Conservation genetics of red-listed medicinal plant species in the Western Ghats, India.

Sofia Hemmilä

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Biology Education Centre and Department of Evolutionary Functional Genomics
Supervisors: Martin Lascoux and Susanne Gustafsson
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Summary

Tropical forests provide not only timber and fuel, but also a wide variety of non-timber forest products (NTFP). In the tropics, many local small-scale industries as well as human communities rely on the forests for the harvest of food, medicine and other extractive products. Western Ghats in India is a hot spot for biodiversity but its forests are under enormous pressure. The primary threats are logging for conversion of forests to agricultural land and the harvest of NTFP. Among the red-listed tree species in the area are *Dysoxylum malabaricum* and *Myristica malabarica*, which are harvested for several purposes. In order to put forward a species recovery program for threatened medicinal plant species in the Western Ghats, a study on the species and the human livelihood demands on them has been initiated at the University of Agricultural Sciences, Bangalore.

This study has two objectives; to review the biodiversity and conservation situation in the Western Ghats today and to develop microsatellite markers for the tree species *Dysoxylum malabaricum* and *Myristica malabarica*. The microsatellite markers are to be used in a future study of the genetic diversity and population structure of tree populations in the Western Ghats.

By reviewing the literature I conclude that the populations of medicinally important trees in the Western Ghats are small and declining. Furthermore, I show that attempts to carry out integrated development and conservation programs often fail and that it is extremely important for a positive outcome to make a thorough study on both the species and the communities before initiating such a program.

I develop microsatellite markers for *Dysoxylum malabaricum* and *Myristica malabarica* using DNA extracted from frozen leaf samples. I use an internet-based software to design primers for the microsatellite sequences. I obtained primers for 16 of the *D. malabaricum* microsatellites and for the 70 longest *M. malabarica* microsatellites.
Introduction

The conservation of species is a critical issue for any attempts to manage sustainable land use. The rapid increase in demands for resources is a global problem that constantly gives rise to conflicts of interests. This is particularly true for the regions where the local people are depending on natural resources for both substantial and commercial use. Often, this is the case in tropical forests, which constitute some of the main hot spots for biodiversity in the world. Even though forests cover less than 6% of the earth's total surface area, they provide habitat for a majority of the earth's known species. On one hand there is the short-term survival of the local people, and on the other hand the long-term conservation to ensure ecological stability and the well being of future generations. Thus, as human populations grow and exploit new resources, it becomes more and more important to establish conservation guidelines that take into account the needs of the local people (Hunter 2007).

The tropical forests provide not only timber and fuel, but also a wide variety of so called Non Timber Forest Products (NTFP). Local small-scale industries as well as human communities in the area rely on the forests for the harvest of food (edible fruits, nuts, spices etc.), extractive products (gums, dyes, essential oils etc.) and medicine (Kaveriappa & Shetty 2001). Despite the long history of the importance of NTFP it is only recently that conservationists have realized the potential of using NTFP management as a means of conserving biodiversity. It has been shown by Peters et al. (1989) that the long-term profit from harvesting NTFP is greater than the profit from the logging and conversion of forests to agricultural land. Thus, this new perspective on conservation has justified the sustainable management of forests also from a social and economic point of view as well as for biological reasons. Although the NTFP approach to conservation can be successful in the long run, NTFP harvest has its effects on the ecological systems and those are not yet fully understood. For example, NTFP harvest can lead to degradation of the gene pool through the selective harvest of the most vigorous individuals. It can also lead to nutrition depletion of the soils and soil erosion when nutritious and structurally important plants are removed (Hiremath 2004).

The effects of forest fragmentation on biodiversity

The well-known and widely studied theory on the species-area relationship states that there is a relation between the area of a given island and the number of species (trees, birds, insects or any other taxa) on that island. An island can be a real island surrounded by water or any other patch of habitat surrounded by some barrier, which means that an island can be e.g. a hilltop, a pond or a forest fragment surrounded by logged land. This theory has been used by ecologists to predict the effects of habitat loss on species diversity and to distribute resources for conservation. The species-area relationship can be described by the power law

\[ S \approx cA^z \]

where \( S \) is the number of species, \( c \) is a constant specific to the taxon, \( A \) is the area of the island and \( z \) is a constant that equals ¼ for many ecosystems. However, this simplification may give less accurate predictions when the area is very large or small (May & Stumpf 2000).

Both logging and the harvest of NTFP contribute to the fragmentation of forests. Studies on fragmentation in tropical forests have revealed a relationship between patch area and species diversity and that the smaller a patch is, the fewer species it can hold. In forest fragments, it
has been shown that rare tree species, as opposed to common tree species, are exceptionally sensitive to the fragment size. The proportion of rare tree species increases with forest area, while common species constitute the main part of the total forest stand (Hill & Curran 2001).

Fragmentation can cause species loss through several mechanisms; both genetic and demographic. When forests are lost, some species will by chance lose their habitat since species occurrence, especially in the tropics, often is patchy. The remaining forest patches will also experience a decrease in species richness. This is due to the smaller area and the isolation of the fragments, which leads to smaller population sizes, less immigration of native species, more immigration of exotic species and more edge effects (i.e. more disturbance from the surroundings) (Turner, 1996).

Population genetics in threatened species
Studies on a variety of wild animal species suggest that genetic factors can contribute to endangerment and extinction (Frankham 1995). Although demographic and environmental variation, habitat loss and overexploitation may contribute most to extinction, genetic factors such as inbreeding and loss of genetic variation can affect populations that are already small in a negative way. Out of these two genetic factors, inbreeding seems to be the most acute and severe one. Genetic factors can also in turn affect demographic factors by altering birth and death rates and their variation (Frankham 1995). A small population size can initiate a negative circle where the decreasing population size leads to loss of genetic variation, which weakens the population. This can be manifested as inbreeding depression; that is, when a population is small enough, mating between closely related individuals will accumulate detrimental alleles in the offspring. In an outbred population, the carrier would normally not be negatively affected by such a mutated allele since they generally are recessive. But as inbred individuals are more often homozygous for the allele, their fitness will likely be reduced (Amos & Balmford 2001; Frankham et al. 1999). Inbreeding depression has been shown in a variety of wild animal and plant species and it has also been shown that inbreeding depression increases the extinction rates in several species. Interestingly, in the species studied there seem to be a threshold level of inbreeding for extinction. Low levels of inbreeding are related to low extinction rates, whereas the extinction rate has a steep increase at intermediate levels of inbreeding. What we can learn from this is that populations in the wild may not show much signs of being on the verge of extinction before they actually go extinct due to inbreeding depression (Frankham 1995).

The degree of inbreeding in a tree population depends to a large extent on the ability of pollen and seed dispersal between populations. There are different dispersal modes among trees, e.g. by birds, insects or by wind. Pollen is often dispersed over long distances but seeds are probably not, though the seed dispersal rate differs among species depending on seed size and dispersal mode. Fragmentation changes the seed and pollen movement and thereby alters gene flow (Hamilton 1999). Seed dispersal mode in tropical trees varies. For instance, seeds from bird-dispersed species can reach longer distances whereas populations of gravity-dispersed species are found in tighter clusters. This might have implications for conservation since the short distance dispersed species would become more isolated than the long distance species because of fragmentation (Seidler & Plotkin 2006). However, Young et al. (1996) emphasize in a literature review that the genetic effects of habitat fragmentation on plant species are hard to predict because of the wide variety of sexual and asexual reproductive systems, dispersal modes, longevity, generation time, natural abundance, storage of genetic material as seeds and interactions with pollinators and dispersal vectors.
Besides leading to accumulation of detrimental alleles, breeding within a small-sized population can lower the overall heterozygosity of the population. The genetic variation in a population is the sum of all genetic variation among the individuals that constitute the population. It is commonly measured as either allelic richness or expected heterozygosity (Young et al. 1996). Most endangered species have low levels of genetic variation (Frankham 1995). When fragmentation has reduced the size and increased the isolation of a population, genetic variation can be reduced by larger effects of random genetic drift, increased inbreeding, reduced gene flow between populations and elevated probability of local extinction of populations in a metapopulation system (Young et al. 1996). The amount of genetic loss will primarily depend on how few individuals the population is reduced to and for how many generations it is held at around that level. It has been shown that decreased heterozygosity can lower the fitness of a population by for example lower the fertility or increase the susceptibility to disease and parasites (Frankham 1995). It can also weaken the ability for the population to adapt to novel challenges (i.e. low heterozygosity populations will be less successful in evolution) (Amos & Balmford 2001; Frankham et al. 1999). However, loss of heterozygosity is a slow process and it is more likely that inbreeding depression will have a larger effect on the population. In the case that heterozygosity itself is more advantageous than homozygosity, heterozygosity will be favored through higher reproductive success and/or higher survival, and thereby a higher degree of heterozygosity than expected may be maintained in the population (Amos & Balmford 2001; Young et al. 1996). Dramatic declines in population size, so called bottlenecks, decrease not only the population size but also the vigor over time and this negative circle might eventually make a population go extinct, although this statement is somewhat controversial (Hunter 2007). Some research indicates that loss of genetic diversity will not by itself endanger the population but in a population that is already endangered, low genetic variation will increase the extinction risk (Holsinger et al. 1998). It has also been argued by Holsinger et al. (1998) that changes in genetic composition puts a population at risk only in cases where self compatibility alleles are lost or when a population hybridizes with a close relative species. However, when populations are small, demographic problems can also have an impact. By chance, age and sex ratios may be imbalanced and thus reproduction may suffer. With all facts taken into consideration, it is desirable to prevent inbreeding and loss of genetic variation when managing threatened populations (Frankham 1995). The expected proportion of heterozygosity in a population, $H_e$, is given by the equation

$$H_e = H_0 (1 - 1/[2N_e/N])$$

where $H_0$ = initial heterozygosity, $N$ = population size, $N_e$ = effective population size and $t$ = time in number of generations. A dissection of the equation shows that in theory, genetic variation can be promoted in a population by maximizing initial heterozygosity (i.e. having as many founder individuals as possible), maximizing population size, maximizing $N_e/N$ ratio (e.g. by equalizing sex ratios) and by minimizing generation time. Inbreeding can be prevented by similar means (Frankham 1995).

A recent study by Jha et al. (2005) on Indian dry deciduous forests concludes that forest patches of decreasing size experience an accelerated rate of species loss. Furthermore, it concludes that the decrease in patch sizes is related to the degree of human activity and the governmental protection of the areas. A literature review by Turner (1996) concludes that evergreen tropical rain forests are particularly susceptible to species loss by fragmentation because of the large number of species that are rare and intolerant of changes in conditions.
Similarly, Mathachen et al. (2006) show that density of medicinally important threatened tree species is higher in undisturbed evergreen forests compared to disturbed evergreen forests in Karnataka, India. These species are often under high pressure since they experience both habitat loss and regeneration loss due to the harvest of seeds. Mathachen et al. conclude that medicinally important threatened tree species are highly sensitive to forest disturbance. Thus, tropical rainforests constitute some major centers for biodiversity but they are also continuously diminishing and in an enormous need for protection.

Models for forest conservation

Often, the most important contribution to the conservation of species is to protect individuals of the species itself and their habitat. Preferably, enough habitat in either one single large area or several small is protected to preserve the species in situ. In natural populations of relatively large sizes, one does not need to worry much about demographic and genetic factors. But when populations are small, one may also need to manage the populations from a genetic point of view. A classical rule of thumb is the 50/500 rule. Soulé (1980) proposed that the minimum effective population size to avoid acute extinction through inbreeding is 50 individuals. Furthermore, the rule states that the effective population size required in order to maintain genetic variation at the level of a natural population is 500 individuals, since that is where, in theory, mutation balances genetic drift. However, this is only a very general estimation. Some animal breeding programs have succeeded with much less than 50 founder individuals while other species may need far more than 50 individuals (Hedrick & Miller 1992).

There are a variety of ways to conserve forests. Early government-run attempts often focused on protecting areas by shutting out all human activities and sometimes even relocating the residents of the areas. Naturally, this idea has been criticized because of the lack of consideration of the forest-dependent communities. In developing countries where people depend on the natural resources for subsistence economy, the classical conservation models have disadvantaged local communities. Also, the success of such programs has been limited because it often triggers a negative attitude towards conservation among the community residents. Thus, more recent attempts have used a variety of models where local communities are involved in the management and where protected areas are divided into zones for different levels of use. Today, most conservation programs also incorporate some kind of incentive for the local people. Incentives may be governmental economic compensation directly related to the conservation effort by the local people. There may also be other economic incentives such as revenue-sharing, employment and tourism development. Unfortunately, these models have not accomplished as much as intended because it is difficult to make the benefits for the locals sufficient and fairly distributed (Spiteri & Nepal 2006).

A review of conservation programs in more than 15 African countries shows that many integrated development and conservation programs fail both the development and conservation objectives. It is argued that the failures depend on erroneous generalisations made by the designers of the programs. Often little is known about the complex ecological and social dynamics in the area of interest. In other words, in order for a program to be successful, more consideration for the specific case is needed when designing the program (Newmark & Hough 2000). Similarly, some researchers argue that integrated development and conservation programs have not reached their full potential (Barret & Arcese 1995). Salafsky et al. (2001) conclude that community-based conservation programs are not
sufficient to promote biodiversity conservation on their own but they can be important contributors under certain conditions.

However, some community-based programs have been successful. For instance, a program that started in 1988 in Nepal has accomplished biodiversity protection by integrating the surrounding communities in the management of the Makalun-Barun national park. It has helped the communities develop ecotourism, trade and links to new markets for local products (Kapoor 2001). Also in the Annapurna conservation area of Nepal an integrated development and conservation program has been successfully carried out. Even though only a few of the community inhabitants experienced direct economic benefit from tourism, most respondents experienced other benefits such as better infrastructure and improvements in health, social services and sanitation. Some costs such as crop damage and predation on livestock were reported, but the overall benefits outweighed the costs (Bajracharya et al. 2006). A final example of successful community-based conservation programs is that of Quintana Roo, Mexico. A forest protection program was initiated in the mid-1980's to protect a number of forest reserves, some specific forest types and one commercially important non-timber forest product. The program was a result of both local and external initiatives. In the most exposed areas, old forest was protected by the plantation of other commercial timbers that could be logged. An evaluation of the program showed a forest loss of only 0.6-0.7% per year (Dalle et al. 2006). According to Brooks et al. (2006), some important factors for the success of a community-based conservation program are permitted use of natural resources, market access and a great community involvement in the project.

**Biodiversity and conservation in the Western Ghats**

Western Ghats is a 1600 km long hill chain area along the west coast of India (between 8 °N and 15 °N). The area occupies only about 5 per cent of the land area of India but through its wealth of different habitats it hosts about 4000 plant species, which is 27 per cent of the country's total plant species. It is estimated that up to half of them are endemic to the Western Ghats area. (Shaankker et al. 2006) Western Ghats is one of the 25 hot spots identified in the world upon the richness in endemic species. Many tree species from several families are of great economic importance to this region as they provide a wide spectrum of NTFP.

In India, forests cover less than 11% of the total land area but the coverage required for ecological sustainability in a longer perspective is 33%. The awareness about the dangers of deforestation has been growing stronger the recent years both among the policy makers and the public. The Department of Applied Botany of Mangalore University was founded in 1994 and has since carried out research on plant conservation, ecology and environment. One of their main objectives has been the *ex situ* conservation of plants of the Western Ghats. An arboretum has been established in order to help preserve more than 100 endemic species with a total of over 2000 plants. The department has also helped establishing an arboretum at Pilikula Nirsarga Dhama, about 15 km from Mangalore. This arboretum hosts some 200 species in an area of 35 hectares. The best thing though, from a conservation perspective, would be *in situ* conservation. Today there are 80 National Parks and 441 Sanctuaries in India, covering 4.5% of the land area. (Kaveriapppa & Shetty 2001)

Medicinal plant species contribute to as much as 30 to 60% of the household incomes in Indian forest-dependent communities. Over the last few decades the liberalization of the markets and the demand for herbal medicine has led to increased pressure on the forests and its products. Since most of the extraction is from natural populations, this enormous pressure
is a true threat towards the plant populations and consequently also to the survival of the species. Successful long-term conservation efforts require thorough investigation of the species distribution, genetic status and anthropological use (Shaanker et al. 2006). Bawa (2004) emphasizes the importance of flexible and locally adapted conservation polices. They should be based on modern science as well as local practices and involve local institutions. The overall goal with conservation should be to promote the well-being of human communities as well as the natural resources they rely upon.

Despite the long tradition of extensive harvest of NTFP in countries like India, and the potential biodiversity implications of it, few attempts have been made to assess the effects. Dr. R. Uma Shaanker, Department of Crop Physiology of the University of Agricultural sciences in Bangalore, India, has initiated a study in order to propose a species recovery program for red-listed medicinal plant species in the Western Ghats. The intention is to put forward a conservation program that is based on thorough genetic and demographic studies, and that takes into consideration the well-being of the forest-dependent communities. The study focuses on seven different tree species. There are five aims in the project, of which the three first have been fulfilled so far. The aims are:

1. to map the spatial distribution of red-listed medicinal plant species in the Western Ghats and to identify geographically distinct populations,
2. to assess the livelihood demands of the communities on medicinal plant species through participatory appraisals in the Western Ghats,
3. to characterize the identified populations with respect to their demographic profile, and to assess the genetic diversity of the populations in the Western Ghats,
4. to evaluate the population structure and genetic diversity of the species in protected and adjoining non-protected areas in Central Western Ghats and
5. to formulate and initiate protocols for restoration and conservation of red-listed medicinal plant species in the Western Ghats (Shaanker et al. 2006).

The role of microsatellite markers in conservation

Microsatellites are stretches of repetitive, non-coding DNA within the genome. They consist of units of 1-4 base pairs that are repeated a number of times, e.g. (CA)$_n$. Because the mutation rate of microsatellite loci is often high, the number of repeat units can vary between individuals in a population or species. This is the key feature that makes them a useful tool if one wants to incorporate population genetics into a conservation program as it provides a way of measuring e.g. relatedness between individuals. Microsatellites can be seen as neutral genetic markers; i.e. DNA that does not contribute to fitness and thus does not undergo selection, but still possesses variability within a species or population.

Microsatellite markers are used in conservation genetics for several reasons. They can be used to investigate the current demographic profile in a species e.g. by performing an assignment test, where individuals are assigned to a population according to the likelihood of finding a certain genotype in different populations (Cornuet et al. 1999). Determining the origin of an individual is also used within forensics, if one wants to hinder illegal trading with plant parts from protected populations. The accuracy in such applications is high (Wasser et al. 2004). Furthermore, microsatellite markers are used to trace back the demographic history to survey the duration and severity of previous bottlenecks. For instance, so called coalescent-based methods are used to calculate historical effective population sizes (Aló & Turner 2005). For example, coalescent-based methods have been used to detect and quantify the effect of recent
human-induced deforestation and habitat fragmentation on orangutan populations on Borneo (Goossens et al. 2006). Those historical inferences can help us to foresee the way populations would react to future declines in population sizes. A final advantage with microsatellite markers, though not as reliable as the other applications, is that they can be viewed as an indirect measure of adaptive genetic variation. By mapping the genetic variance among subspecies or populations, one can define so called evolutionary significant units, i.e. units that show significant allelic divergence and thereby should be given priority in conservation (Moritz 1994). For example, if two populations have been isolated from each other for some time, it is likely that they have evolved different adaptations that may be worth preserving. The amount of adaptive genetic divergence between the populations may be reflected in the non-adaptive microsatellites, but one should be careful to not rely completely on this method as it is the adaptive variation we want to conserve and not the variation at the microsatellite markers themselves (Hedrick 2001).

Objectives

The present study has two objectives. The first objective is to review the work that has been carried out by Shaanker and colleagues on the species conservation in the Western Ghats, with focus on the two species *Dysoxylum malabaricum* and *Myristica malabarica*. I will review the distribution of the two species, the use as NTFP and the demography.

The second objective is to develop microsatellite markers to be used in a future evaluation of the population structure and genetic diversity of the two species mentioned, according to the fourth goal of the ongoing conservation project by Shaanker et al.
Materials and methods

Objective 1. Review of the conservation of red-listed tree species in the Western Ghats.

In this review I will focus on two species: *Dysoxylum malabaricum* and *Myristica malabarica*. The review was part of the annual report from the Ashoka Trust for Research in Ecology and the Environment (ATREE). The work behind the report was carried out in the Western Ghats in 2006. The three first objectives of the study have so far been fulfilled. The distributions of the red-listed tree species were mapped by using geographic information system (GIS). The livelihood demands of the communities on medicinal plant species were examined through interviews with inhabitants of a forest-dependent community. The subjects were asked about what medicinal tree species they knew of and used and their perception of the extent to which these species were endangered. The demographic profile, recruitment and regeneration status of *Dysoxylum malabaricum* was studied in distinct populations throughout the whole Western Ghats, both along a longitudinal and a latitudinal gradient. This was done by measuring the girth of all *D. malabaricum* individuals in a number of quadrats as a way of counting the number of saplings and adults. The populations were classified as either small, medium or large according to the number of adult (i.e. full-grown) trees. Also the density of trees in the quadrants was measured (Shaanker et al. 2006).

Study system

*D. malabaricum* (white cedar) is an endemic tree species only found in the evergreen forests of the Western Ghats (figure 1). It is a large canopy tree that grows up to 40 m in height and 5 m in girth. It has a straight undivided bole and a dense crown with compound leaves measuring up to 35 cm. The bark is pale grey and the lustrous wood has a yellowish orange colour and a sweet scent. It is harvested for its timber and as an ingredient in some traditional Indian medicines (Shaanker et al. 2006).

*M. malabarica* (wild nutmeg) is an evergreen tree that grows to 15-20 m in height. It is frequent in swamp forests of the Western Ghats but also found in South India and Sri Lanka (figure 2). The bark and leaves is used in traditional medicines in India and in other countries. Besides, *M. malabarica* is a close relative to *Myristica fragrans* (nutmeg) and therefore the arils surrounding the fruits are harvested and used as a spice (Shaanker et al. 2006). The IUCN classifies *M. malabarica* as Vulnerable. The main threat towards this species is habitat loss due to agriculture and ground water extraction from the swamps. The Western Ghats Biodiversity Information System also stresses the importance of the overharvesting of fruits and seeds (Western Ghats Biodiversity Network 2006).

Objective 2. Development of microsatellite markers

Extraction of DNA

I extracted DNA from fresh leaves of *Dysoxylum malabaricum* and *Myristica malabarica*. The leaves were collected in March 2005 from tree individuals in the Western Ghats area and frozen while still fresh. Since the leaves of these two species are rich in phenolics and polysaccarides, which inhibit polymerase chain reactions, I used a modified CTAB extraction method (Sambrook et al. 1989).
Processing and size-selection of DNA
I used the extracted DNA to construct an enriched genomic library selecting for high proportions of tandem repeat inserts. A genomic library is a collection of bacteria colonies that contain one specific known insert sequence each. It can be used for efficient amplification of a desired genomic DNA sequence. In this case, I needed the insert sequences to contain microsatellites. This is accomplished by digesting the genomic DNA, size-selecting fragments of appropriate lengths, ligate them into plasmids and to transform *Escherichia coli* cells with the plasmids. For the construction of the genomic library I employed the streptavidin-biotin bench version (Fleisher, R. C. and S. Loew, 1995; M. Hamilton and R. Fleisher, personal communication). I processed 5 µg each of isolated DNA from the two species by digestion with 10 units of restriction enzyme MboI, which leaves a 5′GATC overhang. I dephosphorylated the 5′ sticky ends to prevent self-ligation of the DNA. In order to obtain DNA fragments of appropriate lengths, I size-selected them on agarose gel, selecting fragments of 300-1500 base pairs length. These fragments were the ones that I used as inserts for further construction of the genomic library. I ligated the inserts to double stranded SAULA-SAULB linker at a linker to genomic fragment molar end ratio of approximately 2:1. As the amounts of genomic DNA were not large, I amplified it directly from the ligation in a polymerase chain reaction (PCR). After the PCR amplification, I purified the product using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare).

Enrichment of fragments containing repeat arrays
I wanted to increase the proportion of genomic DNA fragments that contained repeat arrays. Therefore I hybridised the genomic DNA fragments to fragments of six different oligonucleotide repeats: (ACT)_n, (AAC)_n, (ATC)_n, (AAG)_n, (AC)_n and (AG)_n. The oligos were labeled with biotin and this makes them bind strongly to streptavidin. Thus, I used streptavidin-coated iron beads to capture the DNA fragments that contained repeat arrays that were complementary to the oligos. I used a magnet to separate the iron beads from the hybridization mixture. I washed the beads four times to remove the DNA that did not contain repeats. The washes were once at room temperature, twice at intermediate temperature (45°C) and once at stringent temperature (55°C). I kept the temperatures relatively low to decrease the stringency and avoid washing away DNA fragments that contained repeats. However, low stringency is expected to entail some false positives to still remain after washing. To make the single-stranded DNA double-stranded and to increase the amount of DNA I carried out a PCR.

Ligation and transformation
I carried out the ligation and the transformation by following the manufacturer's protocol for pGEM®-T and pGEM®-T Easy Vectors (Promega). I used the pGEM®-T Easy vector, which is a plasmid of 3015 base pairs size with a coding region for β-galactosidase. The genomic DNA is inserted within that coding region. To ligate the genomic DNA fragments with the vectors I simply set up a ligation reaction and incubated it overnight at 4°C. I then transformed competent *E. coli* cells with the vectors in a heat-shock procedure as described in the manufacturer’s protocol.

Cultivation of *E. coli* colonies
I grew colonies of the transformed *E. coli* cells on 150 mm agar plates prepared with 100 µg/ml ampicillin, 250 µl IPTG and 50 µl X-gal. IPTG induces activity of β-galactosidase, an enzyme that promotes lactose utilization, by inducing lacZ gene expression. X-gal is used to
indicate whether a bacterium expresses the β-galactosidase enzyme. X-gal is cleaved by β-galactosidase, forming a bright blue coloured product. Thus, if X-gal and IPTG are contained within an agar medium on a culture plate, one can easily distinguish which colonies have a functioning lacZ gene. Blue colonies still have a functioning lacZ, while white colonies do not. Only colonies with a disrupted lacZ contain genomic DNA inserts.

I grew at least three plates each of the twelve solutions of transformed bacteria (one solution for each oligonucleotide repeat and species). I spread out 150 µl, 200 µl and 250 µl respectively of bacteria solution on the three plates. After incubation at 37°C overnight I selected the white colonies for screening.

Screening for positive clones
For the screening I picked a small amount of the bacteria from the white colonies on the agar plates and performed a PCR on it. Along with the M13F and M13R primers I added the plate-specific oligonucleotide repeat as a primer to each PCR in order to more easily discriminate between transformants that contained microsatellite arrays and those that did not. I ran the PCR product on 1,5% agarose gel to check for positive transformants. Colonies that showed two or more bands on the gel image were considered positive since these two bands indicate amplification of DNA from both the whole genomic DNA insert and the region of the repeat array.

Sequencing
I prepared the positive colonies for sequencing by running a PCR with M13 primers and ET terminators on small samples from the colonies and purifying the product. I employed MegaBACE 1000 for sequencing and searched the obtained sequence files for microsatellite arrays.

Primer design
In order to find and amplify the corresponding microsatellite arrays in other individuals, one needs to have the right primers. I designed primer sequences for the sequenced inserts that contained microsatellite arrays. Before designing the primers I removed all the non-genomic parts of the sequences, i.e. the parts that belonged either to the vector or the linkers. I used the internet-based software Primer3 (Rozen S. and Skaletsky H., 2000) that picks out and presents a number of suitable primer sequences. I divided the microsatellite sequences into two groups. For the first group I designed primers that gave a total product size of about 160 base pairs and for the second group the product size was about 240 base pairs. The primers I chose were about 20 base pairs of length and had annealing temperatures close to 60°C.

Before using the microsatellites for studying e.g. genetic diversity in a population one also needs to determine which ones of the microsatellites that are polymorphic within the population. This work was performed by Dr. Uma Shaanker, University of Agricultural Sciences in Bangalore, India.
Results

Objective 1. Review of the status of red-listed tree species in the Western Ghats

Though *D. malabaricum* is endemic to the Western Ghats, it is widely distributed in the area. The highest density is in the south (figure 1). The distribution of *M. malabarica* is concentrated to the central Western Ghats (figure 2).

![Figure 1. The distribution of *Dysoxylum malabaricum* in the Western Ghats.](image1)

![Figure 2. The distribution of *Myristica malabarica* in the Western Ghats.](image2)
The interview study on the livelihood demands of a forest-dependent community Charmadi, Western Ghats, shows that many different plant species are extracted in the area. The local societies’ extraction for commercial medicinal use is the major threat towards the endangered plant species in the Western Ghats and the extraction for the local’s own use also puts pressure on the species. The one most common species to be extracted is *M. malabarica*, whereas *D. malabaricum* is not on the list of the 21 most commonly harvested species. Since medicinal plants are scarce in this area, the locals are only able to harvest every alternate year. 69% of the respondents even answered that they harvest only some years. This is a clear indication of the decline in these plant populations. 56% of the people also responded that they have perceived a decline of medicinal plants in Charmadi. Furthermore, the respondents considered the vast majority of the species difficult or very difficult to locate. Very few of the species have large populations and most of the species are represented by only a few individuals. There are restrictions regarding the collection and trade of medicinal species in the wild but they are poorly followed. One problem is that the trade is hard to control since most medicinal species are sold under their trade name and in a processed drug form. Therefore, a more efficient conservation strategy would be to ban the collection of plants in the wild and to let commercial users cultivate them.

The demographic profile is important to the stability of future generations. Two medicinal plant tree species were examined, one of which was *D. malabaricum*. In the small-scale study of nine adjacent but distinct populations along a longitudinal gradient there was no significant difference in size class distribution between the populations. However, there was a significant lack of individuals in the advanced growth stage (mean girth at breast height 10-30 cm). This might affect population survival in the future. The lack of differences in size class distribution across the populations may be due to the fact that the species is dispersed via birds whose home ranges are larger than the distances between the populations. This means that even though many populations are small, the dispersal mode of the species may counteract the negative consequences of small population sizes.

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The same parameters were measured in 22 populations along a latitudinal gradient. The results show that there is no difference in size class distributions among the populations. Many of the individuals fall into the size class more than 300 cm girth at breast height. The size class 10-30 cm is absent in the populations along the latitudinal gradient as well. Regeneration (saplings per adult) and density of saplings increase with latitude.

**Objective 2. Development of microsatellite markers**

*Transformation of E. coli with genomic DNA fragments*

I found transformant colonies for all repeats from both of the species. For *Dysoxylum malabaricum*, on average 14% of the colonies on the agar plates were transformants and the total number of colonies on each plate was about 300. For *Myristica malabarica* the proportion of transformants was 21% and the total number of colonies was about 550 on each plate.

*Screening for microsatellite sequences*

Out of the transformants I found a number of microsatellite sequences by analyzing the gel images from the screening procedure. Clones with two or more bands on the gel image were considered positive transformants, i.e. colonies that had a microsatellite sequence inserted (figure 3).
Figure 3. Gel images showing the screening of 2 x 24 transformant colonies from *Myristica malabarica*. The wells marked with "X" indicate the colonies that were considered positive transformants.

For *D. malabaricum*, I found on average 34 transformants that appeared positive from each repeat. For *M. malabarica*, the number was on average 48 transformants.

**Design of primers for the microsatellites**

I designed primer sequences for the 16 microsatellites I found in *D. malabaricum* (table 1) and for the 70 longest microsatellites in *M. malabarica* (table 2).

### Table 1. Primer sequences for *Dysoxylum malabaricum*.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Repeat</th>
<th>Product length (bp)</th>
<th>Left and right primer sequence 5′-3′</th>
<th>T$_m$ (°C)</th>
</tr>
</thead>
</table>
| D1 34 | (ACT)$_6$ | 154 | L: CCACAGACACCTCATCAC  
  R: CGTCACTTTTATTTTGACGAAC | 60.59 |
| D1 46 | (AAC)$_7$ | 207 | L: CGAGAAAAATTTCACTGGGTCT  
  R: TGGGGTTTGTGATCTGACGAT | 59.63 |
| D1 82 | (AAC)$_8$ | 160 | L: ACGGTGCTGTGGAGAGAAC  
  R: GAAATCCGAAACGACGAAA | 60.31 |
| D2 7  | (AAC)$_7$ | 162 | L: CACCGTGAAGATGATGACG  
  R: CCGTTAGACCCAAATAAACAA | 58.86 |
| D2 38 | (AAC)$_8$ | 162 | L: CACCGTGAAGATGATGACG  
  R: CCGTTAGACCCAAATAAACAA | 58.86 |
| D2 48 | (AAC)$_7$ | 166 | L: TCTATTTTGTACCGCACA  
  R: TTTTGTCGATGTTATGTTGG | 59.48 |
| D2 50 | (AAC)$_8$ | 240 | L: GCAGGTTTCAAATCCGTTT  
  R: CTGCAAAACTCTTTGCTGCT | 59.93 |
| D2 67 | (AAC)$_8$ | 158 | L: AGAAATCCGAAACGAGACA  
  R: AGCGTGCTGGAGGAGAAACCA | 59.81 |
| D3 12 | (ATG)$_3$ | 241 | L: GAACCTCTTCGTTGGCCACT  
  R: CAGGAAAATCCGCTTTTCTT | 59.45 |
| D4 12 | (AAG)$_2$ | 242 | L: TGATTCCGAAATAACTGAA  
  R: CTGATTTGAGCTTACGGCCA | 59.45 |
| D4 29 | (AAG)$_1$ | 241 | L: CGGAGGAGTCAATCCCGACT  
  R: TCGGTGACCTCCTACATGGA | 60.26 |
| D4 30 | (AAG)$_2$ | 241 | L: GATCCTCGAGTGAATGTTGATTCC  
  R: TGACGGGTCTCGTTAATCTTG | 59.41 |
### Table 2. Primer sequences for *Myristica malabarica*.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Repeat</th>
<th>Product length (bp)</th>
<th>Left and right primer sequence 5′-3′</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1s 19</td>
<td>(TGA)$_6$ (GGA)$_6$</td>
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<td>L:AAGACGTGAAAACCTATGGTACAA&lt;br&gt;R:TGAGTGGGTCCTCCATCTCCCT</td>
<td>59.14</td>
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<tr>
<td>M1s 20</td>
<td>(ACT)$_6$ (ACT)$_6$</td>
<td>184</td>
<td>L:GACACTGGCCAATATTGACG&lt;br&gt;R:GCAGCAGCAAGGGAGTTA</td>
<td>60.45</td>
</tr>
<tr>
<td>M4s 8</td>
<td>(GAA)$_1$</td>
<td>240</td>
<td>L:AAGCTCCGAGCGTCGTCT&lt;br&gt;R:GGCCCTCATCGTCTCCT</td>
<td>60.27</td>
</tr>
<tr>
<td>M4s 9</td>
<td>(TGA)$_6$</td>
<td>249</td>
<td>L:GGAAGGCCTAAAAAGACATGA&lt;br&gt;R:CCAAAGAGTGCTTTACCC</td>
<td>60.82</td>
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<tr>
<td>M4s 14</td>
<td>(GT)$<em>6$ (GA)$</em>{28}$ (GA)$_{14}$</td>
<td>208</td>
<td>L:GTTCACACGATGGCAGAG&lt;br&gt;R:GCACATTGAGTGGACAGCA</td>
<td>59.37</td>
</tr>
<tr>
<td>M4s 17</td>
<td>(GT)$<em>{11}$ (ATG)$</em>{10}$</td>
<td>247</td>
<td>L:ACACACTTTGTCCTGGA&lt;br&gt;R:GCTGCATTGGTACTTGTGC</td>
<td>58.29</td>
</tr>
<tr>
<td>M4s 18</td>
<td>(AAG)$_{12}$</td>
<td>249</td>
<td>L:TGCCTATCACCAACCGTTC&lt;br&gt;R:GCTGCAAGAACCCAGCAT</td>
<td>60.74</td>
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<tr>
<td>M4s 34</td>
<td>(CT)$<em>{10}$ (CA)$</em>{15}$</td>
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<td>60.16</td>
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<tr>
<td>M4s 40</td>
<td>(AAG)$_{12}$</td>
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<td>60.32</td>
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<td>M4s 50</td>
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<td>59.53</td>
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<tr>
<td>M4s 53</td>
<td>(ATG)$_{16}$</td>
<td>168</td>
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<td>60.16</td>
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<tr>
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<tr>
<td>M4s 77</td>
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<td>59.53</td>
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<tr>
<td>M4s 95</td>
<td>(ATG)$_{6}$</td>
<td>233</td>
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<td>60.16</td>
</tr>
<tr>
<td>M5r 33</td>
<td>(ATG)$_{12}$</td>
<td>157</td>
<td>L:TGCCTATCACCAACCGTTC&lt;br&gt;R:GCTGCAAGAACCCAGCAT</td>
<td>60.74</td>
</tr>
<tr>
<td>Gene</td>
<td>Start (nt)</td>
<td>End (nt)</td>
<td>Forward Sequence</td>
<td>Reverse Sequence</td>
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<tr>
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<td>------------</td>
<td>----------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>M5r 41</td>
<td>(ATG)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>244</td>
<td>L: TTCTTGAGCTGACCCCATTT</td>
<td>R: CCGTAGCCTTTCGATTTTC</td>
</tr>
<tr>
<td>M5r 59</td>
<td>(ATG)&lt;sub&gt;3&lt;/sub&gt; (GT)&lt;sub&gt;10&lt;/sub&gt; (AG)&lt;sub&gt;5&lt;/sub&gt;</td>
<td>255</td>
<td>L: AGCTGACCCAGCTAATTGGA</td>
<td>R: GTGTGATGAACATGAGGGTTTA</td>
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<tr>
<td>M6s 65</td>
<td>(AG)&lt;sub&gt;19&lt;/sub&gt;</td>
<td>160</td>
<td>L: TTTCGAGAAGCATGAACCCT</td>
<td>R: CGGCAAGGAAAGACCACTT</td>
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<tr>
<td>M2s 7</td>
<td>(GTT)&lt;sub&gt;5&lt;/sub&gt;</td>
<td>163</td>
<td>L: CAGTAAAGCAACTCCAGAAGGTC</td>
<td>R: CCGAACCTTGGATCTACCT</td>
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<td>M2s 12</td>
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<td>L: GTTCACACTCTGGCCCTT</td>
<td>R: GATGCACTTTAGTAAGGTTTCAAGC</td>
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<td>M2s 14</td>
<td>(CAA)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>160</td>
<td>L: TGAACCTGACAGTCGCTC</td>
<td>R: CCGGAGCTTGGATCTACCT</td>
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<tr>
<td>M2s 17</td>
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<td>L: CCCATATAGCTAATTTGAGGA</td>
<td>R: TGGTGAGCTCAGATGAGGGTTTA</td>
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<td>M2s 91</td>
<td>(GTT)&lt;sub&gt;13&lt;/sub&gt;</td>
<td>159</td>
<td>L: AAAACGGTCACACATCCATA</td>
<td>R: TTTCTTGAAATCCCTTTTT</td>
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<tr>
<td>M2s 93</td>
<td>(GTT)&lt;sub&gt;5&lt;/sub&gt;</td>
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<td>L: CAGTAAAGCAACTCCAGAAGGTC</td>
<td>R: CGGCAAGGAAAGACCACTT</td>
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<tr>
<td>M2r 6</td>
<td>(CAA)&lt;sub&gt;11&lt;/sub&gt;</td>
<td>154</td>
<td>L: GTTCACACTCTGGCCCTT</td>
<td>R: GATGCACTTTAGTAAGGTTTCAAGC</td>
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<tr>
<td>M2r 9</td>
<td>(GT)&lt;sub&gt;20&lt;/sub&gt;</td>
<td>218</td>
<td>L: CTCTCCACATGTCTGGAGGA</td>
<td>R: AGGTGATATGGCCCATTTTT</td>
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<tr>
<td></td>
<td>(AG)&lt;sub&gt;17&lt;/sub&gt;</td>
<td>160</td>
<td>L: TCTCAGGCTGCTTGGCTCT</td>
<td>R: CTAACCTCTCCCCCTGGCTT</td>
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<tr>
<td>M2r 24</td>
<td>(CAA)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>166</td>
<td>L: CCCCAGTGTGACATGATTGTTT</td>
<td>R: TCATCATCACCCTTGTAGCACAG</td>
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<tr>
<td>M2r 31</td>
<td>(GT)&lt;sub&gt;16&lt;/sub&gt;</td>
<td>158</td>
<td>L: AGCTGAGTGGTGCTGAGTATG</td>
<td>R: CGGCCCAAATTCGACTACAA</td>
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<tr>
<td></td>
<td>(AG)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>156</td>
<td>L: TACCTCTCCCCCTGGCTT</td>
<td>R: CTAAGGTCTGGCTTCTCTTCT</td>
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<tr>
<td>M2r 32</td>
<td>(GTT)&lt;sub&gt;5&lt;/sub&gt;</td>
<td>164</td>
<td>L: TGGAGTTGTTGGATTCTCCTTTC</td>
<td>R: AAAACGGTCACACATCCATA</td>
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<tr>
<td>M2r 40</td>
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<td>L: CCCCAGTGTGACATGATTGTTT</td>
<td>R: TCATCATCACCCTTGTAGCACAG</td>
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<td>M2r 44</td>
<td>(GTT)&lt;sub&gt;13&lt;/sub&gt;</td>
<td>166</td>
<td>L: GAAAAGATAACGGTCACACATCC</td>
<td>R: TCTCAGGCTGCTGAGTATG</td>
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<tr>
<td>M2r 53</td>
<td>(GTT)&lt;sub&gt;5&lt;/sub&gt;</td>
<td>167</td>
<td>L: GAAAAATCCAGAAGCAAGAACAT</td>
<td>R: TGGTTAATTCAACTCACTCAGT</td>
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<td>M2r 72</td>
<td>(GTT)&lt;sub&gt;5&lt;/sub&gt;</td>
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<td>L: CAGTAAAGCAACTCCAGAAGGTC</td>
<td>R: CGGCAAGGAAAGACCACTT</td>
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<tr>
<td>M2r 76</td>
<td>(CAA)&lt;sub&gt;8&lt;/sub&gt;</td>
<td>177</td>
<td>L: GGTGGATGCTGGGCTTATTTC</td>
<td>R: ACGTTCACAGATCCCCATA</td>
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<td>M1s 38</td>
<td>(TGG)&lt;sub&gt;2&lt;/sub&gt; (TAG)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>248</td>
<td>L: AACCCCCACTTGGTTTCC</td>
<td>R: CAGCAGCAGAGGATTTACTA</td>
</tr>
<tr>
<td>M1s 41</td>
<td>(CTA)&lt;sub&gt;9&lt;/sub&gt;</td>
<td>156</td>
<td>L: TGATTTCAGCCACGCAGT</td>
<td>R: CACACTGGAGAAGATGGGAAC</td>
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<tr>
<td>M1s 75</td>
<td>(TGG)&lt;sub&gt;5&lt;/sub&gt; (TAG)&lt;sub&gt;9&lt;/sub&gt;</td>
<td>164</td>
<td>L: AAGAAAATGGGGCAGACAGT</td>
<td>R: TGCTGCTAATTCTCTTCTTCTGAT</td>
</tr>
<tr>
<td>M1s 77</td>
<td>(AAG)&lt;sub&gt;5&lt;/sub&gt;(TAG)&lt;sub&gt;9&lt;/sub&gt;(AAG)&lt;sub&gt;5&lt;/sub&gt;</td>
<td>159</td>
<td>L: ATGCCAAAAATACCTCACA</td>
<td>R: TTGGTGTAAGCTTCTTCTTCTT</td>
</tr>
</tbody>
</table>

18
<p>| M1r 6 | (AGT)$<em>{10}$ (GAA)$</em>{10}$ | 164 | L: CCGACAACTGGAGAAGATGG | 59.71 |
| M1r 10 | (ACT)$<em>{10}$ (CT)$</em>{8}$ | 155 | L: GATCTTGGGTGAAGCTTTC | 57.82 |
| M1r 11 | (ACT)$<em>{11}$ | 249 | L: GACGACGACTCTTTCTCT | 58.14 |
| M1r 12 | (TAG)$</em>{2}$ (AGG)$<em>{2}$ | 163 | L: TTGCTCATTCTCAATGTGA | 58.52 |
| M1r 13 | (CT)$</em>{11}$ (CT)$<em>{4}$ (CT)$</em>{8}$ | 237 | L: CACAGCTCACCCTCTCTT | 59.96 |
| M1r 19 | (TGG)$<em>{2}$ (TAG)$</em>{2}$ | 239 | L: CTGTTAGCCTCACCCTT | 60.05 |
| M1r 38 | (TAG)$<em>{2}$ (TAG)$</em>{2}$ | 168 | L: CATTAACCCAGGTACTTGACC | 59.35 |
| M3s 8 | (CAT)$<em>{8}$ | 158 | L: TGCACAGCCAGCTCCTG | 60.11 |
| M3s 21 | (ATG)$</em>{10}$ | 243 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |
| M3s 22 | (GAA)$<em>{8}$ (GAT)$</em>{10}$ | 243 | L: TGATCTTGGGTGAAGCTTTC | 57.82 |
| M3s 33 | (TGA)$<em>{10}$ | 156 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |
| M3s 35 | (ATG)$</em>{8}$ | 244 | L: TTGCTCATTCTCAATGTGA | 58.52 |
| M3s 36 | (ATC)$<em>{8}$ | 253 | L: CCGACAACTGGAGAAGATGG | 59.71 |
| M3s 45 | (ATG)$</em>{8}$ | 169 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |
| M3s 67 | (ATG)$<em>{8}$ | 247 | L: TTGCTCATTCTCAATGTGA | 58.52 |
| M3s 68 | (ACT)$</em>{11}$ | 247 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |
| M3s 75 | (ATG)$<em>{10}$ | 245 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |
| M3s 77 | (ATG)$</em>{8}$ | 247 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |
| M3s 78 | (AGT)$<em>{8}$ | 246 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |
| M3s 79 | (ATG)$</em>{8}$ | 247 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |
| M3s 84 | (ATG)$<em>{8}$ | 168 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |
| M3s 85 | (ATG)$</em>{8}$ | 251 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |
| M3s 88 | (ATG)$<em>{11}$ | 156 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |
| M3s 96 | (ATG)$</em>{8}$ | 161 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |
| M3r 10 | (ATG)$_{10}$ | 247 | L: AAAGGAAGACCTAAAATGACATGG | 59.79 |</p>
<table>
<thead>
<tr>
<th>M3r 2</th>
<th>(ATG)$_{10}$</th>
<th>160</th>
<th>R:GGTTGCAAATGAGAGTGTGG</th>
<th>59.14</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>L:GCCACAGTTTGATGTCAGCTT</td>
<td>60.31</td>
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<td></td>
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<td>R:AGCCTCCGCTCTACCATCTC</td>
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<td>250</td>
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</tr>
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<td></td>
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<td>R:TGGAGCCACATTAGAATGACA</td>
<td>59.14</td>
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<tr>
<td>M3r 34</td>
<td>(ATG)$_{14}$</td>
<td>162</td>
<td>L:TTATGGCTTTAAAAAGGACCATAGC</td>
<td>58.15</td>
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<td>M3r 71</td>
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<td>150</td>
<td>L:AAACAGAATGGAATGCGAAG</td>
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<td>(TGG)$_{9}$</td>
<td>155</td>
<td>R:TCTCCTCCTCAGAAAAAGCAAG</td>
<td>59.24</td>
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<tr>
<td></td>
<td>(ATG)$_{9}$</td>
<td></td>
<td>R:TCTGACACCTGAAAATCTTGTTTGC</td>
<td>58.29</td>
</tr>
</tbody>
</table>
Conclusion

The results from the demographical study show that the populations of *D. malabaricum* are already suffering from demographic imbalance. Regeneration in some populations is poor. It would be of interest to conduct a similar study for *M. malabarica* as well. The restriction of the harvest of fruits, in combination with the promoting of commercial cultivation of the species might be part of a solution.

Before initiating any conservation program on the species it would be useful to carry out a thorough study of the population structure and genetic diversity of the species. One could then compare the differences between populations in protected and non-protected areas. The microsatellites developed for *M. malabarica* may be a good base for such a study, while the microsatellites for *D. malabaricum* may be too few at this point.
Acknowledgements

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References


