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Population Genetic Structure and Mating System of *Swietenia macrophylla* King (Meliaceae) in the Brazilian Amazon: Implications for Conservation

by

Maristerra R. Lemes

A thesis submitted to the University of Stirling for the degree of Doctor of Philosophy

Department of Biological Sciences
University of Stirling
Stirling, UK
July 2000

"My dream is to see this entire forest conserved because we know it can guarantee the future of all people who live in it..."

Francisco (Chico) Mendes

(Rubber tapper, trade union leader and
ecologist murdered in 22/12/88 because
of his ideas and struggle to protect
the Amazon rainforest)

ACKNOWLEDGEMENTS

It was a long journey from the conception to the completion of this study. During this journey, I was blessed with help of many people and institutions who direct or indirectly made this work possible.

First, I would like to thank the Brazilian Ministry of Science and Technology (PADCT/CNPq - project# 62.0059/97.4) and the World Wildlife Fund (WWF-Brazil - Program Nature and Society, project# CSR 95-033) for the essential financial support to this study. Funding was also partially provided by the European Commission (INCO project # ERBIC18CT970149) for which I am grateful. I extend my thanks to the Brazilian Council for Research (CNPq) who sponsored my PhD grant and fees in UK and Brazil (Process RHAE# 260021/94-6 and 160039/96-7), and the Fundação Botânica Margaret Mee for supplementary grant.

I acknowledge the Instituto Nacional de Pesquisas da Amazônia (INPA) for support in many ways, the Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis (IBAMA) for logistical support during field work, and the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA - Recursos Genéticos e Biotecnologia) for logistical support during laboratory work.

I wish to thank Professor John Proctor for supervision, especially for the help during the thesis writing. I acknowledge also to Dr. Richard Abbott (University of St. Andrews) and Dr. Mike Wyman (University of Stirling) for the acceptance to participate of my examination committee.

I would like to express my gratitude to Dr. Dario Grattapaglia and Dr.Márcio Elias Ferreira for welcomed me to work on their lab (Plant Genetics Laboratory - EMBRAPA, Brasília, Brazil) and

generously providing logistical support and scientific advice during my stay there.

Very special thanks to my friend and molecular biologist Rosana Brondani who trained and helped me in the development of the microsatellite markers. Her technical advice, constant encouragement, patience, and friendship were essential during all laboratory work.

I am very grateful to José Ribeiro my field assistant for his dedication carrying out fieldwork. During field expeditions José and I counted with the assistance of many Amazonian forest workers ("mateiros") who I would like to express my appreciation and gratitude, especially: Orlandino Candiotto, Jurandir Reis Galvão and Jerônimo M. de Souza.

My thanks to Jimmy Grogan for logistical support during field collection at the Marajoara Management Project, in Southeastern Pará, and for providing information on the mapping of mahogany trees in Marajoara.

Thanks to Dr. Spartaco Astolfi Filho for the support during the initial phase of the project in Brazil, Jaime Tadeu França (IBAMA) for providing arrangements to collect mahogany in some study areas, Dr. Lynn Loveless for kindly providing some mahogany seeds from Marajoara, Antonieta N. Salomão and Rosângela C. Mundin (EMBRAPA) who kindly helped me with the mahogany seed germination, Chris Dick for providing important literature, and Jorge A. L. Costa for help with the figures.

I would like to thank all my colleagues at the Plant Genetics lab (EMBRAPA) with whom I spent an enjoyable time during my stay in Brasília, for their friendship and help in many ways. Special thanks to Rosane Collevatti for the discussions on population genetics and help, and also to Alessandra Maria Reis for encouragement and friendship.

I wish to thank my parents, brother, sister and nephew for their constant support especially during our stay in Brasília.

My very special thanks to Dinete M. Nascimento for her dedication, patience and commitment caring after my sons and taking care of our home for so many years. Thanks also to Lucineide for her help with the kids during our stay in Brasília.

I would like to thank my dear sons Pedro and Daniel for their resignation, understanding and patience with this thesis business and mum´s lack of time.

Finally, I wish to thank my husband Rogério for the unfailing support, encouragement, extreme patience, understanding, help and love without whose this work could not have been possible. He was an enthusiastic for the project and made many valuable contributions to the thesis. I thank him for sharing his knowledge, feelings and life with me.

I declare that the thesis has been composed by myself and that it embodies the results of my own research or advanced studies. Where appropriate, I have acknowledged the nature and extend of work carried out in collaboration with others included in the thesis.

Maristerra R. Lemes

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ABSTRACT

Mahogany, *Swietenia macrophylla* (Meliaceae) is the most valuable hardwood species in Neotropics and is seriously threatened owing to over-exploitation and habitat destruction. The population genetic structure and mating system of S. *macrophylla* were studied in the Brazilian Amazon for conservation purposes. Ten highly polymorphic microsatellite markers were developed from an enriched genomic library of *S. macrophylla* and combined in three multiplexed fluorescence-based genotyping systems. The number of alleles per locus ranged from 11 to 25 (mean = 15.8). The probability of genetic identity ($7x10^{-15}$) and the probability of paternity exclusion (0.999998) found over all loci indicate the high discriminating power of these markers.

The genetic structure was investigated in seven populations 8-2,103 km apart. High genetic diversity was detected within populations (mean H_e = 0.761, range 0.719-0.800) and a significant level of inbreeding was found (f = 0.046, P<0.0001, range 0.014-0.097) indicating nonrandom mating of individuals within populations. Genetic differentiation among populations was significant (θ = 0.12 and ρ = 0.14, P<0.0001), but no clear pattern of isolation by distance was found. Conservation strategies for mahogany should take into account the existence of important genetic structuring of populations.

S. macrophylla seems to have adaptations that preferentially produce outcrossed progeny but also allows for selfing. The high

multilocus outcrossing rate (t_m = 0.958) estimated for one population indicated that, although there was a prevalence of outcrossing, selfing was not negligible. Around 4-6% of seedlings in the population were likely to have resulted from self-fertilization and substantial biparental inbreeding was denoted by the significant difference between the multilocus and singlelocus estimates (t_m - t_s = 0.14). Owing to the species pre-adaptation to colonize newly open, disturbed habitats, many of the remaining trees in logged areas may persist as viable individuals which could be very important for population recovery and genetic conservation programmes.

Chapter 1

General Introduction

Deforestation and habitat destruction of tropical forests

The destruction of tropical forests has been occurring at an alarming annual rate of 100,000 to 200,000 km^2 (Katzman & Cale, 1990; Whitmore, 1997). If impacts such as selective logging are considered, the rate of tropical deforestation exceeds 200,000 km^2 per year, or about 1.2% of the total extent of tropical forests in the world (Laurance & Bierregaard, 1997). The tropical forests occupy only 7% of the earth's land surface and support more than half of the biodiversity of the planet making them the most biologically diverse and ecologically complex biomes of the earth (Myers, 1986). Tropical moist forests constitute 86% of the natural tropical forests and the lowland evergreen rainforests represents nearly 50% of the moist forests with the largest extent located in the Americas, especially in the Amazon basin (Whitmore, 1997). Such forests are characterized by not less than 100mm of precipitation in any month for two out of three years, with a mean temperature of 24°C and usually occur at altitudes below 1300m (Myers, 1986).

The Brazilian Amazon comprises an area of approximately five million km^2 (about the size of Western Europe) of which 4 million km^2

are covered by forests (Brazil, INPE 2000). Brazil is unquestionably the richest country in the world in terms of overall terrestrial and freshwater diversity. It is considered the most diverse country in number of species of angiosperms plants (60,000), mammals (524), and freshwater fishes (>3,000), the second one in number of species of amphibians (517) and butterflies (3132), third in birds (1622), and fifth in reptiles (468) (Mittermeyer *et. al.*, 1997). In Central Brazilian Amazonia more than 1,000 tree species were recorded in 2000 ha at the Ducke Reserve, near Manaus (Ribeiro *et al.*, 1999). Despite the great biodiversity of the Brazilian Amazon forest, this biome is being destroyed at an alarming speed. The deforested area in the Brazilian Amazon by 1998 had reached 551782 km based on LANDSAT satellite data interpreted at Brazil's National Institute for Space Research (Brazil, INPE 2000). The size of the deforested area in the Brazilian Amazon is now equivalent to the area of France.

The human activities causing most of the deforestation in the Brazilian Amazon are cattle ranching, agriculture, and commercial logging. The opening of the Brazilian Amazon frontier, and the beginning of forest destruction, started in the early 1970's with the building of roads into the forest and the starting of a colonization process based on slash-and-burn agriculture and cattle ranching in conjunction with a policy of incentive concessions for these practices implemented by the Brazilian government.

Logging has become an increasing economic activity in the Brazilian Amazon region over the last two decades and it is expected to increase even more rapidly in the medium-term future owing to the diminishing of timber resources of the Asian forests. Generally, logging in the Brazilian Amazon is highly selective focusing on a few species of high commercial value both in national and international markets (Uhl *et al.*, 1991). Most of the timber exploitation in the Brazilian Amazon is for the export market (Verissimo *et al.*, 1998). In *terra firme* (dryland) forests hundreds of kilometers of roads are being built by logging companies to transport the logs to sawnmills and act as a catalyst for deforestation by encouraging haphazard settlement, agriculture, and pastures. Recently, Malaysian and other Asian timber companies have started timber exploitation in the Brazilian Amazon and are a source of great concern due to their aggressive operations which have already lead to the severe depletion of the timber stocks in their home countries (Whitmore, 1998). These companies already own or control about 4.5 million of ha of the Brazilian Amazon. They are buying large tracts of forest by means of concessions received from the Brazilian government or purchasing interests in local timber companies (Laurance, 1998).

To date, allied to logging activities, the spread of the soybean cultivation into large areas of the Brazilian Amazon forest is considered a great threat because this practice justifies government

incentives for the cultivation and building of an infrastructure for soybean transportation such as roads, railways and hydroways. Fearnside (1999) considered that this infrastructure will cause more destruction to the forest than the actual clearing of forest areas for the soybean plantations.

Logging has deleterious effects on tropical forests at local to regional scales and includes damage and mortality of trees, significant canopy loss, soil erosion, frequent occurrence of fire, invasion by vines and grasses, destabilization of watershelds, depletion of biodiversity, increase of deforestation, climate change, and threats to indigeneous peoples (Smith, 1981; Uhl & Vieira, 1989; Verissimo *et al.*, 1992; Laurance, 1998).

Habitat destruction and fragmentation resulting from human activities has been recognised as the primary cause of the loss of biological diversity, especially in tropical forests (Ehrlich, 1988). Early, the studies were focused mainly on the ecological effects of habitat destruction and fragmentation on species richness and population composition and dynamics (Lovejoy *et al.*, 1986; Powell & Powell, 1987). Recently, attention has been directed to the effects of these disturbances on population genetics and their implications for species conservation (Young *et al.*, 1996).

Population genetics and conservation

Genetic diversity provides the variability through which a species can evolutionarily adapt to environmental changes and is essential for the long-term survival of species (Fisher, 1958). Deforestation and habitat destruction may result in losses of genetic variation within species leading to the extinction of locally adapted populations as well as reducing gene flow and sizes of effective populations (Bawa, 1994; Hall *et al.*,1996).

The level of genetic diversity and differentiation among populations is affected by factors such as random genetic drift, inbreeding, gene flow, and mating systems (Wright, 1931, 1943). Together with demographic and environmental stochasticity, these factors determine the persistence of populations in the long term (Ellstrand & Elam, 1993). Thus, an understanding of the overall level of genetic diversity and its distribution within and among populations is fundamental for species conservation.

Demography and genetics have been considered important subjects in conservation analyses of natural populations (Lande, 1988; Simberloff, 1988; Alvarez-Buylla *et al.*, 1996). Demographic criteria refer to the size and temporal change of populations and the genetic ones refer to the genetic variation and its distribution within populations (Alvarez-Buylla *et al.*,1996). The significance of size of populations for their breeding structure, genetics and evolutionary

dynamics has been the central theme in conservation biology (Barrett & Kohn, 1991).

Effective population size and genetic drift - The population sizes important to conservation genetics may not be necessarily equivalent to the number of individuals in a population (Barrett & Kohn, 1991). The number of breeding individuals are usually different to the total population size due to factors such as temporal fluctuations in numbers, nonrandom mating, high variance in reproductive success, unequal sex ratios, age and size structure, and gene flow among populations (Kimura & Crow, 1963; Crawford, 1984).

The concept of effective population size (N_e), first proposed by Wright (1931) refers to the size of an idealized population in which individuals contribute equally to the gamete pool and have the same amount of inbreeding or variation in allele frequencies as the "real" population considered (N). It has been recognized as a general indicator of the rate at which genetic drift proceeds to alter the genetic composition of a population.

Genetic drift is an evolutionary process which produces changes in allelic frequencies due to random sampling of gametes (Wright, 1969). It tends to diminish genetic variation; ultimately, leading to the fixation of one allele at each polymorphic locus (Kimura & Crow, 1963; Lande & Barrowclough, 1987). Thus, the effective size of a population is central to the rate of loss of genetic variation. The

rate of loss of genetic variation due to genetic drift will be higher in populations with smaller effective sizes, whereas in large populations its effects are trivial (Alvarez-Buylla *et al.*, 1996).

The measures of effective population sizes are difficult to obtain because estimates of N_e assumes that a discrete population can be identified which, in most cases, is difficult to delimit objectively in natural conditions (Alvarez-Buylla *et al.*, 1996). Usually, information on the size of a population is provided by estimates of neighborhood size (N_b) , defined as a group equivalent to a panmictic unit within a continuous distribution of individuals. Estimates of effective population (N_e) and neighborhood size (N_b) are scarce for plants but they have been reported for some tropical tree species (Eguiarte *et al.*, 1993; Boshier *et al.*, 1995a; Alvarez-Buylla *et al.*, 1996).

Inbreeding - Inbreeding occurs due to matings between related individuals or by self-fertilization. The main genetic consequence of inbreeding in a population is to increase the frequency of homozygous genotypes at the expense of the frequency of heterozygous genotypes (Hartl, 1987). Populations of outcrossing species carry a genetic load of deleterious mutations which is expressed in homozygotes, but masked by the more frequent, dominant alleles in heterozygous combination (Ledig, 1986). In the short term, a loss of heterozygosity can reduce individual fitness (inbreeding depression) and population viability (Charlesworth &

Charlesworth, 1987; Ellstrand & Elam, 1993; Nason & Hamrick, 1997). Inbreeding depression is defined as a reduction in fitness, due to the expression of deleterious alleles in homozygosity, of inbred/selfed offspring compared with outbred/outcrossed offspring and is influenced by factors such as the mating system and population size (Charlesworth & Charlesworth, 1987; Barrett & Kohn, 1991).

The loss of heterozygosity or increase of the level of inbreeding (F) in a population is a function dependent on the effective population size (N_e), ($\Delta F = 1/(2N_e)$, Wright, 1931; Falconer, 1981) for each generation. Small populations have increased rates of inbreeding when compared to larger populations (Barret & Kohn, 1991). From a conservation biology point of view, large populations are necessary to keep the level of inbreeding low and maintain high levels of heterozygosity. Natural populations of outbreeding species maintained in very small sizes would in the long-term collapse or be fixed for midly deleterious genes diminishing their reproductive capacity and consequently increasing the risk of extinction. In this way, the species ability to respond to changing selection pressures can be limited (Ledig, 1986; Templeton *et al.*, 1990).

Gene flow – Gene flow, movement of genes among populations, plays a critical role in determining the genetic structure of populations (Slatkin, 1985). Restricted gene flow diminishes effective population

size leading to more inbreeding within populations and consequently more differentiation due to selective forces and genetic drift. On the other hand, extensive gene flow will homogenize genetic differences among populations even under selective pressure (Jain & Bradshaw, 1966).

The rate of gene flow is strongly related to the size of populations and the distances between them. The gene exchange among populations declines with increasing geographic distance. Thus, widely isolated populations are likely to be more differentiated than closely spaced populations (Bawa, 1994). As gene flow is important in determining the distribution of genetic variation within and among populations, its measurement has a high priority for population genetics, management and conservation issues. In plant species, the measurement of gene flow in natural populations has been inferred by direct estimates of pollen and seed dispersal distances. This method attempts to describe gene movement within plant populations by monitoring the movement of pollen or seed vectors or following marked pollen or seeds (Levin & Kerster, 1974). The major criticism of this method is that the direct measure of dispersal alone does not necessarily reflect movement of genes if the breeding success of migrants is not taken into account (Whitlock & McCauley, 1999). Other measurements have been made by using indirect methods to estimate gene flow from the distribution of

genetic diversity among populations or from allele frequency data using molecular markers (Wright, 1931; Slatkin, 1985; Slatkin & Barton, 1989; Hamrick *et al.*, 1995).

Studies using indirect estimates of gene flow among populations of tropical tree species have shown that the gene movement via pollen and seeds is extensive enough to overcome the effects of genetic drift in these species, which is indicated by the relatively low levels of genetic differentiation among populations (Loveless, 1992; Hamrick, 1994)

Mating systems – Mating systems determine the mode of transmission of genes from one generation to the next (Brown, 1989). The system of mating of a species is described by the union of male and female gametes in a population and their genetic dynamics. The principal issue in mating systems is the degree of inbreeding which will strongly influence the distribution of genetic variation within and among populations (Bawa, 1994). The mating system is a major factor determining the genetic structure of plant populations and their susceptibility to loss of genetic diversity after habitat destruction and fragmentation (Nason *et al.*, 1997).

In plants, mating systems have been mainly characterized by quantification of the degree of outcrossing. Outcrossing rates have been estimated by segregation analysis of marker genes in progeny arrays examining genotypes of half-sib families (offspring from a

maternal plant) from mother plants of known or assigned genotype, and calculating the relative proportion of seeds resulting from inbreeding and outcrossing (Bawa, 1994). Most tropical tree species are strongly outcrossed. Studies on reproductive biology have shown that the great majority of tropical plants species are dioecious or self-incompatible (Bawa, 1974; Chan, 1981; Bawa *et al.* 1985a, 1985b). Additionally, studies using isozymes markers have confirmed the prevalence of outcrossing in tropical tree species (O´Malley & Bawa, 1987; O´Malley *et al.*, 1988; Murawski *et al.*, 1990; Murawski & Hamrick, 1991; Eguiarte *et al.*, 1992; Boshier *et al.*, 1995b; Loveless *et al.*, 1998). Although dioecious and self-incompatible species are not self-fertile, inbreeding may still occur due to mating among close relatives and limited gene dispersal.

The use of molecular markers for assessment of genetic variation in tropical tree species

Molecular marker technologies are increasingly being used to analyse the organization of genetic variability in natural populations of plants (Rafalski *et al.*, 1996; Grattapaglia *et al.*, 1997). An understanding of the levels and distribution of genetic variation is fundamental for the effective planning of *in situ* and *ex situ* genetic conservation. The use of molecular markers in genetic studies presents many advantages over analysis of morphological characters:

(1) Molecular markers are neutral and not subject to environmental effects because they have a genetic basis, (2) usually the protocols and analytical processes are universal being readily applied to any species, (3) a high level of polymorphism is found per locus compared with the low level observed for morphological markers, (4) non-destructive sampling of biological material (5) The genotype can be studied from cells or tissues at different stages of the life cycle (Ferreira & Grattapaglia, 1995).

A wide array of molecular markers are being applied to the genetic analysis of populations. Two major classes of markers can be identified: (1) markers that classify individuals into nominal genotypic categories, but the categories can not be ordered or grouped. The defining characteristics of this class of markers are that frequency data are available for each genotypic category, but that the categories cannot be ordered or grouped in any way, (2) markers which classify individuals into genotypic categories that themselves may be grouped according to degree of relationship (Milligan *et al.*, 1994). In the first class are included isozymes, restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), and simple sequence repeats (SSR, also called microsatellites). The second class includes haplotype-based markers such as DNA sequences and restriction site markers. The choice of the marker should be driven

mostly by the particular application and interest in mind followed by considerations of the necessary technical skills and facilities, costs and limitations on the access to the technology (Grattapaglia *et al.*, 1997).

Isozymes have often been used to assess levels of genetic variation within and among populations of tropical plant species (Hamrick & Loveless, 1986; Loveless, 1992). Also, they have been routinely used to examine evolutionary factors affecting the genetic structure of plant populations such as mating systems (Hamrick, 1989; Barret & Eckert, 1990). Most of the studies on population genetic structure and mating systems of tropical tree species were carried out in Central America. The patterns of genetic organisation found in these studies showed that most of the species had highly outcrossed breeding systems and that levels of genetic variation within populations were significantly higher than among populations (Eguiarte *et al.*, 1992; Boshier *et al.*, 1993; Hall *et al.*, 1994a; Chase *et. al.*, 1995; Loveless, 1998).

Among the DNA-based genomic marker technologies, RAPD (random amplified polymorphic DNA) markers have been a very popular tool used to assess genetic diversity within and between populations of tropical tree species (Chalmers *et al.*, 1992; Russell *et al.*, 1993; Gillies *et al.*, 1997, 1999; Schierenbeck *et al.*, 1997), because of their ease of use, low cost and accessibility. However,

RAPD markers present limitations for plant population genetic studies because of their low information content per locus. They typically display a dominant inheritance and, technically, only detect one allele at a locus.

Microsatellites or SSR (simple sequence repeats) are expected to be the ideal class of genetic markers for population genetic studies because of their high information content (multiallelic nature), co-dominant inheritance, high abundance and wide distribution in the genome, and the ease of detecting polymorphisms by PCR assay (Rafalski *et al.*, 1996). The allelic variability observed at SSR loci allows the unique genetic characterization of individuals in natural populations and the estimation of genetic factors crucial for genetic conservation and management of tropical trees under intensive human effects. In recent years, the development and characterization of microsatellite markers have been increasingly reported for tropical tree species (Chase *et al.*, 1996; White & Powell, 1997; Aldrich *et al.*, 1998; Ujino *et al.*, 1998; Collevatti *et al.*, 1999; Dayanandan *et al.*, 1999; Dick & Hamilton, 1999; Rossetto *et al.* 1999; Miwa *et al.*, 2000; Rodriguez *et al.*, 2000). The perspective for the near future is an increase in the use of this tool for population genetic investigation of tropical tree species because of the novel library enrichment strategies and rapid fluorescence-based automatic sequence technologies (Rafalski *et al.*, 1996; Grattapaglia *et al.*, 1997).

The case of mahogany

Swietenia macrophylla is one of three species in the genus *Swietenia* (Meliaceae) known as American or "true" mahoganies, and currently is the most commercially valuable hardwood species in the Neotropics: one cubic meter of export-quality sawn wood is valued at about US$ 700 in the international market (Verissimo *et al.*, 1995). The high-value of mahogany wood is related to its easy workability, dimensional stability, durability and above all its attractive colour (Lamb, 1966). Mahogany is mainly used as solid wood or veneer for furniture and for doors and window frames, (Lamb, 1966; CITES, 1996). With the costs increasing and supplies diminishing the greater usage in commerce is now as a veneer.

The Brazilian mahogany (*S. macrophylla*) has a wide geographic range from Mexico through Central America and across the southern Amazon basin of Bolivia and Brazil (Lamb, 1966; Pennington, 1981; Rodan *et al.*, 1992) and the largest reserves are in the Brazilian Amazon (Lamb, 1966; Barros *et al.*, 1992). It is an emergent tree, 30 - 50 m tall (Fig. 1.1), commonly with a straight and cylindrical trunk, with frequent buttresses. The leaves are alternate, usually pinnate with opposite or occasionally alternate pinnate (Lamb, 1966). The species has wide ecological tolerance and occurs in a variety of habitats in wet to seasonally dry, evergreen to deciduous, tropical to

subtropical forests, with typically 800 - 2,500 mm of annual rainfall and at altitudes from 0 to 1,400 m (Lamb, 1966; Whitmore, 1983). The species reaches its optimum natural development under climatic conditions of the tropical dry forest formation of Holdridge's system (Holdridge *et al.*, 1971; Lamb, 1966). Mahogany grows naturally in various types of soils varying from deep, poorly drained, acidic clay and swampy soils to well drained alkaline soils of the limestone highlands, including soils derived from igneous and metamorphic rocks (Lamb, 1966).

S. macrophylla is a monoecious species pollinated by bees and moths (Styles & Khosla, 1976). It is leafless during the dry season when the fruits mature and the seeds are dispersed by wind (Whitmore, 1983). Mahogany tends to occur in widely scattered patches and the average density in natural forests is about less than one commercial-size tree per hectare (Whitmore, 1983, Verissimo *et al.*, 1995; Barros *et al.*, 1992; Gullison & Hardner, 1993). This patchy pattern of distribution is probably related to its mode of regeneration that demands light-exposed areas. Mahogany regenerates in open areas primarily after major disturbances such as river courses changes, hurricanes, blowdowns, fire and the stands are made up of one or few cohorts (Snook, 1996; Gullison *et al.*, 1996). Studies on growth rates of *S. macrophylla* indicate that trees take 105-150 years to reach commercial size (80 cm at dbh) in natural forests of Bolivia

(Gullison & Hubbel, 1992; Gullison *et al.*, 1996). Snook (1993) calculated that *S. macrophylla* requires on average, 120 years to reach the 55 cm commercial diameter limit in Mexico.

S. macrophylla has been exploited throughout its natural range since the beginning of the 20th century (Lamb, 1966; Rodan *et al.*, 1992). In the last decades, with the depletion of natural stands in Central America, most of the extraction has turned to populations in South America, especially in the Brazilian Amazon. Brazil is the main exporter of mahogany in the world. From 1971 to 1992, Brazil exported more than three million cubic meters of mahogany (FUNATURA, 1992), 40% of which was sent to the United States and 35% to the United Kingdom. Basically all mahogany traded in the international market is extracted from primary forests.

Mahogany extraction is based on selective logging, which usually removes only the tallest trees of good form and with a dbh > 80 cm (Verissimo *et al.*,1995; Gullison *et al.*, 1996). Verissimo *et al.* (1995) described in detail the extraction process of mahogany in the Brazilian Amazon. Briefly, the logging operation consists of the identification of mahogany-rich areas (currently reconnaissance of remaining stocks is made using small aircraft; the species is distinguished by its large, shiny, light-green crown), the location and labelling of trees in the forest by woodsmen, tree felling using chainsaws, opening of roads and log landings by bulldozers, and

dragging of the boles to the log landings using skidders. Once at the landings, the boles are cut into appropriated sections for transport to the mills. Parts of the mahogany extraction process are illustrated in Fig. 1.2.

In recent years, the conservation status of *S. macrophylla* has been the subject of increasing concern due to over-exploitation and habitat destruction (Rodan *et al.*, 1992, Rodan & Campbell, 1996; CITES, 1996). In Central America the species is already considered commercially extinct. In Bolivia and Brazil regional commercial extinctions have been reported and are increasing (Collins, 1990; Verissimo *et al.*, 1992; Gullison, 1995). In Brazil, *S. macrophylla* is considered vulnerable. The Brazilian Botanical Society has included *S. macrophylla* in a list of species in danger of extinction (Sociedade Brasileira de Botânica, 1992).

Logging selectively removes the best individuals in terms of growth and/or form, possibly resulting in genetically depleted populations. Population reductions and genetic erosion have already been reported in two of the three mahogany species (*Swietenia humilis* and *Swietenia mahogani*), which are presently listed in Appendix II of the Convention of International Trade in Endangered Species (CITES) (Styles, 1981; Rodan *et al.*, 1992; CITES, 1996). *Swietenia macrophylla* is not so protected. Inclusion in Appendix II would control international trade in *S. macrophylla* by ensuring that

trade is not detrimental to the survival of the species (Rodan & Campbell, 1996). Selective and intensive logging of *S. macrophylla* populations may have a significant impact on its genetic structure and population size, compromising the viability as evolutionary units. The listing *of S. macrophylla* in Appendix II of the CITES has been proposed but has failed on each occasion in the last decade.

Other impacts of mahogany logging are the damage caused to the remaining vegetation by the felling of trees and opening up of logging roads and landings, and the increased susceptibility to fire of the remaining vegetation. The indirect impacts include the acceleration of the deforestation process in the region of mahogany logging and social impacts caused by illegal logging in Indian reserves. Logging acts as a catalyst for deforestation owing to the construction of roads, colonization along the opened areas, and the conversion of the forest to agriculture use and pastures (Verissimo *et al.*, 1995; Fearnside, 1997). With the exhaustion of *S. macrophylla* stocks in private lands, illegal extraction has been reported in nature and Indian reserves in many Central and South American countries (CEDI, 1993; Heringer, 1993; Verissimo *et al.*, 1995; Watson, 1996, Snook, 1996). Veríssimo *et al.* (1998) reports that about 70% of the protected areas in Pará State, Brazil, are economically accessible for timber extraction. In the Brazilian Amazon, one-third of the distribution range of *S. macrophylla* coincides with Indian reserve

lands (Barros *et al.*, 1992; Verissimo *et al.*, 1995). In 1987, 69% of the mahogany exported from Brazil came from the Indian Kayapó reserve in the eastern Amazon (CEDI, 1992). In 1992, mahogany extractions were recorded in all 15 Indian reserves located in Southern Pará, Brazil (CEDI, 1993). The social impacts of illegal mahogany logging in Indian lands is a dramatic picture and constitutes a matter of great concern for the maintenance of the cultural integration of these traditional peoples (Watson, 1996).

Objectives of the study

Despite its importance, information on the extent and distribution of genetic variation and the understanding of the processes maintaining this variation in natural populations of mahogany is scarce. Gillies *et al.* (1999) assessed the genetic diversity of natural populations of *S. macrophylla* in Central America using dominant RAPD markers. More recently, a study evaluated genetic variation in a fragmented population of *Swietenia humilis* in Honduras using microsatellite markers (White *et al.*, 1999).

The main goal of the present study is to understand the extent and distribution of genetic variation, the mating system and gene flow in natural populations of *S. macrophylla* in the Brazilian Amazon for conservation purposes. Chapter 2 reports on the development and characterization of highly informative, semi-

automated multilocus genotyping systems based on fluorescent labelled multiplexed microsatellite markers for S. *macrophylla* to comply with the need for large scale genetic analysis of natural populations. Chapter 3 presents the estimates of genetic diversity for seven populations of mahogany sampled at a broad geographical scale, the partitioning of the genetic variation within and among populations, the levels of genetic differentiation among them, and the patterns of gene flow based on microsatellite loci. Chapter 4 reports on the spatial genetic structure and the mating system of one population of *S. macrophylla* based on the analysis of microsatellite genotypes of adults (mothers) and half-sib seedlings. The patterns of population genetic structure, gene flow, and the mating system are discussed in terms of the conservation of *S. macrophylla.*

Figure 1.1 – Adult tree of mahogany (*S. macrophylla*) in Southeastern Pará, Brazil (Photo: M.R.Lemes).

Figure 1.2 – Mahogany logging operation in the Rondônia State, Brazilian Amazon, showing: (A) tree felling using chainsaw, (B) felled mahogany with 2.17 m of dbh, (C) skidder used to drag the boles, and (D) log landing with mahogany boles. (Photos: M. R. Lemes).

Chapter 2

Development and Characterization of Multiplexed Fluorescence-based Systems of Microsatellite Markers for *Swietenia macrophylla*

INTRODUCTION

The understanding of the organisation of genetic variation in natural populations is a prerequisite for efficient conservation strategies in tropical tree species. In the last two decades, genetic markers have been developed and used to generate important data on the population genetics of plants. Isozymes, the most widely used markers to detect genetic variation in tropical tree species (Hamrick & Loveless, 1986, Loveless, 1992), refer to biochemical forms of an enzyme identified by electrophoresis. The technique involves the extraction of proteins, electrophoretic separation of the molecules on a gel, and histochemical coloration so that polymorphisms can be detected by allelic variation in the gene product (Wendel & Weeden, 1989). The main advantages of isozymes rely on their low cost and accessibility and the codominant Mendelian inheritance of loci. However, this class of markers shows limitations especially those due to the lack of sufficient polymorphisms provided by loci to analyse

critical genetic factors such as levels of heterozygosity, inbreeding, and parentage relatedness of individuals in populations.

A number of DNA-based marker technologies have been developed after the polymerase chain reaction (PCR, Mullis & Faloona, 1987). Among these molecular markers, microsatellites (also called Simple Sequence Repeats, SSR) present the highest levels of polymorphism making them potentially the most informative class of markers for studies of genetic variation in plant populations (Rafalski *et al.*, 1996).

Microsatellites are tandemly repeated sequences of DNA with repeat lengths of 1 to 6 base pairs (bp) found in a wide variety of eukaryote genomes (Hamada *et al.*, 1982; Litt & Luty 1999; Tautz & Renz, 1984; Tautz, 1989; Weber & May, 1989; Lagercrantz *et al.*, 1993; Wang *et al.*, 1994; Jarne & Lagorda, 1996; Powell *et al.*, 1996; Rafalski *et al.*, 1996). They are readily analysed using PCR and locus specific primers complementary to sequences flanking the repeat region. The microsatellite loci exhibit mutation at a notably higher rate than do non-repetitive sequences (Goldstein & Pollock, 1997). The rates of mutation are of the orders of 10^{-5} - 10^{-2} per generation, two or three orders of magnitude higher than values for isozymes (Weber & Wrong, 1993).

The mutational process that generates polymorphism at microsatellite loci is poorly understood (Estoup *et al.*, 1995). The

predominant source of variation at SSR loci appears to be slippage during DNA replication (Levinson & Gutman, 1987) which increases or decreases the current number of repeats leading to high levels of polymorphisms. However larger mutational steps are possible presumably generated by processes other than slippage, such as unequal crossing over (Weber & Wong, 1993; Stephan & Cho, 1994). There is a debate about which models of mutation are most appropriate for population genetic data analysis in microsatellites (Jarne & Lagoda, 1996). Two models have been more frequently applied to microsatellite data: the infinite allele model (IAM, Kimura & Crow, 1964) in which each mutation creates a new allele state with no known relationship to other allelic states and the stepwise mutation model (SMM, Ohta & Kimura, 1973) in which mutation increases or decreases the allele value by one conceiving in this way information about ancestral allelic states. Under the SMM similarly sized alleles at microsatellite loci are less different in terms of mutational steps than alleles of very different sizes (Jarne & Lagoda, 1996)

Microsatellites have become an attractive tool for population genetic studies in plants (Condit & Hubbell, 1991; Morgante & Olivieri, 1993; Powell *et al.*, 1996) because they are neutral, inherited in a codominant Mendelian fashion, show extraordinarily high allelic diversity with expected heterozygosities well over 50% and are

uniformly and widely dispersed in plant genomes (Morgante & Olivieri, 1993; Wang *et al.*, 1994). The development and characterization of plant microsatellites was first reported in tropical tree species by Conditt & Hubbell (1991). Since then, their use for genetic studies in tropical tree species has been increasing (Chase *et al.*, 1996, White & Powell, 1997a, Brondani *et al.*, 1998; Aldrich *et al.*, 1998; Ujino *et al.*, 1998, Collevatti *et al.*, 1999; Dayanandan *et al.*, 1999, Dick & Hamilton, 1999, Rosseto *et al.*, 1999, Miwa *et al.*, 2000, Rodriguez *et al.*, 2000) but is still limited probably due to the costs and technical difficulties of obtaining such markers. The variability observed at SSR loci allows individuals to be uniquely genotyped in natural populations and the estimation of genetic factors that have fundamental importance for the genetic conservation and management of tropical trees under intensive human pressure.

Multiplexing PCR is the simultaneous amplification of several genetic markers in a single reaction (Mitchell *et al.*, 1997). The semi-automated , fluorescence-based allele sizing system consists of the amplification by PCR of a microsatellite locus in which one of the primers is fluorescently labeled. The PCR products are analysed by polyacrylamide gel electrophoresis using an automated DNA sequencer which detects fluorescence emitted by the PCR products. The multiplex PCR allied to fluorescence-based, semi-automated allele size technology allow the simultaneous analysis of several SSR

loci in a single lane gel increasing substantially the efficiency and speed for genotyping plant genetic resources. Another advantage of this system is the accuracy and reliability of allele sizing.

Amplification of SSR loci across closely related species has been reported both in animals and plants, (Moore *et al.*, 1991; Olsen *et al.*, 1996; Wu & Tanksley, 1993; Thomas & Scott, 1993; Kijas *et al.*, 1995; Dayanandan *et al.*, 1997; Ujino *et al.*, 1998; Collevatti *et al.*, 1999), because at many microsatellite loci, the priming sequences flanking the tandem repeat motifs are conserved among related species. This frequently allows the transferability of primers among closely related species (Moore *et al.*, 1991). Transferability among closely related taxa is very advantageous considering the costs and time needed for the development of microsatellite markers. To date, a set of microsatellite markers for *Swietenia humilis* from Central America has been reported (White & Powell, 1997a). Despite the reported cross-species amplification of SSR loci developed for *S. humilis* for 11 species of the Meliaceae (White & Powell, 1997b), this approach failed for *S. macrophylla* in which most of the loci were not informative.

In this chapter I report the development and characterization of ten highly informative microsatellite markers for S. *macrophylla*. I also developed high throughput semi-automated multilocus genotyping systems based on fluorescent labelled multiplexed SSR

loci for this species to comply with the need for large-scale analysis of natural populations. The major aim is the application of this powerful tool to understand the patterns of population genetic structure, mating system and gene flow in *S. macrophylla* for conservation purposes.

MATERIALS AND METHODS

Plant material and DNA isolation

Total genomic DNA was extracted from fresh expanded leaves from a single individual of *S. macrophylla* to develop an (AG) enriched genomic library. For the characterization of SSR loci, total genomic DNA was extracted from fresh and/or dried expanded leaves of 121 individuals of *S. macrophylla* collected from four natural populations in the Brazilian Amazon. Total genomic DNA extraction followed standard CTAB procedure (Doyle & Doyle, 1987).

SSR-enriched genomic library construction

Genomic library construction was performed following protocols developed at Du Pont (Rafalski *et al.*, 1996) and optimised for tropical tree genomes at EMBRAPA - Genetic Resources and Biotechnology, Brasília, Brazil (Brondani *et al.*, 1998). Genomic DNA was digested with three different restriction enzymes, *Mse*I, *Sau*3A I and *Tsp*509, according

to the manufacturer's instructions, aiming to select one that produced a large amount of fractionated DNA in the 280-600 bp range. *Sau*3A I gave the best results and was used thereafter. Approximately 50 μg of genomic DNA was digested with *Sau*3A I (GATC) and fragments were separated by electrophoresis in 1.5% agarose. Fragments between 280 and 600 bp were recovered by DEAE-cellulose NA-45 membrane (Scheleicher and Schuell, NY) via electrophoresis. DNA fragments (around 30 μg) were ligated to adaptors at the *Sau*3A I restriction site. The fragments containing SSR sequences were selected by hybridization with biotinilated oligonucleotides complementary to the repetitive sequence AG/CT and recovered by streptavidin-coated magnetic beads. Fragments were amplified by PCR and cloned into plasmid vector pGEM-T (Stratagene, CA) and then transformed by electroporation into *E. coli* strain XL1-Blue and grown on agar plates containing ampicilin and tetracycline. Transformants were picked out, streaked on 132-mm plates (100 per plate) and regrown at 37°C for 12h. Duplicate plates containing colonies from these transformants were stamped onto positively charged nylon membranes (Hybond N, Amersham Pharmacia), grown, lysed, denatured, neutralized and UV cross-linked.

Selection and sequencing of positive clones and primer design

Transformants having SSR were identified by hybridization with a poly (dA-dG) probe labelled with Digoxigenin-11-ddUTP, using a DIG oligonucleotide 3'-end labelling kit (Boeringer Mannheim) according to the manufacturer's instruction. The temperature used for prehybridization and hybridization was 65°C for the poly AG/TC oligonucleotide. Processed membranes were exposed to X-ray film for 2-3 hours at 37°C. Positive clones were picked and grown overnight in liquid ampicilin LB media. Plasmid DNA was extracted by miniprep. An anchored-PCR strategy was performed to determine the presence of the SSR repeat and its position within the cloned insert (Taylor *et al.*, 1992; Rafalski *et al.*, 1996). Agarose-gel analysis was used to reveal clones containing SSR inserts and the direction within the vector from which they were to be sequenced. DNA inserts were sequenced on an Applied Biosystems 377 sequencer using dye-terminator fluorescent chemistry (Applied Biosystems Incorporated). Primer pairs complementary to sequences flanking the repeat unit were designed using the PRIMER program (Lincoln *et al.*, 1991). Stringent criteria in primer design were observed in order to minimise problems with spurious banding patterns generated during amplification and to allow further development of single-reaction multiplex PCR: (1) a primer T_m of 72°C; (2) a maximum of 3°C difference in T_m between primer pairs; (3) GC content ranging from

40% to 60%; and (4) absence of complementarity between primers. Primers were synthesized by Operon Technologies Incorporated (Alameda, California). The locus designation has sm which is an abbreviation for "*Swietenia macrophylla*", followed by the colony number from which they were sequenced.

PCR amplification and screening of SSR loci

Microsatellite-marker amplification for primer screening was carried out in a 13 µl reaction, containing 0.9 µM of each primer, 1 unit Taq DNA polymerase, 200 µM of each dNTP, 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), DMSO (50%), and 7.5 ng of template DNA. PCR- amplifications were performed using a MJ Research PT-100 thermal controller using the following program for all loci: an initial denaturation at 96°C for 2 min, followed by 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, and a final elongation step at 72°C for 7 min. The PCR products were analysed in 3.5% Metaphor agarose gels containing 0.1 µg/ml of ethidium bromide in 1X TBE buffer (89 mM Tris-borate, 2mM EDTA pH 8.3) and sized by comparison to a 1Kb DNA ladder standard (Gibco, MD). A total of 8 individuals of *S. macrophylla* was used for primer screening on 3.5% agarose gels. The synthesised primers that showed clear and robust band amplification in agarose were selected for analysis of polymorphisms in PAGE (Polyacrylamide

gel). The amplified products were resolved on 4% PAGE stained with silver nitrate (Bassam *et al.*, 1991) and sized by comparison to a 10 bp DNA ladder standard (Gibco, MD). A total of 16 individuals of *S. macrophylla* was analysed for preliminary detection of polymorphisms on PAGE.

Multiplexed fluorescence-based assays

The microsatellite markers that showed clearly interpretable polymorphisms in silver stained denaturing PAGE were selected for fluorescent-dye labelling. One primer of each pair was labelled at the 5' end with fluorescent dye, either 6-carboxyfluorescein (6-FAM), tetrachloro-6-carboxyfluorescein (TET) or hexachloro-6-carboxyfluorescein (HEX). The choice of the fluorescent-dye label for each microsatellite primer selected was based on its observed allelic range size previously detected in silver stained PAGE. Labelled primers had PCR and fluorescence detection optimised in single tube reaction before multiplexing assays. Up to three loci with overlapping allele sizes could be multiplexed by labelling single primers with different dyes. Primers that amplified products with non-overlapping allelic range sizes were labelled with the same fluorescent dye. Three multiplexed systems of microsatellite markers were developed and optimised using 10 pairs of primers. Labelled primers were synthesized at Operon Technologies Incorporated (Alameda,

California).

PCR was performed in a final volume of 25 µl for multiplexed reactions containing 1.25-2.0 µM of each forward and reverse primer, 1 unit Taq DNA polymerase, 200 µM of each dNTP, 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), BSA (2.5 mg/ml), 5.0 ng of template DNA, and dH_2O. For primers in single PCR's, reactions were performed in a final volume of 10 µl containing 1.25-2.0 µM of each forward and reverse primer, 1 unit Taq DNA polymerase, 200 µM of each dNTP, 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), BSA (2.5 mg/ml), 5.0 ng of template DNA, and dH_2O. Fluorescence detection was optimised for the ten microsatellite markers by decreasing or increasing primer concentration in multiplexed and single assays to resolve the most accurate products. The standard PCR profile described in the previous section was used for all loci.

Following PCR, one µl of 1:10 diluted reaction of each multiplex was added to 0.25 µl of GeneScan 500 ROX internal size standard labelled with a fourth fluorescent label (Applied Biosystems Incorporated), 0.45 µl of loading buffer (25 mM EDTA and 50mg/ml Blue-Dextran) and 2.3 µl of deionized formamide. The reactions were heated to 95°C for 3 minutes, chilled on ice and electrophoresed in 5% denaturing PAGE in an ABI Prism 377 DNA sequencer (Applied Biosystems Incorporated). Each gel was run for 2 hours at 3000V and

50-30 mA. Following the gel run, data collection and analysis, as well as automatic sizing of amplified products, was done using GeneScan 672, version 1.1 (ABI, 1993).

Sizing of fluorescence-based amplified products

The scoring of amplified products was carried out using the Genotyper, version 1.1 (ABI, 1994). To check accuracy and reproducibility of allele scoring, I evaluated intra and inter-gel variation in sizing of fragments. DNA of 8 individuals at 5 loci was amplified, electrophoresed and GeneScan run 4 times in the same gel. This procedure was performed in three gels to evaluate inter-gel variation in sizing of fragments. An analysis of variance (ANOVA) was performed to evaluate intra and inter-gel differences in allelic sizing using the program Statistica (STATSOFT, 1995).

Analysis of inheritance and characterization of microsatellite loci

In order to evaluate the inheritance of the microsatellites, I performed a segregation analysis in two open-pollinated half-sib families. I collected and germinated 16 seeds per mother tree. DNA was extracted from fresh leaves for seedlings and dried leaves for mother trees using the protocol cited above. PCR amplification for analysis of allele segregation was performed as described previously

for the multiplex assays. For characterization of the ten microsatellite loci developed, 121 individuals of *S. macrophylla* from four populations were used. The mean number of alleles per locus, allelic frequency, and observed and expected heterozygosities estimated under the Hardy-Weinberg equilibrium were computed for each locus and averaged over all loci using Genetic Data Analysis program (GDA - Lewis & Zaykin, 1998). We estimated two parameters of genetic information content for parentage studies for each locus: (1) probability of genetic identity (I) (Paetkau *et al.*, 1995) and (2) Paternity exclusion probability (Q) (Weir, 1996). The probability of genetic identity (I) was calculated for each locus using the formulae:

$$I = \sum_{i} p^4_i + \sum_{i} \sum_{j>i} (2p_i p_j)^2$$

where p_i and p_j are the frequencies of the ith and jth alleles in a given population. The probability of paternity exclusion (Q) was calculated for each locus using the formulae:

$$Q = \sum_{u} p_u (1 - p_u)^2 - \tfrac{1}{2} \sum_{u} \sum_{v \neq u} p^2_u p^2_v (4 - 3p_u - 3p_v)$$

where p_u and p_v are the frequencies of the uth and vth alleles in a given population, considering $u \neq v$. The combined probability of genetic identity, $IC = \prod I_i$, and the combined probability of paternity exclusion, $QC = 1-[\prod (1-Q_i)]$, were also estimated for the combined loci set.

RESULTS

SSR-enriched genomic library, sequence characterization of SSR and primer design

Digestion of *S. macrophylla* DNA with three restriction enzymes revealed that *Sau* 3AI produced the most adequate digestion profile for library construction with a range of fragments between 280 and 600 bp. The genomic library was screened with the dinucleotide repeat (AG). After the enrichment step, approximately 300 clones were screened from which 168 were scored as positive (56%). From these positive clones, 126 (75%) were selected for sequencing after anchored-PCR analysis. A total of 43 (34%) useful sequences allowed the design of primers using PRIMER program version 0.5 (Lincoln *et al.*, 1991).

Primer screening of SSR loci

Using a single PCR program, twenty (46%) of the 43 pairs of primers designed and tested showed very clean and easily interpretable PCR products, and 17 pairs of primers (39%) amplified clearly interpretable PCR products but also showed non-specific amplification of secondary bands in agarose. A total of 6 primer pairs (14%) did not amplify any product at all. In total 85% of the primer

pairs yielded clearly interpretable PCR products under a single set of PCR conditions.

The set of 20 pairs of primers that showed very clean and clearly interpretable PCR products in agarose was selected to evaluate polymorphism in 16 individuals of *S. macrophylla* in silver-stained denaturing PAGE using the same PCR conditions. Two of the 20 primer pairs tested were monomorphic. A total of 18 pairs of primers showed polymorphism and 12 of them were selected for fluorescent-dye labelling. Fig. 2.1 shows polymorphism detected in 6 SSR loci during screening of primers in PAGE.

Multiplexed fluorescence-based microsatellite systems

I developed and optimised three multiplex systems of microsatellite markers for *S. macrophylla* using 10 of the 12 pairs of primers selected for fluorescent-dye labelling as following: *Multiplex 1*: One triplex single reaction (sm01-TET, sm34-FAM and sm47-FAM) plus 1 biplex single reaction (sm22-TET and sm40-HEX). The triplex and biplex were combined before electrophoresis and loaded in a single lane gel creating a pentaplex system. *Multiplex 2*: Composed of two single primer reactions (sm31-FAM and sm46-FAM) that were combined before electrophoresis and loaded in a single lane gel. *Multiplex 3*: One biplex single reaction (sm45-TET and sm51-HEX)

plus 1 single primer reaction (sm32-FAM) were combined before electrophoresis forming a triplex set.

Sizing of alleles

Variation in fragment sizes were not significant, neither within (F=0.00038, p=0.99999, ANOVA) nor between (F=0.001016, p=0.974578, ANOVA) gels when analysed by Genotyper. A priori the scoring of amplified products using Genotyper is accurate and reproducible.

Characterization of Microsatellite Loci and Analysis of Inheritance

All repeat sequences found were simple and perfect, like the categories of repeat motifs identified by Weber (1990), with repeat motif size ranging from 18 to 31 (Tab. 2.1). For the ten microsatellite loci evaluated I identified a total of 166 alleles among 121 individuals of *S. macrophylla* and the two opened-pollinated half-sib families (32 individuals). Considering only the 121 adult individuals genotyped, an average of 15.8 alleles was detected per locus. All ten loci were hypervariable, with the least and most variable loci showing 11 and 25 alleles respectively. The mean expected heterozygosity was 0.84 and the mean observed heterozygosity was 0.73. The allele frequency distributions for the 10 loci are shown in Fig. 2.2. For some loci 2 or

3 alleles were particularly frequent. At other loci, i.e. sm31, sm32, sm45 and sm46 alleles were distributed more uniformly.

Analysis of codominant Mendelian inheritance of the ten microsatellite loci was performed with two heterozygous mother trees and their open-pollinated half-sib families. All loci exhibited patterns expected under Mendelian inheritance. Each of the 16 sibs shared at least one allele with the mother for all 10 loci analysed. Fig. 2.3 and 2.4 exhibit electropherograms showing inheritance and segregation in the families 21301 and 30704 (heterozygous mother and 5 sibs) analysed for locus sm01 and sm47, respectively.

The paternity exclusion probability (Q) was estimated for each locus and showed high values for all loci (Tab. 2.2). The combined probability of paternity exclusion (QC) using all 10 loci was 0.999998, which indicates that there is a probability of 99.9998% of correctly excluding a random father in the population who is not the true father. Probability of genetic identity ranged from 0.009 to 0.12 with a combined value of 7.0×10^{-15} considering all loci.

DISCUSSION

This study has shown that (AG) microsatellites are present in *the S. macrophylla* genome and can be used to as highly polymorphic markers. Before developing microsatellite markers, I attempted to

transfer SSR markers developed for *Swietenia humilis* (White & Powell,1997a) to samples of *S. macrophylla.* Transferability of these markers was reported for 11 species of Meliaceae, including *S. macrophylla* (White & Powell, 1997b). However tests have showed that for 12 *S. humilis* SSR markers analysed, 7 (58%) did not present polymorphism, 3 (25%) did not amplify and only 2 (17%) were informative presenting polymorphism for *S. macrophylla* in silver-stained 4% PAGE. In total, 83% of the SSR markers developed for *S. humilis* were not informative for *S. macrophylla.* These results indicate that although homology of flanking regions of simple sequence repeats exist among the Meliaceae species, which allows amplification of products, it does not necessarily indicate the presence of a microsatellite between these flanking regions and in consequence polymorphism was not observed.

The method used was very efficient in developing microsatellite markers for *S. macrophylla.* After the enrichment step, I was able to detect 56% of positive clones. Of these, 75% were selected for sequencing after using an anchored-PCR strategy and 34% of these sequences allowed primer design. In total, 85% of the primer pairs designed yielded clearly interpretable PCR products under a single set of PCR conditions. The overall efficiency of microsatellite marker development in *S. macrophylla* was thus 14.3% (0.56x0.75x0.34) for yield of primer sequences and 12.1% (0.56x0.75x0.34x0.85) for

obtaining informative loci. The efficiency in obtaining sequences suitable for primer design and informative loci for *S. macrophylla* was 3.5 and 12 times higher, respectively, than that found for *S. humilis* by White & Powell (1997a). Other studies with tropical tree species that allowed estimates of the same parameters showed an efficiency of 8.8 for yield of primer sequences and 6.3% yielding informative loci in *Eucalyptus* spp. (Brondani *et al.*, 1998) and 2.7% and 1.4% respectively, for *Caryocar brasiliense* (Collevatti *et al.*, 1999).

Considering the efficiency from data sequencing to the obtaining of operationally useful SSR loci (loci that generated clearly interpretable PCR products), this study showed an efficiency of 29% (0.34x0.85). This value is higher than that found for *S. humilis* 16% (0.68x0.24) and *C. brasiliense* 7% (0.14x0.54) but lower than the efficiency of 63% (0.87x0.72) found for *Eucalyptus spp.* (Brondani *et. al.,* 1998). The higher efficiency of SSR development found for *Eucalyptus* spp. (Brondani *et al.*, 1998) and *S. macrophylla* (present study) is probably due to the use of an anchored-PCR screening strategy in these two studies, which improves the yield considerably by eliminating false positives and positive clones with repeats positioned too close to the vector (Rafalski *et al.*, 1996).

To date this study represents the first report on the development and characterization of multiplex PCR and fluorescence-based systems of microsatellite markers for a neotropical tree species.

Multiplexed assays have been documented using SSR loci in many eukaryotes, such as humans (Edwards *et al.*, 1991; Urquhart *et al.*, 1995), cattle (Glowatzki-Mullis *et al.*, 1995), polar bear (Paetkau *et al.*, 1995), trout (Olsen *et al.*, 1996, Wenburg *et al.*, 1996), and in plants (Kijas *et al.*, 1995, Mitchell *et al.*, 1997, Rosseto *et al.*, 1999). High-throughput genotyping systems based on fluorescent-labelled multiplexed SSR loci were developed for *S. macrophylla* to comply with the need for large scale analysis of natural populations. Multiplexing is the combination of allele fragments from more than one locus from an individual in a single lane of an electrophoretic gel. This can be achieved by co-amplification of multiple loci in the same PCR, mixing of amplified loci after PCR, or both (Olsen *et al.*, 1996). Here I considered both approaches. I was able to co-amplify up to three loci in a single PCR tube and was able to resolve up to five loci in a single gel lane.

The use of SSR markers combined with fluorescence-based DNA detection and semi-automated fragment analysis has the potential to act as a powerful tool to improve plant genetic conservation considering accuracy, informativeness, automation and cost effectiveness (Mitchell *et al.*, 1997). Multiplexed systems of fluorescently-labelled SSR loci represent a tremendous potential increase in speed and efficiency of genetic data collection relative to manual methods of single-locus analysis, such as allozymes

(Wenburg *et al.*, 1996), and even multilocus analysis, such as SSR markers in silver stained denaturing polyacrylamide gels.

Accuracy and reproducibility of DNA fragment sizing is essential for correct genotyping with microsatellites (Haberl & Tautz, 1999). The Genescan and Genotyper softwares consider that the maximum difference between estimates of allele sizing should be ≤ 0.5 bp for accurate evaluation. However, it should be considered that when evaluating dinucleotide microsatellites this maximum difference may be up to 1 bp and that allelic variation will be at least 2bp. Attention should be given to consistent differences across PCR reactions in allele sizing, usually by plus or minus one nucleotide. These single base pair differences may be caused by incorporation of dATP to the 3` end of PCR products by DNA Taq polimerase during amplification (Clark, 1988). The differences in size estimates of fragments did not vary significantly, either within or between gels (respectively p=0.99999 and p=0.974578) demonstrating the high accuracy and efficiency in allele sizing which allows reliable and repeatable genotyping.

The informativeness of microsatellites tends to increase with the increasing numbers of repeats (Weber, 1990). This means that loci with greater numbers of repeats are more likely to be polymorphic. This was observed in *S. macrophylla*. The sm31 locus, the longest in number of repeats $(AG)_{31}$, was the most informative of

the loci developed, detecting 25 alleles. The mean number of alleles per locus was 15.8 for 10 loci (range 11-25), higher than that observed in *S. humilis* for the same number of loci analysed (9.7 alleles per locus – range 4-23) (White & Powell, 1997a). The mean expected (He) and observed heterozygosity (Ho) for *S. macrophylla* was 0.84 and 0.73, respectively. These values are amongst the highest found for tropical tree species (Tab. 2.3).

We found a combined probability of identity – probability that two individuals selected at random from a population would have identical genotypes – of $7.0x10^{-15}$, considering all 10 loci. Collevatti, *et al* (1999) found a value of 3.1×10^{-17} for *C. brasiliense*, also analysing 10 loci. Paetkau *et al.* (1995) found overall probability of identity ranging from 1.0×10^{-6} to 2.1×10^{-7} within four populations of polar bears (*Ursus maritmus*) for 8 SSR loci. These results are quite impressive and show the potential usefulness of microsatellite markers for genetic studies, especially for breeding structure and paternity.

Combined with this, very high values for single locus (0.52 to 0.87) and combined probability of paternity exclusion (0.999998) were found. Collevatti *et al.* (1999) also found a very high combined probability of paternity exclusion (0.99999995) and single locus probability of paternity exclusion ranging from 0.69 to 0.95 for 10 SSR loci surveyed in *C. brasiliense* in Central Brazil. Dick & Hamilton

(1999) found expected exclusion probabilities for single loci varying from 0.23 to 0.87, with a multilocus expectation of > 0.995 for an Amazonian tree, *Dinizia excelsea* (Fabaceae). Dayanandan *et al.* (1999) found paternity exclusion probability for SSR loci in *Carapa guianensis*, a species of Meliaceae, ranging from 0.14 to 0.77, with an overall joint paternity exclusion probability for the three SSR loci studied of 0.93. These results indicate the tremendous power of the microsatellite loci developed for population genetic structure, gene flow, breeding structure and parentage studies in *S. macrophylla.*

Table 2.1 - Characteristics of the ten microsatellite loci developed for *Swietenia macrophylla*: SSR locus denomination, repeat motif, forward and reverse primer sequences from sequencing data, annealing temperature (°C), expected fragment size (bp), allelic range size (bp), and number of alleles detected per locus (A).

SSR Locus	Repeat	Primer sequences (5' - 3')	Anneal. Temp. (°C)	Expected fragment size (bp)	Allelic range size (bp)	A
sm01	$(AG)_{19}$	5'-GCGCGATTGATTGACTTC-3' 5'-GCGCTTAGCATTATTCTCC-3'	56	280	261-295	17
sm22	$(AG)_{18}$	5'-TCTGCTACAGAGCTGGATGC-3' 5'-GTATGCTCGAAGAAGTCGTTG-3'	56	147	119-161	16
sm31	$(AG)_{31}$	5'-CTTCTAATGTTCTGATGCCTG-3' 5'-AGCAACTCGTGAGGAATTTAC-3'	56	128	80-138	25
sm32	$(AG)_{20}$	5'-CACCTTATGTACACCACACAG-3' 5'-GAAGGAGACACCAGCAATC-3'	56	171	146-184	15
sm34	$(AG)_{19}$	5'-GCACTCAAGGTACACTATGAT-3' 5'-TACGTGTGAATGCGTCTAT-3'	56	87	40-96	16
sm40	$(AG)_{19}$	5'-TGCTACTGTCAAGAGTGTAT-3' 5'-GACAAACATGTACCACAAG-3'	56	138	120-146	12
sm45	$(AG)_{21}$	5'-CCTTATGTTCACCACACAGTA-3' 5'-GAGACACCAGCAATCCAG-3'	56	167	140-178	15
sm46	$(AG)_{20}$	5'-GCAGTACTCGCCTATCTTCA-3' 5'-TGAGAACTGCAGAATCCTTT-3'	56	207	190-226	16
sm47	$(AG)_{24}$	5'-GCCATTGGTCTCAATCTTAC-3' 5'-GGAAGAGTCTTAGAACACAG-3'	56	147	114-150	14
sm51	$(AG)_{22}$	5'-GCAATTTCCAGAAGAAACC-3' 5'-CTGTAGGCGATAACAATCAG-3'	56	161	138-182	20

Table 2.2 - Genetic parameters of ten SSR loci of *S. macrophylla* evaluated on 121 individuals: A - number of alleles; H_o - observed heterozygosity; H_e - expected heterozygosity; Q - probability of paternity exclusion, QC - combined probability of paternity exclusion; I - probability of genetic identity, IC - combined probability of genetic identity.

Locus	A	H_o	H_e	Q	I
sm01	17	0.65	0.80	0.65	0.06
sm22	14	0.72	0.82	0.66	0.06
sm31	25	0.85	0.94	0.87	0.009
sm32	15	0.74	0.91	0.81	0.02
sm34	12	0.32	0.72	0.52	0.12
sm40	13	0.69	0.75	0.56	0.10
sm45	15	0.89	0.91	0.81	0.02
sm46	17	0.83	0.88	0.77	0.03
sm47	11	0.73	0.84	0.69	0.05
sm51	19	0.86	0.88	0.76	0.03
Mean	15.8	0.73	0.84	QC= 0.999998	IC= 7.0×10^{-15}

Table 2.3 - Comparison of microsatellite loci characteristics from tropical tree species. Sample size (n), number of loci, mean number of alleles (A), mean expected heterozygosity (H_e), and mean observed heterozygosity (H_o).

Species	n	n° loci	A	H_e	H_o	Reference
Melaleuca alternifolia	500	5	19.6	0.78	0.72	Rossetto *et al.*, 1999
Melaleuca cajuputi	31	11	5.7	0.66	0.61	Miwa *et al.*, 2000
Carapa guianensis	170	3	14.7	0.64	0.66	Dayanandan *et al.*, 1999
Caryocar brasiliense	123	10	16.0	0.89	0.73	Collevatti *et al.*, 1999
Dinizia excelsea	121	7	11.6	0.75	0.53	Dick & Hamilton, 1999
Eucalyptus grandis	32	15	11.0	0.83	0.56	Brondani *et al.*, 1998
Eucalyptus urophylla	32	15	11.0	0.86	0.59	Brondani *et al.*, 1998
Pithecellobium elegans	52	4	6.5	0.64	0.71	Chase *et al.*, 1996
Shorea curtisii	40	8	7.9	0.64	?	Ujino *et al.*, 1998
Simphonia globulifera	914	3	18.3	0.83	0.72	Aldrich *et. al.*, 1998
Swietenia humilis	88	10	9.7	0.55	0.41	White & Powell, 1997
Swietenia macrophylla	121	10	15.8	0.84	0.73	Present study

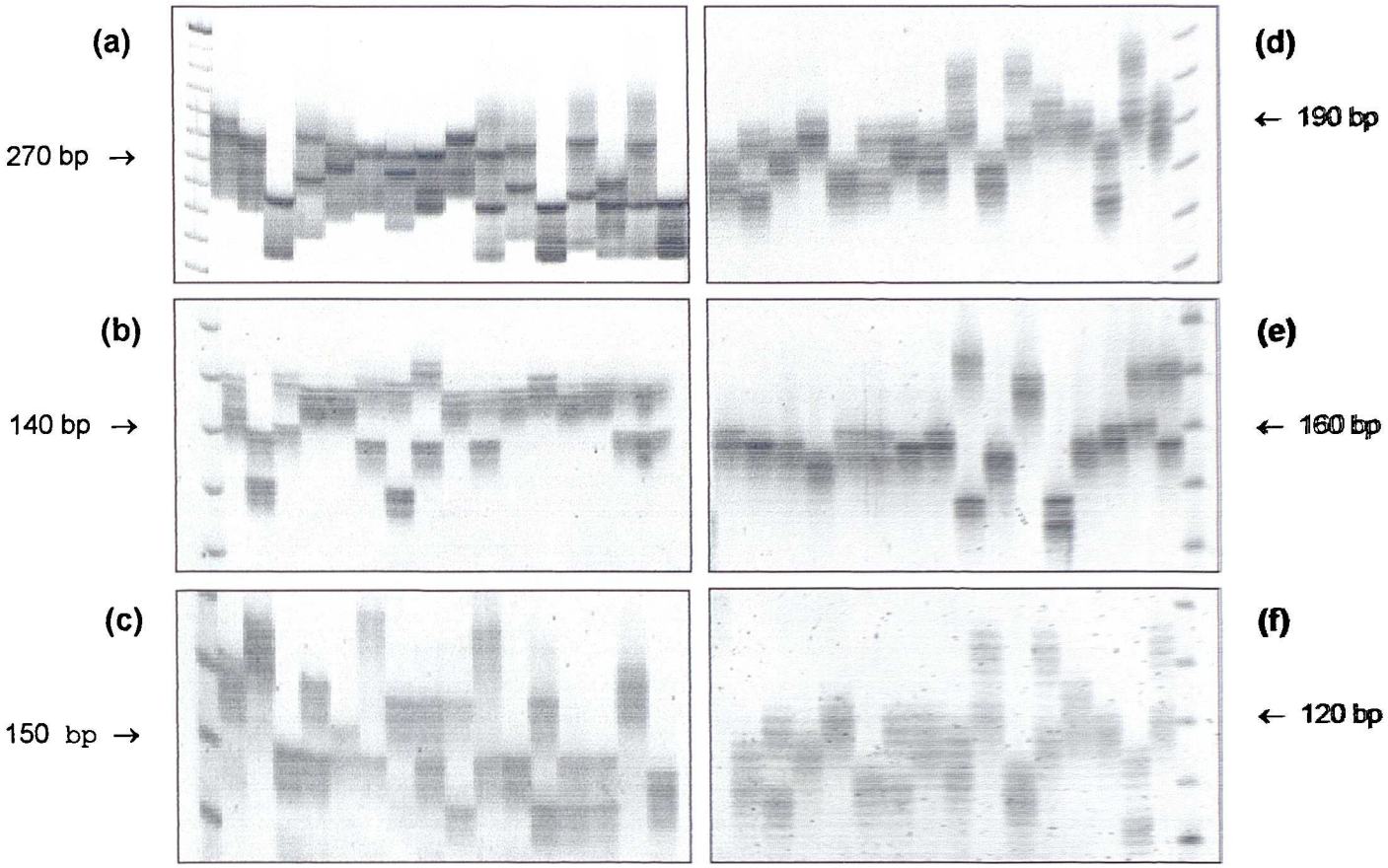


Figure 2.1 - Screening of 6 SSR loci in silver-stained denaturing polyacrylamide gels showing DNA polymorphisms in 16 individuals of *S. macrophylla*. First lane of panels (a), (b), and (c) and last lane of panels (d), (e), and (f) are 10 bp ladder (Gibco). Panels: (a) locus sm01, (b) locus sm22, (c) locus sm45, (d) locus sm32, (e) locus sm51, and (f) locus sm31.

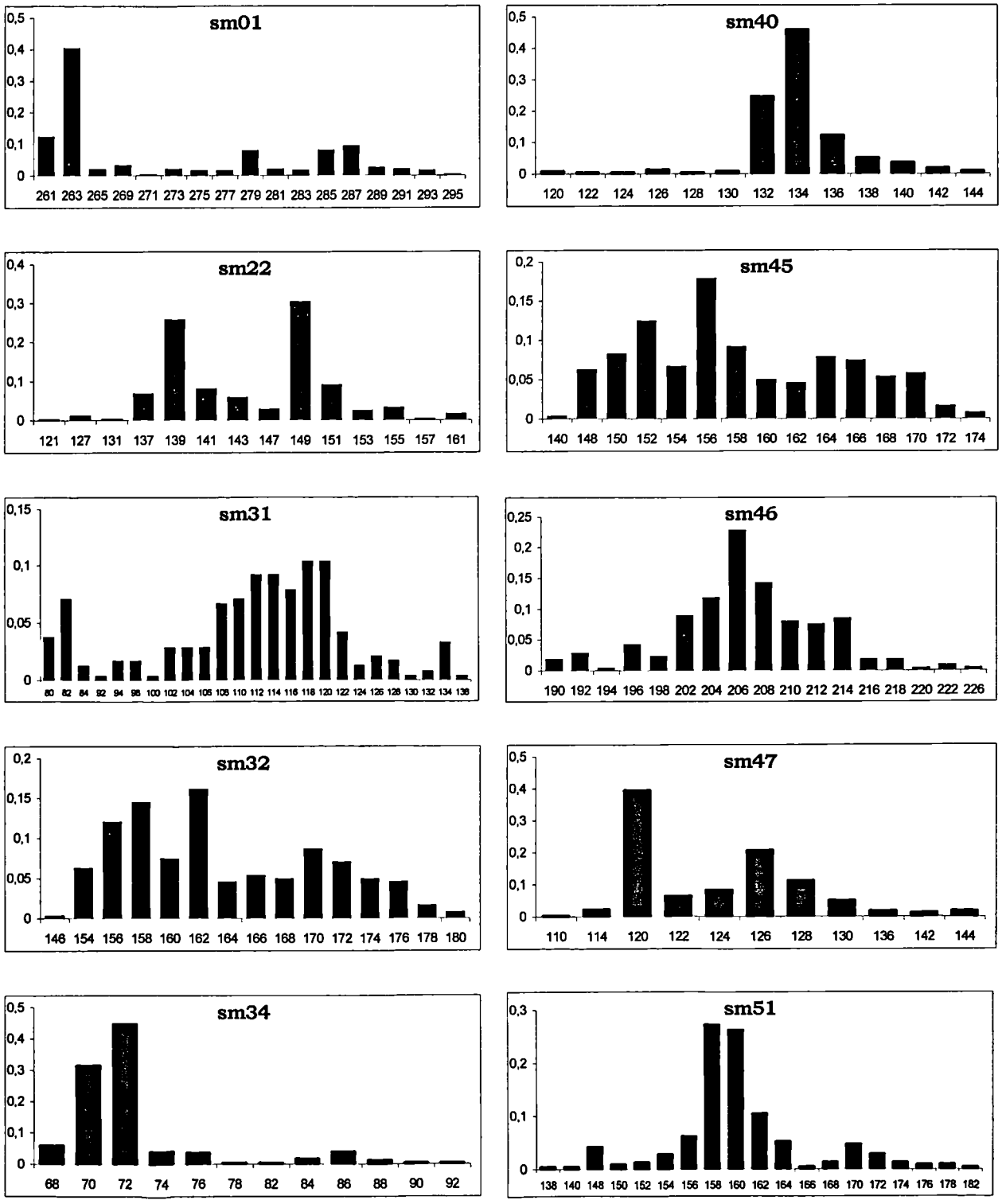


Figure 2.2 – Patterns of allele frequency distribution in 10 microsatellite loci of *S. macrophylla*. X-axis – allele size in base pairs, Y-axis allele frequency.

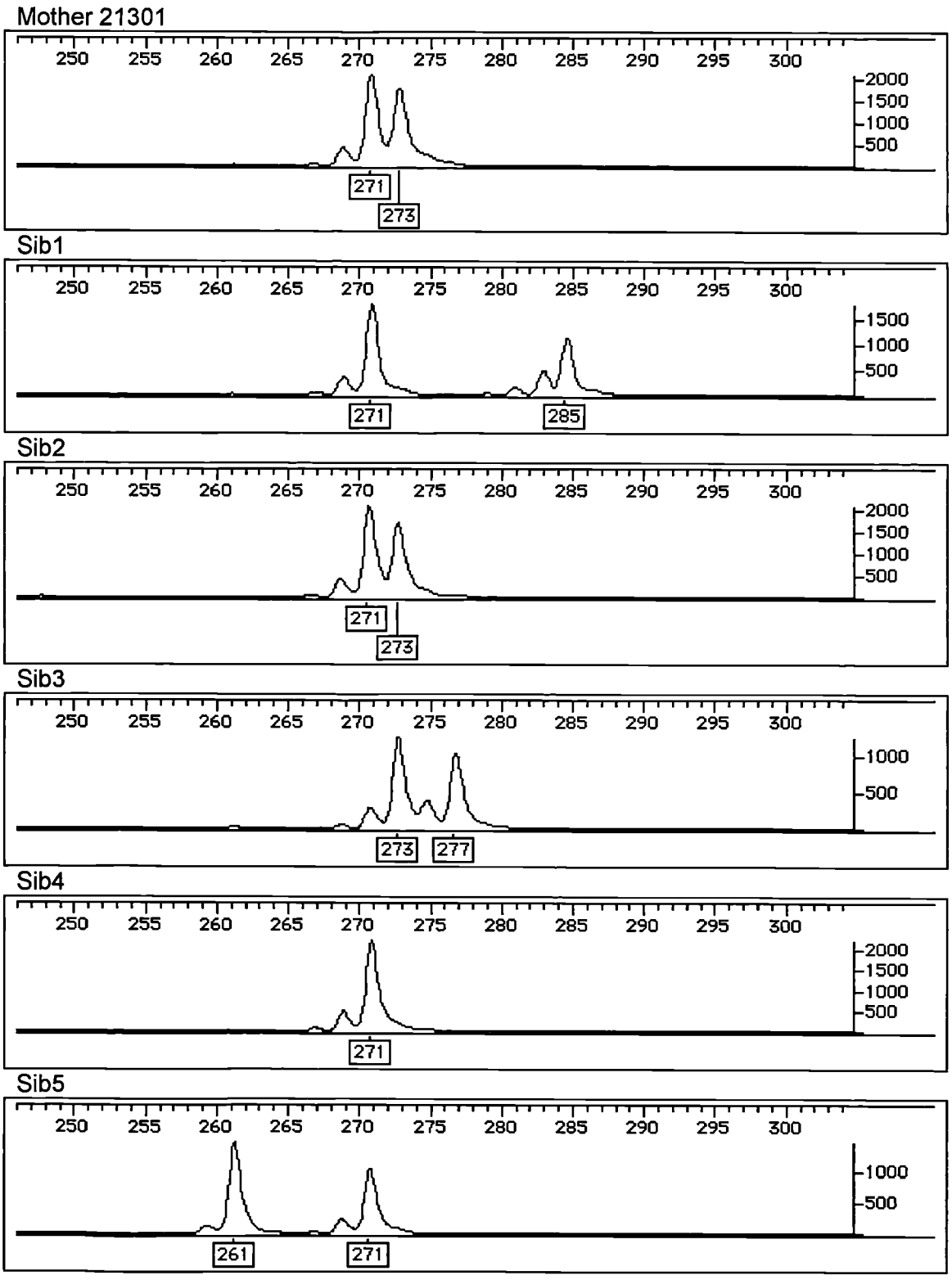


Figure 2.3 - Electropherograms showing pattern of segregation in an open-pollinated progeny of *S. macrophylla* tree 21301 (heterozygous mother and 5 sibs), using fluorescence-based microsatellite marker (locus sm01-TET). The scale at the top of each panel indicates fragment sizes in base pairs. The scale at the right of each panel indicates fluorescence intensities of the peaks.

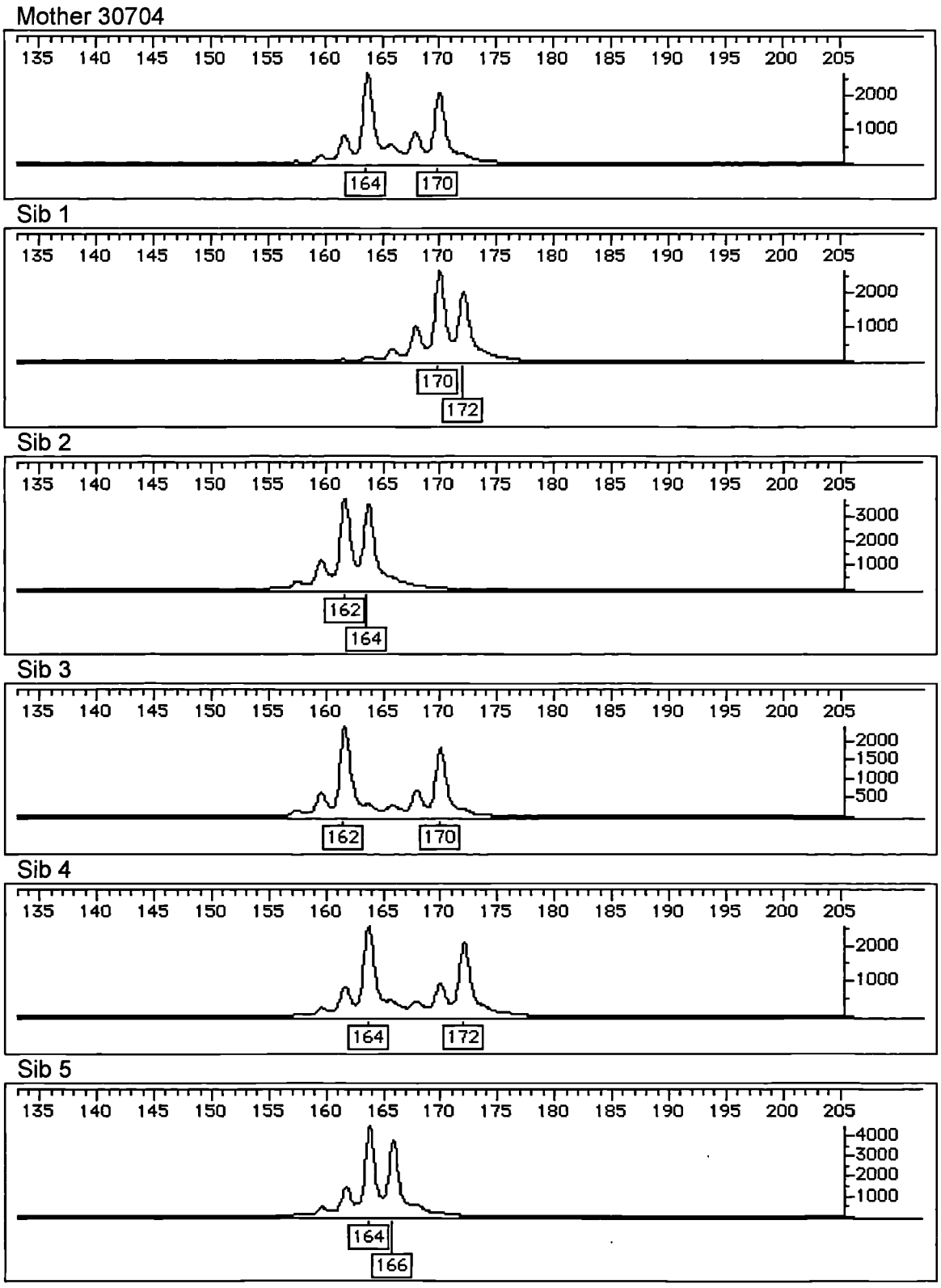


Figure 2.4 - Electropherograms showing pattern of segregation in an open-pollinated progeny of *S. macrophylla* tree 30704 (heterozygous mother and 5 sibs), using fluorescence-based microsatellite marker (locus sm47-FAM). The scale at the top of each panel indicates fragment sizes in base pairs. The scale at the right of each panel indicates fluorescence intensities of the peaks.

Chapter 3

Population Genetic Structure and Gene Flow in *Swietenia macrophylla* in the Brazilian Amazon

INTRODUCTION

The rates of deforestation and habitat fragmentation of tropical forests worldwide have been increasing alarmingly in the last decades (Katzman & Cale, 1990; Whitmore, 1997; Bawa & Seidler, 1998). The consequences of habitat destruction, fragmentation, and also selective logging represent a significant threat to the maintenance of biodiversity and biological processes in tropical forest ecosystems (Bawa, 1994, Young *et al.*, 1996). Genetic diversity in tropical forest trees is endangered as a consequence of such habitat disturbances. Deforestation and fragmentation of tropical forests can lead to losses of genetic variation of tree species by causing extinction of locally adapted populations as well as reduction of effective sizes and gene flow, and disruption of their pollen and seed vector associations (Bawa, 1994; Hall *et al.*, 1996; Nason *et al.*, 1997). Selective logging practice also threatens genetic diversity of tree species causing dysgenic selection due to continuous exploitation of large, superior individuals and increasing level of inbreeding due to reduction in

stand density (Bawa, 1994). In this context, the understanding of the distribution and levels of genetic variation within tropical tree species is crucial for devising effective conservation and sound management strategies.

Genetic structure refers to the distribution of genetic variation in space and time. Many historical and evolutionary factors influence the distribution of gene frequencies in populations and shape their genetic structures, among which dispersal (gene flow), mutation, selection, genetic drift, and the mating system play fundamental roles (Wright, 1931, 1937, 1943). Mutation, natural selection, and genetic drift will lead to genetic differentiation while gene flow will counteract differentiation among populations by exchange of gametes, individuals, or groups of individuals in space (Slatkin, 1987). Population differentiation may occur by a well-characterised evolutionary process known as isolation by distance (Wright, 1943) where pairs of populations that are located more distantly geographically would show greater genetic distance, assuming constant rates of migration. The mating system refers to the mode of transmission of genes from one generation to the next. By the analyses of the plant's mating system it is possible to determine levels of inbreeding in populations, a crucial component of population structuring (more fully discussed in Chapter 4). Beside the strictly genetic events, ecological and life history traits must also be taken

into account as important features in determining genetic structure of plant populations (Loveless & Hamrick, 1984; Loveless, 1998).

Population genetic studies of tropical trees have shown that most of the species exhibit high levels of genetic diversity and gene flow, genetic variation mostly occurs within rather than among populations, and the species are mainly outcrossed (Hamrick & Loveless, 1989; Alvarez-Buylla *et al.*, 1996). The great majority of these studies were developed over relatively small spatial scales and have employed isozymes as genetic markers to quantify genetic diversity and estimation of population genetic structure parameters (Hamrick & Loveless, 1986; Hamrick & Murawski, 1991; Loveless, 1992, 1998; Alvarez-Buylla & Garay, 1994; Murawski & Bawa, 1994; Hall *et al.*, 1994a, 1994b).

With the advent of PCR (Polymerase Chain Reaction) in the late 1980´s, a number of DNA marker technologies have been developed. Among these RAPD markers (Random Amplified Polymorphic DNA, Welch & McClelland, 1990; Williams *et al.*, 1990) have been increasingly used and have contributed significantly to the rapid assessment of genetic variability in many organisms. The RAPD procedure does not require prior knowledge of the target genome, only small amounts of DNA are required, and the technique is relatively simple and inexpensive. The main disadvantage of RAPD as a molecular marker for population genetic studies is its dominant

inheritance. Compared to co-dominant markers, this peculiarity reduces the accuracy of the estimation of allele frequencies necessary for population genetic analysis with RAPD´s. However, this problem can be reduced by indirect methods available to estimate allele frequencies from dominant fragment frequencies (Lynch & Milligan, 1994; Isabel *et al.*, 1999). Most of the population genetic studies of tropical trees species using RAPD´s have highlighted genetic diversity and the hierarchical analysis of the distribution of genetic variation within and among populations (Chalmers *et al.*, 1992; Russel *et al.*, 1993; Gillies *et al.*, 1997; Schierenbeck *et al.*, 1997).

Comparisons have been made between dominant RAPDs and codominant markers such as isozymes and RFLP to evaluate genetic diversity and distribution of genetic variation within and between populations in plant species. Although caution should be taken in such comparisons, in general both dominant (RAPD) and codominant (RFLP and isozymes) markers have shown congruence in the evaluation of the partitioning of genetic variation in plant populations (N´Goran *et al.*, 1994, Isabel *et al.*, 1995, Buso *et al.*, 1998).

In the last few years, microsatellites or simple sequence repeats (SSR) have become an attractive tool for population genetic studies in plants owing to their codominant inheritance, multiallelic nature and abundance and wide distribution in plant genomes. The variability observed at SSR loci allows the accurate characterization of

individuals in natural populations and estimates of genetic parameters such as levels of inbreeding, heterozygosity, gene flow, and mating system, that have fundamental importance for the genetic conservation and management of tropical trees under intensive human pressure. Recently, the development and characterization of microsatellite markers have been reported for tropical tree species (Chase *et al.*, 1996; White & Powell, 1997; Aldrich *et al.*, 1998; Ujino *et al.*, 1998; Collevatti *et al.*, 1999; Dayanandan *et al.*, 1999; Dick & Hamilton, 1999) allowing application in population genetic studies. The perspective for the near future is an increase in the use of this tool for population genetic investigation of tropical tree species due mainly to novel library enrichment strategies and rapid fluorescence-based automatic sequence technologies (Rafalski *et al.*, 1996).

The Brazilian mahogany (*Swietenia macrophylla*) is the most valuable hardwood species from the Neotropics: one cubic meter of export-quality sawn wood is valued at about US$ 700 (Verissimo *et al.*, 1995) in the international market. The production of mahogany is about 500,000 m^3/year in the Brazilian Amazon. Most of mahogany exploitation has been conducted in a non-sustainable way. In recent years, the conservation status of *S. macrophylla* has been the subject of increasing concern due to overexploitation and selective logging over natural populations. A listing of this species in Appendix II of the Convention on International Trade in Endangered Species (CITES)

has been proposed but has failed many times in the last few years. There is an urgent need for effective conservation and management strategies of this high-value forest resource and estimates of population genetic parameters are critical in this context. Despite its importance, little information exists on the extent and distribution of genetic variation in natural populations of *S. macrophylla* over their geographical range (but see Gillies, 1999).

In this chapter I report the use of a battery of fluorescent labelled, multiplexed microsatellite markers developed for *S. macrophylla* to estimate population genetic parameters for seven populations of mahogany sampled at a broad geographical scale. Also, the estimates of population differentiation based on dominant RAPD markers were compared with co-dominant microsatellites for two populations. The main aim of this study is understanding the extent and distribution of genetic variation at microsatellite loci and the patterns of gene flow in populations of *S. macrophylla* in the Brazilian Amazon.

MATERIALS AND METHODS

Population sites, collection of samples and DNA extraction

Mature trees of *S. macrophylla* were sampled from seven populations in the native range of the species near the southern boundary of the Brazilian Amazon region. Sample sites were located 8-2103 km apart. The location of the populations is illustrated in Figure 3.1: (1) Município de Água Azul do Norte (A. Azul) *ca.* 06° 54.0'S, 50° 16.0'W, (2) Marajoara Management Project (Maraj) *ca.* 07° 50.0'S, 50° 16.0'W, (3) Município de Pimenta Bueno (P.Bueno) *ca.* 12° 21.6'S, 61°26.4'W, (4) Fazenda Cachoeira Parecis A (Cach.A) *ca.* 12° 30.1'S, 61° 29.9'W, (5) Fazenda Cachoeira Parecis E (Cach.E) *ca.* 12° 34.4'S, 61° 29.9'W, (6) Reserva Extrativista Chico Mendes, (C.Mendes) *ca.* 10° 25.0'S, 69° 18.0'W, and (7) Município de Pontes e Lacerda (P. Lacerda) *ca.* 15° 04.6'S, 59° 09.4'W). The collections were made between May 1996 and September 1998. Leaf samples were collected from 24-34 individuals per population. The leaves were dried, preserved in silica gel, and stored at -20°C until DNA extraction. Total genomic DNA was extracted from the leaves following standard CTAB procedure (Doyle & Doyle, 1987). DNA quantification was performed by comparison with standard concentrations in ethidium bromide-stained 1% agarose gels.

Microsatellite analysis

Microsatellite analysis of all 194 individuals from seven populations was made using 10 sets of microsatellite loci developed and optimised for *S. macrophylla* using Applied Biosystem's fluorescence-based technology. Multiplexing of six reaction (multiplexed or single primers) mixes allowed genotyping one individual into three gel lanes (see Chapter 2). In multiplexed reactions PCR amplifications were carried out in a final volume of 25 µl containing 1.25-2.0 µM of each forward and reverse primers, 1 unit Taq DNA polymerase, 200 µM of each dNTP, 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), BSA (2.5 mg/ml), 5.0 ng of template DNA, and dH_2O. In single primers reactions each amplification was performed in a volume of 10 µl containing 1.25-2.0 µM of each forward and reverse primers, 1 unit Taq DNA polymerase, 200 µM of each dNTP, 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), BSA (2.5 mg/ml), 5.0 ng of template DNA, and dH_2O. The PCR conditions were as follows: an initial denaturation at 96°C for 2 min followed by 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min; and a final elongation step at 72°C for 7 min. Amplified products were electrophoresed in 5% denaturing polyacrilamide gel with internal standard in an ABI Prism 377 sequencer. Fluorescent products were automatically analysed using GeneScan 672 version 1.1 and Genotyper version 1.1 softwares

(ABI, 1993, 1994).

RAPD analysis

Genetic parameters using RAPD and microsatellite markers were analysed for the same individuals in two populations of *S. macropylla* (Maraj and P. Bueno, 1323 km apart) in order to compare estimates of population genetics using dominant and co-dominant markers. A total of 271 primers from Operon Technologies (Alameda, CA, USA) primer kits OPA, AB, C, D, G, I, J, K, L, N, O, P, R, U, X, W, Y and Z were screened with *S. macrophylla* DNA in eight individuals. Of these, only 45 primers that provided very consistent amplification products, signal strength, resolution of bands, and polymorphism were selected to use in the population analysis. Twenty-four individuals were analysed from each population. RAPD amplification were carried out as described in Ferreira & Grattapaglia (1995). Each 10 µl reaction mixtures contained 7.5 ng of genomic DNA, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 1.0 µg/µl of BSA, 0.2 mM of each dNTP, 0.4 µM 10-mer primer and 1U of *Taq* DNA polymerase. Amplifications were performed using a MJ Research PT-100 thermal controller programmed for 40 cycles of : 1 min at 92 °C, 1 min at 35 °C, 2 min at 72 °C; and a final elongation step at 72 °C for 7 min. Amplification products were separated by electrophoresis in 1.5% agarose gels in 1X TBE (Tris-Borate-EDTA) buffer system. Gels were

stained with ethidium bromide, and then visualised and photographed under UV light. Band sizes were determined by comparison with a 1Kb ladder in each gel. For microsatellite analysis the ten pairs of primers developed for *S. macrophylla* were used as described in the previous section.

Data analysis

To estimate overall levels of genetic diversity, the following measures were calculated for all populations: the mean number of alleles per locus (A), the Ho and He, the mean observed and expected heterozygosities respectively. The genetic parameters were calculated for each locus and averaged over all loci using the GDA program (Lewis & Zaykin, 1999). Tests for departure from the Hardy-Weinberg equilibrium (HW) were made by using the U-test (Raymond & Rousset, 1998) and the inbreeding coefficient (f) (Weir & Cockerham, 1984) considering both hypotheses of heterozygote deficiency and excess. These analyses were performed using Genepop version 3.1.b program (Raymond & Rousset, 1998). Estimation of exact-P values was determined by the Markov chain method (Guo & Thompson, 1992).

The extent of the genetic differentiation of populations was investigated by calculating fixation indices based on two models: the Infinite Allele Model (IAM) (Kimura & Crow, 1964) and the Stepwise

Mutation Model (SMM) (Ohta & Kimura, 1973). The IAM considers that mutation creates a new allele state with no known relationship to other allelic states while the SMM conceives information about ancestral allelic states (Jarne & Lagoda, 1996). Under the SMM similar size alleles at microsatellite loci are less different in terms of mutational steps than alleles with very different size (Jarne & Lagoda, 1996). The unbiased estimates of Wright F-statistics (Weir & Cockerham, 1984) was calculated under the former model by the FSTAT program version 2.9.1 (Goudet, 2000). Analogous to Wright's F_{ST}, θ measures divergence in allele frequencies among populations, whereas f (similar to F_{IS}) measures the correlation of allele frequencies within individuals within populations and F (similar to F_{IT}) is related to the correlation of allele frequencies within individuals in different populations (Cockerham, 1969). Values of θ were calculated over all loci and populations and for each pairwise comparison of populations. Statistical significance of the observed θ values was tested using the exact G-test (Goudet *et al.*, 1996) and by bootstrapping over loci with 95% nominal confidence interval (Weir, 1996). P-values were obtained after 1000 permutations. Significance tests of differentiation between each pair of populations were carried out after considering Bonferroni corrections implemented by FSTAT program version 2.9.1 (Goudet, 2000). Genetic differentiation under the SMM was assessed by ρ, an estimator of Slatkin's R_{ST} (Slatkin,

1995) analogous to θ. The estimate of ρ was obtained by calculating the between – and within – population components of variance using allele sizes expressed in terms of the standardized number of repeats. It was calculated using the program R_{ST} CALC, version 2.2 (Goodman, 1997) over all loci and populations and for pairwise comparisons of populations. Significance levels were determined after 1000 bootstraps with 95% nominal confidence intervals and permutation tests (Lynch & Crease, 1990) were made to determine if observed values of ρ were significantly different from zero.

Indirect estimates of gene flow among populations were calculated based on the mean number of migrants among populations per generation (*Nm*). Values of *Nm* were estimated by two methods: (1) Estimates from θ (similar to Wright's F_{ST}) and ρ (similar to Slatkin's R_{ST}) by the relationship: $Nm = 1/4(1/X_{ST} - 1)$ where N is the effective population size, m is the rate of migration, and X_{ST} is either the coefficient of genetic differentiation between populations θ or ρ; (2) Private allele method (Slatkin, 1985a; Barton & Slatkin, 1986) which considers the average frequency of rare alleles for estimating the value of *Nm*. The estimates of overall and pairwise *Nm* based on θ and private alleles methods were calculated using Genepop version 3.1 b program (Raymond & Rousset, 1998). Estimates of overall and pairwise *Nm* based on the ρ estimator were calculated using program R_{ST} CALC version 2.2 (Goodman, 1997).

The isolation-by-distance model (Wright, 1943) was tested by correlating values of the population pairwise θ and ρ estimators against the $\log_{10}$ of the population pairwise geographical distance values. The statistic used was the Spearman Rank correlation coefficient and significance was determined after 1000 permutations of a Mantel procedure (Mantel, 1967). The Mantel test was made using the program GENEPOP version 3.1.c (Raymond & Rousset, 1998).

A comparison between two classes of markers was performed in order to evaluate the extent of genetic variation in the exact same individuals (24 individuals from each population) of two populations of *S. macrophylla* (Maraj and P.Bueno 1323 km apart) using dominant RAPD and co-dominant microsatellite markers. Data regarding RAPD markers were recorded as presence and absence of fragments, and the data were entered into a binary data matrix. Individuals exhibiting a band were interpreted as homozygous for the dominant allele (AA) or heterozygous (Aa), whereas individuals with no fragment were considered as recessive homozygous (aa). Genetic parameters based on RAPD were calculated considering the loci for which fragment frequencies were less than $1 - 3/n$, where n is the population sample size (Lynch & Milligan, 1994). The allele frequencies used to estimate RAPD's F_{ST} were corrected for deviations from the Hardy-Weinberg equilibrium using the inbreeding coefficient

(f) estimated from microsatellite loci (Isabel *et al.,* 1999). To assess the partitioning of genetic variation within and between populations an analysis of molecular variance (AMOVA) was conducted as described by Excoffier *et al.* (1992) using the program ARLEQUIN (Schneider *et al.*, 1997). Two AMOVA's were made one using RAPD and the other using microsatellite data.

RESULTS

Genetic variation

The multiplexing approach employed in this study allowed the analysis of up to five loci into a single gel lane (Fig. 3.2). All microsatellite loci surveyed for *S. macrophylla* were highly polymorphic. A total of 181 alleles were detected for 194 individuals from the seven populations. The average number of alleles over all loci was 18.1 alleles (range 13 – 27) (Tab. 3.1), whereas the mean number of alleles observed per locus per population over all populations was 9.3 (range 7.6-10.3) (Tab. 3.2). The total number of alleles detected in a single population, considering all loci, varied from 76 for P.Bueno to 103 for Cach.A. Generally alleles showed a relatively even distribution of frequencies (Appendix I). Forty-two private alleles (alleles restricted to a single population) were found in mahogany from the seven populations surveyed at the 10

microsatellite loci. Private alleles constituted 23.2% of all alleles. All populations contained private alleles and the number detected per population varied from 4 alleles for A.Azul and P.Bueno to 11 for C.Mendes (Tab. 3.2). Except for alleles 152 (locus sm32), 68 (locus sm34) and 146 (locus sm45) detected in the P.Lacerda population, which occurred at frequencies of 27.3%, 28.3% and 25% respectively, all the private alleles occurred with a relatively low frequency varying between 1.5 to 12.5% (Appendix I). The mean observed heterozygosity (H_o) was 0.73 and ranged from 0.37 for locus sm34 to 0.90 for locus sm45. The mean expected heterozygosity (H_e) was generally higher than H_o (with only three loci showing the opposite trend). The mean H_e was 0.76 and ranged from 0.51 for locus sm34 to 0.89 for locus sm31 (Tab. 3.1). The observed heterozygosity (H_o) per population ranged from 0.69 to 0.77 and the expected heterozygosity (H_e) per population ranged from 0.72 to 0.80 (Tab. 3.2).

Hardy-Weinberg Equilibrium

Of the 70 tests of conformity to Hardy-Weinberg proportions that were made, twelve showed a significant departure from expected proportions at the 5% level. Based on the inbreeding coefficient, f (Weir and Cockerham, 1984), eleven of the deviations were due to a deficit of heterozygotes and only one, in locus sm45 in the C.Mendes population, was due to an excess of heterozygotes (Tab. 3.3). The

mean overall f considering all populations was 0.046. Although it represents a low value, it is significantly different from zero and suggests a not negligible amount of inbreeding detected in *S. macrophylla* populations.

Population differentiation, substructuring, and gene flow

The unbiased estimates of Wright's F-statistics (Weir & Cockerham, 1984) and the estimate of genetic differentiation ρ (Goodman, 1997) calculated overall populations of *S. macrophylla* for each and over all loci are presented in Tab. 3.4. Overall, the mean coefficient of inbreeding f was low but significantly different from zero (f = 0.046, 95% C.I. 0.0004–0.0918, $p<0.0001$) indicating a nonrandom mating of individuals within populations. The value of F, the measure of inbreeding that considers both the effects of non-random mating within and among populations, was different from zero (F= 0.155, 95% C.I 0.091-0.241, $p<0.0001$)) indicating population substructuring.

The two overall measures of genetic population differentiation θ and ρ showed close values for the combined data from all populations each significantly greater than zero (θ = 0.116, 95% C.I 0.073–0.176 and ρ= 0.144, 95% CI 0.134–0.195, $p<0.0001$) suggesting that there is a significant degree of genetic differentiation among populations of *S. macrophylla* in the Brazilian Amazon.

Despite consistent patterns of genetic differentiation over all populations using both estimators θ and ρ , non congruent values of θ and ρ were found in the pairwise comparisons between mahogany populations (Tab. 3.5).

For all loci combined and all populations pooled, the number of individuals assumed to migrate among populations per generation (*Nm*) was very similar calculated using either F_{ST}/R_{ST} based approaches (Wright, 1951; Slatkin, 1995) or the private allele method. The values found were *Nm*[θ] = 1.905, *Nm*[ρ] = 1.584 and Nm[pa] = 1.932, respectively. In the pairwise comparisons among populations, the highest number of migrants per generation was found between A.Azul and Maraj (107 km apart) considering both the θ and ρ methods (*Nm* = 6.43 and 12.45, respectively). For the private alleles method, the highest value of *Nm* in pairwise comparisons was observed between Cach.A and P.Bueno (*Nm* = 2.11) populations 17 km apart. Therefore, the estimates of migration between populations of *S. macrophylla* in the Brazilian Amazon showed no consistent patterns with low to high levels of gene flow (range 0.685 - 12.455) occurring in pairwise population comparisons (Tab. 3.5).

Isolation-by-distance

The test of isolation by distance showed no significant correlation between pairwise comparisons of either θ or ρ *versus* $\log_{10}$

distance (p = 0.786 and 0.841 respectively) for 21 pairwise combinations of seven populations. If the population Cach.E is excluded from the analysis (this population, located in a deep valley at the slopes of the Chapada dos Parecis mountains, exhibited a high differentiation from the two neighbouring populations, Cach.A and P. Bueno) the correlation was positive but still not significant (p = 0.582 and 0.626, respectively). Therefore, there was no clear evidence of a pattern of isolation by distance for the *S. macrophylla* populations in the Brazilian Amazon.

Comparison of dominant RAPD and codominant microsatellite markers

A total of 78 polymorphic RAPD fragments were scored in 48 individuals of two populations studied using 45 pairs of primers. The mean number of RAPD fragments detected per primer was 1.7. The 78 polymorphic RAPD loci showed frequencies less than 1-3/n and all were considered for calculations of genetic parameters. Correcting fragment frequencies from the Hardy-Weinberg equilibrium using an average within population inbreeding coefficient (f) estimated from microsatellites had almost no effect in the estimation of RAPD F_{ST} (F_{ST}= 0.2432918 and 0.2432925 without and with correction respectively).

The hierarchical analysis of the genetic structure based on an analysis of molecular variance (AMOVA) using 78 dominant RAPD markers in two populations studied were congruent with other AMOVA data using ten co-dominant microsatellite loci for the same individuals (Tab. 3.6). Most of the genetic variation (75% for RAPD analysis and 92% for microsatellites) was detected within populations. However, a significant proportion of the variation was found between populations (24.7% $p<0.0001$ for RAPD and 8% $p<0.0001$ for SSR).

DISCUSSION

The present study has highlighted the utility of microsatellite markers for investigating the genetic variability and structuring of *S. macrophylla* populations. The fluorescence-based technology used allied to the multiplexing of up to five microsatellite loci from an individual in a single lane gel greatly improved the speed and efficiency of population genotyping of *S. macrophylla.* A more extensive use of this genetic tool will certainly improve knowledge on genetic structure and mating system of tropical trees in the near future and provide a genetic basis for policies of conservation and management of valuable natural resources such as mahogany.

Genetic Diversity and the Hardy-Weinberg Equilibrium

The levels of genetic diversity observed for *S. macrophylla*, mean number of alleles detected per locus (A= 18.1) and the average gene diversity (H_e = 0.76), are within the range observed for other tropical tree species using microsatellite markers (Aldrich *et al.*, 1998; Ujino *et al.*, 1998; Dayanandan *et al.*, 1999; Rosseto *et al.*, 1999; Collevatti *et al.*, 1999; White *et al.*, 1999). These studies found that the mean number of alleles per locus ranged from 7.9 to 18.3 and the average value of expected heterozygosity (H_e) ranged from 0.55 to 0.83. As expected for microsatellite loci with high mutation rates, the values are substantially higher when compared with other kinds of markers such as isozymes. Loveless & Hamrick (1987) studying genetic variation in eight tree species in Barro Colorado, Panama found the number of alleles per polymorphic locus ranging from 1.43 to 2.64 and values of gene diversity (H) ranging from 0.106 to 0.273. Loveless (1992) in a review about isozyme variation in 37 tropical tree species found an average gene diversity (H) ranging from 0.038 to 0.216.

Deviations from Hardy-Weinberg genotypic expectations were found in this study with a significant deficit of heterozygotes in eleven of 70 tests made. In only one case (locus sm45 at population C. Mendes) was there observed a departure from HWE due to a significant excess of heterozygotes. As observed in Tab. 3.3, four out

of seven populations analysed had values of f (coefficient of inbreeding) significantly greater than zero indicating an excess of homozygotes. In the other three populations values of f were also positive but not statistically significant.

Several explanations could be put forward for the finding of a deficit of heterozygotes. One is the possibility of the occurrence of null alleles. Null alleles are undetectable alleles due to a mutation in the flanking primer sequences (Callen *et al.,* 1993). Some studies have documented the presence of null alleles at microsatellite loci occurring at frequencies of up to 15% (Callen *et al.,* 1993; Paetkau & Strobeck, 1995; Pemberton *et al.*, 1995; Jarne & Lagoda, 1996). However it seems unlikely to be occurring in *S. macrophylla* because no null/null homozygotes were detected in any loci when genotypes of 400 progenies and 25 mother trees were analysed in a mating system study carried out in the Maraj. population (M.R. Lemes, unpublished data). Also no mismatches between a mother and her offspring were observed (i.e. all individual offspring displayed at least one maternal allele).

Heterozygote deficits can also be due to the existence of undetected breeding subunits within populations, the Wahlund effect or assortative mating which generally increases the frequency of homozygous genotypes in the population at the expense of heterozygous genotypes (Hartl, 1987). In fact, inbreeding seems to be

the most probable cause for the heterozygote deficit in *S. macrophylla.* The low, but not negligible, level of inbreeding detected in *S. macrophylla* populations is in agreement with data from the mating system study of the Maraj population (Chapter 4), where 4.2% of seeds resulted from self-fertilizations or correlated matings. The excess of heterozygotes detected in one locus (sm45) at population C.Mendes was probably a sampling artefact caused by a relatively small sample size. The great number of alleles detected in microsatellite loci usually occurring at low frequencies may lead to bias when the sample size is small. Theoretical studies have highlighted the effect of sample sizes on the performance of different genetic parameters estimated for loci with multiple alleles (Ewens, 1972; Sjögren & Wyöni, 1994). Ruzzante (1998) using a microsatellite data set has showed that the variance in estimates of some genetic distance and structure measures may be influenced by the number of individuals and loci surveyed.

When compared to the other SSR loci in this study, the locus sm34 exhibited the highest coefficient of inbreeding for four out of seven populations studied and over all populations (Tab. 3.3). This result is intriguing and suggests that selection could be acting at this SSR locus favouring individuals with allele sizes 70-72 base pairs, which are much more frequent than other alleles in all populations (Appendix I).

Genetic differentiation and gene flow

Genetic differentiation among populations of *S. macrophylla* were analysed by calculating θ (Weir & Cockerham, 1984) and ρ (Goodman, 1997) estimators which are based on the assumption of the Infinite Allele Model (Kimura & Crow, 1964) and the Stepwise Mutation Model (Ohta & Kimura, 1973), respectively. It has been suggested that for microsatellites the stepwise model might be more appropriate (Valdes *et al.*, 1993; Goldstein *et al.*, 1995; Slatkin, 1995; Goldstein & Pollock, 1997) because it takes into account the mutational properties of microsatellite loci. The multilocus values of genetic differentiation based on θ (0.12) and ρ (0.14) were very close in this study, indicating a moderate but significant ($p<0.0001$) degree of differentiation among populations of *S. macrophylla* in the Brazilian Amazon which is in accordance with the tendency observed for tropical tree species in general (Hamrick, 1994). The value of θ estimator (0.12) obtained for *S. macrophylla* was similar to the mean G_{ST} (0.11) value found for 37 different tropical taxa using isozymes (Loveless, 1992). However, caution should be taken when comparing estimates of genetic differentiation among studies owing to variation in geographical scale, life history traits and class of genetic marker used.

Few studies have reported assessment on genetic variation in natural populations of tropical tree species using microsatellites (Chase *et al.*, 1996; Aldrich *et al.*, 1998; Rosseto *et al.*, 1999; White *et al.*, 1999; Dayanandan *et al.*, 1999). The two species of Meliaceae studied with microsatellites so far, Ca*rapa guyanensis* in Costa Rica (Dayanandan *et al.*, 1999) and *Swietenia humilis* in Honduras (White *et al.*, 1999), exhibited much lower levels of genetic differentiation (ρ = 0.041 and 0.032, respectively) among populations (or fragments of population) than that found for *S. macrophylla* using the same estimator. These differences should be interpreted with caution since the spatial scale of the studies with *C. guianensis* and *S. humilis* (populations up to 44 km apart) contrasts with the extensive sampling along of the geographic distribution of *S. macrophylla* in this study (up to 2100 km). The spatial scale is important because patterns of differentiation may reflect factors such as mating systems, selection, population size and pollen and seed dispersal distances (Alvarez-Buylla *et al.*, 1996).

The moderate, but statistically significant, genetic differentiation among populations of *S. macrophylla* may reflect the balance of several ecological factors which historically have influenced gene flow distances in this species. Mahogany is a monoecious species with minute flowers pollinated by a diverse array of generalist insects, such as small-sized bees, moths and thrips

(Styles, 1972; Styles & Khosla, 1976; Howard *et al.*, 1995). Although little is known about the effectiveness of these small generalist insects as long-distance pollinators, it is generally accepted that they have limited foraging ranges. The pollination by small diverse insects probably promotes pollen flow at less extensive distances than other more specialised systems, such as pollination by bats or by large or medium-sized bees (Frankie *et al.*, 1976; Bawa, 1990). The short distance pollination systems enhance genetic differentiation because the opportunities for gene exchange declines between populations.

Among several life history traits examined in a review of patterns of genetic organisation in tropical plants, Loveless (1992) found that seed dispersal mechanisms contributed to significant levels of population differentiation between species. Abiotic modes of dispersal (wind and gravity) showed more than twice as much population differentiation (mean G_{ST} = 0.14) as species dispersed by biotic agents (mean G_{ST} = 0.05). The genetic differentiation observed among populations of *S. macrophylla*, an anemochorous species with median seed dispersal distance of only 32-36 m (Gullison *et al.*, 1996), is in accordance with the common tendency observed for tropical tree species abiotically dispersed.

Gene flow greater than one individual per generation can prevent neutral alleles being fixed due to random genetic drift (Wright, 1931; Slatkin, 1985b). The estimates of *Nm* obtained using

different statistics derived from θ , ρ, and calculated by the private alleles were in agreement (averaging 1.8 migrants per generation). This value, however, seems not to be high enough to totally override the effects of drift, resulting in a moderate level of differentiation among populations of *S. macrophylla.* Genetic evidence suggest that gene flow among local populations is high in tropical tree species, but geographically distant populations show moderate levels of genetic differentiation (Loveless,1992). High *Nm* values may also indicate substantial movement of genes by pollen and seeds among populations. The *Nm* estimates varied considerably (range 0.43-29.94) in a study carried out with 14 tropical tree species in Barro Colorado, Panama (Hamrick & Loveless, 1989). Generally it depends on dispersal seed ecology, pollination syndrome and mating system (Ellstrand, 1992).

Slatkin (1995) proposed that allele-frequency-based (θ) statistics should overestimate gene flow relative to R_{ST} (ρ), however in the present study the overall *Nm* estimate derived from ρ was not lower than *Nm* from θ. Here the biases proposed were not observed. The same tendency on estimates of migrants per generation using different approaches were also found in other studies with microsatellite data (Goodman ,1998). Although many studies have addressed the estimation of gene flow in natural populations by indirect methods, the usefulness of these indirect estimates is a

matter of controversy. Whitlock & McCauley (1999) pointed out that only rarely can genetic data based on Wright's F-statistics be translated into an accurate estimate of gene flow (*Nm*). The authors highlighted the violation of the assumptions of the island model (Wright, 1931) underlying the translation of F_{ST} statistics into *Nm* in natural systems and the consequences on the quantitative and qualitative conclusions from indirect studies of gene flow.

The geographical distance among populations was not significantly correlated with genetic differentiation suggesting that other processes than simple isolation by distance are acting to structure populations of *S. macrophylla*. Other tropical tree species have shown similar tendencies of deviation from isolation by distance model (Hall *et al.*, 1994b; Chase *et al.*, 1995; Loveless, 1998). The more conspicuous failure of the isolation by distance model is represented by the pair of populations Cach.A and Cach.E, only 8 km apart, but exhibiting the second highest value of θ in pairwise comparisons (Tab. 3.5). Topographic barriers may restrict gene flow and have an important role in the genetic isolation of these populations, since the former is located in a flatland *plato* of the Brazilian Shield whereas the later is set in a deep valley at the undulate slopes of the Serra dos Parecis mountains. Another possible explanation is that distinct selective pressures in the two different habitats lead to population differentiation, despite geographical

proximity. Many geographical features can restrict gene flow between sets of populations such as rivers and mountains (Whitlock & McCauley, 1999). In all pairwise population comparisons ρ values indicate significant differentiation between populations at level $p<0.001$ except for the pair Agua Azul X Marajoara that showed significant differentiation at $p<0.05$. This pair of populations that are 107 km apart showed the lowest genetic difference and largest migration rate estimated either by θ and ρ.

Dominant RAPD *versus* codominant microsatellite markers

In the present study the distribution of genetic variation inferred by the two different classes of markers (RAPD and microsatellites) were in accordance. Most of the genetic variation was present within rather than among populations which is in agreement with most tropical trees species (Heywood & Fleming, 1986; Buckley *et al.*, 1988; Eguiarte *et al.*, 1992; Hamrick, 1994, Hall *et al.*, 1994a; Hall *et al.*, 1996; Schierenbeck *et al.*, 1997; Gillies *et al.*, 1997). Nevertheless, significant genetic differentiation was found among populations. Such results are expected for species with a patchy distribution pattern such as mahogany. Gillies *et al.* (1999) using RAPD markers to assess genetic diversity in populations of *S. macrophylla* from Central America also found the same tendency.

Although both classes of molecular markers have shown a

significant differentiation among populations, estimates are rather different in magnitude (F_{ST} = 0.25 and 0.08, for RAPD and SSR respectively). While RAPD markers are biallelic and do not allow direct estimation of heterozygosities, microsatellite markers are highly multiallelic. As microsatellite markers typically present high heterozygosities within populations the magnitude of differentiation estimated by F_{ST} tends to be small (Hedrick, 1999). This fundamental difference in the genetic basis of the two classes of markers implicates in a non-totally legitimate interpretation of values in direct comparisons.

Implications for conservation

The Brazilian mahogany (*Swietenia macrophylla*) is threatened throughout its range due to over-exploitation and habitat destruction which has clearly reduced its population sizes. Reduction may become so intense that these populations may not constitute viable units in the long term owing to the prevalence of genetic drift and inbreeding causing loss of genetic variation (Bawa & Ashton, 1991). Therefore the long-term survival of this high-value forest resource requires urgent protection of their populations over its geographical distribution.

The knowledge of the levels and distribution of genetic variation is of fundamental importance for the establishment of

effective and efficient conservation strategies for tropical tree species under intense human pressure. Results from a genetic study based on RAPD markers suggest that selective logging reduces genetic diversity within *S. macrophylla* populations in Central America (Gillies *et al.*, 1999). The current study, based on highly informative microsatellite markers, reports high genetic diversity within populations and the existence of significant genetic structuring of *S. macrophylla* populations along the Brazilian Amazon.

Such findings indicate the urgent need for conservation of multiple populations along the distribution range, with a relatively large number of individuals per population to ensure that allelic and genotypic diversity will be maintained in *S. macrophylla* populations. The reserve areas for canopy tropical tree species that occur at densities of one adult or less per hectare (Bawa & Ashton, 1992) such as *S. macrophylla* (Barros *et al.*, 1992; Verissimo *et al.*,1995) should be very large to maintain the high intrapopulation genetic variation and to avoid inbreeding and genetic drift (Eguiarte *et al.*, 1992). Furthermore, since genetic differentiation seems not to be correlated with geographic distance, populations of *S. macrophylla* only 8-15 km apart can exhibit a high level of differentiation if physical barriers avoid gene flow among them. The occurrence of such microgeographical differentiation emphasizes the importance of maintaining populations in their diverse habitats, especially in areas

with mosaic of topography and soils.

As pollen and seeds of *S. macrophylla* can not be dispersed over long distances, the alarming fragmentation caused by selective logging practice and by conversion of the forest into plantations and pastures is likely to reduce the chances for colonization of new sites, despite the ability of this species to regenerate in disturbed habitats (Snook, 1996).

Studies on genetic variation of tropical plants have emphasized that the breeding system significantly influences genetic diversity within and among populations (Hamrick & Loveless, 1989). According to Hamrick *et al.* (1991) the first step in designing an effective management strategy for an endangered tree species should be the determination of its breeding system. The mating system, a major component of the breeding system is the subject of the investigation reported in the next chapter for *S. macrophylla.*

Efforts for *ex situ* conservation strategies seems not to be very practical for conservation of genetic diversity of tropical forest trees (Eguiarte *et al.*, 1992). The main reasons for the ineffectiveness of *ex situ* conservation for most tropical forest species are: (1) Difficulties in collecting the quantities of seeds necessary to preserve at least part of the genetic variation present in a population, (2) problems with long-term storage of seeds, (3) limited capabilities of many large woody species for cultivation, and (4) possible lack of maintenance of

mechanisms of pollination *in ex situ* conditions (Bawa & Ashton, 1991; Eguiarte *et al.*, 1992).

In the case of Brazilian mahogany (*S. macrophylla*) most efforts should be directed towards *in situ* conservation strategies allied to effective policies to regulate its exploitation. With depletion of natural stocks in private lands, illegal extractions of mahogany have been reported from National Parks, and Indian reserves in Brazil and other countries (Rodan *et al.*, 1992). Unfortunately, there is no reason for optimism about the conservation of the mahogany in the Brazilian Amazon. The distribution range of *S. macrophylla* coincides with the areas of higher deforestation rates such as south Pará, Tocantins, north Mato Grosso, Rondônia and Acre States. The paucity of official conservation policies and the weakness of the Brazilian environment agencies contrast with the strength of the economic pressures which lead to uncontrolled logging and replacement of the forest by extensive cattle ranch pastures or soybean monoculture for exportation. To date, these are the main threats for effective viability of *S. macrophylla* populations in the Brazilian Amazon .

Table 3.1 - Genetic parameters of ten microsatellite loci in seven populations of *S. macrophylla*. The repeat motif, total number of alleles, H_e (expected mean heterozygosity) and range, H_o (observed mean heterozygosity) and range.

Locus	Repeat motif	Number of alleles	H_e (range)	H_o (range)
sm01	$(AG)_{19}$	18	0.707 (0.222-0.904)	0.697(0.235-0.920)
sm22	$(AG)_{18}$	17	0.759 (0.525-0.837)	0.698(0.500-0.823)
sm31	$(AG)_{31}$	27	0.893 (0.807-0.922)	0.830(0.783-0.967)
sm32	$(AG)_{20}$	17	0.854 (0.774-0.900)	0.772(0.559-0.880)
sm34	$(AG)_{19}$	17	0.508 (0.358-0.775)	0.366(0.208-0.581)
sm40	$(AG)_{19}$	13	0.704 (0.629-0.769)	0.739(0.592-0.840)
sm45	$(AG)_{21}$	17	0.853 (0.802-0.889)	0.902(0.781-0.970)
sm46	$(AG)_{20}$	17	0.841 (0.790-0.901)	0.824(0.760-0.875)
sm47	$(AG)_{24}$	17	0.688 (0.379-0.836)	0.693(0.360-0.853)
sm51	$(AG)_{22}$	21	0.803 (0.715-0.903)	0.750(0.500-0.909)
Mean over all loci		18.1	0.761 (0.719-0.800)	0.727(0.665-0.766)

Table 3.2 - Microsatellite diversity in seven populations of *S. macrophylla* averaged over ten microsatellite loci. N, sample size per locus; A, number of alleles per locus; P_A , number of private alleles; H_o, observed heterozygosity ; H_e, expected heterozygosity, and coefficient of inbreeding (f).

Population	N	A	P_A	H_e	H_o	f
A. Azul	29.7	8.7	4	0.758	0.747	0.014
Cach.A	32.0	10.3	6	0.749	0.728	0.028
Maraj	24.9	9.4	5	0.800	0.740	0.076
P.Lacerda	23.3	9.4	7	0.789	0.766	0.029
C.Mendes	34.0	10.0	11	0.719	0.688	0.044
Cach.E	24.0	9.6	7	0.777	0.754	0.031
P.Bueno	23.3	7.6	4	0.735	0.665	0.097
Over all populations	27.3	9.3	43	0.761	0.727	0.046

Table 3.3 - Coefficient of inbreeding (f) (Weir & Cockerham, 1984) for each and over all loci in seven populations of *S. macrophylla.*

Locus	A.Azul	Cach. A	Maraj	P. Lacerda	C.Mendes	Cach. E	P. Bueno	Over all
sm01	0.074	-0.034	-0.018	-0.172	-0.062	0.007	0.302**	0.015
sm22	-0.055	-0.012	0.131	0.082	-0.009	0.106	0.314**	0.082*
sm31	-0.050	0.062**	0.083	0.139	0.141	0.103*	0.031	0.072***
sm32	0.033	0.018	0.021	0.095	0.281	0.151*	0.060	0.097*
sm34	0.035	0.362*	0.333**	0.596*	0.347*	0.113	0.208	0.283***
sm40	0.232*	-0.053	-0.125	-0.169	0.025	-0.179	-0.119	-0.050
sm45	0.021	0.049	-0.090	-0.090	-0.213***	-0.096	0.013	-0.058
sm46	0.020	0.009	0.091	-0.058	0.087	0.082	-0.095	0.021
sm47	0.197	0.029	0.275***	0.006	-0.026	-0.126	-0.004	-0.008*
sm51	0.038	-0.004	0.171	-0.033	-0.049	0.123	0.345***	0.068
Over all	0.014	0.028**	0.076**	0.029	0.044	0.031*	0.097***	0.046***

Significant departures from Hardy-Weinberg expectations at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$
Estimation of exact P-values by the Markov chain method

Table 3.4 - Unbiased estimates of Wright's F-statistics (Weir and Cockerham, 1984) and estimate of genetic differentiation ρ (Goodman, 1997) for each and over all loci in seven populations of *S. macrophylla*. Significance is based on permutation testing (1000 times) with 95% confidence intervals (CIs) for all estimates over all loci.

Locus	f (SE)	F (SE)	θ (SE)	ρ
sm01	0.015 (0.048)	0.226 (0.103)	0.214 (0.092)	0.191
sm22	0.073 (0.044)	0.174 (0.037)	0.109 (0.033)	0.089
sm31	0.072 (0.028)	0.106 (0.029)	0.037 (0.008)	0.139
sm32	0.095 (0.040)	0.166 (0.054)	0.078 (0.021)	0.195
sm34	0.277 (0.071)	0.522 (0.069)	0.340 (0.065)	0.022
sm40	-0.039 (0.057)	0.051 (0.043)	0.087 (0.033)	0.123
sm45	-0.058 (0.040)	0.019 (0.025)	0.073 (0.021)	0.184
sm46	0.019 (0.027)	0.071 (0.024)	0.053 (0.009)	0.084
sm47	-0.008 (0.037)	0.150 (0.045)	0.157 (0.055)	0.145
sm51	0.055 (0.049)	0.105 (0.045)	0.053 (0.017)	0.190
Over all	0.046* (0.024)	0.155* (0.041)	0.116* (0.028)	0.144*(0.001)
Upper Bound	0.0918	0.2413	0.1757	0.1950
Lower Bound	0.0004	0.0908	0.0730	0.1340

*$p<0.0001$

Table 3.5 - Pairwise multilocus estimates of θ, ρ, and *Nm* considering the seven populations of *S. macrophylla*. Calculations of *Nm* were based on θ, ρ, and private alleles methods. 95% confidence intervals for ρ and *Nm(ρ)* are shown in parenthesis.

Population Comparison	Distance (km)	$\theta^{(*)}$	$\rho^{(**)}$ (95% CI)	*Nm* [θ]	*Nm* [pa]	Nm [ρ]$^{(**)}$ (95% CI)
Cach.A - Cach.E	8	0.165	0.110 (0.090-0.166)	1.26	0.97	2.01 (1.244-2.519)
Cach.A - P.Bueno	17	0.043	0.105 (0.072-0.170)	5.60	2.11	2.13 (1.205-3.170)
Cach.E - P.Bueno	24	0.152	0.074 (0.060-0.130)	1.40	0.96	3.12 (1.677-3.901)
A.Azul - Maraj	107	0.037	0.020 (0.006-0.073)	6.43	1.96	12.45 (3.054-28.075)
P.Lacerda - Cach.E	375	0.081	0.074 (0.044-0.142)	2.83	1.14	3.13 (1.495-5.321)
Cach.A - P.Lacerda	381	0.142	0.078 (0.051-0.140)	1.51	0.70	2.95 (1.526-4.595)
P.Lacerda - P.Bueno	389	0.144	0.111 (0.078-0.176)	1.49	0.73	2.00 (1.148-2.919)
Cach.A - C.Mendes	882	0.148	0.177 (0.132-0.242)	1.44	1.00	1.16 (0.775-1.615)
C.Mendes - Cach.E	884	0.142	0.072 (0.049-0.133)	1.51	0.68	3.23 (1.627-4.736)
C.Mendes - P.Bueno	884	0.133	0.060 (0.046-0.110)	1.63	1.30	3.89 (2.022-5.136)
P.Lacerda - C.Mendes	1216	0.139	0.115 (0.078-0.185)	1.55	0.81	1.93 (1.096-2.881)
Maraj - P.Lacerda	1258	0.063	0.175 (0.132-0.250)	3.72	1.10	1.18 (0.750-1.625)
A.Azul - P.Lacerda	1307	0.083	0.150 (0.104-0.228)	2.75	0.74	1.41 (0.838-2.122)
Maraj - P.Bueno	1323	0.080	0.155 (0.117-0.227)	2.92	1.17	1.36 (0.851-1.883)
Cach.A - Maraj	1334	0.084	0.136 (0.102-0.197)	2.71	1.38	1.58 (1.010-2.146)
Maraj - Cach.E	1337	0.082	0.088 (0.055-0.154)	2.81	1.72	2.60 (1.357-4.276)
A.Azul - P.Bueno	1342	0.108	0.140 (0.091-0.208)	2.07	0.95	1.55 (0.948-2.466)
A.Azul - Cach.A	1355	0.129	0.117 (0.090-0.173)	1.70	1.10	1.88 (1.178-2.529)
A.Azul - Cach.E	1358	0.103	0.104 (0.075-0.170)	2.17	0.95	2.16 (1.218-3.078)
Maraj - C.Mendes	2101	0.130	0.186 (0.152-0.243)	1.73	1.18	1.09 (0.777-1.396)
A.Azul - C.Mendes	2103	0.170	0.223 (0.178-0.291)	1.25	1.10	0.87 (0.608-1.153)

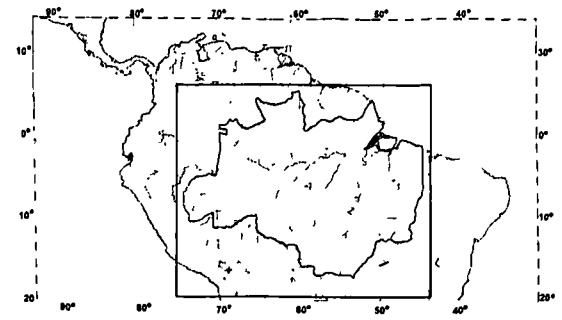
* For all pairwise comparisons of θ, non-adjusted p-values, $p<0.001$. For corrected p-values using standard Bonferroni procedure, $p<0.05$.

**For all pairwise comparisons of ρ and Nm[ρ] $p<0.001$,except for pair A.Azul X Maraj., where $p<0.05$.

Table 3.6 - Comparison of genetic differentiation estimates based on AMOVA data in two populations of *S. macrophylla* using RAPD and SSR markers. The data show the degrees of freedom, sum of squared deviation (SSD), mean squared deviation (MSD), variance component estimate, percentage of total variation contributed by each component.

Source of variation	d.f.	SSD	MSD	Variance components	% Total
RAPD					
Between populations	1	98.15	98.15	3.63	24.66
Within population	46	509.87	11.08	11.08	75.34
Total	47	608.02	12.9	14.71	
SSR					
Between populations	1	18.02	18.02	0.30	8.25
Within population	94	318.69	3.39	3.39	91.75
Total	95	336.70	3.54	3.69	

F_{ST} (RAPD) = 0.246
F_{ST} (SSR) = 0.082



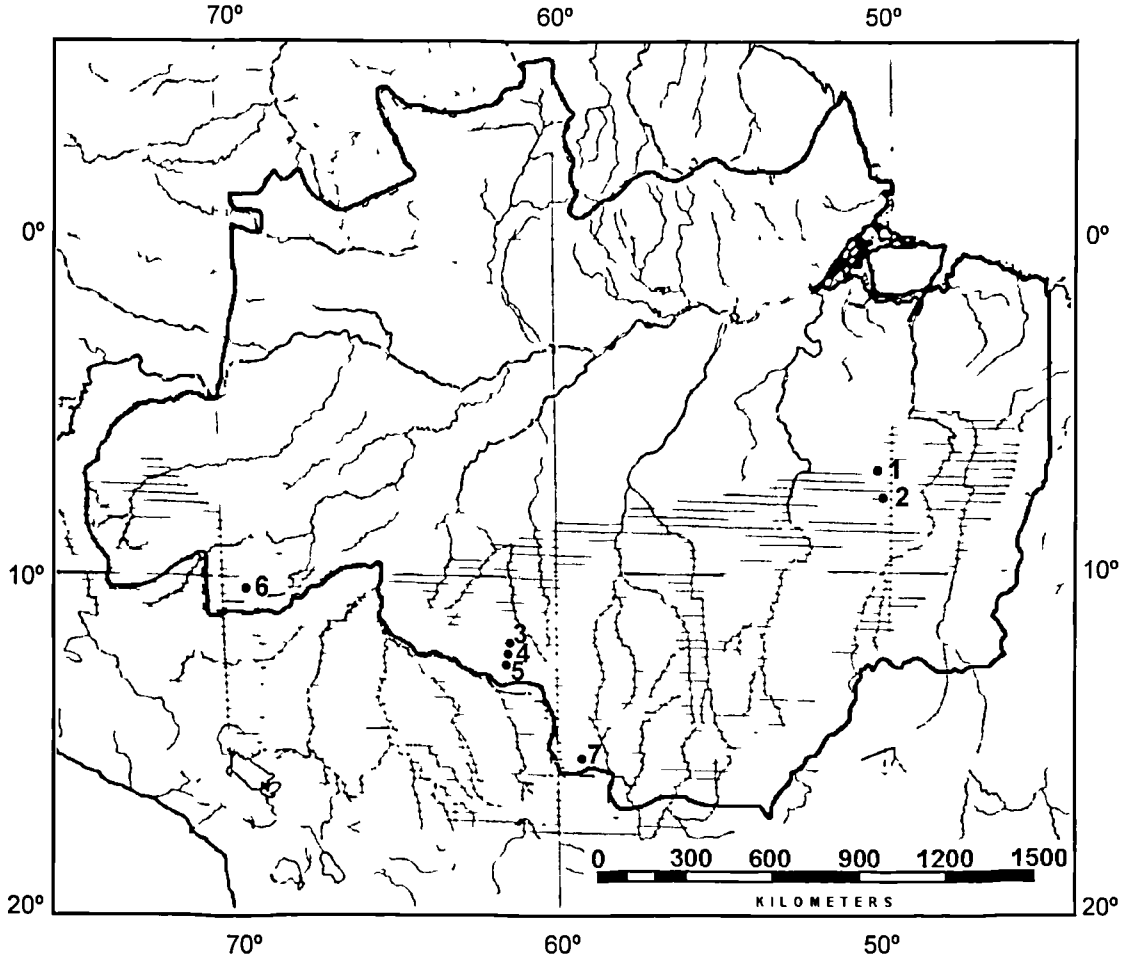


Figure 3.1 - Range of *Swietenia macrophylla* in South America and the locations of the seven populations sampled: (1) Água Azul, (2) Marajoara, (3) Pimenta Bueno, (4) Cachoeira Parecis A, (5) Cachoeira Parecis E, (6) Chico Mendes, and (7) Pontes e Lacerda

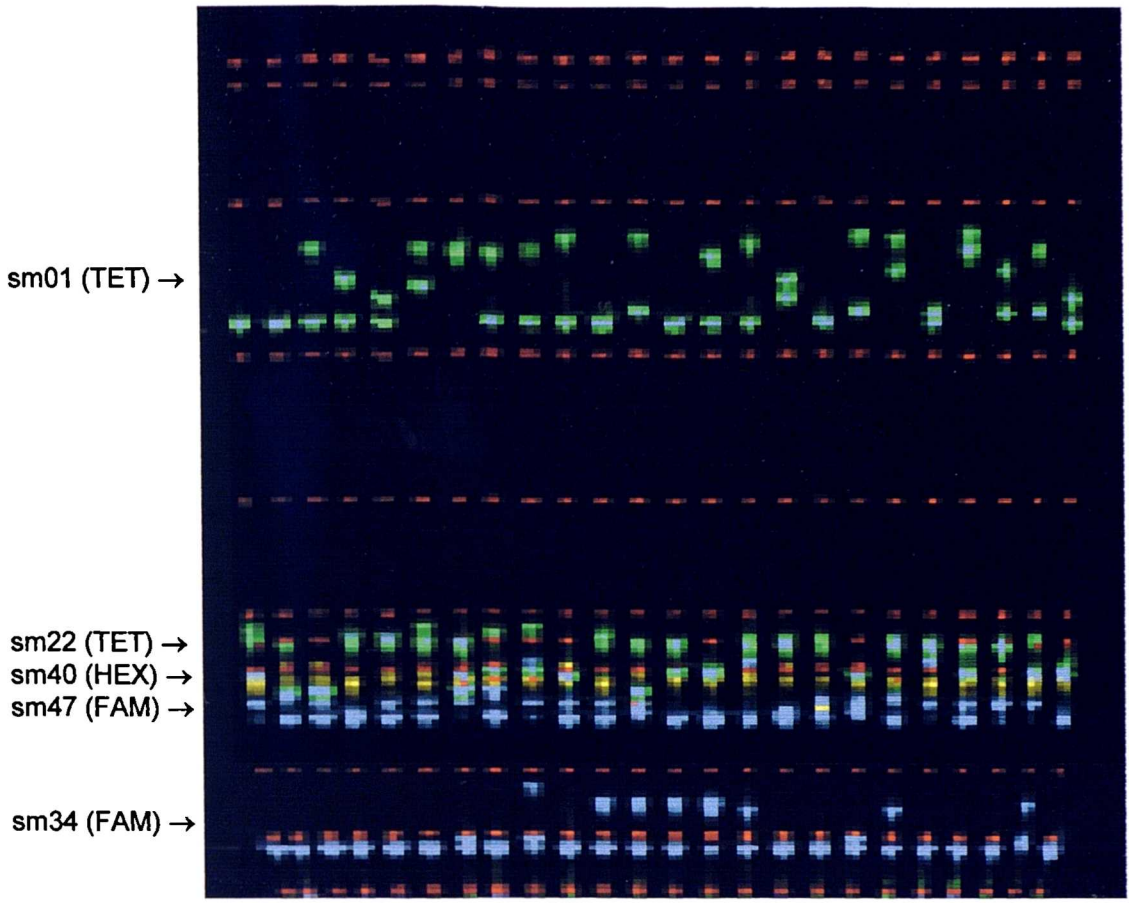


Figure 3.2 - Polyacrylamide gel image showing resolution of 5 SSR loci in multiplex fluorescence-based system for 24 individuals of *S. macrophylla* in population Cach.E, Rondônia, Brazil. Locus identification and dye label are specified on the left. Red fluorescence corresponds to Genescan 500 Rox internal size standard.

Chapter 4

Mating System, Genetic Diversity, and Spatial Genetic Structure in a Population of *Swietenia macrophylla* in the Brazilian Amazon.

INTRODUCTION

The mating system determines how the transition of genotypic frequencies from one generation to the next occurs in a population (Ritland, 1988). The classification of the mating system in plants is based on the genetic relatedness between the male and female gametes (derived from the pollen and the ovule respectively) in the newly formed zygotes (Ritland, 1983), and it can be measured as the proportion of mature seeds resulting from selfing or outcrossing events (Murawski & Hamrick, 1991). Mating system is an important factor determining how genetic variation is distributed among subdivisions of a population and among individuals in those subpopulations, as well as among progenies and among individuals within progenies, with a direct impact on the long-term maintenance of genetic variation in existing populations. The susceptibility of a population to the loss of genetic diversity and inbreeding depression

following logging or habitat fragmentation depends, to a considerable extent, on the characteristics of its mating system.

The pattern of mating in tropical trees is probably very complex, reflecting the influence of several genetic and ecological factors such as the demography of adult trees, flowering intensity, synchronism of flowering phenology among and within individuals, nature and intensity of the self-incompatibility mechanisms, foraging behaviour of pollinators, and selective abortion of young fruits. Although many plant populations and species appear to produce mixtures of selfing and outcrossing progeny (Barrett & Husband, 1990), most tropical trees exhibit morphological and physiological adaptations which promote outcrossing and prevent or reduce selfing (Bawa, 1990). As a result, the mating system of the majority of tropical trees investigated so far using allozymes showed predominant or complete outcrossing (O'Malley & Bawa, 1987; O'Malley *et al.*, 1988; Murawski *et al.*, 1990; 1994b; 1994b; Murawski & Hamrick, 1991; 1992a; 1992b; Eguiarte *et al.*, 1992; Alvarez-Buylla & Garay, 1994; Hall *et al.*, 1994b; 1996; Boshier *et al.*, 1995b; Doligez & Joly, 1997; James *et al.*,1998; Loveless *et al.*, 1998). Additionally, many studies have shown that the inbreeding of biparental origin is very low or undetectable among tropical trees (Eguiarte *et al.*, 1992; Murawski & Hamrick, 1992a; 1992b; Hall *et al.*, 1994b; Boshier *et al.*, 1995b; Doligez & Joly, 1997; James *et al.*,

1998; Loveless *et al.*, 1998). These findings contrast with previous ideas stating that autogamy and inbreeding would be favoured in the widely spaced conspecific tropical forest trees (Fedorov; 1966). Based on data available so far, only species belonging to the guild of the fast growing, gap-colonizer tropical trees, with light-requiring juveniles and adults persistent in the mature forest, as the bombacaceous *Ceiba pentandra* and *Cavanillesia platanifolia* and the dipterocarp *Shorea trapezifolia*, exhibit a mixed mating system (Murawski & Hamrick; 1991; 1992a; 1992b; Murawski *et al.*, 1994).

Swietenia macrophylla is the most valuable hardwood species in the Neotropics. The conservation status of this species has been the subject of increasing concern due to the over-exploitation by selective logging and increasing habitat destruction. Despite the high socio-economic importance of *S. macrophylla*, information on the processes maintaining genetic variation in natural populations of this species is still scarce. In this study, the outcrossing rate and other mating system parameters were estimated for a population of *S. macrophylla* in the Brazilian Amazon using ten highly polymorphic microsatellite loci. The main aim is to understand how the mating system may be contributing to shape and maintain the genetic structure of the population and to infer how the logging and habitat fragmentation may affect the reproduction of remaining individuals and the recovering capacity of the population. These findings,

coupled with information on pollination and seed dispersal mechanism, may have important implications for the long term conservation and management of this valuable hardwood species.

MATERIALS AND METHODS

Study site

Field work was conducted in the Marajoara Management Project (*ca.* 07° 50'S, 50° 16'W) in the south of Pará State, Brazil. The management area of the project has around 4,100 ha (13 km long by 3.15 km wide) of selectively logged forest sub-divided into 13 plots with approximately 340 ha (1.08 km by 3.15 km each). Genetic samples for the present study were only collected from trees in plots 1, 2, and 3, at the eastern half of the project's area. The western half of the project's area was logged for mahogany in 1985, at an unknown intensity. Plots 1, 2, and 3, were selectively logged between 1992-1994 by the SEMASA logging company, which harvested 268 mahogany stems, leaving at least 108 standing stems as seed trees (J. Grogan, personal communication). Therefore, overall stand density in these three plots was reduced from approximately one tree per 2.7 ha (0.37/ha) to one tree per 9.4 ha (0.11/ha). At the study area, *S. macrophylla* is nearly always found at the greatest densities on low ground, flat areas with poorly drained soils, or along the banks of

small streams, sharing this habitat with the palm species *Orbignya phalerata* and *Maximiliana maripa* (J. Grogan, personal communication; M. R. Lemes, personal observation). Mahogany is not usually found on the denser and taller up-slope or mid-slope forest communities.

Spatial genetic structure

Twenty-two mapped adult trees on plots 2 and 3 were used to analyse the spatial genetic structure within the adult population. Leaves were collected and genomic DNA extracted following standard CTAB procedure (Doyle & Doyle, 1987). All trees were genotyped at ten microsatellite loci combined in multiplexed fluorescence-based systems developed for *S. macrophylla* (Chapter 2). PCR conditions were as described in Chapter 2. PCR products were electrophoresed in 5% polyacrylamide gel in a ABI Prism 377 sequencer and analysed using Genescan and Genotyper programs (ABI, 1993; 1994).

Mating system analysis

Seeds and leaves were collected from 25 adult trees in the study area. Seeds from a given family were sampled from the same capsule and germinated in a greenhouse. For the polymorphism assay and estimates of mating system parameters, genomic DNA was extracted from the leaves of 400 seedlings of 25 open-pollinated

families (16 seedlings/family) and the 25 maternal trees using conventional CTAB procedure (Doyle & Doyle, 1987). All seedlings and mother trees were genotyped at ten microsatellite loci as described above.

Data analysis

The occurrence of spatial genetic structuring was tested by a pairwise correlation analysis between the spatial distance and the number of alleles in common among the trees. A given pair of trees could share 0, 1 or 2 alleles per locus, or 0 to 20 when considering the 10 SSR loci together. Thus, a positive correlation coefficient would be expected if the related individuals (i.e., trees bearing a higher number of alleles in common) are spatially closer than unrelated ones.

The inbreeding coefficient (f) for each locus for the parent and seedling generations was calculated by the formula $f = 1 - (H_o/H_e)$, where H_o is the observed proportion of heterozygotes and H_e is the expected heterozygosity in Hardy-Weinberg equilibrium. Deviations (excess or deficiency) of heterozygotes from the Hardy-Weinberg proportions were tested for adults and seedlings by calculating the U-test (Raymond & Rousset, 1998) and the inbreeding coefficient (Weir & Cockerham, 1984) using Genepop version 3.1.b program (Raymond

& Rousset, 1998). Estimation of exact-P values was determined by the Markov chain method (Guo & Thompson, 1992).

Mating system analysis was based on the mixed mating model of Ritland & Jain (1981) by using the multilocus mating system program MLTR (Ritland, 1996). The MLTR program only allows the analysis of up to eight alleles. When the number of alleles exceeded eight, the alleles were sorted by frequency and the following criteria were applied to pool the alleles into the eight allelic classes allowed by MLTR: allelic classes from 1 to 6 were represented respectively by the alleles with 1st to 6th higher frequencies in the locus; allelic class 7 by the 7th and 8th alleles pooled, and allelic class 8 by the remaining alleles pooled.

MLTR estimation method is based upon the maximum-likelihood procedure (Ritland & Jain, 1981; Ritland & El-Kassaby, 1988). The expectation-maximization method was used for maximizing the likelihood equation for both population and individual family estimates. The MLTR program estimated the following mating system parameters: multilocus outcrossing rate (t_m), minimum variance single locus outcrossing rate (t_s), average single locus inbreeding coefficient of maternal parents (f), correlation of outcrossing rate within progeny arrays (r_t), correlation of outcrossed paternity within progeny arrays (r_p), and gene frequencies of the pollen and ovule pools (p). The correlated mating system estimates (r_p and r_t) were based on the progeny-pair model (Ritland, 1989). The variances were estimated

based on 1000 bootstraps re-sampled within progeny arrays, in the case of population estimates, and individuals within progeny arrays, in the case of individual family estimation.

The chi-square test for goodness of fit of progeny frequencies to expectations proposed by Ritland (1983), present in MLT, was omitted from MLTR due to the ambiguous results generated (MLTR readme file, Ritland, 1996). If a high number of polymorphic loci are used in the analysis, like the present study, the multilocus estimates tend to be robust and the violations of the assumptions of the mixed-mating system model have a minor impact in the estimates of the mating system parameters.

The detected outcrossing rate (t_d) estimate for a given fruit was obtained by dividing the number of detected ('unambiguous') outcrossing progenies by the total number of progeny genotypes in that fruit. The presence of an allele in a progeny genotype that was not present in the maternal tree indicated that this progeny unambiguously resulted from an outcross event (Shaw *et al.* 1981).

RESULTS

Genetic variation in adults and seeds

Nearly all 25 adult trees and 400 offspring were genotyped for the 10 SSR loci developed *for S. macrophylla.* All offspring individuals

displayed at least one maternal allele, suggesting Mendelian inheritance and showing no evidence of null alleles in this population. The pattern of segregation in the open-pollinated progeny of the tree 66 (mother tree and six sibs) at locus sm01 is shown in Fig. 4.1.

Data on number of alleles, heterozygosity and inbreeding coefficient per locus for the adult and seedling cohorts are shown in Tab. 4.1. The number of alleles per locus ranged from 4 to 13 (average 9.4) among the seed parents and from 8 to 21 (average 15.3) among the seedlings. Heterozygozity, or the expected proportion of heterozygous individuals under the Hardy-Weinberg equilibrium, was very similar ($H \cong 0.80$) in the adult and offspring stages. No excess of heterozygotes (i.e., f significantly negative) was found among seedlings or adults for any of the 10 loci. The inbreeding coefficient of nine out of 10 loci were significantly positive for seedlings, suggesting an excess of homozygous individuals at this stage, whereas only three loci showed significant positive departures from the Hardy-Weinberg proportions, for adults. Over all loci, the inbreeding coefficient was significantly positive for adults and seedlings, pointing out an overall heterozygote deficiency for both stages if the sampled population is considered to be a single panmictic unit. The inbreeding coefficient in seedlings was slightly higher than in adults ($f = 0.109$ and 0.076

respectively) suggesting no (or weak) selection favouring heterozygotes from seed to the adult stage.

Spatial genetic structure

Spatial distance and number of alleles in common among the 22 trees (231 pair combinations) had a range of 29-2,889 m and 0-10 alleles respectively (Fig. 4.2). No significant correlation was found between distance and number of alleles in common ($r = -0.0115$, $P = 0.862$) indicating that the spatial distribution of adult genotypes could not be distinguished from a random one at least on the scale of the sampled area. Even pairs of trees more highly related genetically (*e.g.* those trees exhibiting 8 to 10 alleles in common) were not systematically associated with short distance between them.

Mating system

Estimates of multilocus (t_m) and single locus (t_s) outcrossing rates using 10 SSR loci (Tab. 4.2) suggest that *S. macrophylla* is a predominantly outcrossed species ($t_m = 0.958 \pm 0.025$; mean $t_s = 0.819 \pm 0.025$). The values of t_s for each locus were consistently lower than t_m, except for the locus sm31. The large difference between the multilocus and single locus estimates ($t_m - t_s = 0.139 \pm 0.029$) provides evidence that, in spite of the high outcrossing rate, a considerable inbreeding of biparental origin (caused by correlated or 'consanguineous' matings)

contributed to the genetic structure of this population. The correlation of outcrossed paternity within the progeny arrays was relatively high and had low variance (multilocus r_p = 0.555, range 0.428-0.660), indicating that the probability of a randomly chosen pair of progeny from the same capsule being full sibs is slightly higher than 50%.

Multilocus outcrossing rates for individual trees varied from 0.38 to 1.00 (Tab. 4.3). The inbreeding in the population was concentrated mainly in two trees (trees 3 and 18, t_m = 0.68 and 0.38 respectively) whereas the remaining trees were highly or totally outcrossed. Genotypic data analysis from the progeny arrays found that 377 seedlings (94% of the total) unambiguously resulted from outcrossed matings, exhibiting at least one locus with a different allele from those present in the maternal parent. The remaining 23 seedlings had genotypes consistent with a self-fertilization origin, but may also result from outcrossing to an individual having a similar genotype (cryptic outcrossing). The detected outcrossing in maternal trees 3 and 18 was also relatively low (t_d = 0.44 and 0.38 respectively). The mean family estimate of detected outcrossings (t_d = 0.94) was similar to the value obtained for the population multilocus estimate (t_m = 0.958). Values of t_d and t_m family estimates were strongly correlated (r = 0.979, P = 0.0002, N = 25). Apomixis was ruled out in this population of *S. macrophylla* since none of the 400 seedlings had multilocus genotype identical to its mother tree.

DISCUSSION

Genetic diversity in adults and seeds

An excess of heterozygotes has been commonly reported for natural populations of temperate trees (Bush & Smouse, 1992) and for some tropical trees (Eguiarte *et al.,* 1991; Doligez & Joly, 1996), whereas a significant excess of homozygotes among adult trees has been rarely found (but see White *et al.,* 1999; Gibson & Wheelwright, 1995). The excess of homozygous seeds in *S. macrophylla* for a high proportion of the loci, despite a high outcrossing rate, is likely to have arisen via self-fertilization in a few individuals and/or consanguineous matings (see below). This homozygote excess suggests some level of self-compatibility and/or weak selection against the homozygous zygote genotypes. The slightly higher inbreeding coefficient in seeds than in adults could also be a reflection of an increase in the selfing rate produced in the population subsequent to logging, but further evidence is necessary for this conclusion, since no pre-logging estimate is available.

The inbreeding coefficient found in the adult cohort (f = 0.076) is considerably higher than that expected if the inbreeding was only due to selfing (f_{eq} = 1 - t / 1 + t = 0.021), suggesting that population has not reached inbreeding equilibrium. The high proportion of

homozygous genotypes in the adult stage, although less than in the seed generation, also suggests that selection against inbred seedlings and juveniles, if any, seems not to be strong enough to totally overcome the excessive production of homozygous seeds by maternal trees. The Wahlund effect, which is a sampling error showing deficiency of heterozygotes relative to the Hardy-Weinberg expectations owing to undetected population subdivision, seems not to be the main reason for the heterozygote deficit, since no spatial genetic structuring was found on the scale of the area studied. The presence of null alleles has been detected in microsatellite markers and has also been cited as a possible cause for the deficiency of heterozygotes (Jarne & Lagoda, 1996). As no mismatches between progeny and maternal tree were detected for the 10 microsatellite loci assayed among the 25 families, it is unlikely that null alleles had any influence in the estimate of the proportion of homozygotes in the Marajoara population.

The clumped distribution of mahogany trees probably reflects the species adaptation for colonizing new open areas resulting from large hydrological disturbances, fires, or blow-downs (Gullison *et al.*, 1996). Mahogany populations established in new areas are likely to have experienced demographic bottlenecks due to the putative small number of founders. Previous theories predict that after demographic bottlenecks and/or prolonged inbreeding, most of the recessive

deleterious mutations would be exposed to selection and purged from the inbred populations, reducing the genetic load (Lande & Schemske, 1985; Uyenoyama, 1986). Therefore, a plausible explanation for the current excess of homozygous trees and the putative weak selection against homozygotes observed for this population of *S. macrophylla* may be that individuals with lower levels of recessive detrimental or lethal mutations (consequently, with more tolerance for selfing) had adaptive advantages during the sporadic colonization events.

It is noteworthy that other species of mahogany from Central America, *S. humilis*, with similar life history traits, also exhibited a significant deficit of heterozygotes among adult trees (White *et al.*, 1999). *Ocotea tenera* (Lauraceae), a gap colonist tree, also exhibited a heterozygote deficiency in Costa Rica (Gibson & Wheelwright, 1995). In contrast, populations of two other Meliaceae species, *Carapa procera and C. guianensis*, with distinct life history traits (animal-dispersed seeds, shade tolerant juveniles with mid-story regeneration) showed no departure from Hardy-Weinberg genotypic proportion (Hall *et al.*, 1994b; Doligez & Joly, 1996; Dayanandan *et al.*, 1999).

Spatial distribution of genotypes

Based on the relatively short distance pollination and seed dispersal mechanisms one would predict that some spatial pattern could be found in the distribution of maternal tree genotypes, as a

result of a within-population isolation by distance process. Several other factors which are not mutually exclusive may explain the lack of genetic structure found among adults of *S. macrophylla* in the Marajoara population, such as: (1) the predominance of outcrossing in most trees combined with an asynchrony between male and female flower maturation and among individual flowering periods are likely to allow mating between trees from different neighbouring areas; (2) dispersal of small pollinator insects can be wind-mediated (Nason *et al.*, 1996; Byrne *et al.*, 1988) promoting pollen flow at distances long enough to blur the structure existing in the founding population after a few generations; (3) distances of the established saplings and juveniles from the maternal tree may be larger than those experimentally detected by Gullison *et al.* (1996) for seed dispersal (mean of 32-36m), due to factors such as lower availiability of suitable sites or higher seed predation in the vicinity of the fruiting adult, contributing to the erosion of the original spatial structure; (4) multiple founder or distinct colonization events could shape complex genetic structures which are currently indistinguishable from a random pattern.

On the other hand, it is also possible that some weak spatial structure was present at the Marajoara adult population, but it could not be detected because of the small number of genotyped trees (around 6% of the trees originally present in the stand) used in the

pairwise analysis. Thus, further work genotyping a higher proportion of trees in the stand would be necessary to produce a finer scale view of the genotypic distribution in this population. Using less polymorphic isozyme markers, Doligez & Joly (1996) also found no spatial pattern in established individuals of another Meliaceae species, *Carapa procera*, in French Guiana. Similarly, Hamrick *et al.* (1993) did not find any clear pattern of genotypic distribution in adults of *Platypodium elegans* and *Alseis blackiana*, both species, like *S. macrophylla*, pollinated by small insects and wind-dispersed. In contrast, other neotropical tree species such as *Cordia alliodora* (Boshier *et al.*, 1995b), *Ocotea tenera* (Gibson & Wheelwright, 1995), and *Swartzia simplex* (Hamrick *et al.*, 1993) exhibited significant spatial genetic structure in adults. In sum, it is still difficult to predict which and how ecological and historical factors determine the degree of genetic structure and shape the genetic architecture within adult populations of tropical trees.

Mating system

There is no other study on the mating system of tropical trees using microsatellite markers to make comparisons with the data presented here for *S. macrophylla*. The high outcrossing estimate for 23 out of 25 trees points out that most individuals of *S. macrophylla* in the Marajoara population are completely or predominantly

outcrossed. High estimates of outcrossing rate have also been reported for the majority of the rain forest tree species in the neotropics investigated so far using isozyme markers (O'Malley & Bawa, 1987; O'Malley *et al.*, 1988; Murawski *et al.*, 1990; Murawski & Hamrick, 1991; Eguiarte *et al.*, 1992; Alvarez-Buylla & Garay, 1994; Hall *et al.*, 1994b; Boshier *et al.*, 1995b; Doligez & Joly, 1997; James *et al.* 1998; Loveless *et al.*, 1998). The multilocus outcrossing estimate found here for *S. macrophylla* using microsatellites (t_m = 0.958) was similar to the ones recorded for the Meliaceae species *Cedrela odorata* (t_m = 0.969) and *Carapa guianensis* (t_m = 0.967 and 0.986) in Costa Rica (James *et al.*, 1998; Hall *et al.*, 1994b), but considerably higher than the one found for *Carapa procera* (t_m = 0.78) in French Guiana (Doligez & Joly, 1997), using isozymes.

Multilocus outcrossing rates (t_m) estimated among families were not evenly distributed among maternal trees. It was as high as 1.0 or as low as 0.38, implying that the degree of self-incompatibility, or the level of dichogamy, may be variable among individuals. A wide range in individual outcrossing rates have also been observed in other neotropical tree species, such as *Ceiba pentandra* and *Cavallinesia platanifolia* (Murawski & Hamrick, 1992a; 1992b; Murawski *et al.*, 1990). These two bombacaceous species are, like *S. macrophylla*, early successional trees that colonize large gaps, with light-requiring juveniles and the long life cycle individuals persisting in the mature

forest as emergent trees. The ability to reproduce by selfing may be advantageous for tree species that disperse seeds into spatially and temporally unpredictable environments (Murawski *et al.*, 1994) such as open areas formed by tree-fall gaps, blow-downs or landslides. Data on the mating system of *S. macrophylla, C. pentandra,* and *C. platanifolia* suggest that tropical trees belonging to this ecological class probably have plastic mating systems which permit that even single individuals will eventually set seeds when colonizing large gaps.

Evidence of inbred matings in the Marajoara population was given by the significant excess of homozygote seeds (f = 0.11) and by the considerable biparental inbreeding denoted by the difference between the multilocus and the mean singlelocus estimate of outcrossing rate ($t_m - t_s = 0.14$). Both mating parameters suggest that gametes are not uniting totally at random from adults to seed generations probably due to factors such as self-fertilization and/or 'consanguineous' mating (Ritland & Jain, 1981).

Given that *S. macrophylla* is a monoecious species, self-pollination is potentially possible. Monoecism enhances outbreeding but does not necessarily ensure outbreeding as effectively as dioecism or as an efficient self-incompatibility mechanism. Although temporal separation between maturation of male and female flowers has been cited in the Meliaceae at inflorescence level (Styles, 1971), it is

probable that some overlapping of flowering times of the two sexes may occur on individual trees, allowing self-pollination. The relatively high biparental inbreeding denoted by the t_m - t_s estimates is an intriguing point, since no spatial genetic structure among adults was found within the population. It could, however, be explained if the assortative mating would instead reflect the existence of a higher overlapping in the flowering phenology between genetically related individuals, as suggested for *Carapa procera* (Doligez & Joly, 1996).

The genetic relatedness between pairs of progeny sampled from the same capsule was strongly positive and did not differ significantly among capsules (correlation of outcrossing paternity r_p = 0.555, ranging from 0.428 to 0.660), suggesting that pollen loads were composed of pollen from a small number of sources. In consequence, individual flowers were fertilized by few, probably from one to three, pollen donors. A decrease in r_p, however, would be expected if the sib-pairs were sampled from different capsules on the same tree, since these capsules were likely to be derived from flowers pollinated on different days.

Measures of outcrossing based on the number of detected outcrossers provide a reliable and useful indication of the minimum number of outcrossed progeny (Shaw *et al.*, 1981; Brown *et al.*, 1985), despite the simplistic approach. When a large number of highly polymorphic loci are used, as in the current study of *S.*

macrophylla, it is likely that the detected outcrossing represents a close estimate of the actual outcrossing. Owing to the high exclusion probability (Weir, 1996) of the ten microsatellite loci used here (P > 0.9999), the fraction of undetected (cryptic) outcross progeny was probably very low. This is likely to be the main reason for the strong correlation found between t_m and t_d among families. Consequently, the estimated selfing rate ($s = 1 - t_m = 4.2\%$) in this study probably represents principally matings resulting from self-fertilization. The unique family discrepancy found, the considerably higher t_m than t_d in tree 3 (0.63 *versus* 0.44), was probably caused by the high allele frequencies of this maternal tree in the pollen pool, which inflate the estimates of the expected proportion of undetected outcross events (Cruzan *et al.* 1994; Brown *et al.*, 1985; Shaw *et al.*, 1981).

Overall, this population of *S. macrophylla* seems to have adaptations that preferentially produce outcrossed progeny but also allow for selfing. Around 80% of maternal trees had 100% of the seeds in the sampled fruit resulting from outcrossing. Despite the high outcrossing, the potential for inbreeding in this population was not negligible. For some families the estimated and detected outcrossing rate was lower, suggesting the occurrence of variable degrees of self-incompatibility or different levels of dichogamy among individuals. In the Marajoara population, around 4-6 % of the seeds probably originated by self-fertilization, whereas the difference

between t_m and t_s suggested that nearly 14% were derived from biparental inbreeding due to correlated or 'consanguineous' mating. The origin of the biparental inbreeding exhibited by the trees in the Marajoara population is still unclear since no spatial genetic structuring was found among adults. A possible explanation may be a temporal genetic structuring caused by a higher flowering synchrony among genetically related trees, but further studies comparing flowering phenologies and demographic genetics are necessary to test for this hypothesis.

The findings described above on the mating system of *S. macrophylla* have some consequences for its conservation biology. Owing to its generalist pollination system and some level of tolerance for selfing, *S. macrophylla* seems to be very resilient to environmental disturbances, setting fruits and seeds even at low densities of adult trees. Although the effect of logging on the seed output is still an unanswered question, the remaining trees in the Marajoara population have maintained their capacity of annually setting fruits with predominantly outcrossed seeds, even in an intensively logged area, where tree density dropped to less than 30% of the one originally found. In fact, fruit- and seed-setting in *S. macrophylla* has been observed in anthropic landscapes located outside of its distribution range, as in the urban areas of Brasília, in Central

Brazil, and Manaus, in Central Amazon (M. R. Lemes, pers. observation).

Despite the observed high genetic diversity within populations, this species seems to have the ability to cope with some levels of inbreeding, as suggested by the excess of homozygotes found among adult trees and by the significant proportion of selfed offsprings in some progeny arrays. These traits, likely reflecting the gap-colonist life history of *S. macrophylla*, suggest that population reductions due to logging and fragmentation may not seriously affect the reproductive potential of all remaining individuals, as occurs with most tropical rain forest tree species, which probably kept high genetic loads (Alvarez-Buylla *et al.*, 1996). Moreover, since the pollen and seed vectors of *S. macrophylla*, widespread small generalist insects and wind respectively, are most likely not affected by logging activities, the pollination and dispersal capabilities persist even in these already exploited, disturbed areas.

In conclusion, many of the remaining trees in logged areas and relict fragments may persist as viable individuals and would be very important in the future for long term population recovery and genetic conservation programmes. Therefore, intensive logging followed by the replacement of the forest habitat by pastures and extensive plantations, as has been occurring at alarming rates throughout the Brazilian Amazon, seems to be the main short term threat for the

maintenance of *S. macrophylla* populations. On the other hand, low intensive logging management projects, leaving a proportion of reproductive trees, followed by economic activities which conserve the remaining arboreal covering (e. g. extraction of non-timber forest products, agroforestry) may represent the best strategy for combining conservation and economic usage of the Brazilian mahogany in areas with increasing human pressure. Additionally, initiatives for the establishment of new public and private reserves and protected areas are urgently necessary for the genetic conservation of the Brazilian mahogany and of many other highly threatened valuable tropical tree species.

Table 4.1 - Heterozygosity (H) and coefficient of inbreeding (f) of the adult trees and offsprings for ten microsatellite loci of *Swietenia macrophylla* at Marajoara area.

Locus	Generation	N	No. alleles	H	f
sm01	parents	25	13	0.904	-0.018^{ns}
	offspring	393	17	0.903	0.079**
sm22	parents	25	9	0.826	0.131^{ns}
	offspring	398	17	0.724	0.120*
sm31	parents	25	13	0.914	0.083*
	offspring	398	19	0.911	0.018***
sm32	parents	25	10	0.899	0.021^{ns}
	offspring	396	14	0.892	0.071**
sm34	parents	25	10	0.775	0.333**
	offspring	387	21	0.782	0.435***
sm40	parents	25	10	0.749	-0.125^{ns}
	offspring	394	14	0.786	0.099***
sm45	parents	24	10	0.881	-0.090^{ns}
	offspring	393	18	0.896	-0.011^{ns}
sm46	parents	25	8	0.834	0.091^{ns}
	offspring	392	11	0.823	0.095*
sm47	parents	25	4	0.494	0.275***
	offspring	393	8	0.465	0.200***
sm51	parents	25	7	0.722	0.171^{ns}
	offspring	396	14	0.723	0.064***
Over all loci	parents		9.4	0.799	0.076***
	offspring		15.3	0.800	0.109***

Significance levels as: ^{ns} $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 4.2 - Singlelocus (t_s) and multilocus (t_m) outcrossing rate estimates, index of correlated matings (r_p), and maximum likelihood estimates of ovule and pollen frequency of the most common allele of *S. macrophylla* in Marajoara area. Standard deviations are shown in parentheses.

Locus	t_s	r_p	ovule	pollen
sm01	0.876 (0.076)	0.640 (0.094)	0.200 (0.065)	0.161 (0.049)
sm22	0.721 (0.077)	0.449 (0.085)	0.220 (0.060)	0.291 (0.060)
sm31	0.982 (0.026)	0.428 (0.067)	0.220 (0.052)	0.229 (0.042)
sm32	0.822 (0.065)	0.499 (0.081)	0.180 (0.051)	0.077 (0.027)
sm34	0.291 (0.057)	0.660 (0.093)	0.320 (0.083)	0.086 (0.068)
sm40	0.724 (0.105)	0.618 (0.135)	0.400 (0.060)	0.327 (0.066)
sm45	0.908 (0.053)	0.475 (0.083)	0.240 (0.047)	0.055 (0.021)
sm46	0.852 (0.080)	0.579 (0.077)	0.280 (0.074)	0.233 (0.060)
sm47	0.537 (0.183)	0.515 (0.079)	0.667 (0.050)	0.697 (0.142)
sm51	0.891 (0.069)	0.492 (0.121)	0.373 (0.073)	0.433 (0.055)
average	t_s = 0.819 (0.046)			
multilocus	t_m = 0.958 (0.025)	r_p = 0.555 (0.050)		
	$t_m - t_s$ = 0.139 (0.029)			

Table 4.3 - Number of unambiguous outcrossed seedlings and estimates of outcrossing rate (t_m) for 25 families of *Swietenia macrophylla* in the Marajoara area.

Tree	No. of seedlings	No. of unambiguous crossed seedlings (%)	Outcrossing rate (± SE)
1	16	16 (100)	1.00 (0.00)
2	16	16 (100)	1.00 (0.00)
3	16	7 (43.75)	0.63 (0.13)
4	16	16 (100)	1.00 (0.00)
5	16	16 (100)	1.00 (0.00)
6	16	15 (93.75)	0.94 (0.06)
7	16	16 (100)	1.00 (0.00)
8	16	16 (100)	1.00 (0.00)
9	16	16 (100)	1.00 (0.00)
10	16	16 (100)	1.00 (0.00)
11	16	16 (100)	1.00 (0.00)
12	16	16 (100)	1.00 (0.00)
13	16	16 (100)	1.00 (0.00)
14	16	16 (100)	1.00 (0.00)
15	16	14 (87.50)	0.88 (0.09)
16	16	16 (100)	1.00 (0.00)
17	16	16 (100)	1.00 (0.00)
18	16	6 (37.50)	0.38 (0.13)
19	16	16 (100)	1.00 (0.00)
20	16	16 (100)	1.00 (0.00)
21	16	15 (93.75)	0.94 (0.06)
22	16	16 (100)	1.00 (0.00)
23	16	16 (100)	1.00 (0.00)
24	16	16 (100)	1.00 (0.00)
25	16	16 (100)	1.00 (0.00)

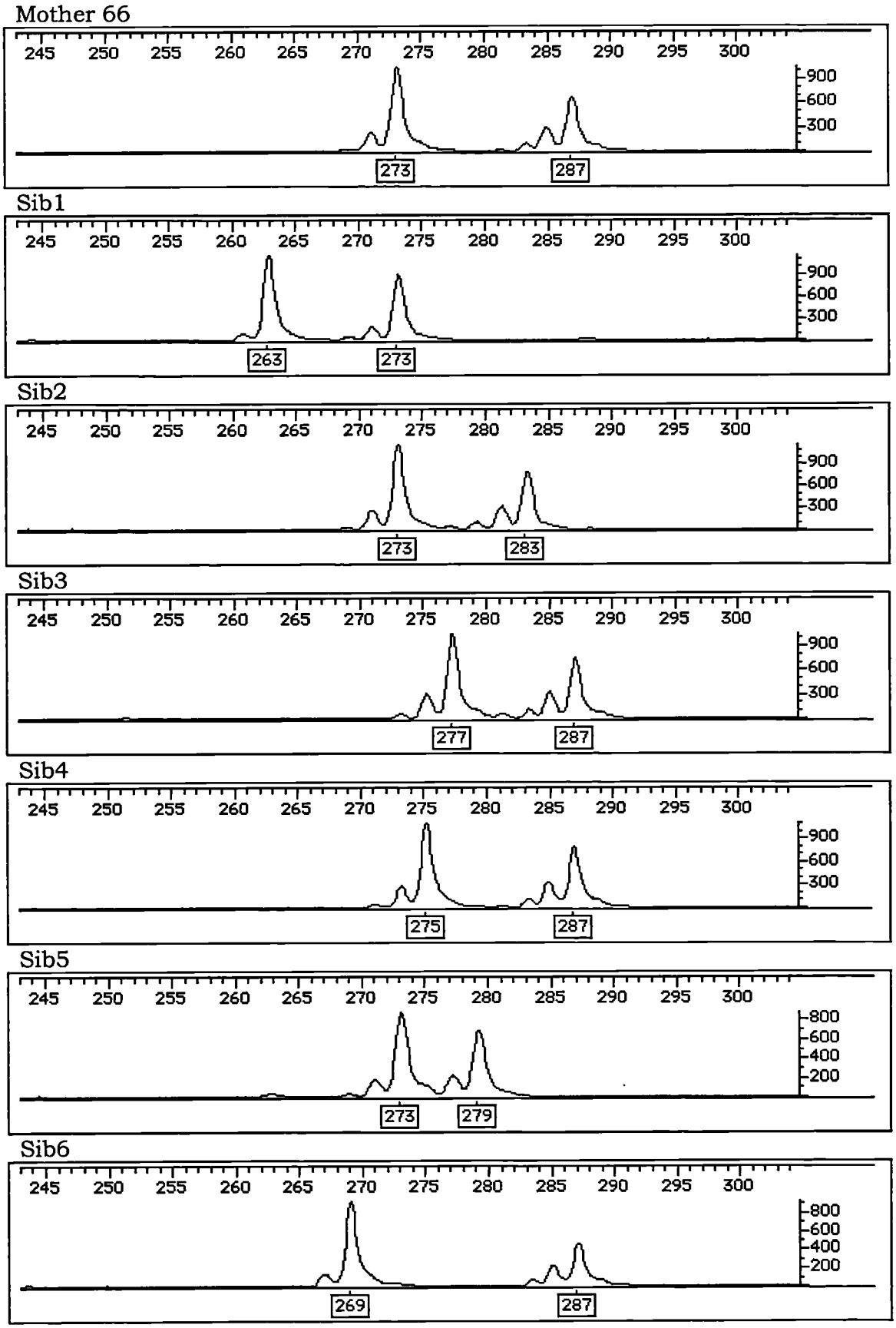


Figure 4.1 - Electropherograms showing pattern of segregation in a open-pollinated progeny of the tree 66 (mother and 6 sibs) using fluorescence-based microsatellite marker sm01(TET). The scale at the top of each panel indicates fragment sizes in base pairs. The scale at the right indicates fluorescence intensities of the peaks.

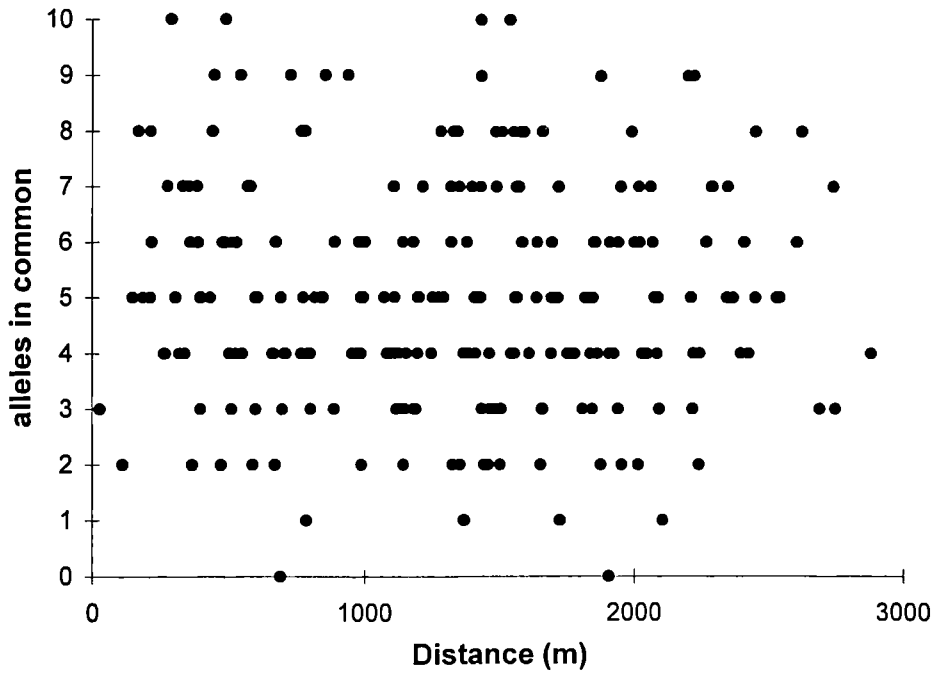


Figure 4.2 - Number of alleles in common for 10 microsatellite loci related to distance between pairs of *Swietenia macrophylla* trees at the Marajoara population. N= 22 trees.

Chapter 5

Concluding Remarks

1. This study revealed the presence of (AG) microsatellite loci in the *Swietenia macrophylla* genome. The set of ten highly polymorphic microsatellite markers developed and characterized for *S. macrophylla* constitutes a very useful tool for the understanding of the levels and distribution of genetic variation which is crucial for effective conservation genetics programmes and management of natural populations of this species.
2. Using an enriched genomic library and anchored-PCR screening prior to sequencing, the efficiency of microsatellite marker locus development for *S. macrophylla*, from data sequencing to operationally useful SSR loci, was 29%, a high value compared to other tropical tree studies.
3. The three high throughput semi-automated multilocus genotyping systems based on fluorescent labelled multiplexed microsatellite loci developed for this species allowed a large-scale analysis of natural populations. The number of alleles per locus ranged from 11 to 25 with a mean value of 15.7 and expected heterozygosity varied from 0.72 to 0.91 (mean = 0.84). These values are amongst the highest ones found for tropical tree species.

4. The probability of genetic identity over all loci was 7×10^{-15} and the combined probability of paternity exclusion was 0.999998, indicating the high discriminating power of these genotyping systems for genetic relatedness studies. All microsatellite loci showed Mendelian inheritance and segregation in open-pollinated families.
5. The multilocus values of genetic differentiation (θ = 0.12 and ρ = 0.14, $P<0.0001$) indicate a moderate but significant degree of differentiation among populations of *S. macrophylla* in the Brazilian Amazon, which is in accordance with the tendency observed for most tropical tree species. Since *S. macrophylla* is a gap-colonist tree, the level of population differentiation would be inflated by historical factors which enhance inbreeding and drift such as population bottlenecks and founder effects. The short distance pollination and seed dispersal systems probably also have contributed to the genetic isolation and differentiation among populations.
6. The inbreeding coefficient (f) was positive for all populations and the mean overall value of f (0.046, $P<0.0001$) was low but significantly different from zero. The heterozygote deficit detected in the overall population probably resulted from population substructuring and some level of inbreeding.

7. Tests of isolation by distance revealed that there was no positive pairwise correlation between geographic distance and genetic differentiation among populations, estimated by either θ or ρ. The indirect estimates of gene flow obtained using different statistics derived from θ , ρ, and calculated by the private alleles were in agreement (mean *Nm* = 1.8). This value seems not to be high enough to totally override the effects of drift, resulting in a moderate level of differentiation among populations of *S. macrophylla.*
8. The hierarchical analysis of the genetic structure based on analysis of molecular variance (AMOVA) using dominant RAPD markers was congruent with AMOVA data using co-dominant microsatellite loci for the same individuals. Most of the genetic variation was detected within rather than between populations suggesting the existence of significant structuring of the genetic variation in *S. macrophylla* populations. These results are expected for a species with a patchy distribution such as mahogany.
9. Conservation strategies for mahogany in the Brazilian Amazon should take into account the existence of important genetic structuring of populations throughout its geographical range. This population differentiation leads to the need to conserve a representative number of populations with a relatively large

number of individuals per population, since most of the genetic variability occurs within populations.

10. Genetic differentiation seems not to be necessarily correlated with geographic distance, since populations of *S. macrophylla* only 8-15 km apart can exhibit a high level of differentiation if physical barriers prevent gene flow among them. The occurrence of such microgeographical differentiation emphasizes the importance of maintaining populations in their diverse habitats, especially in areas with a mosaic of topography and soils.

11. *Swietenia macrophylla* seems to have adaptations that preferentially produce outcrossed progeny but also allow for selfing. The high estimate of the multi-locus outcrossing rate (t_m = 0.958 ± 0.025) for the 25 trees of the Marajoara population using 10 SSR loci indicates that *S. macrophylla* is a predominantly allogamous species.

12. Levels of outcrossing were not evenly distributed among maternal trees, ranging from 0.38 to 1.00. The inbreeding in the sampled population was concentrated mainly in two trees which exhibited t_m values of 0.68 and 0.38 respectively, whereas the remaining 23 trees were highly or totally outcrossed. These data suggest the occurrence of a variable degree of self-incompatibility and/or dichogamy among individual trees of *S. macrophylla*. The ability to

cope with some levels of inbreeding is likely to be a reflection of the gap-colonist life history of *S. macrophylla.*

13. Genotypic data analysis from the progeny arrays found that 94% of the seedlings in the sampled population were unambiguously resulted from outcrossed matings. The remaining 23 seedlings had genotypes consistent with a self-fertilization origin. Apomixis was ruled out since none of the 400 seedlings had a multilocus genotype identical to its mother tree.

14. The large difference between the multi-locus and single-locus outcrossing estimates (t_m - t_s = 0.139 ± 0.029) provides evidences that, in spite of the high out-crossing rate, a considerable inbreeding of biparental origin has contributed to the genetic structure of this population. Although spatial genetic structure among adults was not detected, the relatively high biparental inbreeding denoted by the t_m - t_s estimates could be explained if the assortative mating would reflect the existence of a higher overlapping in the flowering phenology among genetically related individuals.

15. The genetic relatedness between pairs of progeny sampled from the same capsule was strongly positive and did not differ significantly among capsules (correlation of outcrossing paternity r_p = 0.555, range 0.428-0.660), suggesting that pollen loads were composed of pollen from a small number of sources. In

consequence, the probability of a randomly chosen pair of progeny from the same capsule being full sibs is slightly higher than 50% and individual flowers were likely to have been fertilised by only 1-3 pollen donors.

16. Despite a high outcrossing rate, *S. macrophylla* exhibited an excess of homozygous individuals in the seed and adult cohorts for a high proportion of loci. The excess of homozygotes among seeds is likely to have arisen via self-fertilization in the few self-compatible trees or by consanguineous' matings. Given that *S. macrophylla* is a monoecious species, self-pollination is potentially possible. Historical factors related with the gap-colonizer life strategy of *S. macrophylla*, such as founder effects and population bottlenecks, are likely to have exposed populations for prolonged inbreeding and also contributed to the observed homozygote excess in the current adult generation.

17. Due to its generalist pollination system and some level of tolerance for selfing, *S. macrophylla* seems to be resilient to environmental disturbances such as those caused by logging, setting fruits and seeds even at low densities of adult trees. Therefore, the remaining individuals in logged areas or in relict fragments may be very important for long term population recovery and genetic conservation programmes.

The knowledge of the levels and distribution of genetic variation is of fundamental importance for the establishment of effective and efficient conservation strategies for tropical tree species under intense human pressure such as *Swietenia macrophylla.* The high genetic diversity detected within populations and the existence of significant structuring of *S. macrophylla* populations along the Brazilian Amazon, found in the current study, indicate the urgent need for conservation of multiple populations along the distribution range, with a relatively large number of individuals per population to ensure that allelic and genotypic diversity will be maintained in *S. macrophylla* populations.

Another important factor in designing an effective management strategy for an endangered tree species should be the determination of its breeding system. (Hamrick *et al.,* 1991). The high multilocus outcrossing rate (t_m = 0.958) estimated for one population in this study, indicated that, although there was a prevalence of outcrossing, selfing was not negligible. Owing to the species pre-adaptation to colonize newly open, disturbed habitats, many of the remaining trees in logged areas may persist as viable individuals which could be very important for population recovery and genetic conservation programmes. Therefore, intensive logging followed by the replacement of the forest habitat by pastures and extensive plantations, as has been occurring at alarming rates throughout the Brazilian Amazon,

seems to be the main short term threat for the maintenance of *S. macrophylla* populations, because pollen and seeds dispersal mechanisms can not occur over long distances.

Initiatives for the establishment of new public and private reserves and protected areas are urgently necessary for the genetic conservation of the Brazilian mahogany and of many other highly threatened valuable tropical tree species.

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Appendix I. Allelic frequencies for ten microsatellite markers in seven populations of *S. macrophylla.* Mean observed and expected heterozigosities , H_o and H_e, are listed for each population and each locus and also mean expected heterozygosity over all populations for each locus. Private (rare) alleles are present in bold.

Locus	Allele size (bp)	A. Azul	Cach.A	Maraj	P.Lacerda	C.Mendes	Cach. E	P.Bueno	Mean H_e
sm01	261	0.000	0.453	0.040	0.000	0.000	0.000	0.563	
	263	0.222	0.000	0.160	0.478	0.882	0.417	0.021	
	265	0.000	0.047	0.000	0.022	0.015	0.000	0.042	
	267	0.000	0.000	0.000	0.000	0.000	0.104	0.188	
	269	0.056	0.063	0.000	0.022	0.000	0.000	0.042	
	271	0.000	0.000	0.020	0.022	0.000	0.063	0.000	
	273	0.093	0.000	0.100	0.000	0.000	0.000	0.000	
	275	0.056	0.016	0.040	0.000	0.000	0.021	0.042	
	277	0.037	0.000	0.180	0.000	0.029	0.042	0.000	
	279	0.185	0.078	0.040	0.043	0.000	0.000	0.000	
	281	0.056	0.000	0.000	0.043	0.000	0.042	0.000	
	283	0.019	0.047	0.100	0.000	0.000	0.000	0.042	
	285	0.074	0.172	0.060	0.043	0.029	0.063	0.021	
	287	0.167	0.047	0.140	0.152	0.029	0.125	0.042	
	289	0.019	0.063	0.020	0.022	0.000	0.021	0.000	
	291	0.000	0.016	0.080	0.087	0.000	0.083	0.000	
	293	0.000	0.000	0.020	0.065	0.015	0.021	0.000	
	295	**0.019**	0.000	0.000	0.000	0.000	0.000	0.000	
	H_o	0.815	0.781	0.920	0.875	0.235	0.792	0.458	
	H_e	0.879	0.756	0.904	0.740	0.222	0.800	0.652	0.702
sm22	119	0.019	0.000	0.000	0.000	0.000	0.063	0.000	
	121	0.000	0.000	0.000	0.000	0.000	0.000	**0.021**	
	125	0.000	0.000	0.000	0.000	**0.044**	0.000	0.000	
	129	0.000	0.000	0.040	0.000	0.000	0.104	0.000	
	131	0.000	0.016	0.020	0.000	0.000	0.000	0.188	
	135	0.000	0.000	0.000	0.000	0.015	0.063	0.000	
	137	0.000	0.031	0.220	0.273	0.029	0.167	0.063	
	139	0.352	0.156	0.140	0.205	0.309	0.000	0.354	
	141	0.019	0.000	0.000	0.091	0.206	0.000	0.000	
	143	0.000	0.000	0.020	0.000	0.206	0.000	0.000	
	145	0.000	0.000	0.000	0.000	0.029	0.000	0.021	
	147	0.000	0.031	0.100	0.068	0.015	0.092	0.000	
	149	0.333	0.672	0.300	0.182	0.103	0.208	0.354	
	151	0.167	0.063	0.140	0.000	0.000	0.042	0.000	
	153	0.111	0.000	0.000	0.000	0.029	0.063	0.000	
	155	0.000	0.031	0.020	0.091	0.015	0.000	0.000	
	161	0.000	0.000	0.000	**0.091**	0.000	0.000	0.000	
	H_o	0.778	0.531	0.720	0.783	0.823	0.750	0.500	
	H_e	0.738	0.525	0.826	0.846	0.816	0.837	0.724	0.760
sm31	80	0.000	0.031	0.000	0.196	0.000	0.042	0.000	
	82	0.000	0.031	0.000	0.130	0.132	0.042	0.000	
	84	0.000	0.000	0.000	**0.022**	0.000	0.000	0.000	
	92	0.000	0.000	0.000	**0.022**	0.000	0.000	0.000	
	94	0.017	0.000	0.020	0.000	0.044	0.000	0.000	
	96	0.000	0.031	0.000	0.000	0.000	0.083	0.000	
	98	0.000	0.016	0.000	0.000	0.029	0.063	0.000	

	100	0.000	0.016	0.040	0.000	0.000	0.000	0.000	
	102	0.000	0.063	0.000	0.022	0.000	0.000	0.000	
	104	0.000	0.000	0.060	0.022	0.088	0.000	0.000	
	106	0.067	0.016	0.000	0.022	0.029	0.021	0.000	
	108	0.133	0.031	0.040	0.022	0.059	0.000	0.065	
	110	0.067	0.000	0.060	0.065	0.147	0.250	0.043	
	112	0.067	0.141	0.140	0.087	0.074	0.083	0.304	
	114	0.133	0.094	0.060	0.000	0.118	0.042	0.000	
	116	0.100	0.000	0.180	0.152	0.088	0.021	0.000	
	118	0.117	0.109	0.060	0.109	0.088	0.000	0.196	
	120	0.083	0.219	0.140	0.087	0.088	0.208	0.261	
	122	0.100	0.047	0.080	0.000	0.000	0.083	0.065	
	124	0.050	0.000	0.040	0.000	0.000	0.021	0.022	
	126	0.033	0.031	0.000	0.000	0.015	0.021	0.000	
	128	0.017	0.000	0.080	0.022	0.029	0.021	0.000	
	130	**0.017**	0.000	0.000	0.000	0.000	0.000	0.000	
	132	0.000	0.000	0.000	0.022	0.015	0.000	0.000	
	134	0.000	0.125	0.000	0.000	0.000	0.000	0.022	
	136	0.000	0.000	0.000	0.000	0.000	0.000	**0.022**	
	138	0.000	0.000	0.000	0.000	**0.015**	0.000	0.000	
	H_o	0.967	0.844	0.840	0.792	0.794	0.792	0.783	
	H_e	0.921	0.898	0.914	0.909	0.922	0.880	0.807	0.893
sm32	146	0.000	**0.016**	0.000	0.000	0.000	0.000	0.000	
	152	0.000	0.000	0.000	**0.273**	0.000	0.000	0.000	
	154	0.000	0.000	0.000	0.205	0.000	0.167	0.000	
	156	0.032	0.172	0.000	0.023	0.132	0.063	0.313	
	158	0.081	0.047	0.000	0.023	0.397	0.000	0.042	
	160	0.000	0.031	0.140	0.114	0.221	0.250	0.125	
	162	0.113	0.359	0.160	0.045	0.074	0.104	0.188	
	164	0.081	0.016	0.040	0.000	0.015	0.125	0.104	
	166	0.177	0.016	0.140	0.000	0.059	0.083	0.000	
	168	0.000	0.016	0.120	0.159	0.015	0.063	0.021	
	170	0.129	0.078	0.140	0.068	0.059	0.042	0.063	
	172	0.113	0.078	0.100	0.068	0.029	0.021	0.083	
	174	0.113	0.078	0.060	0.000	0.000	0.000	0.000	
	176	0.113	0.063	0.060	0.000	0.000	0.063	0.063	
	178	0.032	0.016	0.040	0.023	0.000	0.000	0.000	
	180	0.016	0.016	0.000	0.000	0.000	0.000	0.000	
	184	0.000	0.000	0.000	0.000	0.000	**0.021**	0.000	
	H_o	0.871	0.812	0.880	0.739	0.559	0.750	0.792	
	H_e	0.900	0.827	0.899	0.854	0.774	0.880	0.841	0.854
sm34	40	**0.048**	0.000	0.000	0.000	0.000	0.000	0.000	
	44	0.000	0.000	**0.040**	0.000	0.000	0.000	0.000	
	68	0.000	0.000	0.000	**0.283**	0.000	0.000	0.000	
	70	0.613	0.000	0.340	0.630	0.000	0.750	0.000	
	72	0.032	0.766	0.320	0.000	0.794	0.083	0.696	
	74	0.000	0.000	0.020	0.022	0.118	0.000	0.043	
	76	0.145	0.078	0.120	0.000	0.000	0.000	0.174	
	78	0.000	0.000	0.000	0.000	**0.015**	0.000	0.000	
	82	0.016	0.000	0.000	0.022	0.000	0.000	0.000	
	84	0.016	0.000	0.020	0.043	0.000	0.042	0.000	
	86	0.016	0.156	0.000	0.000	0.015	0.125	0.043	
	88	0.081	0.000	0.040	0.000	0.044	0.000	0.022	
	90	0.000	0.000	**0.060**	0.000	0.000	0.000	0.000	
	92	0.000	0.000	0.000	0.000	**0.015**	0.000	0.000	
	94	0.000	0.000	0.020	0.000	0.000	0.000	0.022	

	96	**0.032**	0.000	0.000	0.000	0.000	0.000	0.000	
	106	0.000	0.000	**0.020**	0.000	0.000	0.000	0.000	
	H_o	0.581	0.250	0.520	0.208	0.235	0.375	0.391	
	H_e	0.601	0.389	0.775	0.518	0.358	0.422	0.492	0.508
sm40	120	0.000	0.000	**0.040**	0.000	0.000	0.000	0.000	
	122	0.000	0.000	0.000	0.000	0.000	**0.042**	0.000	
	126	0.000	0.000	0.020	0.000	0.029	0.021	0.000	
	128	0.000	0.000	0.000	0.000	0.015	0.021	0.000	
	130	0.000	0.000	0.020	0.000	0.000	0.125	0.000	
	132	0.204	0.047	0.240	0.136	0.441	0.542	0.130	
	134	0.389	0.469	0.440	0.591	0.221	0.146	0.413	
	136	0.167	0.281	0.060	0.045	0.191	0.021	0.348	
	138	0.111	0.047	0.040	0.045	0.074	0.083	0.022	
	140	0.000	0.156	0.060	0.114	0.000	0.000	0.065	
	142	0.019	0.000	0.020	0.068	0.000	0.000	0.000	
	144	0.111	0.000	0.060	0.000	0.029	0.000	0.000	
	146	0.000	0.000	0.000	0.000	0.000	0.000	**0.022**	
	H_o	0.592	0.719	0.840	0.739	0.706	0.792	0.783	
	H_e	0.769	0.683	0.748	0.629	0.723	0.674	0.701	0.704
sm45	140	0.000	**0.016**	0.000	0.000	0.000	0.000	0.000	
	146	0.000	0.000	0.000	**0.250**	0.000	0.000	0.000	
	148	0.000	0.000	0.000	**0.068**	0.000	0.000	0.000	
	150	0.016	0.156	0.000	0.045	0.132	0.208	0.341	
	152	0.032	0.047	0.000	0.159	0.353	0.000	0.045	
	154	0.000	0.031	0.104	0.023	0.088	0.208	0.136	
	156	0.145	0.375	0.208	0.159	0.029	0.125	0.182	
	158	0.097	0.016	0.021	0.000	0.221	0.000	0.045	
	160	0.161	0.016	0.125	0.000	0.015	0.083	0.000	
	162	0.000	0.016	0.083	0.114	0.059	0.125	0.023	
	164	0.097	0.078	0.208	0.068	0.074	0.104	0.068	
	166	0.145	0.078	0.083	0.045	0.029	0.042	0.091	
	168	0.097	0.078	0.063	0.045	0.000	0.021	0.000	
	170	0.161	0.063	0.063	0.000	0.000	0.063	0.068	
	172	0.032	0.016	0.042	0.023	0.000	0.000	0.000	
	174	0.016	0.016	0.000	0.000	0.000	0.000	0.000	
	178	0.000	0.000	0.000	0.000	0.000	**0.021**	0.000	
	H_o	0.871	0.781	0.958	0.956	0.970	0.958	0.818	
	H_e	0.889	0.821	0.881	0.876	0.802	0.876	0.829	0.853
sm46	190	0.000	0.078	0.000	0.091	0.088	0.000	0.021	
	194	0.000	0.000	0.000	0.000	**0.088**	0.000	0.000	
	196	0.000	0.250	0.000	0.068	0.015	0.083	0.000	
	198	0.000	0.000	0.000	0.000	**0.015**	0.000	0.000	
	200	0.000	0.000	0.000	0.023	0.000	0.042	0.042	
	202	0.000	0.188	0.180	0.227	0.074	0.042	0.000	
	204	0.274	0.141	0.200	0.068	0.044	0.271	0.208	
	206	0.161	0.047	0.280	0.250	0.074	0.146	0.208	
	208	0.306	0.125	0.120	0.023	0.221	0.042	0.313	
	210	0.097	0.141	0.080	0.023	0.103	0.021	0.000	
	212	0.129	0.016	0.100	0.114	0.029	0.208	0.167	
	214	0.032	0.000	0.000	0.114	0.132	0.063	0.042	
	216	0.000	0.000	0.020	0.000	0.059	0.000	0.000	
	218	0.000	0.016	0.020	0.000	0.044	0.021	0.000	
	220	0.000	0.000	0.000	0.000	**0.015**	0.000	0.000	
	222	0.000	0.000	0.000	0.000	0.000	**0.042**	0.000	
	226	0.000	0.000	0.000	0.000	0.000	**0.021**	0.000	
	H_o	0.806	0.844	0.760	0.869	0.823	0.792	0.875	

	H_e	0.790	0.852	0.834	0.852	0.901	0.862	0.800	0.842
sm47	100	0.000	0.000	**0.040**	0.000	0.000	0.000	0.000	
	110	0.000	0.000	0.000	0.000	**0.015**	0.000	0.000	
	114	0.000	0.000	0.000	0.000	**0.074**	0.000	0.000	
	118	0.000	0.031	0.000	0.000	0.000	0.396	0.000	
	120	0.774	0.313	0.680	0.283	0.088	0.063	0.438	
	122	0.000	0.031	0.000	0.022	0.147	0.000	0.104	
	124	0.032	0.031	0.060	0.022	0.250	0.313	0.083	
	126	0.161	0.203	0.220	0.152	0.265	0.063	0.229	
	128	0.000	0.031	0.000	0.326	0.088	0.000	0.021	
	130	0.000	0.125	0.000	0.109	0.074	0.021	0.063	
	136	0.000	0.016	0.000	0.000	0.000	0.083	0.000	
	138	0.000	0.031	0.000	0.000	0.000	0.000	0.021	
	140	0.000	0.000	0.000	0.000	0.000	0.063	0.042	
	142	0.000	0.016	0.000	0.087	0.000	0.000	0.000	
	144	0.000	**0.125**	0.000	0.000	0.000	0.000	0.000	
	146	0.032	0.016	0.000	0.000	0.000	0.000	0.000	
	148	0.000	**0.031**	0.000	0.000	0.000	0.000	0.000	
	H_o	0.452	0.812	0.360	0.792	0.853	0.833	0.750	
	H_e	0.379	0.836	0.494	0.785	0.831	0.742	0.747	0.688
sm51	138	0.000	0.000	0.000	0.024	0.015	0.000	0.000	
	144	0.000	**0.016**	0.000	0.000	0.000	0.000	0.000	
	146	0.000	0.016	0.000	0.000	0.132	0.000	0.000	
	148	0.000	0.000	0.000	0.000	**0.015**	0.000	0.000	
	150	0.065	0.047	0.020	0.000	0.044	0.000	0.000	
	152	0.000	0.000	0.000	0.000	**0.044**	0.000	0.000	
	154	0.000	0.000	0.020	0.048	0.000	0.063	0.000	
	156	0.016	0.047	0.320	0.119	0.162	0.083	0.000	
	158	0.210	0.156	0.400	0.190	0.324	0.354	0.318	
	160	0.468	0.125	0.160	0.238	0.103	0.250	0.364	
	162	0.048	0.109	0.060	0.143	0.074	0.000	0.068	
	164	0.161	0.000	0.000	0.000	0.015	0.021	0.091	
	166	0.000	0.047	0.000	0.024	0.015	0.021	0.114	
	168	0.000	0.063	0.000	0.071	0.029	0.000	0.000	
	170	0.000	0.063	0.000	0.071	0.000	0.083	0.000	
	172	0.016	0.156	0.020	0.048	0.015	0.042	0.023	
	174	0.000	0.000	0.000	0.000	0.015	0.021	0.000	
	176	0.016	0.141	0.000	0.024	0.000	0.000	0.000	
	178	0.000	0.000	0.000	0.000	0.000	0.042	0.023	
	180	0.000	**0.016**	0.000	0.000	0.000	0.000	0.000	
	182	0.000	0.000	0.000	0.000	0.000	**0.021**	0.000	
	H_o	0.742	0.906	0.600	0.909	0.882	0.708	0.500	
	H_e	0.715	0.903	0.722	0.879	0.842	0.806	0.757	0.803