Microsatellite markers were designed to investigate the genetic structure of the remaining natural populations of *L. maldivica*.
- ***Methods and Results:*** We developed 12 polymorphic and three monomorphic microsatellite markers for this species, with a mean number of alleles per locus of 13.2 (range 5–21) and expected heterozygosity values ranging from 0.31–0.91 for the polymorphic loci.
- ***Conclusions:*** These markers enable us to study the patterns of genetic diversity, contemporary seed dispersal, and the fine-scale spatial genetic structure of this important conservation flagship species.

PRIMER NOTE

Lodoicea maldivica (J. F. Gmel.) Pers. (Arecaceae; coco de mer) is an evolutionarily and ecologically distinct dioecious palm (Edwards et al., 2002, 2015) that holds several botanical records, among which are the largest female flowers in any palm and the largest seeds in the plant kingdom (Leishman et al., 2000). The species was once widespread across two Seychelles islands, Praslin and Curieuse (Malavois, 1787, quoted in Fauvel, 1909), but now persists in only four main semiconnected populations—at Vallée de Mai, Fond Peper, and Fond Ferdinand on Praslin, and also on Curieuse Island (Fleischer-Dogley et al., 2011).

The total *L. maldivica* population on Praslin and Curieuse was estimated at 24,376 individuals in 2004, but despite the relatively large population size, reproductive female trees make up only a small proportion (15.6%) of the population (Fleischer- Dogley, 2006). The recent population reduction is due to habitat degradation arising from several serious fires and lumber harvest (Bailey, 1942). Although *L. maldivica* nut kernel has been listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which prohibits exportation without a license, unsustainable harvesting and poaching of nuts continues to threaten the species, as natural regeneration is severely limited (Rist et al., 2010). Fleischer- Dogley et al. (2010) used amplified fragment length polymorphisms to assess genetic diversity in *L. maldivica*, but the dominant nature of the markers did not permit detailed genetic analyses. By developing microsatellite markers, we provide the foundation for in-depth molecular research on the ecology and population genetics of the species, and a tool for the conservation and sustainable production of *L. maldivica* nontimber products. This study reports the isolation and characterization of 12 polymorphic and three monomorphic microsatellite loci in *L. maldivica.*

METHODS AND RESULTS

Size-selected fragments from genomic DNA were enriched for simple sequence repeat (SSR) content using magnetic streptavidin beads and biotinlabeled CT and GT repeat oligonucleotides. The SSR-enriched library was made by the company ecogenics (Balgach, Switzerland) and analyzed on a Roche 454 platform using the GS FLX Titanium reagents (454 Life Sciences, a Roche Company, Branford, Connecticut, USA). The 6607 reads had an average length of 143 base pairs. Of these, 617 contained a microsatellite insert with a tetra- or a trinucleotide of at least six repeat units or a dinucleotide of at least 10 repeat units. Primer design was done using the Primer3 core (Rozen and Skaletsky, 1999). Suitable primer design was possible in 212 reads. Seventy-eight primer pairs were tested, and the most reliable polymorphic candidates were optimized. Genomic DNA was extracted from silica gel–dried *L. maldivica* leaf or flower tissue (*n* = 1252) following the DNeasy 96 Plant Kit (QIAGEN, Hombrechtikon, Switzerland) manufacturer's protocol, except that grinding was carried out at four cycles of 30 s at 30 Hz, and the first incubation step was extended to 1 h at 65°C. Leaf tissue samples from *L. maldivica* individuals from each population are located at the Tissue Collection of the Royal Botanic Gardens, Kew, Richmond, Surrey, United Kingdom (Appendix 1).

Table 1. Characteristics of the 12 polymorphic and three monomorphic microsatellite loci in *Lodoicea maldivica*[a].

Locus	Primer sequences (5'–3')	Repeat motif	Allele size range (bp)[b]	Fluorescent dye	Multiplex[c]	Conc. (μM)	GenBank accession no.
Multiplex PCR							
Lm4716	F: TGGAGAGTACAATAGATGAAATGCC	$(CA)_{12}$	128–140	YY	MP1	0.30	KT897315
	R: AACGGAGTTATCATGCTTGC					0.30	
Lm2630	F: AAATAAGAGCAACCAGAGAAGTC	$(GA)_{16}$	121–157	ATTO565	MP1	0.30	KT897316
	R: GCAGGTGTCTCA ATC AAG GC					0.30	
Lm8853	F: CTATGGTCTAGGTGGACGCC	$(ATGT)_{9}$	193–231	ATTO550	MP1	0.20	KT897317
	R: GGCTGGACATGCGTTCTATG					0.20	
Lm5648	F: CCAAGACTGTAACTTGTTCCCC	$(TATC)_{12}$	235–285	FAM	MP1	0.20	KT897318
	R: AGGCTTAGTGTTCAGGACCG					0.20	
Lm6782	F: GGTCTAAAACTATTGGAGCAAATCAA	$(TATG)_{12}$	252–334	ATTO565	MP2	0.30	KT897319
	R: AGACTCTTAAGTGGGCGAAC					0.30	
Lm1153	F: TTGGGATACATGAGAGCGGG	$(GA)_{13}$	120–166	FAM	MP2	0.30	KT897320
	R: AGATCAGTTGACTATTTGTTACTCTC					0.30	
Singleplex PCR[d]							
Lm4293	F: TCACCTTAGAGATGGTGCAGG	$(GTAT)_{7}$	138–200	FAM	1	0.08	KT897321
	R: TGCACTTGAAGGTTACGTATG					0.32	
Lm1750	F: AGTACTTAGGCATAGGCCAGC	$(TACA)_{10}$	218–234	ATTO565	1	0.08	KT897322
	R: ATGACATGGCCTGGAAGAGC					0.32	
Lm2407	F: GGGATCCTCATCCCATGCTC	$(ACAT)_{9}$	84–112	FAM	1	0.06	KT897323
	R: TCGTACCGCCTAAGCCTAAC					0.24	
Lm6026	F: AGAGCACTTTTTGCCAACCC	$(TATG)_{8}$	147–225	YY	1	0.06	KT897324
	R: ACATCTCATGTGAGGGCATTC					0.24	
Lm0144	F: GCGCGTGCACACATAGATAG	$(TAGA)_{8}$	244–280	ATTO550	1	0.06	KT897325
	R: CATGCTCTCCGCTAAAACCC					0.24	
Lm2071	F: CCATCTCCGCCATTTTTCCC	$(GA)_{13}$	104–138	FAM	2	0.08	KT897326
	R: TACGCACCTACGTTCCTTCC					0.32	
Lm7170	F: ACGCATGGGAAGGATCTCAC	$(ATAC)_{9}$	213[c]	FAM	2	0.08	KT962232
	R: ATGGGGGCTTGTCCATTAGG					0.32	
Lm1012	F: GTCGATGGTGCTTCTAGCTG	$(TACA)_{7}$	251[c]	ATTO565	2	0.08	KT962233
	R: CCTGCTTACCATGAAAGGTCG					0.32	
Lm5950	F: ACCGAATGGAACAAAGTCACAC	$(TATC)_{7}$	180[c]	ATTO565	2	0.08	KT962234
	R: CGTTAGAAACATAGGAAACAGCC					0.32	

[a] Values based on samples collected from the four populations across the natural range on Praslin and Curieuse (1252 samples for the polymorphic markers and 64 samples for the monomorphic markers).

[b] Range of allele sizes includes M13 tail (5′-TGTAAAACGACGGCCAGT-3′) attached to the forward primer.

[c] Mix for the multiplex PCR (MP1 and MP2) or pseudo-multiplex mix (1 and 2) for fragment analysis (using singleplex PCR products).

[d] The singleplex PCRs used forward primers with M13 tails (5′-TGTAAAACGACGGCCAGT-3′) at the 5'-ends (as described by Schuelke, 2000) and reverse primers and M13-primer universal tails marked with either FEM, ATTO565, ATTO550, or YY (Microsynth).

[e] Monomorphic microsatellite marker.

Two methods were used for PCR reactions: two multiplex PCRs were used to amplify six primers, and the remainder of the primers were amplified in singleplex. Multiplex PCRs (MP1 and MP2) were carried out using primers labeled with either FAM, ATTO565, ATTO550, or Yakima Yellow (YY) (Microsynth, Balgach, Switzerland) (Table 1). PCR amplifications were carried out in 10.3-Ml reactions containing 1× PCR Buffer (colorless Flexi GoTaq PCR buffer), 0.2 mM dNTPs, 3.1 mM MgCl2, 0.05 U/µL *Taq* Polymerase (all Promega Corporation, Zürich, Switzerland), 0.18 µg/µL bovine serum albumin (BSA; BioConcept, Allschwil, Switzerland), 1.3 µL DNA, labeled forward primers, and unlabeled forward and reverse primers (for primer concentrations see Table 1).

Touchdown PCRs were carried out on a Bio-Rad Dyad Cycler (Bio-Rad Laboratories, Hercules, California, USA) with the following conditions: initial denaturation 95°C/4 min; 12× (denaturation 95°C/30 s, starting annealing temperature 62°C/30 s, decreasing by 0.5°C/cycle, extension 72°C/30 s); 29× (MP1)/28× (MP2) (denaturation 95°C/30 s, annealing 56°C/45 s, extension 72°C/30 s); and final extension 72°C/30 min and storage at 10°C. PCR product (2.5 µL) was added to 10 µL of HIDI formamide and 0.25 µL GeneScan 500 LIZ Size Standard (Applied Biosystems, Waltham, Massachusetts, USA).

The singleplex PCRs used forward primers labeled with M13 tails (5′-TGTAAAACGACGGCCAGT- 3′) at the 5′ ends (as described by Schuelke, 2000) (Table 1). PCRs occurred in 11-µL reaction volumes containing 1× PCR Buffer, 0.2 mM dNTPs, 2.5 mM MgCl2, 0.025 U/µL *Taq* Polymerase, 0.18 µg/µL BSA, 1.0 µL DNA, forward primers with M13 tails, reverse primers and M13- primer universal tails labeled with either FAM, ATTO565, ATTO550, or YY (Microsynth) (for primer concentrations see Table 1). Cycling for singleplex PCRs was as follows: initial denaturation 95°C/5 min; 12× (denaturation 95°C/30 s, starting annealing temperature 62°C/30 s, decreasing by 0.5°C/cycle, extension 72°C/30 s); 25× (denaturation 95°C/30 s, annealing 56°C/45 s, extension 72°C/30 s); 8× (denaturation 95°C/30 s, annealing 53°C/45 s, extension 72°C/45 s); and final extension 72°C/30 min and storage at 10°C. PCR products were combined to create two pseudo-multiplex mixes (Table 1). For each PCR product (Lm4293, Lm2407, Lm6026, and Lm0144 were diluted 20× first), 1.2 µL were added to 10 µL of HIDI formamide and 0.15 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems). Singleplex and multiplex products were denatured for 3 min at 92°C and run on an ABI 3730xl automatic capillary sequencer (Applied Biosystems). Electropherograms were scored with GeneMarker 2.6.0 (SoftGenetics, State College, Pennsylvania, USA).

The number of alleles, deviations from Hardy–Weinberg equilibrium (HWE), and observed and expected heterozygosity values were calculated (Table 2) using GenAlEx 6.5 (Peakall and Smouse, 2006). Linkage disequilibrium was tested in GENEPOP (Raymond and Rousset, 1995). The 12 polymorphic loci revealed between five and 21 alleles, with a total of 158 alleles across all *L. maldivica* individuals (Table 2). Significant deviation from HWE was seen in the majority of loci in all populations (Table 2). Expected heterozygosity values ranged from 0.399–0.896 (mean ± SE: 0.687 ± 0.048) for the polymorphic markers. No significant linkage disequilibrium was detected between loci pairs after sequential Bonferroni correction ($\alpha = 0.05$) (Holm, 1979). The putative presence of null alleles in 11 loci (all except the monomorphic loci and Lm4716) was detected using MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004); however, these are unlikely to affect HWE at such low frequencies (Dakin and Avise, 2004). There was no evidence for large allele dropout.

CONCLUSIONS

We developed 12 highly polymorphic and three monomorphic loci for *L. maldivica*, with allele numbers ranging from five to 21 for the polymorphic loci. The pattern of homozygote excess can be observed across almost all loci in all populations. This can likely be explained by high inbreeding levels due to the very clustered growth patterns observed in the species. These markers will provide a useful tool in investigating the natural population structure, seed dispersal patterns, and fine-scale genetic structure of this highly charismatic and important endemic palm species (Morgan et al., in prep.).

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Table 2. Genetic properties of 12 *de novo* microsatellite markers in the four extant *Lodoicea maldivica* populations.[a,b]

	Vall*ée de Mai (n = 482)*				Fond Peper (n = 293)				Fond Ferdinand (n = 265)				Curieuse (n = 212)			
Locus	A	H_O	H_E	HWE[c]	A	H_O	H_E	HWE[c]	A	H_O	H_E	HWE[c]	A	H_O	H_E	HWE[c]
Lm4716	4	0.525	0.514	3.243 ns	5	0.455	0.467	5.893 ns	3	0.457	0.500	3.749 ns	7	0.476	0.548	518.001***
Lm2630	17	0.570	0.879	1824.687***	17	0.543	0.909	1103.038***	18	0.598	0.895	807.148***	16	0.612	0.880	384.399***
Lm8853	6	0.454	0.540	32.452**	5	0.579	0.586	19.938*	6	0.481	0.563	46.053***	5	0.566	0.608	23.262**
Lm5648	13	0.797	0.857	99.149 ns	12	0.806	0.834	67.738 ns	12	0.820	0.841	54.122 ns	11	0.768	0.820	159.926***
Lm6782	15	0.429	0.747	1213.166***	13	0.463	0.716	536.756***	13	0.398	0.699	623.970***	17	0.401	0.743	738.903***
Lm1153	18	0.482	0.831	1148.177***	16	0.569	0.802	711.396***	16	0.537	0.823	1013.648***	15	0.398	0.846	720.193***
Lm4293	9	0.155	0.437	1791.297***	7	0.310	0.519	294.419***	9	0.191	0.415	1046.787***	14	0.194	0.602	1163.556***
Lm1750	5	0.573	0.649	53.724***	5	0.657	0.675	15.885 ns	4	0.564	0.633	14.284*	5	0.524	0.632	24.213**
Lm2407	6	0.258	0.309	66.480***	6	0.337	0.369	21.129 ns	6	0.354	0.406	35.106**	7	0.448	0.597	97.316***
Lm6026	11	0.412	0.791	1526.267***	11	0.444	0.758	824.227***	10	0.361	0.791	704.062***	8	0.341	0.754	432.905***
Lm0144	8	0.374	0.603	343.146***	8	0.425	0.646	689.482***	6	0.395	0.648	176.941***	9	0.320	0.706	370.609***
Lm2071	15	0.838	0.852	161.679***	15	0.771	0.823	110.342 ns	13	0.817	0.853	94.963 ns	13	0.830	0.830	77.941 ns

Note: A = number of alleles; H_E = expected heterozygosity; H_O = observed heterozygosity; HWE = Hardy-Weinberg equilibrium; *n* = number of individuals sampled.

[a] Geographic co-ordinates for the populations are: Vallée de Mai = 4°19'43"S, 55°44'11"E*;* Fond Peper = 4°20'01"S, 55°44'17"E; Fond Ferdinand = 4°21'02"S, 55°45'39"E; and Curieuse = 4°16'45"S, 55°43'25"E*.*

[b] Sixteen individuals were tested from each population using the three monomorphic loci.

[c] Deviations from HWE using χ^2 tests: *$P \le 0.05$, ** $P \le 0.01$, *** $P \le 0.001$; ns = not significant.

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APPENDIX

Appendix 1. Locations and DNA bank information for populations of *Lodoicea maldivica* used in this study.[a]

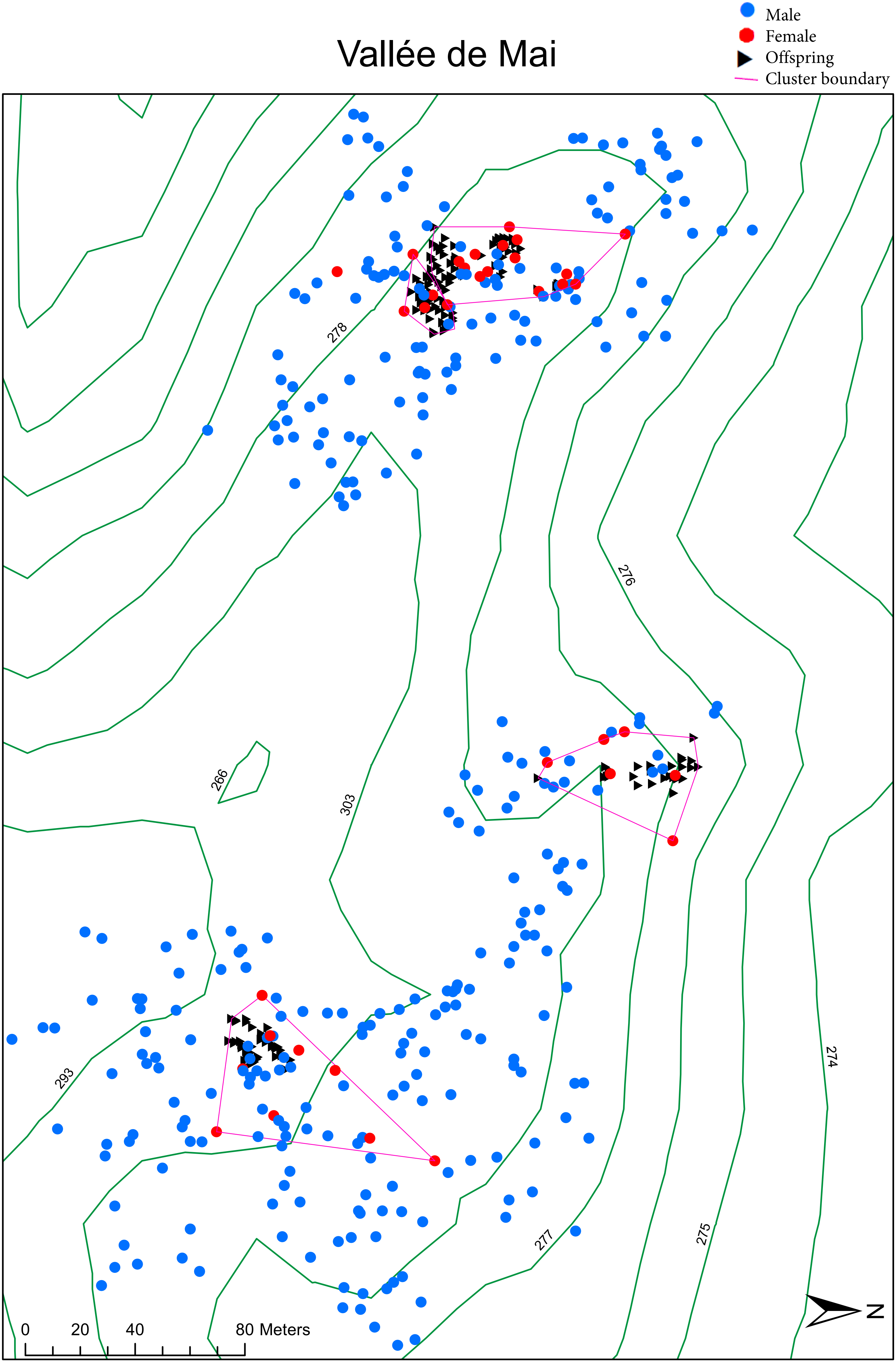
Population	Cohort	UTM coordinates[b]	Tissue collection no.
Vallée de Mai, Praslin	Adult male	359634.8mE, 9521289.06mN	6091
Vallée de Mai, Praslin	Adult male	359660.3mE, 9521279.96mN	6092
Fond Peper, Praslin	Adult male	359871.3mE, 9520653.71mN	6093
Fond Peper, Praslin	Juvenile	359634.8mE, 9520672.20mN	6094
Fond Ferdinand, Praslin	Adult female	361575.2mE, 9518670.34mN	6095
Fond Ferdinand, Praslin	Juvenile	361494.4mE, 9518728.30mN	6096
Curieuse Island	Juvenile	358386.5mE, 9526223.40mN	6097
Curieuse Island	Immature	358391.0mE, 9526213.75mN	6098

[a] Silica gel-dried leaf samples deposited at the Tissue Collection of the Royal Botanic Gardens Kew, Richmond, Surrey, United Kingdom.

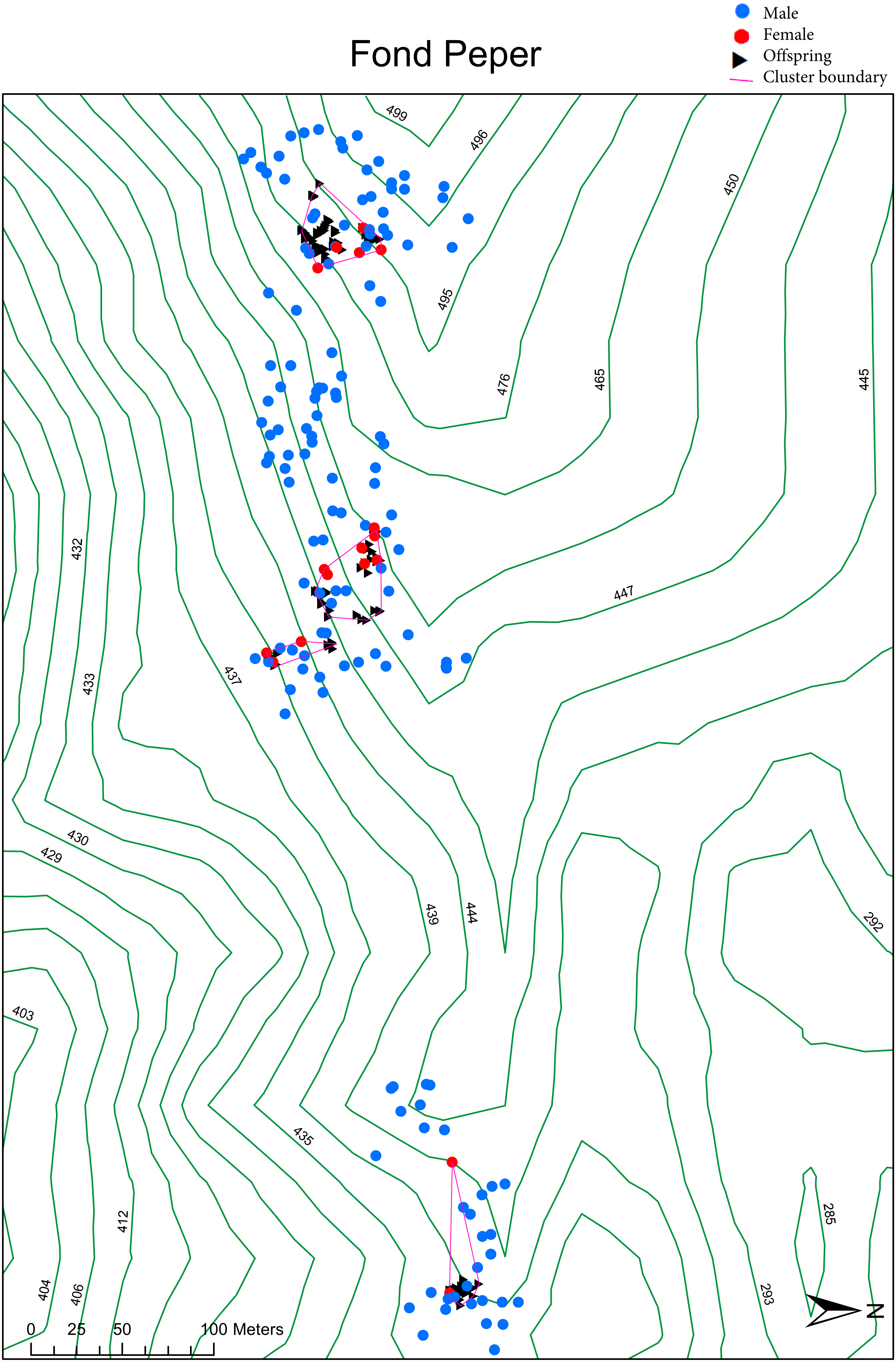
[b] Universal Transverse Mercator coordinates: WGS 84, UTM Zone 40S.

APPENDIX B

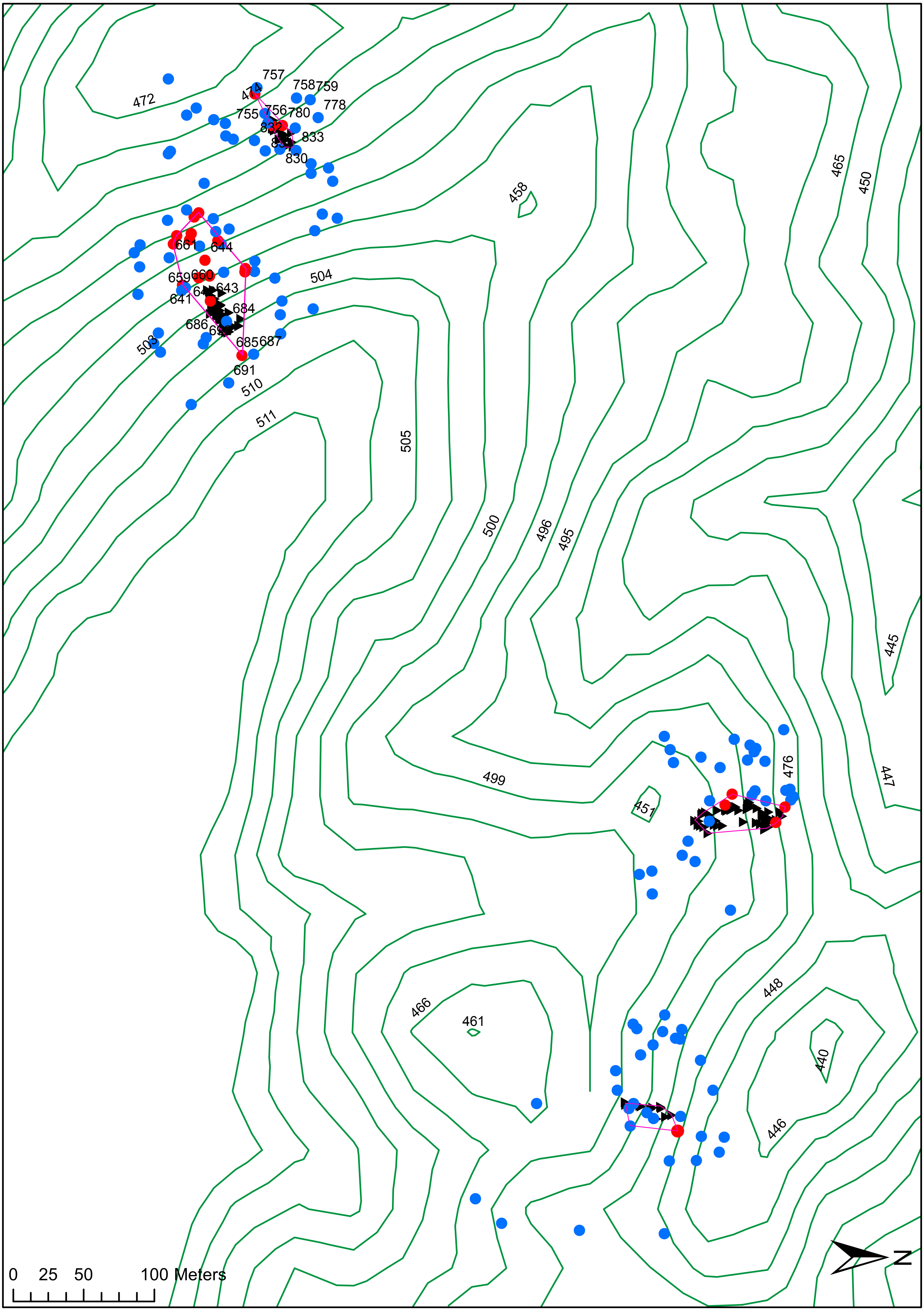
Sampling sites

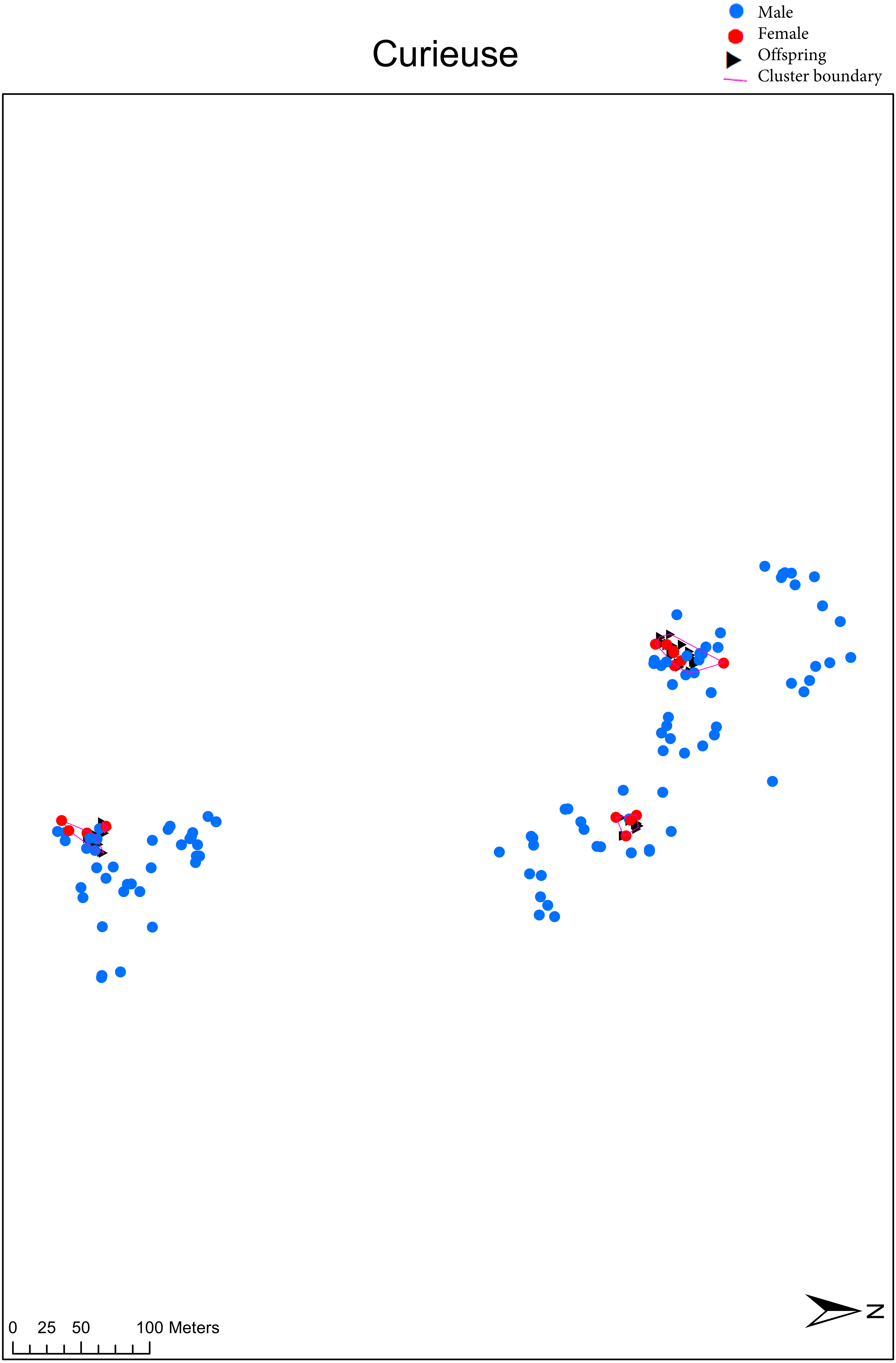

Vallée de Mai
Male
Female
Offspring
Cluster boundary
278
276
266
303
293
277
275
274
0
20
40
80 Meters
N

Fond Peper


Male
Female
Offspring
Cluster boundary
499
496
495
476
465
450
445
447
432
433
437
430
429
439
444
292
435
403
404
406
412
285
293
0
25
50
100 Meters
N

Fond Ferdinand




Curieuse
● Male
● Female
▶ Offspring
Cluster boundary
0 25 50 100 Meters
N

APPENDIX C

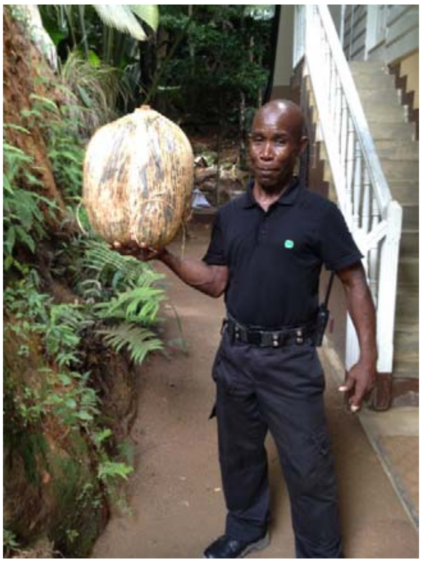
Photos from the field







14/05/2015 12:56




Welcome
to
Curieuse

APPENDIX D

Archived data

All archived data can be found in:

N:/EM_archive/Emma Data/

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Although my name is on the front of this thesis, many, many people have been involved in making my PhD a success. I have learnt many new skills during the time of my PhD and I would like to thank some of the people who helped me by name (but not necessarily in order of importance. It’s too late for those kinds of decisions).

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