

Krakatau: genetic consequences of island colonization

(Krakatau: genetische gevolgen van eiland kolonisatie)

(met een samenvatting in het Nederlands)

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This thesis is for Mum
and dedicated to Dad.

Visit <http://www.krakatau.org>

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General Introduction

Relevance of the Study

The objective of this study is to examine patterns of genetic diversity and genetic differentiation in populations of tree species on islands after a recent colonization event. This work has been carried out on the Krakatau islands, Indonesia. The Krakatau islands provide a unique opportunity to study the restoration of a tropical rainforest system as vegetation on these islands was completely destroyed by volcanic eruptions in 1883 (Verbeek, 1885; Docters van Leeuwen, 1936; but see Backer, 1929). The islands have since re-vegetated in species-poor secondary monsoonal forest (Whittaker *et al.*, 1989) and now exist as a protected nature reserve system (see Thornton, 1996). Given that we currently are facing a massive biodiversity crisis as human activity drastically diminishes natural habitat (Wilson, 1992; Myers, 1993) such a study is highly pertinent. Studies that document and define existing systems, and identify processes important in their evolution, will aid in the preservation of natural systems.

The study of the genetics of populations is of importance for two reasons. First, genetic variation is the building block of evolution (Frankel and Soulé, 1981). Genetic variation allows for adaptation to and exploitation of novel conditions and is therefore important to plant success in a variable environment. The loss of such variation due to the effects of reduced population size, chance events and inbreeding has been associated with decreased fitness at various levels (for example, increased susceptibility to disease and pests, see Barrett and Kohn, 1991; disease and infertility, e.g. *Acinonyx jubatus*, O'Brien *et al.*, 1985 and *Felis concolor*, Roelke *et al.*, 1993; inbreeding depression, see Barrett and Charlesworth, 1991 and Koelewijn, 1998) and has been associated with increased rates of population extinction (e.g. Glanville fritillary butterfly, Saccheri *et al.*, 1998; e.g. *Drosophila*, Bijlsma *et al.*, 2000). Therefore, given the negative impact of the loss of genetic variation, it is important to understand what forces shape, maintain and deplete genetic variation, and how that variation is structured in natural populations.

Second, one of the fundamental tenets of population genetics is that the history of a population is reflected in the distribution and partitioning of present day variation (Wright, 1969). A considerable amount of theory and analytical technique has been developed that

explain how evolutionary forces shape patterns of genetic variation (Maynard Smith, 1998). Therefore, a study of the distribution of that variation will give insight into the processes that have formed it. The study of natural populations allows for the testing and implementing of this theory.

This introduction will be used first to introduce genetic variation, how it arises and how it is shaped. Next, islands and their importance to studies of the evolution of diversity at both the species and genetic level are discussed. The genetic consequences of colonization are then outlined and patterns of genetic variation in tropical plants and factors important for shaping that variation are introduced. Next, the study system, the Krakatau islands, Indonesia is described and the methods used to assess genetic variation are then outlined. Finally, the aims of this study will be outlined by presenting the central questions put forward in each chapter.

Genetic Variation

Genetic variation is the difference in genetic constitution between individuals in a population and can be quantified at the genomic or phenotypic level. Due to the difficulty in determining the relative contributions of genetic and environmental effects on the phenotype, most population studies directly examine genomic variation using molecular markers. For population studies genetic variation can be quantified as the number and frequency of alleles per locus and is partitioned into hierarchical levels; the individual, within the population, between populations and between regions. Within population variation is measured by the number of alleles at a locus, the number of polymorphic loci and levels of heterozygosity. Variation between populations is usually measured as the variation in allele frequency differences between populations and is referred to as population differentiation (Wright's fixation index, F_{ST}) (Wright, 1969).

Genetic variation arises through mutation and recombination, and is shaped by the processes of population foundation, genetic drift, migration (gene flow), breeding structure and selection (Wright, 1969; Crow and Kimura, 1970). Foundation is the formation of a new population. The number of founding individuals is important in determining the amount of variation within the new population as diversity is greatly reduced as population size becomes small. After establishment, random genetic changes in allele frequency (genetic drift) can occur and result in increasing differentiation between populations. Genetic drift will be of greater effect when population size is small ($N < 200$, Falconer, 1981). The movement of individuals into a population is called migration. The number of migrants exchanged between

populations depends greatly on the distance between the populations. Migration results in gene flow (the effective movement of genetic material) and the cohesion of a species. Reductions in gene flow lead to population differentiation and, if severe enough, breeding isolation and speciation. If populations remain small or migrants are highly related, inbreeding occurs and results in the decrease in genetic variation within populations (increased homozygosity). Natural selection determines the survival of genotypes most suited to a particular environment and usually results in the reduction of genetic variation within populations. If environments differ between populations, selection will lead to population differentiation.

Islands as Systems to Study Evolutionary Processes

Islands provide suitable places to study the processes of evolution for two reasons. First, islands have long been considered hot-spots for evolution as they contain many endemic species. Radiation in many groups has been rapid and has produced forms not seen on continental areas (e.g. Weller *et al.*, 1990; Schultz and Ganders, 1996; also see Whittaker, 1998). Therefore, factors must be operating on islands that promote species diversification. Second, due to their finite size, isolated nature, diversity of habitats and number, islands provide simplified, replicated natural experiments to study the processes of evolution.

Properties of islands that affect organismal diversity are summarized in Figure 1.1, where island size and barriers to dispersal are important. The importance of island isolation and population size in developing and maintaining species diversity was first formalized in the equilibrium theory of island biogeography (MacArthur and Wilson, 1967) and was later applied to genetic polymorphism (Jaenike, 1973) (Figure 1.2). Island size determines population size and hence will affect extinction probability and the number of species that can co-exist. Island isolation will determine the level of breeding isolation in new populations and species number as distance will affect the number, rate and genetic composition of propagules arriving on an island. The rate at which new species immigrate and existing species go extinct are decreasing functions of island isolation and size, respectively (Figure 1.2). Big islands should maintain larger populations and a greater number of habitats and therefore contain more species as the risk of extinction is reduced. On islands close to a source region immigration should be high and therefore a large species number should be maintained. As an island becomes more distant immigration events will be less frequent and immigrant numbers fewer. Island isolation will exclude species with limited dispersal ability,

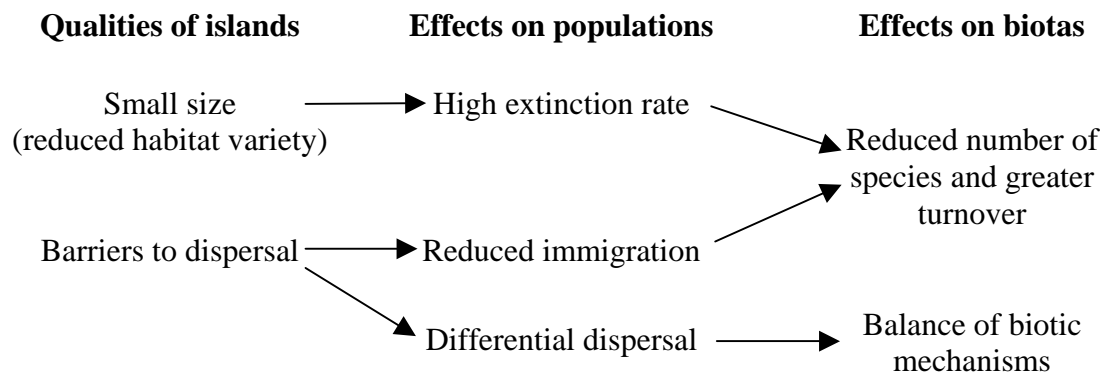


Figure 1.1: A scheme outlining the qualities of islands and their effect on populations and biotas (after MacArthur and Wilson, 1967). For explanation see the text.

resulting in island biotas containing disproportionate numbers of species with high dispersal ability (Figure 1.1) (MacArthur and Wilson, 1967).

Empirical evidence supports the importance of distance and size in determining species number and genetic diversity on islands. Comparative studies show that islands closer to continental landmasses have higher numbers of species, and share more species in common with continents than their oceanic counterparts. While oceanic islands have fewer species per area they possess higher numbers of endemic species than any other habitat (see Whittaker, 1998), thus, demonstrating the importance of restricted gene flow and drift on population differentiation. On average, island populations and endemic island species demonstrate lower genetic diversity than mainland counterparts, with genetic diversity decreasing with decreasing islands size and increasing degree of isolation (Frankham, 1996; Frankham, 1997). The magnitude of these differences are related to organism dispersal ability (Frankham, 1997), again highlighting the importance of gene flow in maintaining genetic diversity. Higher extinction rates in island species of animals (also high in plants) over the last 400 years than in any other habitat (Reid and Miller, 1989; World Conservation Monitoring Centre, 1992) demonstrates the susceptibility of island populations to perturbations. High extinction rates following disturbance on island versus mainland populations likely reflect their susceptibility to stochastic affects but also lower levels of genetic diversity (Frankham, 1997; Frankham, 1998).

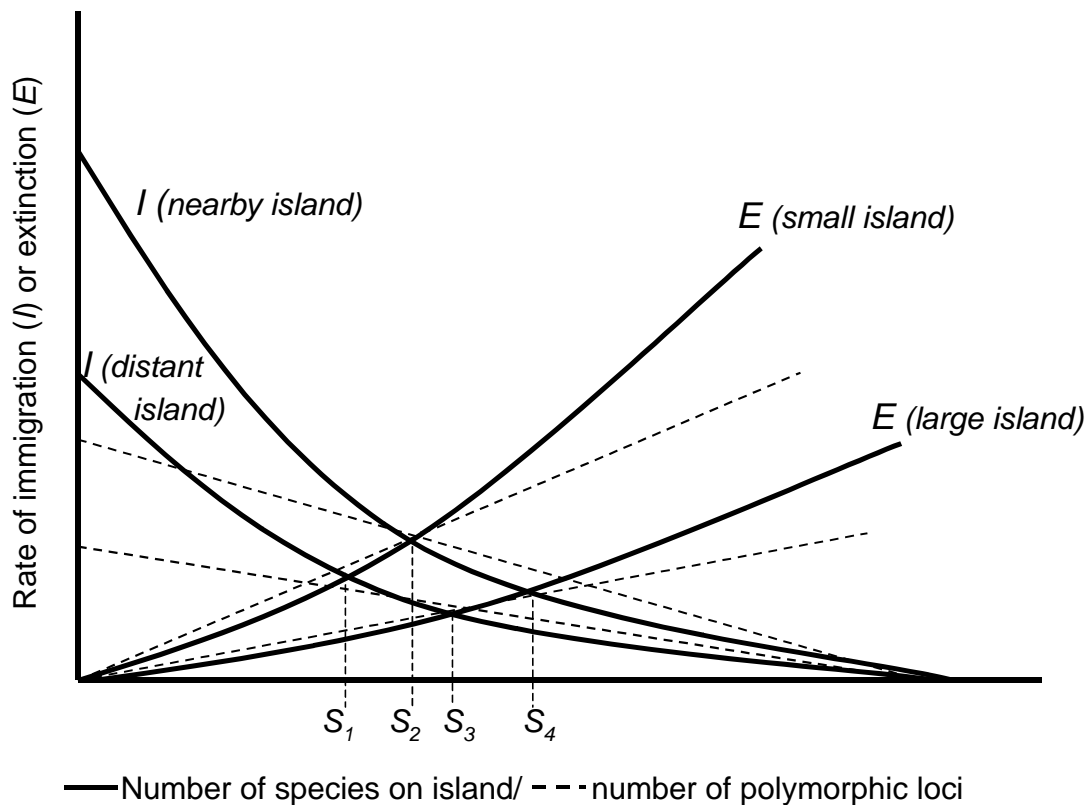


Figure 1.2: Island biogeography theory (MacArthur and Wilson, 1967) predicts that on islands with the same habitat, species number will increase due to immigration and decrease as species already present go extinct. Where these rates are equal the number of species will be at equilibrium. Rates of immigration and extinction are a function of the distance an island is from sources and island size, respectively. Therefore, each island should have its own equilibrium number of species (S). Jaenike (1973) predicted that the same relationship for species diversity will also exist for genetic polymorphism.

Island isolation leading to breeding isolation may facilitate speciation between islands or islands and sources, however diversification within an island is more complicated. Theories of speciation on islands are diverse and both genetic and ecological factors are important and may in fact work together. Genetic revolutions associated with founder events have often been proposed as important in species divergence in island systems (Mayr, 1963; Carson and Templeton, 1984). In these models founder events rearrange preadapted gene complexes. Founder events, isolation, and new ecological opportunities in the few colonists that make it to islands has been implicated with the radiation in Hawaiian groups such as the silversword alliance (Carr *et al.*, 1989), lobelioids (Lammers, 1990), honeycreepers (Freed *et al.*, 1987) and Drosophilids (Carson, 1992).

Most of the major examples of evolution on islands come from older, very isolated islands, such as Hawaii, the Galapagos and the Canaries. Is it possible to detect evolutionary changes on islands of shorter temporal and spatial scales? Cody and Overton (1996) demonstrated that selection for reduced dispersibility occurred within five generations in some weedy, short-lived, wind-dispersed species of Asteraceae on inshore islands in British Columbia, Canada. Given the rapid change in organism population size and distribution due to human habitat destruction and fragmentation it is of increasing importance to study island systems at a smaller spatial and shorter temporal scale. The Krakatau islands in Indonesia provide a rare opportunity to do this.

Genetic Effects of Colonization

Colonization involves the movement of propagules into a habitat currently not inhabited by the species. This process is important as it offsets the loss of populations due to extinction. Many species exist in a metapopulation of interacting groups linked by the dispersal of individuals where a balance between the extinction of populations and the colonization of new sites exists (see Hanski and Gilpin, 1991).

The genetic effects of colonization are complex as each case of colonization involves a unique set of circumstances due to variation between environments and species natural history (Barrett and Husband, 1989; McCauley, 1991). However, factors important in effecting genetic structure during colonization can be divided into two categories; those factors which greatly affect within population diversity (small population size, Nei *et al.*, 1975) and those which affect differentiation between populations (gene flow, Slatkin, 1977; Wade and McCauley, 1988). Small population size may occur during the formation of new populations (founding), and will increase rates of drift (random changes in allele frequency) and inbreeding (mating of closely related individuals). The movement of individuals (migration) will result in the exchange of genetic material (gene flow). Theory that outlines the effect of these factors is understood and is outlined below.

Population Size

Colonization of new islands is often associated with small population sizes as only a small number of propagules arrive (founder events), habitat area may be reduced compared to mainland sites and disturbance afterwards may keep population size small for several generations after founding (Falconer, 1981). Finite population size can have a dramatic effect on genetic diversity. First, the initial founding event only samples a subset of individuals and

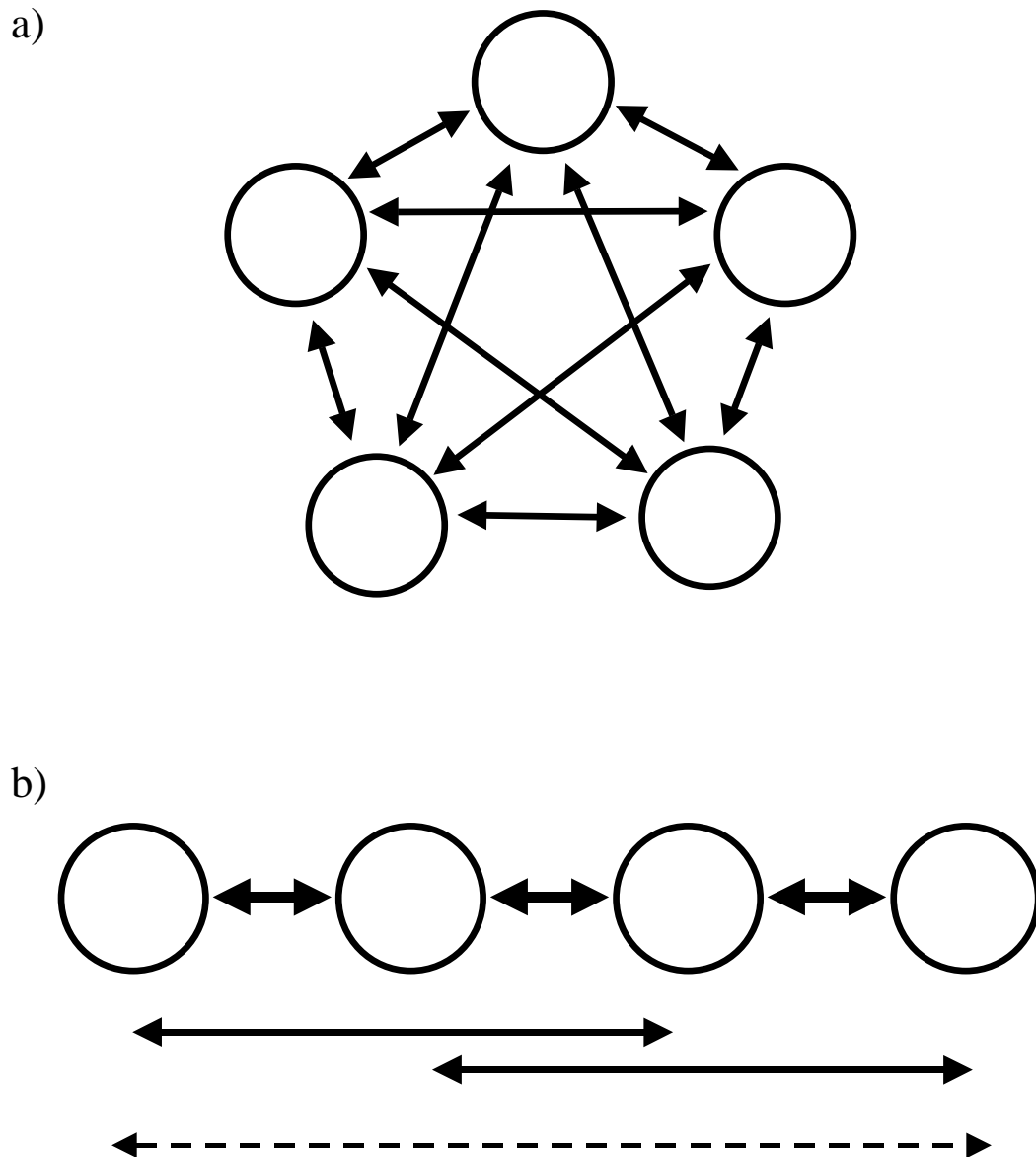


Figure 1.3: Two simple models that describe migration between islands and effects on genetic differentiation (after Wright 1931 and 1943). a) The ‘island model’ predicts that all populations contribute migrants to all other populations equally. Population genetic differentiation (F_{ST}) is a function of the proportional number of migrants each generation, $F_{ST}=1/4Nm+1$, where N is the effective population size and m the rate of migration per generation. b) The ‘stepping-stone’ model predicts that nearest neighbors contribute most to each other’s migrant pool and therefore are more similar than more distant populations. Therefore, when distance is a limiting factor for dispersal genetic differentiation increases with distance between sites (isolation-by-distance).

Chapter 1

genetic variation from the source population (Mayr, 1963). This predominantly results in the loss of rarer alleles, and also changes allele frequencies in populations (Wright, 1969). Second, random genetic drift results in the fixation of alleles and therefore reduces allele number and heterozygosity. The proportional rate of loss of heterozygosity due to drift each generation is $1/(2N_e)$, where N_e is the effective population size. The loss of heterozygosity will be of significance where N_e is small (order of 20 individuals or less) and will be more so when population size remains small for several generations. If population size increases rapidly, the loss of heterozygosity will be limited, even when initial founder sizes are small (Nei *et al.*, 1975). Empirical studies have shown that in very small populations changes in allele frequency and heterozygosity were consistent with that theoretically predicted for neutral loci under the affect of genetic drift (e.g. Ryegrass, *Lolium multiflorum*, Polans and Allans, 1989; e.g. housefly, *Musca domestica*, McCommas and Bryant, 1990).

The chance of inbreeding is higher in small populations due to the limited number of available mates. Any form of inbreeding, whether self-fertilization or sib-mating, will result in the increase of homozygosity (Wright, 1931). The rate at which the level of inbreeding (F) increases in a population over time is proportional to the effective population size (N_e) as $\Delta F = 1/(2N_e)$ (Falconer, 1981). Therefore, smaller populations will become inbred more rapidly than larger ones, even when mating is random. The rate of inbreeding in a population will be reduced in populations where sexes are on separate individuals compared to hermaphrodite systems. Inbreeding will be promoted in newly colonized populations if the initial colonists share kinship. This will occur where dispersal is in clusters of seeds from the same mother (Cherplick, 1993). Due to the variety of breeding systems in plants and the large number of species which have the capacity for some level of self-fertilization, the frequency and intensity of inbreeding between plant species will be variable, and the effect of inbreeding on fitness will be hard to predict without knowledge of the breeding system (Charlesworth and Charlesworth, 1987).

Gene Flow

The movement of individuals between populations is important as it facilitates gene flow (the exchange of genetic material) and hence species cohesion. A breakdown in gene flow will result in increased genetic differentiation and if severe enough may eventually result in speciation (Mayr, 1963). Factors which determine the level of gene flow are those that effect the movement of individuals between populations; distance between populations,

the dimensionality of dispersal (direction of movement) and the dispersal capability of the species in question. Various models have been proposed that explain patterns, processes and in some cases the genetic effects of colonization. The island model (Wright, 1931) states that genetic differentiation (F_{ST}) between populations is a simple function between the gain of genetic variation through migration (m , the rate of migration per generation) and the loss of variation due to random events (genetic drift which is proportional to (effective) population size, N), $F_{ST}=1/(4Nm+1)$. In this model, all populations are equally likely to provide migrants to other populations regardless of population size or distance between populations (Figure 1.3a). However, in many circumstances equal contribution of all populations to the migrant pool is unlikely. Wright (1943) later refined the island model by including the effect of distance on structuring genetic variation between populations. Dispersal in all species is to some extent limited by distance. Therefore, neighboring populations contribute significantly more to each others' gene pool than to those further away; differentiation between sites being an increasing function of distance (Figure 1.3b). This effect has been termed isolation-by-distance (Wright, 1943). Isolation-by-distance works only when dispersal becomes increasingly limited with distance and when there are no other barriers to dispersal operating over the study area. The concept of isolation-by-distance was further extended into the stepping-stone models of colonization where gene flow between distant populations can be facilitated by those populations between (Maruyama and Kimura, 1980).

The number of migrants and the origin of these migrants are also important in determining genetic structure between populations (Slatkin, 1977; Wade and McCauley, 1988). Slatkin (1977) outlined the Propagule and Migrant Pool models to illustrate the extreme ends of the colonization spectrum and their effect on population genetic structure. In the Propagule model, the founding event (small population size during colonization) is severe and colonists are drawn from a single source. This results in increased differentiation between sites. The other extreme is the Migrant model (Slatkin, 1977), where colonists are drawn from many populations, with the effect of decreasing overall differentiation between populations. Therefore, it is assumed that migration from a limited number of source pools will create structure between populations.

Measuring Gene Flow and Patterns of Dispersal Throughout the Range of a Species

Direct measurement of gene flow between populations is difficult to achieve. Therefore, indirect estimates are often made. Wright's (1931) equation, $F_{ST}=1/(4Nm+1)$, is frequently used to find Nm (the number of migrants per generation) from F_{ST} , as F_{ST} can be easily

estimated from population genetic data. Wright's F_{ST} is measured as the reduction in heterozygosity in the subpopulation relative to the total population, $F_{ST}=(H_T-H_S)/H_T$. H_T and H_S are the expected heterozygosity of an individual in the total population and subpopulation, respectively. However, there are various assumptions and problems associated with the estimation of Nm in this manner (Whitlock and McCauley, 1999). First, one simplifying and unrealistic assumption in this model is that all populations in a region are equally likely to contribute to each others' gene pool regardless of spatial scale. Further, the model assumes that populations are at equilibrium between immigration and drift and that this is constant between all populations throughout the range of a species. Therefore, different processes should not be functioning at different spatial scales in the range of a species. Another problem with the use of this equation is that it is difficult to determine the relative importance of immigration and drift as these factors are confounded in the product, Nm . Further, inaccuracies in measuring differentiation (F_{ST}) can lead to a disproportionate effect on the estimated value of Nm , as the relationship between the two is not linear. Whitlock and McCauley (1999) advise that in most cases genetic differentiation as measured by F_{ST} will be an adequate measure of gene flow. Qualitative estimates of gene flow can be determined using rules-of-thumb suggested by Hartl and Clark (1989): when F_{ST} is less than 0.05 gene flow is high, gene flow is more restricted at values of F_{ST} between 0.05 and 0.15, greatly restricted between than 0.15 and 0.25 and extremely restricted when F_{ST} is greater than 0.25.

Untangling the relative importance of drift and migration in population differentiation is difficult. Pairwise comparisons of differentiation between populations can give insight into differences in gene flow between populations throughout a region. Slatkin (1993) applied Wright's (1943) model of isolation-by-distance to demonstrate that when groups of populations reach an equilibrium between drift and migration, a positive correlation should exist between genetic differentiation and geographical distance. When not at equilibrium because the relative effects of drift or migration become dominant, no relationship will exist. Where distance is not limiting to dispersal no patterns will exist as migration is extensive and populations will not be differentiated. On the other hand, when distance or some other barrier is greatly limiting, dispersal will break down and differentiation will increase due to genetic drift (Hutchison and Templeton, 1999). Hutchison and Templeton (1999) demonstrate that the relationship between genetic structure and distance can vary over the range of the species as the relative extent of drift and dispersal will vary depending on local conditions. Various other empirical studies have used this principle to determine past patterns of dispersal and gene flow within a species and to identify which factors lead to the breakdown of gene flow

(e.g. Giles and Goudet, 1997; Gaudeul, *et al.*, 2000). The scale at which distance becomes limiting will be a function of species dispersal abilities. Therefore, the effect of distance to a new site on genetic differentiation will vary between species.

Other Genetic Effects-Interspecific Hybridization

Interspecific hybridization results in new genetic forms and in some cases can lead to speciation when genetic recombinants exploit new niches and are able to maintain breeding isolation. The large number of occurrences of hybridization among a diverse range of plants and animals (Arnold, 1992; Abbott, 1992; Rieseberg and Wendel, 1993) supports its evolutionary importance. Introgressive hybridization will play an important role in maintaining or generating genetic diversity in populations on islands and disturbed habitats when population size is low and will allow for the exploitation of new ecological opportunities. For example, hybridization has been shown to occur between some Galapagos finch species with small population sizes (*Geospiza fuliginosa* and *G. scandens*, Grant and Grant, 1997) and maintains genetic variability in these populations despite their small size, as the loss of variation due to drift is counteracted through gain by gene flow (*G. scandens* and *G. fortis*, Grant and Grant, 1992). Gene exchange in the finches only occurred after extreme environmental disturbance (El Niño) when hybrid fitness was enhanced (Grant and Grant, 1993). Numerous other examples of hybridization in small populations and disturbed environments exist. Increased levels of hybridization have been reported in some small populations of the tree species, *Eucalyptus argutifolia* (Kennington and James, 1997). Introgressed hybrid plant taxa have been documented in areas where exotic species had invaded and hybridization with local species had occurred (Abbott, 1992). Introgressive hybridization has been implicated in the evolution of a number of groups of plants occurring on oceanic islands; for example, *Bidens* (Asteraceae), *Scaevola* (Goodeniaceae) and *Pipterus* (Urticaceae) on the Hawaiian Islands (Gillet, 1972; Carr, 1995), *Argyranthemum* (Asteraceae) in Macaronesia (Francisco-Ortega, 1996) and several species in the New Zealand flora (Rattenbury, 1962; Raven, 1972).

Genetic Diversity, Gene Flow and Life-History in Tropical Trees

Tropical forests house an enormous wealth of species, much greater than any other ecosystem on earth (Parker, 1982; Wilson and Peter, 1988). Early authors predicted that tropical rain forest trees should be predominantly inbred or self-fertilizing as low tree population densities would lead to high selfing rates (e.g. Corner, 1954). Thus, genetic diversity in tropical trees

should be low. However, studies of allozyme diversity have demonstrated that tropical rain forest trees are at least as genetically diverse as temperate species (Loveless, 1992). Analyses of mating systems through direct observation (Bawa *et al.*, 1985) and molecular markers (Murawski and Hamrick, 1991; Loveless, 1992; Stacy *et al.*, 1996) have shown that outcrossing is the predominant mode of reproduction in tropical tree species. Such high outcrossing rates are achieved because of the high proportion of self-incompatibility in hermaphroditic species (Bawa *et al.*, 1985) and of dioecious species in tropical floras (Bawa, 1980).

Other life-history characters are also important for determining patterns of genetic diversity and structure in tropical tree species. Genetic variation within populations (lower versus higher) has been associated with growth form (shrubs versus trees), geographical range (restricted versus widespread) and dispersal type (abiotic versus biotic), with dispersal type being most important in determining genetic structure between populations. Species with abiotic seed dispersal demonstrate on average more than twice as much population differentiation than species with biotically-dispersed seeds (Loveless, 1992). Hamrick and Loveless (1989) found a significant negative correlation between population differentiation and the genetic mobility of species (determined by pollination and seed dispersal characteristics) in 14 tree species on Barro Colorado Island, Panama. Therefore, species dispersal ability is important in determining genetic diversity within and between populations.

Gene flow in tropical trees can be high over small spatial scales. Numerous studies have observed little genetic differentiation (F_{ST} less than 0.05) in many tropical tree species at scales of less than 10 km, inferring that gene flow is extensive at such scales (e.g. Hamrick and Loveless, 1989; Alvarez-Buylla and Garay, 1994; Schierenbeck *et al.*, 1997). Paternity studies also support that pollen dispersal is extensive at smaller spatial scales (hundreds of meters) in many species (e.g. Chase *et al.*, 1996; Stacy *et al.*, 1996). In some species pollen dispersal can be extensive at larger spatial scales. Paternity analysis of a number of *Ficus* species on Barro Colorado Island, Panama demonstrated that pollen dispersal between trees routinely occurs over distances of up to 14 km and that the number of different pollen donors contributing to a single crop within a tree is high, despite low tree population density (Nason *et al.*, 1996). At larger spatial scales patterns of genetic differentiation can vary considerably between species. For example, high levels of differentiation have been observed between remnant populations of *Caesalpinia echinata* in Brazil ($F_{ST}=0.413$, Cardoso *et al.*, 1998) and in some populations of *Abies* species in Mexico and Guatemala ($F_{ST}=0.271$ in *A. hickeli*,

Aguirre-Planter *et al.*, 2000). However, in other tree species gene flow has been inferred to be extensive over larger spatial scales. For example, in the tropical pioneer species *Cecropia obtusifolia* very little population differentiation was observed over a range of 0.05 to 107 km (Alvarez-Buylla and Garay, 1994).

While numerous studies have examined the effect of habitat fragmentation on genetic diversity and gene flow between populations, little is known about the formation of new populations that are located at distances greater than tens of kilometers from source populations.

The Krakatau Islands- a natural experiment in island colonization

The Krakatau islands, Indonesia (Figure 1.4) are a unique and invaluable case of ecosystem destruction and rebuilding in the tropics. A series of extreme volcanic events, concluding on 23rd August 1883, covered the three existing islands, Rakata, Sertung and Panjang in a thick layer of hot pyroclastic ash (up to 100m thick in places); greatly altering their landscapes. Due to caldera formation two-thirds of Rakata sunk into the ocean (Verbeek, 1885). All life on the islands is thought to have been destroyed and no propagules were likely to have survived (Docters van Leeuwen, 1936; reviewed by Whittaker and Bush, 1993; but see Backer, 1929). Therefore, all life returning to the Krakataus did so from outside the system and must have bridged the 30 km or more of sea from the nearest coasts of Java and Sumatra. Thus, a completely empty environment for the development of new populations in a discrete, insular environment was created.

Various scientific expeditions have provided a remarkable record of the return of life to the Krakatau islands (reviewed by Docters van Leeuwen, 1936; Whittaker *et al.*, 1989; Partomihardjo, 1995). These expeditions, though sporadic in time and research aim, can provide insight into the approximate arrival times of the species (and age of populations) and in some cases give information about the structure of the early populations. Due to these records and extensive recent research, descriptions and models of the restructuring of the forests have been developed (Docters van Leeuwen, 1936; Whittaker *et al.*, 1989; Whittaker and Jones, 1994a).

Reforestation has followed a successional pathway shaped by the dispersal characteristics of the colonists (Whittaker *et al.*, 1989; Whittaker and Bush, 1993). Patterns of succession were similar on all three islands for the first 50 years after the 1883 eruptions (Docters van Leeuwen, 1936; Whittaker *et al.*, 1989). Expeditions in 1886 and 1887 recorded

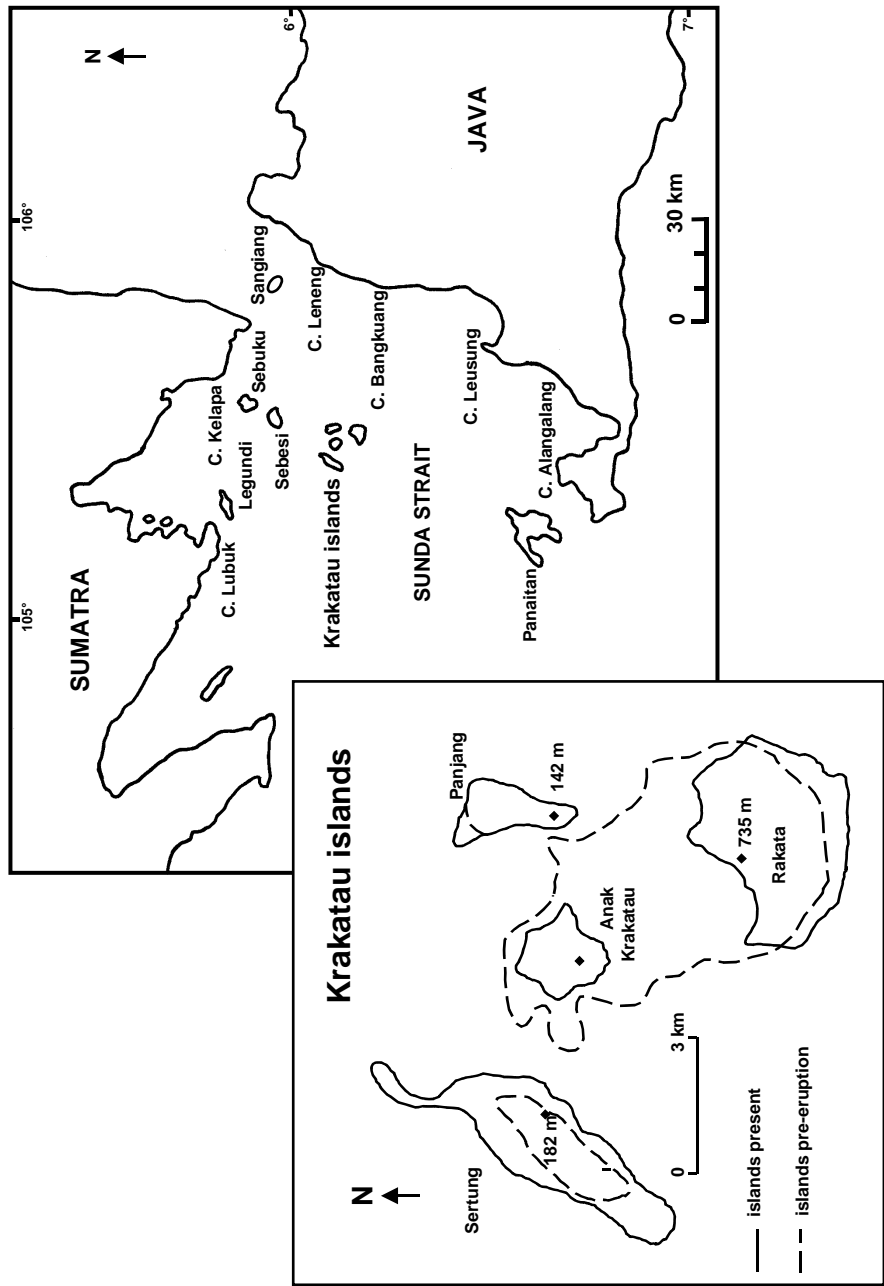


Figure 1.4: A map of the Krakatau islands and the Sunda Strait region, Indonesia. The dashed line represents the Krakatau islands before 1883 and the full line the current islands. The distances between the Krakatau islands and several mainland sites are given in Table 1.1.

Table 1.1: Distances (km) between the Krakatau islands and selected sites in the Sunda Strait region, Indonesia (refer to Figure 1.4 for location of the sites). Distances are after Whittaker and Jones (1994b). Symbols for regions are; (K)rakatau islands, other (I)slands in the Sunda Strait, (S)umatra and (J)ava.

Locality (Region)	Rak.	Sert.	Panji.	Anak	Sebe.	Sebu.	Leg.	Panai.	Sang.	C.Kel.	C.Lub.	C.Ala.	C.Leu	C.Ban.
Rakata (K)														
Sertung (K)	6													
Panjang (K)	3	5												
Anak Krakatau (K)	3	2	1.5											
Sebesi (I)	17.5	12.5	12	14										
Sebuku (I)	25.5	20.5	19.5	22.5	2									
Legundi (I)	36.5	27	33	31.5	24	22								
Panaitan (I)	44.5	47	50.5	50	65.5	73.5	75							
Sangiang (I)	40.5	48.5	42.5	46.5	34.5	33	59.5	88.5						
Cape Kelapa (S)	37.5	33.5	31.5	35	14.5	7	31.5	85.5	12					
Cape Lubuk (S)	45	35.5	42	40.5	33.5	34	5.0	79	72	42.5				
Cape Alangalang (J)	53	58.5	60.5	60	75	83	89	9.5	90.5	93.5	95			
Cape Leusung (J)	40.5	50.5	46.5	47.5	58	64	81.5	42.5	57	70.5	90	35		
Cape Bangkuang (J)	41	49	42.5	45	44	47.5	73	71	25	36	83	69.5	33	
Cape Leneng (J)	51.4	57	50	54	45.5	45.5	72.5	89.5	10	26.5	84.5	89.5	54	20.5

that pioneer vegetation was establishing (Treub, 1888). The first communities established quickly. Strandline vegetation, consisting of sea-dispersed species, was one of the first communities to develop. By 1906 this coastal woodland had already established (Ernst, 1908), had obtained all species and has since shown virtually no turnover (Whittaker *et al.* 1989). Grasslands developed inland and consisted mostly of highly dispersive ferns and grasses (wind and animal dispersed). Coastal forest establishment attracted larger and more diverse frugivore populations, resulting in an increase in animal-dispersed plant species diversity and the dispersal of trees inland. Patches of woodland of secondary forest species, rich in *Macaranga* and *Ficus* had started to appear in the grassland by 1908 (Backer, 1909). The gradual invasion of tree species inland resulted in the replacement of early-successional grasslands with species-poor mixed secondary forest (Docters van Leeuwen, 1936). Forest closure occurred at the end of the 1920s (Docters van Leeuwen, 1936; Whittaker *et al.* 1989). Due to Rakata having different topography than both Panjang and Sertung, habitat above 200m is available on this island and has seen the predominance of other species (Whittaker *et al.*, 1989). Data for Rakata demonstrates that by 1934, 48 tree species had colonized, of which 27 were animal-dispersed, three by wind and 18 by sea (of which 20 were part of the coastal woodlands) (Docters van Leeuwen, 1936). The development of forests provided habitat for a new group of wind-dispersed species, forest epiphytes. By 1983 the total number of tree species recorded on the islands had risen to 86 (Whittaker *et al.*, 1992a). Of the new arrivals since 1934, 26 were animal-dispersed, one sea-dispersed (and one extinction) and two were wind-dispersed. *Ficus* plays an important role in forest diversity, with 22 species on the islands and the genus accounts for 63% of animal-dispersed species (Whittaker and Jones, 1994b).

After the initial 50 years, considerable forest structure divergence occurred between the islands (Whittaker *et al.*, 1989). Resumption of volcanic activity in the caldera between the Krakatau islands in the late 1920s and the birth in 1930 of a new volcanic island, Anak Krakatau, is considered one of the main driving forces in island forest differentiation (Whittaker *et al.*, 1992b; Schmitt and Partomihardjo, 1997; Schmitt and Whittaker, 1998). Sporadic volcanic activity has resulted in considerable periodic ash deposits on both Sertung and Panjang with Sertung being the most effected (Whittaker *et al.*, 1992b). The effects of ashfall on plant growth is great and has resulted in the resetting of successional clocks of these islands backward (Whittaker *et al.*, 1989; Bush *et al.*, 1992; Schmitt and Partomihardjo, 1997; Schmitt and Whittaker, 1998). Such disturbance is thought to be the primary factor contributing to the dominance of a few species, such as *Timonius compressicaulis* and

Dysoxylum gaudichaudianum on these islands (Whittaker and Bush, 1993). The forest of Rakata is considerably more diverse than that of either Sertung or Panjang (Whittaker *et al.*, 1989; Schmitt and Partomihardjo, 1997). Forest has colonized Anak Krakatau but remains in an early successional state because of periodic destruction from volcanic activity and is confined to a small coastal site (Partomihardjo *et al.*, 1992). Apart from volcanic ash disturbance, differences in forest composition between islands is also influenced by variation in topography (Rakata reaches over 700m and therefore has habitat for sub-montane species and different hydrological and stability characters), extreme climatic events (El Niño-Southern Oscillation events, Schmitt and Whittaker, 1998), volcanic disturbance other than ashfall such as earthquakes, landslips and storm activity (Schmitt and Whittaker, 1998) and chance differential arrival time of species (Docters van Leeuwen, 1936; Tagawa, 1992).

At present, the Krakatau islands are covered in species-poor secondary monsoonal forest and as a whole are depauperate of inland tree species with the canopy remaining dominated by relatively few, early seral species (Whittaker *et al.*, 1989; Whittaker and Bush, 1993). Further diversification of this forest may be seriously slowed due to habitat loss on Java and Sumatra (Whittaker and Bush, 1993) and the sea barrier which prevents dispersal of certain groups to the islands (large canopy trees with large, winged and wind-scattered seeds and large seeded animal-dispersed species, Whittaker and Jones, 1994a; Whittaker *et al.*, 1997). Thus, without high levels of ongoing species recruitment forest on the Krakatau islands may be locked into an early successional stage longer and maintain larger populations of early colonist species than would normally occur on mainland sites. The Krakatau islands, unlike other Sunda Strait islands such as Sebesi (Figure 1.4) and many mainland sites, have remained relatively free from human influence (Whittaker *et al.*, 1989) and thus offer a rare opportunity to study plant colonization and population genetics in a tropical ecosystem little disturbed by human activity.

Although the successional processes in which forest communities have restructured on Krakatau are well studied (Whittaker *et al.*, 1989; Whittaker *et al.*, 1992a; Whittaker and Bush, 1993; Whittaker and Jones, 1994a) nothing is known about the origins of new populations and how colonization has affected the patterns of genetic variation within these populations. Despite the increasing availability of molecular marker techniques, to date no studies have examined the genetic structure of the new Krakatau populations.

The geographical location of the islands and species dispersal ability will be of fundamental importance in the colonization of the islands by tree species. The islands are located approximately equidistant from two large landmasses, Java and Sumatra with the

nearest points on these landmasses from the Krakatau islands being approximately 31 and 40 km distant, respectively (Figure 1.4 and Table 1.1). If dispersal is not limited by distance and suitable habitat is available on both landmasses, then equal contribution of migrants would be expected from both Java and Sumatra. However, if dispersal is restricted by distance then the nearest landmass (Sumatra) is expected to contribute the majority of colonists. In species with poor dispersal ability the distance to the Krakatau islands may be greatly limiting colonization. In such species chance events and factors such as wind and sea currents may play an important role in colonization events.

Dispersal to the Krakatau islands, and hence gene flow, may be further facilitated by the many small islands that lay between the Krakataus and Sumatra, if such islands act as stepping-stones to colonization. Sebesi, the nearest island to the Krakataus (12 km north of Panjang) may be one such island (Docters van Leeuwen 1923; Whittaker *et al.*, 1992a; though Thornton *et al.*, 1996, assumes a less significant role for Sebesi). Despite the fact that vegetation on this island was greatly damaged by the Krakatau eruptions, by 1921 floral communities were much further advanced than those on Krakatau (Docters van Leeuwen, 1923). However, since the nineteenth century Sebesi has been used extensively for agricultural purposes (Thornton, 1996) and by the 1970s the natural vegetation on Sebesi was confined to the upper regions of the island. Whittaker *et al.* (1992a) concluded that the island may now no longer be functioning as a stepping-stone for colonization and patterns of relationship between Sebesi and the Krakataus are likely lost.

The surrounding coastlines of Java and Sumatra were also greatly affected by the volcanic activity of Krakatau. Several tidal waves, of up to 50 m in height, devastated large areas of the coastline, killing an estimated 36,000 people. Ash fall was also recorded throughout the Sunda Strait coastlines (Verbeek, 1885; Simkin and Fiske, 1983). Due to the proximity of undamaged forest, coastal populations are expected to have rapidly re-established relative to island sites. Therefore, we can assume in this study that coastal mainland populations can be used in comparison with the new populations of the Krakatau islands to examine the genetic effects of colonization over a greater distance. Nonetheless, it must be noted that past and current land use at mainland sites has greatly reduced natural habitat (Whitten *et al.*, 1996) and that such sites may themselves be facing increasing insularity.

Measuring Genetic Variation, Molecular Techniques and Population Biology

With the advent of molecular markers, studies into population genetic processes have expanded as variation at the level of the genome can be sampled directly and efficiently. Today there are a great variety of molecular techniques available, each with their own benefits and drawbacks. Therefore, when starting a research project one must decide, given the questions to be asked and the limits of the project, which method will be most suitable. Traditionally, population studies examine allelic variation at a locus using co-dominant markers (where individual alleles can be identified) such as allozymes. More recently other co-dominant markers such as microsatellites have become available. A wealth of analytical techniques and extensive theoretical background exists that uses allelic frequency, diversity and heterozygosity to examine patterns in the partitioning of genetic variation. However, certain practical limitations apply for both allozymes and microsatellites. Allozymes have problems associated with material storage (usually requiring fresh leaf material) and generally produces relatively low levels of polymorphism. Microsatellites, while generating considerably greater numbers of polymorphic markers (especially many more alleles per locus), require expensive and time laborious development. In this project we have used Amplified Fragment Length Polymorphism (AFLP, Vos *et al.*, 1995) and polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) in non-coding chloroplast DNA to examine genetic polymorphism within and between populations and species.

AFLP and Population Studies

Amplified fragment length polymorphism (AFLP, Vos *et al.*, 1995) examines total genomic DNA variation using a combination of restriction digestion and PCR amplification (Figure 1.5). DNA is digested into small fragments (approximately 50-800 basepairs long) with two restriction enzymes, one rare and one frequent cutter such as *Eco* RI and *Mse* I (though other enzymes can be used, for example, *Pst* I and *Taq* I). Concurrent with digestion, adapters for primers are ligated to the ends of the new fragments. Two series of PCR using primers that extend out into genome sequence (with increasing selectivity in the second reaction) are then performed to create a highly polymorphic multilocus fingerprint. Typically the first series of amplification (the pre-amplification step) only uses one nucleotide extension on each primer, thus reducing the likelihood of mismatch and increasing band reproducibility (Vos *et al.*, 1995). The second series of amplification increases the number of selective nucleotides to either two or three, depending on plant genome size and the number of bands produced in the fingerprint (typically three extension nucleotides on both forward and reverse primers are

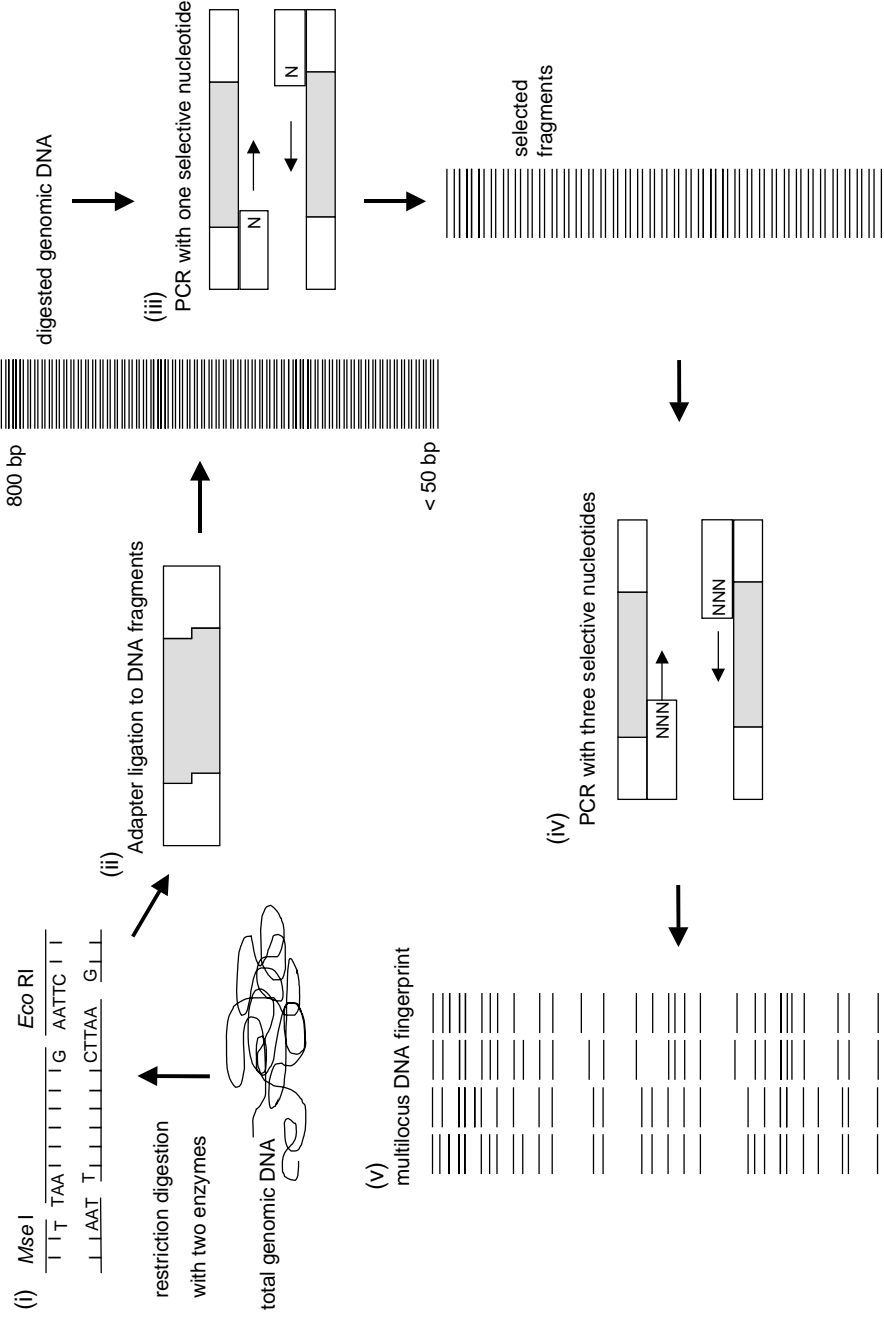


Figure 1.5: Outline of the methodology of Amplified Fragment Length Polymorphism (AFLP). (i) Total genomic DNA is digested with two restriction enzymes (one frequent and one rare cutter). (ii) During the digestion reaction adapters with primer sites are ligated to the ends of the fragments. (iii) PCR using primers with one selective nucleotide extending into the genome fragment is performed. This reduces the number of fragments and improves reproducibility of final fingerprints. (iv) A second series of PCR follows using two or three selective nucleotides on each primer, thus (v) generating a highly polymorphic DNA fingerprint.

selected for plants with larger genomes. This is reduced to two selective nucleotides on one of the primers in plants with smaller genomes).

There are a number of reasons why the AFLP method is highly suited to population studies. First, as variation is sought through restriction digestion and adapter ligation, no previous knowledge of the species genome is required and little species specific fine-tuning is needed. Typically, large numbers of highly polymorphic markers sampled over the entire genome are produced with each primer combination (more than for other commonly used techniques such as Random Amplified Polymorphic DNA, RAPD (Griffiths and Orr, 1999)), thus generating large data sets with each experiment. AFLPs have been shown to exhibit Mendelian inheritance (*Lycopersicum*, Vos, *et al.*, 1995; *Pinus sylvestris*, Lerceteau and Szmidt, 1999). Plant material for DNA extraction can be quickly and readily preserved and stored for long periods of time. Once extracted, DNA can be used for a variety of analysis techniques. One negative aspect of AFLP is cost. Acrylamide sequencing gels and radio-labels or fluorescent detection sequencing machines are required and are more expensive than the agarose based detection systems used in other multilocus dominant methods such as RAPD. However, the more reproducible nature of AFLP and considerably larger number of polymorphic markers generated per primer combination make the method more reliable and attractive than RAPD (Griffiths and Orr, 1999).

AFLP data are dominant (presence of a band prevents the detection of the null allele). Thus, heterozygotes cannot be identified and allelic frequency cannot be directly determined (unless Hardy Weinberg Equilibrium (HWE) is assumed or determined with other genetic techniques). This reduces the applicability of traditional analysis techniques. However, a variety of population analysis methods are readily available and combined with the high levels of polymorphism generated make AFLP a powerful tool that is becoming increasingly popular in population biology. Measures of genetic diversity such as percentage of polymorphic loci, number of unique alleles and various diversity indices are available to compare within population diversity. If HWE is assumed, values of expected heterozygosity can be calculated using Nei's (1978) unbiased estimate, $H_e = [2n/2n-1](1-\sum_i p_i^2)$, where n is the number of individuals used for analysis and p_i is the frequency of an allele at the i th locus. Allelic frequencies can be determined as the null-phenotype (band absent) represents the homozygote recessive and is equal to q^2 where $1=p+q$. Genetic variation can also be determined through measures of DNA-fingerprint similarity (the fraction of bands shared), $S_{xy} = 2n_{xy}/n_x + n_y$, where n_{xy} is the number of bands shared by individuals x and y , and n_x and n_y

the number of bands present in individual x and y , respectively. This similarity index provides upwardly biased estimates of population homozygosity (Lynch, 1990) and has been shown to correlate with levels of inbreeding (e.g. in chickens, Kuhnlein, 1990). A rough estimate of heterozygosity can be determined when pairwise differences in bandsharing are used to calculate dissimilarity (Gilbert *et al.*, 1991). Differentiation between populations has been traditionally measured using Wright's (1969) fixation index, F_{ST} calculated using allele frequencies. This can also be done with dominant AFLP data, assuming HWE within the populations. Alternatively, estimates of the genotypic F_{ST} can be calculated for dominant molecular data by conducting an analysis of molecular variance (AMOVA) where pairwise genetic distance comparisons between individuals can be used to examine partitioning of genotypic variance within and between populations and regions (Excoffier *et al.*, 1992).

Chloroplast DNA Studies

The chloroplast genome has uniparental, cytoplasmic inheritance (predominantly maternal) and does not undergo effective recombination (Mogensen, 1996). Therefore, it provides an alternative genetic history to the nuclear genome. Due to increased effects of drift, lack of recombination and low levels of mutation, chloroplast DNA shows low levels of polymorphism (Birky, 1988). However, the conserved nature of the chloroplast genome and knowledge of the entire chloroplast sequence of a number of diverse plant species has led to the development of PCR primers that span non-coding regions (the most polymorphic regions in the chloroplast genome) which are of universal application in plants (Taberlet *et al.*, 1991; Demesure *et al.*, 1995). Such primers have been used extensively for population and systematic studies (e.g. Ferris *et al.*, 1993; Gielly and Taberlet, 1996). Use of chloroplast markers are particularly useful when the history of the seed parent is to be followed in hybridization studies (e.g. Reiseberg and Soltis, 1991).

Thesis Aims

The main focus of this thesis is the genetic consequences of colonization in five tree species on the Krakatau islands. There are four scientific chapters in this thesis, each dealing with a specific set of questions regarding the genetic and ecological effects of island colonization and the use of molecular markers in population studies. The first scientific chapter (Chapter Two), 'Genetic Diversity of Five Tree Species on the Krakatau islands, Indonesia; 116 years after habitat destruction and rebuilding' examines patterns of genetic diversity on island and mainland populations using AFLP markers. The central aim of this chapter is to determine whether there has been a decrease in within population genetic diversity after colonization of the islands. Patterns of genetic diversity are also examined in relation to species dispersal abilities. Chapter Three, 'Genetic Differentiation in Five Tree Species in Sites From Java, Sumatra and Krakatau: inferences about gene flow between isolated populations' examines partitioning of diversity between populations and identifies whether distance is an important factor in structuring genetic diversity. An attempt is made to identify source regions of colonists. In the next chapter, 'Genetic Evidence for Natural Hybridization Between Species of Dioecious *Ficus* on Island Populations', hybridization is identified in species of *Ficus* in disturbed island habitats, thus highlighting a potential genetic consequence associated with expansion into new sites. Chapter Five, 'Identification of a Male-Specific AFLP Marker in a Functionally Dioecious Fig, *Ficus fulva* Reinw. ex Bl. (Moraceae)', does not deal with issues related to island colonization, but rather documents the fortuitous identification of a sex marker in *F. fulva* and highlights the applicability of AFLP markers for a broad range of questions in biology. Finally, concluding remarks are made in Chapter Six.

Genetic Diversity in Five Tree Species on the Krakatau Islands: 116 years after forest destruction and recolonization.

Abstract

Patterns of genetic diversity within and between populations of five tropical tree species, *Ficus fulva*, *F. fistulosa*, *F. pubinervis*, *Dysoxylum gaudichaudianum*, and *Oroxylum indicum* are examined on the Krakatau islands in comparison with other sites on Java and Sumatra and islands in the Sunda Strait, Indonesia. Complete deforestation of the Krakatau islands occurred in 1883 (due to extreme volcanic eruptions). The subsequent reforestation of the islands (in species poor secondary monsoonal forest) has provided a unique chance to study the population genetic effects of colonization in the tropics. AFLP markers were used for analysis as they are selectively neutral, produce highly polymorphic, multilocus and reproducible markers without prior genomic information and require little development time.

In contrast to expectation four of the five species studied (*Ficus fulva*, *F. pubinervis*, *D. gaudichaudianum*, and *O. indicum*) showed indistinguishable or higher levels of genetic diversity on the Krakatau islands in relation to mainland sites. This mainly reflects large population sizes on the Krakatau islands and moderate to high dispersal ability of three of these species (*Ficus fulva*, *F. pubinervis*, and *D. gaudichaudianum*), as well as habitat fragmentation and isolation at some mainland sites. However, *O. indicum* is a water/wind-scattered species of low dispersal ability with small population size on Krakatau but shows an increase in genetic diversity on Krakatau relative to all other sites. Despite a large population size in *F. fistulosa* on most of the Krakatau islands, levels of genetic diversity in the one population studied on the Krakatau islands are lower than at some mainland sites, suggesting increased levels of inbreeding in this population. In all species the lowest levels of genetic diversity were observed at a number of mainland sites or other islands in the Sunda Strait closer to the mainland than the Krakatau islands. Habitat fragmentation and disturbance are thought to be causes for these lower levels of genetic diversity.

Patterns of genetic diversity within and between populations and between species follow patterns observed in tropical plant species as a whole. That is, species with biotic dispersal have higher within population diversity and less structuring between populations, than species with abiotic dispersal. Levels of genetic variation within and between

populations at the species level was related to species dispersal ability (genetic mobility). Species dispersal ability is not necessarily a predictor of the genetic results of colonization on Krakatau as ecological and chance events may also be important in structuring genetic diversity during new population establishment.

Introduction

The colonization of islands is often expected to be associated with the loss of genetic diversity due to distance limiting the number of migrants entering a population (Founder Principle, Mayr, 1942; MacArthur and Wilson, 1967) and to genetic drift associated with small population size (Nei *et al.*, 1975). As the natural landscape becomes increasingly fragmented into smaller habitat islands it is increasingly important to study island systems in order to determine if and how genetic diversity is maintained. The preservation of genetic variation within and between populations is important for plant success in a variable environment and is the building block of evolution (Frankel and Soulé, 1981) as genetic variation is the raw material for adaptation by natural selection. Loss of genetic variation has been shown to reduce the ability of populations to adapt to a changing environment and increases susceptibility to such factors as disease and infertility (*Acinonyx jubatus*, O'Brien *et al.*, 1985), can lead to inbreeding depression (Barrett and Charlesworth, 1991; *Plantago coronopus*, Koelewijn, 1998) and increases extinction risk (Glanville fritillary butterfly, Saccheri *et al.*, 1998). Therefore, it is important to understand what forces shape and maintain genetic diversity and how genetic diversity is structured in natural populations.

In tropical plant species genetic variation within a species has been correlated with such characteristics as (lower versus higher), growth form (shrubs versus trees), geographical range (restricted versus widespread) and dispersal type (abiotic versus biotic) (Loveless, 1992). Dispersal type is important in determining genetic structure between populations (Hamrick and Loveless, 1989; Loveless, 1992). Hamrick and Loveless (1989) ranked 14 species of tropical tree on Barro Colorado Island, Panama according to their ability to move genes (genetic mobility, which was based on seed and pollen dispersal characters) and found a significant negative correlation between species-ranked genetic mobility and population differentiation. Therefore, ability to disperse (and hence colonize new sites) is important in determining the amount and structuring of genetic diversity within a species.

At some level all species disperse. Dispersal is important for the maintenance of a species, as for a species to survive, the colonization of new sites must offset the loss of populations due to extinction. Given the variety of life-histories observed in the natural world

and spatial variation in landscapes, the pattern, range and frequency of dispersal varies greatly between species. Dispersal may even vary within a species (loss of dispersal abilities on islands, Cody and Overton, 1996). Consequently, the effect of colonization and extinction on genetic variation and structure within and between populations is complex (McCauley, 1991). However, mathematical models have demonstrated that the number of individuals involved in the initial founding event, and the number of source populations from which colonists are sampled, are important factors in structuring patterns of genetic diversity within and between populations (Nei *et al.*, 1975; Slatkin, 1977; Wade and McCauley, 1988). Small founding population size, limited gamete sampling from the source pool, low population growth rate and long distance dispersal are known to increase founding effects, drift and inbreeding; all of which have negative effects on genetic diversity and the likelihood of population survival (Nei *et al.*, 1975; reviewed in Barrett and Husband, 1989). When founding numbers are small, significant losses in genetic polymorphism occur, the more so when populations remain small for several generations (Nei *et al.*, 1975). Island populations, due to their isolated nature (limiting dispersal) and finite size (small population size) are assumed to be more likely to be affected by such processes than populations on larger landmasses.

Island biogeography theory provides a qualitative model for how island isolation and area determine species diversity (MacArthur and Wilson, 1967). As islands become further away from a large landmass, species number decreases as the rate of immigration is affected by distance (Figure 1.4). As island size decreases, so too does species number as the probability of extinction heightens in each case. Isolation and limited habitat area mentioned above will effect levels of genetic polymorphism on islands in the same way (Figure 1.4) (Jaenike, 1973). Empirical evidence supports this theory: island populations of species from a broad range of taxa show, on average, lower genetic diversity than their mainland counterparts, with the effect being greatest on small and isolated islands (Frankham, 1997). Continental islands have higher levels of genetic polymorphism than do comparable oceanic islands and depending on their degree of isolation may not be distinguishable from their mainland counterparts. The magnitude of these differences is related to species dispersal ability (Frankham, 1997).

The Krakatau islands in Indonesia (Figure 2.1) provide a unique opportunity to study the effects and processes of colonization in action in the tropics. The 23rd August 1883 saw the conclusion to a series of extreme volcanic eruptions that covered the three Krakatau islands, Rakata, Sertung and Panjang in a thick layer of hot pyroclastic ash and destroyed all

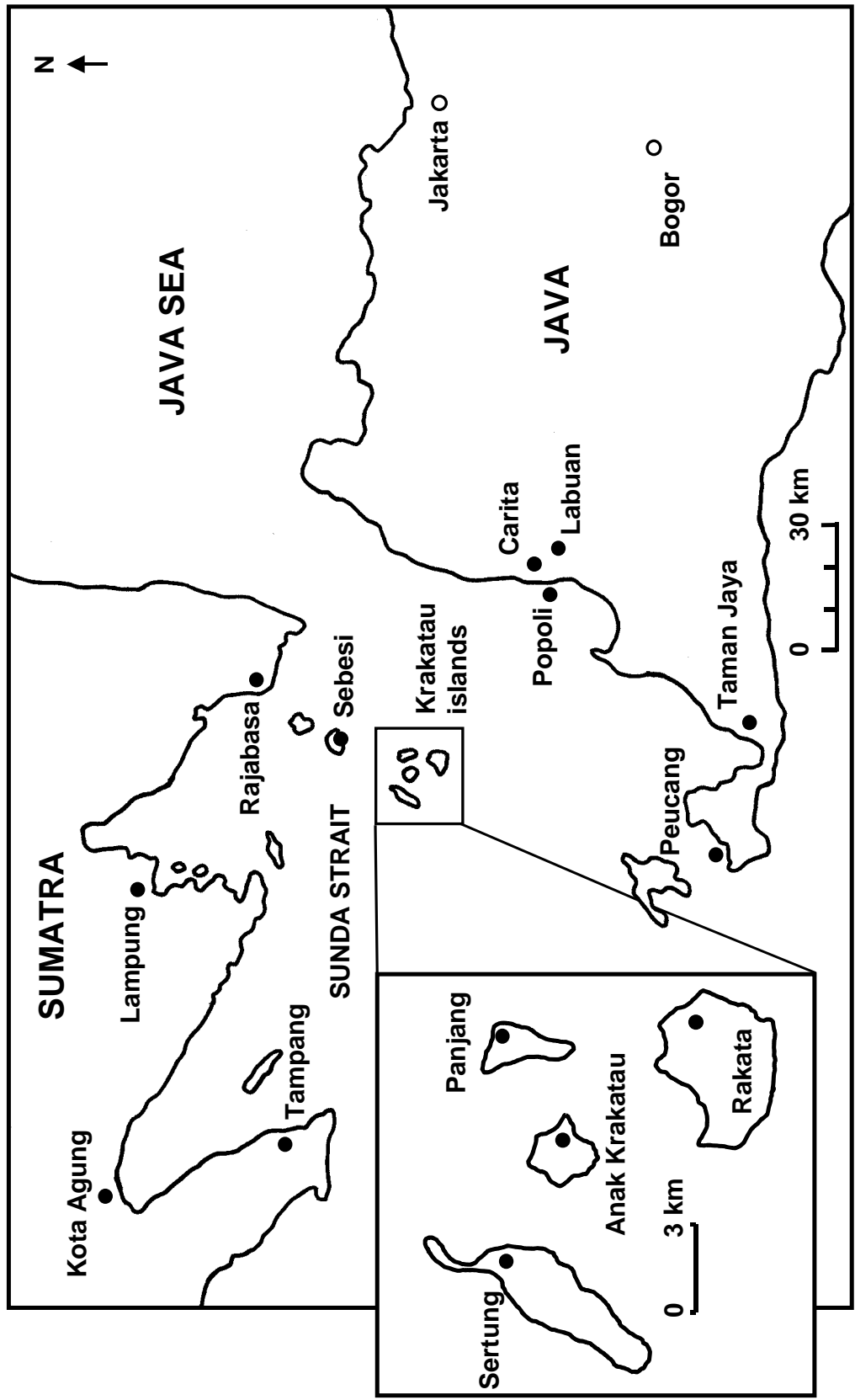


Figure 2.1: Map of the Sunda Strait region in Indonesia, the Krakatau islands and collection sites (filled-in circles).

life (Verbeek, 1885; reviewed by Docters van Leeuwen, 1936; reviewed by Whittaker *et al.*, 1989; but also see Backer, 1929, who argued against the complete sterilization of the islands). Thus, in effect a completely clean slate for the development of new populations in a discrete, insular environment was created. Life has subsequently returned to the Krakatau islands. The islands are now covered in species poor secondary monsoonal forest and, as a whole, remain depauperate of inland tree species, with the canopy remaining dominated by relatively few, early seral species (Whittaker *et al.*, 1989; Whittaker and Bush, 1993). It is assumed that all life subsequently returning to the Krakataus did so from outside the island system and must have bridged the 30 km or more of sea from the nearest coasts of Java and Sumatra.

Various scientific expeditions have provided a record of the return of life to the Krakatau islands (reviewed by Docters van Leeuwen, 1936; Whittaker *et al.*, 1989; Partomihardjo, *et al.*, 1992; Partomihardjo, 1995; Thornton, 1996) and thus, the approximate arrival time of species can be estimated (Table 2.1). Empirically derived models describing the restructuring of the forests, based on this record, have been developed (Whittaker *et al.*, 1989; Whittaker *et al.*, 1992a; Whittaker and Bush, 1993; Whittaker and Jones, 1994a). Reforestation has followed a successional pathway shaped by the dispersal characteristics of the colonists (Whittaker *et al.*, 1989), with similar patterns occurring on all three islands for the first 50 years (Whittaker and Bush, 1993). Initial colonists arrived mainly by sea or wind; the first tree communities being coastal (on the strand line and nearby flats) with grasslands developing inland (Whittaker and Jones, 1994a). Coastal forest establishment attracted larger and more diverse frugivore populations, resulting in increased plant species diversity. The secondary dispersal of trees inland replaced early successional grasslands and forest closure occurred by the end of the 1920s (Whittaker and Bush, 1993). A fourth volcanic island, Anak Krakatau, formed in the late 1920s in the caldera between the three older Krakatau islands. Ashfall from this island has had a dramatic effect on the forest of both Panjang and Sertung (Whittaker *et al.*, 1992b). There is now considerable forest divergence between the islands with the forest of Rakata being more diverse than that of Panjang and Sertung (Whittaker *et al.* 1989). Factors such as differences in volcanic and other abiotic disturbance (Schmitt and Partomihardjo, 1997; Schmitt and Whittaker, 1998; Whittaker *et al.*, 1992b; Whittaker *et al.*, 1998), topography (Whittaker *et al.*, 1989) and arrival sequence of species (Tagawa, 1992) have been important in shaping forest structure and divergence.

Species have continued to accumulate over the last 50 years, though further diversification may be seriously slowed due to habitat loss on Java and Sumatra (Whittaker and Bush, 1993; Whittaker *et al.*, 1997) and the sea barrier which prevents dispersal of

certain groups (Whittaker and Jones, 1994a). Large canopy trees with large, winged, wind-scattered seeds, and large seeded animal-dispersed species are poorly represented on the Krakatau islands (Whittaker and Jones, 1994a; Whittaker *et al.*, 1997). Thus, the Krakatau islands may be locked into an early successional stage longer than would normally occur on mainland sites. Fortunately, the Krakatau islands, unlike other Sunda Strait islands such as Sebesi (Figure 2.1) and many mainland sites, have remained relatively free from human influence, with pumice and land-crab collection being the main non-natural disturbance on the island (Docters van Leeuwen, 1936; Parrish, pers. observ.). As a result, the Krakatau islands offer a rare opportunity to study plant colonization and population genetics in a tropical ecosystem little influenced directly by humans.

Traditionally, studies of genetic diversity have focused on systems where allelic patterns can be determined. However, systems such as allozymes can be limited by the restricted availability of fresh material and other methods such as microsatellites depend on the costly development of the markers before studies can be undertaken. Where many species are to be examined other techniques that involve less species-specific fine-tuning and expense are required. One such method is Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.*, 1995). This method combines both restriction digestion and PCR to produce selectively neutral, highly polymorphic, Mendelian inherited, multilocus, dominant markers which are highly reproducible (Vos *et al.*, 1995). This technique has already been used to assess patterns of genetic variation and structure in a number of tropical tree species (*Calycophyllum spruceanum*, Russell *et al.*, 1999; *Moringa oleifera*, Muluvi *et al.*, 1999). AFLP studies have documented in some species a positive relationship between genetic diversity and population size (*Eryngium alpinium*, Gaudeul *et al.*, 2000; Hawaiian mint, *Haplostachys haplostachya*, Morden and Loeffler, 1999) and a decrease in genetic variation in newly formed populations after the introduction of a species to new areas (*Moringa oleiflora* in Africa, Muluvi *et al.*, 1999; and *Rubus alceifolius* in Australia and the Indian Ocean Islands, Amsellem *et al.*, 2000).

In this Chapter the population genetic diversity of five tree species, *Ficus fulva*, *F. fistulosa*, *F. pubinervis*, *Dysoxylum gaudichaudianum*, and *Oroxylum indicum* in populations throughout the Sunda Strait region, Indonesia, is estimated using AFLP. First, levels of genetic diversity are compared between populations on Krakatau, Java, Sumatra and other islands in the Sunda Strait to determine if recent long-distance colonization has had an effect on genetic diversity within the Krakatau populations. It is hypothesized that genetic diversity will be lower in the Krakatau island populations relative to mainland sites because founding

events have been severe on the islands. Second, the effect of genetic mobility (of which dispersal ability is a major component) on genetic diversity is examined. Species relative genetic mobility is estimated and the species ranked into genetic mobility groups. Overall levels of genetic diversity and differentiation are compared between species in relation to this genetic mobility. It is hypothesized that population diversity will be positively correlated with genetic mobility and differentiation between populations will decrease as genetic mobility increases.

Material and Methods

Species

The five tree species used in this study, *Ficus fulva*, *F. fistulosa*, *F. pubinervis*, *Dysoxylum gaudichaudianum*, and *Oroxylum indicum*, were chosen to represent species with a variety of estimated arrival times onto the islands and dispersal modes (Tables 2.1 and 2.2). These species share some aspects of their life-history in common (growth form and geographical range) that are correlated with levels of genetic diversity in tropical tree species (see Loveless, 1992) and allows for the effects of differing dispersal characters on genetic diversity to be compared. They are all common widespread tree species in the Malayan region, and four of the five species are known to have obligate outcrossing breeding systems. The species are listed in order of arrival time on the Krakatau islands, earliest to latest.

Ficus fulva

Ficus fulva (Moraceae) is a small functionally dioecious (therefore obligate outcrossing) tree species. The species is distributed throughout India and West Malaysia (Corner, 1988) and is an early colonizer of open, disturbed sites. Seeds are small (<2 mm) and are dispersed by bats and birds (Thornton *et al.*, 1996).

Ficus fulva colonized the Krakatau islands very early and was first collected on Panjang and Rakata in 1896 and 1897, respectively (Docters van Leeuwen, 1936). In 1906, within 10 years of first being recorded, the species was amply fruiting, though still thought to be uncommon (Ernst, 1908). However, by 1919 the species was already common and was one of the dominant early woodland species invading the grasslands of the interior (Docters van Leeuwen, 1936). The species remains common on all islands. Populations are dense and large on Rakata and Panjang, estimated at 15,000+ and 20,000 individuals, respectively (N. Mawdsley, pers. comm.) Population sizes have not been estimated on Sertung for any of the

Table 2.1: The collection record of the five study species on the Krakatau islands. This record can provide an estimate of the relative arrival times of species on to the Krakatau islands. It must be noted however, that collections were not uniform in time or the islands visited. Absence of species from an island or collection trip may reflect this collection inconsistency. For more detailed accounts of species collection and early populations refer to Docters van Leeuwen, 1936 and Whittaker *et al.*, 1989.

Species	Collection Record ¹													
	1886	1887	1908	1920	1922	1924	1929	1932	1934	1951	1979	1982	1983	1990s ²
<i>Ficus fulva</i>	R-P	R-P	R-P	RS-	R--	R--	RSP	RSP	R--	R--	R--A ²	RSPA ²	RSPA ²	RSPA
<i>Ficus fistulosa</i>		R-P	R-P	RS-	R--	R--	RSP	RSP	---	R--	R--	RSP-	RSP-	RSPA
<i>Ficus pubinervis</i>					R--	R--	R--	R--	---	R--	R--	RSP-	RSP-	RSPA
<i>Dysoxylum gaudichaudianum</i>								--P	---	---	R--A ²	RSP-	RSPA ²	RSPA
<i>Oroxylum indicum</i>														R---

Rakata, Panjang, Sertung, Anak Krakatau. 1. Whittaker *et al.*, 1989; 2. Partomihardjo, 1995

Table 2.2: Dispersal, reproductive and pollination systems of the study species.

Species	Main Seed Disperser	Fruit Characters	Seed Characters	Pollinator	Breeding System
<i>Ficus fulva</i>	bat/bird ¹	fleshy syconium c25 mm	<2 mm	Species-specific wasp	functionally dioecious
<i>Ficus fistulosa</i>	bat ¹	fleshy syconium c20 mm	<2 mm	Species-specific wasp	outcrossing functionally dioecious
<i>Ficus pubinervis</i>	bird ²	fleshy syconium c10 mm	<2 mm	Species-specific wasp	outcrossing monoecious outcrossing
<i>Dysoxylum gaudichaudianum</i>	bird ³	capsule	10mm with fleshy aril	Insect/moth ⁵	±dioecious ⁵ outcrossing
<i>Oroxylum indicum</i>	wind/water ⁴	pod	large flat 10mm wide, papyrus wing 100mm	Single species of bat Not plant species- specific ⁶	hermaphrodite ?

1. Thornton *et al.*, 1996; 2. Partomihardjo, 1995; 3. Whittaker and Turner, 1994; 4. Whittaker and Jones, 1994a; 5. Mabberley *et al.*, 1995; 6. Start and Marshall, 1976

study species. *Ficus fulva* is common in the early successional woodland of Anak Krakatau (the new volcanic island which began to form in the late 1920s) and is one of the few tree species colonizing open ash fields on the island (Parrish, pers. observ.). The breeding population of *F. fulva* on Anak Krakatau, estimated at 200 individuals in 1994 suffers from highly reduced levels of pollination, thought to be due to severe pollinator limitation (plant populations too small to support wasp populations) or environmental effects (Compton *et al.*, 1994).

Ficus fistulosa

Ficus fistulosa (Moraceae) is a small functionally dioecious tree species with cauliflorous fruit which grows along streams and open forest throughout India, Southern China and Malesia (Corner, 1988). Seeds are small (<2 mm) and bats are the predominant dispersal agents (Thornton *et al.*, 1996).

Ficus fistulosa was an early colonist on the Krakatau islands, being first recorded on Rakata in 1905 by Valeton (Docters van Leeuwen, 1936). By 1906 the species was considered common (Backer, 1929; Ernst (1934) and was already fruiting (Ernst, 1908). Currently, population sizes on the islands are large, estimated at 60,000+ and 10,000+ on Rakata and Panjang, respectively (N. Mawdsley pers. comm.).

Ficus pubinervis

Ficus pubinervis (Moraceae) is a large, monoecious tree which can grow to heights in excess of 30 m. It is distributed throughout Indonesia from Sumatra to Molucca (Corner, 1965). Monoecious *Ficus* species typically produce crops synchronously within the tree and hence outcrossing is ensured (Ramirez, 1970). The small fruits of *F. pubinervis* are dispersed by various birds (Partomihardjo, 1995) and, like the other fig species in this study, seeds are small.

Ficus pubinervis was first recorded on the Krakatau islands on Rakata as a single 'well-developed' individual in 1922 and by 1928 was still uncommon (Docters van Leeuwen, 1936). The species was not recorded on Panjang and Sertung until 1982 (Whittaker *et al.*, 1989); this reflects the lack of surveys into the interior of these islands after 1928-1934. The species is now common on the older Krakatau islands and forms large dominant stands in some areas (Parrish, pers. observ.). Populations sizes have been estimated at over 115,000 and 5,000 individuals on Rakata and Panjang, respectively (N. Mawdsley pers. comm.).

Dysoxylum gaudichaudianum

Dysoxylum gaudichaudianum (Meliaceae) is a common, large secondary rain forest tree and is distributed east from Sumatra to the Philippines, the Lesser Sunda Islands, Moluccas, Christmas Islands, Papua New Guinea, Queensland, the Solomon Islands and New Hebrides (Mabberley, *et al.*, 1995). The genus is predominantly dioecious and rarely may contain hermaphroditic flowers (Mabberley *et al.*, 1995). The fruits of *D. gaudichaudianum* are large with an average diameter of 10 mm and are predominantly dispersed on Krakatau by two species of pigeon (Green Imperial Pigeon, *Ducula aenea* and Black-naped Fruit-dove, *Ptilinopus melanospila*) and a bulbul (Yellow-vented Bulbul, *Pycnonotus goiavier*) (Whittaker and Turner, 1994). Bats may also have a role in dispersal (Docters van Leeuwen, 1936). However, large pigeons such as *Ducula* are the only species likely to be involved in long distance dispersal as this genus is the only disperser visiting the islands with a digestive tract large enough to pass the seeds (Whittaker and Turner, 1994).

Dysoxylum gaudichaudianum was first recorded on Panjang in 1932, where a few older trees were fruiting and were surrounded by younger trees (Docters van Leeuwen, 1936). The species was not recorded on the other islands until later expeditions; Rakata in 1979 and Sertung in 1982 (Whittaker *et al.*, 1989). It must be noted, though, that Sertung, of all the islands was little explored or completely ignored in various scientific expeditions between 1928 and 1982. *Dysoxylum gaudichaudianum* is common on Panjang and Sertung, where it is a canopy dominant over large areas of the islands (Whittaker *et al.*, 1989). While common in Rakata lowland forest the species does not reach such extensive canopy dominance as on the other Krakatau islands (Schmitt and Whittaker, 1998).

Oroxylum indicum

Oroxylum indicum (Bignoniaceae) is an evergreen or partly deciduous small tree growing to 20 m. The genus is monotypic and is widely distributed throughout India, Southern China, and in Malesia to the Philippines, the Celebes and Timor in the east (Corner, 1988). The tree is common in disturbed habitats such as villages and rice fields and along streams (Ridley, 1930; Corner, 1988). The flowers are nocturnal, white, hermaphroditic, bell-shaped and bat-pollinated (Corner, 1988; Start and Marshall, 1976). Nothing could be found by the author regarding the breeding system of this species. Pollination of *O. indicum* is performed by one species of nectivorous bat, *Eonycteris spelaea* (Gould, 1978). However, examination of pollen taken from *E. spelaea* faecal samples found that *O. indicum* pollen is typically poorly represented, indicating pollination services are not specific to *O. indicum* (Start and Marshall,

1976). The seeds are large flat discs (1.5 cm across) surrounded by a large papyrus for wind scattering or water dispersal (Ridley, 1930; Corner, 1988). Local information suggests that the plant has multiple uses ranging from green vegetable, goat feed, hedgerows, lightning rods (Parrish, pers. observ.) and medicinal (Corner, 1988).

Oroxylum indicum was first recorded on Krakatau in 1994 (Partomihardjo, 1995). It is known from only one highly disturbed coastal site at Handl's Bay on Rakata and fewer than 20 adults were identified there between 1996 and 1999. Some adult trees were already senescent, indicating that initial colonization must have occurred before the 1990s.

Photographs of the fruit of the study species can be seen in Appendix II.

Study Sites and Material Collection

Leaf material for DNA analysis was collected on the Krakatau islands and surrounding islands and coastlines in the Sunda Strait region of Java and Sumatra during the dry seasons (July-August) from 1996-1998. Study sites (Figure 2.1, detailed description in Appendix I) were selected over as wide an area of the coastline as possible. Each species was collected from a minimum of four populations with at least one population sampled from the Krakatau islands, Java and Sumatra. Due to differences in species distribution or time available for sampling, not all species were collected from the same sites or as broadly as would be desired. Depending on local availability of a species, 6-17 individuals were sampled from each population. At some sites an individual species was not locally abundant and hence fewer individuals were collected. Where possible, similar aged individuals were sampled within a species.

Leaf material for DNA extraction was preserved in a NaCl/CTAB solution (Rogstad, 1992). This method proved useful as leaves could be sampled directly into bottles and stored at ambient temperatures for several weeks until placed at -20°C for long-term storage.

Given the early colonizing nature of the study species and the shrinking nature of undisturbed forest on mainland sites, most collection sites in Java and Sumatra were situated in disturbed areas, usually in the vicinity of farmland or timber reserves. However, the sites at Taman Jaya and Peucang are situated within the Ujung Kulon National Park and seem relatively undisturbed by humans. Population sizes for mainland sites were not determined. However, the mainland sites and other islands in the Sunda Strait tended to be more fragmented (small patches of habitat separated by cultivated land) than the Krakatau island sites (except in Ujung Kulon National Park and for *O. indicum*). Nearly all sites were coastal or located within 10 km of the coast.

Ficus fulva was collected from three Krakatau populations Anak Krakatau, Panjang and Rakata in order to examine the effects of early colonization on Anak Krakatau. The species was collected at one site on both Java and Sumatra, from open, disturbed roadside locations that had been previously used for agriculture. Material was collected predominantly from mature individuals.

Due to time limits, genetic analysis of *F. fistulosa* was performed from only one island on Krakatau Sertung. This island was chosen because of its highly disturbed history. Population size has not been estimated but the species is common on this island. All mainland sites were situated along or nearby streams that are bordered by cultivation or timber reserves. Leaf material was collected at all sites from a range of fruiting and immature plants.

Ficus pubinervis was also collected from Sertung for the same reasons as above. Due to the disturbed nature of this collection site only immature trees of this species were available for collection. Population size was not estimated, though the species is common on the other older Krakatau islands. It was difficult to find large stands of advanced forest at many mainland sites. Only a small number of saplings were collected at the Rajabasa site from within a narrow gully where only a few adult trees were present. The Carita site was located in a small patch of forest along a stream within the Taman Wisata Alam Forest Reserve. Only saplings were collected. The populations of Taman Jaya and Pulau Peucang being situated within Ujung Kulon National Park were extensive and showed little sign of disturbance. There was very little recruitment in these two populations and the trees may date from a period around the time of the Krakatau eruptions. Only adult material was collected from these sites.

To make comparisons between the Krakatau islands, *D. gaudichaudianum* was collected on Panjang, Sertung and Rakata. Due to the large size of *D. gaudichaudianum* and the disturbed nature of many sites, immature trees or saplings were predominantly sampled. Collections on Sumatra (Rajabasa and Tampang) and Sebesi (an island off the coast of Sumatra) were made at disturbed, sandy, coastal flats which are heavily influenced by human activity. These sites predominantly featured juvenile trees of this species. At Popoli island (a small coral island 1 km from mainland Java) material from juvenile trees only was collected. This site was being recolonized after cultivation. A small patch of large adult trees (<20 individuals) was present but they were too tall for collection.

Only one small population of *O. indicum* is known from the Krakatau islands at a disturbed coastal site on Rakata. At the time of sampling on Rakata mature *O. indicum* were deciduous. There was considerable recruitment at this site and therefore only saplings could

be sampled. Mainland collections were made in highly disturbed agricultural sites. Immature trees of *O. indicum* were uncommon in the mainland sites studied and so predominantly mature trees were sampled.

DNA extraction

To compare DNA quality between different extraction protocol techniques, DNA from an individual was initially extracted using two different extraction protocols, QIAGEN and a hot CTAB method (slightly modified from Rogstad, 1992). Individual AFLP profiles were identical between both extraction methods. The CTAB method was selected as it is cheaper. Two slight modifications were made to the Rogstad (1992) CTAB DNA extraction protocol to create a miniprep method; small quantities of leaf material (less than 2x2cm) were mixed in 600 µl of extraction buffer and incubated at 65 °C for approximately two hours. The rest of the protocol was scaled according to this volume. DNA for population analysis was then extracted from preserved leaf material.

AFLP Primer Screening

Initial screening for suitable primer combinations was done with three selective nucleotides per primer on two individuals per species. Sixty-three primer combinations were screened in each of *D. gaudichaudianum*, *F. fistulosa*, *F. pubinervis* and *O. indicum*, and 25 in *F. fulva*. Relatively few bands were observed in *F. fulva* and *O. indicum*. Therefore, only 9 and 15 primer combinations with two selective nucleotides on the Mse or the Eco primer were used for further screening in *F. fulva* and *O. indicum*, respectively. Further screening using five individuals per species and a subset of primer combinations was performed to select two or three primer combinations per species for later use in population studies. Selection aimed to maximize the number of bands while ensuring that patterns remained readily scorable. Reproducibility of banding patterns in the chosen primer combinations was determined before use in population studies. Two or three independent DNA extractions were made from a number of individuals per species. Subsequent AFLP profiles within an individual were compared and high reproducibility was found.

AFLP Protocol

The AFLP protocol followed was similar to that of Vos *et al.* (1995). DNA was first digested with *Eco RI* and *Mse I* restriction enzymes, primer adapters were then ligated and two rounds of selective PCR followed. Total genomic DNA (300 ng) was digested with 5U of each *Eco RI* and *Mse I* (Life Technologies) using New England Laboratories buffer number 4 (NEB) in 40 µl for 1 hour at 37 °C. Dual digestion/ligation was then undertaken by the addition of a ligation mixture (10 µl) which contained 5 pmol of *Eco RI* adapter and 50 pmol of *Mse I* adapter, 1 µl NEB, 2 U of T4 DNA ligase and 1.0 mM ATP for 3 hours at 37 °C. This was then diluted to 200 µl with TE⁻⁴ solution (10 mM Tris-HCl, 1mM EDTA, pH 8.0).

Two rounds of selective PCR amplification, to create an AFLP fingerprint, were then performed on this primary template. Primers with one and three extra extension nucleotides were used in the first and second round of amplification, respectively. The first round of amplification reaction (20 µl) contained 5 µl of diluted primary template, 50 ng of each primer, 0.2 mM of each dNTP, 0.25 U *Taq* polymerase (Goldstar, Eurogentec), 1.5 mM Magnesium Chloride, 75 mM Tris-HCl (pH 9.0), 20 mM (NH₄)SO₄ and 0.01% Tween 20. These preamplification products were then diluted 20-fold with TE⁻⁴ and stored at -20 °C until used. The second round of amplification used the same PCR reaction protocol with the exception of 10 µl of diluted first round PCR product and 5ng of δ33P radiolabelled *Eco* primer. For *D. gaudichaudianum*, *F. fistulosa*, *F. pubinervis* and *O. indicum*, both rounds of amplification were performed on a Perkin-Elmer 9600 thermal cycler using the following touchdown protocol; cycle 1, 94 °C at 30 sec, 65 °C at 30 sec, 72 °C 1 min; cycles 2-13, as for cycle 1 but with a 0.7 °C touchdown per cycle for the annealing temperature; cycles 14-36, 94 °C at 30 sec, 56 °C at 30 sec, 72 °C at 60 sec. Amplification in *F. fulva* was done on a PTC-200 (MJ Research) thermal cycler with the following protocol being used for both rounds of amplification; cycle 1, 94 °C at 1 min, 65 °C at 1 min, 72 °C 1:30 min; cycles 2-10, as for cycle 1 but with a 1 °C touchdown per cycle for the annealing temperature; cycles 11-32, 94 °C at 30 sec, 56 °C at 30 sec, 72 °C at 60 sec; cycle 33, 72 °C at 5 mins. Five microliters of products were separated on a 6% polyacrylamide sequencing gel.

Band Scoring

Autoradiographs were made of the gels and scored manually. A locus was considered polymorphic where at least one individual showed the presence or absence of a band across all populations examined. Loci which were unclear, and hence difficult to score, were excluded from analysis. The regions of the gels used for analysis began one band before the first discernable polymorphic locus and one band after the last discernable polymorphic locus.

Statistical Analysis

Genetic diversity was quantified as the proportion of polymorphic loci (P), average expected heterozygosity (H_e), number of unique bands and average proportion difference in band-sharing (APD) within populations. The proportion of polymorphic loci was determined as the number of polymorphic loci within a population over the total number of loci within the species (polymorphic and fixed). Average expected heterozygosity was determined taking the population average of Nei's (1978) unbiased expected heterozygosity at a locus assuming that the populations were in Hardy-Weinberg equilibrium. Nei's unbiased expected heterozygosity per locus was determined as $H_e = [2n/2n-1](1-\sum_i p_i^2)$, where n is the number of individuals used for analysis and p_i is the frequency of the i th allele at the locus under consideration; H_e was then averaged across loci. Allelic frequencies were determined assuming the frequency of null-phenotype (band absent) represents the homozygote recessive and is equal to q^2 and $p=1-q$. Though there was no evidence to support the assumption that populations were at Hardy-Weinberg equilibrium, analysis was performed as a comparative base to other studies. A count of unique bands was also made. A band was considered unique when it occurred exclusively in a population.

Overall within population dissimilarity was measured as the average proportion difference (APD) in pairwise comparisons of band-sharing between individuals within a population. The proportion difference in band-sharing between individuals is $PD = N_{AB}/(N_A + N_B)$, where N_{AB} is the number of bands that differ between individuals A and B, and N_A and N_B are the number of bands in individuals A and B, respectively (Gilbert *et al.*, 1991). All bands present within the species (even those fixed in all populations) were used for analysis. APD is the mean of pairwise comparisons of PD between all individuals within a population and can range from 0 (individuals exactly identical) to 1 (individuals

completely different). APD can be considered a rough downward estimation for heterozygosity (Gilbert *et al.*, 1991).

The patterns of genetic variance partitioning within and between populations were examined using AMOVA (Analysis of Molecular Variation) using the Arlequin ver. 2.0 program (Schneider *et al.* 2000). F_{ST} , a measure for population differentiation based on genetic correlations (see Chapter 3), was determined as the percentage of total variation explained by population differences in the AMOVA analyses.

Mean population genetic diversity for P, H_e and APD was determined for each species. An ANOVA (analysis of variance) and Tukey HSD test were performed using SYSTAT (Wilkinson, 1999) to determine if there were significant differences in genetic diversity between species.

Species Genetic Mobility

To compare patterns of genetic diversity and structure with dispersal capability, species were ranked according to their genetic mobility as determined by the authors. This has been previously done in studies of genetic diversity in tropical tree species where associations between breeding system and genetic diversity have been made (Hamrick and Loveless, 1989). Genetic mobility was assessed using the following characters; seed dispersal mode, number of seed dispersal agents, seed size, importance of species to disperser populations, breeding system, pollinator characters, and arrival time of species on the islands.

Results

AFLP Banding Patterns

Table 2.3 lists the primer combinations used for population studies, the number of bands produced per combination and the number of bands polymorphic at the species level. The number of bands produced per primer combination ranged from 32 to 95 and averaged 55.3 across all species. The percentage of polymorphic bands per primer combination ranged from 25% to 75%. *Oroxylum indicum* showed the lowest level of polymorphism, 29.3%; with the three *Ficus* species, *F. fistulosa*, *F. fulva* and *F. pubinervis* showing the highest levels at 63.3%, 50% and 64.7% respectively. *Dysoxylum gaudichaudianum* had 46.4% of bands polymorphic.

Patterns of Genetic Diversity Between Populations

Population sample sizes and values of genetic diversity within populations of the study species are given in Table 2.4. In some cases, due to difficulties in finding adequate numbers of individuals at a site, small collection sample sizes (less than 12 individuals) were made. Resampling analysis of AFLP data indicated that more than 95 percent of the polymorphic loci were detected within a sample size of 10 individuals; larger sample sizes detected little more polymorphism (results not shown).

When the consistency of population differences across P, He and APD is taken into consideration several trends are clear. In *F. fulva* and *O. indicum* values of genetic diversity for all measures are consistently higher in the Krakatau populations than all mainland sites, despite the Krakatau population of *O. indicum* and the Anak Krakatau population of *F. fulva* being relatively small. Values of genetic diversity for *F. fistulosa* are lower in the Krakatau island population (Sertung) than three mainland sites (Rajabasa, Kota Agung and Carita), but values of P and He in Sertung are higher than in the fourth mainland site (Lampung). Two species, *D. gaudichaudianum* and *F. pubinervis* show equivalent levels of genetic diversity to mainland sites. The picture emerging is that contrary to our expectation, the Krakatau populations for most of the five species do not show a loss of genetic variability, but show a trend to equal or higher genetic variability compared to their mainland populations.

Comparing the mainland populations from Java and Sumatra and other islands in the Sunda Strait (closer to the mainland than Krakatau), four populations appear to have consistently lower than average values for P, He and APD; the populations of *F. pubinervis* at Rajabasa, *D. gaudichaudianum* from Tampang and Sebesi Island and *O. indicum* at Taman

Table 2.3: AFLP primer combinations used in this study and the levels of polymorphism observed within species.

Selective Nucleotides	Number Bands Polymorphic	Number Fixed	Total Number Bands
<i>Ficus fulva</i>			
<i>Eco</i> -AGG, <i>Mse</i> -CA	36	26	62
<i>Eco</i> -AGA, <i>Mse</i> -CT	27	37	64
Total All Primers	63 (50 %)	63 (50 %)	126
<i>Ficus fistulosa</i>			
<i>Eco</i> -AAG, <i>Mse</i> -CTG	25	19	44
<i>Eco</i> -AAC, <i>Mse</i> -CTA	25	10	35
Total All Primers	50 (63.3%)	29 (36.4%)	79
<i>Ficus pubinervis</i>			
<i>Eco</i> -AGA, <i>Mse</i> -ACT	40	26	66
<i>Eco</i> -ATC, <i>Mse</i> -ACT	26	10	36
Total All Primers	66 (64.7%)	36 (35.3%)	102
<i>Dysoxylum gaudichaudianum</i>			
<i>Eco</i> -AGA, <i>Mse</i> -AGG	30	28	58
<i>Eco</i> -ATC, <i>Mse</i> -AGC	36	35	71
<i>Eco</i> -AGA, <i>Mse</i> -ACT)	38	57	95
Total All Primers	104 (46.4 %)	120 (53.6 %)	224
<i>Oroxylum indicum</i>			
<i>Eco</i> -AC, <i>Mse</i> -CGA	8	24	32
<i>Eco</i> -AG, <i>Mse</i> -CTG	14	35	49
<i>Eco</i> -AT, <i>Mse</i> - CGA	17	35	52
Total All Primers	39 (29.3%)	94 (70.7%)	133

Jaya. The low levels of dissimilarity (APD) observed in these four populations indicate that individuals in these populations have higher levels of relatedness than in other populations of the same species. In *O. indicum* values of genetic diversity (across all measures) are more than 1.5 times greater in the Krakatau population (Rakata) than in the mainland population of Taman Jaya. However, some peculiarities of two of these populations, which will effect values of genetic diversity, should be noted. The *F. pubinervis* Rajabasa population was extremely small and collected from an isolated gully where there were few mature trees. In combination with this only six saplings were sampled. This population, though situated in Sumatra, has no unique bands which suggests that it is not geographically distinct from the Java or Krakatau populations. Low levels of P in *D. gaudichaudianum* population on Sebesi are likely influenced by the small sample size (N=6).

Genetic Mobility

Prior to genetic analysis the genetic mobility of species was estimated based on various aspects of their breeding biology and dispersal system (Table 2.2). Genetic mobility is, to a certain extent, difficult to quantify as various factors are involved in determining individual species dispersal ability. *Ficus* species were ranked as the most genetically mobile (Table 2.5) because they have endozoochorous dispersal, small seed size, a large number and variety of dispersal vectors (Thornton *et al.* 1996), a large foraging range of some of their dispersers (Shilton *et al.*, 1999; Thornton *et al.*, 1996), keystone importance to frugivore populations (Lambert and Marshall, 1991), ubiquity in tropical forests, an outcrossing breeding system, a species-specific pollination system, potential long-distance dispersal of pollinators (Nason *et al.*, 1996; Pemberton, 1934) and early arrival time onto the Krakatau islands (Whittaker *et al.*, 1989). These are all factors that would increase gene flow and the effectiveness of gene flow between populations. *D. gaudichaudianum* was ranked second to *Ficus* in genetic mobility for a variety of reasons. Though the species is also endozoochorous, its seed size is larger. The number of dispersers and the importance of the species to frugivore populations are considerably less than that for *Ficus* populations. *Dysoxylum gaudichaudianum* has an obligate outcrossing breeding system (Parrish, unpublished data) but pollination is performed by insects (Mabberley *et al.*, 1995) that are probably non-specific. The later arrival of *D. gaudichaudianum* onto the islands compared to early colonist *Ficus* species is indicative of a more limited dispersal capability rather than of differences in succession time. This is justified as *D. gaudichaudianum* has been observed to colonize highly disturbed sites simultaneously with early colonist *Ficus* species where a source population of *D.*

Table 2.4: Populations sampled (number of individuals), proportion and total number of polymorphic loci (P), average expected heterozygosity across loci (H_e , where (N) is number of loci) (\pm SE), dissimilarity measure (APD) and number of unique bands in populations of the five studied species.

	Population (n)	P	H_e (N loci)	APD	Unique
	<i>Ficus fulva</i>				
			(63)		
K	Anak (16)	0.397 (50)	0.231 \pm 0.023	0.106	0
K	Panjang (14)	0.405 (51)	0.232 \pm 0.023	0.110	1
K	Rakata (16)	0.389 (49)	0.253 \pm 0.025	0.109	0
S	Rajabasa (13)	0.365 (46)	0.210 \pm 0.024	0.101	3
J	Labuan (14)	0.365 (46)	0.211 \pm 0.023	0.100	2
	<i>Ficus fistulosa</i>				
			(50)		
K	Sertung (15)	0.380 (30)	0.156 \pm 0.025	0.116	0
S	Rajabasa (13)	0.392 (31)	0.163 \pm 0.026	0.124	0
S	Lampung (12)	0.342 (27)	0.144 \pm 0.025	0.117	1
S	Kota Agung (11)	0.456 (36)	0.197 \pm 0.025	0.150	4
J	Carita (13)	0.481 (38)	0.197 \pm 0.026	0.146	8
	<i>Ficus pubinervis</i>				
			(66)		
K	Sertung (15)	0.529 (54)	0.198 \pm 0.021	0.146	2
S	Rajabasa (7)	0.324 (33)	0.163 \pm 0.025	0.111	0
J	Carita (10)	0.402 (41)	0.183 \pm 0.024	0.141	1
J	Taman Jaya (15)	0.490 (50)	0.193 \pm 0.021	0.143	3
J	Peucang (15)	0.461 (47)	0.206 \pm 0.022	0.154	1
	<i>Dysoxylum gaudichaudianum</i>				
			(104)		
K	Panjang (16)	0.290 (65)	0.168 \pm 0.018	0.071	1
K	Sertung (17)	0.272 (61)	0.171 \pm 0.019	0.063	2
K	Rakata (16)	0.277 (62)	0.160 \pm 0.019	0.058	2
S	Rajabasa (14)	0.268 (60)	0.163 \pm 0.018	0.070	4
S	Tampang (15)	0.192 (43)	0.117 \pm 0.017	0.048	4
S	Sebesi (6)	0.143 (32)	0.118 \pm 0.019	0.052	1
J	Popoli (16)	0.295 (66)	0.157 \pm 0.017	0.068	5
	<i>Oroxylum indicum</i>				
			(39)		
K	Rakata (15)	0.218 (29)	0.271 \pm 0.033	0.041	6
S	Rajabasa (13)	0.188 (25)	0.233 \pm 0.035	0.039	2
S	Sebesi (14)	0.165 (22)	0.221 \pm 0.036	0.040	1
J	Taman Jaya (14)	0.143 (19)	0.141 \pm 0.030	0.027	2

gaudichaudianum was nearby (Parrish, pers. observ). *O. indicum* was ranked with the lowest genetic mobility for a variety of reasons; mainly large wind-scattered seeds and late arrival time of the species in the islands despite being a colonist of open sites elsewhere. Comparative studies between island and mainland floras support the assumption that large-seeded wind dispersed species have poor dispersal ability relative to other dispersal groups as they are underrepresented on islands in comparison to mainland sites (Ridley, 1930; Whittaker *et al.*, 1997). Little is known about the species breeding system. *Oroxylum indicum* is pollinated by a single bat, *Eonycteris spelaea* (Gould, 1978). However, pollination services are likely to be relatively poor as *E. spelaea* does not exclusively feed on *O. indicum* (*O. indicum* pollen is proportionally poorly represented in *E. spelaea* guano samples) (Start and Marshall, 1976).

Table 2.5: Average population values of genetic diversity within species (mean±se) and the significance level based on an ANOVA test for differences among species. Small letters indicate significant differences (P<0.05) based on Tukey HSD tests. Species with the same letter are not significantly different from each other. Species genetic mobility ranking is also given.

Species	P	He	APD	Gen. Mob. Ranking
F. fulva	0.384±0.008 a	0.227±0.008 a	0.105±0.002 a	1
<i>F. fistulosa</i>	0.410±0.025 a	0.171±0.011 bc	0.131±0.007 b	1
<i>F. pubinervis</i>	0.441±0.036 a	0.189±0.007 abc	0.139±0.007 b	1
<i>D. gaudichaudianum</i>	0.248±0.022 b	0.151±0.009 c	0.061±0.003 c	2
<i>O. indicum</i>	0.179±0.016 b	0.217±0.027 ab	0.037±0.003 d	3
ANOVA	P=0.000	P=0.001	P=0.000	

Genetic Diversity and Population Differentiation Within a Species In Relation to Genetic Mobility

Measures of average population genetic diversity within a species and species genetic mobility ranking are shown in Table 2.5. The general trend in species mean population measures of P and APD is that as species genetic mobility decreases so too does genetic diversity. Differences between each mobility group are significant for APD. A significant difference exists between mean population values of P for *Ficus* species (mobility group 1) and the other study species, *D. gaudichaudianum* (mobility group 2) and *O. indicum* (group 3) but not between *D. gaudichaudianum* and *O. indicum*. There is no clear trend in values of H_e . This may reflect the fact that this value is an estimate of heterozygosity (assuming Hardy-Weinberg equilibrium) rather than actual observed heterozygosity.

Values of overall population differentiation (F_{ST}) within species were 0.046, 0.049, 0.052, 0.162 and 0.213 for *F. fulva*, *F. fistulosa*, *F. pubinervis*, *D. gaudichaudianum* and *O. indicum*, respectively (Table 2.6). Patterns of differentiation between populations within a species show the converse to patterns of population genetic diversity (Table 2.5); as species mobility increases, differentiation between populations decreases. There is very little genetic differentiation between *Ficus* populations, which indicates high levels of gene flow between populations (justifying their genetic mobility ranking). There are moderate levels of differentiation between populations of *D. gaudichaudianum* (genetic mobility group 2). *Oroxylum indicum* demonstrates higher levels of differentiation between populations, thus demonstrating greater restrictions in gene flow in this species. It must be noted that the F_{ST} value for *F. pubinervis*, 0.052, was estimated excluding the Rajabasa population because this population is thought to be highly inbred and may be non-representative of the species as a whole. Inclusion of this population in analysis resulted in an F_{ST} value of 0.129

Table 2.6: AMOVA (Analysis of Molecular Variation) showing the subdivision of variation within and between populations and the corresponding F_{ST} estimate.

Species	Source of Variation	d.f	Sums of Squares	Variance Components	Percentage Variation
<i>F. fulva</i>	Among Populations	4	59.204	0.41954	4.61
	Within Populations	68	590.659	8.68616	95.39
	Total	72	649.863	9.10570	
	$F_{ST}=0.046$				
<i>F. fistulosa</i>	Among Populations	4	36.394	0.28428	4.94
	Within Populations	59	322.70	5.46949	95.06
	Total	63	359.094	5.75377	
	$F_{ST}=0.049$				
<i>F. pubinervis</i> *	Among Populations	3	42.761	0.44944	5.24
	Within Populations	51	414.367	8.12484	94.76
	Total	54	457.127	8.57427	
	$F_{ST}=0.052^*$				
<i>D. gaudichaudianum</i>	Among Populations	6	209.450	1.80826	16.23
	Within Populations	93	868.020	9.33355	83.77
	Total	99	1077.470	11.14181	
	$F_{ST}=0.163$				
<i>O. indicum</i>	Among Populations	3	58.729	1.10696	21.29
	Within Populations	52	212.789	4.09209	78.71
	Total	55	271.518	5.19505	
	$F_{ST}=0.213$				

*NB: Rajabasa has been removed from analysis because it is highly differentiated relative to other populations. It is uncertain whether this is a real geographical phenomenon or the product of population size and inbreeding. If included in analysis; within population variation=12.88, among population variation=87.02 and $F_{ST}=0.129$.

Discussion

Patterns of Genetic Diversity on Krakatau versus Mainland Sites

Colonization of islands is usually associated with small founding numbers and small subsequent population size due to island isolation and smaller habitat patch size. Therefore, genetic variation on islands will be determined by the net effects of loss at foundation, subsequent loss caused by finite population size since foundation and gains arising from secondary immigration and mutations (Jaenike, 1973). Small founding population size has a major effect on levels of genetic diversity, the more so when populations remain small for several generations (Nei *et al.*, 1975). Empirical evidence indicates that, on average, island populations have lower genetic diversity than mainland populations (Frankham, 1997). However, this relationship depends greatly on the degree of island isolation (in time and space), island size and species dispersal ability (Frankham, 1997). Have populations of trees on the Krakatau islands experienced a severe enough founding event to exhibit negative effects associated with colonization?

Four of the five species in this study, *D. gaudichaudianum*, *O. indicum*, *F. fulva* and *F. pubinervis*, show equivalent or higher levels of genetic diversity on the Krakatau islands in comparison with mainland sites on Java and Sumatra, indicating no negative genetic effect associated with colonization. This is in contrast to our initial expectations. Various reasons can be proposed for why these species have built up and maintained high levels of genetic diversity after colonization of the islands. Given that three of the species, *D. gaudichaudianum*, *F. fulva* and *F. pubinervis*, presently have large population sizes (tens of thousands of individuals) on the Krakatau islands these results are not greatly surprising. Maintaining large population sizes during colonization reduces the likelihood of negative genetic effects (Nei *et al.*, 1975; see Barrett and Husband, 1989). The early populations of both *D. gaudichaudianum* and *F. fulva* were most likely large. The first report of *D. gaudichaudianum* (1932) is of several trees with clusters of juveniles underneath (Docters van Leeuwen, 1936). *F. fulva* was fruiting within the first ten years of being recorded on the islands and within another 13 years it was one of the most common woodland tree species (Docters van Leeuwen, 1936). The formation and maintenance of large populations in some early colonist species are likely promoted by the empty post-eruption Krakatau environment (Verbeek, 1885), the sea barrier to some dispersal groups (Whittaker *et al.*, 1997) (which may lock the islands into a longer early successional stage) and post-colonization disturbance (Schmitt and Whittaker, 1998).

In contrast, many mainland populations are likely smaller than those of Krakatau as they are mosaics of different habitat patches in different successional states and suffer continued habitat fragmentation and disturbance by human activity (Parrish, pers. observ.). For example, forests dominated by many species common on Krakatau such as *Dysoxylum* are seldom seen on Java (Kartawinata, 1977). In fact, in all five species in this study (*F. fulva*, *F. fistulosa*, *F. pubinervis*, *D. gaudichaudianum* and *O. indicum*) the lowest levels of genetic diversity have been recorded in mainland populations. In some cases these values are one and a half times lower than those observed on the Krakatau islands (eg. *O. indicum* at Taman Jaya (Java) and *D. gaudichaudianum* at Tampang (Sumatra)).

Distance and positioning of the Krakatau islands from source populations and distance between islands in the archipelago is another factor maintaining the high levels of genetic diversity in some species (see also Chapter 3). The colonization distance from the mainland to the Krakatau islands is minimally 30 km and most likely is not a significantly large barrier to dispersal for many early-colonist species, especially to small-seeded endozoochorous species such as *Ficus*. *Ficus* have numerous bird and bat dispersal agents on the Krakatau islands (Thornton *et al.* 1996; Whittaker and Jones, 1994b). Species of bats on Krakatau have large foraging ranges and long gut retention times which would make it possible for extensive seed dispersal between mainland and island sites (Shilton *et al.*, 1999). Pollen flow distances greater than 10 km are known to be routine in monoecious species of *Ficus* from Central America (Nason *et al.*, 1996) and wasp colonization of new sites has been recorded at over 100 km (Hawaii islands, Pemberton, 1934). Low overall genetic differentiation between all *Ficus* populations in this study (F_{ST} values around 0.05) indicates that gene flow is high over the scale of the study area (even at distances of over 100 km) (see also Chapter 3). The maintenance of high levels of genetic diversity in the newly establishing population of *F. fulva* on Anak Krakatau (despite severe pollinator limitation and small population size, Compton *et al.*, 1994) likely reflects high seed dispersal rates between the islands and the short time since population establishment. The position of the Krakatau archipelago approximately equidistant between two landmasses, Java and Sumatra, will also have a positive effect on genetic diversity as two different and geographically separate colonization source regions are potentially available (Chapter 3).

Considering the importance attributed to population size (Nei *et al.*, 1975) and dispersal ability (Loveless, 1989) in establishing and maintaining genetic polymorphism, *O. indicum* provides an interesting set of results. This species is wind/water-scattered with limited dispersal capability (Ridley, 1930; Corner, 1988), shows high population

differentiation ($F_{ST}= 0.213$) over the scale of the study and exists in one small, restricted population on Krakatau; yet, surprisingly this population has levels of genetic diversity higher than all mainland populations. If severe sampling during founding had occurred (which seemed probable in this population) then the converse, a decrease in genetic variation, would be expected. There are several possible explanations for the observed maintenance of genetic diversity. First, there is the possibility of multiple colonist sources for the Krakatau population. Given the high population genetic differentiation in the species ($F_{ST}=0.213$), colonization by multiple sources may have the effect of a hybridization event where combination of alleles close to or fixed in source populations will lead to increases in polymorphism. The limited dispersal capabilities and high population differentiation in this species may make multiple colonist sources seem unlikely. However, formation of new populations in more isolated sites (such as the Krakatau islands) may be greatly affected by extreme dispersal events (cyclonic winds or storms, for example) which will have an important influence on the genetic diversity in new populations and may draw propagules from a large area. Further, the recent arrival time of the species means that few generations have elapsed since founding and so there has not been time for post-establishment small population effects to manifest.

Escape from human influence may be another factor important in maintaining the levels of genetic diversity observed in *O. indicum* on Rakata. Multiple human uses have been reported for this species, ranging from animal feed to lightning rods (Parrish, pers. observ.) and also medicine (Corner, 1988). In Taman Jaya (Java), where this species has considerably lower levels of genetic diversity than Rakata or Rajabasa (Sumatra), trees were planted in places as hedgerows. Selected planting of trees may lead to closely related individuals or genetic clones being propagated and planted in the same neighborhood. Genetically identical individuals have been found in some mainland populations (Parrish, unpublished data), thus suggesting that clonal propagation may occur.

In contrast with the above results, *F. fistulosa* on the Krakatau island, Sertung showed relatively lower levels of genetic diversity and higher levels of relatedness relative to some mainland sites. Levels of disturbance on Sertung due to ashfall from the nearby volcano, Anak Krakatau may play a role in these patterns as ashfall greatly increases overall plant mortality on Sertung (Schmitt and Partomihardjo, 1997; Schmitt and Whittaker, 1998). Population size of *F. fistulosa* (which has not been estimated on Sertung) may be kept small if regular ashfall increases mortality in this species, which in turn could lead to inbreeding or genetic drift resulting in a lowered genetic variability. *F. fistulosa* has large populations on

other Krakatau islands (which unfortunately have not been sampled). Comparisons of genetic diversity between islands will be instrumental in determining if patterns observed on Sertung are typical for the other Krakatau populations. It must be noted that in contrast to *F. fistulosa* the population of *F. pubinervis* on Sertung has high levels of genetic diversity. The relatively longer life span and later reproductive onset in *F. pubinervis* would mean fewer generations have passed since colonization and the relative effects of inbreeding would be less than for *F. fistulosa*.

Has the Krakatau experiment been running long enough to see effects?

The negative genetic effects of small population size and post-colonization processes may take some time to manifest in trees with long life spans. Loss of genetic diversity will occur during the initial founder event and in following generations if population size remains small (Nei *et al.*, 1975). Therefore, one could raise the question of whether the Krakatau experiment has been running long enough to see the genetic effects of colonization in the species studied. Limited time since fragmentation has been proposed to maintain genetic diversity in populations of sugar maple, *Acer saccharum* (Ontario, Canada, Young *et al.*, 1993). On the Krakatau islands this is most likely the case in such species as *F. pubinervis* and *D. gaudichaudianum*, which do not reproduce until they are large trees. Some are long-lived (*F. pubinervis*). Many trees of *F. pubinervis* on the Krakataus are therefore likely to be the original colonists. However, species such as *F. fistulosa* and *F. fulva* are likely to have seen multiple generations since founding. These species were fruiting within 10 years of colonizing Krakatau (observed in 1908, see Docters van Leeuwen, 1936) and can reproduce at less than 10 cm diameter at breast height (dbh) (Parrish, pers. observ.). Therefore, time since colonization may be long enough to see post-colonization effects and this is likely the case for *F. fistulosa*. The population of *O. indicum* on Rakata was first recorded in 1994 (Partomihardjo, 1995), though the presence of adult and senescent adult trees proves an earlier time of colonization. The population is small which likely lead to it being overlooked in earlier surveys. However, it seems unlikely that many generations have passed.

Patterns of diversity, differentiation and genetic mobility

Loveless (1992), in a review of factors important in structuring genetic diversity in tropical tree species, showed that geographical range (endemic versus widespread), growth form (shrubs versus trees) and mode of dispersal (abiotic versus biotic) had significant effects on genetic diversity within populations of tree species (P and H). Dispersal type was the only

factor that showed a significant relationship with population differentiation (G_{ST} in Loveless, 1992) with biotic-dispersed species having lower levels of differentiation between populations than species with abiotic-dispersal agents. Hamrick and Loveless (1989) found a negative correlation between genetic mobility (estimated from seed and pollen dispersal characters) and population differentiation in 14 tropical tree species on Barro Colorado Island, Panama.

The results of this study support the findings of Hamrick and Loveless (1989) and Loveless (1992) that species with different genetic mobility and dispersal modes (abiotic versus biotic) show different levels of population genetic diversity and differentiation. Species with high genetic mobility (*Ficus*) showed high population genetic diversity and low differentiation between populations, with genetic diversity decreasing and population differentiation increasing as mobility decreased. This pattern was evident in all measures used in this study (P , APD and F_{ST} , except H_e). The observed relationship was likely further strengthened by the fact that all species in this study have features of their life-history in common that have been shown to affect patterns of diversity in plant species as a whole (breeding system (though unknown in *O. indicum*), growth form and geographical range) (Hamrick and Godt, 1989; Hamrick *et al.*, 1992). In addition, sampling was carried out over comparable geographical areas.

Interestingly, despite the importance of dispersal ability in structuring genetic diversity within species, it was not in all cases a predictor for the genetic consequences of colonization in this study. For example, the small Krakatau island population of *O. indicum* had relatively high levels of genetic polymorphism in comparison with mainland sites despite apparently poor dispersal ability. *F. fistulosa*, with higher dispersal ability and an early colonist, had decreased levels of diversity in the Krakatau island population studied relative to some mainland sites. These results highlight the importance of ecological processes and chance events in shaping patterns of genetic diversity during colonization.

Conclusions

Colonization of new sites is essential for the perpetuation of a species. Success in colonization or ability to disperse will vary greatly between species depending on their natural history and the situation at hand. The Krakatau islands provide a unique case study in the restructuring of populations and ecosystems. Complete habitat destruction provided a clean slate for population rebuilding and a model where all individuals immigrated from outside the island system. The island study site provides a discrete, isolated experimental area. Has colonization led to a loss of genetic diversity in populations from a selection of tree species on Krakatau?

The conclusion from this study is that most species show little genetic effect of colonization to the Krakatau islands and have maintained comparable to higher levels of genetic variation relative to mainland sites. Such a result reflects mainly the young age and large size of populations in most of the study species on Krakatau, the moderate to high dispersal abilities of the species and the increased habitat fragmentation at some mainland sites. However, for two species the converse is true. Despite having small population size on Krakatau and poor dispersal ability, *O. indicum* has higher levels of genetic diversity compared with all mainland sites. The one population of *F. fistulosa* examined on the Krakatau islands has relatively lower levels of genetic diversity compared to some mainland sites, despite the species having high dispersal ability. Various factors have been proposed to explain these patterns, highlighting the need to consider individual species biology and population history when trying to make sense of results.

This study supports observations from various other studies that the genetic mobility of a species (related to dispersal mode) is important in determining patterns of genetic diversity within and between populations. The general rule is that as genetic mobility increases, genetic diversity within populations also increases and structuring between populations decreases.

This study has been a preliminary survey of the patterns of genetic diversity on the Krakatau islands and has provided an interesting background for further, more extensive research on the processes important for colonization on the islands.

Appendix I: A description of study site localities.

Region	Site Name	Lat/Long	Description
Krakatau	Rakata	S06° 08.825, E105°27.979	Collections made on the coast predominantly in the area of Owl Bay and Handels Bay
	Panjang	S06° 05.263, E105°27.886	North-eastern coastal flats
	Sertung	S06° 04.590, E105°23.967	Coastal flats on northern tip of island
Java	Carita	S06° 16.846, E105°51.490	Path to Curug Kendang Waterfall, Taman Wisata Alam Forest Reserve, Carita
	Popoli Island	S06° 23.337, E105°49.084	Small island off coast of Lampai (lat/long is for Lampai)
	Taman Jaya		Forests and fields directly behind village, Ujung Kulon National Park
	Peucang Island	S06° 44.701, E105.15.735	Center of island, north and northeastern coastal flats
	Labuan	S06° 11.246, E106.08.617	Along road between Labuan and Serang near Cadas Sari, Pandeglang
Sumatra	Bogor		Town, between railway station and Ramayana market
	Rajabasa	S05° 45.170, E105°38.037	Coastal collections made just north of Beralung Simpung hot springs, coastal road south of Kalianda
		S05° 46.685, E105°38.096	Collections made along a stream and near a waterfall inland from the site above
	Kota Agung		Way Lalaan Reserve next to road, less than 10 km east of Kota Agung on road to Bandar Lampung.
	Lampung		Nature reserve approximately 10 km south of Bandar Lampung on coastal road east of Gunung Ratai
	Sebesi Island		South eastern slopes near Pak Babats cottage, overlooking the Krakatau islands.
	Tampang		Coastal flats at Tampang village, Bukit Barisan Selatan National Park

Appendix II: The fruit of some of the species studied in this thesis. *Dysoxylum gaudichaudianum* fruits (a) are approximately 25 mm in diameter and contain bright red seeds (10mm in diameter) with a fleshy aril that is attractive to birds. Seeds of *Oroxylum indicum* (b) are flat disks approximately 15 mm broad, are surrounded by a large papyrus that is up to 150 mm wide and are dispersed by wind and water. The seeds of *Ficus* species examined in this study are small (<2 mm) and are surrounded by a fleshy syconium (fruit) that is attractive to bats and birds. Fruits of *F. fulva* (c) and *F. septica* (d) are approximately 25 mm in diameter and are positioned as pairs in the axil of terminal leaves. In *F. pubinervis* (e) the fruits are somewhat smaller (10 mm). Fruits of *F. fistulosa* (f) are positioned on short branchlets along the trunk (cauliflory) and are approximately 15 mm in diameter.





Genetic differentiation in five tree species in sites from Java, Sumatra and Krakatau: inferences about gene flow between isolated populations.

Abstract

Patterns of genetic differentiation between populations are examined to gain insight into colonization processes and levels of gene flow in five tree species, *Ficus fulva*, *F. fistulosa*, *F. pubinervis*, *Dysoxylum gaudichaudianum* and *Oroxylum indicum* on the Krakatau islands and surrounding areas using Amplified Fragment Length Polymorphism (AFLP). If populations exist in an equilibrium between migration and genetic drift, and distance is the only factor limiting dispersal, then a positive and monotonic relationship should exist between genetic differentiation (F_{ST}) and geographical distance (isolation-by-distance) (Slatkin, 1993; Hutchison and Templeton, 1999). A lack of correlation indicates that either migration or drift is dominant in determining genetic structure and that distance either is not restricting dispersal (no population differentiation) or some barrier to dispersal exists (high differentiation). F_{ST} is used as a comparative estimate of gene flow in this study. In order to identify possible source regions for Krakatau colonists, relationships between populations were examined using principal coordinate analysis.

Low values of population differentiation in *Ficus* species (overall F_{ST} of 0.046 in *F. fulva*, 0.049 in *F. fistulosa* and 0.052 in *F. pubinervis*) indicate high levels of gene flow and are reflective of the species' high dispersal capabilities. Non-significant levels of differentiation were observed between some island and mainland populations thus indicating panmixia between these sites. High levels of gene flow indicate that the distance to the Krakatau islands was not greatly limiting migration in these species. Isolation-by-distance was observed in *F. fistulosa* and *F. fulva*. Patterns of ordination show no differentiation between sites, further supporting the idea that gene flow is extensive. Therefore, colonists for the new Krakatau populations of *F. fistulosa* and *F. fulva* were likely drawn from an extensive area; from both landmasses of Java and Sumatra and possibly from other islands in the Sunda Strait region. Genetic differentiation in *F. pubinervis* was not associated with distance. Small population effects and the contribution of a small number of populations to

the migrant pool are suggested as factors determining current patterns of genetic differentiation.

Isolation-by-distance was demonstrated in *D. gaudichaudianum*, but because gene flow is more restricted in this species (overall $F_{ST}=0.163$), the nearest landmasses are contributing significantly more to the gene pools of the Krakatau island populations. Due to the East-West positioning of the islands, the populations of Java and Sumatra have varying levels of influence on the gene pools of the different islands. Sertung is more highly related to populations in Sumatra than those of the neighboring Krakatau islands. Panjang, further east, demonstrates higher levels of differentiation to Sumatra and lower to Java relative to that of Sertung.

High overall population differentiation ($F_{ST}=0.213$) and no correlation between genetic differentiation and geographical distance was observed in *O. indicum*. Gene flow is greatly restricted over the distances in this study due to the species' poor dispersal capabilities. High differentiation between Krakatau and the other sites indicates that genetic drift plays an important role in structuring genetic differentiation between populations and formation of colonies. Due to this drift no patterns of relationship between the Krakatau population and other populations could be found.

The importance of stepping-stone islands in the Sunda Strait for mediating dispersal to the Krakatau islands is discussed.

Introduction

When examining the establishment of new populations after colonization it is of interest to determine which factors have been important during colonization and what effect there has been in the structuring of genetic variation. Various questions can be asked. What was the relative contribution of migration and genetic drift in structuring the new populations? At what distance does dispersal to new sites become limited? Were there any other barriers to dispersal? From where did new colonists originate and how extensive were migrant numbers? Given the current rate of destruction and fragmentation of natural habitats due to human activities, it is becoming increasingly important to understand which factors are important in colonization and maintenance of gene flow between sites. Islands provide a simplified system in which to tease apart some of these processes.

Various models have been developed that evaluate processes that are important in colonization and their effect on genetic structure. Wright (1931), in the island model, demonstrated that genetic differentiation (F_{ST}) between populations is a simple function, $F_{ST}=1/(4Nm+1)$, of the gain of genetic variation through migration (m , the migration rate) and the loss of variation due to random events (genetic drift, which is proportional to effective population size, N_e). Wright (1943) later refined the island model by including the effect of distance in structuring genetic variation between populations. Dispersal in all species is to some extent limited by distance. Therefore, neighboring populations contribute significantly more to each other's gene pools than to those further away, with differentiation between sites being an increasing function of distance. This effect has been termed isolation-by-distance (Wright, 1943). Slatkin (1977) outlined the Propagule and Migrant Pool models to illustrate the extreme ends of colonization patterns and their effect on population genetic structure. In the propagule model, the founding event (small population size during colonization) is severe and colonists are drawn from a single source. This results in increased differentiation between sites. The other extreme is the migrant model (Slatkin, 1977), where colonists are drawn from many populations, with the effect of decreasing overall differentiation between populations. Therefore, it is assumed that migration from a limited number of source pools will create structure between populations and stochasticity will lead to a breakdown in patterns of relationship between populations.

Examining the spatial structuring of genetic differentiation throughout the distribution of a species can provide important insights into the relative importance of distance, migration and genetic drift (Slatkin, 1993; Hutchison and Templeton, 1999). Slatkin (1993) applied Wright's (1943) principle of isolation-by-distance to demonstrate that when groups of

populations reach an equilibrium between drift and migration a positive and monotonic correlation should exist between genetic differentiation and geographical distance. When not at equilibrium, the relative effects of drift or migration become dominant, and thus no correlation will occur (Hutchison and Templeton, 1999). Where distance does not limit dispersal, no patterns will exist, as migration is extensive and populations will not be differentiated (low F_{ST}). On the other hand, when distance or some other barrier is very limiting, dispersal rates will be greatly reduced and differentiation increases (high F_{ST}) due to genetic drift (Hutchison and Templeton, 1999).

Various empirical studies have used the above principle to determine past patterns of dispersal within a species and identify which factors lead to the breakdown of gene flow. Hutchison and Templeton (1999) demonstrated that the relationship between genetic structure and geographic distance in eastern collared lizards (*Crotaphytus collaris collaris*, eastern USA) can vary over the range of the species as the relative extent of drift and dispersal varies depending on local regional conditions. In a rare alpine herb, *Eryngium alpinum*, high genetic differentiation ($F_{ST}=0.40$) and the absence of a correlation between distance and genetic structure between alpine valley populations indicate that dispersal between alpine valleys is poor and structure between populations is determined greatly by drift (Gaudeul *et al.*, 2000). However, isolation-by-distance occurs within valleys, indicating that gene flow is not limited over shorter distances. Giles and Goudet (1997) found that genetic structure in *Silene dioica* in some parts of the Skeppsvik Archipelago, Sweden, is determined by factors that affected dispersal, such as wind-direction and sea currents.

In order to estimate the amount of gene flow between populations, Wright's (1931) equation, $F_{ST}=1/(4Nm+1)$, is frequently used to find the number of migrants per generation (Nm), as F_{ST} can be easily estimated from population genetic data. However, there are various assumptions and problems in this equation that limit its use (Whitlock and McCauley, 1999). First, one simplifying and unrealistic assumption of this model is that all populations in a region are equally likely to contribute to each other's gene pool regardless of spatial scale. Further, the model assumes that populations are at equilibrium between migration and drift and that these processes are constant between all populations throughout the range of a species. Therefore, different processes cannot be functioning at different spatial scales of a species. Another problem with the use of this equation is that it is difficult to determine the relative importance of migration and drift as these factors are confounded in the product Nm . Further, inaccuracies in measuring differentiation (F_{ST}) can lead to a disproportionate effect on the estimated value of Nm , as the relationship between the two is not linear. Whitlock and

McCauley (1999) advise that in most cases genetic differentiation as measured by F_{ST} will be an adequate measure of gene flow. Qualitative estimates of gene flow can be determined using rules-of-thumb suggested by Hartl and Clark (1989): when F_{ST} is less than 0.05 gene flow is high, gene flow is more restricted at values of F_{ST} between 0.05 and 0.15, greatly restricted between than 0.15 and 0.25 and extremely restricted when F_{ST} is greater than 0.25.

The Krakatau islands in Indonesia are a rare example of a well-documented case study of ecosystem destruction and colonization in an island system. The vegetation of the Krakatau islands, Rakata, Sertung and Panjang for all practical purposes was completely destroyed by volcanic eruptions in August 1883 (Docters van Leeuwen, 1936). Since then, vegetation has been returning to the islands in a successional manner and the islands are now covered in relatively species-poor secondary monsoonal forest (Whittaker *et al.*, 1989). A fourth volcanic island, Anak Krakatau, was formed in 1930 (Docters van Leeuwen, 1936). This island exists at an earlier successional state due to volcanic disturbance (Partomihardjo *et al.*, 1992) and has had a great effect on succession on other Krakatau islands (Schmitt and Partomihardjo, 1997; Schmitt and Whittaker, 1998; Whittaker *et al.*, 1992b).

The order of plant species return (Docters van Leeuwen, 1936; Whittaker *et al.*, 1989) and the successional processes in which plant communities have restructured on Krakatau (Whittaker *et al.*, 1989; Whittaker and Jones, 1994a) have been well-studied. However, little is known about the origins of new populations and how the distance to the islands and their positioning has affected population restructuring. The positioning and distance of the islands relative to other larger landmasses and dispersal characteristics of individual species will be important in determining patterns of colonization.

The Krakatau islands are located in the Sunda Strait, approximately midway between Sumatra (to the North and West) and Java (to the East). The nearest points of these landmasses from the Krakatau islands are approximately 31.5 and 40.5 km distant, respectively (Table 1.1, Whittaker and Jones, 1994b). Various authors have considered whether the distance between Krakatau and mainland sites is limiting to dispersal and question whether dispersal would be further facilitated by islands acting as stepping-stones. Several such small islands lay between the Sumatra coast and the Krakatau islands, with Sebesi island to the north of Krakatau considered one of the most important (Dammerman, 1948; Whittaker *et al.*, 1992a; Thornton, 1996). Despite considerable damage occurring during the 1883 eruptions (over one meter of ash was deposited and forest defoliated (Verbeek, 1885)) Sebesi was less severely affected than the Krakatau islands. Within nine months of the eruptions plants were re-shooting from underground parts/seeds (Cotteau,

1886) and by 1921 floral communities were much further advanced than those on the Krakatau islands (Docters van Leeuwen, 1923, Dammerman 1948).

Considering the theories of Wright and Slatkin mentioned above, three scenarios for the colonization patterns of tree species on the Krakatau islands, depending on species dispersal ability, can be proposed. First, in highly mobile species, the distance between Krakatau and Java and Sumatra will not be limiting and therefore both landmasses will contribute colonists with about equal frequency (provided suitable source forests are present in each region). Patterns of colonization between the islands should be similar if habitats are available on all islands. Population genetic diversity should remain high and differentiation between populations be low (Slatkin's 1977 Migrant Pool model). Second, in species that are less mobile, distance will become more important and the nearest landmass will contribute colonists at a higher frequency than those further away. Due to chance events, colonization histories between the islands may vary. Given the lower dispersal ability, differentiation will be greater between populations and greater genetic variation will be observed between new populations. Third, in species where dispersal is highly restrictive, chance events during founding and reduced gene flow will result in high population differentiation (Slatkin's 1977 Propagule Pool model).

The objective of this chapter is to determine which factors have been important throughout colonization in some tree species on the Krakatau islands by examining patterns in the structuring of genetic polymorphisms. Amplified Fragment Length Polymorphisms (AFLP) are examined in populations of five tree species, *Ficus fulva*, *F. fistulosa*, *F. pubinervis*, *Dysoxylum gaudichaudianum*, and *Oroxylum indicum* on the Krakatau islands and surrounding areas in the Sunda Strait region. These species can be divided into three different dispersal groups (Chapter 2), where *Ficus* species have the highest dispersal ability and *O. indicum* the lowest. In this study, levels of gene flow between populations (measured as genetic differentiation (F_{ST})) are determined and discussed in the context of the species' dispersal characteristics. Then, patterns of correlation between differentiation and geographical distance are examined to determine if distance is important in structuring genetic diversity (isolation-by-distance). This will provide insight into the relative contributions of gene flow and drift in the newly structured populations. Finally, multivariate patterns of relationships and levels of differentiation between the Krakatau islands, other islands in the Sunda Strait and mainland sites are examined, to identify possible source regions of colonists and the importance of stepping-stone islands for dispersal of species to Krakatau.

Materials and Methods

Study Sites and Sampling

Analyses in this chapter use the same samples and data set as in Chapter 2. Refer to that chapter for a more extensive methodological outline. A map of the Sunda Strait region, the Krakatau islands and collection sites is given in Figure 2.1. Sample sizes and collection sites for each species are found in Table 3.1. A minimum of four populations per species were sampled, and at least one from each of Krakatau, Java and Sumatra. Sampling was limited by the local distribution of the species, the availability of the species at a site and the time available for sampling. For details about material preservation, refer to Chapter 2.

Study Species

The five tree species, *Ficus fulva*, *F. fistulosa*, *F. pubinervis*, *Dysoxylum gaudichaudianum*, and *Oroxylum indicum* used in this study were selected to represent species with a variety of arrival times on the Krakatau islands, dispersal abilities and life-histories, and can be divided into three distinct genetic mobility groups (Table 2.5).

The species with greatest genetic mobility, *F. fulva*, *F. fistulosa* and *F. pubinervis* all have small (less than 2 mm) endozoochorous seeds and numerous probable dispersal agents to the islands (Thornton, *et al.*, 1996; Whittaker and Jones, 1994b). The species were first recorded on the Krakatau islands in 1897, 1905 and 1922, respectively (see Docters van Leeuwen, 1936; Whittaker *et al.*, 1989). *Ficus* species are known to have extensive seed (Shilton *et al.*, 1999) and pollen dispersal (Nason *et al.*, 1996) and hence, high genetic mobility (Chapter, 2). All *Ficus* species are outcrossing due to separation of sexes (dioecy, *F. fulva* and *F. fistulosa*) or synchronous crop production within monoecious species (*F. pubinervis*) (Ramirez, 1970). The *Ficus* species in this study are common on the older Krakatau islands and have large population sizes (Chapter 2).

Dysoxylum gaudichaudianum also exhibits endozoochorous dispersal but has a large seed measuring approximately 10 mm in diameter, with only large pigeons (*Ducula*) as the likely long-distance dispersal agent to the islands (Whittaker and Turner, 1994). The genus is predominantly dioecious (Mabberley *et al.*, 1995) and dioecy is the likely breeding system of *D. gaudichaudianum* (Parrish, unpub. data). Pollination is most likely performed by non-specific insects such as moths (Mabberley *et al.*, 1995). The species was first recorded on Sertung in 1932 (Docters van Leeuwen, 1936) and is now a common canopy dominant on both Sertung and Panjang (Whittaker *et al.*, 1989). While widespread and common over a

large part of the Rakata lowland forest, *D. gaudichaudianum* does not reach the same canopy or numerical dominance as the other islands (Schmitt and Whittaker, 1998). *Oroxylum indicum* is a late arriver on to Krakatau and was first recorded on Rakata in the 1980s (Partomihardjo, 1995). The late arrival of the species is most likely due to its limited dispersal ability by water and wind-scattering (Ridley, 1930). Flowers are nocturnal and are pollinated by non-species specific bats (Start and Marshall, 1976; Corner, 1988).

Oroxylum indicum is still only known on the Krakatau islands from one small population on Rakata.

All species in this study are common and widespread throughout areas of south-east Asia and can be termed as early colonizers. A more extensive outline of species biology and colonization of the Krakatau islands has been given in Chapter 2.

AFLP procedure and Protocols

Genetic data were obtained using the AFLP procedure described by Vos *et al.* (1995) using *EcoRI* and *MseI* digestion enzymes and two to three selective nucleotides. The primer selection procedure, exact AFLP protocols and genetic diversity results used in this study have been set out in Chapter 2.

Data Analysis

The population genetic structure of the AFLP data was analyzed with the AMOVA (Analysis of Molecular Variance) procedure of the Arlequin software package (Schneider *et al.*, 2000). In this procedure summed pair-wise differences between AFLP genotypes are calculated, by assigning a 1 when the alleles of the *i*-th locus were identical and a 0 when otherwise. The AMOVA procedure partitions the genotypic variance, resulting in an F_{ST} which refers to the correlation of genotypes rather than to the correlation of genes as in the classical F_{ST} . Nevertheless, assuming that the mating pattern is identical in all studied populations, this measure is informative with respect to the differentiation between populations.

Pairwise F_{ST} for each population combination and overall differentiation within a species were determined. Significance of F_{ST} values was calculated from 100 permutations using the non-parametric permutation approach of Excoffier *et al.* (1992). Because multiple pairwise F_{ST} comparisons were made, a Bonferoni correction, based on Holmes' sequential comparisons test (Rice, 1989), was applied to reduce the experimentwise error rate. Population pairwise F_{ST} was then plotted against geographical distance between sites. The geographical distance was calculated from the Tactical Pilotage Chart M-10C, first edition

(1971), Ministry of Defence, United Kingdom, Scale: 1:500,000 map. The significance of the association between genetic differentiation and geographical distance in each species was determined using a Mantel Test (Arlequin, vers. 2).

The patterns of genetic relationship within and between populations of species were further investigated using principal coordinate analysis (PCoA). A pair-wise similarity matrix was constructed by counting the number of shared phenotypic states (band absence and presence) between individuals. PCoA analysis was undertaken using CANOCO, ver. 4 (Ter Braak and Šmilauer, 1998). Scatterplots were constructed from the first two ordination axes to inspect the relatedness among populations.

Results

Overall Population Differentiation

Values of overall F_{ST} , between population pairwise F_{ST} and geographical distance for all species are shown in Table 3.1. Maps illustrating F_{ST} values between populations for each species are shown in Figure 3.1. Species dispersal ability affects the number of migrants between populations and therefore plays an important role in determining gene flow and structuring genetic variation between populations. Species with high inferred dispersal capability, *F. fulva*, *F. fistulosa* and *F. pubinervis*, have low differentiation (overall F_{ST} of 0.046, 0.049 and 0.052, respectively) and hence high inferred gene flow between populations. Gene flow is interpreted to be more restricted in the less mobile *D. gaudichaudianum* ($F_{ST}=0.163$) and highly restricted in the wind and water-scattered *O. indicum* ($F_{ST}=0.213$).

Between Population Differentiation in F. fulva and F. fistulosa

Ficus fulva and *F. fistulosa* show very similar patterns of differentiation and relationships between populations. Pairwise F_{ST} values between populations are low and range narrowly from -0.003 and 0.098 and -0.007 to 0.095, respectively, thus demonstrating high levels of gene flow between populations of these species over the entire area covered in this study (distances of 4 to 164 km). Non-significant or negative values of F_{ST} indicate panmixia between populations. Comparisons between populations of *F. fulva* on the Krakatau islands, Anak Krakatau, Panjang and Rakata demonstrated no significant differentiation between these populations, indicating that these islands are panmictic. Higher values (though not greatly different) were observed between the more distant mainland sites and between mainland and island sites. In *F. fistulosa*, no significant difference was observed between the

Table 3.1: Genetic differentiation (F_{ST}) and geographical distance (km) matrices for the five study species. Lower left triangle: genetic distance. Upper right triangle: geographic distance. F_{ST} values that are significantly different at least at the $P=0.05$ level after Bonferoni correction are indicated in bold (calculated from 100 permutations). Also shown is the F_{ST} as calculated over all populations together. Regions are given as K(rakatau), J(ava) and S(umatra). Collection sites and sample sizes are given for each species.

<i>F. fulva</i>		<i>Overall $F_{ST}=0.046$</i>						
		Anak	Panj	Rak	Lab	Raj		
K	Anak (16)	0	4.0	6.0	79.0	40.0		
K	Panjang (14)	0.015	0	5.7	76.5	37.5		
K	Rakata (16)	-0.001	-0.003	0	75.0	43.0		
J	Labuan (14)	0.096	0.057	0.098	0	82.5		
S	Rajabasa (13)	0.045	0.035	0.063	0.058	0		
<i>F. fistulosa</i>		<i>Overall $F_{ST}=0.049$</i>						
		Sert	Car	Raj	Lamp	K. A		
K	Sertung (15)	0	55.0	39.5	66.5	108.0		
J	Carita (13)	0.019	0	67.0	111.0	164.0		
S	Rajabasa (13)	0.025	0.047	0	47.0	111.0		
S	Lampung (12)	0.067	0.095	-0.007	0	70.0		
S	K. Agung (11)	0.081	0.086	0.020	0.052	0		
<i>F. pubinervis</i>		<i>Overall $F_{ST}=0.052$ (including Rajabasa, 0.129)</i>						
		Sert	Car	T. J	Peuc	Raj		
K	Sertung (15)	0	55.0	81.0	75.0	39.5		
J	Carita (10)	0.035	0	65.0	79.0	67.0		
J	T. Jaya (15)	0.019	0.071	0	27.5	114.0		
J	Peucang (15)	0.056	0.086	0.056	0	113.0		
S	Rajabasa (7)	0.286	0.355	0.273	0.318	0		
<i>D. gaudichaudianum</i>		<i>Overall $F_{ST}=0.163$</i>						
		Rak	Panj	Sert	Pop	Raj	Seb	Tamp
K	Rakata (16)	0	5.7	9.5	48.0	43.0	19.5	90.5
K	Panjang (16)	0.123	0	7.0	52.5	37.5	14.0	87.5
K	Sertung (17)	0.073	0.100	0	57.0	39.5	16.0	82.5
J	Popoli (16)	0.179	0.157	0.237	0	73.5	59.0	136.0
S	Rajabasa (14)	0.168	0.112	0.107	0.229	0	24.5	105.0
S	Sebesi (6)	0.100	0.096	0.043	0.204	0.066	0	89.0
S	Tampang (15)	0.250	0.134	0.173	0.254	0.195	0.225	0
<i>O. indicum</i>		<i>Overall $F_{ST}=0.213$</i>						
		Rak	TJ	Raj	Seb			
K	Rakata (15)	0	73.0	43.0	19.5			
J	T. Jaya (14)	0.231	0	114.0	92.5			
S	Rajabasa (13)	0.230	0.239	0	24.5			
S	Sebesi (14)	0.222	0.261	0.074	0			

Krakatau population Sertung and Carita and Rajabasa (55 and 39.5 km, respectively). Nor was any significant difference observed between a number of mainland populations, some of which were 111 km distant. Comparisons between the Krakatau islands could not be made in this species as only one Krakatau population was analyzed.

For both species, the correlation between genetic differentiation and geographical distance (Figure 3.2) was positive and significant (in *F. fulva*, Mantel test, $r_m=0.886$, $P=0.008$; *F. fistulosa*, Mantel test, $r_m=0.667$, $P=0.036$).

Ordination patterns in both *F. fulva* and *F. fistulosa* show no regional differentiation between populations from Krakatau, Java and Sumatra, with all populations forming a single cloud of samples (Figure 3.3). This further supports the low levels of differentiation observed in F_{ST} values and that gene flow is extensive over the scale of this study. The lack of differentiation observed between mainland and island populations indicates that the islands are not more closely related to any one region.

Between Population Differentiation in F. pubinervis

Quite different patterns were observed in *F. pubinervis*. In all populations except one, *F. pubinervis* has low levels of F_{ST} (0.019 to 0.086) and therefore like the other *Ficus* species in this study has high levels of gene flow between populations (Table 3.1). However, the one population from Sumatra in this study, Rajabasa, shows very high levels of differentiation from all other populations (F_{ST} ranges from 0.273 to 0.355). This difference is likely due to small sample number, small population size and inbreeding with subsequent low levels of genetic diversity (Chapter 2). High differentiation due to geographical isolation is ruled out as no unique AFLP-bands are observed (Chapter 2). The Krakatau population, Sertung, showed no significant differentiation from Carita and Taman Jaya (Java) despite being 55 and 81 km distant, respectively. Only one Krakatau population was used for analysis.

Due to the unusually high levels of differentiation observed between the Rajabasa population and all other populations, tests for the correlation between distance and genetic structure were performed including and excluding this population (Figure 3.2). No significant correlation was observed in either (Mantel test, $r_m=0.340$, $P=0.137$, including Rajabasa; $r_m=0.014$, $P=0.498$, excluding Rajabasa), thus indicating that isolation-by-distance does not operate between the sites in this study.

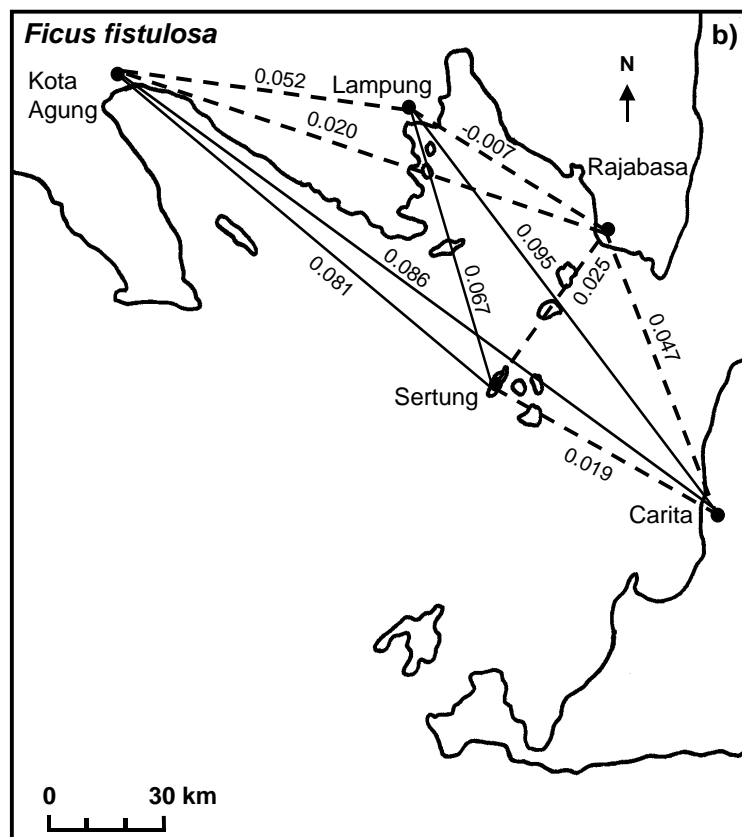
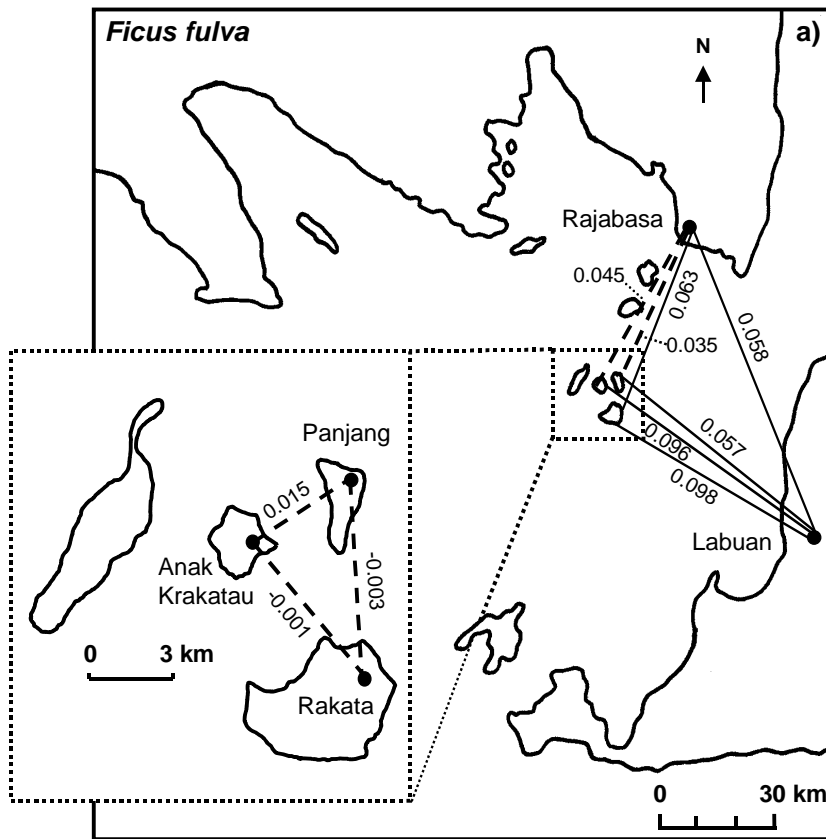
Differentiation between Rajabasa (Sumatra) and all other *F. pubinervis* populations was also observed in PCoA analysis (Figure 3.3). This differentiation is unlikely to be due to geographical division for reasons previously mentioned.

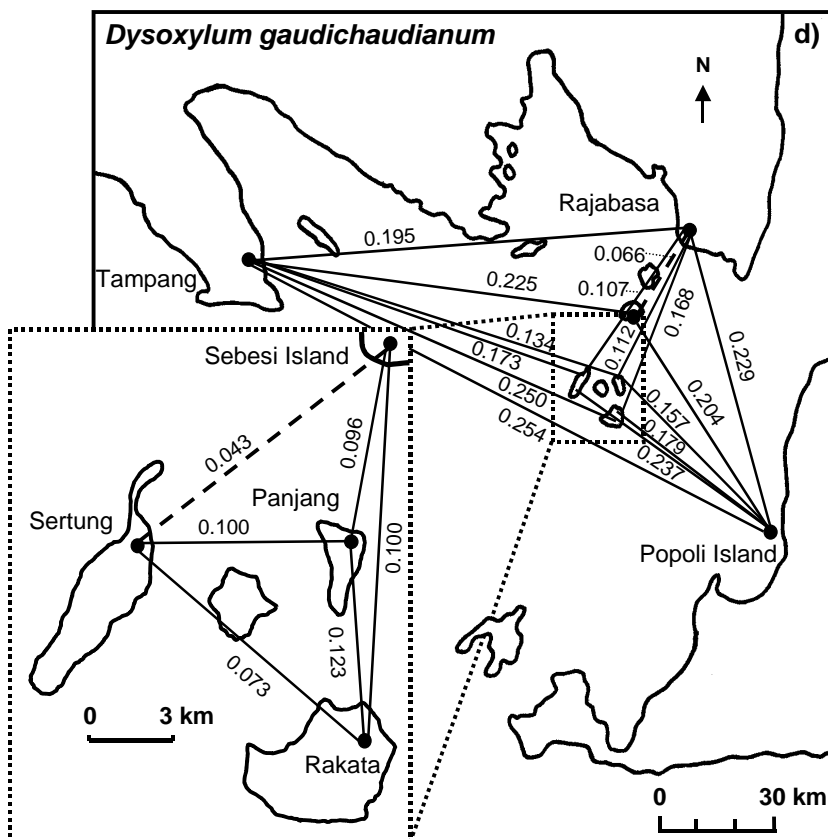
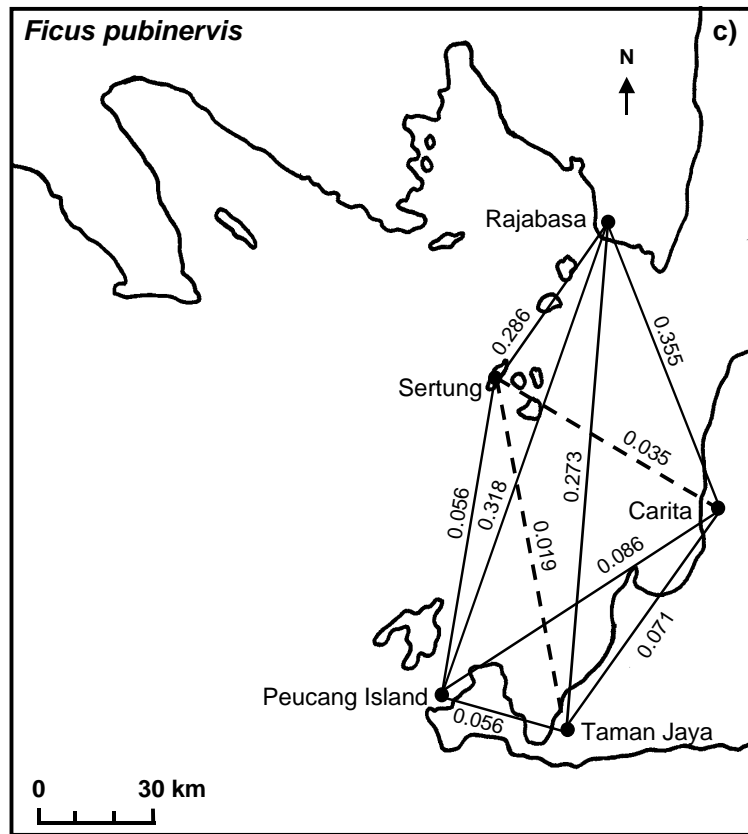
Between Population Differentiation in D. gaudichaudianum

Overall differentiation between populations of *D. gaudichaudianum* ($F_{ST}=0.163$) is considerably greater than that for the *Ficus* species, thus indicating considerably more restriction in gene flow in this species (Table 3.1). Population pairwise F_{ST} values between the Krakatau island populations and mainland ones ranged widely from 0.043 to 0.254; all except two showed significant levels of differentiation. Levels of differentiation between the individual Krakatau islands (Sertung and Rakata, $F_{ST}=0.073$; Panjang and Sertung, $F_{ST}=0.100$; Rakata and Panjang, $F_{ST}=0.123$) in this species were greater than those observed in *F. fulva*. Non-significant differentiation was observed between Sertung and Sebesi Island (the latter lies 16 km to the north of Sertung) and between Sebesi and Rajabasa (24.5 km to the north of Sebesi) despite these sites being further apart than the Krakatau islands. Sebesi showed relatively low levels of differentiation from Panjang ($F_{ST}=0.096$) and Rakata ($F_{ST}=0.100$). On average, Rakata demonstrated the highest differentiation from mainland sites and Panjang the least of all Krakatau islands.

There was a significant and positive correlation between genetic differentiation and geographical separation between populations (Mantel test, $r_m=0.806$, $P=0.004$) (Figure 3.2), indicating that distance is important in structuring genetic variation.

Populations of *D. gaudichaudianum* from Java and Sumatra form distinct ordinal clusters (Figure 3.3), hence demonstrating differentiation between the two regions. It must be noted that only one population from Java was analyzed. The Krakatau populations tend to cluster only with the Sumatra populations, indicating that these populations are more closely related to Sumatran populations than to that from Java. However, Panjang has some overlap with the Java population Popoli and shows the greatest spread of all the Krakatau populations.





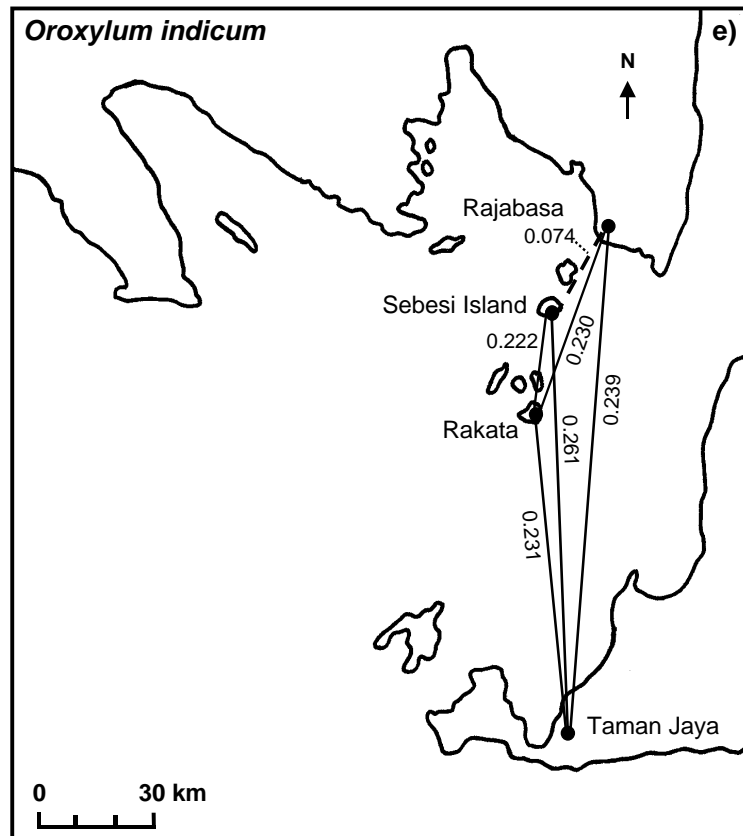


Figure 3.1: Maps illustrating the genetic differentiation (F_{ST}) between populations for each study species. Hashed lines indicate that the two linked populations are not genetically differentiated.

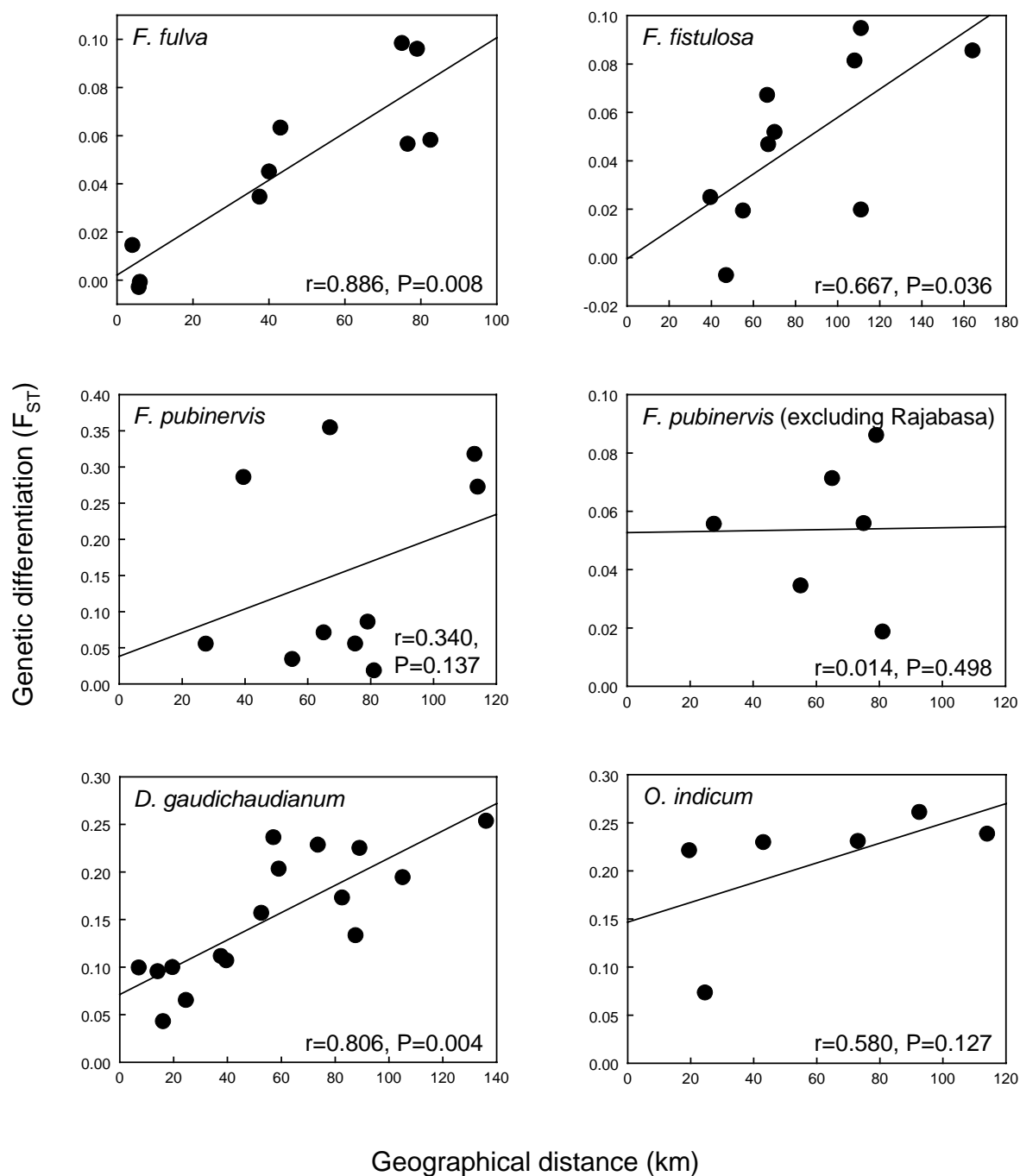


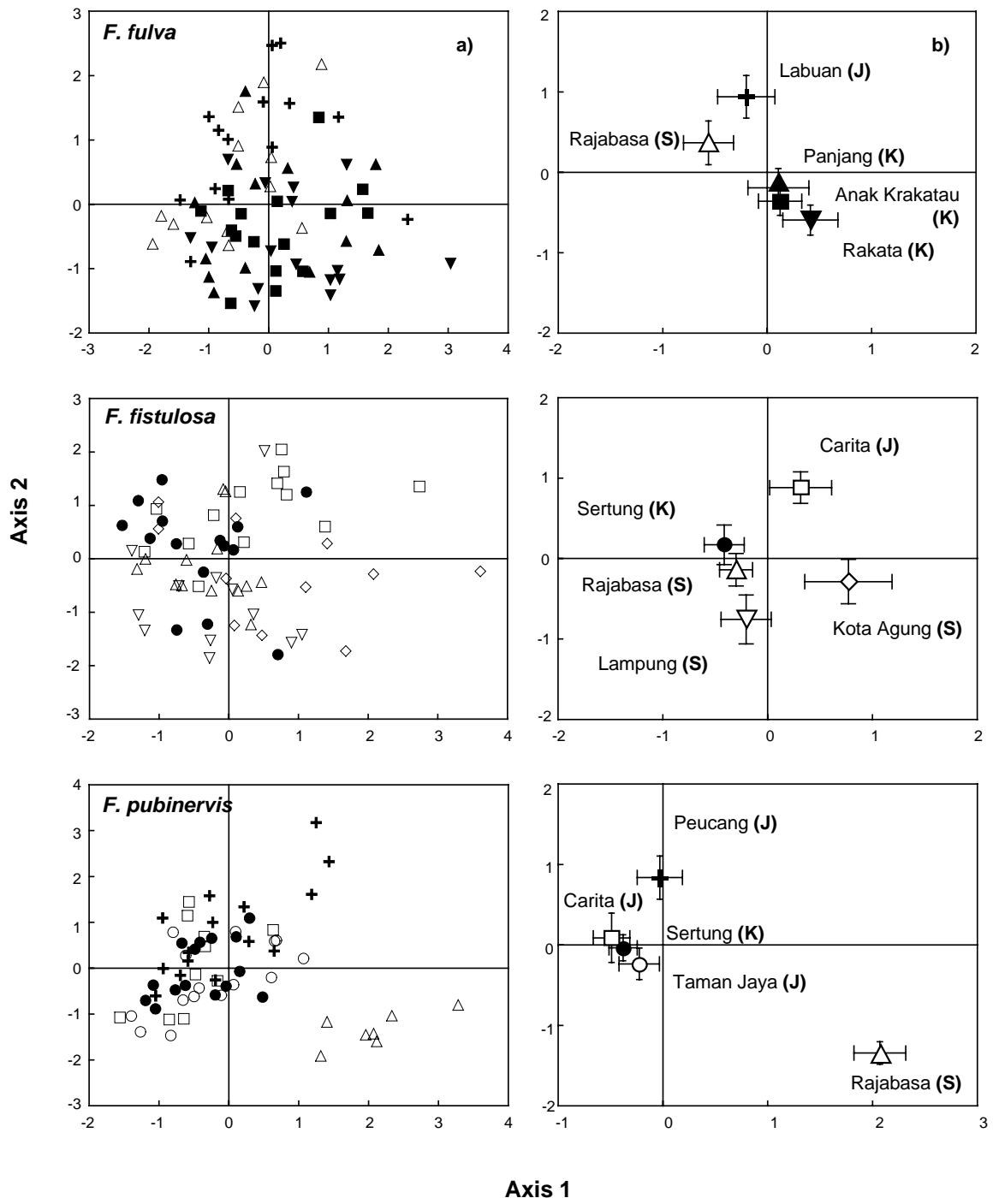
Figure 3.2: Correlations between geographical distance (km) and genetic differentiation (F_{ST}) across populations for five different species. The significance of the correlation between the genetic and geographic distance matrices, P is determined by a Mantel test (Arlequin, vers. 2, Schneider *et al.*, 2000). Note, the regression line is added as a visual cue only as regression cannot be performed on non-independent data such as used here.

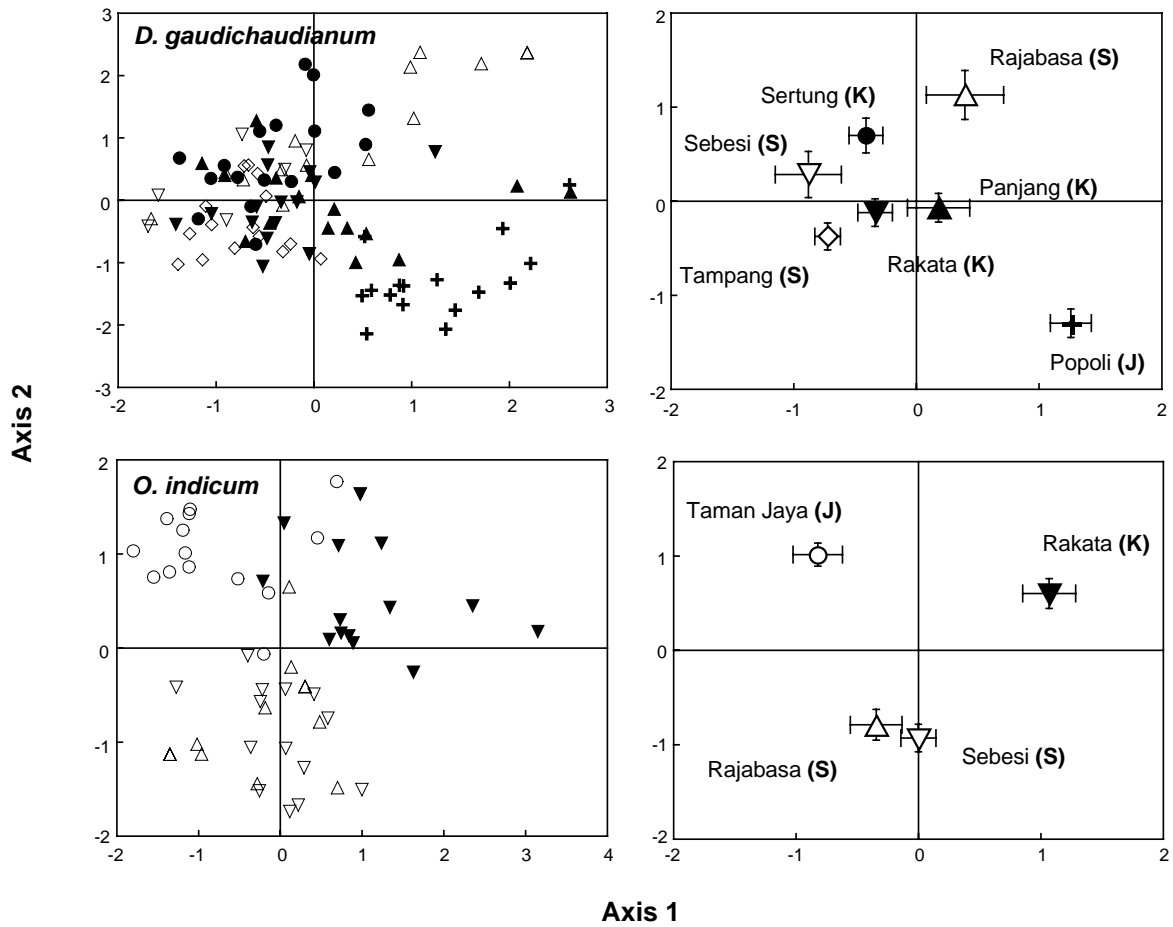
Between Population Differentiation in *O. indicum*

Of all species in this study *O. indicum* has the highest overall levels of population differentiation ($F_{ST}=0.213$), indicating more severe restrictions in gene flow which reflects the poor dispersal ability of this species (Table 3.1). Pairwise population F_{ST} values range from 0.222 to 0.261, with one exception, the pairing of Sebesi and Rajabasa which showed no significant differentiation ($F_{ST}=0.074$). Rakata shows no particular relation to any mainland populations and exhibits similar levels of genetic differentiation with respect to all other sites. All pairwise comparisons between Rakata and all other sites show significant differentiation between populations. *Oroxylum indicum* is found in only one site on the Krakatau islands, and therefore, comparisons could not be made between islands.

No significant correlation was observed between genetic differentiation and geographical separation ($r_m=0.580$, $P=0.127$) (Figure 3.2). Populations of *O. indicum* (Figure 3.3) from Java, Sumatra and Krakatau occupy distinct ordinal space, thus demonstrating regional differentiation between the populations sampled from these three areas. Some scatter points of Rakata, however, lie within the space occupied by the Taman Jaya population (Java). The Rakata population cluster is more scattered than any mainland site.

The Sumatran population, Rajabasa and the nearby island, Sebesi cluster tightly together (they have overlapping standard errors in both axes), supporting the low genetic differentiation and hence high relatedness observed between the populations (F_{ST} not significant).





Krakatau	Sumatra	Java
▼ Rakata	△ Rajabasa	+ Popoli, Peucang and Labuan
■ Anak Krakatau	▽ Sebesi and Lampung	□ Carita
▲ Panjang	◇ Kota Agung and Tampang	○ Taman Jaya
● Sertung		

Figure 3.3: The first two axes of a Principal Coordinate Analysis showing relationships between populations of the five species. a) all individuals included and b) population mean (\pm s.e.). Axis scales are different between species plots. Regions are given as (K)rakatau, (J)ava and (S)umatra.

Discussion

Gene Flow, Patterns of Colonization and Origins of the Krakatau Colonists

Isolation-by-distance was observed in two species with high inferred dispersal ability, *F. fulva* and *F. fistulosa*, indicating that there are no barriers to dispersal throughout the study area and that distance between sites is important in determining genetic structure in these species. Gene flow between populations is extensive over the entire area of this study (Table 3.1 and Figure 3.1) and reflects the high dispersal capabilities of these species. In some cases, island and mainland populations were not significantly differentiated and can therefore be considered panmictic. Some mainland populations of *F. fistulosa* more than 100 kms distant also showed no significant differentiation. High levels of gene flow have been reported over similar distances in the tropical pioneer tree species *Cecropia obtusifolia* (Alvarez-Buylla and Garay, 1994). Ordination analysis also showed no structure between populations over the entire study area. It can be concluded that the distance to the Krakatau islands is not limiting dispersal and within the normal dispersal range of these two *Ficus* species. The ability to form interacting populations over large fragmented areas is of great importance, considering the increasing levels of isolation faced by many *Ficus* populations and plants as a whole (Mawdsley *et al.*, 1998). In *F. fulva* there is no significant inter-island differentiation between the Krakatau islands, indicating that Krakatau populations are panmictic. Given these observations it can be concluded that the number of migrants and source populations providing migrants to the new Krakatau populations were extensive and likely drawn from populations on both Java and Sumatra. Genetic drift had little effect on the gene pool of new populations. This resembles Slatkin's (1977) migrant model of colonization.

Interestingly, a more complicated pattern of colonization appears in *F. pubinervis*. Given the high dispersal ability and gene flow observed in this and other species of *Ficus* no barriers to dispersal would be expected. However, there was no correlation between distance and genetic structure in this species. Regional differentiation between Java and Sumatra, and clustering of the Krakatau population with Java was observed. This would suggest a directional pattern involved in colonization, with colonists to the Krakatau population most likely originating from Java. However, this conclusion must be viewed with caution. The high levels of differentiation observed between the one Sumatran population used in this study (Rajabasa) and all other populations may be due to the effects of small collection sample size, small population size and high levels of relatedness between individual trees at this site rather than regional differentiation (Chapter 2). Increased levels of genetic

differentiation in aging populations heading toward extinction have been reported in *Silene dioica* (Giles and Goudet, 1997). Therefore patterns of differentiation could be obscuring relationships.

Nonetheless, when Rajabasa was excluded from analysis isolation-by-distance was still not observed between the Java and Krakatau populations, further indicating some additional breakdown in dispersal or biased relationship between areas. One factor that may be determining these results is the varying levels of damage suffered by different mainland populations during the Krakatau eruptions. Given the relatively long time to the onset of reproduction in this species (pers. observ.), damaged or reestablishing populations would not be able to contribute to the early Krakatau gene pool. Krakatau shows no significant differentiation from Carita or Taman Jaya. These populations are unlikely to have been damaged by the Krakatau eruptions due to their distance and elevation relative to the coast and may have been a source of colonists for the new island populations. Peucang Island (which is more differentiated from the Krakatau population) is low lying: populations there were greatly effected by ashfall and tsunamis during the Krakatau eruptions (Hommel, 1987) and therefore would likely have been unable to contribute to the new Krakatau gene pool. More extensive sampling from both Java and Sumatra would be instrumental in further trying to elucidate patterns of relationships between populations.

Isolation-by-distance between populations of *D. gaudichaudianum* was demonstrated and indicates that there is no extreme barrier to dispersal for this species throughout the area of study. However, given the more restricted dispersal ability and gene flow observed in this species ($F_{ST}=0.163$) dispersal will become more limited at smaller spatial scales than for *Ficus* species. Therefore, the positioning and proximity of landmasses relative to the Krakatau islands should have a greater influence in structuring genetic variation in populations of *D. gaudichaudianum*. This is essentially what is observed. Krakatau populations have a greater relationship with their nearest neighboring Sumatran populations of Rajabasa and Sebesi than with other more distant mainland populations. In fact, levels of differentiation observed between these two populations and the Krakatau islands are less than those observed between some of the islands themselves.

Unlike in populations of *F. fulva* on Krakatau, *D. gaudichaudianum* shows considerable variation in levels of differentiation within the archipelago and with the mainland sites. This suggests there are differences in patterns of colonization (differences in the source pool of colonists) despite their geographical proximity. These differences are most likely due to positioning of the islands, chance events during colonization and habitat

differences between the islands. For example the high relatedness between Sertung and Sebesi and high differentiation between Sertung and Popoli (Java) reflects the closer positioning of Sertung to Sumatra and a likely dominant role of Sumatra in providing colonists. In contrast Panjang, while being approximately equally close to Sumatra is further east and closer to Java, shows some ordination overlap with Java and has the lowest average differentiation from mainland sites, thus indicating a broader source of colonists.

No correlation between genetic differentiation and geographical distance and high overall differentiation between populations in *O. indicum* indicates that there are barriers to gene flow in this species throughout the study area. Gene flow is restricted in this species over the study area and genetic drift (influenced by the small number of founders and sources) should have been predominant in the development of new populations, resulting in high differentiation. The observed pattern of genetic differentiation in *O. indicum* is consistent with its late arrival at Krakatau. High levels of differentiation between populations have been reported in tropical trees with abiotic dispersal as a whole (Loveless, 1992). Given the restricted dispersal ability in this species, the distance between most study sites (greater than 40 km) is the most likely reason for this breakdown in gene flow.

Interestingly, a non-significant level of differentiation was observed between populations of *O. indicum* from Rajabasa and Sebesi Island. These sites are the most closely situated among the *O. indicum* sites in the study (24.5 km) and at this scale relatively high levels of gene flow may occur. Migration of *O. indicum* to Sebesi may also have been facilitated by humans as this species has some uses with the local people (Parrish, pers. observ.; Corner, 1988) and hence may have been planted on the island. Humans have occupied the island since the 1890s (Thornton, 1996). The most likely route of introduction by people would be from the Rajabasa area as boat access to the islands (via ferry and fishermen) is predominantly from this area. Further sampling between more closely situated populations in both Java and Sumatra would determine the distances at which gene flow is less restricted.

Source regions for colonist of *O. indicum* to the Krakatau island, Rakata, are difficult to elucidate due to high differentiation (F_{ST} ranging from 0.222 to 0.231) and separate clustering of Rakata in ordinal space from all other populations. These patterns most resemble Slatkin's (1977) Propagule Pool model where the small number of migrants and source pools providing these migrants increases differentiation between sites.

Sebesi as a Stepping-Stone

Sebesi Island is the nearest landmass to the Krakatau islands (14 km north of Panjang) and its potential as a stepping-stone for colonists to the Krakatau islands has often been discussed (Dammerman, 1948; Whittaker *et al.*, 1992a; Thornton, 1996). Despite considerable damage occurring during the 1883 eruptions (over one meter of ash was deposited and forest defoliated (Verbeek, 1885)) the island was less severely effected than the Krakatau islands. Within nine months of the eruptions some plants were reestablishing from buried plant parts/seeds (Cotteau, 1886) and by 1921 floral communities were much further advanced than those on the Krakatau islands (Docters van Leeuwen, 1923). However, agriculture has been practiced on Sebesi since the 1890s (Thornton, 1996) and by the 1970s vegetation on Sebesi was confined to the upper regions of the island. Whittaker *et al.* (1992a) was thus led to the conclusion that the island may now be considered a greatly reduced or lost stepping-stone and that patterns of relationship were lost.

The results of this study do not conclusively support the idea of Sebesi as an important stepping-stone in facilitating the colonization of the Krakatau islands. First, in species such as *F. fulva* and *F. fistulosa* gene flow is extensive between mainland and island populations. Therefore, distance does not limit dispersal in these species and the relative contribution from islands such as Sebesi is likely to be minimal. Further, *F. fulva* has never been recorded on Sebesi (Docters van Leeuwen, 1923; Whittaker *et al.*, 1992a; Parrish, pers. observ.). Sebesi may have played a more important role in *D. gaudichaudianum*. The Krakatau islands have low levels of differentiation to and cluster in ordinal space with Sebesi and Rajabasa. Further, differentiation between Sebesi and Sertung (one of the closest Krakatau islands to Sebesi) is lower than that between Sertung and the other Krakatau islands. Therefore, it could be concluded that Sebesi is a plausible stepping-stone for *D. gaudichaudianum* to the islands. However, pattern does not necessarily equal process and the role of Sebesi as a stepping-stone is questionable. It must be noted that *D. gaudichaudianum* was not recorded on Sebesi in expeditions there in 1921 (Docters van Leeuwen, 1923) or 1979 (Whittaker *et al.*, 1992a) and was only found in 1996 (during this study) from a small stand of individuals in a highly disturbed site situated in coconut plantation. The low differentiation observed between Sebesi and Krakatau populations could reflect secondary colonization of Sebesi or high rates of migration from the Krakatau islands.

High differentiation between the Rakata population of *O. indicum* and Sebesi (equal with differentiation to all mainland sites) rules out this island as a stepping-stone for the colonization of Krakatau in this species. This result is interesting given that *O. indicum* was

recorded on Sebesi (Docters van Leeuwen, 1923) approximately 70 years before its identification on Rakata and that there is low differentiation between populations of *O. indicum* on Sebesi and the nearest site on the Sumatra coast, Rajabasa (24.5 km). However, it seems likely that the poor dispersal ability of *O. indicum*, the small size of Sebesi and the distance to Krakatau from there would preclude Sebesi as an important link to dispersal.

Conclusions

Migration and colonization are important processes because they determine the cohesion of the species' gene pool. These processes will become even more important in the future, as habitats become more and more fragmented and the distances between neighboring populations increase due to human activities. Genetically isolated populations may be vulnerable to genetic erosion caused by genetic drift and inbreeding. Whether geographic isolation leads to genetic isolation is a matter of scale, depending on species-specific dispersal capacities. Direct observations of effective dispersal are difficult and are not often made. The documented recolonization of the Krakatau islands by plant species is a rare exception. However, as this study illustrates, indirect inferences about effective dispersal can also be drawn from population genetic analyses with AFLP markers. Such information is important to evaluate the long-term genetic consequences of increasing insularity.

Genetic Evidence for Natural Hybridization Between Species of Dioecious *Ficus* on Island Populations

with M. Kruijt

Abstract

Natural hybrids between *Ficus septica* and two closely related dioecious species, *F. fistulosa* and *F. hispida*, are confirmed in this study using Amplified Fragment Length Polymorphisms (AFLP) and chloroplast DNA markers. *Ficus* species have a highly species-specific pollination mutualism with Agaonid wasps. The identification of cases where breakdown in this sophisticated system occurs and the circumstances under which it happens is, therefore, of interest. Various studies have confirmed that *Ficus* species are able to hybridize and that pollinator-specificity breakdown can occur under certain conditions. This study is the first example where hybrid identity and the presence of hybrids in the natural distribution of parental species for *Ficus* have been confirmed with molecular markers. Hybrid individuals were identified on three island locations in the Sunda Strait region of Indonesia. These findings support Janzens' (1979) hypothesis that breakdown in pollinator specificity is more likely to occur on islands. We hypothesize that hybrid events can occur where the population size of pollinator wasps is small or has been small in one of the parental species. Later generation hybrids were identified, indicating that backcrossing and introgression does occur to some extent and that therefore, hybrids can be fertile. The small number of hybrids found indicates that there is little effect of hybridization on parental species integrity over the study area. Though hybrid individuals are not common, their presence at multiple sites indicates that the hybridization events reported here were not isolated incidences. Chloroplast DNA haplotypes of hybrids were not derived solely from one species, indicating that the seed donor was not of the same parental species in all hybridization events. This study highlights the usefulness of molecular markers for determining whether morphotypes within some *Ficus* species may be the result of hybrid events and whether hybridization is of evolutionary importance in this genus. The predominant character used to identify hybrids, fruiting morphology, needs to be further investigated if this character is to be used reliably to identify particular hybrid morphotypes.

Introduction

Ficus is one of the most specious and diverse genera of woody plants with approximately 750 species spanning the tropical and sub-tropical regions of the world (Berg, 1989). *Ficus* species exhibit two breeding systems, either monoecy (c400 species) or functional dioecy (c350 species) (Berg, 1989). The pollination system of *Ficus* is highly specialized with an obligate mutualism existing between *Ficus* and their pollinator wasps, Agaonidae. With few exceptions each species of *Ficus* is pollinated by a single associated species of Agaonidae (Ramirez, 1970). Flowers are enclosed in a fleshy syconium (fruit structure formed from the receptacle) with pollinators gaining entry to the flowers through a tiny bract-barred opening (ostiole). Such a breeding system allows for high quality pollination services to the plant and supposedly ensures high levels of breeding isolation between closely related co-occurring species. How this specificity is maintained is not well understood but it is thought to involve species-specific attractant volatiles and physical factors (Janzen, 1979).

Despite the strong mutualism between wasp and plant, breakdown in pollinator specificity occasionally occurs. Erroneous pollination events are known from isolated trees that are located at a considerable distance from conspecifics (Ramirez, 1970; Compton, 1990) or from trees that are introduced to an area where they lack their natural pollinator (Ramirez, 1994). Such breakdowns are not common, and are considered the exception and not the rule (Ramirez, 1970). For example, an isolated individual of *F. lutea* in Grahamstown South Africa (500 km outside of its normal range) was visited by two alien pollinator wasp species (Compton, 1990; Ware and Compton, 1992). One of these species reproduced successfully. Seeds were also produced, germinated but died shortly after the cotyledon stage (Compton, 1990). Ramirez (1970) found that 5% of pollinated syconia in an isolated individual of *F. turbinata* in Venezuela were pollinated by an unknown wasp species. The hybrid seeds were not viable.

Hybridization and the production of viable hybrids is known to be possible within *Ficus*. On a number of occasions artificial interspecific hybrids of *Ficus* (in some cases from taxonomically diverse groups) have been formed and viable offspring produced (eg. *Ficus pumila* x *F. carica* producing viable seeds, Condit, 1950; *F. glabrata* x *F. sycomorus*, non-viable seeds and *F. glabrata* x *F. radula*, viable seeds, Ramirez, 1986). However, natural hybrids in *Ficus* are rare (Corner, 1958; Ramirez, 1970) and hybridization is considered of no importance in *Ficus* evolution (Ramirez, 1986). Ramirez (1970) reports a single putative hybrid individual between *F. teurckheimii* x *F. jimenezii* from a natural population, based on

morphological characters. However, Janzen (1979) indicates that hybrid individuals may be overlooked in the field due to high similarity between parental species. Hybrid events have been recorded more frequently in species outside of their natural range (*F. benjamina* (Old World) x *F. padifolia* (New World), Ramirez and Monterro, 1988; hybrid seedlings of *F. religiosa* (Old World) x *F. aurea* (New World) in Miami and *F. septica* (New World) x *F. religiosa* (New World) in Manila, The Phillipines, Ramirez, 1994). To date the presence of natural hybrids has not been genetically ascertained and the frequency of such individuals in natural populations is unknown.

Janzen (1979) predicted that pollinator specificity breakdown could occur in areas where the pollinator of a species has gone extinct or is temporarily absent in an area and/or where reduced host availability selects for latitude in wasp host selection. Such events could occur on islands or in harsh mainland environments (ie. the edge of a species range) for the host species. Therefore, sites such as the Krakatau islands, in Indonesia (Figure 2.1) are suitable places to search for the presence of natural hybrids. The Krakatau islands were denuded of their vegetation during the infamous 1883 volcanic eruptions and thus provide an interesting case for colonization. New colonists must cross a sea barrier of at least 30 km. A variety of dioecious early colonizing *Ficus* species has established in this new environment, in some cases forming sympatric populations. These species had varied establishment times and initial population sizes (Docters van Leeuwen, 1936). Evidence suggests that early *Ficus* populations may have suffered initial pollinator limitation as pollinator populations had not established by the time fruiting commenced (Backer, 1929; Ernst, 1934). Currently, pollinator limitation occurs in newly establishing populations of *Ficus* on Anak Krakatau, the new volcanic island formed in the late 1920s (Compton *et al.*, 1994).

During field work on the Krakatau islands in 1996, Dr Nick Mawdsley (University of Leeds, UK) kindly pointed out two individual trees that were considered morphological hybrids of *F. septica* and *F. fistulosa*. Further field work in 1999 identified a variety of different putative hybrid phenotypes in a number of localities in the Sunda Strait region surrounding Krakatau. Based on their morphology most hybrids appeared to be crosses between *F. septica* and *F. fistulosa*. However, one individual appeared to be a hybrid between *F. septica* and *F. hispida*. In this paper we provide molecular evidence (using Amplified Fragment Length Polymorphism (AFLP) and chloroplast PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)) that confirms hybridization events between *F. septica* and two other species, *F. fistulosa* and *F. hispida*, on the Krakatau islands and two other island localities in the Sunda Strait region in Indonesia.

Molecular analysis provides a reliable method to identify the hybrid nature of morphological intermediate individuals and to determine with high certainty parental species. The identification of species-specific parental genetic markers (and hence parental DNA fingerprints) allows for the exclusion of all non-parental species. AFLP markers are highly suited to this type of study as they are highly reproducible, show Mendelian inheritance and produce a large number of markers (Vos *et al.*, 1995). The dominant nature of AFLP allows for the identification of first generation hybrids (F1), as these individuals should contain all bands fixed in both parental species. Chloroplast DNA markers can provide an additional line of genetic evidence for hybridization. In angiosperms chloroplasts are predominantly uniparentally inherited (usually maternally, Mogensen, 1996) and therefore, are of particular use in hybridization studies as the identity of the seed parent and the direction of gene flow in the initial hybridization event can be elucidated.

Materials and Methods

Species Choice, Sampling and Collection Sites

Based on the putative hybrid morphology, *F. fistulosa*, *F. hispida* and *F. septica* were considered the most likely candidates for parental species and therefore are the main focus of this study. In order to exclude other likely parental species four other dioecious species, *F. fulva*, *F. montana*, *F. ribes*, and *F. variegata* were included in analysis. All seven species are early colonists to the Krakatau islands (Whittaker *et al.*, 1989), are lowland tree species (except *F. ribes*) and have populations of varying degrees of sympatry on the islands and mainland sites. *F. ribes* typically occurs in montane sites on Krakatau and was not observed elsewhere on the mainland in populations sympatric with the other species (Parrish, pers. observ.). *F. fistulosa*, *F. hispida*, *F. septica*, *F. ribes* and *F. variegata* are all pollinated by species of Agaonid wasp from within *Ceratosolen* subgenus *ceratosolen*, whereas the pollinators of *F. fulva* and *F. montana* are from different genera (*Blastophaga* and *Liporrhopalum*, respectively) (Wiebes, 1994).

Field expeditions were undertaken on the Krakatau islands and surrounding regions of the Sunda Strait coastlines during July to September in 1996 and 1999, during which the authors were specifically searching for and making collections of hybrid *Ficus* individuals and their putative parental species. Putative hybrids were identified based on their fruiting morphology, growth form and leaf characters, and were categorized into four groups (see

results section). Though morphological characters were used to identify putative hybrids, actual morphometric measurements were not taken.

Collection sites are listed in Table 4.1 and shown in Figure 2.1. Two further populations of *F. fistulosa* in Sumatra (Kota Agung and Lampung, used in Chapter 2 for population studies) were examined but no putative hybrid individuals were identified. In total, five mainland and four island sites were examined for putative hybrids. Leaf material for *F. montana* was supplied by Dr S.G. Compton (University of Leeds, UK) and the *F. variegata* individual from Tutong, Brunei was collected by Dr E. Joussein (CNRS, Montpellier, France). Leaf material for molecular analysis was collected and preserved in a CTAB/NaCl solution (Rogstad, 1992).

Putative hybrids were identified at five localities; the Krakatau islands (Panjang and Rakata), Bogor (on Java), Popoli Island and Peucang Island. It must be noted that four of these five sites are islands. On Panjang and Rakata putative hybrids of *F. fistulosa* x *F. septica* (type (i)) were identified (for an explanation of typology i/iv see results). Both *F. septica* and *F. fistulosa* currently maintain large population sizes on the Krakatau islands. Popoli is a small coral and sand island approximately 1-2 km off the west coast of Java. This site was formerly a market garden and is highly disturbed. Currently early secondary forest is colonizing and the island is dotted with new woodland. *F. septica* is the most common *Ficus* species on the island. A small stand of *F. hispida* was also identified. Two hybrid morphologies were identified, one putative *F. fistulosa* x *F. septica* (type (ii)) and one putative *F. septica* x *F. hispida* (type (iii)). Peucang Island is part of Ujung Kulon National Park, Java and lies approximately 1 km off the west coast. This island was disturbed by ashfall and tsunamis during the Krakatau eruptions in 1883 (Verbeek, 1885; Hommel, 1987). Forest is now in a more advanced successional state (Kartawinata *et al.*, 1985) than that of Krakatau. *F. septica* is common on Peucang, though *F. fistulosa* and *F. hispida* were not identified there by the authors and, as a whole, are uncommon throughout Ujung Kulon (Hommel, 1987). A single large *F. variegata* individual was identified and genotyped. Putative hybrids of *F. fistulosa* x *F. septica* on Peucang were categorized into a fourth morphological group (type (iv)). Some putative hybrids were collected from the inland town of Bogor (West Java) and resembled those found on Krakatau (type i). In Bogor *F. septica* is a common weedy species, growing on road verges and in drains. *F. fistulosa* was also identified in similar habitat but was far less common.

Where possible, foundress wasps were sampled from putative hybrid fruits and stored in 96% ethanol. Comparative samples of emergent pollinator wasps were also collected from parental species.

AFLP and Chloroplast DNA Typing

Methods of leaf preservation, DNA extraction and AFLP procedures used in this study are outlined extensively in Chapter 2. DNA was extracted using a hot CTAB extraction procedure based on the methods of Rogstad (1992). The AFLP protocol used in this study follows the basic principles of Vos *et al.* (1995) and used *EcoRI* and *MseI* restriction enzymes. Two AFLP primer combinations (with extension nucleotides *EcoRI*-AAG, *MseI*-CTG (primer 1) and *EcoRI*-AAC, *MseI*-CTA (primer 2)), previously used for population studies in *F. fistulosa* (Chapter 2), were selected for this study.

The PCR-RFLP method was used to identify species-specific chloroplast haplotypes in *F. hispida*, *F. fistulosa* and *F. septica*. Non-coding chloroplast DNA regions were amplified using universal primers designed by Demesure *et al.* (1995). PCR reactions were performed in a 50 µl reaction volume containing 2-20 ng of DNA, 5 µl Pharmacia PCR reaction buffer, 0.2 mM dNTPs, 10 pmoles each of forward and reverse primer and 2.5 U Pharmacia Taq Polymerase. PCR was performed on a Hybaid Omnigene thermal cycler from Biozyme using the following thermal cycle protocol; denaturing at 94 °C 5 min, then 1 min at 94 °C, 1 min at 52°C, and 3 min at 72 °C for 35 cycles, followed by 5 min at 72°C. Restriction digestion was performed directly on diluted PCR product following enzyme specifications of the manufacturer. Polymorphism patterns from nine chloroplast loci digested with eight (four or five base) restriction enzymes each were examined. Haplotypes were viewed on a 1% agarose gel for polymorphisms between the species.

Analysis

Individual AFLP genotypes were scored and recorded as band presence or absence in the seven putative parental species and hybrid individuals. Co-migrating bands within a gel between different individuals were considered to be homologous. In order to avoid mistakes in assigning band identity, individuals from different species and putative hybrid individuals were run close to each other on a gel.

To identify hybrid genotypes, AFLP banding patterns were analyzed in two different ways. First, the banding patterns within and between species were examined and species-

specific or species-exclusive patterns were identified. Bands were considered species-specific if they were only present in that species. Bands were considered species-exclusive if they were absent in a species but present in various combinations of other species. The presence or absence of these bands was then examined in putative hybrid individuals. An individual was identified as a hybrid if it contained at least one band from two species. Given that AFLP markers are dominant, an individual was considered an F1 hybrid if it contained all bands fixed in both parental species. The patterns of relationship between species and putative hybrids were investigated using Principal Coordinate Analysis (PCoA). A pair-wise similarity matrix was constructed from the 0/1 AFLP data matrix using Jaccard's index (SYSTAT, Wilkinson, 1999) and PCoA analysis performed using the CANOCO program ver. 4 (Ter Braak and Šmilauer, 1998). Scatterplots were constructed from the two main principal axes that explained most of the variation in the data, to inspect the relatedness among populations. Hybrid individuals are expected to contain a mixture of markers from parental species and therefore should cluster between the ordinal clouds of the parental groups. The patterns of genetic relationship between the seven study species (and an eighth species, *F. padana*, collected from Carita and Rajabasa; *F. padana* was included in analysis as it is a close relative of *F. fulva* and may add further resolution to relationships within the study species) were further examined in a UPGMA tree (nearest neighbor joining) using SYSTAT (Wilkinson, 1999). This tree was constructed using euclidean distances calculated from the AFLP data matrix.

Chloroplasts are typically inherited through the maternal parent and therefore can identify which species was the seed parent during a hybridization event. The chloroplast haplotype was, where possible, identified in putative and hybrid individuals.

Results

Species and Putative Hybrid Morphology

Putative hybrid individuals were found at several localities. Based on morphology of these putative hybrid individuals three species, *F. septica*, *F. fistulosa* and *F. hispida* were considered to be the most likely candidates as hybrid parents. Descriptions of characters typical for these species in the Sunda Strait region that were used to identify potential hybrids are as follows (Parrish, pers. observ.). *F. septica* has large, glabrous, discolourous, elliptic leaves which are dark shiny green on the upper surface with a prominent white midrib. Stipules are white to pale green. The fruits are paired and axillary. The species is widespread

and common, growing in light areas as an open-branching tree. *F. fistulosa* leaves are typically subobovate to oblong or elliptic and often slightly asymmetric. Young shoots and stipules are often red. Fruit grow on short cauliflorous branchlets on the trunk and branches with numerous fruit on each branchlet. The species is widespread but more shade tolerant and less common than *F. septica* and is often found under the canopy and along streams. *F. hispida* has hairy, serrate subobovate to elliptic leaves. Fruit are positioned on long branchlets (up to 1 meter in length) along the trunk. The species is widespread though tends to have patchy distribution and, when present, locally abundant in both open and closed forest. Morphological characters were used merely as a guide for the identification of putative hybrids and not used further for morphometric analysis.

Four different putative hybrid morphologies were identified;

- (i) growth form and leaf morphology most similar to *F. septica* but had cauliflorous fruiting morphology most similar to that of *F. fistulosa*. The extent of branchlet size and fruit number varied greatly from occasional paired fruit on branch buds along the trunk to extensive bunches of fruits clustered on a distinct branchlet.
- (ii) leaf morphology and colour most similar to *F. fistulosa* but had paired, axillary fruit and growth form of *F. septica*.
- (iii) sapling leaf shape of *F. hispida* but lacking hairs and had glabrous shiny surface resembling *F. septica*
- (iv) uncertain morphology, leaves intermediate between *F. fistulosa* and *F. septica*, cauliflorous fruits.

In total 22 putative hybrids were identified at five sites, the Krakatau islands (Panjang and Rakata), Popoli Island, Peucang Island and Bogor. Three, ten and four individuals (17 in total) with morphology type (i) were identified and collected on Panjang, Rakata and Bogor, respectively. On Popoli Island two putative hybrids were identified, one with morphology type (ii) and another with type (iii). Three individuals with morphology type (iv) were identified on Peucang Island. Except at Bogor no putative hybrids were found at mainland sites, despite five localities being examined.

Molecular Markers and Hybrid Identification

Two AFLP primer combinations were used to identify species-specific DNA fingerprints in the study species (see Materials and Methods). The number of individuals used to construct these fingerprints and source locations of the specimens is given in Table 4.1. Given that *F. fistulosa* and *F. septica* were considered to be the most likely parental species of hybrids,

Table 4.1: The seven species of dioecious *Ficus*, the number of individuals (N) and collection sites used in AFLP analysis to establish species-specific banding patterns. Two AFLP primer combinations (primer combinations 1 and 2, see Materials and Methods) generated a total of 226 bands across all species. The numbers of species-specific bands per species are shown below. More bands were considered diagnostic as they excluded certain combinations of species and are shown in Table 4.2. The collection sites of the putative hybrids are also listed. Letters after collections sites designate the region of origin ((K)rakatau islands, (S)umatra, (J)ava and (B)runei).

Species	(N)	Collection Sites (Region)	# Species Specific Bands
<i>F. fistulosa</i>	16	Rajabasa (S), Rakata (K), Carita (J)	7
<i>F. fulva</i>	3	Rajabasa (S), Labuan (J)	20
<i>F. hispida</i>	6	Rajabasa (S), Carita (J), Panjang (K), Rakata (K)	15
<i>F. montana</i> †	3	Bogor (J), Rakata (K)	29
<i>F. ribes</i>	6	Rajabasa (S), Carita (J), Rakata (K)	12
<i>F. septica</i>	20	Rajabasa (S), Sebesi (S), Popoli (J), Panjang (K), Rakata (K)	8
<i>F. variegata</i>	10	Rajabasa (S), Carita (J), Peucang (J), Panjang (K), Rakata (K), Tutong (B)*	48
Putative hybrids	22	Popoli (J), Peucang (J), Bogor, (J)Panjang (K), Rakata (K)	

Collected by † S.G. Compton (University of Leeds, UK) and by * E. Jouselin (CNRS, Montpellier, France) from Tutong, Brunei.

considerably more individuals of these species were examined. A total of 226 clearly distinguishable bands were produced across all seven species of which 7, 20, 15, 29, 8, 12 and 48 were specific to *F. fistulosa*, *F. fulva*, *F. hispida*, *F. montana*, *F. septica*, *F. ribes*, and *F. variegata*, respectively (Table 4.1).

Nine chloroplast DNA regions were amplified using primers from Demesure, *et al.* (1995) and digested with eight restriction enzymes in order to identify diagnostic RFLP haplotypes between *F. fistulosa*, *F. hispida* and *F. septica*. Eight polymorphisms were detected, of which the intergenic spacer region between *trnM* to *rbcL* (primers sequences are for *trnM* 5'-TGCTTTCATACGGCGGGAGT-3' and *rbcL* 5'-GCTTTAGTCTCTGTTTGTGG-3'), when digested with *Hinf* I, produced diagnostic

haplotypes for each of the three species. A number of individuals per species was examined to determine that these patterns were consistent across the study area of the species (23 individuals of *F. septica* from Panjang, Rakata, Sebesi, Rajabasa, Popoli and Bogor; 19 individuals of *F. fistulosa* from Carita, Sertung, Lampung and Rakata and four individuals of *F. hispida* from Panjang and Rakata). Chloroplast haplotypes were not determined in the other dioecious species in this study, *F. fulva*, *F. montana*, *F. ribes* and *F. variegata*. The chloroplast haplotypes of hybrid individuals (as confirmed with AFLP) are shown in Table 4.2.

All 22 putative hybrids were fingerprinted with AFLPs. Eight putative hybrids contained AFLP-bands typical of more than one species. The remaining fourteen putative hybrids contained only AFLP bands typical of *F. septica*. These 14 plants also carried a cpDNA haplotype typical of *F. septica* and were therefore considered not to be hybrids. A ninth individual, collected as an adult *F. hispida* specimen, also contained extra-species bands. A summarized genotype of these individuals is given in Table 4.2 (only bands that were present in hybrid individuals are included. Bands present in all species, and hence non-diagnostic, are excluded). In some cases bands not unique to a species could be considered diagnostic as they were shared between species but absent in others (species exclusive). For example in some cases a band was absent in *F. septica* but present in various other species, such as *F. fistulosa*, thus indicating that individuals most similar to *F. septica* contained bands not typical of this species. In no cases were bands specific to *F. fulva* and *F. ribes* identified in putative hybrids, thus excluding these species as potential parental species.

Seventeen putative hybrids with morphology type (i) (*F. septica* x *F. fistulosa*) were collected from Krakatau (Panjang and Rakata) and Bogor. Of these, only 3 individuals from Rakata (H1, H2 and H3, Table 4.2) proved to possess hybrid markers. H1 and H2 contained markers unique to *F. septica* and *F. fistulosa*, and contained none specific to other species, thus confirming the parental species predicted from hybrid morphology. The chloroplast haplotypes of H1 and H2 were that of *F. fistulosa* and *F. septica*, respectively (Table 4.2). Assuming uniparental chloroplast inheritance in *Ficus*, this implies that the seed donor parents were different. The identity of the parental species of H3 was not so obvious. H3 contained markers specific to *F. septica* but none specific to any other one species. This individual contained two bands shared by *F. fistulosa* and *F. ribes* and two shared by *F. fistulosa*, *F. hispida* and *F. ribes*. Therefore, the second parental species could not be unambiguously identified. H3 contained a haplotype typical of *F. fistulosa* but as the chloroplast haplotype of *F. ribes* was not determined the identity of the second parental

species remains ambiguous, though most likely *F. fistulosa*, considering the parental species found for other hybrid individuals. All three of these hybrid individuals are unlikely to be first generation hybrids (F1) as they do not contain all bands specific to parental species as expected given the dominant nature of AFLP. This indicates that hybrids are later generation hybrids. F1's are thus fertile and backcrossing occurs. The fact that 14 of 17 putative hybrids in this morphology class were not genetically identified as hybrids indicates that the morphological character used to identify hybrids (fruiting morphology) needs to be further assessed and refined if hybrids are to be identified more reliably in the field.

On Popoli Island both putative hybrids were confirmed as hybrids using AFLP, one individual being a cross between *F. septica* and *F. fistulosa* (H4, morphology type (ii), with *F. septica* chloroplast haplotype), and one being a cross between *F. hispida* and *F. septica* (H5, morphology type (iii), *F. hispida* chloroplast haplotype) (Table 4.2). These individuals are likely F1 hybrids as they possess all bands fixed in the parental species and contain a number of other bands found in the parental species. The total band number in these individuals is high (H4=55, H5=57) and outside the range observed in a sample of individuals from each species (*F. fistulosa*=38-48, *F. hispida*=40-48 and *F. septica*=35-44, Tables 4.2 and 4.3). H4 possessed a single band of which it was difficult to elucidate if it was unique or shared with *F. variegata*. A third individual containing bands from more than one species was identified on Popoli Island (H6). This individual was collected as an adult tree of *F. hispida* and contained all bands fixed and unique to *F. hispida*, as expected, but also contained a band shared by *F. fistulosa*, *F. ribes* and *F. septica*, and a second shared by *F. septica* and *F. variegata*. Such results may reflect past hybridization with *F. septica*, misidentification of markers or rare bands not detected in the *F. hispida* sample used in this

Table 4.2: The summarized genotypes of hybrid individuals, collection sites, morphology type, parental species as identified by AFLP and chloroplast haplotype. Only bands present in the hybrid individuals are shown. The species identity of bands, F=*F. fistulosa*, H=*F. hispida*, M=*F. montana*, R=*F. ribes*, S=*F. septica*, V=*F. variegata* and U=unidentified/unique is given to the right of the table. Loci fixed within the species are underlined, those of greater than 80% frequency are in bold and less than 80% in plain text. No hybrid individuals contained bands specific to *F. fulva* and *F. ribes*. The total number of bands (including those fixed in all species) in each individual's fingerprint for both primer combinations are given at the bottom of the table and can be compared to species averages in Table 4.3. Dashes indicate missing data due to non-amplification.

Chapter 4

Collection Site	H1	H2	H3	H4	H5	H6	H7	H8	H9	Species Identity of Band
Morph. Type	Rak (i)	Rak (ii)	Rak (i)	Pop (ii)	Pop (iii)	Pop H	Peu (iv)	Peu (iv)	Peu (iv)	
Parental Species	FXS	SXF	FXS	SXF	HXS	?XH	SXF	SX?	?X?	
Chloroplast Type	F	S	F	S	H	-	S	S		
Pr 1	0	0	0	1	0	0	1	0	0	F
(Each line represents a separate locus)	0	0	0	1	0	0	0	0	0	F,R
	0	0	0	0	1	1	0	0	0	H
	0	0	0	0	1	1	0	0	0	H,V
	1	1	1	1	1	0	1	1	1	S,F,R
	1	1	1	1	1	1	1	1	1	S,H,V,R
	0	0	0	0	1	1	0	0	0	H
	1	1	1	0	0	0	1	1	1	S
	0	0	0	1	0	0	1	0	0	F
	0	0	0	0	1	1	0	0	0	H,V
	1	0	1	1	0	0	1	0	0	S
	0	0	0	0	1	1	0	1	0	H
	0	0	0	0	0	0	0	1	1	V
	0	0	0	0	1	1	0	0	0	H
	1	1	1	1	1	0	1	1	1	S,F,R
	1	1	1	1	1	0	1	1	1	S
	1	1	1	1	0	0	1	1	1	F,R
	0	0	0	0	1	0	0	0	0	S
	1	1	1	1	1	1	1	1	1	F,H,R
	0	0	0	0	0	0	0	0	0	S
	0	0	1	1	0	1	0	1	1	S,F,R
	0	0	0	0	0	0	0	0	0	S,F
	1	0	1	1	1	1	1	1	1	S,F,H
	1	1	1	0	1	0	1	1	1	S
	1	1	0	1	1	1	1	1	1	F,H,R
	1	1	0	1	1	1	1	1	0	F,H,R
	1	1	0	1	0	0	1	0	0	F,V
Pr 2	1	0	0	1	0	0	1	0	-	F
	0	0	0	0	1	1	0	0	-	H,V
	1	1	1	1	1	0	1	1	-	S,F,V,R
	1	0	1	1	1	0	1	1	-	S
	0	0	0	0	1	1	0	0	-	F,H,R
	0	0	0	0	0	0	0	1	-	U
	0	1	0	1	0	0	1	0	-	F
	0	0	0	0	0	0	0	1	-	U
	1	0	1	0	1	1	0	0	-	F,H,R
	1	0	0	1	0	0	1	0	-	F
	1	1	1	1	1	0	1	1	-	S,V
	0	1	0	1	0	0	1	1	-	S,F,R
	0	0	0	1	0	0	0	0	-	U or V?
	1	0	1	0	0	0	1	0	-	F,R
	0	0	0	0	1	1	0	0	-	H
	0	0	0	0	1	0	0	0	-	U
	0	0	0	0	0	1	0	0	-	M
	1	1	1	1	1	0	1	1	-	S,F,V,R
	0	0	0	0	1	1	0	0	-	H
	1	1	1	1	1	0	1	1	-	S,F,R
	0	0	0	1	1	1	0	0	-	F,H,V
	0	0	0	0	1	1	0	0	-	H
	1	0	1	0	0	0	0	0	-	U
	0	0	0	0	1	1	0	0	-	H
	1	1	1	1	1	0	1	1	-	S,F,R
	0	0	0	0	0	1	0	0	-	H
	0	0	1	0	0	0	0	0	-	S
	0	0	0	0	1	1	0	0	-	H,V
	0	0	0	0	1	1	0	0	-	H
	0	0	0	0	1	1	0	0	-	F,H
	1	1	1	1	1	0	1	1	-	S,F,R
	0	1	0	1	0	0	1	1	-	F,H,V,R
	0	1	0	1	0	1	1	0	-	S,V
# Bands/Indiv.	47	42	44	55	57	45	53	48	27	

study. A single *F. montana*-typical band was identified in this individual and was considered to most likely be a comigrating non-homologous band.

All three putative hybrids collected on Peucang possessed bands from more than one species (H7, H8 and H9). These individuals had leaf characters intermediate to *F. fistulosa* and *F. septica* and cauliflorous fruiting behavior. H7 is likely an F1 hybrid between *F. septica* and *F. fistulosa* as it contains only bands specific to, and most fixed in, these species and possesses a larger number of bands (53) than normally observed in these species (Tables 4.2 and 4.3). The chloroplast haplotype of this individual was typical of *F. septica*. The genotypes of H8 and H9 are more complicated as they possess bands from more than two species. H8 contained all bands fixed and a number of bands specific to *F. septica*, one specific to each of *F. hispida* and *F. variegata* and one shared between *F. fistulosa* and *F. ribes*. H9 also contained all bands fixed and a number of bands specific to *F. septica*, one specific to *F. variegata* but other bands shared by *F. fistulosa*, *F. hispida* and *F. ribes* which were mutually exclusive of *F. variegata*. Data is missing for primer combination 2 in this individual due to non-amplification. Both H8 and H9 had chloroplast haplotypes typical of *F. septica*. The hybrid status of these two individuals is questionable as very few bands not specific to *F. septica* were observed. These bands may be rare bands not observed in the population samples of *F. septica* used in this study.

Table 4.3: The average number (\pm standard error) and range of bands in AFLP fingerprints from (N) individuals of *F. septica*, *F. fistulosa*, and *F. hispida*.

Species	N	Mean # Bands/Indiv. \pmse	Range
<i>F. septica</i>	20	37.4 \pm 0.5	35-44
<i>F. fistulosa</i>	16	42.3 \pm 0.6	38-48
<i>F. hispida</i>	6	44.9 \pm 1.0	40-48

Foundress Wasps

Of the nine individuals identified to contain DNA markers specific to more than one species foundress wasps were found in only two individuals, H1 and H4. Both these individuals were hybrids between *F. fistulosa* and *F. septica* and contained foundress wasps of *Ceratosolen bisulcatus*, the pollinator wasp of *F. septica*. H1 and H4 were both female trees but developing seeds were only found in H4. Individuals H2, H3 and H9 were collected in 1996 and their fruits were not examined. H5 was a prefruiting sapling. The fruits of H6 were not examined. H7, while fruiting and possessing fruits large enough for pollination, contained no pollinators and H8 and H9 were not fruiting.

PCoA

Principal coordinate analysis was performed on the AFLP data from a subsample of *F. fistulosa*, *F. hispida*, *F. ribes* and *F. septica* (in order to limit cluster group size) and eight of the nine individuals identified to contain bands typical of more than one species. Hybrid 9 was excluded from analysis due to the large amount of missing data from this sample (no amplification in primer combination 2). A scatterplot of the first and second axes are shown in Figure 4.1. Axes one and two explain 75.8 and 13.3 percent of the total variance in the sample, respectively. The plot shows that all four species occupy distinct ordinal space, though *F. fistulosa* and *F. ribes* cluster very close. *F. fistulosa* x *F. septica* hybrid individuals, H1- H4, and H7 occupy distinct ordinal space intermediate to the clusters of these two species. H8 shows overlap with the *F. septica* cluster. *F. hispida* x *F. septica* individual, H5 is intermediate between the clusters of *F. hispida* and *F. septica*, while H6 clusters within the *F. hispida* cloud. These results question the hybrid status of both H6 and H8. In an additional PCoA analysis (data not shown), putative hybrid individuals of *F. fistulosa* and *F. septica* (morphology type (i)) which showed no genetic evidence for hybridization clustered within the *F. septica* cloud, as would be expected.

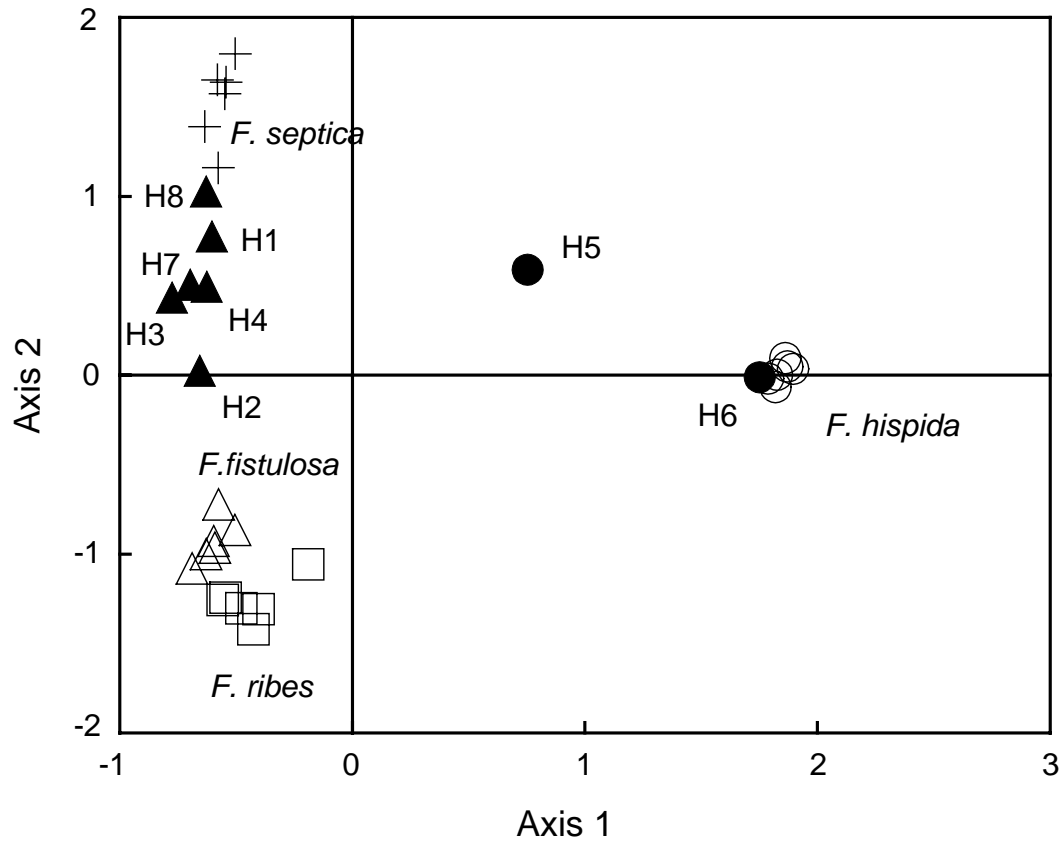


Figure 4.1: Principal coordinate analysis results demonstrating the relationships between the species and hybrid individuals (open symbols are the species; plus=*F. septica*, upward triangle=*F. fistulosa*, square=*F. ribes* and circle=*F. hispida*; closed symbols are hybrid individuals, upward triangle=H1-4, H7, H8 and circle=H5, H6. Plots are made from axes 1 and 2, which explained, 75.8 and 13.3 percent of the total variance in the data, respectively.

UPGMA

Two independent UPGMA trees were constructed from the data from primer combinations one and two and both gave comparable results. The tree from primer combination one is shown in Figure 4.2. All species form discrete clusters, except *F. fistulosa* and *F. ribes*, which show some overlap in their clusters. *F. septica* (the one species involved in all hybrid events documented in this study) clusters with three other species, *F. fistulosa*, *F. hispida* and *F. ribes*, that are also pollinated by species of *Ceratosolen*. This indicates that *F. septica* is genetically closely related to the two species it hybridizes with, *F. fistulosa* and *F. hispida*. In contrast, *F. ribes*, which is equally genetically related, was not demonstrated in this study to be hybridizing with *F. septica*. This likely reflects the different ecological distribution of *F. septica* and *F. ribes* in the study area. *F. septica* occupies lowland habitat while *F. ribes* occupies montane. The fifth species pollinated by *Ceratosolen* wasps, *F. variegata*, appeared more genetically similar to *F. fulva* and *F. padana* which are pollinated by a different genus of wasp, *Blastophaga*. *F. montana* is genetically the most distinct species in this tree and is pollinated by a third genus of Agaonidae, *Liporrhopalum*.

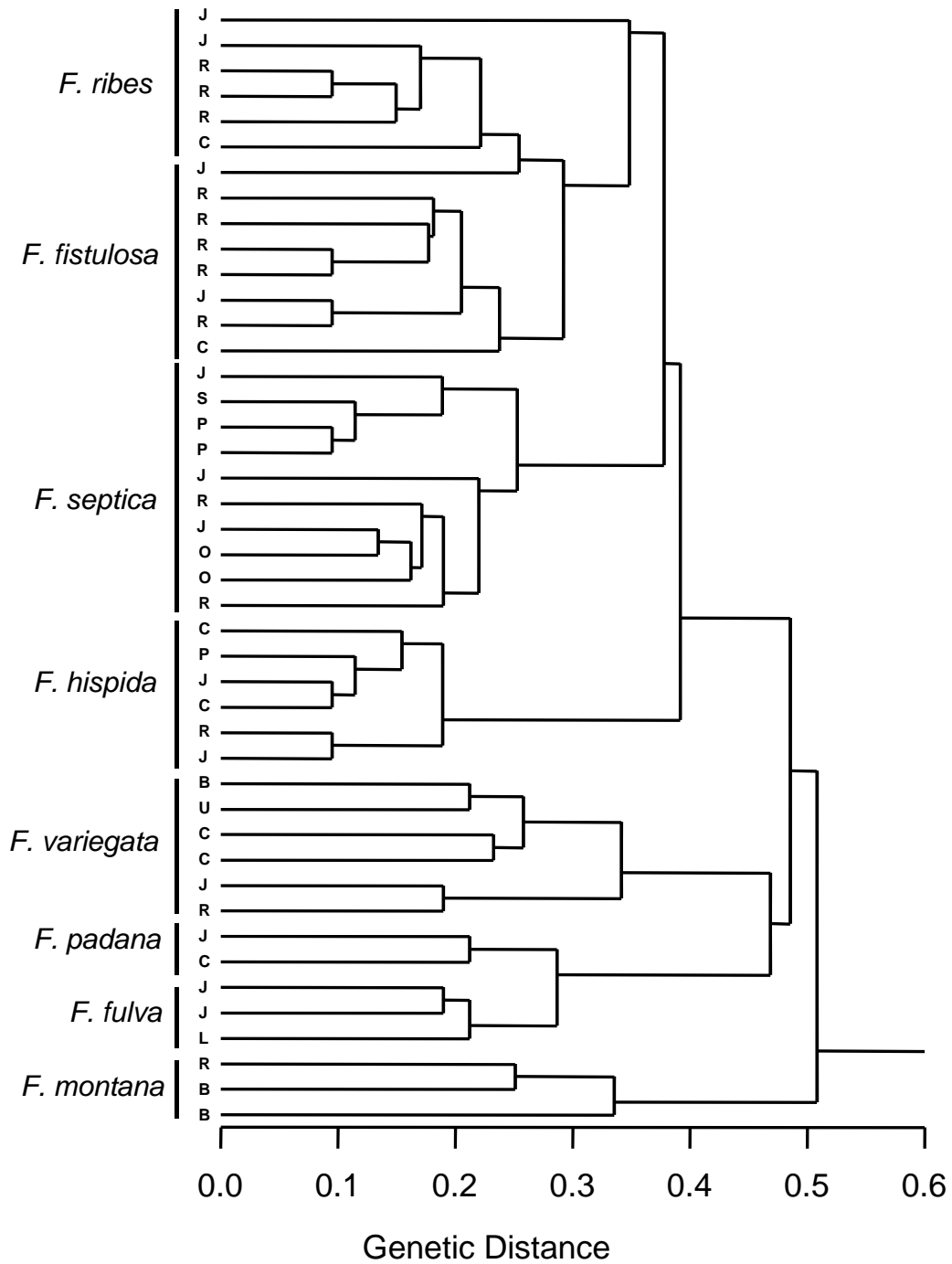


Figure 4.2: An average linkage clustering UPGMA tree constructed using euclidean distances of data from primer 1 demonstrating patterns of genetic dissimilarity between the study species. *F. septica* clusters between the two species it was found to be hybridizing with in this study. Small letters indicate collection sites of samples where, B=Brunei, C=Carita, J=Rajabasa, O=Popoli Island, R=Rakata, S=Sertung, and U=Peucang Island.

Discussion

In this paper we provide genetic evidence that hybridization events are occurring in natural populations of dioecious *Ficus* species, *F. septica* and two other closely related species *F. fistulosa* and *F. hispida*. Nine individuals were found to possess bands typical of more than one species. Of these individuals, six are without doubt hybrid (H1-H5, H7) because they contain a large number of bands that are typical of more than one species and cluster between the parental species in PCoA. However, the hybrid status of three other individuals (H6, H8 and H9) can be questioned and may be individuals of *F. septica*. Despite containing bands from more than one species, only a small number of bands not typical for *F. septica* were observed. These bands were further not consistently attributable to any other single species. Such bands may in fact be rare bands of *F. septica* not observed in the samples used in this study. H6 and H8 clustered in ordination with *F. hispida* and *F. septica*, respectively. H9 remains unclear because of a lack of data due to poor PCR amplification in one primer combination.

It has long been known that hybridization can occur between *Ficus* species, but most examples exist between rather phylogenetically disparate species in non-natural situations (Condit, 1950; Ramirez, 1986; Ramirez and Montero, 1988; Ramirez, 1994). Hybridization or cases of pollinator-specificity breakdown in *Ficus* have been reported where one species is outside its natural range (Compton, 1990; Ramirez and Montero, 1988; Ramirez, 1994), from isolated individuals (Compton, 1990; Ramirez, 1970; Ware and Compton, 1992) or from artificial crosses (Condit, 1950; Ramirez, 1986). To date, observations of putative natural hybrids are rare (Ramirez, 1970) with determination being solely based on morphological characters. Overall morphological similarity between *Ficus* species may result in hybrids being overlooked in the field (Janzen, 1979). Therefore, it is of significance that hybrid events have been genetically confirmed in natural populations where the species involved naturally occur in sympatry, have similar ecological tolerances and are inside their natural distribution. It has been suggested that hybridization between unrelated species is brought about by a breakdown in wasp specificity due to chance convergent volatile profiles that have evolved independently (Janzen, 1979; Ramirez, 1988). In closely related species it would be expected that breeding isolation be maintained by divergent chemical profiles. In this study we show that hybridization events between closely related *Ficus* species have occurred repeatedly.

Only a few hybrids over a large collection area were identified in this study, suggesting that natural hybrids are rather rare. Hybrids were nonetheless observed in three

separate localities, thus indicating that hybridization is not unique to the Krakatau situation and occurs repeatedly at different localities. The three sites where hybridization has been identified are all islands with a history of disturbance. Janzen (1979) suggests that extinction or absence of pollinators would be an important factor on island populations. It seems likely that reduced pollinator wasp numbers in one *Ficus* species due to island isolation or small plant population size will drive hybridization events. Latitude in accepting the wrong pollinator in isolated individual plants may be an advantage as it may be better to hybridize than go extinct.

The study species are early colonists that establish populations at different rates and times (Parrish, pers. observ.; for Krakatau see Docters van Leeuwen, 1936; Whittaker *et al.*, 1989). Therefore, pollinator populations in different *Ficus* species will likely establish at different times due to factors such as plant population size, island isolation and wasp dispersal capabilities. The rate of establishing will be greatly affected on islands, as small plant populations, which cannot support a reliable local pollinator population, may not be able to easily recruit wasps from nearby populations.

While current populations of many *Ficus* species on Krakatau are large there is evidence suggesting that there were initial differences in *Ficus* species abundance (see Docters van Leeuwen, 1936) and that pollinator limitation may have been occurring in early populations (Backer, 1929; Ernst, 1934). In 1906, more than 20 years after the eruption, species such as *F. fistulosa* were already common on the Krakatau islands, but while amply fructifying only unripe and not completely developed fruits were observed (Backer, 1929; Ernst, 1934). At this time *F. septica* was considerably less abundant (see Docters van Leeuwen, 1936). In addition, no fig wasps were collected during the first zoological expedition in 1908 (Jacobson, 1909) despite extensive invertebrate collections being made. However, in 1922 (some 15 years later) fig wasps (including *Ceratosolen bisulcatus*, the pollinator wasp of *F. septica*) were very abundant and present in nearly all male figs examined (Dammerman, 1922, 1948). It appears that between about 1910 and 1920 the fig wasp populations on Krakatau have expanded dramatically. Pollinator limitation occurs today in newly establishing populations of *Ficus* on Anak Krakatau (Compton *et al.*, 1994). On both Popoli and Peucang islands *F. septica* is very common while the other two species are uncommon or not observed by the authors. Therefore, it is most likely that the pollinators of *F. fistulosa* and *F. hispida* are absent in these newly establishing populations or, if present, are colonists from other sites.

Chloroplasts are typically maternally inherited in angiosperms (Mogensen, 1996) and it is assumed here to also be the case in *Ficus*. Chloroplast DNA markers can therefore identify the seed parent in a cross and indicate the direction of gene flow when species-specific haplotypes have been identified. Hybrid individuals of *F. septica* and *F. fistulosa* were found to have chloroplast haplotypes of either species, indicating that both species can function as seed parents and that gene flow is bidirectional. It is unknown, however, which pollinator species was the mediator. In the two cases where foundress wasps were collected from hybrid individuals, the pollinator of *F. septica*, *C. bisulcatus*, was found. However, this does not give insight into initial hybrid events as hybrid individuals would most likely have different morphology and chemical profiles compared to parental species. Interestingly, F1 hybrids between *F. septica* and *F. fistulosa* on both Popoli and Peucang islands contained chloroplast DNA haplotypes of *F. septica* indicating that this species was the maternal plant in initial hybrid events and the pollen parent was *F. fistulosa*. Therefore, either pollinators of *F. fistulosa* were blown in from other sites or an isolated (and currently unobserved) male *F. fistulosa* plant produced pollinators somewhere in the neighborhood.

The presence of later generation hybrids in this study indicates that backcrossing occurs, that hybrids can be fertile and can establish in the field. However, the rarity of hybrid individuals indicates that hybridization will have little effect on species integrity in the present study environment. Hybridization is considered of little significance in the evolution of *Ficus* as a whole (Corner, 1958; Ramirez, 1970). If hybrid events are to have an effect on species evolution then one should examine more isolated islands where there is increased chance of long-term pollinator absence or extinction in one of two co-occurring species (Janzen, 1979). Hybridization is increasingly recognized as an important evolutionary process in some island groups (Gillet, 1972; Raven, 1972; Carr, 1995; Francisco-Ortega, *et al.*, 1996). Recent studies have demonstrated that pollinator populations of dioecious *Ficus* species may be more sensitive to extreme environmental perturbations and are likely to be subject to extinction events more frequently than pollinators in monoecious species (Harrison, 2000). Therefore, dioecious species may be more prone to hybridization in island settings. Corner (1975) notes that variation in dioecious *F. scabra* in the New Hebrides may be due to hybridization.

The morphological character of cauliflory needs further consideration. Numerous *F. septica* trees with cauliflorous fruits were identified on Krakatau and Bogor (Java); however, only three of these trees proved to be hybrid. The extent of cauliflory varied greatly between individuals, from paired fruits on the trunk subtended by a leaf to actual small branchlets

bearing fruit (data not shown). In all hybrid individuals that were cauliflorous, the fruit were born on distinct branchlets. The nature of cauliflory and its incidence throughout the range of *F. septica* and hybrid morphology needs to be further investigated and quantified if characters are to be identified that can be reliably used in the field.

Various forms of fruit arrangement are reported throughout the range of *F. septica* (Berg, in prep.). For example, a cauliflorous form of *F. septica* with fruit on long branchlets occurs in the Solomon Islands and Australia (Berg, in prep.). This may be a potential case for hybrid origin, though remains to be investigated. Molecular markers based on AFLP and chloroplast-PCR provide powerful tools to answer such questions.

Identification of a Male-Specific AFLP Marker in a Functionally Dioecious Fig, *Ficus fulva* Reinw. ex Bl. (Moraceae).

Abstract

A male-specific AFLP-marker has been identified in the functionally dioecious fig species, *Ficus fulva*. A total of 89 polymorphic fragments from three primer combinations (used for population studies on the Krakatau islands, Indonesia) were produced, of which one (246 bp) was present in all males (N=23) and absent in all females (N=24). Further analysis showed that this marker segregated in open-pollinated progenies from natural populations in a 1:1 ratio (N=156). The marker was isolated, cloned and sequenced. Searches on GenBank BLAST found no sequences with homology to the fragment. Two pairs of PCR primers were designed in an attempt to create a more cost-effective way of assigning sex. However, amplification products of similar size were produced in both males and females of *F. fulva*, indicating high sequence homology between the sexes and that differences in AFLP patterns are due to restriction site loss. Examination of chromosome preparations showed no evidence for morphologically distinct sex chromosomes. The importance of a sex-specific marker to *Ficus* research is discussed.

Introduction

Plants exhibit a wide variety of breeding systems ranging from hermaphroditism, through various forms of monoecy, to dioecy (Sakai and Weller, 1999). Hermaphroditism is the most common form of breeding system in plants (c72% of species) (Yampolsky and Yampolsky, 1922). However, dioecy is not insignificant. Four percent of species are truly dioecious, 7% are either gynodioecious or androdioecious and 75% of plant families have some dioecious members (Yampolsky and Yampolsky, 1922; though Bawa (1980) argues for much higher figures for dioecy, given higher rates of dioecy in tropical floras and their relatively unstudied status). The diverse independent phylogenetic incidence of dioecy implies that this breeding system has evolved numerous times (Charlesworth, 1991), which explains the great diversity of sex determination systems in plants.

The sexual phenotype in plants can be determined by genetic and environmental factors (Grant, 1999). According to Grant (1999) genetic determination systems can be diverse and controlled by 1) a single locus, 2) a group of tightly linked loci on an autosome, 3) a number of unlinked loci on autosomes or 4) loci on heteromorphic sex-chromosomes (eg. *Silene spp.*).

If sex determination is predominantly under the control of a single gene or a group of tightly linked genes then it should be possible to identify sex-specific DNA-markers. The development of molecular marker methods that have the potential to examine the whole genome, require no previous knowledge of species DNA sequence and are easy to implement, has resulted in increasing numbers of sex-linked markers being reported. Randomly Amplified Polymorphic DNA (RAPD) is widely used to identify sex-related markers in both plants (*Pitacia vera*, Hormasa *et al.*, 1994; *Salix viminalis*, Alstrom-Rapaport *et al.*, 1998; *Cannabis sativa*, Mandolino *et al.*, 1999; *Atriplex garrettii*, Ruas *et al.*, 1998; *Silene latifolia*, Zang *et al.*, 1998) and animals (*Parus major* and *Haematopus ostralegus*, Lessells and Mateman, 1998). RAPD is popular because of its relative ease and cheapness of use but can have problems associated with reproducibility. Currently, Amplified Fragment Length Polymorphism (AFLP; Vos *et al.*, 1995) technology is also becoming widely applied for linkage studies because of the large number of markers generated and the relatively more reproducible nature of the technique. The AFLP method is increasingly being used in the identification of sex-linked markers (e.g. Terauchi and Kahl, 1999) and in some cases it successfully identifies markers where other techniques have failed (*Struthio camelus* and *Phalacrocorax aristotelis*, Griffiths and Orr, 1999).

There are approximately 750 species of *Ficus* throughout the tropical and subtropical regions of the world. *Ficus* exhibits two breeding systems, either monoecy (c400 species) or gynodioecy (referred to here as functional dioecy, c350 species) (Berg, 1989). Functional dioecy is restricted to the subgenus *Ficus* (distributed in the Palaeotropics with Malesia as center of diversity) where it is paraphyletic and thought to have evolved once or twice with two reversions back to monoecy (Weiblen, 2000).

The *Ficus* breeding system is particularly interesting because of the tight pollination mutualism with Agaonidae wasps. A trade-off exists between seed production and wasp reproduction as wasps also utilize female flowers for larvae development. *Ficus* flowers are unisexual and arranged in an urn-shaped receptacle (syconium). In monoecious species male and female flowers exist in the one syconium with female flowers being divided between seed and wasp production. In functionally dioecious species, female trees produce syconia

containing only female flowers that only produce seed. Upon entering the syconium the female wasp usually cannot leave and therefore provides only pollination services to the plant and does not reproduce. In this character functionally dioecious *Ficus* is unique in the plant kingdom (Valdeyron and Lloyd, 1979). Male trees, while being functionally male (produce anthers), also produce female flowers that are almost exclusively used for wasp reproduction. The exact mechanism that controls different female flower function between the sexes in *Ficus* is unknown (difference in style length between female flowers in the two sexes may be of importance, Storey, 1975) and is obviously of great importance in determining the functional sex.

Breeding studies in *Ficus carica* L. (the edible fig) led Storey (1955 and 1975) to hypothesize that sex in *Ficus* is genetically determined by two closely linked loci; one locus controlling anthenogenesis and the second controlling female function by preventing wasp ovipositioning in female plants (longer style length in female plants would exceed wasp ovipositor length). Storey's experiments also found that males are the heterogametic sex (1955). Evidence suggests that there is little genetic difference between males and females. Homogametic males formed through male/male crosses are fully fertile (males of dioecious *Ficus* retain female flowers and, in *F. carica*, some ability to produce seed, Storey 1975 and Valdeyron and Lloyd, 1979). Further, there is no evidence for morphologically distinct sex chromosomes in this genus (where $2n=26$ is the common diploid chromosome number in *Ficus*) (Condit, 1964).

F. fulva Reinw. ex Bl (subgenus *Ficus*, section *Ficus*, Corner, 1965) is a small, early successional, functionally dioecious tree distributed widely throughout South-east Asia and India. This species is the object of a study on colonization, population and breeding biology on the Krakatau islands, Indonesia (Figure 1.4) using AFLP markers. In this article we (i) identify a sex-specific marker in *F. fulva* generated with AFLP markers, (ii) examine segregation of this AFLP marker in open-pollinated seedlings from natural populations of *F. fulva*, (iii) determine the marker sequence and search for homology at Genbank, (iv) attempt to design PCR primers that amplify the male-specific fragment and v) examine chromosome preparations to determine if morphologically distinct sex chromosomes are evident.

Materials and Methods

Materials

Leaves and fruits of adult trees of *Ficus fulva* were collected on the Krakatau islands (Rakata, Panjang and Anak Krakatau) in Indonesia for population and pollination studies. Trees were sexed according to the presence or absence of male flowers (anthers around the ostiolar opening in the syconium) or to the development of seeds. In total, 23 male and 24 female trees were used for genetic analysis (8 males and 8 females from each of Rakata and Panjang, and 7 males and 8 females from Anak Krakatau). Leaf samples for DNA analysis were preserved in a NaCl/CTAB solution (Rogstad, 1992). Seeds were sampled from five females (a total of eight fruits per island) from both Rakata and Anak Krakatau. These were later germinated on wet filter paper (12 hour day, at 27 °C day/25 °C night) and then transferred to 4:1 peat/sand mix.

DNA Extraction

DNA was extracted from both adult and seedling leaf material following the hot CTAB method of Rogstad (1992). Two slight modifications were made to this protocol; small quantities of leaf material (2 cm x 2 cm) were mixed in 600 µl of extraction buffer and incubated at 65 °C for approximately two hours. The rest of the protocol was scaled according to this volume.

AFLP Protocols

The AFLP protocol followed was similar to that of Vos *et al.* (1995). DNA was first digested with two restriction enzymes, primer adapters were then ligated and two rounds of selective PCR followed. Total genomic DNA (300 ng) was digested with 5U each of *Eco* RI and *Mse* I (Life Technologies) using New England Laboratories buffer number 4 (NEB) in 40 µl for 1 hour at 37 °C. Dual digestion/ligation was then undertaken by the addition of a ligation mixture (10 µl) which contained 5 pmol of *Eco* RI adapter and 50 pmol of *Mse* I adapter, 1 µl NEB, 2 U of T4 DNA ligase and 1.0 mM ATP, for 3 hours at 37 °C. This was then diluted to 200 µl with TE⁻⁴ solution (10 mM Tris-HCl, 1mM EDTA, pH 8.0).

Two rounds of selective PCR amplification, to create an AFLP fingerprint, were then performed on this primary template. Primers with one and two or three extra extension nucleotides were used in the first and second round of amplification, respectively. The first

round of amplification reaction (20 μ l) contained 5 μ l of primary template, 50 ng of each primer, 0.2 mM of each dNTP, 0.25 U *Taq* polymerase (Goldstar, Eurogentec), 1.5 mM magnesium chloride, 75 mM Tris-HCl (pH 9.0), 20 mM (NH₄)SO₄ and 0.01% Tween 20. These preamplification products were then diluted 20 times with TE⁻⁴ and stored at -20 °C until used. The second round of amplification used the same PCR reaction protocol with the exception of 10 μ l of diluted first round PCR product and 5ng of 33P radiolabeled *Eco* primer. A PTC-200 (MJ Research) thermal cycler, with the following protocol, was used for both rounds of amplification: cycle 1, 94 °C at 1 min, 65 °C at 1 min, 72 °C 1:30 min; cycles 2-10, as for cycle 1 but with a 1 °C touchdown per cycle for the annealing temperature; cycles 11-32, 94 °C at 30 sec, 56 °C at 30 sec, 72 °C at 60 sec; cycle 33, 72 °C at 5 mins. Five micro-liters of products were separated on a 6% polyacrylamide sequencing gel.

Screening of AFLP markers

AFLP primers were screened for population studies. Different combinations of primer selective-nucleotide numbers were tested in order to optimize band number and ease of gel reading. Initial screening used three selective nucleotides for the *Eco* RI primer and two or three selective nucleotides for the *Mse* I primer. As the sex of all individual plants was known, populations were subsequently examined for markers that amplified in only one sex and not the other.

Testing Marker Association and Reliability

To determine whether a sex association could have arisen by chance a Fisher's-Exact test was performed. Null-alleles may arise due to mutation or recombination, resulting in males with two such alleles being incorrectly assigned as female. A 95% binomial confidence interval (Rohlf and Sokal, 1969) was determined to test the reliability of the marker for assigning sex. To test for the likelihood of Mendelian segregation of a marker, marker frequency was examined in seedlings from natural populations. A chi-square test for deviations from the expected 1:1 sex ratio among the progenies was performed.

AFLP Band Isolation and Sequencing

The sex-specific fragment was excised from a dried sequencing gel and eluted in water at 65 °C for several hours. PCR was performed on 5 μ l aliquots using the same AFLP primer combination used to generate the marker and 15 μ l PCR reaction mix (as used for first round

AFLP amplification, above). Thermal cycles were performed as follows: denaturing at 94 °C 2 min, then 1 min at 94 °C, 1 min at 56°C, and 1 min 30 sec at 72 °C for 30 cycles. PCR products were purified using the Qiagen PCR Purification Kit and resuspended in 30µl ddH₂O. The PCR fragment was ligated into a 'T' overhang vector using the P Gem-T Easy cloning kit (Stratagene). Transformation into XL1 Blue competent cells were performed by the heat-shock method as in Sambrook *et al.* (1989). Cells were grown by shaking at 200 rpm for 90 minutes in 3ml LB, and then plated onto selective media containing ampicillin, x-gal, and IPTG. Plates were incubated at 37°C over night. Six white colonies were selected and grown up overnight in 2 ml LB containing ampicillin and tetracycline at 37°C overnight with shaking. 1.5 ml of this culture was taken and minipreps were performed by the lysis-by-boiling method as in Sambrook *et al.* (1989). An aliquot of this was digested with 0.5 U of the restriction endonuclease *Eco* RI for 120 minutes and run on a 0.9% agarose gel containing 1X TAE Buffer to check for the presence of an insert. In all the white colonies an insert of the correct size was observed. Minipreps were then further purified using the Qiagen Miniprep Kit. Two of the clones were then sequenced using T7 and SP6 primers by the University of Leeds Biomolecular Sciences DNA Sequencing Facility.

Primer Design for Direct PCR of the Fragment

The sequences obtained for the two clones were identical in length. In order to design a more direct PCR method for the amplification of the male-specific fragment, forward and reverse PCR primers were designed against the sequence data and used in a PCR reaction containing 0.2 mM dNTP's, 3mM MgCl, 5 pmol each primer, 50 ng genomic DNA, and 0.4 U *Taq* polymerase (Promega) in a final volume of 20 µl. Thermal cycles were performed as follows: denaturing at 94 °C 4 min, then 30 sec at 94 °C, 1 min at 56°C, and 1 min at 72 °C for 35 cycles, followed by extension step of 2 mins at 72 °C and cooling of 2 mins at 25 °C. PCR products were visualized on a 1% agarose gel containing 1X TAE.

Chromosome preparations

For chromosome counts young root tips were pre-treated in a 1 % aqueous solution of 8-hydroxyquinoline at 4 ° C for 6 hours and fixed and stored in a 1:3 mixture of acetic acid and ethanol (Carnoy) at -20 ° C. Chromosome spread preparations were made according to Pijnacker and Ferwerda (1984). Chromosomes were visualized with DAPI (4',6 diamidino-propyl-indole) and counted under a fluorescence microscope.

Results

AFLP Marker Identification

Initial screening of AFLP primers using three selective nucleotides for both the *Eco* RI and *Mse* I primers produced a low number of bands. Therefore, further studies were carried out using two selective nucleotides for the *Mse* I primer. A total of nine primer combinations were screened before three were chosen for population studies.

The three primer combinations used produced 89 readily scorable polymorphic bands in three populations on the Krakatau islands (Panjang, Rakata and Anak Krakatau) (Table 5.1). One primer combination (*Eco*-AGG, *Mse*-CA primer # 2, Table 5.1) produced a marker that was present in all males (N=23, from three populations) but absent in all females (N=24, from three populations) (Figure 5.1). The probability that such a sex association could arise by chance is small (Fisher's Exact test: $P=0.62 \times 10^{-14}$). As no size markers were used in AFLP analysis this band was excised, re-amplified and viewed on an agarose gel. The resulting band was 246 bp in length.

If sex is determined by a single locus with one of the sexes heterogametic, a 1:1 ratio of male and females among the offspring of either controlled or open-pollinated crosses would be expected. To test the likelihood of Mendelian inheritance, the frequency of the male-specific marker was examined in an array of open pollinated seedlings. Seedlings were raised from five open-pollinated mothers (a total of eight fruits per population) from each of two populations (Rakata and Anak Krakatau). Seedling samples were pooled for each population, as within-fruit seedling sample sizes were too small for individual statistical analyses. When both populations were pooled, the segregation ratio did not differ significantly from a 1:1 ratio (78 individuals with the marker present from 156), expected for a single dominant locus ($\chi^2=0.00$, $P=1.00$, Table 5.2).

Sex-Marker Sequence and Homology

The sequence of the cloned sex-marker is given in Figure 5.2. The fragment is 224 basepairs in length and is shorter than that observed on gels because the sequence generated from the adapters used in AFLP have not been included. A search on the Genbank BLAST website (<http://www.ncbi.nlm.nih.gov/BLAST>) identified no homologous sequences.

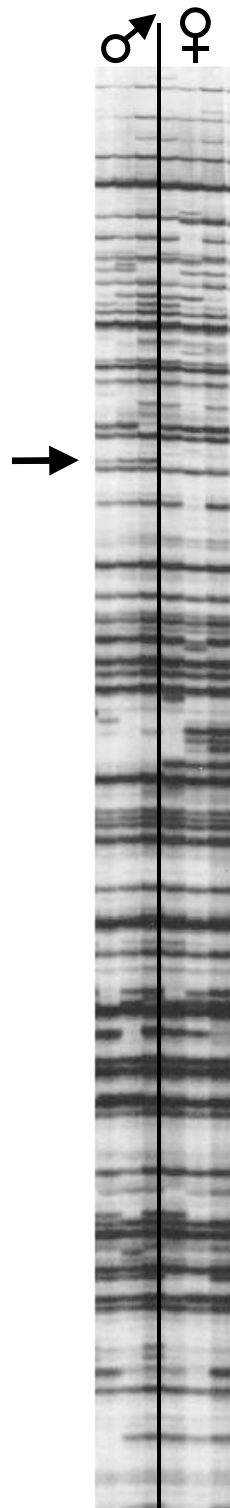


Figure 5.1: An AFLP profile showing the 246 bp male-specific band (arrow) in a population sample of three males and three females.

Table 5.1: AFLP selection sequences used in population studies and screened for male-specific markers. Primer combination 2 produced a male-specific marker. The number of polymorphic and fixed bands that were generated is listed.

Primer Comb. #	<i>Eco</i> Extension	<i>Mse</i> Extension	# Bands Polymorphic	# Bands Fixed
1	5'-AGA-3'	5'-CT-3'	36	26
2	5'-AGG-3'	5'-CA-3'	27	37
3	5'-ATC-3'	5'-CA-3'	26	36
Total Number of Bands			89	99

Table 5.2: Segregation of the marker in 16 fruits (approximately 10 seeds per fruit) from five mothers from each of two populations, Anak Krakatau and Rakata. Also indicated is a chi-square test for deviations from the expected 1:1 sex ratio among the progenies.

Population	# female trees	# fruits	# progeny	Male marker	Female marker	X² (d.f.=1)	P
Rakata	5	8	77	40	37	0.117	0.73
Anak	5	8	79	38	41	0.114	0.74
Total All	10	16	156	78	78	0.000	1.00

Primer Design and Amplification

In order to develop a direct PCR technique to amplify male-specific DNA two primer sets based on the cloned sequence were designed (Figure 5.2). Primer amplification was tested on DNA from 3 male and 4 female *F. fulva*. However, amplification products (of the same size) were observed in both sexes, thus indicating that there is considerable sequence homology between males and females.

Karyology

In six seedlings the somatic chromosome number of 26 was counted. The chromosomes were small and in none of the six seedlings was a pair of heteromorphic homologous chromosomes observed.

```

1  TTAACATCAT  ACTTGAAAAT  ATTTTCTTAC  TTGAAACCAA  ATCTCATTCT
51 TATCATGTGA  ATATTATTAG  TGAGCATGCT  TCATGATGTC  ACAACAACAT
101 AGCATAAACC  ATAAGCAACT  CAACATGTAT  GCATGAGCTT  GAAAACATAC
151 AAGCACTCAA  AACCAATTCTT  TTATCTATGT  TGTAGGCGGA  GTTATACTTA
201 TCGGTTATGC  TAATTCCTGA  ATTC

```

Primer design.

Set 1

5' TAA CAT CAT ACT TG 3' F1 (forward)

5' CAG GAA TTA GCA TAA CCG 3' R1 (reverse)

Set 2

5' CTC ATT CTT ATC ATG TG 3' F2 (forward)

5' GCA TAA CCG ATA AC 3' R2 (reverse)

Figure 5.2: The 224 base-pair sequence of the male-specific fragment obtained through cloning. The sequence length is shorter than the AFLP fragment observed in gels as the adapters used in AFLP have been excluded from the sequence. The ends of the sequence are defined by the *Mse* I and *Eco* RI recognition sites, that were used initially to create the AFLP fragment. Below, are the two primer sets designed from the sequence.

Discussion

In this study a male-specific AFLP marker in the functionally dioecious fig species, *Ficus fulva* has been identified and sequenced. The association between the AFLP marker and the male sex was not established in a cross, but in natural populations. There was strong linkage disequilibrium between the marker and the sex of the trees. Although linkage disequilibrium may be caused by other factors, we consider close chromosomal linkage as the most likely explanation for the linkage disequilibrium. Founder effects and genetic drift could have occurred during the colonization of the Krakatau islands. However, these factors would have generated genome-wide AFLP-sex linkage disequilibria, which is not the case here. Other signatures of founder effects, like loss of rare alleles and loss of heterozygosity are also not obvious in the present AFLP material (Chapter, 2). Moreover, the 1:1 band segregation in the open-pollinated material supports a chromosomal linkage of the marker to the sex-determining factor.

The identification of a male-specific marker for *F. fulva* supports two observations made by Storey (1955) on sex-determination in *F. carica* (the edible fig). Given the dominant nature of AFLP markers this result is in concordance with Storey's (1955) observations in *F. carica* that males in *Ficus* are the heterogametic sex. Second, the identification of a sex-specific marker strongly suggests that sex determination in *F. fulva* is, as hypothesized for *F. carica* (Storey, 1955), under simple genetic control, possibly by a single gene or a small group of tightly linked genes. If sex-determination was under the control of multiple, unlinked loci, recombination would prevent the identification of a single male-specific marker. The chromosome number in *F. fulva* appeared to be 26, which is the common diploid chromosome number in the genus (Condit, 1964). As in other functionally dioecious *Ficus* species (Condit, 1964), we found no evidence for morphologically distinct sex chromosomes.

A primary concern when using PCR or restriction based methods for the assignment of sex is the reliability of the marker. Males may possess two null alleles (because of mutation or recombination between the marker and the sex-determining locus), resulting in such males being incorrectly assigned as female. All 23 males that were screened carried the diagnostic AFLP band. The corresponding 95 percent confidence interval ranges from 88 to 100 percent (Rohlf and Sokal, 1969). The fact that the marker segregated in an exact 1:1 ratio (78 marker present and 78 absent) in 158 seedlings in open pollinated arrays further supports the reliability of the marker for predicting sex. Such a marker can be used to determine sex of pre-adult and non-flowering individuals which is for example useful in studies on sex ratio distortion where the distortion level varies with the life stage (Eppley, 2001).

The male-specific AFLP marker was discovered fortuitously during population studies. Few primer combinations (producing a total of 89 polymorphic bands) were examined before the marker was found. This has not been the case in many other studies searching for markers linked to sex in other plant species. Often hundreds of RAPDs must be screened before a single sex-linked or sex-specific marker is found, as was the case for *Atriplex garrettii* (Ruas *et al.*, 1998), *Pistacia vera* (Hormaza *et al.*, 1994) and *Salix viminalis* (Alstrom-Rapaport *et al.*, 1998) (but see Mulcahy *et al.*, 1992 for a fortuitous case in *Silene latifolia* which has an Y chromosome). While the results from this study may have been fortunate, it highlights that the AFLP technique may be highly suitable for further studies seeking to identify sex-specific markers in *Ficus*. Large numbers of polymorphic loci are generated with each AFLP primer combination, more so than with other techniques such as RAPD (Griffiths and Orr, 1999), allowing for a more extensive screening of the genome with each primer combination. Current AFLP studies in a second *Ficus* species, *F. montana* (used as a model for population studies in *Ficus*), have also identified potential sex-specific markers (S. Ahmed, pers. comm.).

In this study, the male-specific marker was detected by examining individual fingerprint banding patterns in population studies where the sex of individuals was known. However, typically when searching for character-linked DNA it is more efficient to use Bulk Segregant Analysis (BSA) (Michelmore *et al.*, 1991) where DNA from a number of individuals of the same sex is examined in one PCR reaction. This greatly reduces the number of reactions needed when screening for markers linked to sex. Parrish (unpublished data) has identified that AFLP-BSA can be performed on up to ten *F. fulva* individuals while still producing reproducible banding patterns. This will be of value in future studies searching for sex-markers in *Ficus*.

Despite the advantages of AFLP, this method is more expensive and time-consuming than direct PCR. Therefore, if an inexpensive and efficient screening technique for assigning sex is to be developed, PCR primers specific for the cloned sequence should be made. Two sets of sequence-specific primers were designed in this study. However, when tested these primers produced amplification products of the same size in both male and female DNA samples and therefore are not suitable for use in assigning sex. Interestingly, two conclusions can be drawn from these results. First, there is considerable sequence homology between males and females at this chromosomal region. Second, given that one primer overlaps the *Mse* I recognition site, and there is no size difference observed between the fragments

produced in males and females, the absence of the AFLP marker in females is likely due to the absence of the *Eco* RI recognition site.

No sequences homologous to the fragment identified in this study were found in other species when the GenBank sequence database was searched. This result is not surprising considering the diversity of sex-determination systems in plants, or our limited knowledge of the mechanism of sex-determination in plants and the size of the fragment relative to the average plant genome.

Acknowledgements

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Summarizing Discussion

The objective of this study was to examine the genetic effects of colonization of some tree species on the Krakatau islands, Indonesia. As natural habitat is rapidly being reduced in area, and suffering increased isolation due to human habitat destruction, it is becoming increasingly important to document biodiversity and to understand the processes involved in its maintenance. Colonization of new sites plays an important role in natural systems as it offsets loss due to extinction. The study of colonization is becoming increasingly important if conservation becomes centered around restoration.

The effect of colonization on genetic variation and structure within and between populations in a species can be complex and depends greatly on the way a new population is formed (McCauley, 1991). Mathematical models have demonstrated that the number of individuals involved in the initial founding event (Nei *et al.*, 1975) and the number of source populations that colonists are sampled from are important factors in structuring patterns of genetic diversity within and between populations (Slatkin, 1977; Wade and McCauley, 1988). If colonists are drawn from a single source, differentiation between sites is increased. However, when colonists are drawn from many populations, overall differentiation between populations is reduced. Small population size, limited source pool sampling, low population growth rate and long distance dispersal are known to increase founding effects, drift and inbreeding, all of which have negative effects on genetic diversity and the likelihood of population survival (Nei *et al.*, 1975; reviewed in Barrett and Husband, 1989; Falconer, 1981). Island populations, due to their isolated nature (limiting the number of migrants) and finite habitat area (small population size), are more likely to be affected by such processes than populations on larger landmasses.

The Krakatau islands are a unique, well-documented natural experiment site for the study of island colonization in the tropics. A series of volcanic eruptions in 1883 completely destroyed all life on and greatly altered the landscape of the islands (Verbeek, 1884; Docters van Leeuwen, 1936). Numerous scientific expeditions (though sporadic and not uniform in purpose) documented the return of life to the islands and have allowed for the development of models describing the restructuring of the forests. Reforestation has followed a successional pathway shaped by the dispersal characteristics of the colonists (Whittaker *et al.*, 1989;

Whittaker and Bush, 1993). Though the successional processes in which forest communities have restructured on Krakatau is well studied (Whittaker *et al.*, 1989; Whittaker *et al.*, 1992a; Whittaker and Bush, 1993; Whittaker and Jones, 1994a), little is known about the origins of new populations and how colonization has affected their genetic restructuring. This project is the first study to have used population genetic methods to examine patterns and processes in the colonization of the Krakatau islands.

Species Dispersal Ability and Genetic Variation

Various life-history traits have been identified as important in determining patterns of genetic polymorphism within plant species as a whole (Hamrick and Godt, 1989; Hamrick *et al.*, 1992). Loveless (1992) found that in tropical tree species geographical range (endemic versus widespread), growth form (shrubs versus trees) and mode of dispersal (abiotic versus biotic) had significant effects on genetic diversity (P , proportion of polymorphic loci and H , heterozygosity). Dispersal type was the only factor that showed a significant effect on population differentiation (Hamrick and Loveless, 1989) with biotic dispersed species having lower levels of differentiation between populations than for species with abiotic dispersal agents (Loveless, 1992). Another factor, breeding system, has been shown in plants as a whole to be important in determining genetic diversity (Hamrick and Godt, 1989; Hamrick and Loveless, 1989; Hamrick *et al.*, 1992).

Comparisons of levels of genetic diversity between the study species confirmed that dispersal ability is important in determining levels of genetic variation within a species. The study species were grouped into three genetic mobility groups based on their dispersal ability; all *Ficus* species have high genetic mobility, *Dysoxylum gaudichaudianum* moderate and *Oroxylum indicum* demonstrated the lowest genetic mobility. Factors considered important for mobility were the dispersal syndrome (abiotic versus biotic), the relative number of dispersal agents, seed size and pollination system. Genetic diversity was highest in species with high inferred genetic mobility (*Ficus*) and it decreased as a species became less genetically mobile. The study species shared life-history characters that have been shown to have a significant effect on structuring variation (growth form, geographical distribution and breeding system, except *O. indicum* whose breeding system was undetermined) thus excluding the interaction of these factors in patterns of genetic diversity. Species with high inferred genetic mobility demonstrated low levels of population differentiation as gene flow is high. Levels of differentiation between populations increased with decreasing inferred genetic mobility.

Genetic Variation

Levels of genetic diversity on islands are expected to be lower than that on comparable mainland sites due the effects of isolation and population size (Jaenike, 1973). However, this depends on whether distance to an island is outside that normally encountered by the species in question and whether this distance is greatly limiting dispersal. The general conclusion of this study is that colonization has had little effect on genetic diversity in most of the study species on the Krakatau islands. This result is counter to initial expectations. In four of the five study species (*D. gaudichaudianum*, *F. pubinervis*, *F. fulva* and *O. indicum*) levels of genetic diversity in Krakatau populations are equivalent to or higher than that observed in populations on Java, Sumatra and other islands in the Sunda Strait. In three of these species, *D. gaudichaudianum*, *F. pubinervis* and *F. fulva*, this effect is thought to be mainly due to the large size of island populations. Also, the distance to the Krakatau islands was thought not to be greatly limiting dispersal (and hence geneflow) in species with moderate (*D. gaudichaudianum*) to high (all *Ficus* species) dispersal ability. The empty nature of the post-eruption environment (Verbeek, 1885), continuing disturbance regimes (Schmitt and Whittaker, 1998) and isolation reducing the colonization of later seral species (Whittaker *et al.* 1997) are all factors which will work to maintain large population size in certain early colonist groups.

Interestingly, in two cases the size of new populations and species dispersal ability were not good predictors for the consequences of colonization on genetic diversity. Against expectation, high genetic diversity was maintained in the study population of *O. indicum* on Krakatau regardless of small population size and extremely poor dispersal ability of this species. Colonization of Krakatau by propagules from genetically different sources may be the reason for this increase in genetic diversity.

The second unexpected result in this study was that the only species to show decreased levels of genetic diversity on Krakatau was *F. fistulosa*, despite this species having high dispersal abilities and large population size on some of the islands. Only one Krakatau population (Sertung) was examined. It is tentatively proposed that volcanic disturbance on Sertung may greatly effect plant mortality and breeding structure and hence levels of inbreeding in *F. fistulosa* on this island.

Important to note is that the lowest levels of genetic diversity observed in all species were observed in mainland sites. Ongoing habitat fragmentation due to human activity (Whitten *et al.*, 1996) may be the reason for this result. It is of interest to see such patterns in

common, weedy and widespread species such as examined in this study, which would be expected to be more immune to the effects of habitat fragmentation.

Due to the long life span of trees, the negative genetic effects of small population size and post-colonization processes may take some time to manifest. Therefore, one could question whether the Krakatau experiment has been running long enough to see the long-term genetic effects of colonization in the species studied. In species such as *F. pubinervis* and *D. gaudichaudianum*, which do not reproduce until large trees and are long-lived (*F. pubinervis*), this may be the case. Many trees of *F. pubinervis* on the Krakataus may be the original colonists. However, species such as *F. fistulosa* and *F. fulva* are likely to have seen multiple generations since founding. These species were fruiting within 10 years of colonizing Krakatau (Ernst, 1908) and can reproduce at less than 10 cm diameter at breast height (Parrish, pers. observ.). Therefore, time since colonization may be long enough to see post-colonization effects and this is likely the case for *F. fistulosa*. The population of *O. indicum* on Rakata was first recorded in 1994 (Partomihardjo, 1995), though the presence of adult and senescent adult trees indicates an earlier time of colonization. Nonetheless, it seems unlikely that many generations have passed.

Patterns of Colonization

Examining patterns of genetic differentiation between sites and ordination methods have provided considerable insight into colonization processes of the study species to the Krakatau islands. Three factors have been of predominant importance in determining patterns of colonization on the islands: species dispersal capability, distance to the islands, and position of the islands relative to mainland source populations. However, these factors were not of uniform importance in all species. Dispersal characteristics of the species will effect overall gene flow between populations and determines the effect distance has on limiting dispersal. Patterns of colonization to a new site will vary between species due to differences in dispersal ability and other features of their biology. The Krakatau islands are situated approximately mid-way between Java and Sumatra, although areas of the Sumatra coast are approximately 10 kilometers closer. Therefore, if distance is greatly limiting dispersal it would be expected that the Sumatra region be contributing significantly more to the new Krakatau gene pool.

Colonization in *F. fistulosa* and *F. fulva* likely follows the Migrant Pool Model of Slatkin (1977) where an extensive number of propagules sampled from a diverse number of source populations occurred. While isolation-by-distance was observed, gene flow was high, indicating that though distance is important in structuring genetic diversity the distances

covered to the Krakatau islands were not greatly limiting dispersal. No regional structuring was observed between Java and Sumatra or Krakatau populations, thus indicating that there was no structure in colonization and that colonists were likely sampled from both regions. There was no significant differentiation between populations of *F. fulva* on the three Krakatau islands, indicating that these populations act as a single panmictic group and that colonization processes were likely to have been similar between all islands.

Patterns of dispersal in *F. pubinervis* over the study area were unclear. While having high dispersal ability and gene flow between populations like other *Ficus* species, *F. pubinervis* demonstrated no isolation-by-distance. High differentiation was observed between the single Sumatra population examined in this study and all other sites. This differentiation is likely due to small population effects in this population and not to regional differentiation. Low levels of differentiation existed between the Krakatau and Java populations. However, an absence of isolation-by-distance likely reflects unequal contribution from mainland sites to the new Krakatau genepool. Differential damage to mainland sites during the Krakatau eruptions, in combination with the long-lived nature of the species, may be one reason for these patterns.

In *D. gaudichaudianum* patterns of dispersal were much more limited by distance and therefore more directional than that of *Ficus* species. This species demonstrated isolation-by-distance and moderate restrictions to gene flow over the study area indicating that dispersal will be limited over shorter distances than more genetically mobile species such as *Ficus*. Positioning of the islands relative to landmasses has played an important role in determining colonization patterns and levels of genetic structuring in this species. Strong patterns of relationship between Krakatau populations and the nearest landmasses, Sumatra and Sebesi (an island halfway between), were observed indicating that the nearest mainland sites play a predominant role in supplying immigrants to the new Krakatau populations in this species. However, moderate levels of differentiation between islands indicate some divergence in colonization patterns between the islands. Sertung, the westernmost of the Krakatau islands, demonstrates the lowest levels of differentiation with Sumatra (lower than between islands) indicating that colonization to this island has been dominated by immigration from Sumatra. However, Panjang which is east in the archipelago demonstrates some ordination overlap with Java populations, therefore indicating a mixed colonization patterns relative to Sertung.

Drift and chance events have played a major part in the colonization of *O. indicum* to the Krakatau islands as distance is greatly limiting dispersal in this species over the scales of this study. The species most closely resembles Slatkin's (1977) Propagule Pool Model of

colonization where it is expected that a small number of immigrants sampled from few source regions have led to high levels of differentiation between sites. A likely source region for colonists to the Krakatau islands could not be determined as no patterns of relationship could be identified between the single Krakatau population and any mainland site or region.

Sebesi as a Stepping-stone for Colonization to the Krakatau islands

If distance is greatly limiting dispersal, smaller islands between Krakatau and Sumatra may act as stepping-stones that facilitate dispersal and gene flow. Sebesi island, may be one potential stepping-stone (Dammerman, 1922, 1948) because it is closest to the islands and is similar in size to Rakata. The vegetation of Sebesi was greatly damaged during the 1883 eruptions (Verbeek, 1885). However, damage is considered to have been less than on Krakatau as within nine months plants were reshooting from underground seeds/parts (Cotteau, 1886) and by 1921 the development of vegetation was considerably more advanced than that of Krakatau (Docters van Leeuwen, 1923). However, agriculture has been practiced on Sebesi since the 1890s (Thornton, 1996) and by the 1970s vegetation on Sebesi was confined to the upper regions of the island. Whittaker *et al.* (1992a) thus reached the conclusion that the island may now be lost as a stepping-stone and patterns of relationship will be hard to trace.

Of the species in this study only one, *O. indicum*, was recorded on Sebesi prior to its arrival on Krakatau (Whittaker *et al.*, 1992a). This species has poor dispersal ability and therefore stepping-stone islands would be expected to play an important role in dispersal in this species. However, the results of this study do not support Sebesi as a stepping-stone to the colonization of Krakatau by *O. indicum* as levels of differentiation between Krakatau and Sebesi are high despite a strong pattern of relationship between Sebesi and the Sumatra mainland.

Low levels of differentiation between Sertung, Sebesi and Rajabasa would seem to support Sebesi as a stepping-stone to colonization in *D. gaudichaudianum*. However, caution must be exercised in interpreting these results. *D. gaudichaudianum* was not observed on Sebesi in expeditions there in 1921 (Docters van Leeuwen, 1923) and 1979 (Whittaker *et al.*, 1992a) and was only collected during this study from a small population in 1996. Given that populations of *D. gaudichaudianum* are large on Krakatau, the close relatedness observed between Sebesi and Krakatau may in fact represent colonization of Sebesi from Krakatau.

Given the high level of gene flow observed in *Ficus* species and the absence of regional structure throughout the study area, islands such as Sebesi, while facilitating gene

flow are unlikely to be critical in determining levels of dispersal to the Krakatau islands in *Ficus*.

Other Genetic Consequences - Hybridization

Hybridization between closely related functionally dioecious species of *Ficus* was detected on the Krakatau islands and other disturbed island sites. These results have important implications as *Ficus* species maintain a tight species-specific pollination mutualism with Agaonidae wasps. Hybrid events and breakdown in the pollination mutualism have been previously reported; however, nearly all cases involved phylogenetically and geographically disparate species brought into sympatric populations (Condit, 1950; Ramirez, 1986; Ramirez and Monterro, 1988; Ramirez, 1994) or isolated individuals outside their normal distribution (Compton, 1990; Ware and Compton, 1992). On rare occasions, putative hybrid individuals determined by morphological characters have been identified (Ramirez, 1970) but have not been previously confirmed using genetic techniques. This study is the first to do so.

While the hybrids recognized in this study were rare, they were detected at three different sites, indicating that conditions suitable for hybridization were present at more than one site. Hybrids were only identified on islands. This suggests that conditions exist on islands that may favor hybrid formation. It is proposed that limited tree population size and island isolation may lead to pollinator limitation and subsequent increased amplitude in plant mate selection. Due to the relatively small numbers of hybrids that are formed, little effect on species evolution is expected. However, this amplitude for mating flexibility may be of more considerable evolutionary importance when colonization occurs at more isolated locations.

Future Research Directions

There is considerable forest structure divergence between the three older islands of the Krakatau archipelago. Rakata is considerably more diverse than Panjang and Sertung, with Sertung being the least diverse. One driving factor in this divergence is disturbance regime. Sertung and Panjang are greatly affected by ash fall from the volcanic island Anak Krakatau, though Sertung is more greatly affected (Whittaker *et al.*, 1989; Whittaker *et al.*, 1992b; Schmitt and Partomihardjo, 1997; Schmitt and Whittaker, 1998). Rakata nonetheless suffers high levels of disturbance due to other factors such as landslides and extreme climatic events (Schmitt and Whittaker, 1998). Ash fall has the effect of setting successional clocks back and has been associated with changing species dynamics (Whittaker *et al.*, 1989; Bush *et al.*, 1992; Schmitt and Partomihardjo, 1997; Schmitt and Whittaker, 1998). Therefore, there is

opportunity within the Krakatau islands to study the genetic effects of disturbance and changing population dynamics. One species greatly benefiting from volcanic disturbance: *Dysoxylum gaudichaudianum* was examined in this study and was observed to maintain high levels of genetic diversity on the islands. However, species such as *Timonius compressicaulus* and *Neonauclea calycina*, though dominant in many areas, are now thought to be suffering population declines (Schmitt, 1997) and may provide an opportunity to study the genetics of populations in decline.

It was tentatively proposed in this study that volcanic disturbance on Sertung may be the cause for the lower levels of diversity observed in *F. fistulosa* on this island. However, levels of genetic diversity have not been estimated for this species on the other Krakatau islands. A further comparative study of genetic variation between islands would be instrumental in determining if disturbance plays an important role in structuring genetic variation. High mortality in plant and mutualist pollinator populations may effect breeding structure in *F. fistulosa*. Pollinator limitation has been observed in some populations of *F. fulva* on the Krakatau islands (Compton *et al.*, 1994; Parrish, unpub. data) and other populations of dioecious *Ficus* suffering environmental perturbation (Harrison, 2000). Therefore, further genetic studies combined with studies of pollinator populations would be of interest. Currently, Monika Zavodna of the Netherlands Institute of Ecology is undertaking such studies in two species of *Ficus*, *F. septica* and *F. montana*. She is developing microsatellite markers for both *Ficus* species and their associated pollinators with the intention of studying colonization processes.

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Thesis Summary

This research has examined the genetic consequences of colonization in five tree species on the Krakatau islands, Indonesia after habitat had been destroyed over a century earlier by extreme volcanic events. The Krakatau islands provide a unique, well-documented example of island colonization in the tropics. The islands are continental and lay approximately midway between Java and Sumatra; the nearest points on these landmasses to the islands being approximately 41 and 31 km, respectively. Currently the islands are covered in species poor, early seral monsoonal forest that is little disturbed by humans. With the increasing levels of habitat destruction occurring in the tropics due to human mediated environmental degradation, it is important to understand the processes involved in ecosystem restoration. This is the first study to use genetic markers to examine the genetic consequences and patterns of colonization on these islands.

Chapter 1 provides a general introduction to the thesis. The theoretical underpinnings of the research, the study system and research objectives are outlined. The effect of colonization on genetic variation and structure within and between populations in a species can be complex. The number of individuals involved in the initial founding event, the number of source populations that colonists are sampled from and the amount of ongoing migration are important factors in determining levels of genetic diversity within and between populations. Small founding population size, limited source pool sampling, low population growth rate and long distance dispersal are known to increase founding effects, drift and inbreeding, all of which have negative effects on genetic diversity and the likelihood of population survival. Island populations, due to their isolated nature (limiting dispersal) and finite habitat area (small population size) are more likely to be affected by such processes than populations on larger landmasses.

The objectives of this study were as follows; i) determine the effect of long-distance colonization on genetic diversity by comparing levels of genetic diversity on the islands after colonization with that of mainland populations, ii) determine if species dispersal ability affects levels of within population genetic diversity, iii) document current patterns of genetic differentiation between populations to determine levels of gene flow between sites, identify patterns of colonization and consider the importance of distance, species dispersal ability and island positioning to colonization and iv) document other genetic effects of colonization.

In **Chapter 2** levels of genetic diversity (using AFLP) in populations of five tree species on the Krakatau islands were compared with that of populations on the mainland and other islands closer to the mainland. Five tree species, *Ficus fulva*, *F. fistulosa*, *F. pubinervis*, *Dysoxylum gaudichaudianum*, and *Oroxylum indicum* were chosen for study. All species have obligate outcrossing breeding systems (except *O. indicum* whose breeding system is unknown) and are common widespread tree species in Southeast Asia. The species can be grouped into three different dispersal groups; high (all *Ficus* species), moderate (*D. gaudichaudianum*) and poor dispersal ability (*O. indicum*).

Counter to expectation genetic diversity in populations on the Krakatau islands in four of the five study species (*F. fulva*, *F. pubinervis*, *D. gaudichaudianum*, and *O. indicum*) was equivalent to or higher than all other sites. The establishment of large population size during colonization and moderate to high dispersal abilities are factors likely to be maintaining genetic diversity in populations of *D. gaudichaudianum*, *F. fulva* and *F. pubinervis* on Krakatau. In contrast *O. indicum*

Summary

has poor dispersal ability and small population size on Krakatau yet has higher genetic diversity on Krakatau relative to all other sites. In *F. fistulosa* genetic diversity in the one Krakatau population (Sertung) studied was considerably lower than many mainland sites despite the species high dispersal capabilities. An increase in inbreeding due to volcanic disturbance and pollinator limitation is posed as the cause for the higher relatedness observed between individuals in this Krakatau population, though more conclusive studies need to be performed. The lowest levels of within population polymorphism in all species were identified in some populations on the mainland or islands closer to the coast than Krakatau. This likely reflects the ongoing human mediated habitat degradation at these sites.

Species dispersal ability was important in determining levels of genetic diversity within a species as is reported for tropical tree species and plants as a whole. Species with higher dispersal ability had higher levels of within population polymorphism than those with poorer dispersal ability. However, dispersal ability was not necessarily able to predict the consequences of colonization in individual populations (as mentioned above).

In **Chapter 3** patterns of colonization to the islands and levels of gene flow between populations were examined in the same species as Chapter 2 (using the same AFLP data set). Colonization of the Krakatau islands is strongly determined by the dispersal ability of each species, distance to the islands and the relative positioning of the islands to other landmasses.

In *F. fistulosa* and *F. fulva* low levels of differentiation between populations indicates that gene flow is extensive over the study area and that the distance to the Krakatau islands does not limit dispersal. No ordinal differentiation was observed between regions thus indicating that migrants to Krakatau were likely drawn from Sumatra and Java with equal frequency. Islands such as Sebesi, that are thought to be possible stepping-stones to colonization, are unlikely to have a significant role in patterns of dispersal in these species.

Patterns of colonization were more complicated in *F. pubinervis*. Isolation-by-distance was not observed and indicates that variation in dispersal patterns over the study area occurred, despite the species having high dispersal ability. The high differentiation observed between the single Sumatra population in this study and all other sites is likely due to small population effects at this site and not regional differentiation. It seems likely that populations from Java contributed unequally to the new Krakatau gene pool due to differential population damage during the Krakatau eruptions.

Levels of gene flow in *D. gaudichaudianum* were more restricted than for *Ficus* species, indicating that distance will become more limiting over smaller spatial scales. A strong relationship was observed between the Krakatau islands and the closest landmasses (Rajabasa and Sebesi Island, Sumatra) indicating that the nearest land mass to the islands will be contributing most significantly to the Krakatau gene pool. Higher levels of differentiation between populations of the Krakatau islands than to nearest landmasses indicate that the relative positioning of the islands to other landmasses will also affect colonization patterns. Low differentiation between Sertung to Sebesi and Rajabasa would indicate that Sebesi is a stepping stone for colonization to Sertung from Sumatra. However, absence of this species from Sebesi in expeditions prior to this study may indicate that the present patterns may instead reflect colonization of Sebesi with propagules from the Krakatau islands.

Gene flow in *O. indicum* is greatly restricted over the area of this study, thus indicating that distance is likely limiting dispersal. Colonization in this species likely involves largely chance events

and is greatly affected by drift. The Krakatau population showed no clear relationship to other regions. Islands such as Sebesi are unlikely to be stepping-stones for colonization.

In **Chapter 4** AFLP and chloroplast markers were used to document hybridization between *F. septica* and two other species of closely related, early colonizing, dioecious *Ficus*, *F. fistulosa*, *F. hispida*. The occurrence of hybridization within natural populations inside the normal range of the species is of significance as *Ficus* species maintain a tight species-specific pollination mutualism with Agaonidae wasps. Previous known cases of hybrid events and break down in the pollination mutualism have involved phylogenetically and geographically disparate species brought into sympatric populations or occurred in isolated individuals outside their normal distribution. Putative hybrids determined by morphological characters are rarely identified and have not been previously confirmed using genetic techniques. This study is the first to do so.

Hybrids as identified in this study, are rare. Nonetheless, identification of a total of six such individuals spread over three independent sites indicates that factors favoring hybridization are not restricted to a single site. Hybrids were identified only on island populations, usually in disturbed or recently disturbed sites. Hybridization events occurred at sites where one of the parental species is or was previously rare or absent, indicating that small population size plays an important role in driving hybridization events. It is proposed that the breakdown in the breeding system specificity is due to pollinator limitation driving individual plants to have reduced selectivity in mate choice. The hybridization events observed in this study have little effect on species integrity. Various authors have concluded that hybridization has an insignificant role in *Ficus* evolution. Nonetheless, hybridization may be important as an evolutionary process in more isolated island systems where dispersal events are greatly limited and where extinction of one species (either host or pollinator) is more likely.

The findings of **Chapter 5** do not relate to issues involved in colonization of islands but rather present some fortuitous results and emphasize the value of markers such as AFLP in population and ecological studies. A 224 basepair length male-specific marker was identified during population studies in the functionally dioecious species, *F. fulva*. The presence of the marker in all males studied and an exact 1:1 segregation ratio in progeny arrays supports a strong linkage of the marker and its suitability for use in assigning sex. The identification of a male-specific marker supports two observations on sex-determination in *F. carica* (and presumably is applicable to *Ficus* as a whole). First, the presence of a sex marker indicates that sex-determination in *Ficus* is under simple genetic control. Second, given the dominant nature of AFLP markers the presence of a male-specific marker confirms that males are the heterogametic sex.

In **Chapter 6** the final conclusions of this study are outlined. Species dispersal ability, distance to the islands, island positioning and ecological factors are all important in structuring genetic diversity. Species with high dispersal ability maintain higher levels of genetic diversity within populations and lower levels of differentiation between populations relative to species with poorer dispersal ability. Dispersal ability alone was not a good predictor for the effect of colonization on genetic diversity within populations as other ecological factors are also important in structuring genetic variation. Counter to expectation, most of the study species maintained equivalent or higher levels of genetic diversity on Krakatau relative to mainland sites and other islands in the Sunda Strait closer to the coast. Small plant population size and island isolation is thought to be an important factor leading to hybridization in some species.

Samenvatting

In dit onderzoek worden de genetische gevolgen van kolonisatie voor vijf boomsoorten op de Krakatau eilanden, Indonesië, onderzocht, nadat dit habitat meer dan een eeuw geleden door extreme vulkanische gebeurtenissen was vernietigd. De Krakatau eilanden vormen een uniek, goedgedocumenteerd voorbeeld van eiland kolonisatie in de tropen. De continentale eilanden liggen ongeveer halverwege Java en Sumatra; de kleinste afstanden tot deze landmassa's zijn respectievelijk ongeveer 41 en 31 kilometer. Tegenwoordig zijn de eilanden bedekt met een soorten-arm, vroeg-succesie moesson-bos dat weinig is verstoord door menselijke activiteiten. In verband met de voortschrijdende vernietiging van habitats in de tropen als gevolg van de aantasting van het milieu door menselijk handelen, is het belangrijk de processen te begrijpen die betrokken zijn bij het herstel van ecosystemen. Dit is de eerste keer dat er gebruik gemaakt wordt van genetische merkers om de genetische gevolgen en patronen van kolonisatie van deze eilanden te bestuderen.

Hoofdstuk 1 geeft een algemene inleiding voor het proefschrift. The theoretische basis voor het onderzoek, de studiesystemen en de onderzoeksdoelstellingen worden uiteengezet. De effecten van kolonisatie op genetische variatie en structuur binnen en tussen populaties van een soort kunnen complex zijn. Het aantal individuen dat bij de oorspronkelijke *founder* gebeurtenis betrokken is, het aantal bron-populaties waaruit kolonisten afkomstig zijn en de migratie na kolonisatie zijn belangrijke factoren, die de mate van genetische diversiteit tussen en binnen populaties beïnvloeden. Een kleine *founder* populatie, een beperkte bemonstering van bron-populaties, een geringe populatiegroeisnelheid en zeldzame lange-afstandsverspreiding zijn alle factoren die *founder* effecten, *genetic drift* en inteelt versterken; processen die op hun beurt alle een negatief effect hebben op de genetische diversiteit en de waarschijnlijkheid van populatie-overleving. Eilandpopulaties hebben, als gevolg van hun geïsoleerde aard (dispersie beperking) en begrensde habitat oppervlak (geringe populatie grootte) een grotere kans door deze processen beïnvloed te worden dan populaties van grotere landmassa's.

De doelstellingen voor dit onderzoek waren de volgende: i) het effect van lange-afstandskolonisatie op de genetische diversiteit te bepalen door de niveaus van genetische diversiteit op de eilanden na kolonisatie te vergelijken met die van de vasteland-populaties, ii) te onderzoeken of het verspreidingsvermogen van een soort de niveaus van genetische diversiteit binnen populaties beïnvloedt, iii) het documenteren van de huidige patronen van genetische differentiatie tussen populaties om hieruit de hoeveelheid *gene flow* tussen populaties, de patronen van kolonisatie, en het belang van afstand, verspreidingsvermogen en eilandligging voor kolonisatie af te leiden, en iv) het documenteren van andere genetische effecten van kolonisatie.

In **Hoofdstuk 2** werden de niveaus van genetische diversiteit (aan de hand van AFLP) in populaties van boomsoorten van de Krakatau eilanden vergeleken met die van populaties op het vasteland en andere eilanden dicht bij het vasteland. Vijf boomsoorten, te weten *Ficus fulva*, *F. fistulosa*, *F. pubinervis*, *Dysoxylum gaudichaudianum*, en *Oroxylum indicum* werden voor dit onderzoek uitgekozen. Al deze soorten zijn obligate kruisbestuivers (mogelijk met uitzondering van *O. indicum*, waarvan het kruisingssysteem onbekend is) en zijn algemene en wijdverspreide boomsoorten in Zuidoost Azië. Gebaseerd op zaad en bestuivingsysteem eigenschappen kunnen de soorten in drie verschillende dispersiegroepen ingedeeld worden: hoog (alle *Ficus*-soorten), middel (*D. gaudichaudianum*) en laag dispersievermogen (*O. indicum*).

In tegenstelling tot de oorspronkelijke verwachting was de genetische diversiteit in populaties van de Krakatau eilanden in vier van de vijf soorten (*F. fulva*, *F. pubinervis*, *D. gaudichaudianum*, and *O. indicum*) gelijk of zelfs hoger dan die van alle andere standplaatsen. De vestiging van grote populaties gedurende kolonisatie en de middelmatige tot hoge dispersievermogens zijn factoren die er waarschijnlijk voor zorgen dat genetische variatie in populaties van *D. gaudichaudianum*, *F. fulva* en *F. pubinervis* op Krakatau gehandhaafd blijft. *O. indicum* heeft een laag dispersievermogen en een kleine populatie op Krakatau. Desondanks is de genetische diversiteit van deze soort op Krakatau groter dan die van alle andere standplaatsen. In *F. fistulosa* was de genetische diversiteit in de enige bestudeerde Krakatau populatie (Sertung) aanzienlijk lager dan die van veel vasteland standplaatsen, ondanks het hoge dispersievermogen van deze soort. Een toename van de inteelt als gevolg van vulkanische versterking en een gebrek aan bestuivers kunnen mogelijke verklaringen zijn voor de hogere verwantschapsgraad binnen deze Krakatau populatie. Verder onderzoek naar de oorzaken is echter noodzakelijk. Voor alle soorten werden de laagste niveaus van binnen-populatie polymorfie aangetroffen in enkele populaties op het vasteland en eilanden dicht bij de kust dan Krakatau. Dit is vermoedelijk het gevolg voortdurende habitat-verslechtering door menselijk handelen in deze gebieden.

Voor tropische boomsoorten en voor planten in het algemeen, is gevonden dat het dispersievermogen in belangrijke mate bepalend is voor de hoeveelheid genetische diversiteit binnen soorten. Ook in dit onderzoek werd gevonden dat soorten met een hoog dispersievermogen hogere niveaus van binnen-populatie polymorfie hadden dan soorten met geringer dispersievermogen. Dispersievermogen alleen was echter niet altijd in staat om de consequenties van kolonisatie in individuele populaties te voorspellen (zie hierboven).

In **Hoofdstuk 3** werden de patronen van kolonisatie van de eilanden en de niveaus van *gene flow* tussen de populaties onderzocht in dezelfde soorten als in Hoofdstuk 2 (gebruik makend van dezelfde AFLP data set). Kolonisatie van de Krakatau eilanden wordt sterk bepaald door het verspreidingsvermogen van iedere soort, de afstand tot de eilanden en de relatieve ligging van de eilanden ten opzichte van de andere landmassa's.

In *F. fistulosa* en *F. fulva* wijst een geringe differentiatie tussen populaties erop dat de *gene flow* binnen het onderzoeksgebied omvangrijk is en dat de afstand tot de Krakatau eilanden niet beperkend is voor dispersie. Er was geen ordinatie-differentiatie tussen de verschillende regio's waarneembaar. Daarom is het waarschijnlijk dat de kolonisten en migranten van Krakatau in gelijke mate afkomstig waren van Sumatra en Java. Eilanden zoals Sebesi, welke mogelijk als tussenstations zouden kunnen fungeren, hebben waarschijnlijk geen belangrijke rol gespeeld in de verspreidingspatronen in deze soort.

De patronen van kolonisatie waren meer gecompliceerd in *F. pubinervis*. *Isolation-by-distance* werd niet waargenomen en dit wijst erop dat variatie in de verspreidingspatronen binnen het onderzoeksgebied voorkomt, ondanks het grote dispersievermogen van deze soort. De sterke differentiatie tussen een Sumatra populatie en alle andere vindplaatsen is waarschijnlijk het gevolg van kleine populatie effecten binnen deze populatie en niet van regionale differentiatie. Het lijkt waarschijnlijk dat populaties van Java in verschillende mate aan de nieuwe Krakatau *gene pool* hebben bijgedragen, als gevolg van verschillen in schade aan populaties, veroorzaakt door de Krakatau uitbarstingen.

Samenvatting

De niveaus van *gene flow* in *D. gaudichaudianum* waren meer beperkt dan die in *Ficus*-soorten, hetgeen er op wijst dat afstand meer beperkend wordt op kleine ruimtelijke schalen. Een sterke verwantschap werd gevonden tussen de Krakatau eilanden en de meest nabije landmassa's (Rajabasa and Sebesi Island, Sumatra). Dit wijst er op, dat de meest nabije landmassa's de grootste bijdrage aan de Krakatau *gene pool* leverden. De differentiatie tussen de Krakatau populaties was groter dan die tussen één van de Krakatau populaties (Sertung) en nabij Sumatra (Sebesi en Rajabasa). Dit duidt er op dat de relatieve ligging van de eilanden ten opzichte van andere landmassa's de kolonisatiepatronen ook heeft beïnvloed. Geringe differentiatie tussen Sertung, Sebesi en Rajabasa suggereert dat Sebesi een tussenstation zou kunnen zijn geweest voor de kolonisatie van Sertung vanuit Sumatra. Echter, het ontbreken van *D. gaudichaudianum* op Sebesi ten tijde van eerdere expedities zou kunnen duiden op een kolonisatie in omgekeerde richting, vanuit Krakatau naar Sebesi.

Gene flow in *O. indicum* is sterk beperkt binnen het studiegebied; afstand is dus waarschijnlijk beperkend is voor dispersie. Kolonisatie door deze soort berust vermoedelijk in hoge mate op toevalsgebeurtenissen en wordt sterk beïnvloed door *genetic drift*. De Krakatau populatie vertoonde geen duidelijke verwantschap met andere gebieden. Eilanden zoals Sebesi waren waarschijnlijk geen tussenstations voor kolonisatie.

In **Hoofdstuk 4** werden AFLP- en chloroplast-merkers gebruikt om hybridisatie tussen *Ficus septica* en twee nauw-verwante, vroeg- koloniserende, tweehuizige *Ficus*-soorten, *F. fistulosa* en *F. hispida*, te documenteren. Het voorkomen van hybridisatie in natuurlijke populaties binnen het normale verspreidingsgebied is belangrijk, omdat *Ficus*-soorten door een strikt soortspecifiek bestuivings-mutualisme met Agaonidae-wespen gekenmerkt worden. Eerder bekende gevallen van hybridisatie en afbraak van het bestuiving-mutualisme hebben betrekking op phylogenetisch en geografisch verschillende soorten welke in sympatrie werden gebracht of hebben betrekking op geïsoleerde individuen buiten hun normale verspreidingsgebied. Vermoedelijke hybriden, gedetermineerd aan de hand van morfologische kenmerken, worden zelden gevonden en zijn niet eerder bevestigd door middel van genetische technieken. In deze studie wordt hiervan voor het eerst gebruik gemaakt.

Hybriden, zoals geïdentificeerd in dit onderzoek, zijn zeldzaam. Desalniettemin, de identificatie van zes van dergelijke individuen verspreid over drie verschillende standplaatsen, betekent dat de factoren die hybridisatie bevorderen niet beperkt zijn tot één enkele plek. Hybriden werden alleen gevonden in eiland populaties, meestal in verstoorde of onlangs verstoorde plekken. Hybridisatie gebeurtenissen vonden plaats op plekken waar één van de oudersoorten zeldzaam of afwezig is of was. Dit wijst erop dat een geringe populatie omvang een belangrijke drijvende kracht in het hybridisatie proces is. Verondersteld wordt dat de afbraak van de specificiteit van het bestuivingsstelsel het gevolg is van bestuiver-limitatie, waardoor de selectiviteit in partner keuze van individuele planten wordt verminderd. De in deze studie vastgestelde hybridisatie gebeurtenissen hebben weinig gevolgen voor de integriteit van de soorten. Verschillende auteurs hebben geconcludeerd dat hybridisatie geen belangrijke rol gespeeld heeft in de evolutie van het *Ficus*-geslacht. Desalniettemin kan hybridisatie een belangrijk evolutionair proces zijn in meer geïsoleerde eiland systemen, waar dispersie gebeurtenissen sterk gelimiteerd zijn en waar het uitsterven van één soort (zowel gastheer als bestuiver) waarschijnlijker is.

De bevindingen beschreven in **Hoofdstuk 5** hebben geen betrekking op eiland kolonisatie, maar zijn nogal toevallige resultaten, die waarde van merkers zoals AFLP voor populatie en ecologische studies benadrukken. Tijdens populatie studies in de functioneel tweehuizige *Ficus*-soort, *F. fulva*, werd een voor mannelijke individuen specifieke DNA-merker van 224 baseparen lengte gevonden. De aanwezigheid van deze merker in alle onderzochte mannelijke bomen en de exacte 1:1 uitsplitsing in nakomelingschappen wijst op een sterke koppeling van de merker met een geslachtsbepalende factor en op de bruikbaarheid van de merker bij het bepalen van het geslacht. De vondst van een mannelijk-specifieke merker ondersteunt twee waarnemingen omtrent geslachtsbepaling in *F. carica* (en die waarschijnlijk gelden voor het hele *Ficus*-genus). Ten eerste, dat de geslachtsbepaling in *Ficus* onder simpele genetische controle staat. Ten tweede, gegeven de dominante aard van AFLP merkers, bevestigt de aanwezigheid van een mannelijk-specifieke merker dat mannelijke bomen het heterogametische geslacht zijn.

In **Hoofdstuk 6** worden de eindconclusies van dit onderzoek getrokken. Het verspreidingsvermogen van een soort, de afstand tot de eilanden, de ligging van de eilanden en ecologische factoren zijn allemaal belangrijk in het structureren van de genetische diversiteit. Soorten met een groot dispersievermogen handhaven hogere niveaus van genetische diversiteit binnen populaties en lagere niveaus van differentiatie tussen populaties, in vergelijking tot soorten met geringere dispersievermogens. Dispersievermogen alleen was geen goede voorspeller voor het effect van kolonisatie op genetische diversiteit binnen populaties, omdat andere ecologische factoren ook van belang zijn voor het structureren van de genetische variatie. In tegenstelling tot de oorspronkelijke verwachting, handhaafden de meeste onderzochte soorten vergelijkbare of zelfs hogere niveaus van genetische diversiteit op de Krakatau eilanden in vergelijking tot het vasteland of de eilanden in de Sunda Straat dicht bij de kust. Geringe populatie omvang en eiland isolatie zijn vermoedelijk belangrijke factoren die in sommige soorten tot hybridisatie kunnen leiden.

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Curriculum vitae

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Born	Nowra, New South Wales, Australia and lived entire childhood nearby in Berry ‘the town of trees’.
1984-1989	Bomaderry High School, Bomaderry, N.S.W. where the Higher School Certificate was awarded.
1990	Year from school working and then travelling for 3 months in Asia.
1991-1994	Bachelor of Science at the Australian National University (ANU), Canberra graduating with First Class Honours. Honours research topic was ‘The Systematics of Two Species of Waratah, <i>Telopea mongaensis</i> (Cheel) and <i>T. oreades</i> (Muell.), Proteaceae’. -various summer research assistant positions with researchers at ANU.
1995	Research assistant at the CSIRO Division of Forestry, Yarralumla, Canberra examining genetic variation in a variety of Australian tree species.
1996-2002	PhD at the Netherlands Institute of Ecology-Center for Terrestrial Ecology at Heteren, the Netherlands.
2001-2002	Research assistant position at the Natural History Museum, London working on genetic determination of species identity and systematics in New World <i>Trema</i> .

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