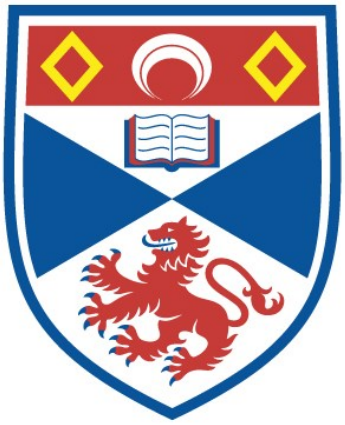
REPRODUCTIVE BIOLOGY OF TWO BOMBACACEOUS TREES IN THE BRAZILIAN CENTRAL AMAZON

Rogerio Gribel

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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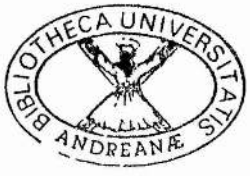
Reproductive Biology of Two Bombacaceous Trees in the Brazilian Central Amazon

by

Rogério Gribel

A thesis submitted to the University of St. Andrews for the degree of Doctor of Philosophy



School of Biological and Medical Sciences

University of St. Andrews

July 1995

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Só sei que nada sei ...
(um filósofo grego)
Mesmo assim não tenho certeza ...
(um ecólogo tropical)

To Aldenora, for her assistance, dedication, and friendship.

(Para Aldenora, por sua assistência, dedicação e amizade)

ACKNOWLEDGEMENTS

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I am very grateful to Dr. Peter E. Gibbs for the supervision and constant encouragement. Dr. Richard J. Abbott provided advice with the use of isozymes and offered a number of valuable comments on the thesis. I would like to thank David Forbes for the help with the electrophoresis at beginning of my period in St. Andrews. This study would not have been possible without the tireless dedication of Aldenora L. Queiróz and Laércio

Marajó (Leo)in carrying out nocturnal field work in the várzea.

Thanks also to all my Brazilian and foreigner friends, members of the 'alien community': Tânia Sanaiotti, Marcelo Vieira, Manuel Claudio Silva Jr., Marcelo Nascimento, Dora Villela, Cláudio Bohrer, Francisco das Chagas Silva, Fábio Scarano, Flávio and Regina Luizão, Apuema and Alexandra Salgueiro, Socorro Leningham, Imelda Udofot, Patricia Teixidor, with whom I enjoyed part of the time in Scotland. Thanks to my parents, my brother, and sisters for the constant support. I am very grateful to Naldite Lemes who gave essential help caring after the kids during our final period in Scotland. Pedro and Daniel Gribel were very patient with the Dad always 'writing up thesis'. Finally, it would not have been possible to finish this work without the assistance, encouragement, and commitment of my wife Maristerra Lemes.

DECLARATION

I, Rogerio Gribel, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Rogerio Gribel

July 1995

STATEMENT

I was admitted to the Faculty of Science of the University of St. Andrews under the Ordinance General No. 12 on November 1st 1991 and as a candidate for the degree of Ph.D. on 30th July 1992.

Rogerio Gribel

July 1995

CERTIFICATE

I hereby certify that the candidate has fulfilled the conditions for the Resolution and Regulations appropriate to the Degree of Ph.D.

P. E. Gibbs

July 1995

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Abstract

Studies were carried out on the reproductive biology of two bombacaceous trees (*Pseudobombax munguba* and *Ceiba pentandra*) which occur in the seasonally flooded areas of the white-water river basins ('várzea') in the Amazon region.

The unique pollinator of *P. munguba*, which has nectarless flowers, is the large-sized phyllostomid bat *Phyllostomus hastatus*. In contrast, the flowers of *C. pentandra* are visited by a wide range of nocturnal (bats, marsupials, night-monkeys, hawk moths) and diurnal (bees, wasps, hummingbirds) animals, but only the phyllostomid bats, especially *Phyllostomus hastatus* and *Phyllostomus discolor*, play a relevant role in the pollination of this mass-flowering species.

Both species appear to be self-incompatible since hand self-pollinated flowers always abscised 5-8 days after pollination, whereas a proportion of cross-pollinated flowers (20-29% in *P. munguba*; 17% in *C. pentandra*) formed fruit. However, analysis of fixed pistils using fluorescence microscopy revealed that in both species the self-pollen germinated normally on the

stigma and the self-pollen tubes penetrated the ovules at the same rate as the cross-pollen tubes.

Mixed-pollinated flowers (self- plus cross-pollen on the stigma) also set some fruits (9-14% in *P. munguba*; 9% in *C. pentandra*). Paternity analyses using isozyme genetic markers indicated that fruits resulting from controlled mixed-pollinations set a few selfed seeds (range of 0-28% in *P. munguba; ca* 2% in the studied tree of *C. pentandra*).

The multilocus estimate of the outcrossing rate (t_m) was calculated for *P. munguba* using data from two isozyme loci of 29 parent trees and 728 progenies. The population outcrossing estimation was high (t_m = 0.948) suggesting that the breeding population is large and the level of inbreeding (both uniparental and biparental) is very low. The proportion of selfed-seeds produced by two neighbouring *C. pentandra* trees, which flowered simultaneously, was estimated using isozyme genetic markers at 9% and 28% respectively. It is considered that a high level of genetic load is the main factor responsible for the self-sterility and the predominant outcrossing mating system observed in both species. The number of lethal equivalents per zygote estimated for

each was high: average of 13.8 (minimum 6.4) in the *P. munguba* population, and 12.3 for the single assessed individual of *C. pentandra*.

Chapter 1

General Introduction

Studies on the reproductive biology of tropical plants can provide essential information for an understanding of how high biological diversity has been maintained over evolutionary time in the tropics. Our knowledge of the reproductive biology of tropical plants, especially tropical trees, has increased considerably over the last two decades. Hundreds of studies have described the pollination mechanisms and the diversity of morphological and functional adaptations of the tropical flowers (see reviews in Faegri and van der Pijl 1979, Prance 1985, Bawa 1990, and Endress 1994). Other studies have investigated breeding systems (through controlled pollinations) and the relative frequency of taxa with different pollination and sexual systems at the community level (Bawa 1974, Bawa and Opler 1975, Zapata and Arroyo 1978, Appanah 1981, Bawa *et al.* 1985a, 1985b, Bullock 1985, Ramirez 1988, Ramirez and Brito 1990). Isozyme markers have been recently used in order to estimate the outcrossing rates and other mating system parameters for several tropical trees (O'Malley and Bawa 1987, O'Malley

et al. 1988, Murawski *et al.* 1990, 1994, Murawski and Hamrick 1991, 1992a, 1992b, Alvarez-Buylla and Garay 1994, Hall *et al.* 1994, Boshier *et al.* 1995). Some general patterns have been revealed from these studies in regard to the following:

(1) Pollination system. Biotic pollination is predominant among tropical plants (reviewed in Bawa 1990). The majority of species are pollinated by a diverse array of insects (bees, beetles, butterflies moths, wasps, thrips, etc.) and, less frequently, by vertebrates (hummingbirds, passerine birds, bats, and different groups of non-flying mammals). Wind-pollination is relatively rare (Bawa and Crisp 1980, Bawa *et al.* 1985b, Bawa 1990).

(2) *Sexual system*. The majority of species of the tropical forests are monomorphic in regard to sexual system (hermaphroditic or monoecious), but around 22-26% of the trees are dioecious (Ashton 1969, Bawa 1974, Bawa *et al.* 1985a, Zapata and Arroyo 1978, Bullock 1985, but see Ramirez and Brito 1990). The frequency of dioecy in understory plants is significantly lower than that found among canopy trees (Bawa 1990).

(3) *Breeding system*. Contrary to the predictions of Fedorov (1959), most tropical trees are found to set no

fruit following self-pollination, or very few compared with cross-pollination (Bawa 1974, Bawa *et al.* 1985a, Zapata and Arroyo 1978, Chan 1981, Sobrevilla and Arroyo 1982, but see Ramirez and Brito 1990). However, many herbs, shrubs, and understory plants in general are inbreeding (Bawa 1990). The proportion of agamospermous species may be relatively high in some tropical forests (Kaur 1978, Ramirez and Brito 1990).

(4) *Mating system*. Almost all tropical trees investigated so far seem to be predominantly outcrossing (outcrossing rate $t_m > 0.8$), but two gap-strategist neotropical bombacaceous trees (*Ceiba pentandra* and *Cavallinesia platanifolia*), and one Dipterocarpaceae from Sri Lanka (*Shorea trapezifolia*) exhibited intermediate outcrossing rates (O'Malley and Bawa 1987, O'Malley *et al.* 1988, Murawski *et al.* 1990, 1994, Murawski and Hamrick 1991, 1992a, 1992b, Alvarez-Buylla and Garay 1994, Hall *et al.* 1994, Boshier *et al.* 1995).

- Reproductive biology of Bombacaceae.

Bombacaceae is a relatively small family of Malvales, with six tribes and approximately 28 genera and 250 species distributed in tropical areas of the world,

especially in Tropical America (Aubréville 1975, Baum and Oginuma 1994). Five tribes occur in the neotropical region: Matisieae (with 8 genera), Ceibeae (2 genera), Adansonieae (5 genera), Catostemmateae (2 genera), and Hampeae (3 genera). Adansonieae also has two genera, *Adansonia* and *Bombax*, which occur in Africa and Australasia. The remaining tribe, the Durioneae (8 genera), stands somewhat apart since it shows similarities with the Sterculiaceae and has an exclusively Asian/Australasian distribution.

The family Bombacaceae is characterised by small chromosomes with a persistent nucleolus throughout the nuclear divisions (Baker and Baker 1968). The 'diploid' number of chromosomes commonly is 2n = 86-92 (Baum and Oginuma 1994). However, some species exhibit an exceptionally high number of chromosomes, such *as* *Rhodognaphalon brevicuspe* (2n ≈ 150), *Adansonia digitata* (2n = 160), and *Eriotheca pubescens* (2n = 276), whereas others from the tribe Durioneae have a lower number (2n = 28, 56) (Baker and Baker 1968, Morawetz 1986, Oliveira et al. 1992, Baum and Oginuma 1994). Ancient polyploidization is the most likely cause of the higher number of chromosomes observed in Bombacaceae in

comparison with other Malvalean families such as Malvaceae, Sterculiaceae, and Tiliaceae (Bawa 1973, Baum and Oginuma 1994).

Our knowledge of the pollination biology of the Bombacaceae is still fragmented. It seems to be one of the few families of angiosperms in which pollination by vertebrates (mainly bat-pollination) prevails over insect-pollination. In the Old World, bat-pollination has been cited for *Ceiba* and *Adansonia* (Baker and Harris 1957, Harris and Baker 1959, Faegri and van der Pijl 1979, Marshall 1983, Elmqvist *et al.* 1993), whereas *Bombax* may be pollinated either by birds or bats (Faegri and van der Pijl 1979).

In Tropical America, a variety of animals visit bombacaceous flowers and have been considered as potential pollinators. Evidence of bat-pollination exists for *Ceiba* (Carvalho 1961, Baker et al. 1971, Heithaus et al. 1975), *Pseudobombax* (Heithaus et al. 1975, Eguiarte *et al.* 1987, Gribel 1988, Gribel *et al.* 1990), and *Ochroma* (Heithaus et al. 1975, Gribel 1988). Non-flying mammals (mainly marsupials) usually visit and may pollinate flowers of *Ceiba*, *Quararibea*, *Ochroma*, and *Pseudobombax* (Janson *et al.* 1981, Gribel 1988). Diurnal

pollination by hummingbirds and non-hovering birds was proposed for *Ceiba* (Baker *et al.* 1971, Toledo 1977). Entomophily seems to be relatively rare in the family, but butterfly-pollination probably occurs in three species of *Chorisia* (Gibbs *et al.* 1988), and large Anthophoridae bees pollinate two species of *Eriotheca* (Oliveira *et al.* 1992).

Studies on the breeding systems of Bombacaceae have been particularly scarce. *Ceiba pentandra* trees in the Old World tropics have been considered as self-fertile (Toxopeus 1950, van der Pijl 1956, Baker 1955, 1965, Elmqvist *et al.* 1992). Bawa *et al.* (1985) obtained 0% and 100% fruit-set following self- and cross-pollination respectively in *Ochroma pyramidale*. Gibbs and Bianchi (1993), working with *Chorisia speciosa* and *C. chodati*, found both species to be self-sterile, yielding no fruit-set by self-, and 90-93% fruit-set by cross-pollination. Oliveira *et al.* (1992) also found that *Eriotheca gracilipes* was self-sterile (fruit-set 0% and 19.4% following self- and cross-pollination respectively). Late-acting self-incompatibility was considered as the cause of the self-sterility observed in *E. gracilipes*, *C. speciosa*, and *C. chodati* (Oliveira *et al.* 1992, Gibbs and

Bianchi 1993). Polyembryony and apomixis were reported for *Bombacopsis glabra* (= *Pachira oleaginea*) and *Eriotheca pubescens* (Baker 1960, Duncan 1970. Oliveira *et al.* 1992).

The observations of fixed self- and cross-pollinated pistils of *Eriotheca* and *Chorisia* under fluorescence microscopy using the aniline-blue staining technique (Martin 1959) suggested that: (1) the self- and cross-pollen tubes have similar rate of growth, (2) both types of pollen tubes achieved similar level of ovule penetration, and (3) selfed and crossed ovules exhibited a resting zygote with initial endosperm development a few days following pollination (Oliveira *et al.* 1992, Gibbs and Bianchi 1993).

Mating system studies, using isozyme genetic markers, with bombacaceous trees in Central America revealed that *Quararibea asterolepsis* was completely outcrossed (t_m = 1.008), whereas the 'gap-strategists' *Ceiba pentandra* (t_m = 0.689) and *Cavanillesia platanifolia* (t_m = 0.213-0.661) exhibited a mixed mating system (Murawski *et al* 1990, Murawski and Hamrick 1991, 1992a, 1992b).

- The problem and the scope

The richness and the diversity of plant species in the Amazon is probably higher than anywhere else in the World. In this region, however, studies on the reproductive biology of plants are still scarce. Probably the main cause of this paucity of information concerns the logistic difficulties involved in studies of floral and pollination biology in the canopy and sub-canopy layers of tropical forests. Such technical difficulties, however, are common for all tropical forests and have been overcome in other studies carried out elsewhere (Perry 1978, Perry and Starrett 1980, Lack and Kevan 1984). The relatively low density of most tree species, a consequence of the high diversity, also makes it difficult to track pollinator (and pollen) movements between conspecific individuals. Furthermore, the enormous richness and complexity of plant reproductive strategies in the Amazon contrasts with the scarcity of resources available for research and with the reduced number of biologists and ecologists effectively working on plant reproduction in this region.

Studies on the reproductive biology of amazonian plants have mainly focused on aspects of the floral

biology and pollinator behaviour for individual species, or for a few species from a given family (Braga 1976, Prance 1976, 1980, 1985, Prance and Arias 1975, Prance and Anderson 1976, Mori *et al.* 1978, Webber 1981, Hopkins 1984, Moritz 1984, Gottsberger 1989, Kress and Stone 1993). Most data available on the reproduction of plants in the Amazon, however, are still anecdotal and descriptive.

In fact, most information on the reproductive biology of neotropical forest plants (especially trees) has been derived from extensive studies conducted outside of the Amazon Basin, particularly at the northern boundary of the neotropical forest distribution in Central America (Bawa 1974, Bawa and Opler 1975, Bawa *et al.* 1985a, 1985b). Although such studies have produced some valuable data for individual species and useful community-wide insights, they have provided a rather superficial view of the complexity of factors which affect the reproductive biology of tree species. More recently, the use of isozyme markers has allowed the estimation of the outcrossing rates for several Central American trees (O'Malley and Bawa 1987, Murawski *et al.* 1990, Murawski and Hamrick 1991, 1992a, 1992b, Alvarez-

Buylla and Garay 1994, Hall *et al.* 1994, Boshier *et al.* 1995), but the biological and ecological factors modulating such mating system characteristics are still poorly understood. For example, several factors such as differences in the distribution pattern, flowering phenology, flower morphology and function, genetic load, self- and cross-incompatibility, pollinator behaviour, or pattern of seed dispersal may concomitantly affect the mating system of the tropical trees.

In the present study I have attempted to approach the reproductive biology of two amazonian bombacaceous trees from several viewpoints, considering the relative influence of the flowering phenology, floral structure, the performance of the self- and cross-pollen, the occurrence of self-incompatibility and/or the genetic load, and the role of pollinator behaviour with regard to fruit- and seed-set, and the mating system. The main objective has been to provide an overall view of the factors affecting the reproductive biology of these two species. However, other components of the reproductive cycle, such as the dispersal of the propagules and seedling establishment and regeneration, were not included in this study. The specific objectives are

enumerated in each Chapter. *Pseudobombax munguba*, which is still very abundant in the Central Amazon, was investigated in more detail. *Ceiba pentandra*, which unfortunately has become very rare in the region due to the over exploitation by the plywood industry, was studied in less detail by using, opportunistically, a few individuals which were logistically accessible.

Chapter 2

Floral Biology, Pollination Ecology, and Breeding System of *Pseudobombax munguba*

2.1 - INTRODUCTION

Pseudobombax (Bombacaceae) is an exclusively neotropical genus of trees and shrubs with 20 species which occur in North Argentina, Paraguay and South and Central Brazil and Central America (Robyns 1963, Abreuville 1974). The *Pseudobombax* species are generally specialised to colonise open habitats which have some soil constraints for other arboreal species, such as long periods of flooding, high water table, superficial lateritic layer, shallow soils in areas of rocky outcrops, etc.

In most *Pseudobombax* species the flowers have a robust structure, large size, and hundreds of stamens which produce a 'brush' appearance to the androecium (Robyns 1963). The rigid structure of the flowers and the production of massive amounts of pollen suggest that pollination by large animals may be common. This inference, based on the morphological characteristics, has been confirmed by the few observations made on the pollination ecology of the *Pseudobombax* species. Heithaus *et al.* (1975), working in Costa Rica, caught six

different bat species (*Phyllostomus discolor, Artibeus jamaicensis, Artibeus phaeotis, Sturnira lilium, Carollia perspicillata,* and *Glossophaga soricina*) carrying pollen loads of *Pseudobombax septinatum* on their fur. Eguiarte *et al.* (1987) observed in Mexico that *Pseudobombax ellipticum* was pollinated primarily by three species of nectarivorous/pollenivorous bats (*Leptonycteris sanborni, Choeronycteris mexicana,* and *Glossophaga leachii*) and secondarily by three species of orioles (*Icterus spp.*). Gribel (1988) observed that, in Central Brazil, *Pseudobombax tomentosum* was regularly visited and pollinated by one arboreal marsupial (*Caluromys lanatus*) and, less frequently, by a large phyllostomid bat species.

The main objectives of this chapter are to describe the floral biology, the pollination ecology, and the breeding system of the Amazonian tree *Pseudobombax munguba*. Two species of *Pseudobombax* from Central Brazil (*P. longiflorum* and *P. tomentosum*), which exhibit different pollination systems, are used for congeneric comparisons with regard to floral morphology and pollination system.

- The study sites

Most of the field work was carried out with 65 *P. munguba* trees located inside a plot of 500 x 150 m in the Ponta do Catalão, a peninsular area where the Negro and Solimões Rivers join to form the Amazon River, about 4.5

km SE of the city of Manaus (ca. 03°08'S, 60°00'W), Amazonas State. Around 50% of the plot area was permanently inundated, without any arboreal vegetation. Additional data on the breeding system and floral visitors were collected in the Lago do Mapixí, Purús River (ca. 05°55'S, 63°59'W, approximately 570 km southwest from the Catalão area). *Pseudobombax munguba* is one of the dominant arboreal species in the Catalão and Mapixí areas, growing in terrains which are annually flooded for 4-7 months.

Anecdotal observations on the floral visitors were also obtained for trees of *P. munguba* growing in the tidal várzea of the Ilha do Combú, at 3 km north of the city of Belém (ca. 01°27'S,48°27'S, 1330 km east of Manaus), Pará State.

Comparative observations on the floral biology and pollination ecology of two congeneric species, *P. tomentosum* and *P. longiflorum*, were made in the Fazenda Agua Limpa, located 18 km south of Brasília (ca. 15°45'S; 47°48'W, altitude 1000-1100m), in the Central Brazil. This region is covered by the "cerrado" (*sensu latu*) vegetation (Eiten 1972), a savanna-type woodland with gallery forests which border the streams which traverse the area. The cerrado vegetation of Brasília was described in Eiten (1984).

The map (fig. 2.1) shows the location of the four study sites. The fig. 2.2 shows an aerial view of the main study area in the Ponta do Catalão.

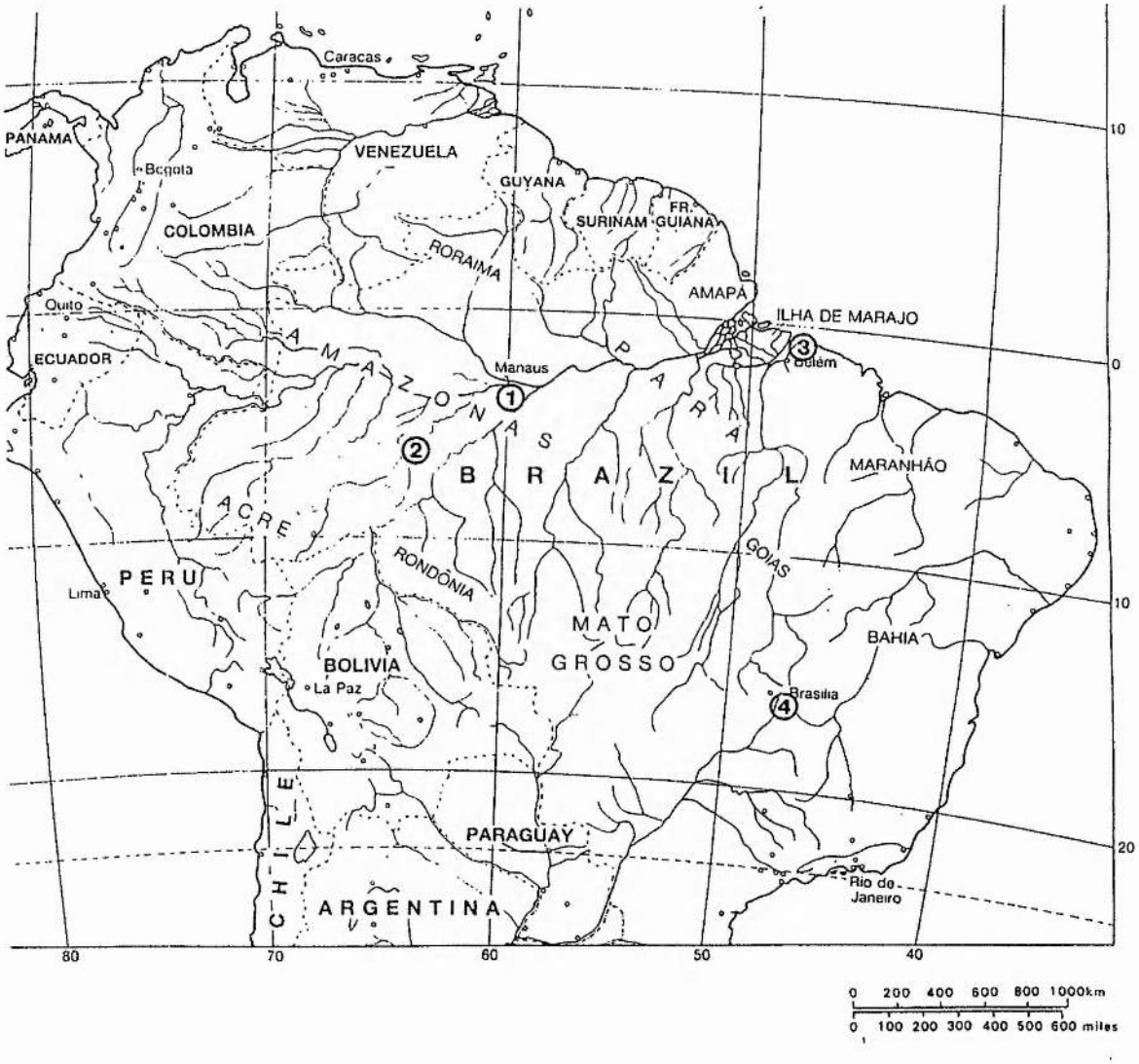

Figure 2.1 - Location of the study sites in Brazil. **(1)** Ponta do Catalão, **(2)** Lago do Mapixí, **(3)** Ilha do Combú, and **(4)** Fazenda Água Limpa.

Figure 2.2 - Aerial view of the Catalão area, located in the meeting of the Solimões River ('white' water) and Negro River ('black' water).

- The Tree

Pseudobombax munguba is the only species of the genus which occurs in the extensive quaternary floodplain of the Amazon, extending from the Peruvian and Colombian Amazonian lowlands to the mouth of the Amazon. It is a tree of up to 40m in height (fig. 2.3a) that typically occurs in the seasonally inundated habitat called 'várzea', which is characteristic of the sedimentary basins of the 'white-water' rivers (Pires 1974, Pires and Prance 1985, Worbes 1992). It is also found in the daily inundated 'tidal várzea' of the rivers near to the Amazon River estuarine region, in Pará and Amapá States of Brazil. The várzea inhabitants use the fiber from the bark of *P. munguba* to make strings and bags, and the silk-cotton from the fruits to fill pillows and cushions. This species is still very abundant and seems to be a keystone plant resource in the várzea habitat, producing annually great quantities of small seeds rich in oils and proteins which are consumed by a large number of commercially important regional fishes. A potential threat to *P. munguba* populations in the near future is that recently its timber has been sold by mills in Manaus as wood to mould concrete in building constructions (madeira de 'azimbre').

Pseudobombax munguba is a deciduous tree, renewing its foliage during the period from flowering to fruit maturation (approximately from June to September). The release of the leaves is less synchronised among the

trees than the flowering, so that there are usually some trees still with old leaves and others totally leafless during the flowering period.

The flowering period lasts for 8-10 weeks, usually starting in early or middle May and finishing at the end of July or beginning of August in the Central Amazon. The flowering coincides with the peak flood period of the várzea. The flowers (fig. 2.3b) are solitary, inclined, large (corolla diameter 10-14 cm), structurally robust and totally white (including anthers and pollen). The short (1.5-2.5 cm), rigid staminal tube and the 1000-1200 stamen filaments give a 'semi-spherical brush' appearance to the flower. The long (6-9 cm) and robust pistil emerges from the inside of the staminal tube and the mass of stamens exposing the stigma 1-3 cm above the level of the anthers. A detailed description of the *P. munguba* flowers is given in Robyns (1963).

The fruits (fig. 2.3c) are elliptic, 15-30 cm long, red capsules which dehisce by 5-7 valves. The capsules have 500-2700 small (2-3 mm diameter), globose seeds. weighing 19-32 mg each, surrounded by the pale yellow silk cotton. Fruit maturation occurs 55-75 days after the flower opens.

2.2 - MATERIALS AND METHODOLOGY

- Production of flowers, fruits, and seeds

The daily number of flowers opened by 65 trees in the Catalão population was counted at 4-7 days intervals

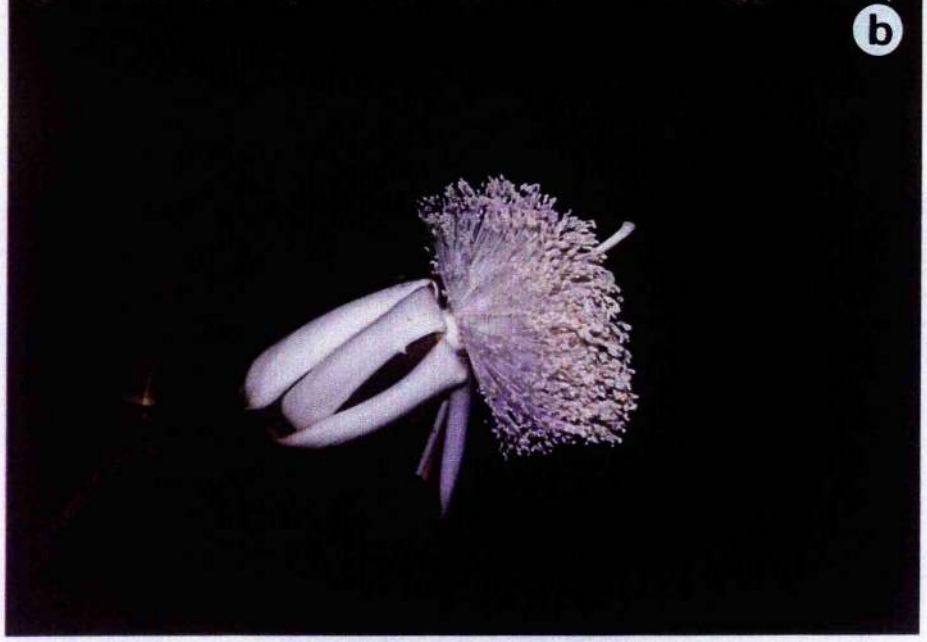

Figure 2.3 - (a) *Pseudobombax munguba* tree in the Catalão area. **(b)** Flower of *P. munguba* after the anthesis. **(c)** Fruits of *P. munguba*, three of them exposing the 'silk-cotton'.

during the 1993 flowering period. The total flower production of the population was estimated from the area under the curve of the graph describing the total number of flowers produced against time. The fruit-set of the population was estimated following the development of tagged flowers during the fruiting periods of 1990, 1991 and 1993 in the Catalão, and of 1990 in the Mapixí area. The number of seeds per capsule was counted in 88 fruits resulting from natural pollinations randomly collected in the Catalão population.

- Pollination tests

Controlled pollinations were used to determine the breeding system of *P. munguba* populations in the Catalão and Mapixí areas. Flowers on the lower branches of the trees were reached by canoe and hand-pollinated. Pollinations were made by touching repeatedly clusters of anthers on the stigma. The following treatments were carried out:

- **Apomixis**: flower-buds were opened, emasculated, and bagged before anthesis, at 17:00-18:00 h. Flowers unbagged the following day around 11:00-12:00 h.
- **Self-pollination**: flower-buds were bagged in the afternoon before anthesis, at 17:00-18:00 h. Flowers were unbagged and a massive self-pollen load was deposited on the stigma at 19:00-20:00 h,

after which the flowers were rebagged. Flowers were unbagged the following day at 11:00-12:00 h.

- **Nocturnal cross-pollination:** flower-buds were opened, emasculated, and bagged at 17:00-18:00 h. Flowers were unbagged and a massive cross-pollen load, from a tree located at least 100 m away, was deposited on the stigma at 19:00-20:00 h. Flowers were kept bagged until the next day at 11:00- 12:00 h.

- **Diurnal cross-pollination:** the treatment was identical to the above, except that the flowers were unbagged, cross-pollinated, and rebagged at 06:00-06:30 h.

- **Open pollination:** Flowers were tagged on the morning following anthesis and left for natural fruit-set.

- Floral biology, observation, and capture of floral visitors

The occurrence of the following floral events were observed in *P. munguba*: beginning of anthesis, odour release, nectar secretion, and pollen liberation. Stigmatic receptivity was visually inferred based on the turgor and the wetness of its surface.

Observations on the behaviour of floral visitors to *P. munguba* were made from a canoe anchored near flowering trees or from platforms placed inside the canopy. Nocturnal observations were made with the aid of

head-lights covered with a red filter. During the observation periods, the size of the bat visitors, the size of the forage group, the occurrence of aggressive interactions, and the behaviour at flowers were noted.

Nocturnal visitors of *P. munguba* flowers were captured with 12 x 2.8 m mist-nets placed near flowering canopies, and suspended by cords from forks of neighbouring trees (usually *Cecropia latiloba*). Samples of the pollen load attached to the floral visitors' fur were prepared after the technique described by Beattie (1971) and compared under the microscope with the pollen collected directly from *P. munguba* flowers.

Comparative data on the floral biology and pollination ecology of *Pseudobombax tomentosum* and *P. longiflorum* were taken from Gribel (1988) and Gribel et al. (1990), as well as additional unpublished personal observations on these species.

2.3 - RESULTS

- Flower, fruit, and seed production

Table 2.1 shows the flowering phenology of 65 trees during the 1993 flowering season in the Catalão area. On the basis of the area under the *number of flowers x time* curve, it was estimated that a total of 18,079 flowers were produced by the 65 trees which flowered in the plot. Approximately 68% of the total flowering was concentrated in only eight individuals (12.5% of the sampled trees).

Table 2.1 - Number of flowers produced per tree in the Catalao area in 1993.

Tree	1.-May	6-May	13-May	20-May	24-May	28-May	3-Jun	11-Jun	17-Jun	21-Jun	25-Jun	28-Jun	5-Jul	9-Jul	total(%)
#0	0	8	42	52	52	40	58	23	24	40	12	22	18	0	11.57
#1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0.03
#3	0	3	8	6	6	4	0	1	1	3	0	3	0	0	1.04
#3B	0	2	4	2	3	2	1	0	0	0	0	0	0	0	0.41
#3A	0	1	2	1	3	1	6	2	1	2	0	3	1	1	0.71
#4	0	0	4	0	10	6	1	3	1	4	0	1	0	0	0.89
#5	0	0	0	0	1	2	5	1	0	2	2	5	1	0	0.56
#6	0	0	1	2	1	6	8	8	3	0	6	3	1	0	1.15
#6A	0	3	8	13	10	6	9	1	1	0	0	1	0	0	1.54
#N14	0	1	2	2	1	1	1	1	0	0	0	0	0	0	0.27
#5A	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0.09
#7	0	0	0	0	0	0	1	0	2	0	7	2	0	1	0.38
#8A	0	0	0	0	0	5	7	2	8	10	3	2	2	0	1.15
#9	0	0	3	2	2	1	0	9	1	3	3	0	0	0	0.71
#9A	0	0	0	2	0	1	1	2	3	2	0	0	0	1	0.34
#9B	0	0	0	0	0	1	0	2	0	0	1	0	0	0	0.12
#9C	0	2	5	1	1	0	1	0	0	0	0	0	0	0	0.29
#10A	0	0	1	0	1	1	3	1	0	0	0	4	1	0	0.36
#10D	0	0	0	1	1	1	3	0	0	7	0	1	0	0	0.41
#10C	0	0	2	0	0	2	8	4	5	3	1	0	0	0	0.74
#11	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0.09
#11A	0	0	0	0	1	0	3	1	1	1	0	0	1	0	0.24
#11B	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0.03
#5B	0	0	1	4	1	3	11	3	4	2	0	0	0	0	0.85
#5C	0	0	2	0	3	1	0	0	0	0	0	0	0	0	0.18
#5D	0	0	1	0	3	7	13	10	8	5	4	0	0	0	1.50
#5E	0	0	0	1	2	1	2	0	0	0	0	0	0	0	0.18
#5F	0	1	1	1	0	8	1	2	1	0	0	0	0	0	0.44
#M7	0	0	1	0	3	1	1	0	0	0	0	0	0	0	0.18
#M8	0	0	0	1	1	0	0	2	1	2	0	7	1	2	0.50
#12	0	13	48	44	50	24	6	0	0	0	0	0	0	0	5.47
#12A	0	0	2	0	1	0	0	1	0	0	0	0	0	0	0.12
#12B	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0.12
#14	0	0	5	7	0	2	0	0	0	2	0	0	0	0	0.47
#12C	0	0	0	0	0	0	0	2	3	1	1	0	0	0	0.21
#N11	0	3	5	7	2	1	1	0	0	0	0	0	0	0	0.56
#N12	0	0	0	0	0	0	1	0	0	1	0	2	0	0	0.12
#13	0	0	6	0	2	4	7	8	1	1	0	1	1	0	0.92
#N13	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0.15
#16	0	0	2	0	0	1	0	0	0	3	0	5	0	10	0.62
#A1	0	15	38	94	120	56	50	14	8	6	0	3	2	0	12.01
#17	0	0	0	3	7	6	17	7	18	48	10	18	14	11	4.70
#18	0	0	0	0	1	0	2	0	0	0	0	1	0	0	0.12
#19	0	0	1	0	2	2	4	6	5	7	6	9	11	7	1.78
#20	0	0	0	0	0	0	6	0	0	3	1	2	0	0	0.36
#21	0	2	4	6	3	9	23	15	10	6	4	3	0	0	2.51
#22	0	0	2	0	0	2	1	2	0	0	0	0	0	0	0.21
#23	0	0	1	6	1	7	1	0	0	0	0	0	0	0	0.47
#24	0	0	0	8	5	4	2	0	0	0	0	0	0	0	0.56
#Y	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0.09
#Y1	0	0	7	1	0	0	0	0	0	0	0	0	0	0	0.24
#Y2	0	0	1	4	2	1	1	0	0	3	0	0	0	3	0.44
#N4	0	1	4	8	54	44	88	21	8	9	4	4	2	2	7.37
#N3	0	1	0	26	26	18	36	11	8	4	0	0	2	0	3.91
#N2	0	0	0	0	3	3	7	4	0	3	0	1	0	0	0.62
#M5	0	0	0	0	4	1	6	8	22	72	78	56	44	14	9.02
#M1	0	3	8	8	5	1	0	1	0	0	0	0	0	0	0.77
#M2	0	1	4	0	2	1	1	0	0	0	0	0	0	0	0.27
#N1	0	0	0	0	3	5	0	0	0	0	0	0	0	0	0.24
#8B	0	0	0	0	1	2	5	10	9	0	0	1	1	0	0.86
#N9	0	0	0	0	4	6	12	1	7	13	2	4	3	3	1.63
#B3	0	0	0	4	6	8	5	3	4	3	1	1	0	0	1.04
#B2	0	0	4	2	0	1	2	0	0	0	0	0	0	0	0.27
#B1	0	0	0	1	2	0	4	4	5	9	1	5	2	1	1.01
#A2	0	0	0	6	40	60	56	96	70	44	50	25	8	11	13.79
Total	0	60	234	327	454	373	493	292	244	325	197	196	118	67	100

The occurrence of a large number of small trees setting few flowers was probably caused by the fact that the sedimentation process of the Catalão region is still actively in progress (Irion *et al.* 1983), resulting in continuous colonisation of new areas by *P. munguba* and, consequently, a relatively high proportion of young trees in the population. Fruits from open-pollinations contained on average 1548.5 ± 427.3 seeds (range 566-2635 seeds, N = 88 fruits). The total weight of seeds per fruit was 36.5 ± 8.9 g (range 11.44-58.03 g, N = 88 fruits).

- Floral biology and breeding system

Anthesis starts soon after dusk, around 18:30 - 18:45h. At this time the flower-bud is swollen because of the internal pressure made by the turgor of the stamens. The petals fold backward successively in sudden movements as their margins are released. The stigma is receptive and the anthers release dusty pollen just after the flowers open. The inner surface of the petals, the whole androecium (including anthers and pollen) and the style/stigma are shiny white. The whole opening process spans 5-10 minutes. The flowers last only one night; the stamens, attached to the staminal tube, and the petals fall during the following morning. No nectar is produced by the *P. munguba* flowers.

The results of the pollination tests, reported in table 2.2, indicate that the *P. munguba* trees in

Table 2.2. Pollination tests in *Pseudobombax munguba*

Local, year/ treatment	flowers pollinated	fruits produced	fruit-set (%)	number of trees
Lago do Mapixí, 1990				
Apomix control	17	0	0	7
Self-pollination	92	0	0	22
Cross-pollination	89	18	20.2	24
Open-pollination	102	2	2.0	22
Ponta do Catalão, 1990				
Apomixis control	39	0	0	11
Self-pollination	61	0	0	16
Cross-pollination	62	14	22.6	13
Diurnal cross-pollination	96	8	8.3	20
Open-pollination	315	9	2.9	24
Ponta do Catalão, 1992				
Self-pollination	51	0	0	11
Cross-pollination	85	25	29.4	18
Open-pollination	189	13	7.0	18
Ponta do Catalão, 1993				
Self-pollination	202	0	0	30
Cross-pollination	465	133	28.6	44
Open-pollination	554	23	4.2	36

both the Catalão and Mapixi populations are seemingly self-incompatible. The fruit-set by nocturnal cross-pollination is around 20-30%, depending on the area and flowering year. Fruit-set by diurnal cross-pollination was significantly lower than by nocturnal cross-pollination (8.3% *versus* 22.6% respectively during the 1990 flowering period in the Catalão area, $\chi^2 = 6.38$, 1*df*, $P = 0.0115$). Comparative data on fruit- and seed-set by cross-, self- , open-, and mixed- (self- + cross-pollen load) pollinations are described in detail in Chapter 4.

Self-pollinated pistils were retained and enlarged somewhat, in the same manner as were cross-pollinated ones, until the 5^{th} - 8^{th} day following pollination, after which they were aborted. Unpollinated pistils from the apomixis control treatment did not present any post-pollination enlargement and were aborted up to two days following anthesis. Almost all pistils from naturally pollinated flowers were also retained and enlarged until the 5^{th} - 8^{th} day following anthesis, which suggests that most these flowers were pollinated (probably as a result of self-pollination, since the flower structure facilitates the auto deposition of pollen).

- Floral visitors and pollination

Only one bat species, *Phyllostomus hastatus*, was observed visiting flowers of *P. munguba* in the Catalão,

Mapixí, and Combú areas during the nocturnal period. This bat is easily differentiated from other phyllostomid flower visitors because of its characteristic vocalisations when foraging in groups, and its large size (weight 80-115g, head/body length 110-130mm, and wingspan aprox. 450-550mm). *Phyllostomus hastatus* is the second largest neotropical bat, smaller only than *Vampyrum spectrum* and around the same size as *Chrotopterus auritus*, both of which are exclusively carnivorous species.

In the three areas where observations were carried out, the more profusely flowering trees of *P. munguba* (with 50-120 opened flowers/night) were usually visited by groups of *P. hastatus* comprising 5-20 individuals soon after nightfall, (i.e. around 18:50-19:30h). Groups of *Phyllostomus hastatus* individuals flew around the tree crowns several times before initiating visits to the flowers. Fig. 2.4 illustrates two general tendencies observed in the visitation by *P. hastatus* in the more copiously flowering trees: (1) group size and number of visits declined throughout the night, and (2) visitation during the beginning of the flowering season tended to be more frequent than during the peak or at the end of flowering. Trees with few flowers were irregularly visited apparently by solitary bats. During visits, *P. hastatus* landed, generally hanging down, and 'embraced' the flowers for 1-3 s, after which the bat released the flower and flew away. On several occasions it was

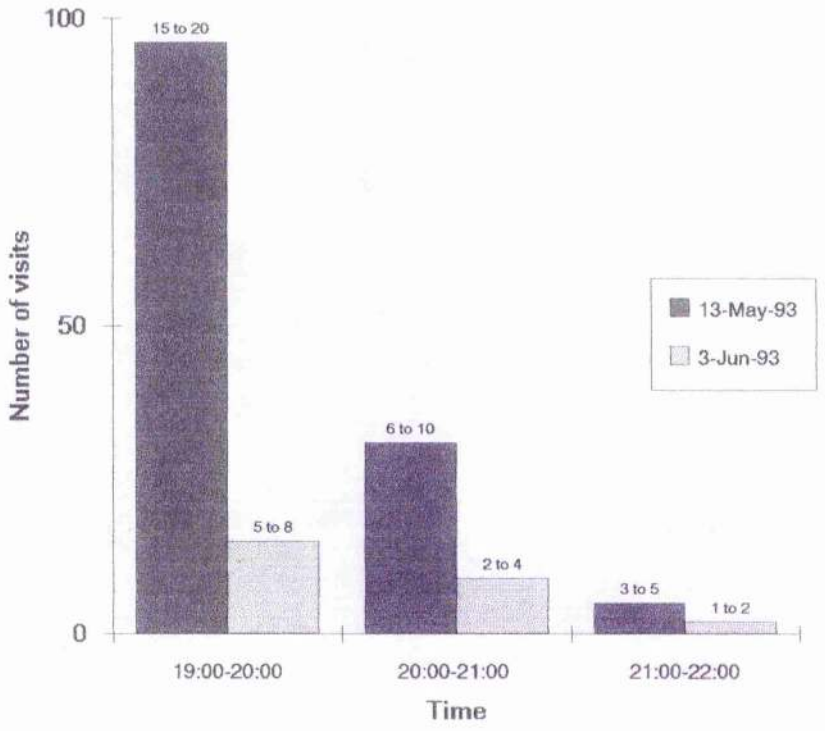

Figure 2.4 - Number of visits of *Phyllostomus hastatus* to flowers of one *Pseudobombax munguba* tree (#0) in the Catalão area at beginning (13 May) and during the peak (3 June) of the 1993 flowering season. Number of open flowers in the tree: 13 May = 42; 3 June = 58. Number at top of bars = estimated group size.

possible to observe, from platforms using head-lights that the ventral surface of *P. hastatus* was heavily covered by white pollen of *P. munguba* following periods of bat visitation

Table 2.3 lists the bats captured near flowering trees of *P. munguba* in the Catalão area during three different flowering seasons. Eleven of the twelve captured individuals of *P. hastatus* had pollen on the fur. The dense pollen load was spread along the ventral parts of the wings, body, and head. Microscopic analysis revealed only *P. munguba* pollen from the samples collected on the *P. hastatus* fur. Faeces collected from three of four *P. hastatus* individuals contained only *P. munguba* pollen, whereas the fourth one contained insect fragments and *Cecropia* seeds. No individuals of the other six captured species had pollen attached to their fur.

During the first hours of the day, before the stamens drop, bees of the genera *Trigona* and *Apis* visited the flowers to collect pollen from the anthers, and rarely touched the stigmata.

- Comparative floral structure and pollination

Pseudobombax tomentosum and *P. longiflorum* are two species from Central Brazil which share some floral characteristics with *P. munguba*, such as large, robust, white flowers with nocturnal anthesis. Both species flower during the dry season. *Pseudobombax longiflorum* is a treelet more commonly found in the 'campo limpo' and

Table 2.3 - Bats captured near flowering trees of *Pseudobombax munguba* in the Catalão area.

Date	Time	Species	load on the fur	load on the faeces
19 June 90	19:10	*Phyllostomus hastatus*	*P. munguba* pollen	-
	19:30	*Phyllostomus hastatus*	*P. munguba* pollen	-
	19:45	*Phyllostomus hastatus*	*P. munguba* pollen	*Cecropia* seeds
20 June 90	19:50	*Artibeus lituratus*	-	-
	20:05	*Carollia perspicillata*	--	-
	20:40	*Uroderma bilobatum*	-	-
06 June 91	19:00	*Molossus molossus*	-	-
	19:30	*Phyllostomus hastatus*	-	*Cecropia* seeds and insects
	20:15	*Artibeus jamaicensis*	-	*Cecropia* seeds
	20:15	*Artibeus jamaicensis*	-	-
	20:30	*Phyllostomus hastatus*	*P. munguba* pollen	-
01 July 92	19:00	*Noctilio albiventer*	-	-
	19:00	*Noctilio albiventer*	-	-
	19:10	*Noctilio albiventer*	-	-
	20:30	*Phyllostomus hastatus*	*P. munguba* pollen	*P. munguba* pollen
	20:30	*Phyllostomus hastatus*	*P. munguba* pollen	*P. munguba* pollen
20 June 93	19:10	*Phyllostomus hastatus*	*P. munguba* pollen	-
	19:10	*Phyllostomus hastatus*	*P. munguba* pollen	-
	19:20	*Phyllostomus hastatus*	*P. munguba* pollen	-
	19:30	*Phyllostomus hastatus*	*P. munguba* pollen	-
	19:30	*Phyllostomus hastatus*	*P. munguba* pollen	*P. munguba* pollen

'campo sujo', which are open grassland formations in the cerrado complex with impoverished woody vegetation. *Pseudobombax tomentosum* is a tree which occurs mainly in the borders of gallery forests and in semi-deciduous forests in the region.

Pseudobombax longiflorum bears flowers in a markedly inclined position, with 200-300 long (8-13 cm), bifurcated filaments attached to a large (4-5 cm long, 0.8-1 cm width) staminal column. The style is long (12-18 cm) and pendent. The flowers have a 'spherical brush' general appearance. The nectar accumulates in the chamber formed by the hypanthium, and the access to the nectar chamber is via the narrow space between the staminal column and the perianth. Each flower produces 500-1000 µl of nectar over night. In Central and Southeast Brazil flowers of *P. longiflorum* are visited by glossophagine bats and by *Phyllostomus discolor*, and, more rarely, by sphingid moths (R. Gribel, unpublished data; I. Sazima and M. Sazima personal communication).

The flowers of *P. tomentosum* are positioned erectly, with a funnel shaped androeceum composed of 700-1000 stamens, and a long (12-15 cm) erect style. The flowers have a 'paint-brush' general appearance. The nectar accumulates inside the relatively short (1.5-2.2 cm height) staminal column, so that the ovary is submersed in the nectar. The access to this nectar chamber is through the middle of the staminal column which is relatively wide (c. 1.5-2.0 cm). The flowers produce a

copious volume of nectar each night (three flowers from the same tree secreted 1012, 1487, and 1500 µl of nectar, respectively, between 19:00 to 24:00 h). The sugar concentration of this nectar was 13-14%. *Pseudobombax tomentosum* flowers were visited and probably pollinated by the arboreal marsupial *Caluromys lanatus* and, much more rarely, by an unidentified large species of phyllostomidae bat (Gribel, 1988).

The floral structure and pollination of *P. munguba*, *P. longiflorum*, and *P. tomentosum* are illustrated in fig. 2.5.

2.4 - DISCUSSION

- Why nectarless flowers?

One universally accepted feature of the syndrome of chiropterophily is the production of a large quantity of nectar (Faegri and van der Pijl 1979). In this respect, the occurrence of nectarless flowers by *P. munguba* is a noteworthy exception among chiropterophilous plants. The first question raised from the observations of the pollination system in *P. munguba* is if there is some adaptive advantage in having nectarless flowers.

In the tropical rain forest the selection for long-distance pollen flow tends to be higher than anywhere else (Janzen 1970, Bawa 1990). This study has shown that *Pseudobombax munguba*, the only species of the genus which has a widespread distribution in the Amazon Basin, is

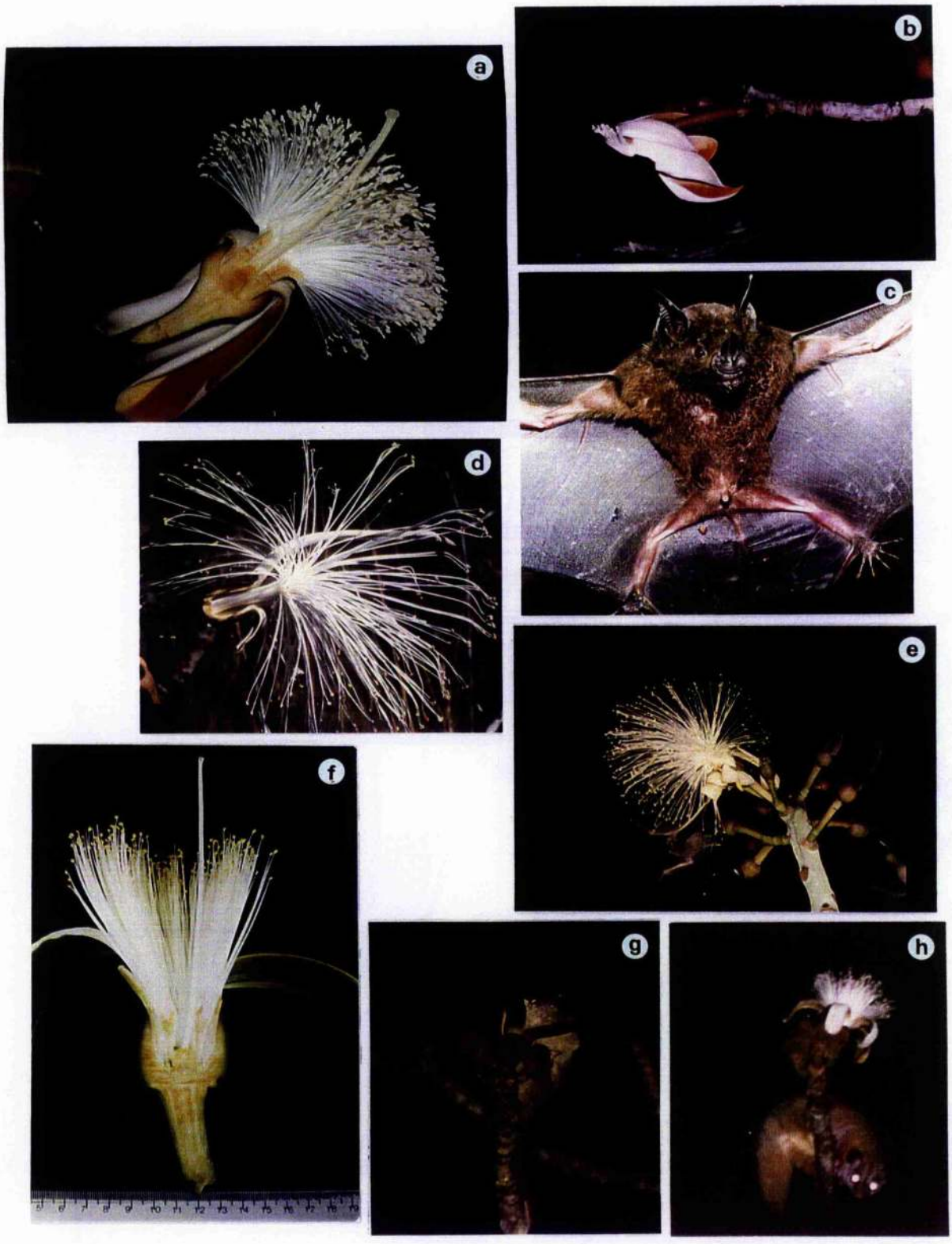

Figure 2.5 - (a) Section of the flower of *P. munguba*. **(b)** *Phyllostomus hastatus* visiting a flower of *P. munguba*. **(c)** General appearance of *P. hastatus*. **(d)** Section of the flower of *P. longiflorum*, showing the narrow access to the nectar chamber. **(e)** Glossophagine bat dropping backwards out the flower of *P. longiflorum* after the visit. **(f)** Section of the flower of *P. tomentosum*. **(g)** The marsupial *Caluromys lanatus* visiting the flower of *P. tomentosum*. **(h)** *Caluromys lanatus* leaving the flower after the visit.

self-sterile. Partial or total cross-sterility among related trees due to biparental inbreeding is, presumably, a common event as well (see Chapter 4). Therefore, fruit- and seed-set in *P. munguba* probably demand the occurrence of extensive pollen flow. Floral adaptations which maximise long-distance pollen shadows (or minimise geitonogamy or near-neighbour pollination) should be strongly favoured by selection.

The nectarless flowers of *P. munguba* seem to attract only *P. hastatus,* which is the largest neotropical flower-visiting bat. The daily forage area of an individual *P. hastatus* may comprise a distance over 10-20 km around the roosting site (Williams and William 1970), and so such bats are likely to be amongst the widest ranging floral visitors in the Neotropical region.

In contrast, the nectar-rich flowers of other *Pseudobombax* species and neotropical Bombacaceae in general are visited by assemblages of vertebrates such as other smaller bats, birds, marsupials, and monkeys (Heithaus et al. 1975, Toledo 1977, Janson *et al.* 1981, Eguiarte *et al.* 1987, Gribel 1988, Gribel *et al.* 1990). All these visitors and potential pollinators of 'nocturnal' Bombacaceae have a lower potential as long distance pollen vectors than *P. hastatus*. Thus, the high specificity of the *P. munguba* pollination system, achieved by producing nectarless flowers, results in a long distance and, presumably, 'high quality' (in the genetic sense) pollen delivery.

A possible evolutionary disadvantage of such a specific pollination system, and perhaps the reason why it is not more widespread among other neotropical Bombacaceae, is that the fruit- and seed-set depend on fluctuations of the pollinator density. Local extinction of the pollinator species can dramatically affect the plant population structure and cause its local extinction as well. Pollinator reliability, therefore, seems to be an essential factor for the evolution of specific pollination systems such as that observed in *P. munguba*.

- *Phyllostomus hastatus* as a pollinator

Phyllostomus hastatus is an omnivorous bat; fruits, nectar, pollen. insects, and small vertebrates are included in its diet (Gardner 1977). Its wide range in the Neotropical region, from Honduras in Central America to Bolivia and southeastern Brazil (Jones and Carter 1976), completely covers the distribution of *P. munguba*. It is usually a solitary forager (McCracken and Bradbury 1981, Kress and Stone 1993), but group foraging has been observed when large amounts of resources are available in temporary patches, as when *Hymenaea courbaril* (McCracken and Bradbury 1981) or *Ceiba pentandra* (see Chapter 5) are in flower.

The visits of *P. hastatus* to *P. munguba* flowers in three widely separated sites suggest that this specific pollination system is probably widespread in the Amazon. The observations of the present study indicate that, at

the beginning of the night, *P. hastatus* forages in groups searching for intensely flowered *P. munguba* trees. Later in the night, smaller foraging groups and solitary foragers are likely to exploit more efficiently the larger number of trees which produce few flowers. Such flexible foraging behaviour promotes almost complete random mating among the trees of the Catalão population (see Chapter 4). However, the much higher fruit-set by hand cross-pollination than by open-pollination (20-30% *versus* 2-7%) suggests that fruit production may suffer from some degree of pollinator limitation.

The fact that *P. hastatus* consistently visits *P. munguba* flowers, a species which only offers pollen in reward, suggests that this bat species probably is able to degrade and absorb the pollen proteins to some extent. The pollen picked up in the fur is probably ingested during the grooming. The germination of pollen in the stomach environment and the absorption of the pollen cellular contents by bats was proposed by Howell (1974, 1976) for *Leptonycteris* and other nectarivorous-pollenivorous bats. The large size of *P. hastatus* and, in consequence, its large gastrointestinal tract, may result in a relatively long stay for the pollen in the stomach environment which may facilitate the pollen germination and absorption process.

- *Pseudobombax munguba* pollination and the várzea food chain

The flowering phenology of *P. munguba* fits the 'cornucopia' flowering type (*sensu* Gentry 1974) which is characterised by the production of moderate number of flowers over a period of several weeks. The estimation of the total number of flowers (18,079) and of the fruit-set (4.2%) give a projection of around 760 fruits and nearly 28kg of seeds (c. 1.2 million of seeds given an average of 1,548 seeds per capsule) produced during the 1993 reproductive season in the studied plot. A much higher level of seed production should be expected in more well established várzeas, normally with older and larger *P. munguba* trees. During the fruit ripening and seed dispersal period (from July to September), the water level of the Amazon and tributaries gets lower, rapidly exposing extensive open areas of moist soil and mud, which are potential sites for colonisation by *P. munguba*. A high proportion of the seeds, however, that are randomly dispersed by wind, fall into the water and are consumed by fishes. Seeds of *P. munguba* in the fish digestive tracts are totally triturated (R. Gribel personal observation), indicating that fish act as seed predators rather than dispersors.

The self-sterile tree *P. munguba* depends exclusively on the floral visits of the bat *P. hastatus* to set seeds, which are important food resources for fishes and, ultimately, for the men of the várzea habitat. This case

study exemplifies how organisms from a tropical habitat are interconnected by the web of biological interactions, and how apparently unlinked events, as bat visits to flowers and human carrying capacity of a given environment, may be related to some extent.

- Congeneric comparisons

Congeneric comparisons between pollination systems are useful because divergence in floral characteristics may reflect mainly the effect of different selective pressures made by pollinators on floral structure and function, rather than phylogenetic differences. The flowers of the three *Pseudobombax* species reported in this study share some features in common, such as nocturnal anthesis, white colour, large size, and strong structure, which suggest pollination by nocturnal mammals. However, a variable array of nocturnal mammals visit and pollinate these *Pseudobombax* flowers, ranging from the small glossophagine bats with hovering flights, to comparatively large species of non-glossophagine bats and marsupials which 'land' on the flowers. The differences in size, shape, and behaviour between the main pollinators of these three *Pseudobombax* species are likely to have modulated some of the observed differences in their floral traits.

Several clues indicate that *P. tomentosum* is one of the few neotropical species adapted to pollination by nocturnal non-flying mammals (Gribel 1988). Effective

pollination of *P. munguba* by *Caluromys* probably occurs because: (1) this marsupial species visits the flowers regularly and in a non-destructive fashion, (2) the relative small number of flowers set per night and the clumped distribution of *P. tomentosum* in deciduous forests or along of the edges of gallery forests permit the movement of this arboreal marsupial, through the canopy, between trees and perhaps between clumps, (3) the general floral architecture - vertically oriented flowers; the long, strong but flexible style which holds the stigma above the level of the anthers; the deep nectar chamber with wide access; and the 'paint-brush' funnel-shaped androecium with near one thousand anthers - all form an appropriate structure to offer nectar and to deposit and collect pollen from the funnel shaped head/snout of marsupial species such as *Caluromys*. Additionally, legitimate visits by bats do not occur or are very rare events in the flowers of this species. Pollination by larger non-glossophagine bats, which normally land on the flowers, seems unlikely because of the large distance separating the stigma from the nectar chamber (c.13-16 cm). Glossophagine bats were never observed foraging on *P. tomentosum* flowers probably because such floral architecture precludes their typical hovering flight visits to take nectar (Gribel 1987).

In contrast, the inclined flowers of *P. longiflorum* with the 'ball' of stamens shape, pendant style, and a very narrow access to the nectar appears to be more

effective for use by flying vectors with fine tongues (or proboscis) as pollinators. Glossophagine bats are probably important pollinators of *P. longiflorum*, since they always touch the anthers and usually touch the stigma when arriving and/or leaving the flowers (R. Gribel, personal observation). *Phyllostomus discolor*, a bat frequently cited visiting the same flowers visited by glossophagines (Heithaus *et al.* 1974, 1975, Ramirez *et al.* 1984, Eguiarte *et al.* 1987, Gribel and Hay 1993), probably reaches the nectar by forcing the staminal column with its snout, so as to open a space to introduce its tongue into the nectar chamber.

The large size and strong structure of the *P. longiflorum* flowers may be an ancient trait inherited from an ancestor pollinated by a larger animal, since flowers pollinated by glossophagines, and even *P. discolor*, usually are smaller and/or more delicate (Vogel 1968, 1969a, 1969b, Heithaus *et al.* 1974, Sazima and Sazima 1975, 1978, 1988, Sazima *et al.* 1982, 1989, 1994, Lemke 1984, 1985, Hokche and Ramirez 1990, Ramirez *et al.* 1984, Buzato and Franco 1992, Gribel and Hay 1993). The structure of the *P. longiflorum* flowers does not preclude the visits by sphingid moths. The rarity of this potential pollinator could be caused by the relatively low nocturnal temperatures during June and July in the Central Brazil, or related with the nature of some unmeasurable attractants such as the flower scent and/or the nectar palatability.

Pseudobombax munguba has many features in common with *P. tomentosum*, such as the strong general structure of the flower (especially the robust gynoecium), the short, wide, and strong staminal column, and the huge amount of powdery pollen produced by around 1000 anthers, which seem to be adaptations for pollination by relatively heavy and large animals. Some of *P. munguba* floral traits are also shared with *P. longiflorum*, such as the inclined orientation of the flowers and the 'ball of stamens' shape of the androecium. These characteristics are probably related with the pollination by flying vectors, which can approach the flower from any direction. In sum, the flowers of *P. munguba* present structural and morphological adaptations compatible with the pollination by a relatively heavy and large flying vector with 'landing-visits' (non-hovering visits).

The presence, in most of the *Pseudobombax* species, of floral characteristics such as large size, strong structure, hundreds of anthers, and white colour, suggest pre-adaptation to pollination by nocturnal vertebrates in the original species of the genus. Considering the traits noted above, and the primitiveness of the group (Aubréville 1975), one can hypothesise that the original pollinators were arboreal marsupials which were common during the period of irradiation of the more primitive groups of angiosperms (Sussmann and Raven 1978). *Pseudobombax tomentosum* may have maintained much of the original floral traits from these putative ancestors.

With the evolution of the neotropical Phyllostomidae in the late Miocene (Smith 1976), species such as *P. munguba* and *P. longiflorum* probably irradiated to exploit different types of these more recent and mobile pollinators. An alternative view is the occurrence of an ancestral pre-adaptation for visitation by megachiroptera bats in plants of paleotropical origin which became incorporated to the neotropical flora (Baker 1973). On this view, floral structures as those of *P. munguba*, adapted to pollination by large bats which land or the flowers like many old-world Pteropodidae, should be considered as more primitive, whereas *P. tomentosum* and *P. longiflorum* could be evolved to explore alternative pollination niches.

Chapter 3

Genetics of the Cytosolic Phosphoglucose Isomerase (PGI) Variation in *Pseudobombax munguba*

3.1 - INTRODUCTION

The current work was initiated to resolve a reliable genetic marker system for use in studies of the breeding and mating system of *P. munguba*. Several recent investigations of the mating system of tropical trees have used isozyme markers to estimate rates of outcrossing (O'Malley and Bawa 1987, O'Malley *et al.* 1988, Murawski and Hamrick 1991, 1992, Murawski *et al.* 1990, 1994, Alvarez-Buylla and Garay 1994, Hall *et al.* 1994, Boshier *et al.* 1995). However, the markers used in these studies were not normally subject to a formal genetic analysis of inheritance (but see Alvarez-Buylla and Garay 1994), probably due to the operational difficulties in regard to hand pollinating tall rain forest trees.

The high chromosome number of *P. munguba* (2n = 84, Morawetz 1986, Baum and Oginuma 1994) and the evolutionary history of the Bombacaceae family suggest a paleopolyploid origin for this species. However, no information about the species' ploidy level nor its putative ancestors is available. In this Chapter I report

the results of a genetic analysis of the cytosolic phosphoglucose isomerase (PGI) phenotypes resolved in a population of *P. munguba* in the Central Amazon. The analysis has led to the detection of two diallelic loci controlling the cytosolic PGI variation in *P. munguba* and the resolution of two sets of genetic markers that are suitable for future studies of the reproductive biology of the species.

3.2 - MATERIALS AND METHODS

- The study site and the trees

The study site was located in the Ponta do Catalão, a peninsular area where the Rio Negro and Rio Solimões join to form the Amazon river, about 4.5 km SE of the city of Manaus (ca. 03°08'S, 60°00'W), Amazonas state, Brazil. The area, covered by the "várzea" vegetation, is annually inundated for 4-7 months during the period of high waters. In this area *P. munguba* is one of the dominant arboreal species.

- Electrophoretic procedures

The following enzyme systems were initially assayed: Phosphoglucose isomerase (PGI, E.C. 5.3.1.9), Phosphoglucomutase (PGM, E.C. 5.4.2.2), Alcohol dehydrogenase (ADH, E.C. 1.1.1), Glucose-6-phosphate dehydrogenase (G6PDH, E.C. 1.1.1.49), Malate dehydrogenase (ME, E.C. 1.1.1.40), Esterase (EST, E.C.

3.1.1.-), and Peroxidase (PER, E.C. 1.11.1.7). Only PGI, PGM, and PER presented clear bands and polymorphisms on starch gels. A progeny analysis showed inconsistency between the genotypes of the parent trees and the seedlings for PER, suggesting that different loci encode this monomeric enzyme and are expressed at different developmental stages. It was decided, therefore, not to investigate PER further as a system suitable for the development of genetic markers in *P. munguba*. Although clear and repeatable banding patterns were obtained on gels stained for PGM, further analysis of this enzyme was also discontinued due to the expense of the staining procedure relative to that used for resolving PGI. To resolve PGM, twice the amount of glucose-6-phospate dehydrogenase was required relative to that used to resolve PGI.

Of the seven enzyme systems initially assayed, therefore, only PGI was investigated further. PGI is a dimeric enzyme that catalyses the reversible isomeration of glucose-6-phosphate and fructose-6-phosphate (Stryer 1988). In plants, PGI is usually compartmentalised in the cytoplasm and in the chloroplast (Schuarrenberger & Oeser 1974, Schuarrenberger *et al.* 1975, Simcox and Dennis 1978, Weeden and Gottlieb 1980, Weeden 1983). The plastid PGIs are generally more anodal and exhibit less electrophoretic variability than PGI from the cytoplasm (Gottlieb and Weeden 1981).

Leaf tissue samples (ca. 1 $cm^{2)}$ were crushed with a glass rod in a chilled microtiter plate using the extraction buffer of Mitton *et al.* (1979). Filter paper wicks containing the crude leaf extract were inserted in a 12% (w/v) starch gel for horizontal electrophoresis. Morpholine-citrate electrode and gel buffers (system 2 in Wendel and Weeden 1989) were used in the electrophoresis after modifying pH to 8.0. An electric current of 40 mA (ca. 250 V) was applied to the gels for four and a half hours, before gels were sliced and stained. The staining protocol for PGI followed that described by Wendel and Weeden (1989).

- Analysis of PGI variation

Adults: Fifty six *P. munguba* trees were randomly chosen for analysis. Branches with young leaves were collected from trees in the early morning, transported to the laboratory, and analysed up to two hours later. Enzymes were extracted from fresh young leaf tissue.

Seedlings: Seeds contained in 30 open-pollinated fruits (one fruit per tree) and also in 14 fruits produced following hand cross-pollinations (see below) were collected at maturity approximately 3 months after flower anthesis. Pairs of trees used in the cross-pollinations had been previously assayed and were of known PGI phenotype. Seeds from each fruit were washed with 30% solution of sodium hypochlorite, rinsed, and sown in Petri dishes on moist filter paper. Nearly all seeds

germinated in 2-3 days. Enzymes were extracted from the cotyledons of seedlings 7-10 days after germination. A total of 754 seedlings from open-pollinated and 668 seedlings from hand cross-pollinated fruits was analysed.

- Controlled pollinations

The lower branches of trees were reached by canoe during the period of 'high waters'. Floral buds were opened, emasculated, and enclosed in paper bags shortly before anthesis at approximately 18:30 h. Massive loads of self- (in self-pollinations) or cross-pollen (in cross-pollinations) were deposited on stigmas at around 20:00 h. Flowers were then re-bagged until 10:00-11:00 h the following morning. *Pseudobombax munguba* produced no fruits following self-pollination (fruit-set = 0%, N = 406 self-pollinated flowers), and, consequently, only seeds from fruits resulting from hand cross-pollinations and open-pollinations were analysed.

3.3 - RESULTS

- Cytosolic PGI phenotypes

Nine different PGI phenotypes (fig. 3.1) were resolved among the 1478 individuals assayed (56 adult trees, plus 754 and 668 seedlings from open- and cross-pollinated fruits respectively). All individuals shared a single band in the most anodal zone (approx. 55 mm from

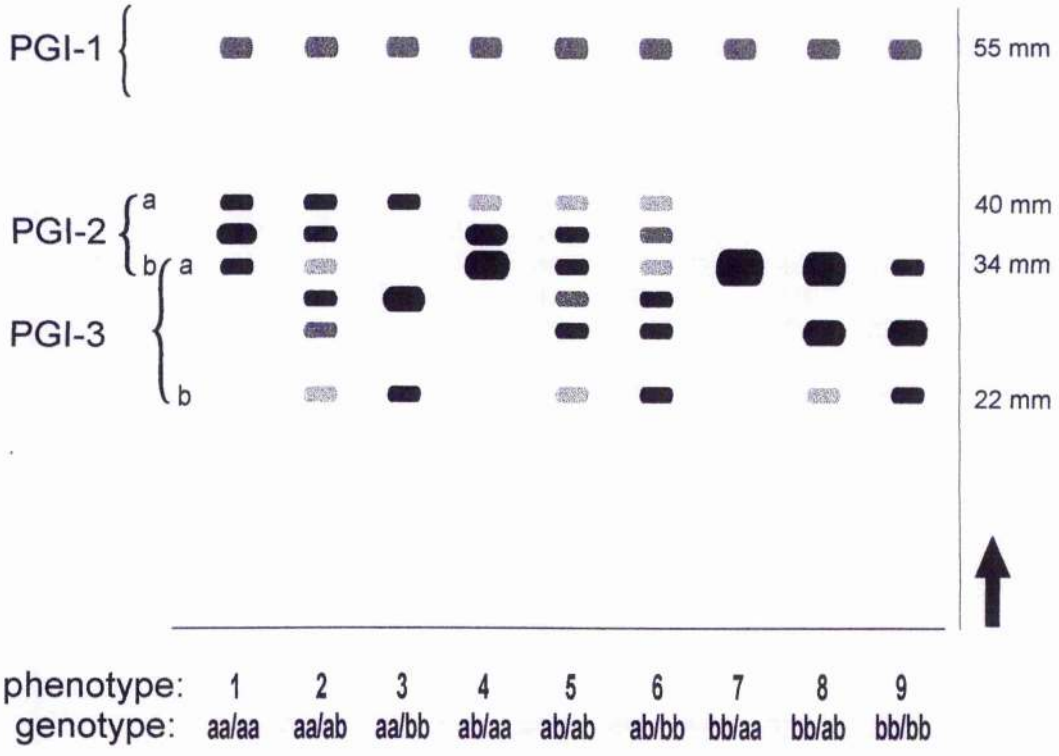

Fig. 3.1 - Eletrophoretic phenotypes and suggested genotypes at *Pgi-2* and *Pgi-3* in *Pseudobombax munguba*.

the origin). This enzyme, designated PGI-1, was not well resolved on most gels, and, by analogy with the findings of studies on other plant species, was inferred to be the chloroplastic PGI (Schnarrenberger *et al.* 1975, Gottlieb and Weeden 1981, Gottlieb 1982, Weeden and Gottlieb 1980).

The less anodal zone presented phenotypes with 1 to 6 well resolved bands of several staining intensities. These bands were separated at regular distances of 3 mm, except the 5^{th} and the 6^{th} bands which were separated by 6 mm. Isozymes resolved in the less anodal zone have been reported as variant forms of cytosolic PGI in other plants (Gottlieb and Weeden 1981). High levels of isozyme variability for cytosolic PGI are usually the result of a gene duplication (Adams and Allard 1977, Gottlieb 1977, 1982, Goldring *et al.* 1985, Lack and Kay 1986, Lumaret 1986, Pascual *et al.* 1988, Cai and Chinnappa 1989, Rovira *et al.* 1993).

A two locus diallelic model would explain the variability of cytosolic PGI phenotypes observed in *P. munguba*. It is proposed, therefore, that the first locus, designated *Pgi-2*, has two alleles ($Pgi\text{-}2^a$ and $Pgi\text{-}2^b$) which code for two subunits (PGI-2a and PGI-2b) with electrophoretic mobility of 40 mm and 34 mm from the origin respectively (fig. 3.1). These two enzyme subunits associate, in heterozygous individuals (genotype $Pgi\text{-}2^{ab}$), to form an intralocus hybrid heterodimer that migrates halfway between the two homodimers.

The second set of cytosolic PGI enzymes, designated PGI-3, may be considered to be coded by the *Pgi-3* locus. Two alleles at this locus ($Pgi\text{-}3^a$ and $Pgi\text{-}3^b$) encode two subunits (PGI-3a and PGI-3b) which migrate 34 and 22 mm from the origin respectively. The intralocus heterodimer produced by these two alleles in heterozygotes exhibits an electrophoretic mobility that is intermediate (28 mm) to that of the two homodimers. The overlap of the faster enzyme of the *Pgi-3* (PGI-3a) and the slower of the *Pgi-2* (PGI-2b) at 34 mm from the origin was not exact. In gels that were run for 5 h (instead of the standard 4½ h) the two enzymes could be separated, with the PGI-3a enzyme migrating to a slightly more anodal position than PGI-2b.

It was interpreted that interlocus hybrid enzymes (interlocus heterodimers), showing intermediate mobility, were formed between the following pairs of homodimers: PGI-2a/PGI-3a, PGI-2a/PGI-3b, and PGI-2b/PGI-3b. These heterodimers were located on gels approximately midway between the respective homodimers (at 37, 31 and 28 mm from the origin respectively). An interlocus heterodimer between PGI-2b and PGI-3a was not observed on most gels due to the overlap of the two homodimeric enzymes, but was visible on gels which ran for more than 5 h.

The model described above allows genotypes to be assigned to each of the nine PGI phenotypes resolved, in the manner shown in fig. 3.1. The relative staining intensities of the bands, which were clearly

distinguishable on all gels, were crucial to the correct designation of genotypes. The postulated genotypes for the nine observed phenotypic patterns can be explained as following:

- Phenotype 1; genotype *Pgi-2*^{aa} *Pgi-3*^{aa} - This homozygous genotype resulted in a balanced three-banded phenotype with the homodimers PGI-2a and PGI-3a at 40 and 34 mm from the origin respectively and each one with regular staining intensity. The intergenic heterodimer was approximately half way between the two homodimers (sometimes slightly beneath the midway point) and had around twice their stain intensity.

- Phenotype 2; genotype *Pgi-2*^{aa} *Pgi-3*^{ab} - This a six-banded phenotype, with the 1st, 2nd and 4th bands more intensely stained than the others. There were three homodimer sub-units: PGI-2a (40 mm, regular staining intensity), PGI-3a (34 mm, faintly stained), and PGI-3b (22 mm, faintly stained). The intralocus heterodimer at Pgi-3 (PGI-3a/PGI-3b at 28 mm) was less stained than the interlocus heterodimers PGI-2a/PGI-3a and PGI-2a/PGI-3b.

- Phenotype 3; genotype *Pgi-2*^{aa} *Pgi-3*^{bb} - This double homozygous genotype produced a balanced three-banded phenotype with homodimer sub-units of regular staining intensity located at 40 and 22 mm from the origin (PGI-2a and PGI-3b respectively). The interlocus heterodimer (PGI-2a/PGI-3b) was placed approximately half-way between the outer bands (at 31 mm from the origin) and stained

approximately twice as intensily as the homodimer sub-units.

- **Phenotype 4; genotype *Pgi-2^{ab} Pgi-3^{aa}*** - This is a three-banded phenotype with unbalanced (or asymmetric) banding pattern. The most anodal sub-unit, the PGI-2a homodimer at 40 mm from the origin, was faintly stained. The PGI-2b homodimer overlapped with the double PGI-3a homodimer forming a broad and very darkly stained band at 34 mm from the origin. The intra and interlocus heterodimers overlapped the half-way between the two homodimers sub-units, at 37 mm from the origin, producing a band slightly less stained than that at 34 mm.

- **Phenotype 5; genotype *Pgi-2^{ab} Pgi-3^{ab}*** - The double heterozygous individuals had a six-banded phenotype with the 2nd, 3rd, and 5th bands more intensely stained. The overlapped homodimeric subunits PGI-2b and PGI-3a at 34 mm, the overlapped intra (PGI-2a/PGI-2b) and inter (PGI-2a/PGI-3a) heterodimers at 37 mm and the overlapped intra (PGI-3a/PGI-3b) and inter (PGI-2a/PGI-3b) heterodimer formed the more darkly stained bands in this phenotype. The faintly stained bands were the homodimers PGI-2a (40 mm) and PGI-3b (22 mm), and the resulting interlocus heterodimer PGI-2a/PGI-3b (31 mm).

- **Phenotype 6; genotype *Pgi-2^{ab} Pgi-3^{bb}*** - This is a six-banded phenotype with the 4th, 5th, and 6th bands more darkly stained than the others. The homodimers PGI-2a (40 mm) and PGI-2b (34 mm) were the faintest bands. The intralocus heterodimer PGI-2a/PGI-2b (37 mm) was slightly

more stained than the respective homodimers. The three bands more intensively stained were the interlocus heterodimers PGI-2a/PGI-3b (31 mm) and PGI-2b/PGI-3b (27 mm), and the double homodimer PGI-3b (22 mm).

- Phenotype 7; genotype *Pgi-2*^{bb} *Pgi-3*^{aa} - This genotype was manifested by an one-banded phenotype resulting from the overlapping of the PGI-2b and PGI-3a homodimers and the putative interlocus heterodimer product. The centre of the very wide and dark band is located approximately 34 mm from the origin.

- Phenotype 8; genotype *Pgi-2*^{bb} *Pgi-3*^{ab} - This genotype showed an unbalanced three-banded phenotype. The most anodal band, resulting from the overlapping of the homodimer PGI-2b with the double homodimer PGI-3a, at 34 mm from the origin, was broad and strongly stained. The less anodal enzyme band PGI-3b at 22 mm from the origin, was faintly stained. Mid-way between those two bands occurred an intermediate band resulting from the overlapping of the interlocus heterodimers PGI-2b/PGI-3b with the intralocus heterodimer PGI-3a/PGI-3b. This intermediate band is less stained than the most anodal and much more intensively stained than the less anodal.

- Phenotype 9; genotype *Pgi-2*^{bb} *Pgi-3*^{bb} - This is a balanced three-banded phenotype. The most anodal band enzyme in this phenotype, at 34 mm from the origin, was the homodimer PGI-2b, whereas the less anodal was the homodimer PGI-3b at 22 mm. The intergenic heterodimer, PGI-2b/PGI-3b, was located mid-way between the two outer

homodimers and stained approximately twice as intensely as those bands.

- Progeny analysis

Based on the two locus diallelic model proposed above, *Pgi-2* and *Pgi-3* genotypes were assigned to maternal trees and the offspring of 30 open-pollinated families surveyed (table 3.1). Twenty two trees were found to exhibit phenotype 1 (fig. 3.1) and were classified, therefore, to be of $Pgi\text{-}2^{aa}$, $Pgi\text{-}3^{aa}$ genotype. Five other trees were also homozygous for $Pgi\text{-}2^{a}$, but were either heterozygous or homozygous for the *b* allele at *Pgi-3*. Due to the rarity in the population of *b* alleles at both loci, only two trees were homozygous for either $Pgi\text{-}2^{b}$ or $Pgi\text{-}3^{b}$.

Offspring genotypes within each open-pollinated family array (table 1) were always in accordance to those expected, based on the genotype assigned to the mother tree and the genotypes assigned to other trees (potential pollen donors) within the population sample. In no instance, therefore, was an offspring genotype recorded that could not have arisen in the family of a maternal tree of assigned genotype.

The results of the controlled crossing programme are presented in table 3.2 with offspring phenotypes illustrated in fig. 3.2. Figure 3.2 illustrates the ease with which different phenotypes were identified in progeny arrays even when differences were based simply on

Table 3.1 - Maternal and offspring genotypes for 30 open-pollinated families of *Pseudobombax munguba* at the *Pgi-2* and *Pgi-3* loci.

Tree	Genotype at *Pgi-2/Pgi-3*	Offspring genotypes at *Pgi-2/Pgi-3* *									
		aa/aa	*aa/ab*	*aa/bb*	*ab/aa*	*ab/ab*	*ab/bb*	*bb/aa*	*bb/ab*	*bb/bb*	Total
#0	*aa/aa*	22	3	-	0	0	-	-	-	-	25
#3	*aa/aa*	25	0	-	0	0	-	-	-	-	25
#3A	*aa/aa*	13	7	-	5	0	-	-	-	-	25
#3B	*aa/aa*	12	4	-	8	0	-	-	-	-	24
#5B	*aa/aa*	24	0	-	0	0	-	-	-	-	24
#5D	*aa/aa*	9	6	-	5	3	-	-	-	-	23
#5F	*aa/aa*	21	1	-	1	2	-	-	-	-	25
#6	*aa/aa*	24	0	-	0	0	-	-	-	-	24
#8A	*aa/aa*	11	10	-	2	1	-	-	-	-	24
#10A	*aa/aa*	15	3	-	3	2	-	-	-	-	23
#10B	*aa/aa*	22	1	-	1	1	-	-	-	-	25
#10D	*aa/aa*	18	6	-	0	0	-	-	-	-	24
#12	*aa/aa*	18	0	-	4	3	-	-	-	-	25
#17	*aa/aa*	24	0	-	0	0	-	-	-	-	24
#21	*aa/aa*	16	6	-	2	0	-	-	-	-	24
#N5	*aa/aa*	16	3	-	2	3	-	-	-	-	24
#N12	*aa/aa*	14	8	-	1	0	-	-	-	-	23
#N13	*aa/aa*	17	3	-	5	0	-	-	-	-	25
#M2	*aa/aa*	20	2	-	0	2	-	-	-	-	24
#M8	*aa/aa*	17	2	-	3	2	-	-	-	-	24
#A2	*aa/aa*	11	9	-	3	0	-	-	-	-	23
#B3	*aa/aa*	19	0	-	6	0	-	-	-	-	25
#11A	*aa/ab*	10	9	2	1	1	1	-	-	-	24
#22	*aa/ab*	9	9	4	0	2	1	-	-	-	25
#B1	*aa/ab*	9	6	3	3	4	0	-	-	-	25
#N2	*aa/ab*	1	5	3	5	10	1	-	-	-	25
#N9	*aa/bb*	-	12	5	-	8	0	-	-	-	25
#5E	*ab/aa*	5	0	-	17	1	-	2	0	-	25
#W	*ab/ab*	6	3	0	5	8	1	1	1	0	25
#N3	*bb/ab*	-	-	-	18	18	6	2	3	1	48
											754

(*). Zero value is included when the genotype indicated could have arisen amongst the offspring of a given tree, although was not found.

Table 3.2 – Genotype segregation for *Pgi-2* and *Pgi-3* in the F_1 progenies from crossing in *Pseudobombax munguba*.

Parent trees	Parent genotypes	Expected progeny	Expected ratio	Observed distribution	Chi-square	*P*
#16 x #10C	*aa/aa x aa/aa*	*aa/aa*	1	48		
#6 x #4	*aa/aa x aa/aa*	*aa/aa*	1	48		
#5F x #12B	*aa/aa x ab/aa*	*aa/aa*	1	37	0.36	0.549
		ab/aa	1	32		
#12 x #N2	*aa/aa x aa/ab*	*aa/aa*	1	25	0.19	0.663
		aa/ab	1	22		
#10C x #N2	*aa/aa x aa/ab*	*aa/aa*	1	25	0.82	0.365
		aa/ab	1	19		
#0 x #11	*aa/aa x ab/bb*	*aa/ab*	1	23	0.08	0.777
		ab/ab	1	25		
#4 x #1	*aa/aa x ab/bb*	*aa/ab*	1	9	1.96	0.162
		ab/ab	1	16		
#5A x #N3	*aa/aa x bb/ab*	*ab/aa*	1	22	0.33	0.566
		ab/ab	1	26		
#A1 x #N3	*aa/ab x bb/ab*	*ab/aa*	1	14	1.13	0.568
		ab/ab	2	20		
		ab/bb	1	12		
#N4 x #N3	*aa/ab x bb/ab*	*ab/aa*	1	11	0.46	0.795
		ab/ab	2	23		
		ab/bb	1	14		
#B1 x #11	*aa/ab x ab/bb*	*aa/ab*	1	10	3.83	0.280
		aa/bb	1	13		
		ab/ab	1	8		
		ab/bb	1	17		
#11 x #N2	*ab/bb x aa/ab*	*aa/ab*	1	16	1.07	0.784
		aa/bb	1	13		
		ab/ab	1	15		
		ab/bb	1	11		
#W x #11	*ab/ab x ab/bb*	*aa/ab*	1	9	4.43	0.489
		aa/bb	1	4		
		ab/ab	2	10		
		ab/bb	2	14		
		bb/ab	1	3		
		bb/bb	1	6		
#11A x #W	*aa/ab x ab/ab*	*aa/aa*	1	4	1.25	0.940
		aa/ab	2	13		
		aa/bb	1	5		
		ab/aa	1	7		
		ab/ab	2	12		
		ab/bb	1	7		

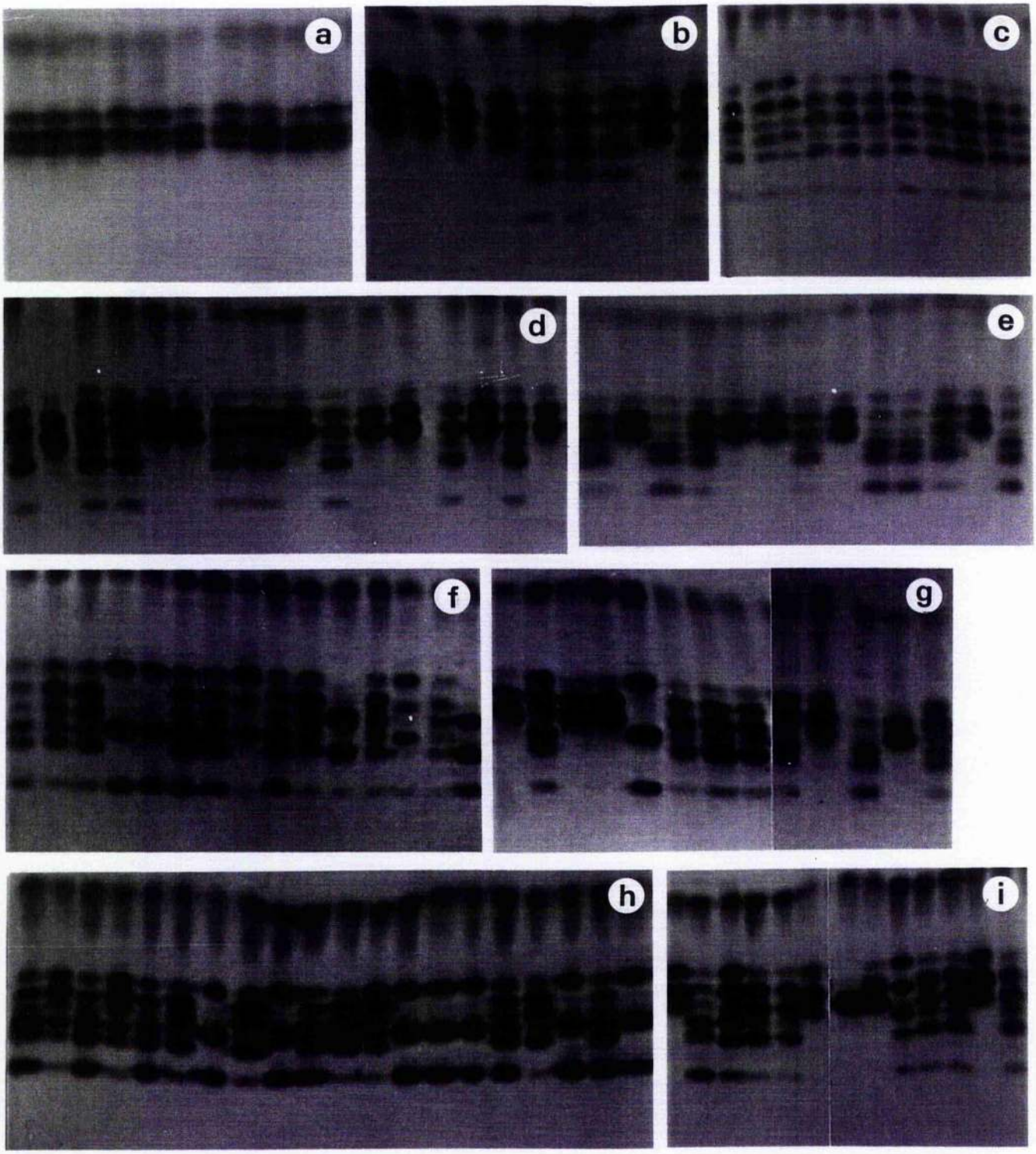

Figure 3.2 - PGI zymograms of progenies from cross-pollinations (a-h) and open-pollination (i) of *Pseudobombax munguba*. Parental trees are noted as *Pgi-2/Pgi-3* genotypes. Progeny phenotype numbers and correspondent genotypes are as given in fig. 1. **(a)** *aa/aa x aa/aa*; progeny exhibiting the 'fixed heterozygous' banding pattern of the phenotype 1; **(b)** *aa/aa x aa/ab*; progeny phenotypes: 1/1/1/1/2/2/2/1/2; **(c)** *aa/aa x ab/bb*; progeny phenotypes: 5/2/2/5/5/5/2/5/5/5/5; **(d)** *aa/aa x aa/ab*; progeny phenotypes: 5/4/5/5/4/4/5/5/4/5/4/4/5/4/5/4; **(e)** *aa/ab x bb/ab*; progeny phenotypes: 5/4/6/5/4/4/5/4/6/6/5/4/6; **(f)** *ab/ab x ab/bb*; progeny phenotypes: 6/4/4/3/3/6/6/2/6/5/8/5/2/5/9; **(g)** *aa/ab x ab/ab*; progeny phenotypes: 4/2/4/4/3/5/5/5/2/1/6/2; **(h)** *aa/ab x ab/bb*; progeny phenotypes: 6/2/6/2/6/5/3/5/ 3/6/5/2/3/3/3/6/5/3/6/3; **(i)** *ab/ab*; progeny phenotypes: 1/6/2/5/5/1/7/4/2/5/5/1/5.

the relative staining intensity of particular bands. Only one of the nine PGI phenotypes presented in fig. 3.1 was not generated by the crosses undertaken. This was the single banded phenotype 7 produced by individuals of genotype *Pgi-2*^{bb} *Pgi-3*^{aa}. However, five seedlings with this phenotype were detected among the open-pollinated progenies surveyed (fig. 3.2i and table 3.1). In contrast, only one seedling homozygous for the rarest allele at both loci (phenotype 9, produced by individuals of genotype *Pgi-2*^{bb} *Pgi-3*^{bb}) was detected among 754 seedlings surveyed from field (table 3.1). Six seedlings with this phenotype, however, were recovered from the cross between genotypes *Pgi-2*^{ab} *Pgi-3*^{ab} and *Pgi-2*^{ab} *Pgi-3*^{bb} (table 3.2).

An examination of the segregation data (table 3.2) showed that deviations from Mendelian expected ratios were not significant for single locus or two locus segregations in any of the families of the 14 crosses examined. Thus, the segregations were as expected based on the two locus diallelic model that is proposed for the variation of PGI-2 and PGI-3 resolved in *P. munguba*.

3.4 - DISCUSSION

The results of the present study support the hypothesis that cytosolic PGI variation in *P. munguba* is controlled by two diallelic loci that assort independently of each other and exhibit disomic

inheritance. Previous studies on the mode of inheritance of phosphoglucose isomerase (PGI) variation in plants have shown that in many species the observed segregation pattern does not depart significantly from Mendelian expectations (Adams & Allard 1977, Gottlieb 1977, Torres *et al.* 1985, Hyon *et al.* 1987, Prentice and Giles 1993, Rovira *et al.* 1993), whereas others have shown that segregation distortion sometimes occurs (Adams and Joly 1980, Cheliak and Pitel 1984, Strauss and Concle 1986, Aravanopoulos *et al.* 1993). PGI duplicated genes have been reported to assort independently in *Clarkia* (Gottlieb 1977, Gottlieb and Weeden 1979, Weeden and Gottlieb 1979) and *Corylus* (Rovira *et al.* 1993), but were found to be linked in studies on the autopolyploid complex of *Dactylis glomerata* (Lumaret 1986).

As segregation distortion in families of the size raised here can be detected only when it is of an extreme kind (Mulcahy and Kaplan 1979), the conclusion that there was no segregation distortion in the families of crosses examined in the present study should be accepted with caution. However, the fact that no deficit or excess of any particular genotype was systematically observed within the 14 families examined is evidence in favour of independent inheritance.

No excess of genotypes identical to the female parent was found among cross- and open-pollinated progenies, suggesting that apomixis did not occur or occurred at an undetectable level in the plants tested.

Furthermore, no apomict seedlings were found among the 215 seedlings assayed from the 6th to the 10th families listed in table 3.2. In these families any agamospermic seedlings would be unambiguously differentiated from those resulting from crossing. Thus, although apomixis has been reported to occur in the Bombacaceae (Baker 1960, Duncan 1970, Oliveira *et al.* 1992), there was no evidence of its occurrence in the present study of *P. munguba*.

The two cytosolic PGI loci of *P. munguba* are likely to be structurally very similar as their homodimeric products associate to form interlocus heterodimeric enzymes. It remains an open question whether the postulated duplication of the PGI gene in *P. munguba* originated by the duplication of a chromosome section by unequal crossing-over (as proposed for *Dactylis* by Lumaret 1986), by chromosome rearrangements (as in *Clarkia* according to Gottlieb (1977)), or as a result of allopolyploidy (as further proposed for *Clarkia* by Gottlieb and Higgins 1984). The high chromosome number and the occurrence of another duplicated isozyme locus (*Pgm*) in the species (Gribel and Abbott per. obs.) could suggest that the latter process (allopolyploidy) may be the more likely cause of duplication of the PGI gene in *P. munguba*.

The non-distorted segregation ratios resulting from the crossing experiments further suggest that there is no detectable difference in fitness among the diverse pollen

Chapter 4

Factors Affecting the Fertility and the Mating System of *Pseudobombax munguba*

4.1 - INTRODUCTION

Most previous studies on the mating system of tropical trees using isozyme markers have provided evidence of high levels of outcrossing in such species (O'Malley and Bawa 1987, O'Malley *et al.* 1988, Murawski *et al.* 1990, Murawski and Hamrick 1991, Alvarez-Buylla and Garay 1994, Hall *et al.* 1994, Boshier *et al* 1995). Two bombacaceous species, however, have been reported with remarkably low outcrossing rate by neotropical standards: in *Cavanillesia platanifolia* t_m varied between 0.213-0.661, while in *Ceiba pentandra* it was 0.689 (Murawski *et al.* 1990, Murawski and Hamrick 1991, 1992a, 1992b).

The high outcrossing rate estimated for most neotropical trees investigated so far have confirmed the findings of previous studies based on pollination experiments which showed the occurrence of partial or

complete self-sterility for many Central American trees following hand self-pollinations (Bawa 1974, Bawa *et al.* 1985, Bullock 1985). Self-incompatibility has been cited as a key factor to explain the self-sterility and the prevalence of outcrossing among neotropical trees (Bawa 1974, Bawa *et al.* 1985, Bullock 1985, O'Malley and Bawa 1987, Murawski *et al.* 1990). The term 'self-incompatibility' has been used by these authors based on circumstantial evidence, since little data is available on the occurrence of typical gametophytic or sporophytic self-incompatibility mechanisms (ie. a multiallelic, single locus controlled mechanism *sensu* de Nettancourt 1977) in such tropical tree species (but see Boshier *et al.* 1995). On the other hand, the possibility that the self-sterility and high outcrossing rate observed in neotropical trees may be caused by genetic load has been overlooked, despite growing theoretical and empirical evidence that genetic load can cause severe post-zygotic embryo abortion following selfing which, in some cases, may mimic self-incompatibility mechanisms (Klekowski 1988, Krebs and Hancock 1991, Manasse and Pinney 1991, Weller and Ornduff 1991, Seavey and Carter 1994, Lande *et al.* 1994)

High mutational loads are to be expected in long-lived, outcrossing plant species, such as most neotropical trees, because (1) deleterious mutations are more unlikely to be purged in outcrossers than in inbreeders (Lande and Schemske 1985, Charlesworth and Charlesworth 1987, Charlesworth *et al.* 1990), and (2) perennials have higher rates of somatic mutation per generation than short-lived plants (Klekowski 1988, Klekowski and Godfrey 1989, Charlesworth 1989b).

The objectives of this Chapter on the reproductive biology *of Pseudobombax munguba* are: (1) to estimate the outcrossing rate and other mating system parameters for the population in the Catalão area; (2) to document the effects of variable proportions of self-pollen loads on the fruit and seed output; and (3) to identify the factors causing the high self-sterility and outcrossing rates observed in trees of this species.

4.2 - MATERIALS AND METHODS

- Intra-ovarian post-pollination events

Observations on the pollen tube growth and penetration in the ovules were made under fluorescence microscopy using the aniline-blue staining technique

(Martin 1959). All observations were made in selfed and crossed pistils fixed in alcohol 70% (v/v) at 24, 36, 48, and 60 hours following the pollination (the procedure used to carry out self- and cross-pollinations was described in Chapter 2). As the overlap of pollen tubes in the ovary precludes precise counting, their relative abundance was subjectively classified into three categories: absent, rare, and abundant. To evaluate the relative performance of the self- and cross-pollen the number of ovules penetrated by pollen tubes was counted in at least 200 ovules/ovary.

The development pattern of the ovules in selfed and crossed pistils fixed five days after pollination was observed under a dissecting microscope, after removing the pericarp wall of the ovaries.

- Experimental mixed-pollinations

Hand mixed- (i.e. self and cross pollen load deposited on the stigma), self-, and cross-pollinations were conducted in flowers of several trees of the Catalão population during the 1993 flowering period. The mixed-pollination procedure was conducted as follows: (a) flower-buds were emasculated and bagged with a paper bag

before anthesis at around 17:30-18:30h; (b) a massive pollen load containing approximately 1:1 or 3:1 ratio of self- and cross-pollen respectively was deposited on the stigma at around 19:30-20:00h, and (c) the flowers were rebagged until 10:00-11:00h next morning. The 1:1 pollen load was obtained by emptying (by shaking) the pollen content of all anthers from one flower of each donor into a petri dish. The 3:1 load mixture of self to cross-pollen was made using only one third of the anthers from the cross-pollen donor flower. The mass of pollen was mixed thoroughly and deposited on stigmata using a small spatula. The self-, cross- and open-pollination procedures were as described in Chapter 2.

Comparisons of the fruit-set between pollination treatments were made using the chi-square test. The difference between the number of seeds per fruit, the total mass of seeds per fruit, and mean weight of the seeds per fruit were tested by one-way ANOVA (Sokal and Rohlf 1981).

- Progeny paternity analysis

The pairs of trees used in the mixed-pollinations were homozygous for a different allele at one of the

duplicated cytosolic phosphoglucose isomerase loci (*Pgi-2* or *Pgi-3*), so that it was possible to identify unequivocally the male parentage of any seedling resulting from the mixed-pollinations (i.e. all seedlings could be unambiguously classified as either selfed or crossed). Sample preparation, electrophoresis procedure, staining technique, and the criteria used to assign to trees and seedlings genotypes at *Pgi-2* and *Pgi-3* were described in Chapter 3.

- Number of lethal equivalents

The genetic load, expressed as the average number of lethal equivalents per zygote (**2B**), was estimated on the basis of the method of Morton *et al.* (1956) as applied to plant population by Sorensen (1969), by the formula:

$$\mathbf{2B = - 4 \ln R}$$

where: **B** is the hidden genetic load which would be expressed fully only at complete homozygosity (inbreeding coefficient F = 1) and is a measure of the number of lethal equivalents per gamete, while **R** is the measure of relative self-fertility. In the present study, relative self-fertility was estimated as the relative survivorship of the selfed zygotes in the mixed-pollinated fruits. It

was assumed, based on observations made under fluorescence microscopy (see below), that there was an equal rate of penetration and fertilisation by self- and cross-pollen. Thus;

$$R = \frac{\text{no. selfed seeds}}{\text{no. crossed seeds}}$$

in progenies resulting from 1:1 mixed-pollinated fruits, whereas

$$R = \frac{\text{no. selfed seeds}}{3 \text{ . no. crossed seeds}}$$

in progenies resulting from 3:1 mixed-pollinated fruits.

In terms of this work all selfing and crossing matings were considered to have inbreeding coefficients of $F = 0.5$ and $F = 0$ respectively.

- Mating system

Electrophoretic variation at the *Pgi-2* and *Pgi-3* loci was assayed for 754 seedlings from 30 open-pollinated families (at least 23 seedlings/family). All maternal genotypes at *Pgi-2* and *Pgi-3* were previously known. The enzyme and the starch gel electrophoresis methods, the assigning of the PGI genotypes, and the genetic basis of the PGI variation were detailed in Chapter 3.

Mating system analysis was based in the mixed mating model of Ritland and Jain (1981) using the Multilocus Population Estimates computer program developed by K. Ritland (Ritland 1990, version updated in 1992). This program uses the maximum-likelihood procedure (Ritland and El-Kassaby 1988) to estimate the multilocus (t_m), and the singlelocus (t_s) outcrossing rate, the multilocus fixation index (F) for the parental trees, and the gene frequencies of the pollen and ovule pools. The standard error estimates were based upon 500 bootstraps resampled within families. Gene frequency departure from the model's assumptions was tested by the chi-square goodness of fit test proposed by Ritland (1983). The chi-square test for heterogeneity of the pollen pool (Brown *et al* 1975, O'Malley *et al*. 1988) was used to detect the occurrence of intrapopulational genetic subdivision.

The fixation index (F) for each locus for the parent and seedling generations was calculated by the formula:

$$F = 1 - (H_{obs}/H_{exp})$$

where H_{obs} is the observed proportion of heterozygotes and H_{exp} is the expected heterozygosity in Hardy-Weinberg equilibrium. The fixation index for the parent generation

was estimated using the allele frequency data from all 56 trees in the Catalão population with known genotype at *Pgi-2* and *Pgi-3*, whereas the F value for the seedling generation was based on the data from the 754 seedlings resulting from open-pollinations used in the estimation of the outcrossing rate. The chi-square test of Li and Horvitz (1953) was used to determine if the fixation index departed significantly from 0;

$$\chi^2 = F^2 N(k-1); \quad df = [k(k-1)]/2$$

where **N** is the total number of trees or seedlings scored at each locus, and **k** is the number of alleles.

4.3 - RESULTS

- Intra ovarian post-pollination events

There were no detectable differences between the self- and cross-pollen tubes regarding their ability to reach the ovary and penetrate ovules (table 4.1). Self- and cross-pollen tubes began to arrive in the top of the ovary 24 hours after pollination, but at this time none or very few ovule penetrations were observed. Massive presence of pollen tubes and 20-30% of ovules penetrated were observed some 36 hours after pollination in both treatments (fig 4.1). The pollen tube penetrations in

Table 4.1 - Comparison of the abundance of pollen tubes into the ovary and proportion of ovules penetrated by pollen tube in selfed and crossed pistils of *Pseudobombax munguba* 24, 36, 48, and 60 hours following pollination.

hours after pollination	pollination treatment	pollen tubes into the ovary	% ovules penetrated mean (range)	n° pistils observed
24	self	absent-rare	2.2 (0.0 - 6.6)	3
	cross	absent-rare	0.0 (0.0 - 0.0)	2
36	self	rare-abundant	12.5 (7.2 - 16.1)	3
	cross	rare-abundant	9.7 (0.5 - 15.0)	3
48	self	abundant	24.6 (22.0 - 31.5)	3
	cross	abundant	27.8 (21.2 - 35.2)	4
60	self	abundant	29.7 (23.5 - 37.9)	3
	cross	abundant	31.0 (24.6 - 33.0)	3

either selfed or crossed pistils reached the maximum level 48-60 hours following pollination. No observations were recorded of more than one pollen tube penetrating an ovule.

The absolute percentage of penetrated ovules should be interpreted with caution since an unknown proportion of them could have been penetrated despite the lack of a visible 'pollen tail'. The data, however, can be used as an estimate of the minimal proportion of penetrated ovules, and as such can be used to compare the relative performance of the self-and cross-pollen tubes, since the bias probably affected both treatments equally.

Ovules in selfed and crossed pistils five days following pollination showed very different developmental patterns. Selfed pistils had a gradient of ovule sizes, with a large variation with regard to the proportion of enlarged developing ovules (embryo + endosperm?) within and between pistils (fig. 4.2a-c). In contrast, all five crossed pistils dissected showed that nearly all of the ovules were homogeneously developed (fig. 4.2d).

- Effect of the different pollen loads

The effect of the different pollination treatments in the fruit-set are compared in fig. 4.3. Fruit-set by 1:1

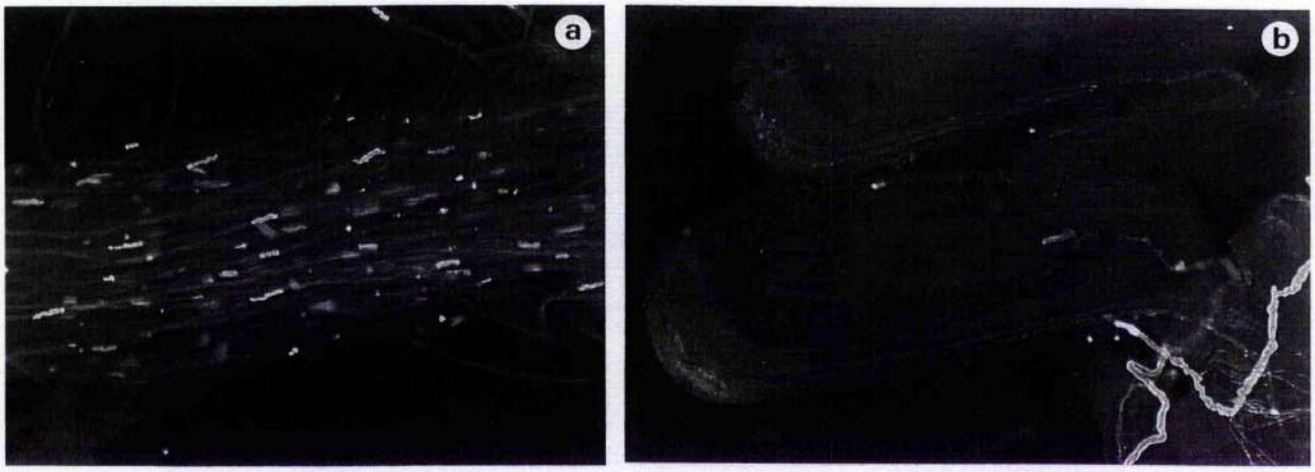

Fig. 4.1 - (a) Pollen tubes growing massively into the ovary of a self-pollinated flower of *P. munguba* 48 hours after pollination; **(b)** pollen tube penetrating the micropyle of an ovule of *P. munguba* 48 hours after self-pollination.

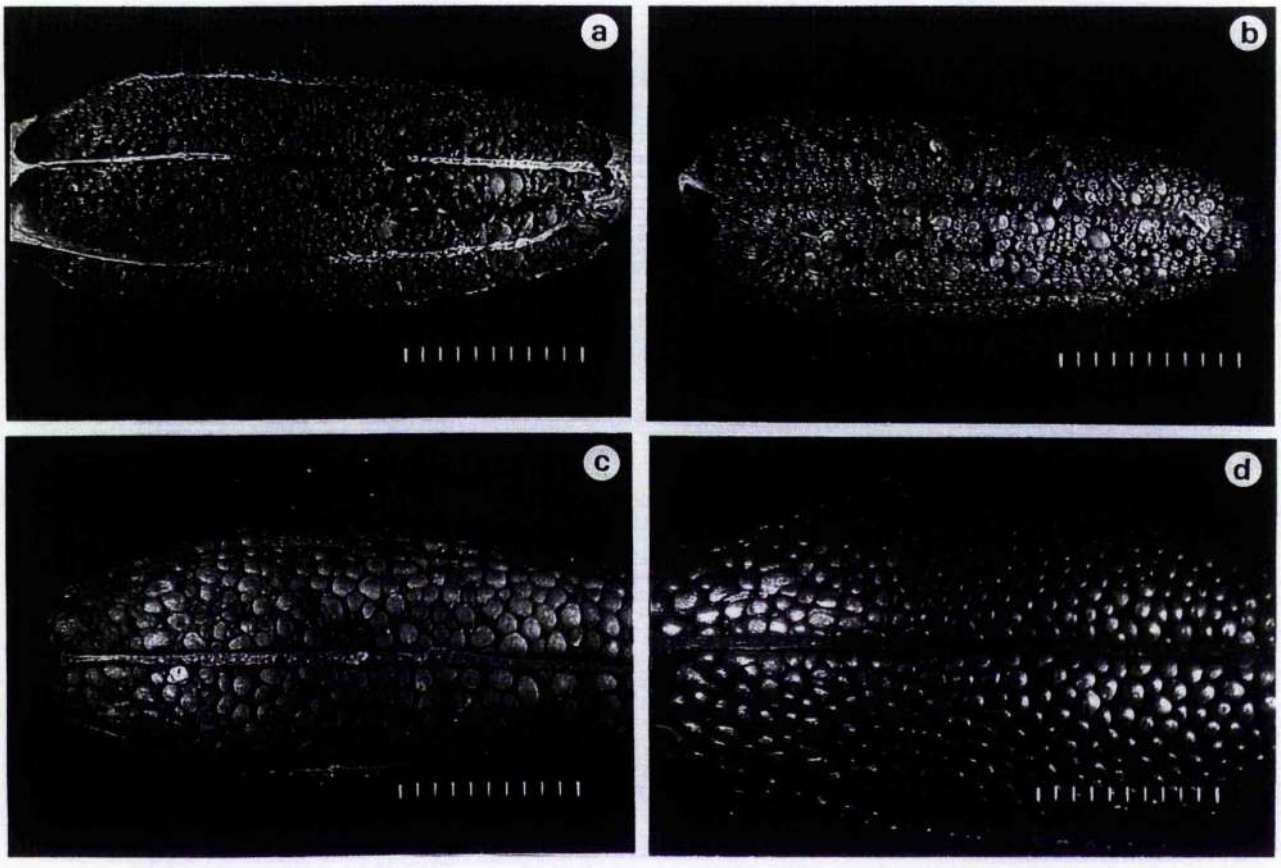

Fig. 4.2 - Ovaries of *P. munguba* with the outer wall removed showing the pattern of development of the ovules six days after pollination; **(a-c)** ovaries from self-pollinated flowers, and **(d)** ovary from a cross-pollinated flower. Scale unit = 0.5 mm.

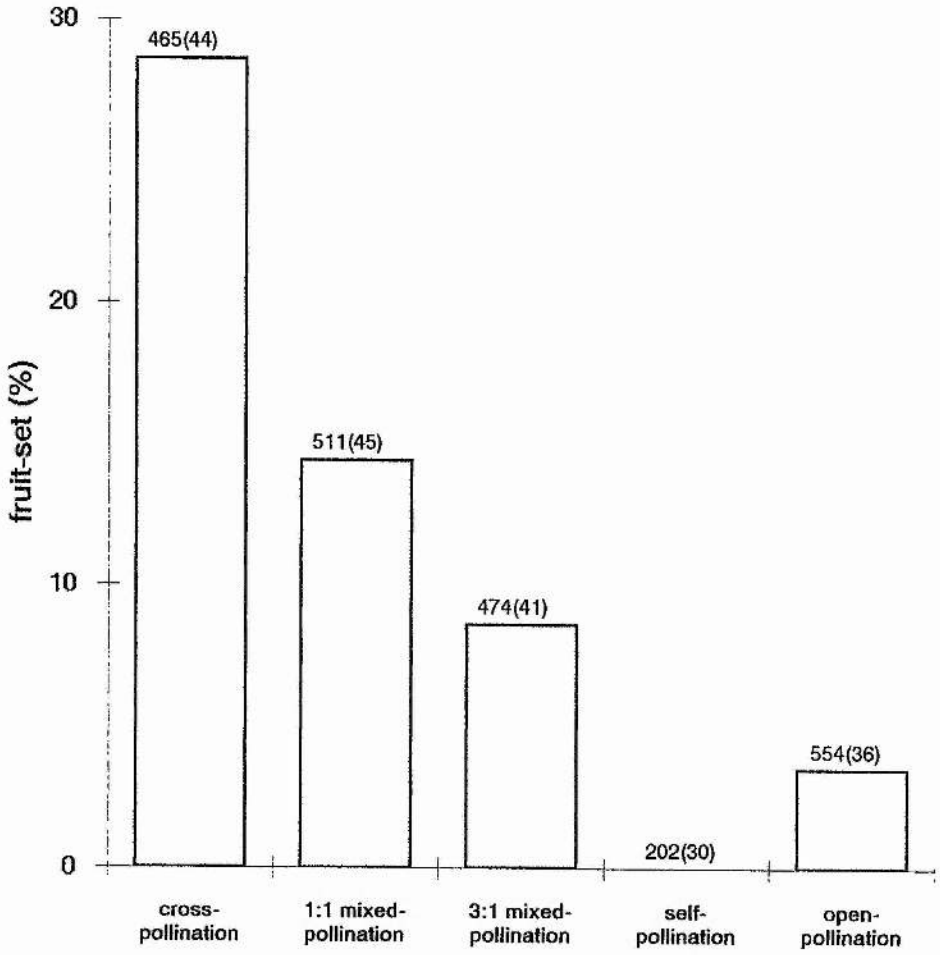

Fig. 4.3 - Fruit-set by the different pollination treatments in *Pseudobombax munguba*. Numbers at top of bars = number of flowers pollinated (number of trees used).

and 3:1 mixed-pollinations yielded around half and one third the number of fruits than cross-pollinations respectively. The difference between the fruit-set by cross-, 1:1 mixed-, and 3:1 mixed-pollination was highly significant (χ^2 = 68.59, 2*df*, P < 0.001). All 30 tested trees were sterile following selfing.

The one-way ANOVA indicated highly significant difference among the four pollination treatments in regard to the number of seeds per fruit and the total weight of the seeds per fruit (F = 36.69 and F = 23.02 respectively, P < 0.001 for both treatments). The cross-pollinated fruits had more seeds and produced a heavier mass of seeds than fruits resulting from open-pollination and both 1:1 and 3:1 mixed-pollinations. In contrast, the seeds of the mixed-pollinated fruits were, on average, heavier than the seeds resulting from cross-pollinations (fig. 4.4).

- Paternity analysis and genetic load

The proportion of selfed seeds in both 1:1 and 3:1 mixed-pollinated fruits varied over a wide range between families (0-12.5% and 0-22% respectively, tables 4.2 and 4.3). Mean proportion of selfed seeds in the 3:1 mixed-

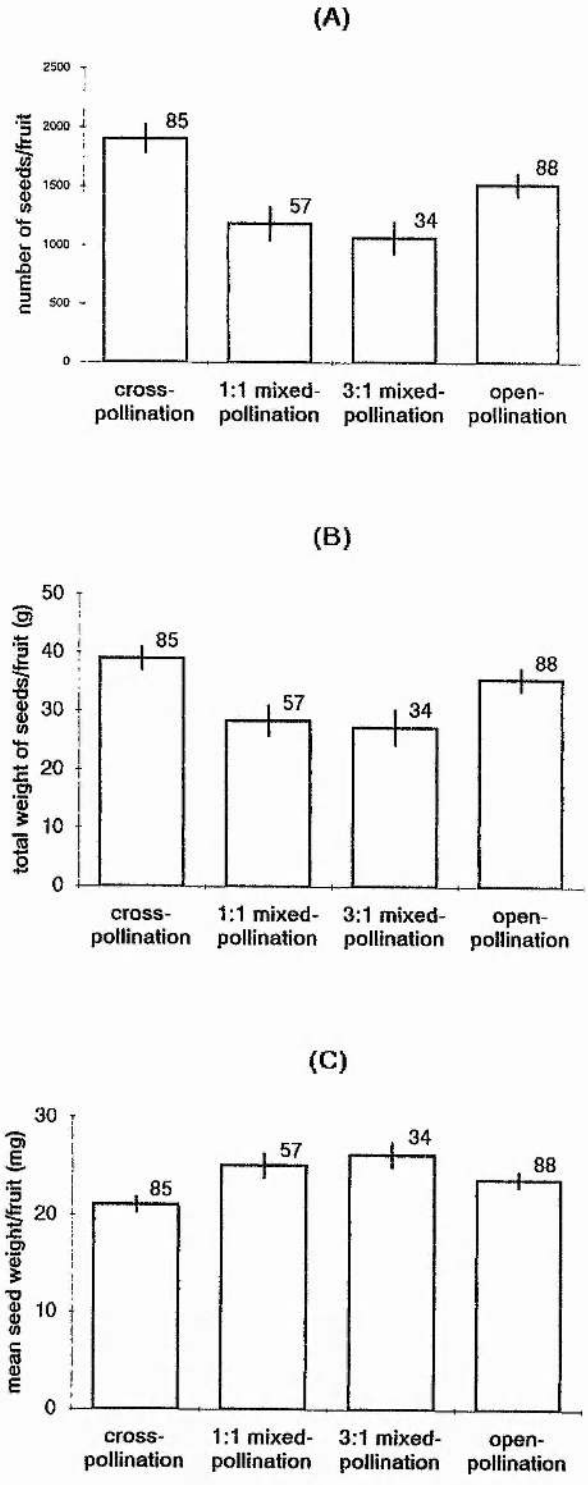

Fig. 4.4 - Effect of pollination treatments in (A) number of seeds per fruit, (B) total weight of seeds per fruit, and (C) mean seed weight of *Pseudobombax munguba*. Error bars = 95% confidence interval. Numbers at top of bars = total fruits in sample.

Table 4.2 - Genotype segregation for the two alleles at *Pgi-2* and *Pgi-3* from mixed-pollinations (1:1 ratio self:cross pollen) of *Pseudobombax munguba*.

Parent trees/ genotypes	Expected progenies by selfing	Observed distribution	Expected progenies by crossing	Observed distribution	n^{o} selfed/ n^{o} crossed (%)
#0 x #11					
aa/aa x ab/bb	aa/aa	0	aa/ab	22	0
			ab/ab	27	
#6 x #11					
aa/aa x ab/bb	aa/aa	0	aa/ab	20	0
			ab/ab	29	
#17 x #11					
aa/aa x ab/bb	aa/aa	1	aa/ab	21	2.08
			ab/ab	27	
#0 x #N3					
aa/aa x bb/ab	aa/aa	0	ab/aa	22	0
			ab/ab	22	
#6 x #N3					
aa/aa x bb/ab	aa/aa	6	ab/aa	25	14.29
			ab/ab	17	
#12A x #N3					
aa/aa x bb/ab	aa/aa	1	ab/aa	20	2.27
			ab/ab	24	
#A1 x #N3					
aa/ab x bb/ab	aa/aa	0	ab/aa	12	0
	2aa/ab	0	2ab/ab	25	
	aa/bb	0	ab/bb	9	
#N4 x #N3					
aa/ab x bb/ab	aa/aa	0	ab/aa	11	0
	2aa/ab	0	2ab/ab	27	
	aa/bb	0	ab/bb	12	
#N11 x #N3					
aa/ab x bb/ab	aa/aa	0	ab/aa	12	0
	2aa/ab	0	2ab/ab	26	
	aa/bb	0	ab/bb	12	
		8		422	2.07 (pooled)

Table 4.3 - Genotype segregation for the two alleles at *Pgi-2* and *Pgi-3* from mixed-pollinations (3:1 ratio self:cross pollen) of *Pseudobombax munguba*.

Parent trees/ genotypes	Expected progenies by selfing	Observed distribution	Expected progenies by crossing	Observed distribution	n^o selfed/ n^o crosses (%)
#0 x #11					
aa/aa x ab/bb	aa/aa	2	aa/ab	22	4.26
			ab/ab	25	
#10B x #11					
aa/aa x ab/bb	aa/aa	3	aa/ab	20	6.38
			ab/ab	27	
#17 x #11					
aa/aa x ab/bb	aa/aa	0	aa/ab	26	0
			ab/ab	24	
#10B x #N3					
aa/aa x bb/ab	aa/aa	3	ab/aa	25	6.38
			ab/ab	22	
#21 x #N3					
aa/aa x bb/ab	aa/aa	0	ab/aa	26	0
			ab/ab	24	
#6A x #N3					
aa/aa x bb/ab	aa/aa	0	ab/aa	22	0
			ab/ab	28	
#N14 x #N3					
aa/aa x bb/ab	aa/aa	3	ab/aa	23	6.25
			ab/ab	25	
#5A x #N3					
aa/aa x bb/ab	aa/aa	0	ab/aa	22	0
			ab/ab	26	
#N9 x #N3					
aa/bb x bb/ab	aa/bb	2	ab/ab	19	4.17
			ab/bb	29	
#N3 x #10B					
bb/ab x aa/aa	bb/aa	1	ab/aa	23	2.04
	2bb/ab	0	ab/ab	26	
	bb/bb	0			
#N2 x #N3					
aa/ab x bb/ab	aa/aa	1	ab/aa	7	28.21
	2aa/ab	6	2ab/ab	24	
	aa/bb	4	ab/bb	8	
#N4 x #N3					
aa/ab x bb/ab	aa/aa	0	ab/aa	12	0
	2aa/ab	0	2ab/ab	22	
	aa/bb	0	ab/bb	15	
		25		572	4.81 (pooled)

pollinations was higher than in the 1:1 treatment (χ^2 = 4.36, 1*df*, P = 0.037).

The relative survivorship of the selfed zygotes, considering the two treatments and the 15 maternal trees pooled, was R = 0.018 (range 0 to 0.145). The average number of lethal equivalents per zygote (i.e. expressed during the embryonic phase) was estimated as 2B = 16.1, ranging from 7.7 to an indeterminable value in the progeny arrays with no selfed seedlings.

It is possible that the method used in the present study overestimated the genetic load. This is because the selective abortion of young fruits was likely concentrated in fruits with a lower number of developing seeds, so that the surviving fruits, with more seeds, probably had a higher proportion of cross-fertilised ovules than the expected average of 50% and 25% resulting from the 1:1 and 3:1 mixed-pollinations respectively.

To compensate for this bias, it was necessary to estimate the proportion of crossed zygotes in mixed-pollinated pistils which had developed into mature fruits. The mean number of seeds in the 34 fruits with the highest seed-set from each pollination treatment was X_{cross} = 2442.9, $X_{1:1\ mixed}$ = 1469.7, and $X_{3:1\ mixed}$ = 1068.4.

Subtracting the 2.11% and 5.05% of selfed seeds found in the 1:1 and 3:1 mixed-pollinated fruits, the estimated number of crossed seeds in fruits resulting from those two treatments was 1438.7 and 1014.4 respectively. Thus, fruits resulting from 1:1 and 3:1 mixed-pollinations suffered, respectively, a reduction of 41.1% and 58.1% in the number of crossed seeds, instead of 50% and 75% expected without the action of selective abortion, assuming equal self- and cross-pollen performance. The estimated number of lethal equivalents, considering the above correction for the calculation of the relative survivorship of the selfed zygotes (R), over the two treatments pooled, is 2B = 13.8 (minimum 6.4 in the #6, table 4.2).

- Mating system

The mating parameters of the *P. munguba* population from the Catalão area are reported in table 4.4. The multilocus and the average single locus outcrossing rate estimates recorded were t_m = 0.943 ± 0.044 and t_s = 0.969 ± 0.045 respectively (mean ± S.E.). These estimates suggest that this species is predominantly outcrossed. The estimated t_m was not significantly different from 1 (based

Table 4.4 - Pollen and ovule gene frequencies, single and multilocus outcrossing rate, and fixation index estimates for the population of *Pseudobombax munguba* in the Catalão area.

Locus	allele	gene frequency		outcrossing rate (SE)	fixation index parents	fixation index seedlings
		pollen pool (SE)	ovule pool			
Pgi-2	*a*	0.832 (0.014)	0.933	1.027 (0.050)	0.238	- 0.039
	b	0.168 (0.014)	0.067			
Pgi-3	*a*	0.815 (0.015)	0.867	0.912 (0.063)	0.153	0.071
	b	0.185 (0.015)	0.133			
Singlelocus (t_s)				0.974 (0.044)		
Multilocus (t_m)				0.948 (0.043)		

upon the confidence interval of ± 2SE), whereas t_s was not. The difference between the multilocus and the single locus outcrossing rates ($t_m - t_s = -0.027 \pm 0.015$) was not positive, which suggests that biparental inbreeding was not occurring in the population (Ritland 1984). The Wright's fixation index of the parental trees was positive for both assayed loci, but not significantly different from zero ($\chi^2 = 3.17$, 1df, P = 0.075 for *Pgi-2*, and $\chi^2 = 1.31$, 1df, P = 0.252 for *Pgi-3*). The seedling generation had a fixation index virtually equal to zero. The positive, although non-significantly different from zero, fixation index of the parent trees would suggest that the proportion of homozygous maternal genotypes was higher than expected by random mating. These results, however, should be interpreted conservatively given the relatively small number of families sampled and the unbalanced allele frequency of both assayed loci.

According the chi-square goodness of fit test (table 4.5) the observed number of offspring genotypes at *Pgi-3* agreed with the assumptions of the mixed mating system ($\chi^2 = 2.74$, 1df, P = 0.098), whereas the genotypic numbers at *Pgi-2* showed a slight departure from the values expected by the model ($\chi^2 = 6.38$, 1df, P = 0.012, critical value of

χ^2 = 3.84). Since this departure from the model was not highly significant (P < 0.01), the data from *Pgi-2* were computed together with that of *Pgi-3* to estimate the mating system parameters.

A highly significant departure from the assumption that there was a homogeneous pollen pool over the maternal trees was found for both loci (table 4.6). However, caution should be taken on the interpretation of these results since the expected numbers of $Pgi\text{-}2^{ab}$ and $Pgi\text{-}3^{ab}$ seedlings fell below five in all families.

4.4 DISCUSSION

- The nature of the self-sterility

The observations on pollen tube growth and rate of penetration of ovules indicated that *P. munguba* lacks a typical sporophytic or gametophytic self-incompatibility barrier to prevent pollen tubes reaching and penetrating ovules. These findings agree with observations on other apparently self-sterile tropical trees (Bawa *et al* 1985), including species of Bombacaceae (Oliveira *et al*. 1992, Gibbs and Bianchi 1993), whereby slight or no detectable differences were found with regard to growth and performance of the self- and cross-pollen tubes.

Table 4.6: Chi-square test for homogeneity of number of offspring genotypes among seed parents homozygous at *Pgi-2* and *Pgi-3* (*).

Seed parents ($Pgi\text{-}2^{aa}$ /$Pgi\text{-}3^{aa}$)	number of seedlings				Total
	$Pgi\text{-}2^{aa}$	$Pgi\text{-}2^{ab}$	$Pgi\text{-}3^{aa}$	$Pgi\text{-}3^{ab}$	
#3A	20	5	18	7	25
#3B	16	8	20	4	24
#5B	24	0	24	0	24
#5D	15	8	14	9	23
#5F	22	3	22	3	25
#6	24	0	24	0	24
#8A	21	3	13	11	24
#10A	18	5	18	5	23
#10B	23	2	23	2	25
#10D	24	0	18	6	24
#12	18	7	22	3	25
#17	24	0	24	0	24
#21	22	2	18	6	24
#N5	19	5	18	6	24
#N12	22	1	15	8	23
#N13	20	5	22	3	25
#M2	22	2	20	4	24
#M8	19	5	20	4	24
#A2	20	3	14	9	23
#B3	19	6	25	0	25
Total	412	70	392	90	482

Pgi-2: $\chi^2 = 44.4$, 19 *df*, $P < 0.001$

Pgi-3: $\chi^2 = 58.3$, 19 *df*, $P < 0.001$

(*). These results should be interpreted conservatively given that all expected numbers of $Pgi\text{-}2^{ab}$ and $Pgi\text{-}3^{ab}$ seedlings are bellow five.

The enlargement of a variable proportion of ovules in the self-pollinated pistils of *P. munguba* (fig. 4.2) suggests that fertilisation and initial zygote and endosperm development probably take place following self-pollen tube penetration, as observed in histological studies of selfed pistils of *Chorisia* (Gibbs and Bianchi 1993). Thus, the failure of most self-penetrated (and, presumably, self-fertilised) ovules to develop into seed seems to be caused by an active post-zygotic rejection mechanism which is genetically controlled by the maternal tree ('late-acting self-incompatibility' *sensu* Seavey and Bawa 1986), or, alternatively, by the action of genetic load reducing the general vigour of the selfed-zygotes ('early acting inbreeding depression'; Wiens *et al.* 1987, Klekowski 1988, Charlesworth 1989b, Krebs and Hancock 1991, Seavey and Carter 1994). Two observations support the view that self-sterility in *P. munguba* accords better with the genetic load model: (1) the observed variety of developmental failure between ovules (zygote + endosperm?) penetrated by the self-pollen tubes which probably reflects the effect of a variable number of deleterious or lethal genes on the embryogenesis (homogeneous failure would be expected under late acting self-incompatibility),

and (2) the variability in the level of self-sterility between trees in the population, revealed by paternity analysis of the mixed-pollinated offspring (species with late acting self-incompatibility should exhibit similar levels of self-sterility among plants). The criteria used to differentiate self-sterility caused by late-acting self-incompatibility or by inbreeding depression have been discussed in detail by Seavey and Bawa (1986), Klekowski (1988), and Sage *et al.* (1994). There is increasing recent evidence, based on empirical observations, that early inbreeding depression is the main cause of the post-zygotic embryo abortion observed in most woody horticultural (review in Sedgley 1994) and many wild plants (Wiens et al. 1987, Bertin et al. 1989, Krebs and Hancock 1991, Manasse and Pinney 1991, Weller and Ornduff 1991, Seavey and Carter 1994, Rigney 1995; but see Waser and Price 1991, and Broyles and Wyatt 1993 for an alternative view).

- Effects of the self-pollination

The controlled pollination program showed that increasing the proportion of self-pollen in the pollen load deposited on the stigmata significantly reduced the

fruit- and seed-set (figs 4.3 and 4.4). Furthermore, all pistils were aborted by the deposition of a load composed by 100% of self-pollen. This decrease in fertility was most likely caused by the absence of an incompatibility barrier to prevent the penetration of self-pollen tubes, and by the high mortality of the selfed zygotes, which remove ovules from the pool of potential outcrossed seeds (Seavey and Carter 1994). The failure of most selfed ovules appears to release resources for the growth of the remaining developing ovules, as suggested by the production of significantly heavier seeds in the mixed-pollinated fruits than in the cross-pollinated ones (fig. 4.4).

Under natural conditions, the *P. munguba* flowers, with the stigma closely surrounded by more than one thousand anthers releasing a powdery pollen (fig. 2.3b and 2.5a in the Chapter 2), probably experience high levels of deposition of self-pollen, either by wind or visitor action. In fact, as emphasised by Charlesworth (1985) and Jarne and Charlesworth (1993), there may be little selective force to prevent self-fertilisation, even if the self-zygotes have low fitness or are unviable, since the early mortality of the selfed zygotes does not affect the

maximum fruit-set (i.e., if sufficient number of crossed zygotes are produced). Thus, the waste of a high proportion of the available ovules due to pollen autodeposition probably does not affect the final seed output, since long-distance and frequent cross-pollen delivery is promoted by a large and mobile bat such as *P. hastatus* (see Chapter 2).

A possible adaptive response to the high levels of self-pollination may be the production of many more flowers than the maximum number of fruits that can reach maturation. The maximum number of fruits in *P. munguba*, as estimated by the fruit-set with cross-pollination, was 28.6% of the total number of flowers produced. By increasing the total number of initiated fruits the plant could have a higher margin to abscise selectively those with lower number of ovules fertilised and/or with lower offspring vigour. Nonrandom fruit abortion may be the dominant selective force maintaining the production of an excess of flowers in many species (Stephenson 1981, Stephenson and Bertin 1983, Stephenson and Winsor 1986, Sutherland and Delph 1984, Sutherland 1986).

The results of the pollination tests, and the high outcrossing rate estimate, suggest that selective abortion

effectively occurs in *P. munguba*, favouring those fruits with more seeds and/or a higher proportion of crossed seeds. In fact, it is not clear if the basis of the abscission process is the number of developing seeds or their genetic composition, since these two factors are correlated (i.e., young fruits resulting from self-pollinations showed fewer developing seeds than those resulting from cross-pollinations, fig. 4.2). Other factors, however, may concomitantly contribute to the overproduction of flowers in *P. munguba* and other plants, such as: (1) uncertainty in pollination success, which could cause pollination limitation; (2) unpredictability of the resources available for fruit maturation; and (3) maximisation of the male function fitness through increasing the quantity of pollen produced (Wilson and Price 1977, Stephenson 1981, Bawa and Webb 1984, Sutherland and Delph 1984, Sutherland 1987, Charlesworth 1989a).

- The high genetic load

The perennial life-form and the large breeding population of *P. munguba* (inferred on the basis of long distance pollen flow, high outcrossing rate, and

undetectable biparental inbreeding) probably contribute to the accumulation of deleterious mutations in the populations of this species. Furthermore, the studied population in the Catalão area is located in the centre of the distribution range of the species, suggesting that a bottleneck in population size, which could increase inbreeding and purge deleterious mutations, has not taken place recently.

On the other hand, the considerable selfing rate experienced by *P. munguba*, due to a high degree of self-pollen deposition and the lack of a self-incompatibility barrier, theoretically should result in load reduction (Lande and Schemske 1985, Charlesworth and Charlesworth 1987, Charlesworth *et al* 1990). This was probably not the case because the selective abortion process in *P. munguba* eliminates virtually all pistils heavily self-pollinated, and the number of selfed seeds in the surviving fruits is very low. Thus, only a very small number of selfed progeny are actually exposed to selection, which reduces the chances of the deleterious mutations being purged from the genomic pool.

The mean of 13.8 recessive lethal equivalents per zygote estimated for *P. munguba* is very high in terms of

angiosperms in general (Crumpacker 1971, Levin 1984). However, more recent investigations have revealed quite high and variable loads among angiosperm species: *Vaccinium corymbosum*, mean 9.6, range 2.2-20 (Krebs and Hancock 1991); *Stylidium spp.*, mean 11, range 1-20 (Burbidge and James 1991); and *Epilobium obcordatum*, mean 11.0, range 2.9-17.6 (Seavey and Carter 1994). In contrast with *P. munguba*, however, all these species exhibited measurable fruit- and seed-set following self-pollination. The comparison between the number of lethal equivalents in *P. munguba* and those three species should be made with caution because of the different manner in which relative survivorship of the selfed and crossed zygotes was calculated (within the same ovary for *P. munguba*, and between different ovaries in those other species).

Previous models of genetic load did not envisage levels of deleterious recessives which could give rise to self-sterility and thus mimic self-incompatibility mechanisms (Lande and Schemske 1985, Charlesworth and Charlesworth 1987). However, the high genetic load estimated for *P. munguba* is consistent with the models recently suggested by Lande *et al.* (1994) to explain the occurrence of species with very high early (embryonic)

inbreeding depression despite intermediate selfing rates. These models postulate that the maintenance of a large number of recessive lethals (>10) would take place in species with genomic mutation rates for recessive lethals typical for long-lived organisms (U = 0.1 to 1.0 per generation), since the primary selfing rate does not exceed a determined threshold. Thus, inter-plant pollinator activity in *P. munguba* may be a key factor in the maintenance of high genetic load, by maintaining primary selfing rates at intermediate levels, counterbalancing factors which promote selfing such as the stamen brush floral structure and the putative lack of self-incompatibility mechanism. The fact that open-pollinated fruits have features (number of seeds, total mass of seeds, and mean weight of individual seeds) somewhat intermediate between the 1:1 mixed- and the cross-pollinated fruits (fig. 4.4), suggests that the naturally pollinated flowers which developed into mature fruit received, on average, more cross-pollen than the 1:1 mixed-pollinated flowers.

- Mating system

The mating system and the breeding structure of the *P. munguba* population in the Catalão area appears to be

modulated by several factors such as (1) the complex pattern of pollen flow, due to the variable foraging behaviour of *P. hastatus* (see Chapter 4, and McCracken and Bradbury 1981), (2) the wind seed dispersal, which affects the patterns of spatial distribution of the progeny, and (3) the high, but variable, level of self-sterility among trees in the population.

The estimate of 95% outcrossing for *P. munguba* is consistent with the findings of the controlled pollinations, whereby a small proportion of selfed seeds were set following deposition of mixed pollen load on the stigma. The combined analysis of the hand-pollination results and the multilocus outcrossing estimate suggest the occurrence of a predominantly outcrossing mating system, with a small (but not negligible) production of selfed seeds. Most of the inbreeding is probably of uniparental origin (caused by autodeposition of pollen followed by self-fertilisation), since agamospermy did not occur (Chapter 3) and the model did not detect biparental inbreeding ($t_m - t_s$ was not significantly positive). Undetectable biparental inbreeding may reflect the long distance pollen flow promoted by *P. hastatus*, and/or a considerable degree of cross-sterility, due to

'outbreeding depression' among related trees in the population which may share a number of recessive deleterious alleles .

The mixed mating models assume that: (1) pollen allele frequencies are homogeneous over all maternal genotypes, (2) the alleles at different marker loci are unlinked, (3) no selection affects the genetic markers from mating to the progeny census, and (4) each locus is in Hardy-Weinberg equilibrium (Brown *et al* 1985, 1989, Hamrick 1989). The data on the genetics of the cytosolic PGI variation in *P. munguba* (Chapter 3) fit the assumptions 2, 3, and 4.

With regard to the first assumption, however, the gene frequency of the pollen pool over the maternal parents departed significantly from homogeneity (table 4.6). The violation of this model assumption was likely derived by factors which cause temporal and spatial substructuring of the population, such as differences in timing of flowering among individual trees (table 2.1, Chapter 2), and/or the tendency observed in *P. hastatus* females to forage in more or less well delimited territories (McCracken and Bradbury 1981). Heterogeneity of the pollen pool has been observed in several other

neotropical trees (O'Malley and Bawa 1987, O'Malley *et al.* 1988, Murawski *et al.* 1990, Murawski and Hamrick 1991). The differences between allele frequency in the ovule and pollen pool may result from (1) immigration, from outside the population, of pollen with higher frequency of the *b* allele at *Pgi-2* and *Pgi-3*, (2) differences in the reproductive effort or in the male and female functions among parental genotypes, and/or (3) an unrepresentative sample of the maternal trees.

The results of this study suggest that the self-sterility and the high outcrossing rate observed in *P. munguba* are achieved via severe post-zygotic embryo abortion (probably caused by high levels of genetic load), in association with long distance pollen flow. Genetic load in *P. munguba* and other species, however, should not be viewed as an adaptation promoting outcrossing, but as an inevitable cost for maintaining genetic variability (Haldane 1957, Klekowski 1988, Charlesworth 1989b, Krebs and Hancock 1991). This reproductive pathway described for *P. munguba* may be widespread in Bombacaceae, since previous studies have similarly indicated self-sterility and lack of a typical incompatibility barrier to preclude self-fertilisation among some species of this family, such

as *Eriotheca gracilipes* (Oliveira *et al.* 1992), *Chorisia speciosa* (Gibbs and Bianchi 1993), and *Ceiba pentandra* (see Chapter 6).

Chapter 5

Floral Biology and Pollination Ecology of *Ceiba pentandra*

5.1 - INTRODUCTION

Ceiba pentandra (L.) (Bombacaceae), the silk cotton or kapok tree, is a species with a pan-tropical distribution; native populations occur in Tropical America and West Africa, while populations from Southeast Asia were probably introduced by Man (Baker 1965). Studies carried out in West Africa, South-east Asia and the Pacific Islands indicate that this species is visited and pollinated by megachiroptera bats (Baker and Harris 1959, Harris and Baker 1959, Wodzicki and Felten 1975, Cox 1983, Elmqvist *et al.* 1992).

In the Neotropical area bats and other vertebrates have been cited as visitors and potential pollinators of the silk-cotton tree. Carvalho (1961) described bats with a large wingspan, which he supposed to have been *Phyllostomus hastatus,* visiting *C. pentandra* flowers in Belém, east Amazon. Villa (1966) registered the bats *Artibeus jamaicensis* and *Leptonycteris nivalis* visiting *C. pentandra* flowers in Mexico. Heithaus *et al.* (1975), working in a seasonal forest in Costa Rica, captured seven bat species with *C. pentandra* pollen attached to the fur. Toledo (1977) registered that three non-flying

mammals, various unidentified bats, seven species of hummingbirds and 26 other birds, as well as several insects, visited the inflorescences of *C. pentandra* in southeast Mexico, and also considered the possibility of pollination by non-hovering birds in this species. In southeastern Peru, Janson *et al.* (1981) observed three diurnal monkeys (*Saimiri sciureus, Cebus apella,* and *Ateles paniscus*) visiting flowers and suggested that these animals may have a significant pollination role in *C. pentandra.*

This chapter aims to describe observations on the floral biology and on the behaviour of the flower visitors made from platforms placed in the canopies of *C. pentandra* trees in two different parts of the Brazilian Central Amazon. The role of each group of visitor in pollination is analyzed critically. Additionally, this chapter aims to produce a gross estimation of some reproductive parameters (total flower and fruit production, fruit set, nectar secretion and sugar concentration) which are still poorly quantified for this species. Data on the breeding and mating system are in the next chapter.

- The Tree

Neotropical populations of *C. pentandra* range from northern Central America and Antilles to the southern boundary of the Amazon Basin in Brazil and Peru. In the Central Amazonian region, *C. pentandra* (locally called

'sumaúma') occurs in the lowland, seasonally flooded habitat (the 'várzea') along the sedimentary quaternary basin of the white water rivers (fig 5.1a). It is a majestic giant emergent tree up to 60 meters high (fig. 5.1b) with large plank buttresses (fig. 5.1c) and the trunk covered with conical, sharply pointed spines. Sumaúma is a deciduous tree, shedding its digitate-compound leaves during the dry season. In the Central Amazon this species flowers irregularly, presenting non-annual cycles. Mass-flowering occurs during the leafless period. In some trees a few isolated branches sometimes set flowers aseasonally, not necessarily in the leafless period.

The inflorescences (fig. 5.1d) are fascicles that are borne mainly on the extremes of the branches, with buds initiated in the axils of leaves just before these are dropped. Flowers have nocturnal anthesis. The number of open flowers per inflorescence per night varies from 1 to 20. The five petals, five staminal filaments and the pistil are creamy white giving a general white colour to the flowers and inflorescence, punctuated by the gold-yellow of the anthers. Most flowers are inclined or hang down thus giving a globose appearance to the whole inflorescence.

The fruits (fig. 5.1e) are elliptic, pendant, brown capsules dehiscing by 5 valves. In each capsule there are 66-250, ovoid, 4-6 mm diameter seeds, weighing 45-65 mg each, surrounded by the pale yellow silk cotton which

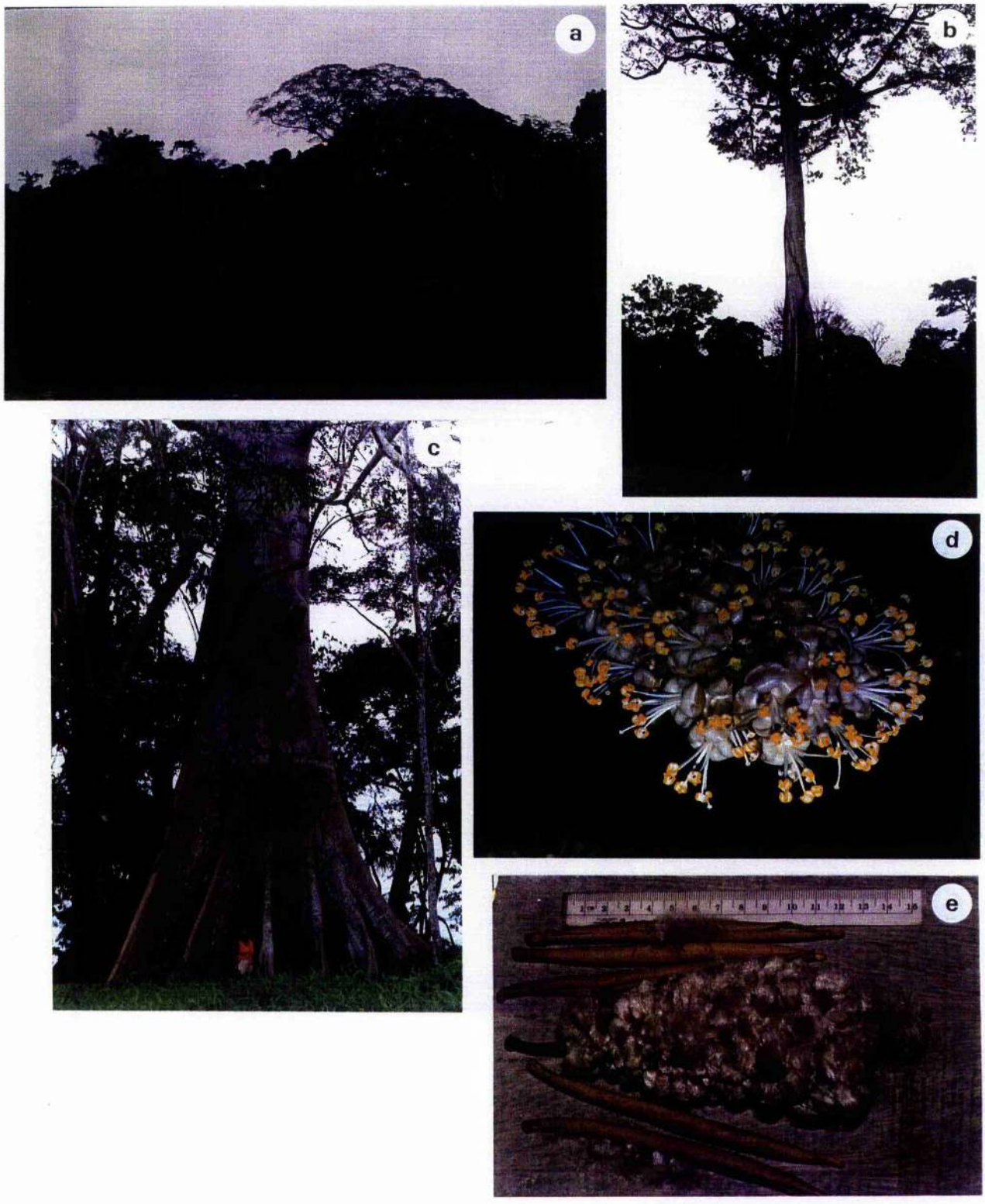

Fig 5.1 - (a) emergent *Ceiba pentandra* tree in the seasonally inundated 'várzea' forest in the margin of the Purús River; **(b)** the #9, a 50 meter high *Ceiba pentandra* tree located in the Catalão area; **(c)** large plank buttresses of the # 12, located in the Amazon River near Manaus; **(d)** inflorescence of *Ceiba pentandra* at the peak of the flowering period; **(e)** mature fruit of *Ceiba pentandra* with the valves dehisced and the fibber and seeds still packed together.

is derived from the pericarp. The seeds have a 'float structure' in their basal portion, indicating that additionally to wind dispersal, they can also be dispersed by water.

The native populations of *C. pentandra* in the Brazilian Amazon have suffered a drastic reduction during the last decade because the trunks of this species became the main source of timber for the plywood industry in the region, replacing the 'ucuúba' (*Virola spp.*) which has become almost commercially extinct.

5.2 - FIELD SITES AND METHODS

- Flowering and Fruiting.

The flowering and fruiting were followed monthly, between 1992 and 1994, in six twenty year old planted trees at the Instituto Nacional de Pesquisas da Amazonia (INPA) campus in the Manaus urban area (trees #1 to #6), and six wild trees located in areas near to the meeting of the Negro and Solimões Rivers (trees #7 to #10) in the Catalão peninsula, tree #11 on the left margin of the Solimões River, and tree #12 in the strip of land separating the Puraquequara Lake and the Amazonas River. The map in fig. 5.2 shows the location of these 12 trees. The flowering was scored as 'massive' when all main branches of the tree set flowers. Alternatively, the flowering was scored as partial when only 1-2

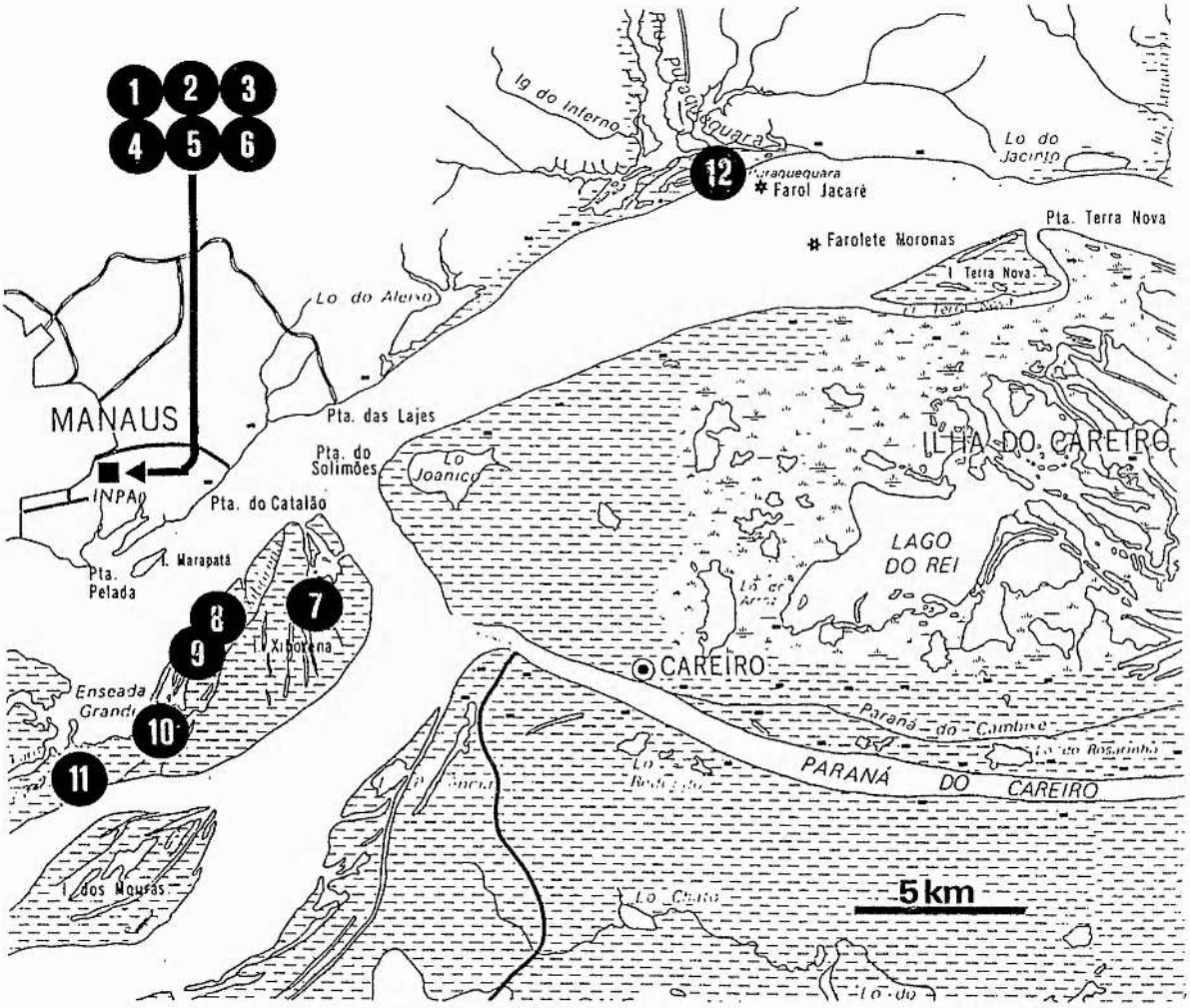

Fig. 5.2 - Location of the twelve *Ceiba pentandra* trees in the study area.

branches set flowers. No intermediate situation was found. The same criterion was used for the fruiting.

The total numbers of flowers and fruits set by trees #1 and #2 were estimated using four 1x1 meter quadrats placed randomly in the pistil and valve shadow area under each tree. The number of pistils, immature fruits, and valves inside each quadrat was counted during the flowering and fruiting periods.

- Floral Biology and Pollination Ecology.

Initial observations and capture of floral visitors were made in a 30 meter high tree located on the margin of the Uraricoera River, Maracá Biological Reserve, Roraima State (ca. 3º 20' N, 61º 20' W) in March 1988. The floral events and the behaviour of the floral visitors were observed from a platform placed between two branches inside the crown and captures of the visitors were made with a 4.0 x 2.8 m nylon mist-net placed beside the platform.

The observations on the floral events and visitor behaviour were also made from a platform placed on the top of an 18 meter scaffolding beside tree #1 at the INPA campus from August to September 1993, a period during which the tree #1 and the tree #2 (located 20 meters away one from each other) flowered. At INPA campus the bats were captured with 12 x 2.8 meter mist-nets suspended from two forks with nylon cords and placed just below the canopy at 18-20 meter high. Bats foraging near the

platform were also caught directly by hand, protected with gloves, whilst they were landing on an inflorescence.

The following sequence of floral events was noted: anthesis, odour release, nectar secretion, pollen liberation, and receptivity of the stigmas. Nectar secretion was measured at two hour intervals with a 15 µl capillary in 10 flowers from tree #1. All measurements of nectar volume in this study are expressed as mean ± standard deviation. The sugar concentration of the nectar was estimated every two hours for the same flowers using a pocket refractometer. Samples from the pollen attached to the bat fur were collected for microscopic analysis using the technique described in Beattie (1971).

5.3 - RESULTS

- Flowering and Fruiting

The results in Table 5.1 show that *C. pentandra* does not flower annually and that partially flowered or isolated flowering trees did not set fruits (e.g. #2 and #12 in 1993, #11 in 1994).

The estimation of the flower production during the flowering peak and the total number of flowers and fruits produced by trees #1 and #2 during the reproductive season of 1992 is shown in Table 5.2. A circular

Table 5.1 - Flowering and fruiting in twelve *C. pentandra* trees in the Manaus area.

Tree	phenological activity	1992	1993	1994
#1	flowering	massive (August)	-	-
	fruiting	massive (October)	-	-
#2	flowering	massive (August)	partial (August)	-
	fruiting	massive (October)	none	-
#3	flowering	-	-	-
	fruiting	-	-	-
#4	flowering	-	-	-
	fruiting	-	-	-
#5	flowering	-	-	-
	fruiting	-	-	-
#6	flowering	-	-	-
	fruiting	-	-	-
#7	flowering	-	massive (August)	-
	fruiting	-	massive (October)	-
#8	flowering	-	massive (August)	-
	fruiting	-	massive (October)	-
#9	flowering	-	massive (August)	-
	fruiting	-	massive (October)	-
#10	flowering	-	massive (August)	-
	fruiting	-	massive (October)	-
#11	flowering	-	massive (August)	partial (April)
	fruiting	-	massive (October)	none
#12	flowering	-	massive (August)	-
	fruiting	-	none	-

Table 5.2 - Estimation of the total number of flowers, total number of fruits, and fruit-set (%) of two *C. pentandra* trees in Manaus, Brazil. Estimates based in four 1 x 1 m quadrat per tree.

Tree	nº flowers/ quadrat (1) (x ± s.d.)	nº fruits/ quadrat (2) (x ± s.d.)	fruit-set /quadrat (%)(3) x (range)	estimated total nº flowers (x ± s.d.)	estimated total nº of fruits (x ± s.d.)
#1	2,084.5 ± 443.8	15.7 ± 9.2	0.71(0.40-1.04)	654,533 ± 139,353	4,930 ± 2,229
#2	845.2 ± 202.7	19.0 ± 8.0	2.22(1.90-2.48)	265,383 ± 63,648	5,890 ± 2,512

(1). nº flowers/quadrat = nº pistils + nº immature fruits + nº valves/5 inside the quadrat.

(2). nº fruits/quadrat = nº valves/5 inside the quadrat.

(3). fruit-set/quadrat (%) = nº fruits/nºflowers inside quadrat x 100%

area with 20 m diameter was considered as pistil and valve shadow surface for each tree.

- Floral biology.

Anthesis starts at the beginning of the night usually around 18:15-18:30. At 18:45-19:15 the flowers open and the anthers and the stigmas are exposed. The stigma is wet and seems to be receptive just after anthesis. Anther dehiscence usually occurs as they are exposed, but under some climatic conditions, such as absence of wind and high humidity, the pollen release can be postponed by up to one hour. The flowers last just one night, and petals, stamens and the style drop during the following morning.

The flowers had 107.5 ± 35.6 µl (n = 10 flowers) of nectar deposited around the ovary just after the petals opened at 19:00h (fig. 5.3). The secretion rate declined from the anthesis to around 02:30 a.m., when it ceased. The total volume secreted by a flower during a whole night was 309.7 ± 75.2 µl (n= 10 flowers). The nectar presented 19.7 % (range 18 - 21%) of sugar concentration at the beginning of the night, and became increasingly more dilute, reaching 11.5% (range 10 - 13%) at the end of the secretion period (fig. 5.3).

- Floral visitors.

Seven mammal species visited the flowers of *C. pentandra* at INPA campus: four phyllostomid bat species

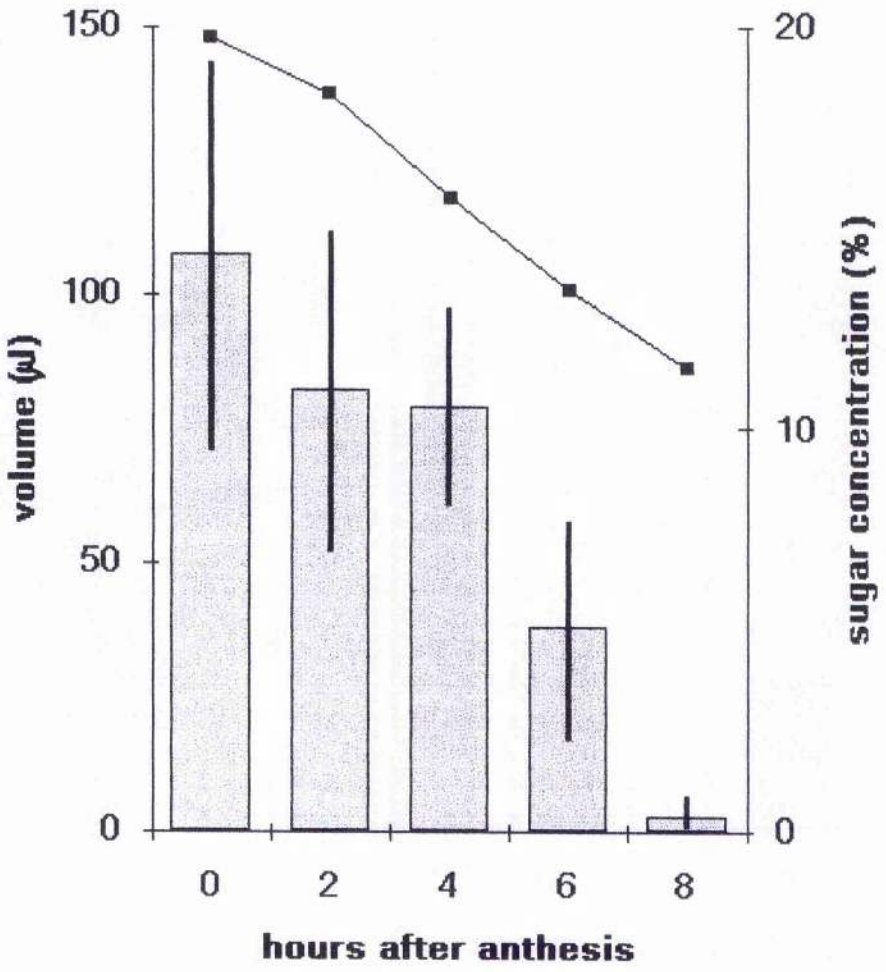

Fig. 5.3 - Mean volume (µl) and sugar concentration (%) in nectar of *Ceiba pentandra*. Columns represent the average of the volume secreted, the error bars represent ± one standart deviation, and the dots represent the average sugar concentration. N = 10 flowers.

(*Phyllostomus discolor, Phyllostomus hastatus, Artibeus jamaicensis,* and *Artibeus concolor*), two species of marsupials (*Caluromys phillander* and *Didelphis marsupialis*) and a species of nocturnal monkey (*Aotus sp.*, which is part of a group formed by individuals released at INPA campus ten years ago). *Phyllostomus hastatus* and *P. discolor* were the unique nocturnal visitors of *C. pentandra* flowers in the Uraricoera River.

Thirty two bats were captured in the mist-nets placed near the crown of the flowering trees at INPA campus: 24 *P. discolor,* four *P. hastatus,* three *A. concolor* and one *A. jamaisensis*. Additionally six *A. concolor* were captured directly by hand in inflorescences near the platform. In the Uraricoera River four *P. discolor* and four *P. hastatus* were captured. All captured bats in the two studied areas had pollen on their fur and microscopic analyses of the pollen load revealed it to contain only *C. pentandra* pollen.

Phyllostomus hastatus (head/body length 110-130 mm, weight 80-115 g) and *P. discolor* (head/body length 70-80 mm, weight 35-45 g) were by far the most frequent flower visitors in the two areas. Flock sizes for these two bat species were visually estimated at 30-50 individuals for *P. hastatus* and more than 100 individuals for *P. discolor* during the flowering peak at INPA campus. In the tree of the Uraricoera River there were more than 100 individuals of each *Phyllostomus* species visiting the flowers at the beginning of night. The flocks of both

species arrived in the trees at dusk, around 18:35-18:45. Both species flew around the crowns several times before initiating flower visits. *Phyllostomus hastatus* activity was much more intense during the first hour after the flowers opened, whereas *P. discolor* had a more or less constant activity until midnight. During their visits these two bat species landed on the inflorescence with the wings extended backwards or 'embraced' the cluster of flowers (fig. 5.4a and b), introduced their head into 1-3 flowers, and lapped the nectar. Each visit lasted 1-5 seconds. Their visits caused a 'rain' of young fruits (= developing ovaries) which fell attached at the calyces and the pedicel, but they apparently did not damage the flowers. Captured individuals of *P. discolor* and *P. hastatus* revealed the pollen load covering the head and ventral surface of body and wings (fig. 5.4c).

Artibeus concolor was most frequently observed and photographed at the end of the flowering season visiting repeatedly the same pair of inflorescences in a restricted part of the crown. This small bat lands and walks on the inflorescence, keeping its wings closed during the visits (fig. 5.4d). They had a large pollen load on the head and ventral parts of the body. During the more intensive period of *P. hastatus* activity, when they vocalize intensely, individuals of *A. concolor* were not observed in the tree. No observations were made on the visiting behaviour of *A. jamaicensis*, but this

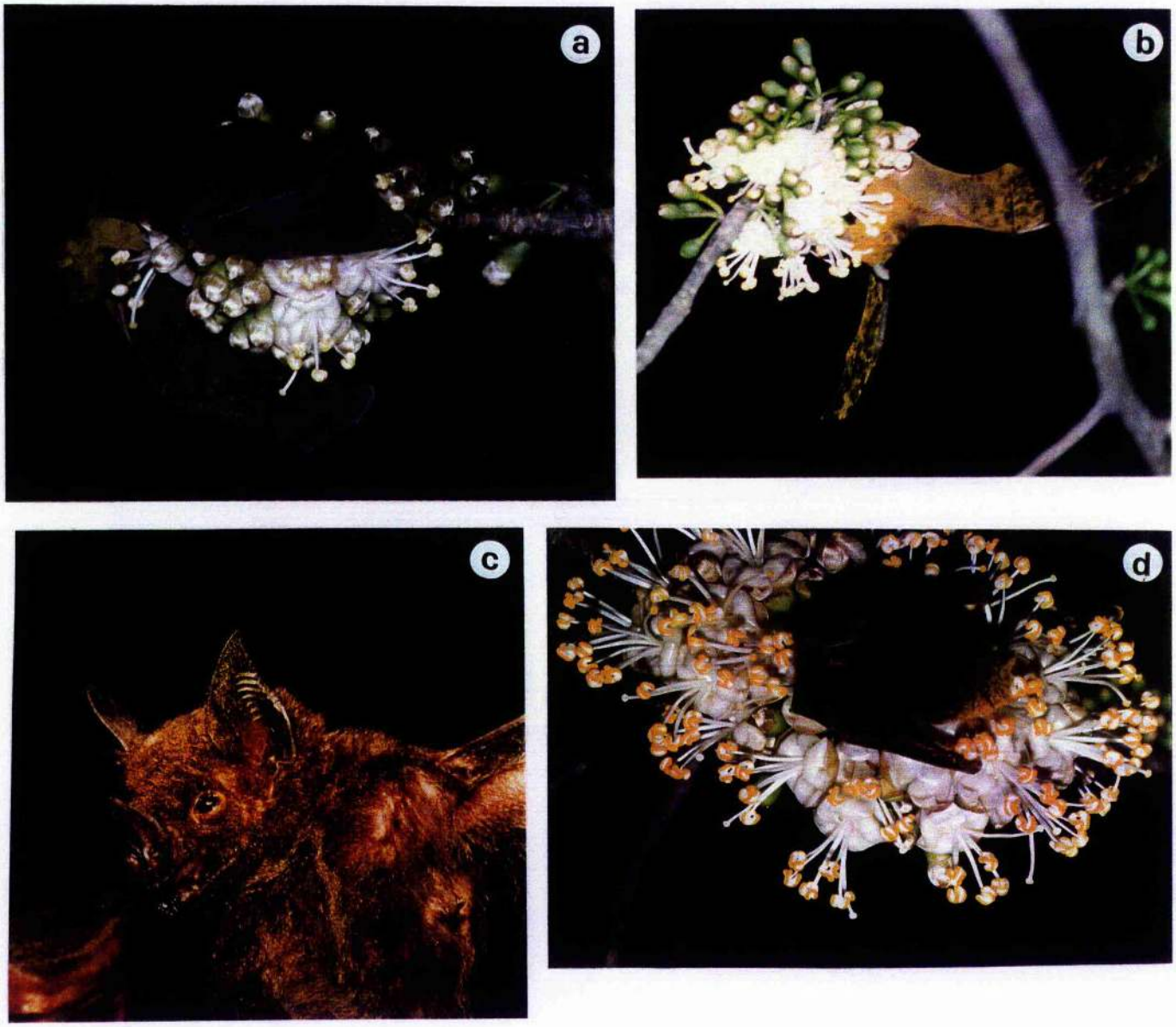

Fig. 5.4 - (a) and **(b)** *Phyllostomus discolor* visiting inflorescences of *Ceiba pentandra* in the Catalão area; **(c)** female of *Phyllostomus hastatus* captured while visiting *Ceiba pentandra* flowers in the Uraricoera River; **(d)** visit of the small bat *Artibeus concolor* to inflorescence of *Ceiba pentandra* in the Manaus area. Note, in all photographs, the pollen load on the fur of the bats.

species could be confused with the other more frequent bat visitors because of its intermediate size between the two *Phyllostomus* species. The single captured individual of *A. jamaicensis* had a very sparse dust of pollen on the fur.

Unidentified hawk moths were observed occasionally visiting the flowers of tree #1 at the end of the flowering season and late in the evening, after the more intensive bat activity period.

Caluromys phillander and *Didelphis marsupialis* walked along branches searching for inflorescences at their extremes. Both species spent up to 30 seconds lapping the nectar in each inflorescence and apparently did not damage the flowers although some developing ovaries dropped during their visits. *Caluromys phillander* was observed foraging alone or in pairs and *D. marsupialis* was observed always alone. Both species visited trees #1 and #2 only during the two weeks of the more intensive flowering.

The night-monkey *Aotus sp* moved more quickly than the marsupial species and frequently jumped between branches to reach the inflorescences. They were always observed foraging in groups of five individuals in both monitored trees at INPA campus. Their activities in the inflorescence apparently did not damage the opened flowers but caused developing ovaries to drop.

Several species of bees (genus *Centris, Xylocopa, Bombus, Trigona, Apis*), wasps (*Polybia*) and an

unidentified hummingbird visit the flowers to collect residual nectar and the pollen during the first hours of the morning.

5.4 - DISCUSSION

- The non-annual mass-flowering and the floral biology

The observations over a three years period on the flowering in *C. pentandra* were not sufficient to produce a clear picture of the frequency of the reproductive cycles in this species, but they do confirm previous observations that the neotropical populations flower in non-annual cycles and/or irregularly (Baker 1965, Frankie *et al.* 1974, Murawski and Hamrick 1992). The data in table 5.1 suggest that the flowering period may be at least in part determined by a genetically controlled 'clock' since the wild and cultivated (with unknown provenance) trees, all exposed to the same climatic stimuli, flowered in different years. The trees in the Maracá and Manaus areas (located at 370 km north and 312 km south of the equatorial line respectively) appear to have their phenology adjusted to flower during the local dry seasons, *i.e.* October-March in Maracá (Thompson *et al.* 1992) and August-October in Manaus (Ribeiro 1976).

Ceiba pentandra is a massive flowering tree that attracts assemblages of different animals during the flowering. The sugar concentration and the volume of

nectar secreted by each flower are within the range of most chiropterophilous species (Heithaus *et al* 1974, Gould 1978, Lack 1978, Faegri and van der Pijl 1979, Voss *et al* 1980, Hopkins 1984, Ramirez *et al* 1984, Lemke 1985, Eguiarte and del Rio 1987, Helversen and Reyer 1984, Kress and Stone 1993). However, the amount of nectar offered by these trees is, to our knowledge, higher than that of any other species. The estimation of around 650,000 flowers set by tree #1 (a relatively young and small tree that flowered for the first time during the study period) means that around 200 litres of nectar (and *ca.* 30 kg of sugars) were available to the floral visitors during the five-week flowering period. Thus, during the flowering peak, a tree such as #1 can probably secrete more than 10 litres of nectar (with over 1.5 kg of dissolved sugar) per night. Older and bigger trees, usually with up to 35 m of crown diameter, certainly produce still more impressive amounts of floral reward.

The very low fruit-set and the fact that less than one among five hand cross-pollinated flowers set fruit (see Chapter 6) indicate that *C. pentandra* produces massively more flowers than necessary for achieving a fruit-set compatible with the available resources. The cost of producing such excess of flowers, nectar, and pollen may be compensated by an increase in male function fertility (Queller 1983). The female fitness may be also favored by the massive flowering because it allows the maternal parent to eliminate (through selective

abortion) immature fruits with few or with genetically poor quality seeds (Janzen 1977, Stephenson 1981). These two selective forces are non-exclusive and may have contributed jointly to the fixation of a massive flowering strategy in *C. pentandra*.

- The role played by each visitor

The combination of floral features (shape, colour, odour, period of nectar production, time of the anthesis) in *C. pentandra* indicates a strong adaptation for pollination by nocturnal animals, especially bats. A remarkable floral event, previously cited by Baker (1983) and by Murawski and Hamrick (1992), is the abscission of the style, together with the petals and anthers, during the morning following the anthesis. Ovaries from diurnal hand pollinations (effected at 06:00 a.m.) presented no pollen tubes, indicating that the pollen tubes do not traverse the length of the style before abscission occurred (see next chapter). Thus, diurnal pollinations are precluded or have no importance in *C. pentandra* reproduction. This strict adaptation for nocturnal pollination differentiates *C. pentandra* from other bat-pollinated Bombacaceae such as *Ceiba acuminata* (Baker *et al.* 1971), and *Pseudobombax ellipticum* (Eguiarte *et al.* 1987), where the diurnal floral visitors may have some influence on fruit- and seed-set. The failure to observe this essential aspect of the *Ceiba pentandra* floral biology by some authors led them to suggest

possible effective pollination by diurnal insects (Toxopeus 1950), birds (Toledo 1977) and monkeys (Janson *et al.* 1981)

In both areas of this study, glossophagine bats were not observed or captured visiting *C. pentandra* flowers although they are frequent visitors of other chiropterophilous plants such as *Couepia longipendula, Caryocar villosum* and *Bauhinia longicuspis* in the Manaus area (R. Gribel, personal observations). At first sight this seems surprising, since *C. pentandra* floral morphology permits glossophagine bats to take nectar during their hovering flight visits (contrasting with others non-glossophagine bat-pollinated or non-flying mammal pollinated Bombacaceae such as *Pseudobombax tomentosum* and *Ochroma pyramidale* which have large, erect and funnel-shaped flowers which preclude such typical glossophagine hovering visits (Gribel 1988, Gribel *et al.* 1990)). However, the absence of glossophagines at C. *pentandra* flowers may be a predator-avoidance response due to the intense activity of *P. hastatus* in these areas. *Phyllostomus hastatus* is an omnivorous bat that can prey on small vertebrates (Gardner 1977), and may include glossophagine bats in its diet, at least in captivity (Dunn 1933). Certainly, glossophagine bats do visit *Ceiba* flowers in other neotropical areas (Alvarez and Gonzales Quintero 1969, Heithaus *et al.* 1975). It is also intriguing that the other bat species cited by Heithaus et al. (1975) as having pollen of *C. pentandra*

in the fur or digestive tract, such as *Carollia perspicillata*, *Sturnira lilium*, and *Artibeus lituratus*, were not captured, photographed or observed visiting this species in this study, despite being very common in the Manaus and Maracá areas (R. Gribel unpublished data).

Although the three nocturnal non-flying mammals observed in this study made legitimate visits (= non-destructive visits, touching the anthers and stigmas) it is likely that they have a minor or negligible role in the *C. pentandra* pollination, when compared with the bats, because of the scarcity of their visits and their lower ability to move between scattered *C. pentandra* trees through a vegetation with a discontinuous canopy and flooded soils during the flowering season (assuming that the cross-pollination is important to the seed-set, see next chapter).

Phyllostomus hastatus and *P. discolor* are by far the main pollinators of *C. pentandra* in both studied areas. The following behavioral feature of both *Phyllostomus* species contribute to the their efficiency as pollinators of this species: (1) the high frequency of their visits and the heavy pollen load always present on their fur (2) foraging in big groups that, presumably, move between trees, (3) their potential to cover large distances, (4) their non-destructive visits on the inflorescences, and (5) their period of foraging concentrated in the first half of the night, permitting the pollen tubes to traverse the style before its

abscission in the morning. The data from the present study, carried out in areas separated by 700 km north/south direction, together with the observations of Carvalho (1960) in Belém (located 1300 km east from Manaus) indicate that the *C. pentandra/Phyllostomus* pollination system may be regionally widespread.

Abundant resources concentrated in small, irregularly distributed patches during ephemeral and unpredictable periods, favour group foraging in phyllostomid bats (Baker 1973, Ayensu 1974, Howell 1979, Fleming 1982, Lemke 1984 and 1985). Group foraging was observed for *P. discolor* in several studies (Heithaus *et al* 1974 and 1975, Sazima and Sazima 1977, Gribel and Hay 1993, Kress and Stone 1993). Foraging in big groups, *P. hastatus* and *P.discolor* share the resources available in a given tree and may be forced to move to other trees to satisfy their energetic demands, promoting cross-pollination. *Phyllostomus hastatus* and *P. discolor* have a high potential as long-distance cross-pollinating vectors. The foraging territory of *P. hastatus* may comprise an area up to 20 km around the roosting site (Williams and Williams 1970; but see McCracken and Bradbury 1981 for a smaller estimation of the *P. hastatus* foraging area). *Phyllostomus discolor* can cover distances greater than 1 km overnight (Heithaus et al. 1975).

Although the palaeotropical trees of *C. pentandra* have been cited as self-compatible (Toxopeus 1950, Baker

1965, Elmqvist *et al.* 1992), cross-pollination can be important in the neotropical populations. Studying the mating system of a population of *C. pentandra* in Panama using allozyme markers, Murawski and Hamrick (1992) showed the occurrence of a mixture of selfing and outcrossing, indicating that cross-pollination can be essential for fruit- and seed-set in a proportion of the eleven studied individuals. The fact, observed in the present study, that isolated flowering trees did not set fruits, indicates that these trees may be self-sterile. Pollination tests carried out on tree #1 have shown that this individual was totally self-sterile (see Chapter 6).

The body size and the visiting behaviour of *P. hastatus* and *P. discolor* resemble some medium- and big-sized Pteropodidae bat species (although the neotropical bats made shorter visits) such as *Nanonycteris veldkampii* and *Epomophorus gambianus* which pollinate *C. pentandra* in Ghana (Baker and Harris 1959, Harris and Baker 1959; compare the photographs of bats on *C. pentandra* flowers in these two papers with the fig. 5.4a and 5.4b). The similarity of the selective pressures exerted by the main pollinators in both Neotropical and Palaeotropical regions may have contributed to the success of *C. pentandra* in colonizing extensive tropical areas and diverse habitats whilst keeping the same basic floral structure and function despite large-scale geographical (and genetical) disjunct populations. It is noteworthy that the *C. pentandra* tree studied by Elmqvist *et al.*

(1992) in Samoa presented a strikingly similar pattern of nectar secretion to tree #1 in Manaus (slightly more than 100 µl of nectar just after anthesis, secretion finishing around 02:30 a.m.).

Chapter 6

Breeding and Mating System of *Ceiba pentandra*.

6.1 - INTRODUCTION

Self-fertility has been cited as a characteristic of *Ceiba pentandra* reproductive biology throughout its distributional range. Toxopeus (1948) stressed that *C. pentandra* is a highly self-pollinating species in Southeastern Asia, and Toxopeus (1950) found that only 16% outcrossing occurred in Java plantations. Baker (1955, 1965) considered this species 'fully self-fertile' and that 'only a single viable seed ... could be the founder of a new colony'. In the Pacific Islands, the fruiting in *C. pentandra* was explained by van der Pijl (1956) on the basis of the introduction of self-fertile forms. Elmqvist *et al.* (1992) cited that 10% of the geitonogamously pollinated flowers developed fruits on a tree in Samoa, but the flowers were not isolated to avoid visits of bats. Murawski and Hamrick (1992) estimated an outcrossing rate of t_m = 0.689 for a population of *C. pentandra* in Barro Colorado Island, Panama, and estimated that some of the studied trees were highly or completely selfed.

This chapter reports a study of the breeding and mating system of a few *C. pentandra* trees in the Manaus area, with observations on pollen tube growth and the developmental pattern of ovules in hand self- and cross-pollinated flowers. In addition, the apparent self-sterility of these plants is discussed in the light of current theories of late-acting self-incompatibility and inbreeding depression.

6.2 - MATERIALS AND METHODOLOGY

- Site location and trees

The study site and the flowering and fruiting behaviour of the trees used in this study are given in the Chapter 5 (fig. 5.2 and table 5.1 respectively).

- Pollination tests

The breeding system was studied by means of hand pollinations made in the lower branches of tree #1 from a platform placed at the top of a 18 m scaffold. The pollen for the cross-pollinations was collected from tree #2 using a 16 m pruning-hook and ladder. The following treatments were carried out:

- **Apomixis**: buds were opened, emasculated and bagged before anthesis at 17:00-18:00h. Flowers were unbagged the following morning at 10:00-11:00h.

- **Self-pollination:** buds were bagged before anthesis. A massive self-pollen load (from #1) was deposited on the stigma at around 20:00h, after which the flowers were rebagged. Flowers were unbagged at 10:00-11:00h next morning.
- **Mixed-pollination:** buds were opened, emasculated and bagged before anthesis. A massive pollen load containing approximately 1:1 ratio of self- (from #1) and cross-pollen (from #2) was deposited on stigmas at around 20:00h, after which flowers were rebagged. Flowers were unbagged at 10:00-11:00h next morning.
- **Cross-pollination:** buds were opened, emasculated and bagged before anthesis. A massive cross-pollen load (from #2) was sited on the stigma at around 20:00h. Flowers were unbagged at 10:00-11:00h of the following morning.
- **Diurnal cross-pollination:** buds were opened, emasculated, and bagged before anthesis. Flowers were unbagged and pollinated with a massive cross-pollen load (from #2) at around 06:00h of the following morning.
- **Open-pollination:** Opened flowers were only targeted in the morning following the anthesis to follow the natural fruit-set.

The 1:1 pollen load for the mixed-pollination was obtained by loading the pollen of five flowers from

tree #1 and five flowers from tree #2 in a petri dish, thoroughly mixing the mass of pollen, and this was deposited on the stigmas with a spatula. The self- and cross-pollinations were made in the same way, but using only a pure load of self- or cross-pollen respectively.

- Fruit and seed collection

Developing fruits resulting from hand-pollinations were enclosed within muslin bags one month after pollinations so that all seeds in those fruits were collected and counted when the valves opened at fruit maturation. The same procedure was followed for 20 open-pollinated fruits. Differences in the number of seeds in fruits resulting from cross-, mixed-, and open-pollination were tested by pairs of treatments by oneway ANOVA (Sokal and Rohlf 1981). Comparisons of fruit-set between treatments were made by the chi-square test.

- Intra-ovarian post-pollination events.

Subsamples of pistils from self-, cross-, and diurnal cross-pollination treatments were fixed in FAA 12, 24, 48 and 72 hours following pollination. The proportion of pollen tube penetrations (identified by the presence of pollen tube `tails' penetrating the micropyles of the ovules) in the hand-pollinated pistils was quantified under fluorescence microscopy using the aniline-blue staining technique (Martin 1959). In

addition, the pericarp wall of six ovaries (three selfed and three crossed) were removed six days after pollination in order to count and observe the size and development patterns of the ovules using a dissecting microscope.

- Electrophoretic procedures.

A preliminary study that attempted to define suitable genetic markers established that the PGI (phosphoglucoisomerase) isozyme presented banding differences between the two trees.

Seeds were collected from the mature capsules resulting from cross-, mixed-, and open-pollinations in tree #1 and from the open-pollinated fruits in tree #2. The seeds from tree #11 were collected from the seed shadow. Seeds were washed in a 30% (v/v) solution of sodium hypochlorite, rinsed, and put to germinate in Petri dishes with moist filter paper. Pieces of fresh leaf tissue (ca. 1 cm^2) from the adult trees and from 15-20 day-old seedlings were crushed in a chilled microtiter plate using a 4 mm diameter glass rod. One drop of the extraction buffer was added to each sample. The extraction buffer was similar to that of Mitton *et al.* (1979) but 5.0 ml of β - mercaptoethanol/liter was used instead of 1.8 ml/liter in order to diminish the intensity of 'ghost' bands. The crude extracts of the leaf tissue were absorbed on 5 x 3 mm chromatography paper wicks and inserted in a 12% (w/v) starch gel. The

histidine-citrate ph 5.7 buffer system (Stuber *et al.* 1977, Wendel and Weeden 1989) was used to prepare the electrode and gel buffers. Staining protocols for PGI enzymes followed Wendel and Weeden (1990). The gels were sliced and stained after 4.5h under constant voltage of 250 volts.

- Interpretation of the PGI banding patterns

Preliminary analysis of parental trees and progenies revealed two staining regions for PGI enzymes. The most anodal (designated PGI-1, located 25 mm from the origin) presented bands with poor resolution and was inferred to be, by analogy with other plant species, the subunits coded by the PGI chloroplast locus (Schnarrenberger *et al.* 1975, Gottlieb and Weeden 1981, Weeden and Gottlieb 1980). The slower region (named PGI-2, located 10 - 15 mm from the origin) exhibits three apparently dimeric enzymatic phenotypes: the three-banded heterozygotes (genotype $Pgi\text{-}2^{ab}$, hereafter designated genotype *ab*), and the fast- and the slow-banded homozygotes (genotypes $Pgi\text{-}2^{aa}$ and $Pgi\text{-}2^{bb}$, hereafter designated genotypes *aa* and *bb* respectively). In terms of this study, *Pgi-2* was interpreted as a diallelic locus coding for the dimeric cytosolic PGI enzyme.

The genetic basis of the *Pgi-2* locus in *C. pentandra* was tested by crossing trees #1 and #2 (genotypes *ab* and *bb* respectively). The progeny genotype array, which consisted of 39 *ab* and 42 *bb* seedlings, was not

statistically different from a Mendelian expectation (χ^2 = 0.11, 1df, P = 0.739). The progeny array presented no excess of the maternal *aa* genotypes, suggesting that apomixis is non-existent or of very low incidence.

- **Estimate of the proportion of outcrossed seeds**

It was assumed that pollen exchange occurred exclusively between the neighbouring trees #1 and #2 (i.e. no other external pollen reached the open-pollinated flowers of both trees), since they flowered simultaneously in 1992 in isolation from other planted or wild conspecific trees (Chapter 5). The proportion of crossed seeds produced by tree #1 in the open-pollinated fruits was estimated by considering the proportion of *aa* genotypes in the progeny by the formula:

% crossed seeds in the tree #1 progeny = 1 - % selfed seeds =

1 - 4 (% *aa* seedlings in the progeny)

The proportion of crossed seeds in the mixed- (self + cross) pollinated fruits of the tree #1 was estimated in the same way.

The estimation of crossing In tree #2 was based on the departure from the 1:1 ratio expected to the *ab* and *bb* progeny genotypes by pure cross-pollination with the tree #1. Thus;

% crossed seeds in the tree #2 progeny = 2 (% *ab* seedlings in the progeny)

In the homozygous tree #11 (genotype *aa*), which flowered in 1993 simultanecusly with several other wild trees (Chapter 5), the proportion of crossed seeds was obtained considering the number of heterozygous seedlings (genotype *ab*) in the progeny, since the two nearest flowering conspecific neighbours (trees #10 and #11) have *ab* genotypes. Thus, the estimation of the percentage of crossed seeds was given by the formula:

% crossed seeds in the tree #11 progeny = 2 (% *ab* seedlings in the progeny)

6.3 - RESULTS

- Intra ovarian post-pollination events

All selfed pistils were retained and enlarged somewhat until the 5th-7th day following pollination when they fell due to an abscission in the pedicel 25-30 mm below the receptacle. Most of the cross- and mixed-pollinated pistils also exhibited this sequence and fate, but a proportion of them (16.7% and 8.7% respectively) persisted to develop into mature fruits. All unpollinated pistils (apomixis controls) and the diurnally pollinated pistils did not undergo any enlargement and they were aborted 24-48 hours following anthesis.

Observations with fluorescence microscopy and leuco-aniline blue staining revealed that both selfed and outcrossed pistils had massive pollen tube growth in the placenta and c. 30% of the ovules were penetrated 24

hours following pollination. The proportion of penetrated ovules in the selfed and crossed pistils slightly increased from the first to the third day following pollination, reaching a maximum of around 40-42% penetrations for both treatments by the 3rd day (fig. 6.1). The diurnal cross-pollinated pistils had no trace of the presence of pollen tubes in the placental region, and likewise no ovules with pollen tube penetrations at 24 hours after pollination (n = 5 pistils).

Fig 6.2 shows the size classes by diameter of the ovules in three self- and cross-pollinated pistils of tree #1 six days after pollination. In the self-pollinated pistils there was a range of sizes but the majority presented little or no enlargement, with only a few showing some degree of enlargement. In the cross-pollinated pistils there were basically two size groups: the small non-enlarged ovules (diameter < 0.4mm) and another group of very enlarged ones, these latter were presumably composed of developing embryos/endosperms. The proportion of these enlarged ovules six days after pollination was considerably lower than the percentage of the penetrated ovules observed by fluorescence microscopy in the crossed pistils 3-4 days after pollination, indicating that the penetration of the cross-pollen tubes in the micropyle may not result in fertilization and/or development of the embryo/endosperm in many cases. However, this bias may be caused by the small sample size for both experiments. The typical appearance of the ovule development in selfed

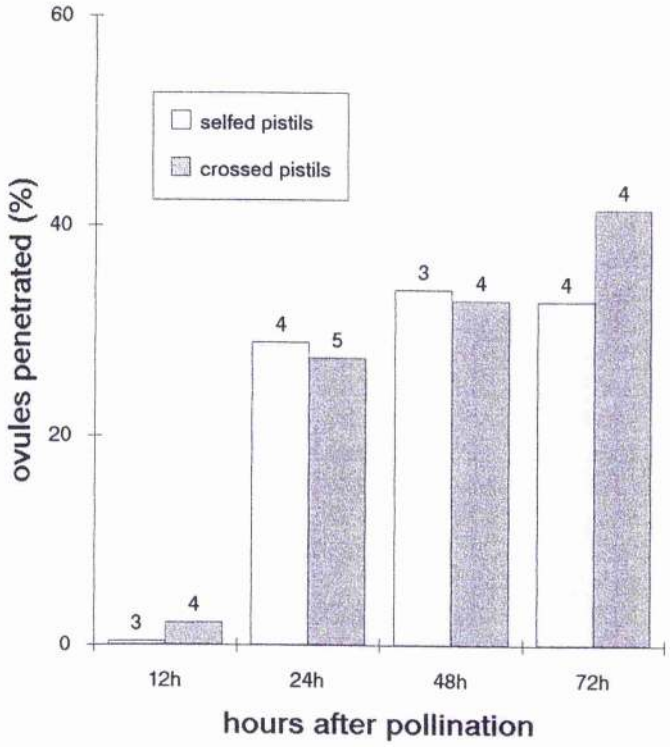

Fig. 6.1 - Percentage of ovules penetrated by a pollen tube in self- and cross-pollinated pistils of *Ceiba pentandra*. Numbers at top of bars = number of pistils observed.

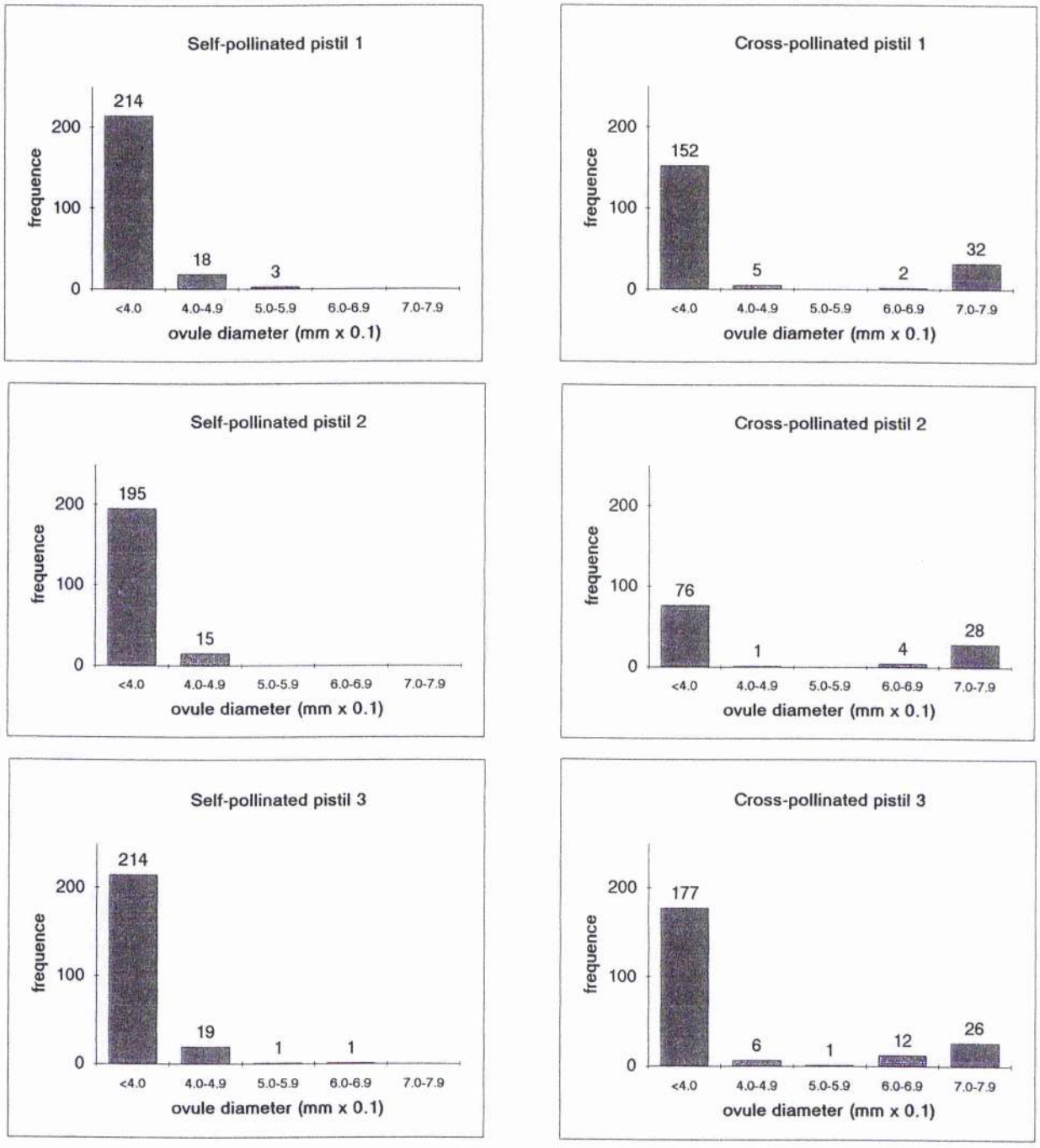

Fig 6.2 -Distribution of ovule size in diameter class in self- and cross-pollinated pistils of Ceiba pentandra six days after pollination.

and crossed pistils, with the ovary wall removed, is shown in fig. 6.3.

- Breeding and mating systems

The result of the controlled pollinations (table 6.1) revealed the following characteristics of the studied tree: (1) it is strongly self-sterile; (2) it does not set fruits when pollinations occur during the diurnal period; (3) cross-pollinated flowers set significantly more fruits than the mixed-pollinated ones (χ^2 = 6.49, 1 *df*, *P* = 0.011); (4) fruits resulting from cross-, mixed-, and open-pollinations are significantly different in regard to the number of seeds per fruit (F = 17.49, *P* < 0.001).

The PGI zymograms of the progeny resulting from cross-pollination (#1 x #2), which exhibit two enzymatic phenotypes (corresponding to genotypes *ab* and *bb*), and from mixed-pollination (#1 x #1,#2), which exhibit three enzymatic phenotypes (corresponding to the genotypes *aa*, *ab*, and *bb*), are shown in the fig 6.4. Notice that the fourth sample in the zymogram of the fig. 6.4b (homozygous for the faster allele, genotype *aa*), must result from selfing.

Table 6.2 shows the estimation of crossed seeds in fruits resulting from controlled mixed-pollinations in the tree #1 and from open-pollinations in the trees #1, #2 and #11. The results suggest that trees #1 and #11 are predominantly outcrossed, whereas tree #2 seems to be somewhat more self-fertile.

Table 6.1 - Pollinations tests, fruit-set and number of seeds per fruit in a tree of *Ceiba pentandra* in Manaus area.

Treatment	pollen load	pollination time	n° flowers treated	n° fruits formed	fruit-set (%)	seeds/fruit (x ± sd)
Apomixis control	-	-	158	0	0.0	-
Self-pollination	100% self	20:00	407	0	0.0	-
Cross-pollination	100% cross	20:00	286	48	16.8	170.5 ± 41.5 (n = 46)
Mixed-pollination	50% self 50% cross	20:00	241	21	8.7	147.0 ± 43.6 (n = 19)
Diurnal cross-pollination	100% cross	06:00	47	0	0.0	-
Open-pollination	?	?	282	2	0.7	106.8 ± 34.1 (n = 20)

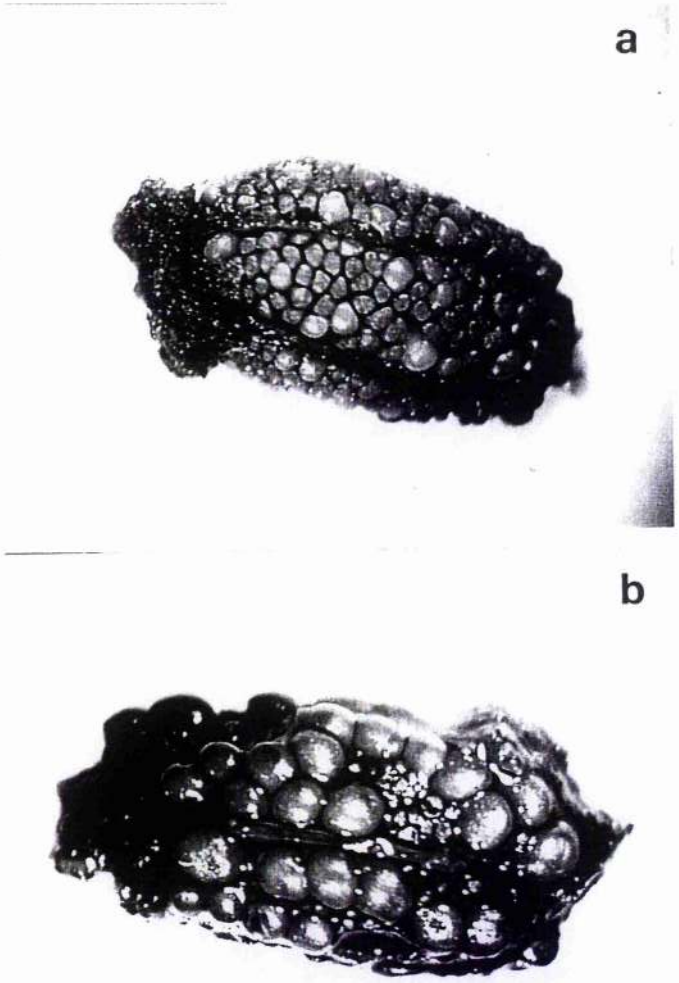

Fig. 6.3 - Ovaries of *Ceiba pentandra* with the outer wall removed showing the pattern of the development of the ovules: **(a)** ovary of a self-pollinated flower, and **(b)** ovary of a cross-pollinated flower. Both ovaries fixed six days after pollination.

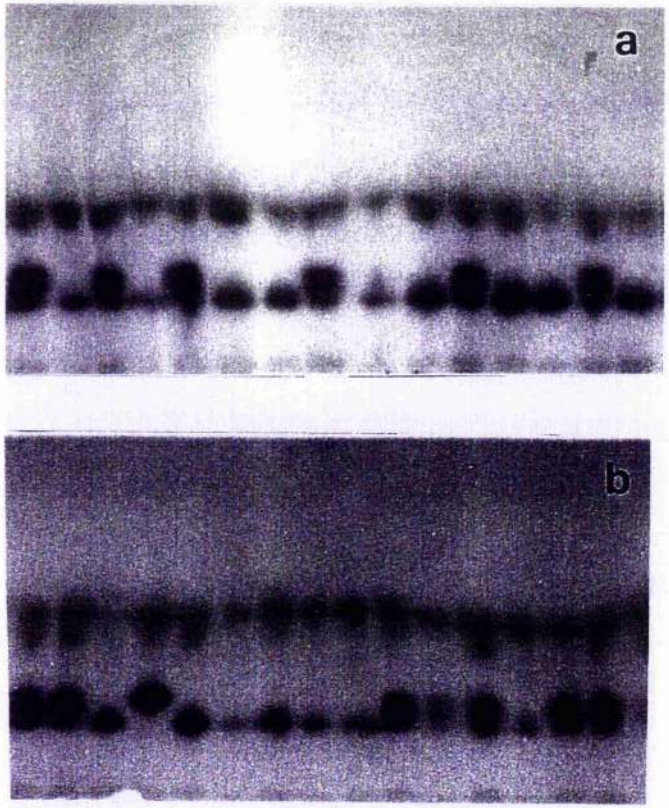

Fig. 6.4 - *Ceiba pentandra* electrophoretic phenotypes of PGI enzymes: **(a)** progeny resulting from the cross-pollination #1 x #2, and **(b)** progeny resulting from the mixed-pollination #1 x (#1,#2). See explanation in the text.

Table 6.2 - Maternal and offspring genotypes at PGI-2 and estimation of the percentage of crossed seeds produced in fruits of *Ceiba pentandra* resulting from hand- and open-pollinations. See explanations in the text.

maternal tree (genotype)	nearest neighbour trees (genotype, distance)	offspring genotypes				estimation of crossed seeds (%)
		aa	*ab*	*bb*	total	
#1 (*ab*)	#2 (*bb*, 20m)					
- mixed-pollination		2	254	241	427	98.1
- open-pollination		5	103	114	222	91.0
#2 (*bb*)	#1 (*ab*, 20m)					
- open-pollination		-	61	110	171	72.3
#11 (*aa*)	#10 (*ab*, 2km), #9 (*ab*, 4.5km)					
- open-pollination		25	23	-	48	95.8

6.4 - DISCUSSION

- The high potential for inbreeding

Self- and geitonogamous-pollinations should be a common event in *Ceiba pentandra* because of (1) the massive flowering and the large amount of nectar available cause the pollinator bats to visit repeatedly flowers from the same tree; (2) the small spatial separation between anthers and stigmata in the cluster of flowers of the inflorescences (low degree of herkogamy), and (3) the stigmatic receptivity and pollen release occurring simultaneously (homogamy).

Thus, *Ceiba pentandra* presents no morphological or functional floral attributes to avoid self-pollination and promote outcrossing. In natural conditions, consequently, this species is exposed to considerably high levels of self-pollination and, presumably, self-fertilization, since there are no constraints (sporophytic or gametophytic SI barriers) to the development of the self-pollen tubes until penetration of the ovules.

Cytological events after self-pollen tube penetration were not covered in the present study, but the initial enlargement of several ovules (embryos + endosperm?) in the selfed flowers indicates that a proportion of ovules may be self-fertilized. Another clue that the fertilization occurs in the self-pollinated flowers is that their pistils are maintained and develop in size until the 5^{th} - 7^{th} day after anthesis, exactly

as happens with most of the crossed flowers, so the physiological stimuli caused by many developing embryos demanding nutrients seems to be similar in both treatments at least during the first days after anthesis. This contrasts with the non-pollinated ones, which exhibit no initial enlargement and are abscised 1-2 days after anthesis. Murawski and Hamrick (1992) using isozyme markers found several *C. pentandra* trees in Panama with low outcrossing rates, including one completely selfed individual, and apomixis was considered absent or negligible by these authors. In the phylogenetically very close genus *Chorisia* (Gibbs *et al.* 1988) fertilization followed by the formation of a resting zygote and initial development of endosperm were observed after self- and cross-pollen tube penetrations (Gibbs and Bianchi 1993).

Therefore, high levels of self-pollen tube penetration presumably followed by self-fertilization seems to be an intrinsic characteristic of the *C. pentandra* breeding system. The higher proportion of selfed-seeds set by tree #1 from open-pollination than by hand mixed-pollination (9.0% *vs.* 1.9% of the total number of seeds respectively, table 6.2) suggests that self:cross pollen ratios in the pollen load naturally deposited on the stigmas was on average much greater than the 1:1 ratio of the controlled mixed-pollinations. This high potential for inbreeding can be at least partially compensated in *C. pentandra* by (1) the very small

seed:ovule ratio and the stronger selective abortion against fruits developing more selfed zygotes, as suggested by the results of the pollination tests; (2) by the great mobility of the pollen vectors; and (3) by the putative efficiency of the combined wind/water dispersal systems.

- Late acting self-incompatibility or early-acting inbreeding depression ?

The virtually equal performance (= capacity to penetrate ovules) of the self- and cross-pollen in *C. pentandra* appears to be a common feature in some predominantly self-sterile species of Bombacaceae (Oliveira et al 1992, Gibbs and Bianchi 1993) and other species of diverse families (Taroda and Gibbs 1982, Bertin and Sullivan 1988, Waser and Price 1991, Lloyd and Wells 1992, Broyles and Wyatt 1993, Gibbs and Bianchi 1993, Seavey and Carter 1994, see more extensive literature reviews in Seavey and Bawa 1986, Gibbs 1988 and 1990, Sage *et al.* 1994). The total or partial failure of the selfed zygotes in these plants has been attributed to the action of embryonic lethal alleles causing an early manifestation of the inbreeding depression (Charlesworth 1985, Klekowski 1988, Krebs and Hancock 1991, Seavey and Carter 1994) or, alternatively, to an active post-fertilization self-rejection reaction under maternal genetic control denominated as 'late-acting self-incompatibility' (Seavey and Bawa 1986, Sage and

Williams 1991, Waser and Price 1991, Broyles and Wyatt 1993, Gibbs and Bianchi 1993).

Two aspects of the reproductive biology of *C. pentandra* should be stressed with regarding to the criteria currently considered to determine whether self-zygote failure is caused by inbreeding depression or late-acting SI: (1) the variety of developmental stages shown by the ovules in self-pollinated flowers just before the pistil abscission; and (2) the variable level of self-sterility between trees, observed in this work and by Murawski and Hamrick (1992) in Panama. Both features are considered to be typically caused by an accumulation of deleterious or lethal recessives rather than by self-incompatibility (Seavey and Bawa 1986, Klekowski 1988, Waser and Price 1991, Sage *et al.* 1994)

A continuum of variability for the degree of self-sterility in a population can be taken as evidence for the occurrence of a variable number of lethal recessives among individuals and also indicates that the failure in the development of the selfed embryos into seeds in these species would be caused by genetic load rather than by late-acting SI (Seavey and Bawa 1986, Klekowski 1988, Waser and Price 1991, Sage *et al.* 1994). The mating system of 11 *C. pentandra* trees studied by Murawski and Hamrick (1992) in Panama ranged from complete selfing to complete outcrossing, indicating that inbreeding depression is likely to be the main cause of the self-sterility observed in a proportion of individuals of this

species. Previous investigations, in contrast with the data of the present study, have indicated that some *C. pentandra* individuals are predominantly self-fertile in other areas of its distributional range (Toxopeus 1948, 1950, Baker 1955, Elmqvist *et al.* 1992).

The strong self-sterility following self-pollination in tree #1, the relatively high (although variable) estimate of outcrossing for the trees #1, #2, and #11, and the fact that the somewhat isolated tree #12 set no fruits despite massive flowering (Chapter 5) suggest, however, that the degree of self-fertility in the Central Amazonian trees is probably lower than in the individuals from those other areas. One possible explanation for this may be that populations in Southeast Asia, the Pacific Islands, and possibly West Africa have suffered a bottleneck in the population size during the colonization phase, with selection favoring individuals with less recessive lethals and consequently reducing the degree of inbreeding depression. On the other hand, in historically large populations located in the 'core' of the distributional range, such as those from the Central Amazon area, higher levels of outcrossing followed by an accumulation of lethal alleles should be expected (Sorensen 1969, Lande and Schemske 1985, Jarne and Charlesworth 1993).

How many embryonic lethals?

The number of embryonic lethals operating in the selfed zygotes of the studied tree can be estimated on

the basis of the following assumptions: (1) the self-pollen tubes penetrate and fertilize the ovules in the same ratio as the cross-pollen tubes in the mixed-pollinated pistils; (2) the inbreeding coefficient between the trees #1 and #2 is near null ($F \approx 0$); and (3) the relative survivorship of the selfed zygote (R), defined as the number of selfed seeds divided by the number of crossed seeds in the mixed-pollinated fruits, can be used to estimate the embryonic genetic load by the method of Morton *et al.* (1956) as applied to plants by Sorensen (1969), Levin (1984), and Seavey and Carter (1994), by the formula:

$$\mathbf{2B = 4 \ln R}$$

where 2B is the 'hidden' genetic load on a zygote basis, expressed as the number of lethal equivalents.

On this basis, considering the production of selfed and crossed seeds by mixed-pollination (table 6.2), there are 15.8 lethal equivalents acting in the selfed zygotes of the tree #1. One could argue that the above value is an overestimate because the selective abortion is concentrated in immature fruits with less seeds (i.e. with probably higher proportion of selfed zygotes), so that the surviving fruits resulting from mixed-pollinations could present a bias towards a higher ratio of cross-:self-fertilization than the 1:1.

This bias can be compensated by estimating the proportion of crossed and selfed zygotes in the mixed-pollinated pistils which developed into mature fruit. The

mean number of crossed seeds in the 19 mixed-pollinated fruits (mean number of seeds minus 1.9% of selfed seeds) was 144.2, whereas the mean number of seeds in the 19 cross-pollinated fruits with highest seed-set was 210.9. Thus, mixed-pollinated fruits had, on average, 31.4% less crossed seeds (instead of 50% less as expected by a 1:1 rate of self:cross fertilization) than fruits resulting from cross-pollination. Considering this correction to calculate the 'realized' R, the estimated number of lethal equivalents was $2B = 12.3$.

With this number of recessive embryonic lethals, the probability of a given self-pollinated pistil having at least 66 lethal-free embryos (which is the minimum number of seeds observed in 85 fruits from tree #1) is virtually nil, even if all ovules are self-fertilized. Thus, with such a high number of embryonic lethals acting, the fruit-set following self-pollination is nil or negligible, and the genetic load effect mimics a SI mechanism. Tree #1, a strongly self-sterile individual, appears to have an exceptionally high genetic load. However, the average number of recessive lethals in other trees may be lower.

The higher fruit-set (estimate of 2.2% *vs.* 0.7% respectively, see Chapter 5) and the higher proportion of selfed-seeds in the naturally-pollinated fruits (28% *vs.* 9% respectively) indicate that tree #2 is more self-fertile than tree #1. It is noteworthy that, despite the lower outcrossing, tree #2 probably received proportionally more cross-pollen than tree #1, since its flowering was

considerably less intense than of its neighbour (Chapter 5). This difference in the degree of self-fertility between these two neighboring trees which flowered simultaneously reinforces the idea that genetic load is likely to be the cause of failure of selfed-embryos in *C. pentandra* rather than a post-zygotic SI reaction (Seavey and Bawa 1986, Klekowski 1988).

- Conservation biology implications

This chapter presented evidence that the *C. pentandra* trees in Central Amazon, which is the center of its native distribution, has a higher degree of self-sterility than in other areas probably because of the accumulation of a genetic load. Consequently, many trees in this region may obligatorily require cross-pollination to set fruits and seeds. The intensive exploitation of this species by the plywood industry in the Brazilian Amazon has caused a dramatic reduction of tree densities, a process already registered for *C. pentandra* in the Peruvian Amazon by Gentry and Vasquez (1988). Large distances may constrain the movement of bats (and consequently pollen) between trees, affecting the reproductive capacity of the non-exploited individuals, usually trees of difficult access or with some trunk defect. Thus, any conservation policy should take into consideration that the recovery of the remnant populations from logging may be limited by the characteristics of the tree mating system in this region, in which outcrossing may be essential for the species propagation.

Concluding Remarks

- Floral biology and pollination ecology

The guild of bat pollinators in the Neotropical region is characterised by a high richness and abundance of bats of the subfamily Glossophaginae. Most chiropterophilous neotropical plants seem to be adapted to pollination by this group of bats (Vogel 1968, 1969a, 1969b, Sazima and Sazima 1978, 1988, Sazima *et al.* 1982, 1989, 1994, Lemke 1985, Ramirez *et al.* 1984, Buzato and Franco 1992, Gribel and Hay 1993). Glossophagine bats are always small (8-15 g), with a long and fine specialised tongue, and visit the flowers in typical hovering flights. In contrast, other more generalist neotropical bats, such as *Phyllostomus*, *Artibeus*, *Vampyrops*, *Sturnira*, and *Carollia*, always land on the flowers to collect nectar or pollen (Carvalho 1960, Heithaus *et al.* 1974, Sazima and Sazima 1975, 1977, Hopkins 1984, Ramirez *et al.* 1984, Gribel and Hay 1993, Kress and Stone 1993). These two groups of bats probably have exerted distinct selective pressures on the bat-pollinated flower structures in the Neotropical region, although the flowers of some plants are visited (and presumably pollinated) by glossophagine and non-glossophagine bats

(Heithaus *et al.* 1974, Sazima and Sazima 1975, Ramirez *et al.* 1984, Gribel and Hay 1993). The two species considered in this study appear to be primarily adapted for pollination by non-glossophagine bats, more specifically for pollination by bats of the genus *Phyllostomus*. Despite the use of similar pollinators, however, *Pseudobombax munguba* and *Ceiba pentandra* exhibit contrasting features with regard to the flowering phenology, and floral structure and function, as for example: 'cornucopia' *vs* 'massive' flowering, nectarless *vs* nectar-rich flowers, and solitary, large, 'shaving brush' flowers *vs* cluster of campanulate flowers as the unit of visitation and pollination. These differences illustrate how distinct phenological, morphological, and functional strategies can evolve in sympatric species of the same family, in similar habits, and using similar pollinators.

On the other hand, the apparently minor variations within the general 'large-robust-white' floral pattern observed in the flowers of diverse *Pseudobombax* species, all of them labelled as 'chiropterophilous' by the criteria of the traditional pollination biology theory (Faegri and van der Pijl 1979), may reflect adaptations

to pollination by distinct groups of nocturnal mammals. For example; glossophagine bats (which are small, with a long and narrow tongue, and 'hovering' visits), non-glossophagine bats such as *P. hastatus* (with large size, non-specialised tongues, and 'landing' visits), and arboreal marsupials (with large size, funnel shaped head with non-specialised tongues, capable of reaching the flowers by walking along the branches), are the main pollinators of *P. longiflorum, P. munguba,* and *P. tomentosum* respectively. These diverse pollinators are likely to have exerted distinct selective pressures and affected differentially the evolution of the floral structures in this genus.

The nectarless flower of *P. munguba* is a very singular case within the Bombacaceae and chiropterophilous plants in general. Nectarless flowers may be rare or exceptional even in the genus, since the other three *Pseudobombax* species investigated to date, *P. longiflorum, P. tomentosum* (Chapter 2), and *P. ellipticum* (Eguiarte *et al.* 1987), have copious nectar production. It is speculated in this study that the nectarless flowers of *P. munguba* could be an adaptation to reduce the guild of generalist visitors which are usually observed

in the nectar-rich chiropterophilous Bombacaceae, selecting only *P. hastatus*, which is probably the most mobile of the neotropical pollinators, as flower visitor. A wider study on the pollination biology of the genus, however, is necessary to clarify whether or not the production of nectarless flowers can be considered as an adaptation to promote long distance pollen flow, or just a physiological adjustment for flowering during the flooded period of this várzea species.

In contrast to the nectarless flowers of *P. munguba*, the nectar-rich flowers of *C. pentandra* are visited by assemblages of animals during the nocturnal and diurnal periods. Among the nocturnal visitors, the bats *P. hastatus* and *P. discolor*, which forage in groups and presumably move between trees, seem to be the main pollinators. Despite the intense diurnal visitation, diurnal pollination has no significance in *C. pentandra* reproduction. This is because the styles drop in the morning following anthesis of the previous evening, so that pollen tubes resulting from diurnal pollination shortly after sunrise do not reach the ovary before the stylar abscission. This result contradicts previous

observations which suggested that monkeys, birds, or bees could be effective pollinators of *C. pentandra*.

- Breeding and mating system

The observations of this and previous studies (Oliveira *et al.* 1992, Gibbs and Bianchi 1993) detected no differences between the self- and cross-pollen tubes with regard to their capacity to grow throughout the style, reach the ovary, and penetrate the ovules. These results suggest that virtually equal performance of the self- and cross-pollen tubes may be a widespread characteristic in Bombacaceae. Histological data from those two studies additionally found that fertilisation takes place following both self- and cross-pollination, since the formation of a resting zygote with initial endosperm divisions was observed in both treatments.

In the present study I find no need to invoke the concept of 'post-zygotic late acting self-incompatibility' (*sensu* Seavey and Bawa 1986) as the mechanism explaining the failure of most selfed ovules and causing self-sterility in *P. munguba* and *C. pentandra*. Empirical observations on the variable level of self-fertility among trees of *P. munguba* and *C. pentandra* suggest that

the self-sterility following self-pollination may be caused by genetic load rather by delayed self-incompatibility. Variable levels of fertility in *C. pentandra* are also cited in the literature (Murawski and Hamrick, 1992). Furthermore, the genetic load hypothesis is reinforced by the observation that, in many selfed-pistils of both species, a proportion of ovules exhibited a considerable, but not homogeneous, degree of enlargement a few days after pollination, indicating the possibility that these ovules were fertilised but subsequently failed probably due to developmental abnormalities. Further embryological studies, however, are necessary to confirm this conclusion based on superficial and empirical observations of the ovule development and breeding system of the trees.

Controlled pollinations in *P. munguba* and *C. pentandra* revealed that no fruit-set occurs when a pure self-pollen load is deposited on a stigma. Furthermore, the deposition of self-pollen mixed with cross-pollen affects negatively the fruit-set and the number of seeds per fruit in both species. Therefore, self-pollination, which is facilitated by the 'shaving brush' flower structure in *P. munguba* and by clusters of flowers in *C.*

pentandra, effectively excludes a high proportion of ovules from the pool which potentially could develop into mature seeds. These results stress the importance of the deposition of cross-pollen, and consequently of the pollinator activity between trees, in the reproduction of both species in the Central Amazon. Data from the literature, however, suggest that higher levels of self-fertility may occur at least in some *C. pentandra* individuals throughout its distribution range (Toxopeus 1948, 1950, Baker 1955, Elmqvist *et al.* 1992, Murawski and Hamrick 1992).

As found for most tropical trees investigated so far, *Pseudobombax munguba* exhibits a predominantly outcrossed mating system. High outcrossing rates in tropical trees have been attributed primarily to the action of self-incompatibility. This study, however, indicates that high levels of genetic load, in association with extensive pollen flow promoted by a very mobile pollinator, are likely to be the main causes of the predominance of outcrossing in *P. munguba*. Levels of mutation rate per generation typical for long-lived organisms and extensive pollen flow, which maintains the selfing rate at intermediate levels, may explain the high

equilibrium frequency of recessive lethals estimated for both species (Lande *et al.* 1994).

These conclusions introduce a novel insight into the possibility of an alternative reproductive pathway inducing high outcrossing rates in some tropical trees. On this view, high levels of outcrossing could be achieved not necessarily by the evolution of an 'adaptation' (i.e. a self-incompatibility mechanism) which promotes outbreeding and heterozygosity, but as an 'inevitable consequence' of the lack of a pre-syngamic barrier together with long distance pollen flow.

Finally, the results of this study have some implications for the conservation of these two species and the management of the várzea habitat. Both species produce large amounts of seeds rich in protein and oil which are consumed by a variety of commercial fishes in the region. Two indirect observations suggest that seeds of these two species should be effectively important to native fish populations in the várzea: (1) local fishermen used to place their nets near *P. munguba* trees releasing seeds because they consider that it is easier to catch fish in such areas; and (2) seeds of *P. munguba* have been successfully used to feed commercial fishes kept in tanks

in the Department of Aquaculture at the Instituto Nacional de Pesquisas da Amazonia in Manaus. The intensive exploitation of *C. pentandra* by the lumber mills for the plywood industry and the recent tendency to use *P. munguba* timber to mould concrete in building constructions, may affect the total seed output of these species and, ultimately, the fish carrying capacity of the várzea habitat. The remaining trees, usually widely spaced, probably receive few or no cross-pollen and have reduced reproductive potential.

On the other hand, the fruit- and seed-set in *P. munguba* and *Ceiba pentandra* appear to be limited by natural pollination (Chapter 2 and 5) and, due to the high levels of self-sterility, to be totally dependent on the bat foraging activities between flowering trees (Chapter 4 and 6). Thus, any human activity affecting the populations of *Phyllostomus* (e.g. destruction or disturbance of the roosting sites which basically consist of trunk holes of large trees and caves) could also influence negatively the fruit and seed production.

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