

Genetic Evidence for Natural Hybridization between Species of Dioecious *Ficus* on Island Populations¹

Tracey L. Parrish, Hans P. Koelewijn, Peter J. van Dijk²

Netherlands Institute of Ecology—Center for Terrestrial Ecology, Boterhoekse Straat 48, 6666GA, Heteren, The Netherlands

and

Marco Kruijt

Laboratory of Phytopathology, Wageningen University, Marijkeweg 22, 6709 PG, Wageningen, The Netherlands

ABSTRACT

Natural hybrids between *Ficus septica* and two closely related dioecious species, *F. fistulosa* and *F. hispida*, were confirmed using amplified fragment length polymorphisms (AFLP) and chloroplast DNA markers. *Ficus* species have a highly species-specific pollination mutualism with agaonid wasps. Therefore, the identification of cases in which breakdown in this sophisticated system occurs and the circumstances under which it happens is 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 in which 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 Janzen's (1979) hypothesis that breakdown in pollinator specificity is more likely to occur on islands. We hypothesized that hybrid events could occur when the population size of pollinator wasps was small or had been small in one of the parental species. Later generation hybrids were identified, indicating that backcrossing and introgression did occur to some extent and that therefore, hybrids could be fertile. The small number of hybrids found indicated that there was little effect of hybridization on parental species integrity over the study area. Although hybrid individuals were not common, their presence at multiple sites indicated that the hybridization events reported here were not isolated incidences. Chloroplast DNA haplotypes of hybrids were not derived solely from one species, suggesting that the seed donor was not of the same parental species in all hybridization events.

Key words: agaonidae; colonization; fig; introgression; Krakatau; monsoonal forest.

FICUS IS ONE OF THE MOST SPECIOSE AND DIVERSE GENERA of woody plants, with *ca.* 750 species spanning the tropical and subtropical regions of the world (Berg 1989). *Ficus* species exhibit two breeding systems, either monoecy (*ca.* 400 spp.) or functional dioecy (*ca.* 350 spp.; 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 putatively ensures high levels of breeding isolation between closely related co-

curing 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 located a considerable distance from conspecifics (Ramirez 1970, Compton 1990) or from trees introduced to an area in which 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 its normal range), was visited by two alien pollinator wasp species (Compton 1990, Ware & Compton 1992). One of these species reproduced successfully. Seeds were also produced and germinated but died shortly after the cotyledon stage (Compton 1990). Ramirez (1970) found that 5

¹ Received 13 May 2002; revision accepted 16 June 2003.

² Corresponding author.

percent 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 several occasions, artificial interspecific hybrids of *Ficus* (in some cases from taxonomically diverse groups) have been formed and viable offspring produced (e.g., *F. pumila* × *F. carica* producing viable seeds, Condit 1950; *F. glabrata* × *F. sycomorus*, non-viable seeds; and *F. glabrata* × *F. radula*, viable seeds, Ramirez 1986). Natural hybrids in *Ficus*, however, are rare (Corner 1958, Ramirez 1970) and hybridization is considered of little importance in *Ficus* evolution (Ramirez 1986). Ramirez (1970) reported a single putative hybrid individual between *F. teurckheimii* × *F. jimenezii* from a natural population based on morphological characters. Janzen (1979), however, indicated 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) × *F. padifolia* (New World), Ramirez & Montero 1988; hybrid seedlings of *F. religiosa* (Old World) × *F. aurea* (New World) in Miami and *F. septica* (New World) × *F. religiosa* (New World) in Manila, the Philippines, 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 in which the pollinator of a species has gone extinct or is temporarily absent in an area and/or reduced host availability selects for latitude in wasp host selection. Such events could occur on islands or in harsh mainland environments (i.e., the edge of a species range) for the host species. Therefore, sites such as the Krakatau islands in Indonesia (Fig. 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

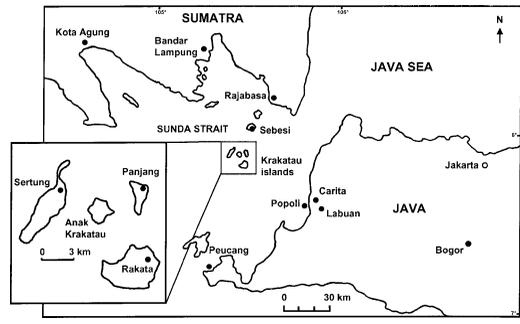


FIGURE 1. Map of the Krakatau islands and the surrounding Sunda Strait region in Indonesia showing the location of study sites (filled circles).

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 fieldwork on the Krakatau islands in 1996, Dr. Nick Mawdsley (University of Leeds, England) identified two individual trees that were considered morphological hybrids of *F. septica* and *F. fistulosa*. Further fieldwork 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*. One individual, however, 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)) which confirmed 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 of Indonesia.

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 were the main focus of this study. To exclude other likely parental species, four other dioecious species, *F. fulva*, *F. montana*, *F. ribes*, and *F. variegata*, were included in the analysis. All seven species were early colonists to the Krakatau islands (Whittaker *et al.* 1989), are lowland tree species (except *F. ribes*), and have populations that demonstrate varying degrees

TABLE 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 (1 and 2) generated a total of 226 bands across all species. The numbers of species-specific bands per species are shown below. The collection sites of the putative hybrids are also listed. Letters after collection sites designate the region of origin: (K)rakatau islands, (S)umatra, (J)ava, and (B)runei.*

Species	N	Collection sites (region)	No. 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> ^a	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) ^b	48
Putative hybrids	22	Popoli (J), Peucang (J), Bogor (J), Panjang (K), Rakata (K)	

^a Collected by S. G. Compton (University of Leeds, England) and ^b by E. Jouselin (CNRS, Montpellier, France) from Tutong, Brunei.

of sympatry on the islands and mainland sites. *Ficus 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. obs.). *Ficus fistulosa*, *F. hispida*, *F. septica*, *F. ribes*, and *F. variegata* are all pollinated by species of agaonid wasps 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 from 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).

Study sites are shown in Figure 1 and populations used for AFLP analysis are listed in Table 1. In total, five mainland (Rajabasa, Kota Agung, Bandar Lampung, Carita, and Bogor) and four island sites (Panjang, Rakata, Popoli, and Peucang) were examined for putative hybrids. Samples from two additional sites, Sebesi Island and Labuan, were used for molecular analysis but no search for hybrid individuals was made at these sites. Leaf material for *F. montana* was supplied by Dr. S. G. Compton (University of Leeds, England) and the *F. variegata* individual from Tutong, Brunei, was

collected by Dr. E. Jouselin (CNRS, Montpellier, France).

Putative hybrids were identified at five localities: the Krakatau islands (Panjang and Rakata), Bogor (on Java), Popoli Island, and Peucang Island. Both *F. septica* and *F. fistulosa* currently maintain large population sizes on the Krakatau islands. Popoli is a small coral and sand island ca. 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. *Ficus septica* is the most common *Ficus* species on the island. A small stand of *F. hispida* was also identified. Peucang Island is part of Ujung Kulon National Park, Java, and lies ca. 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. *Ficus septica* is common on Peucang, although *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. 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.

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

AFLP AND CHLOROPLAST DNA TYPING.—Leaf material for molecular analysis was collected and preserved in a CTAB/NaCl solution (Rogstad 1992). DNA was extracted from this preserved material using a hot CTAB extraction procedure based on the methods of Rogstad (1992). The AFLP protocol used in this study followed the basic principles of Vos *et al.* (1995) and used *EcoRI* and *MseI* restriction enzymes. PCR amplification was performed on a PTC-200 (MJ Research) thermal cycler with the following protocol; cycle 1, 94°C at 1 min, 65°C at 1 min, 72°C at 1:30 min; cycles 2–10, as in 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. 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* (Parrish *et al.*, pers. obs.), 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 and PCR conditions as described in Demesure *et al.* (1995). 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 percent agarose gel for polymorphisms among 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. 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 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 hy-

brid individuals. An individual was identified as a hybrid if it contained at least one band from two species.

The patterns of relationship between species and putative hybrids were also investigated using principal coordinate analysis (PCoA). A pairwise 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 & 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. The patterns of genetic relationship among the seven study species (and an eighth species, *F. padana*, collected from Carita and Rajabasa; *F. padana* was included in analysis because it is a close relative of *F. fulva* and may add more 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, when possible, identified in putative 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. obs.). *Ficus septica* has large, glabrous, discolorous, elliptic leaves that 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. *Ficus 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. *Ficus hispida* has hairy, serrate subovate to elliptic leaves. Fruits are positioned on long branchlets (up to 1 m in length) along the trunk. The species is widespread, although it tends to have a patchy distribution, and when present, is 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 putative hybrid morphologies were identified: (1) Growth form and leaf morphology were 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. (2) Leaf morphology and color were most similar to *F. fistulosa* but had paired, axillary fruit and growth form of *F. septica*. (3) Sapling leaf shape was of *F. hispida* but lacking hairs and had glabrous shiny surface resembling *F. septica*. (4) 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, 10 and 4 individuals (17 total) with morphology type 1 were identified and collected on Panjang, Rakata, and Bogor, respectively. On Popoli Island 2 putative hybrids were identified, 1 with morphology type 2 and another with type 3. Three individuals with morphology type 4 were identified on Peucang Island. Except at Bogor, no putative hybrids were found on mainland sites, despite five mainland localities being examined.

MOLECULAR MARKERS AND HYBRID IDENTIFICATION.—

Two AFLP primer combinations were used to identify species-specific DNA fingerprints in the study species. The number of individuals used to construct these fingerprints and source locations of the specimens is given in Table 1. Given that *F. fistulosa* and *F. septica* were considered to be the most likely parental species of hybrids, considerably more individuals of these species were examined. A total of 226 clearly distinguishable bands were produced across all 7 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 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* when digested with *HinfI*, 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, Bandar Lampung, and Rakata and 4 individuals of *F. hispida* from Panjang and Rakata). Chloroplast haplotypes were not determined for 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 2.

All 22 putative hybrids were fingerprinted with AFLPs. Eight putative hybrids contained AFLP bands typical of more than one species. The remaining 14 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 summarized genotype of these individuals is given in Table 2 (only bands present in hybrid individuals were included. Bands present in all species, and hence non-diagnostic, were excluded). Sometimes bands were 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.

Of the 17 putative hybrids with morphology type 1 (*F. septica* x *F. fistulosa*) collected from Krakatau (Panjang and Rakata) and Bogor, only three individuals from Rakata (H1, H2, and H3; Table 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 2). 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*.

TABLE 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 percent frequency are in bold, and less than 80 percent in plain text. The total number of bands (including those fixed in all species) in each individual's fingerprint for both primer combinations is given at the bottom of the table and can be compared to species averages in Table 3. Dashes indicate missing data due to non-amplification.*

Collection site	H1	H2	H3	H4	H5	H6	H7	H8	H9	Species identity of band
Morph. type	Rak	Rak	Rak	Pop	Pop	Pop	Peu	Peu	Peu	
Parental species	1	1	1	2	3	H	4	4	4	
Chloroplast type	FXS	SXF	FXS	SXF	HXS	?XH	SXF	SX?	SX?	
	F	S	F	S	H	—	S	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	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	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	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	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

TABLE 2. Continued.

Collection site	H1	H2	H3	H4	H5	H6	H7	H8	H9	Species identity of band
Morph. type	Rak 1	Rak 1	Rak 1	Pop 2	Pop 3	Pop H	Peu 4	Peu 4	Peu 4	
Parental species	FXS	SXF	FXS	SXF	HXS	?XH	SXF	SX?	SX?	
Chloroplast type	F	S	F	S	H	—	S	S	S	
	1	1	1	1	1	0	1	1	—	<u>S</u> , <u>F</u> , <u>R</u>
	0	0	0	0	0	1	0	0	—	<u>H</u>
	0	0	1	0	0	0	0	0	—	<u>S</u>
	0	0	0	0	1	1	0	0	—	<u>H</u> , <u>V</u>
	0	0	0	0	1	1	0	0	—	<u>H</u>
	0	0	0	0	1	1	0	0	—	<u>F</u> , <u>H</u>
	1	1	1	1	1	0	1	1	—	<u>S</u> , <u>F</u> , <u>R</u>
	0	1	0	1	0	0	1	1	—	<u>F</u> , <u>H</u> , <u>V</u> , <u>R</u>
	0	1	0	1	0	1	1	0	—	<u>S</u> , <u>V</u>
No. bands/individ.	47	42	44	55	57	45	53	48	27	

Therefore, the second parental species could not be unambiguously identified. H3 contained a haplotype typical of *F. fistulosa* but because the chloroplast haplotype of *F. ribes* was not determined, the identity of the second parental species remains ambiguous; however, it was most likely *F. fistulosa*, considering the parental species found for other hybrid individuals. All three of these hybrid individuals were unlikely to be first generation hybrids (F1) because they did not contain all fixed bands specific to parental species as expected given the dominant nature of AFLP. This indicated that hybrids were later-generation crosses. F1s were thus fertile and backcrossing occurred.

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 2, with *F. septica* chloroplast haplotype), and one being a cross between *F. hispida* and *F. septica* (H5, morphology type 3, *F. hispida* chloroplast haplotype; Table 2). These individuals were likely F1 hybrids as they possessed all bands fixed in the parental species and contained several other bands found in the parental species. The total band number in these individuals was 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 2 and 3). H4 possessed a single band that was difficult to elucidate whether 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*.

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 was likely an F1 hybrid between *F. septica* and *F. fistulosa* because it contained only bands specific to, and most fixed in, these species and possessed a larger number of bands (53) than normally observed in these species (Tables 2 and 3). The chloroplast haplotype of this individual was typical of *F. septica*. The genotypes of H8 and H9 were more complicated as they possessed 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* and one specific to *F. variegata*, but also had other bands shared by *F. fistulosa*, *F. hispida*, and *F. ribes*, which were mutually exclusive of *F. variegata*. Data are missing for primer combination 2 in this individual due to non-amplification. Both H8 and H9 had

TABLE 3. The average number (\pm SE) and range of bands in AFLP fingerprints from (N) individuals of *F. septica*, *F. fistulosa*, and *F. hispida*.

Species	N	Mean No. bands/individ. \pm SE	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

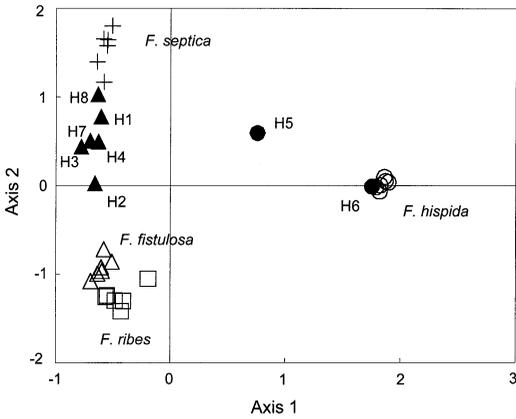


FIGURE 2. Principal coordinate analysis results demonstrating the relationships between the species and hybrid individuals (open symbols are the species; plus = *F. septica*, upward triangle = *F. fistulosa*, square = *F. ribes*, and circle = *F. hispida*; closed symbols are hybrid individuals, upward triangle = H1–H4, H7, H8, and circle = H5, H6. Plots are made from axes 1 and 2, which explained 67.6 and 17.4 percent of the total variance in the data, respectively.

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.

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 of these individuals were hybrids between *F. fistulosa* and *F. septica* and contained foundress wasps of *Ceratosten bisulcatus*, the pollinator wasp of *F. septica*. H1 and H4 were both female trees but developing seeds were only found in H4. Individuals H2 and H3 were collected in 1996 and their fruits were not examined. H5 was a pre-fruiting 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* (to limit cluster group size) and eight of the nine individuals identified as containing bands typical of more than one species. Hybrid 9 was excluded from analysis due to the large amount of missing data from this

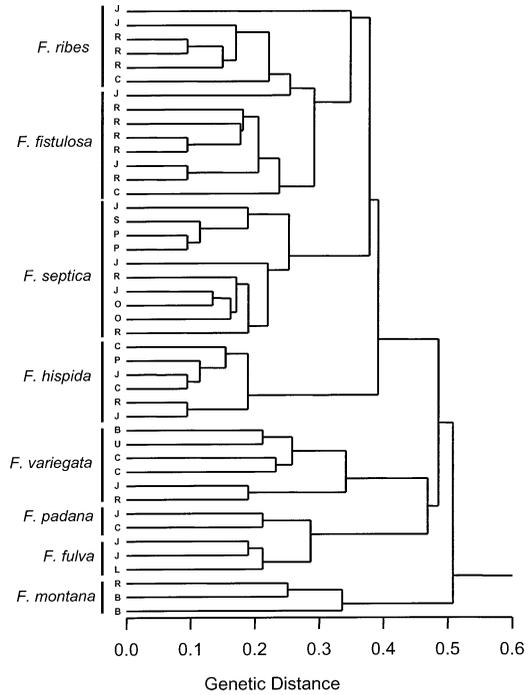


FIGURE 3. An average linkage clustering UPGMA tree constructed using euclidean distances of data from primer 1 demonstrating patterns of genetic dissimilarity among the study species. *Ficus septica* clusters between the two species it was found to be hybridizing with in this study. Small letters indicate collection sites of samples where, B = Brunei, C = Carita, J = Rajabasa, O = Popoli Island, R = Rakata, S = Sertung, and U = Peucang Island.

sample (no amplification in primer combination 2). A scatterplot of the first and second axes is shown in Figure 2. Axes one and two explain 67.6 and 17.4 percent of the total variance in the sample, respectively. The plot shows that all four species occupy distinct ordinal space, although *F. fistulosa* and *F. ribes* cluster very closely. *Ficus 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. The *Ficus 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.

UPGMA.—Two independent UPGMA trees were constructed using the data from primer combinations one and two, and both gave comparable results. The tree from primer combination one is shown in Figure 3. All species formed discrete clus-

ters, except *F. fistulosa* and *F. ribes*, which showed some overlap in their clusters. *Ficus septica* (the one species involved in all hybrid events documented in this study) clustered with three other species, *F. fistulosa*, *F. hispida* and *F. ribes*, which are also pollinated by species of *Ceratosolen*. This indicated that *F. septica* is genetically closely related to the two species with which it hybridizes, *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*. 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*. *Ficus montana* is the most genetically distinct species in this tree and is pollinated by a third genus of Agaonidae, *Liporrhopalum*.

DISCUSSION

In this paper, we provide genetic evidence that hybridization events are occurring in natural populations of dioecious *Ficus* species, between *F. septica* and two other closely related species, *F. fistulosa* and *F. hispida*. Nine individuals were found to possess AFLP bands typical of more than one species. Of these individuals, six are undoubtedly hybrids (H1–H5, H7) because they contained a large number of bands typical of more than one species and clustered between the parental species in PCoA. The hybrid status of three other individuals (H6, H8, and H9), however, may be questionable. These individuals contained only a few alien bands, possibly due to introgression; however, these alien markers may also be rare bands not detected in the *F. septica* or *F. hispida* samples. No hybridization was detected with closely related *F. ribes*. This likely reflects the different ecological distribution of *F. septica* and *F. ribes* in the study area. *Ficus septica* occupies lowland habitat while *F. ribes* occupies montane habitat.

It has long been known that hybridization can occur between *Ficus* species, but most examples exist between rather phylogenetically disparate species in nonnatural situations. 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 importance that hybrid events have been genetically confirmed in natural populations in which the species involved naturally occur in sym-

patry, 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 1986). In closely related species, it would be expected that breeding isolation is maintained by divergent chemical profiles. In this study, we showed 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, indicating that hybridization is not unique to the Krakatau situation and has occurred repeatedly at different localities. The three sites at which hybridization has been identified are all islands with a history of disturbance. Janzen (1979) has suggested 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. In the absence of its own fig species, it is better for a wasp to go to another fig species and try to reproduce rather than to go to no fig at all. This may be facilitated by absence of competition when the other fig species lacks its own pollinator wasp. From the fig's point of view, latitude in accepting the wrong pollinator in isolated individual plants may also be an advantage as it may be better to hybridize than not to reproduce at all.

The study species are early colonists that establish populations at different rates and times (Parrish, pers. obs.; 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 establishment will be greatly affected on islands because 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 (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 com-

mon on the Krakatau islands but were amply fructifying only unripe and incompletely developed fruits (Backer 1929, Ernst 1934). At this time, *F. septica* was considerably less abundant (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. In 1922 (14 years later), however, fig wasps (including *C. 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 ca. 1910 and 1920, the fig wasp populations on Krakatau 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 were 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 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 in which 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 hy-

brids 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 that 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 that they can establish in the field. The rarity of hybrid individuals, however, suggests that hybridization should have little effect on species integrity in the present study environment. Hybridization is considered of little importance 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 than the ones studied here, *i.e.*, where there is increased chance of long-term pollinator absence or extinction in one of two co-occurring species (Janzen 1979). Such a case could be *F. scabra* in the New Hebrides, of which Corner (1975) has noted that morphological variation may be due to hybridization. As our study has shown, however, analysis with molecular markers is necessary to test this hypothesis.

ACKNOWLEDGMENTS

The Indonesian Institute of Sciences (LIPI), the Herbarium Bogoriense and the Indonesian National Parks (PHPA) are greatly thanked for their assistance in this research. Dr. Tukirin Partomihardjo (Herbarium Bogoriense, LIPI), Bogor, Indonesia, provided invaluable field assistance. Dr. Steve Compton and Dr. Emmanuelle Jouselin provided additional samples for analysis. Funding for this Ph.D. project was awarded by the "Biodiversity in Disturbed Ecosystems" priority program of the Netherlands Organization for Scientific Research (NWO). This is publication 3149 NIOO-KNAW, Netherlands Institute of Ecology and publication 61 of the Krakatau Research Programme series.

LITERATURE CITED

- BACKER, C. A. 1929. The problem of Krakatoa as seen by a botanist. Published by the author, Weltevreden, Indonesia.
- BERG, C. C. 1989. Classification and distribution of *Ficus*. *Experientia* 45: 605–611.
- COMPTON, S. G. 1990. A collapse of host specificity in some African fig wasps. *S. Afr. J. Sci.* 86: 39–40.
- , S. J. ROSS, AND I. W. B. THORNTON. 1994. Pollinator limitation of fig tree reproduction on the island of Anak Krakatau (Indonesia). *Biotropica* 26: 180–186.
- CONDIT, I. J. 1950. An interspecific hybrid in *Ficus*. *J. Hered.* 41: 165–168.
- CORNER, E. J. H. 1958. An introduction to the distribution of *Ficus*. *Reinwardtia* 4: 15–45.
- . 1975. *Ficus* in the New Hebrides. *Philosophic. Trans. R. Soc. Lond. B* 272: 343–367.
- DAMMERMAN, K. W. 1922. The fauna of Krakatau, Verlaten Island and Sebesy. *Treubia* 3: 61–112.
- . 1948. The fauna of Krakatau, 1883–1933. *Verhandelingen der Koninklijke Nederlandse Akademie van Wetenschappen Afdeling Natuurkunde (Tweede Sectie)*. 44: 1–594.

- DEMESURE, B., N. SODZI, AND R. J. PETTIT. 1995. A set of universal primers for amplification of mitochondrial and chloroplast DNA in plants. *Mol. Ecol.* 4: 129–131.
- DOCTERS VAN LEEUWEN, W. M. 1936. Krakatau: 1883–1933. E. J. Brill, Leiden, Holland.
- ERNST, A. 1934. Das biologische Krakatauprobem. Vierteljahrsschrift der Naturforschenden Gesellschaft in Zuerich, Switzerland. LXXIX.
- HOMMEL, P. W. F. M. 1987. Landscape ecology of Ujung Kulon (West Java, Indonesia). Ph.D. dissertation. Wageningen University, Wageningen, The Netherlands.
- JACOBSON, E. 1909. De nieuwe fauna van Krakatau. Jaarverslag voor de Topografische Dienst, Batavia, Indonesia.
- JANZEN, D. H. 1979. How to be a fig. *Annu. Rev. Syst. Ecol.* 10: 13–51.
- KARTAWINATA, K., A. APANDI, AND T. B. SUSELO. 1985. The forest of Peucang island, Ujung Kulon National Park, West Java. In D. Sastrapradja et al., S. Soemodihardjo, A.S. Soemartadipura, A. Soegiarto, S. Adisoemarto, K. Kusumadinata, S. Birowo, S. Riswan, and A.B. Lopian (Eds). Proceedings of the symposium on 100 years development of Krakatau and its surroundings, Jakarta, 23–27 August 1983. Volume 1: Natural Sciences. pp. 448–452. LIPI, Jakarta, Indonesia.
- MOGENSEN, H. L. 1996. The hows and whys of cytoplasmic inheritance in seed plants. *Am. J. Bot.* 83: 383–404.
- RAMIREZ, W. R. 1970. Host specificity of fig wasps (Agaonidae). *Evolution* 24: 680–691.
- . 1986. Artificial hybridization and self-fertilization in *Ficus* (Moraceae). *Brenesia* 25–26: 265–272.
- . 1994. Hybridization of *Ficus religiosa* with *F. septica* and *F. aurea* (Moraceae). *Rev. Biol. Trop.* 42: 339–342.
- , AND J. S. MONTERO. 1988. *Ficus microcarpa* L., *F. benjamina* L. and other species introduced in the New World, their pollinators (Agaonidae) and other fig wasps. *Revista de Biología Tropical* 36(2B): 441–446.
- ROGSTAD, S. H. 1992. Saturated NaCl–CTAB solution as a means of field preservation of leaves for DNA analyses. *Taxon* 41: 701–708.
- TER BRAAK, C. J. F., AND P. ŠMILAUER. 1998. Canoco: software for canonical community ordination (version 4). Microcomputer Power, Ithaca, New York.
- VERBEEK, R. D. M. 1885. Krakatau. Staatsdrukkerij, Batavia, Indonesia.
- VOS P., R. HOGERS, M. BLEEKER, M. REIJANS, T. VAN DER LEE, M. HORNES, A. FRIJTERS, J. POT, J. PELEMAN, M. KUIPER, AND M. ZABEAU. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* 23: 4407–4414.
- WARE, A. B., AND S. G. COMPTON. 1992. Breakdown of pollinator specificity in an African fig tree. *Biotropica* 24: 544–549.
- WHITTAKER, R. J., M. B. BUSH, AND K. RICHARDS. 1989. Plant recolonization and vegetation succession on the Krakatau islands, Indonesia. *Ecol. Monogr.* 59: 59–123.
- WIEBES, J. T. 1994. The Indo–Australian Agaonidae (pollinators of figs). Koninklijke Nederlandse Akademie van Wetenschappen Verhandelingen Afdeling Natuurkunde, Tweede Reeks, part 92. Amsterdam, The Netherlands.
- WILKINSON, L. 1999. Systat 9. SPSS, Inc., Chicago, Illinois.