

A Breakdown of Obligate Mutualism on a Small Island: An Interspecific Hybridization between Closely Related Fig Species (*Ficus pumila* and *Ficus thunbergii*) in Western Japan

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Abstract

Ficus (Moraceae) is a well-known group with specific pollination mutualisms, and hybridization is considered to be rare. Here, we report the presence of interspecific hybrids between *Ficus pumila* L. and *F. thunbergii* Maxim. on Okinoshima, a small island offshore of Shikoku, western Japan. AFLP (amplified fragment length polymorphism of genomic DNA) data suggested that more than one-fourth of individuals of morphological *F. pumila* were assigned as intermediate genotypes, suggesting hybrids. The hybridization between the two species was introgressive and unidirectional from *F. thunbergii* to *F. pumila*. The findings of this study, combined with other previous reports, suggest that the breakdown of mutualistic systems can occur in isolated populations such as those on islands.

Keywords

AFLP, Island, Hybridization, *Ficus pumila*, *Ficus thunbergii*, Western Japan

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1. Introduction

Ficus L. (Moraceae) comprises approximately 750 - 800 species mainly occurring in tropical regions throughout the world [1] [2]. The diversity center of the genus is Asia, and more than 450 species are distributed there [1]. Although the conspicuous diversity of this species is a significant point, Asia is also an important region for the evolution of the genus because it houses both of two major sexual expression modes in figs, monoecious and dioecious, with high species diversity in the latter. Most of the approximately 350 species of dioecious figs are distributed in Asia and show extreme variations in life-form, including trees, stranglers, shrubs, and climbing or trailing lianas [1]. These life-form variations are not observed in monoecious figs or figs in other regions. Thus, Asian figs have undergone many novel evolutionary events and are considered an important plant group for studying the evolution of life-form diversity.

Ficus is also well known as a genus with a specific plant-insect relationship. All fig species have their own specific pollinators originally derived from seed parasites (fig wasps, Agaonidae, Chalcidoidea, Hymenoptera). The interaction is a special case of obligate pollination mutualism and has long attracted the interest of many researchers [3]-[12]. This specific mode of pollination demonstrates another consequence of the evolution of figs. Although many plant groups experience frequent hybridization events during their evolution [13] [14], *Ficus* is usually considered an exception because of its specific pollination mode [2]. However, recent estimations from genetic data on six sympatric dioecious fig species in New Guinea suggested that 12% of fig populations included hybrids [15]. Conversely, the genetic identities of sympatric fig species were strictly maintained though fig-wasp species were shared among them [16]. Thus, additional examinations of other fig populations are necessary to confirm whether hybridization plays an important role in the evolution of fig species.

During our studies on population genetic structures and genetic differentiation in Japanese fig species, we identified new instances of hybridization between the closely related fig species *Ficus pumila* L. and *Ficus thunbergii* Maxim. on a small island off the shore of Shikoku, western Japan (Figure 1). *Ficus pumila* is widely distributed in Japan, Taiwan, China, and northern Indochina [17], whereas *F. thunbergii* is almost endemic to Japan (the Japanese archipelago and Cheju Island, Korea). These two species are closely related [17]. This occurrence, along with other cases reported previously, suggests that isolated places such as islands enhance the breakdown of specific relationships and hybridizations in closely related fig species.

2. Materials and Methods

Okinoshima is located off the shore of Kochi Prefecture, Shikoku, western Japan (Figure 2). We collected samples of *F. pumila* and *F. thunbergii* for analysis in 2010. Each sample was collected at least 50 m from neighboring samples to avoid resampling the same genets. Because not all individuals had syconia at the sampling time, we checked the morphological characteristics of leaves for exact identification of the two species when we collected samples: *i.e.*, almost glabrous on the abaxial side and a lateral nerve angle of less than 40° for *F. pumila*, and pubescens on the abaxial side and a lateral nerve angle of more than 50° for *F. thunbergii* [17]. In total, 42 individuals of *F. pumila* and 2 individuals of *F. thunbergii* were collected. Voucher specimens were deposited at



Figure 1. Appearances of two fig species used in this study. Left: *Ficus pumila*, right: *F. thunbergii*. Both photos were taken at Okinoshima, Kochi, western Japan.

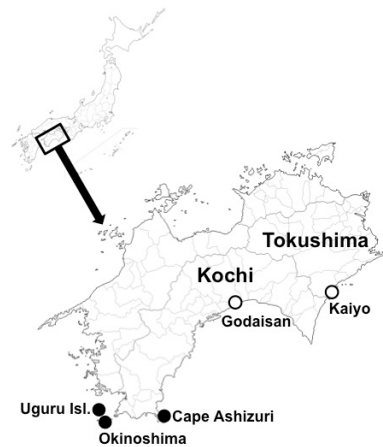


Figure 2. Sampling localities in this study. Solid circles indicate the sampling sites of *F. pumila* and open circles indicate those of *F. thunbergii*. Both species were collected from Okinoshima but main samples are *F. pumila* (42 of 44 samples).

the University Museum, Yamagata University. For comparisons, samples of *F. pumila* were collected from Uguru Island (17 individuals), a similar offshore island without *F. thunbergii*, as well as Cape Ashizuri (23 individuals) (Figure 2). We also collected *F. thunbergii* samples from Mt. Godaisan, Kochi City, Kochi Prefecture (14 individuals), and Kaiyo, a town in Tokushima Prefecture (14 individuals) (Figure 2).

The youngest leaf on the branch tip of each individual of *F. pumila* and *F. thunbergii* was collected and placed in a plastic bag with silica gel for storage until use. DNA extractions were performed using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Extracted DNA was frozen at -20°C until use.

AFLP (amplified fragment length polymorphism) analyses [18] were performed using the Ligation and Pre-selective Amplification Module and selective primers (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Three primer pairs, *EcoRI*-ACA/*MseI*-CAA, *EcoRI*-ACC/*MseI*-CAA, and *EcoRI*-AAG/*MseI*-CAA, were used for selective amplifications. The final amplification products were electrophoresed with the GeneScan™ 500 Rox™ dye Size Standard (Applied Biosystems) using an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). We evaluated the peak pattern of the AFLP band using the analysis software GeneMapper (Applied Biosystems) in the range of 50 - 350 bp. Based on the AFLP data, an assignment test using Bayesian clustering was conducted using Structure 2.3.3 [19] [20]. An analysis was then performed with 25,000 iterations following 25,000 replications of burn-in period.

3. Results and Discussion

In total, 482 polymorphic bands (by three primer pairs) were detected from the five populations of *F. pumila* and *F. thunbergii*. Among these, 260 bands were observed from two populations of *F. pumila* (Uguru Island and Cape Ashizuri), and 274 bands were recorded from two populations of *F. thunbergii* (Godaisan and Kaiyo). In the analysis for $K = 2$ (assuming two genetic clusters representing species, $\text{Ln} = -22262.1$; Figure 3), all individuals from Uguru Island and Cape Ashizuri were assigned as *F. pumila* (0.928 - 0.998), whereas all individuals from Mt. Godaisan and Kaiyo were assigned as *F. thunbergii* (0.968 - 0.998). Among the individuals from Okinoshima, two individuals of morphologically *F. thunbergii* were inferred as the same species, although the values were relatively low (0.900, 0.929). However, 11 of 42 individuals (28.6%) of morphologically *F. pumila* individuals were assigned as intermediates between the two species (assigned to the *F. pumila* cluster: 0.167 - 0.661). This result indicated that interspecific hybridization had taken place between *F. pumila* and *F. thunbergii*; that hybrid individuals of the later generations existed, suggesting less or no hybrid sterility between the two species; and that unidirectional introgression from *F. thunbergii* to *F. pumila* had occurred on the island. This is the first report of the hybridization of *Ficus* in Japan as well as for Section *Rhizocladus*, one of the most species-rich groups in warm-temperate and subtropical Asia.

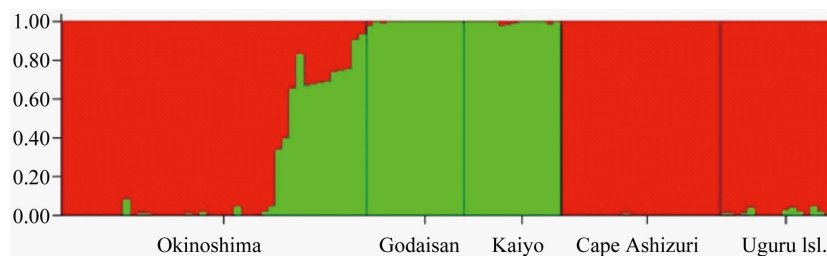


Figure 3. A result of assignment test for the AFLP data of *F. pumila* and *F. thunbergii* ($K = 2$). A genetic cluster of *F. pumila* is indicated as red and that of *F. thunbergii* is as green. Sampling locations are indicated below of the bar plot. Two individuals at the rightmost of the Okinoshima window are *F. thunbergii* and others are *F. pumila*.

One of the reasons for unidirectional introgression may be the body size of fig wasps. The two fig species examined largely differ in the sizes of their syconia: *i.e.*, the syconia of *F. pumila* are larger (3 - 4 cm in diameter) than those of *F. thunbergii* (2 cm). The size of syconia is also related to the size of flowers, and flower size is related to fig-wasp body size. Indeed, wasps from *F. pumila* were morphologically quite similar but slightly larger than those from *F. thunbergii* ([21]; J. Yokoyama, personal observations). Small wasps can enter the large syconia, but large wasps cannot enter the small syconia. This simple mechanism may control the direction of hybridization between these species.

The abundance of each species on the island may be another reason for unidirectional introgression. Among the difference of sampled individuals, the abundance of *F. pumila* was higher than that of *F. thunbergii* at Okinoshima. Under this situation, pollinating wasps of *F. thunbergii* likely encounter the absence of receptive syconia and may be forced to enter syconia of close relatives instead of the original fig partner. How often these mismatches occur should be confirmed in future field observations.

As indicated previously, interspecific hybridization of *Ficus* in nature is considered to be rare because of its specific pollination mechanisms [2]. Hybrid inviability may be another mechanism for preventing interspecific hybridizations [2]. However, recent studies indicate that natural hybridization should occur in closely related fig species. The first case confirmed using a molecular method was the hybridization between *Ficus fistulosa* Reinw. ex Blume and *Ficus hispida* L.f. in Indonesia [22], which also involved island populations. Jansen (1979) suggested that the breakdown of a specific mutualistic system, if it occurs, should be found in small populations such as those on islands [23]. To confirm this hypothesis, additional genetic analyses of figs with closely related sympatric species are needed.

Although the effects of hybridization on the evolution of *Ficus* are still unclear, note that a recent study suggested a hybrid origin of endemic fig species in the Ogasawara Islands, which are oceanic islands separated from the mainland by approximately 1000 km [24] (Note that this implication was based on the discordance of phylogenetic positions between figs and fig-wasps on the Ogasawara Islands, and not on the genomic admixture of figs). This case implies that hybridizations of *Ficus*, as in other plant groups, may have contributed to speciation. Therefore, the examination of other populations of endemic fig species in isolated areas such as oceanic islands is necessary.

4. Conclusion

Due to the nature of specific pollination mutualism, natural hybridization in *Ficus* has been underestimated. However, recent studies have shown that considerable instances in interspecific hybridization may have occurred in the genus. We have presented a new instance of such a hybridization from a small island in western Japan. These occurrences are most likely to be found in restricted areas such as islands where two or more closely related species are distributed. Although evolutionary roles remain unclear, interspecific hybridization in *Ficus* may have contributed to the diversification of the genus, particularly when considering cases in other angiosperms.

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