

# **MORPHOLOGICAL AND MOLECULAR EVIDENCE OF NATURE HYBRIDIZATION BETWEEN TWO RELATED SPECIES, *FICUS RELIGIOSA* AND *FICUS RUMPHII* IN MYANMAR**

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## **Abstract**

*Ficus* is a well known as a genus with a specific plant-insect relationship. All fig species have their own species-specific pollinators fig wasps (Agaonidae, Chalcidoidea, Hymenoptera). Seed dispersal are mediated by generally various kinds of vertebrates. While such ecological characteristics is considered to be associated with a range of gene flow, studies of the genetic structure of *Ficus* plants in tropical Asia have not been well understood in detail. In this study, it is focused on two species of monoecious figs, *Ficus religiosa* and its closely related species *Ficus rumphii*, which are widely distributed in Southeast Asian countries including Myanmar. These two species are generally found in the same environmental condition and also morphologically look like similar and sometimes difficult to distinguish from the morphological outstanding. To determine the genetic differentiation and the level of interspecific hybridization, using Amplified Fragment Length Polymorphism (AFLP) molecular marker is applied. As a result, the genetic differentiation between species and individuals of mixed genotype has been shown. It is considerable that amount of hybrid individuals were present and hybridization were occurred in both directions. Interspecific hybridization of the genus *Ficus* has been thought to be very rare. The results indicated that effects of interspecific hybridization in *Ficus* to its evolution could not be ignored. In addition, it is expected that the study was the first basic information about the genetic diversity of *Ficus* and the results affect the decision of the plant protection in Myanmar.

**Keywords:** AFLP, Hybridization, Morphological, *F. religiosa*, *F. rumphii*, Myanmar

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## Introduction

The figs (*Ficus*, Moraceae) are one of the largest genera of woody flowering plants with about 800-850 species of free standing trees, epiphytes, shrubs, creepers, climbers and stranglers in tropical and subtropical regions worldwide (Corner 1965; Bailey Hortorium 1976). Roughly half of the known *Ficus* species are monoecious, and the rest are functionally dioecious (Berg 2003; Ronsted et al., 2008). Genus *Ficus* is well known to have extremely specialized symbiotic relations, one to one obligate mutualism with fig-wasps. The interactions between figs and fig-wasps (Agaonidae, Chalcidoidea, Hymenoptera) are special plant-pollinator relationships that are highly species-specific (Janzen 1979; Herre et al., 1999; Machado et al., 2001). In relation to the mutualism, *Ficus* plants are well characterized by hanging a flower on the inside wall of spherical inflorescence called a syconium. Generally, 4 different kinds of flowers can be found in the syconia (long-styled female flowers, short-styled female flowers, male flowers and neuter flowers). The only insect which carries pollen to the blooming flower in the inflorescence is the fig-wasp. Females of fig-wasps enter syconia and lay eggs with pollination to flowers. An important aspect of pollination symbiotic relationship of fig/fig-wasp is that a phytophagous insect (seed parasite) is a pollinator at the same time, and this point is greatly different from other relationships between plants and pollinator insects (Ramirez 1974; Wiebes 1979; Yokoyama and Iwatsuki 1998; Herre et al., 1996; Anstett et al., 1997).

There are about 85 species of fig in Myanmar (Kress et al., 2003). The fruits of them are important food sources for many animals including human beings. Some fig tree is planted for ornamental purposes, especially in landscaping due to its aesthetic shape and form. It is also commonly planted in gardens and along roadsides. Some species, as *F. religiosa*, is mostly planted near Buddhist temples as it is referred to as sacred trees in India. Hindus associate the tree with fertility in woman. It is also an important host to lac insects. The tree is also sacred in Myanmar Culture. The Buddha is said to

have gained enlightenment under a sacred fig called the Buddhi Tree (Bawdi-Nyaung).

The *F. religiosa* L. and *F. rumphii* Blume are generally known to be monoecious species, the male, female and gall flowers are borne together in the same syconium and large woody deciduous trees. It can be found in woodland areas with other trees. Flowering and fruiting are observed all year round. These two species are widely distributed in Myanmar. Sometimes, both species are growing together with the same environmental conditions up to elevations 5,000 ft. All of every parts of the tree are useful for as a traditional medicine for various ailments and mature stem-trunks are using as fuels. Generally, these two species are morphologically very similar and complicated (Yamazaki 1983). Although, they are similar natures, they still have different in their characteristics. Pollination is made by species-specific fig wasps and seed dispersal is achieved by birds and mammals.

*F. religiosa* is the famous and essentially important species includes Myanmar (Bawdi-Nyaung) and some neighboring countries throughout the Southeast Asia because this species are well known about the religious sacred tree from the ancient times. Nowadays, this species is grown as an ornamental plant around the world. *F. religiosa* is pollinated by fig-wasps pollinator *Plastyscapa quadriceps* (Galil & Eisikowitch 1968). The mainly native countries are India, Nepal, Chad, Thailand, Myanmar and Southwest China, Vietnam and other Southeast Asia. The related species *F. rumphii* is distributed in India and Malay Peninsula and the Malucca and another described that they occurred in the dry lower slopes of the mountains Panjab; Northern, Western, and Central India; Assam, Myanmar and Malay island (Corner 1965). The local name of *F. rumphii* is Nyaung-Phyu. The pollinating fig wasp is still not known for *F. rumphii*. Breeding systems, pollen and seed dispersal, and life-form are played important roles for the formation of genetic structure and geographical distribution of plant populations. In the cases of the plant species, the ranges of pollen and seed dispersal are greatly influenced by the media, and it is thought that the degree of the influence depend upon what kind of mediator or vectors is used for the dispersion.

There are a lot of studies by many researchers upon morphology, mating systems, or coevolution of figs and their species-specific pollinating wasps (Yokoyama 1995, 2003; Zavodna et al., 2005; Chen et al., 2008; Wang et.al., 2009; Azuma et al., 2010; Lomascolo et al., 2010; Ghara& Borges 2010). However, the studies upon population genetic structure in detail are rarely conducted, especially in Southeast Asia where the species diversity of figs is extremely high. Here, we performed population genetic structure of two closely related species pairs of figs, *F. religiosa* and *F. rumphii* by using AFLP molecular technique. Among the molecular marker technique, AFLP is a novel polymerase chain (PCR) base assay for DNA finger printing and polymorphism detection. In this study, we found extensive hybridization in both species. This findings suggested that hybridization plays an important role of the evolution of *Ficus*, and, at the same time, there is a big question that how they maintain the species identification in the situation that the interspecific hybridization is common. Moreover, genetic relationship among *Ficus* species from Myanmar and other countries will be expected.

## Materials and Methods

### Samples collection

In this study, a total of ca. 600 individuals of *F. religiosa* and *F. rumphii* were randomly collected from 28 different populations throughout the Myanmar in June 2012 and March 2013 (Figure 1). 374 individuals from these 600 individuals were genetic analysis. The collection sites were geo-referenced by using Global Positioning System (GPS). For each population, young leaves were collected from each individual, with an interval > 50m between individuals. Firstly, sampling plants were separated by morphological observation. Secondly, some leaves are wrapped by newspaper and pressed for preservation of specimens. After that, the leaves were measured individually for morphological data. One or two leaves are put into a plastic bag containing silica gel for storage until use.

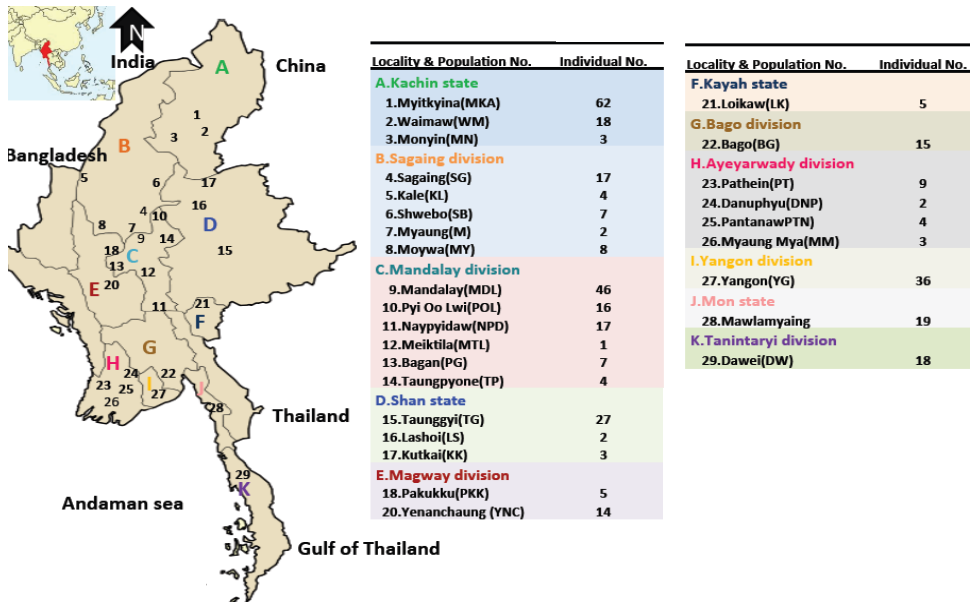
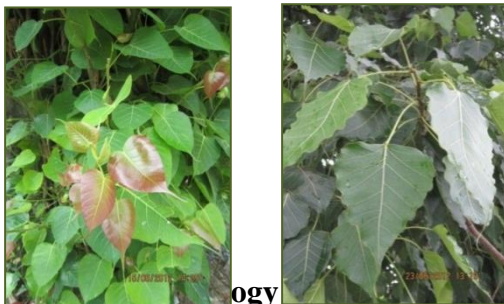


Figure 1. Sampling locations for the genetic studies of *F. religiosa* and *F. rumphii* in Myanmar.

Table 1. Geographic coordinates of sampling locations in Myanmar.

State & Division	Site ID	Latitude	Longitude	Elevation	
Kachin	MKA	25°22'59"N	97°24'00"E	158m(518ft)	
	WM	25°21'00"N	97°25'59"E	165m(541ft)	
	MN	24°46'59"N	96°22'00"E	278(912ft)	
Sagaing	SG	21°52'43"N	95°58'46"E	60m(196ft)	
	KL	23°10'59"N	94°04'00"E	102m(334ft)	
	SB	22°34'09"N	95°41'53"E	111m(364ft)	
	M	21°50'54"N	95°25'28"E	73m(239.5ft)	
	MY	22°06'30"N	95°08'30"E	82m(269ft)	
Mandalay	MDL	21°58'29"N	96°05'00"E	63m(206ft)	
	POL	22°01'59"N	96°28'00"E	1090m(3576ft)	
	NPD	19°44'59"N	96°07'46"E	108m(354ft)	
	MTL	20°52'39"N	95°51'30"E	244m(800ft)	
	PG	21°12'02"N	94°53'11"E	95m(312ft)	
Shan	TP	21°58'29"N	96°05'00"E	63m(206ft)	
	TG	20°47'00"N	97°02'00"E	1400m(4590ft)	
	LS	22°56'00"N	97°45'00"E	836m(2746ft)	
Magway	KK	23°27'00"N	97°56'00"E	1400m(4600ft)	
	PKK	21°20'00"N	95°05'00"E	48m(156.8ft)	
	YNC	24°10'60"N	94°52'60"E	139m(459ft)	
Kayah	LK	19°40'27"N	97°12'33"E	872m(2860ft)	
Bago	BG	17°20'12"N	96°28'46"E	4m(13ft)	
	Ayeyarwady	PT	16°14'58"N	97°57'58"E	12m(39.2ft)
		DNP	17°22'00"N	95°27'00"E	11m(39ft)
		PTN	16°59'00"N	95°28'00"E	7m(26ft)
MM		16°36'00"N	94°55'60"E	11m(39ft)	
Yangon	YG	16°48'19"N	96°09'21"E	13m(42ft)	
	MLM	16°29'29"N	97°37'32"E	30m(98ft)	
Tanintaryi	DW	14°04'59"N	98°12'00"E	5m(16ft)	

**Figure 2.** Appearances of *F. religiosa* L.(left) and *F. rumphii* Blume (right).



## Observations of Morphology

Morphological observations have been done during the sample collecting in the fields. General observation of leaf type of individuals, conditions of tree trunk and their environmental condition and ecosystem characteristics were marked. Five parts of each individual leaf are measured for principal component analysis (PCA) data. Then identifying with the previous morphological and taxonomic literature about *Ficus* and researching data of other neighboring countries in Mandalay University, Myanmar.

## DNA Isolation and AFLP analyses

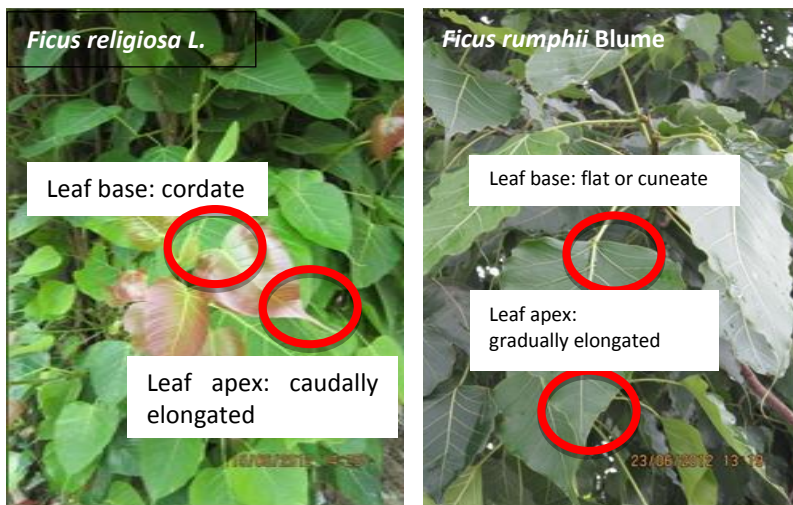
DNA extractions were performed by using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Extracted DNA was frozen at  $-20^{\circ}\text{C}$  until use. AFLP (Amplified Fragment Length Polymorphism) analyses were performed using the Ligation and Preselective Amplification Module and selective primers (Applied Biosystems, Foster City, CA) following the manufacturer's protocol (Vos et al., 1995). Initially, 41 selective primer pairs were tested on 20 individuals to determine those producing the most informative and strong profiles. Finally, three primer pairs, EcoRI-CAT/MseI-ACT, EcoRI-CAA/MseI-AGC, and EcoRI-CAA/MseI-AGG, were used for selective amplifications. The final amplification products were electrophoresed with the GeneScan<sup>TM</sup> 500 Rox<sup>TM</sup> dye Size Standard (Applied Biosystems) using an ABI PRISM<sup>®</sup> 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 (Pritchard et al.,

2000; Falush et al., 2007). An analysis was then performed with 25,000 iterations following 25,000 replications of burn-in period. Based on the AFLP data, the genetic structure analysis with assignment tests were done by analysis software Structure 2.3.1 (Pritchard et al., 2000; Falush et al., 2007). For the assignment tests, a calculation of  $\Delta K$  to estimate the appropriate cluster number was also conducted (Evanno et al., 2005).

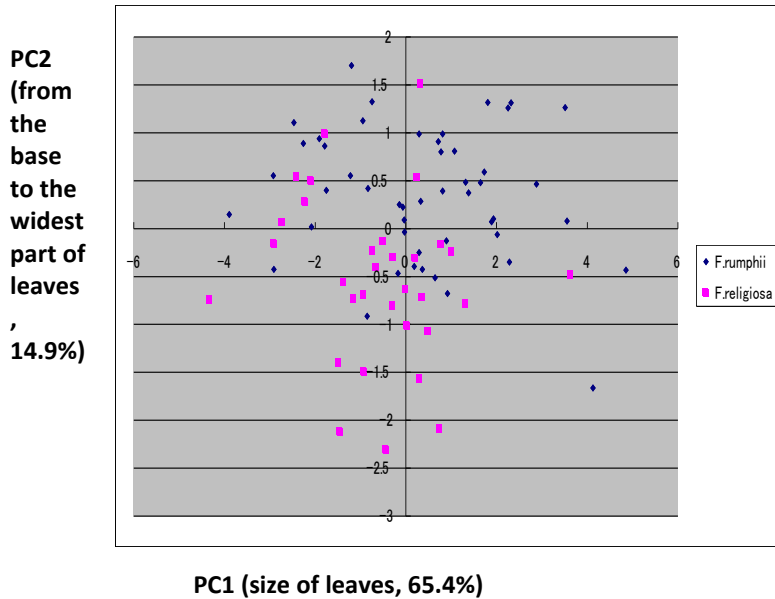
## Results

### Morphological Observation

Generally, *F. religiosa* and their closely related species *F. rumphii* are actually difficult to distinguish from the morphological outlook, but, in the field observation, this two species are clearly separated by the part of leaf-tip length (aristate part was longer in *F. religiosa* than *F. rumphii*, sometimes acuminate but not aristate) and base of leaf-blade (cordate in *F. religiosa* and truncate in *F. rumphii*) (Fig. 3). Due to the principal component analysis (PCA), the widest part of the leaves tended to come more basal position in *F. religiosa* than in *F. rumphii*. But leaf size variation of two species largely overlapped (Fig. 4). This situation may reflect geographic differentiation and/or interspecific hybridization.



**Figure 3.** Morphologically difference part of *F. religiosa* and *F. rumphii*.

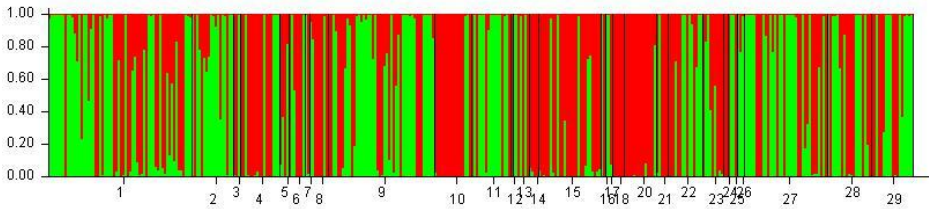


**Figure 4.** Morphological differences between leaves of *F. religiosa* and *F. rumphii*.

### AFLP Analysis

First, the assignment test was performed with all 374 individuals of *F. religiosa* and *F. rumphii* together and analyzed as  $K=2$ . As the result, *F. religiosa* and *F. rumphii* were well divided from each other, suggesting genetic distinction between species (Fig. 5). However, it was shown that many admixture genotypes between the two species also occurred. When we defined the hybrid individuals as 0.2 or more membership value for the less frequent cluster, a total of 42 individuals out of 374 were shown as hybrid individuals. These hybrid individuals were found throughout in the sampled area and morphological characteristics of the were quite variable (Table. 2 and Fig. 6).

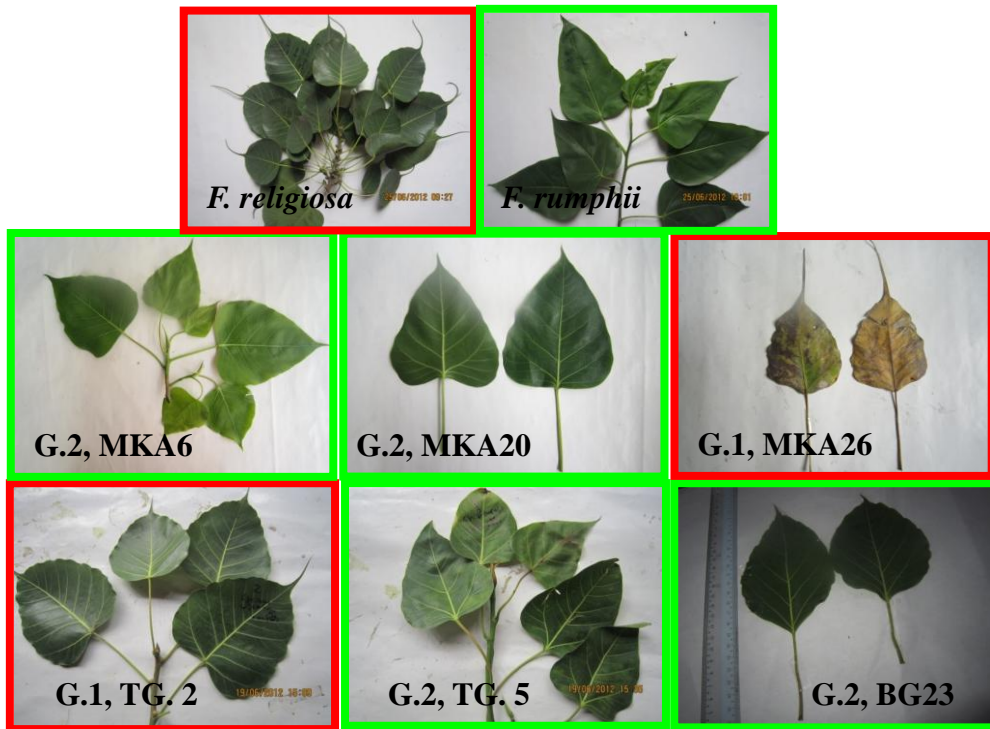




**Figure 5.** A result of assignment test for the combined data of *F. religiosa* and *F. rumphii*, two genetic clusters (K=2). Numbers under the bar plot indicate individual ID and vertical bar is indicated to interspecific hybridization ratio. Two clusters were as follows: (1) *F. religiosa* (red) and (2) *F. rumphii* (green).

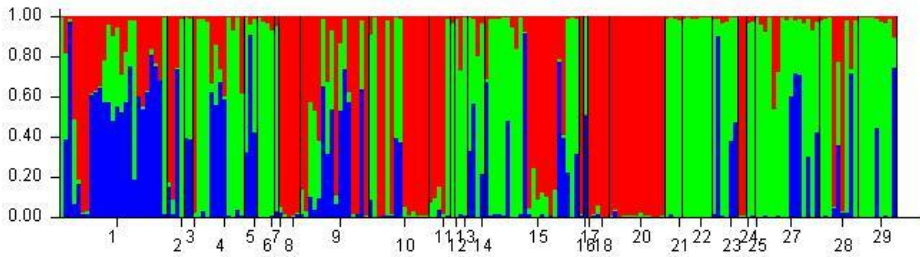
**Table 2.** Summary of individuals assigned as the mixed genotype between *F. religiosa* and *F. rumphii* based on the AFLP analysis. Letters and numbers in parenthesis indicate minor genetic cluster and its frequency. (R-red gene type %, G-green gene type %)

No.	POP No.	Hybrid INDV/siteID	No.	POP No.	Hybrid INDV/siteID
1	1	MKA 20(R. 0.35)	22	9	MDL 27(R.0.35)
2	1	MKA 22(G. 0.25)	23	9	MDL 31(R.0.38)
3	1	MKA25(R.0.50/G.0.50)	24	9	MDL 32(R.0.35)
4	1	MKA 37(R.0.30)	25	9	MDL 36(R.0.50, G.50)
5	1	MKA 42(R.0.38)	26	15	TG 2(G.0.38)
6	1	MKA 43(R.0.35)	27	15	TG 4(R.0.26)
7	1	MKA 47(R.0.20)	28	15	TG 5(R.0.25)
8	1	MKA 56(R.0.40)	29	22	BG 13(R.0.25)
9	1	MKA 58(R.0.42)	30	22	BG 23(R.0.28)
10	1	MKA 6(R.0.20)	31	23	PT 2(R.0.20/18)
11	2	WM 13(R.0.20)	32	23	PT 4(G.0.40)
12	2	WM 15(R.0.25)	33	23	PT 6(R.0.47)
13	2	WM 16(R.0.40)	34	26	MM 2(R.0.25)
14	2	WM 17(R.0.25)	35	27	YG 20(R.0.23)
15	2	WM 4(G.0.40)	36	27	YG 33(G.0.20)
16	5	KL 2(G.0.38)	37	27	YG 34(G.0.38)
17	5	KL 4(R.0.20)	38	27	YG 6(R.0.38)
18	6	SB 3(R.0.45)	39	28	MLM 14(R.0.38)
19	8	MY 2(R.0.20)	40	28	MLM 8(G.0.20)
20	9	MDL 16(R.0.38)	41	29	DW 15(G.0.30)
21	9	MDL 2(G.0.20)	42	29	DW 5(G.0.38)



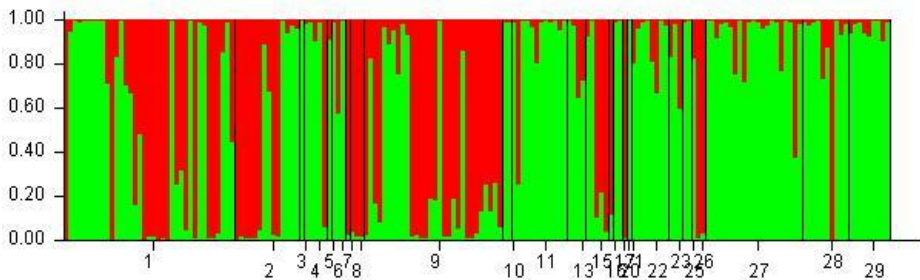
**Figure 6.** Representative individuals with hybrid genotypes between *F. religiosa* and *F. rumphii*

In the next step, *F. religiosa* and *F. rumphii* were analyzed separately to confirm the genetic structure of each species. The results of *F. religiosa* (195 individuals) showed that three genetic types were recognized ( $K=3$ , Fig. 7). Many individuals of the population 1 (MKA), population 9 (MDL) and population 27 (YG) had the least frequent genetic cluster (indicated as blue in Fig. 7). MKA and MDL were the populations where first and second highest numbers of hybrid individuals were recorded (Table. 6). This fact indicated that the genetic cluster represented introgressant fraction of hybrid genotypes. Almost all individuals of the population 20 (YNC), population 8 (MY) and population 10 (POL) were composed of one of the frequent genetic cluster (indicated as red in Fig. 7). However, overall, each population of *F. religiosa* was genetically admixture of individuals of two frequent genetic clusters.



**Figure 7.** A result of assignment test for the data of *F. religiosa*, three genetic clusters (K=3). Numbers under the bar plot indicate individual ID.

Similarly, the result of *F. rumphii* (179 individuals) showed the presence of two genetic clusters (Fig. 8). In population 9 (MDL) and population 1 (MKA), individuals with the less frequent genetic cluster (indicated as red in Fig. 8) were abundant. These two populations was also shown that the least frequent genetic cluster of *F. religiosa* was observed in many individuals (Fig. 8). Thus, the results indicated that the less frequent genetic cluster of *F. rumphii* is also an introgressant fraction of hybrid genotypes. From these two results of assignment tests, both species were genetically mixed extensively in both directions.



**Figure 8.** A result of assignment test for the data of *F. rumphii*, two genetic clusters (K=2). Numbers under the bar plot indicate individual ID.

## Discussion and Conclusion

Two monoecious fig species were examined in this study, *F. religiosa* and *F. rumphii*, were distinguished from each other by leaf morphology. The morphological differences between them were indicated previously by some authors (Corner 1965; Yamazaki 1983) and were confirmed in this study. From the result of the assignment test, genetic differentiation of two species was obvious though these two species were closely related and sympatric throughout the territory of Myanmar. On the other hand, the interspecific hybridization between those two species was also found in this study. Previous study, *F. pumila* L. and relative species *F. thunbergii* Maxim. In Japan hybrid individuals were also found, although few, and hybridization pattern is unidirectional introgression from *F. thunbergii* to *F. pumila*. Characteristics of the hybridization conditions were, however, different from those of the case between *F. pumila* and *F. thunbergii* (Lum Tsai *et al.*, 2015). First, the hybridization between *F. religiosa* and *F. rumphii* in Myanmar was extensively found in many populations. Second, these two species hybridized and introgressed from both directions.

For the first point, factors affected the extensive hybridizations were unclear. However, general flowering phenology of monoecious figs may be influenced in this situation. Flowering and fruiting were generally synchronized in a single individual but not synchronized between individuals. Therefore, there are at least some flowering (receptive) and fruiting (releasing fig-wasps) individuals in a given population of monoecious figs. In this phenological feature, fig-wasps sometime fail to find receptacle figs when the give population of monoecious fig are small (Bronstein *et al.*, 1990). In the situation, individuals of closely related species may be recipients of heterospecific fig-wasps. Occurrence of *F. religiosa* is often affected by human activities and individuals are tended to be isolated from others. The habitats of *F. rumphii* are natural forests but these habitats are recently destructed and fragmented. These situations in both species, combined together, may be basis of extensive hybridization between the two species.

For the second point, introgression occurred in the particular populations though morphological characteristics were rather stable for both species. One of the reasons may be the simple genetic basis of characteristic leaf forms for each species. However, there are no instances of genetic basis of leaf morphology in figs and we do not have information to confirm this hypothesis. On the other hand, morphological identities of species were sometimes maintained even introgressions present in some extent (Tsukaya et al., 2003; Yamaji et al., 2007). The case of two monoecious fig species in Myanmar may be the similar case to those previous studies. Although the influence to morphological features are rather small, these types of introgression should be affected the evolution of given plant groups by introducing genetic sources of different origins and the case of *Ficus* is also not an exception.

The case of *F. religiosa* and *F. rumphii* in Myanmar; generally, both species are morphological very similar in the field observation. These two species were genetically distinguished into two types, even though large number of mixed individuals are existing between both species. Life history is almost the same in both species for such as flowering and fruiting pattern and growing environments. Therefore, under these ecological and environmental conditions, fig and fig-wasp obligate mutualism may be breakdown between *F. religiosa* and *F. rumphii*.

Monoecious fig trees are commonly with large canopies and fig-wasps can fly for long distance. Syconia of the *F. religiosa* and *F. rumphii* are small, therefore easy to feed and carry far away for various birds including small species. These factors were contributed genetic homogenization among populations of *F. religiosa* and *F. rumphii*. It is needed more information about the introgressive hybridizations of figs and further analyses for the nature of introgression in detail should bring more accurate pictures of the evolution and diversification in genus *Ficus*.

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