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Introgressive Hybrids of *Arisaema sikokianum* and *A. tosaense* (Araceae) Confirmed through Nuclear and Chloroplast DNA Comparisons

Hiroshi Hayakawa[1,2], Hidenori Hamachi[1], Kanako Matsuyama[3], Yuko Muramatsu[3], Yukio Minamiya[1], Katsura Ito[1], Jun Yokoyama[4], Tatsuya Fukuda[1*]

[1]Faculty of Agriculture, Kochi University, Nankoku, Japan; [2]United Graduate School of Agricultural Sciences, Ehime University, Nankoku, Japan; [3]The Graduate School of Integrated Arts and Sciences, Kochi University, Nankoku, Japan; [4]Faculty of Science, Yamagata University, Yamagata, Japan.
Email: [*]tfukuda@kochi-u.ac.jp

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ABSTRACT

Morphologically putative introgressive hybrids of Arisaema sikokianum Franch. et Sav. and A. tosaense Makino were newly found in Kochi and Tokushima Prefectures in Japan. All the individuals have the same morphological characteristics as A. tosaense excluding a purple spathe. Molecular analysis using PCR-RFLP of internal transcribed spacer (ITS) in nuclear DNA (nrDNA) indicates that these putative introgressive hybrids have the same pattern as A. tosaense. Moreover, the sequences of chloroplast DNA (cpDNA) of the putative introgressive hybrids were identical to A. sikokianum from Kochi Prefecture and A. tosaense from Tokushima Prefecture. The results suggest that the plants are introgressive hybrids of A. sikokianum and A. tosaense and that they have highly exchanged genes with A. tosaense.

Keywords*: *Araceae, Arisaema, A. sikokianum, A. tosaense, Chloroplast Capture, Introgression, ITS, Molecular Analysis, PCR-RFLP, Trn

1. Introduction

Studies of natural hybridization and introgression and their genetic composition can shed new light on issues concerning reproductive barriers and fitness of them and give important insights into evolutionary processes and the adaptation of species [1].

The genus *Arisaema* Martius (Araceae), which has a large, often colored and conspicuous bract (spathe), subtending and enveloping a bisexual or unisexual spadix with numerous small flowers, comprises 40 - 85 species in Japan [2,3]. Species of *Arisaema* in the section *Pistillata* Nakai have a slender appendage at the base and are mostly distributed in Japan [3]. Section *Pistillata* is included in 35 - 80 species in Japan [3], and presents many taxonomic difficulties caused by the concentration of closely related species with few morphological differences [4].

Sixteen patterns of putative natural hybrids among the species in section *Pistillata* have been reported in *Arisaema* (e.g., [5]). Of them, the hybrids between *Arisaema sikokianum* Franch. et Sav. and *Arisaema tosaense* Makino grow only in Kochi Prefecture in Japan [2,6]. However, Hayakawa *et al*. [7] newly found hybrids of these two species in Tokushima Prefecture and, using molecular analysis, revealed that the hybrids occur bidirectionally and that they have exchanged genes with the two parental species. *A. sikokianum* has a purple upward spathe, a white capitate appendage and leaves with 3 to 5 leaflets (**Figure 1(a)**, See detail **Table 1**). *A. tosaense* has a characteristic green spathe extending approx. 30 - 45 cm before it bends downward to the ground and has leaves with 7 to 11 leaflets (**Figure 1(b)**, **Table 1**). These species have the same chromosome numbers ($2n$ = 28) [8,9]. Sympatry of the two species extends over a wide area in Shikoku, and the hybrids of them share various morphological characters [7,8]. Therefore, it is possible that the parental species could make the hybrid progenies and introduce gene(s) in each other through chloroplast captures, backcrossing and recombination, but introgressive hybrids including near isogenic lines (NILs) and recombinant lines (RLs) have not been made so far.

To clarify chloroplast captures of the introgressive hybrids of *Arisaema sikokianum* and *A. tosaense*, we con-

ducted molecular analysis using nuclear DNA (nrDNA) and chloroplast DNA (cpDNA) sequences. Our findings regarding the introgressive hybrids suggest that chloroplast captures and gene(s) flow may occur in *A. sikokianum* and *A. tosaense*.

2. Materials and Methods

Morphologically, the putative introgressive hybrids of *Arisaema sikokianum* and *A. tosaense* were found at two localities in Kochi and Tokushima Prefectures (**Figure 2**, **Table 2**). Although hybrids of *A. sikokianum* and *A. tosaense* were found with both parental species [7,8], all the putative introgressive hybrids were found with *A. tosaense* (**Figure 2(I)** and **(II)**). Voucher specimens of the introgressive hybrids are deposited in the herbarium of the Makino Botanical Garden, Kochi (MBK).

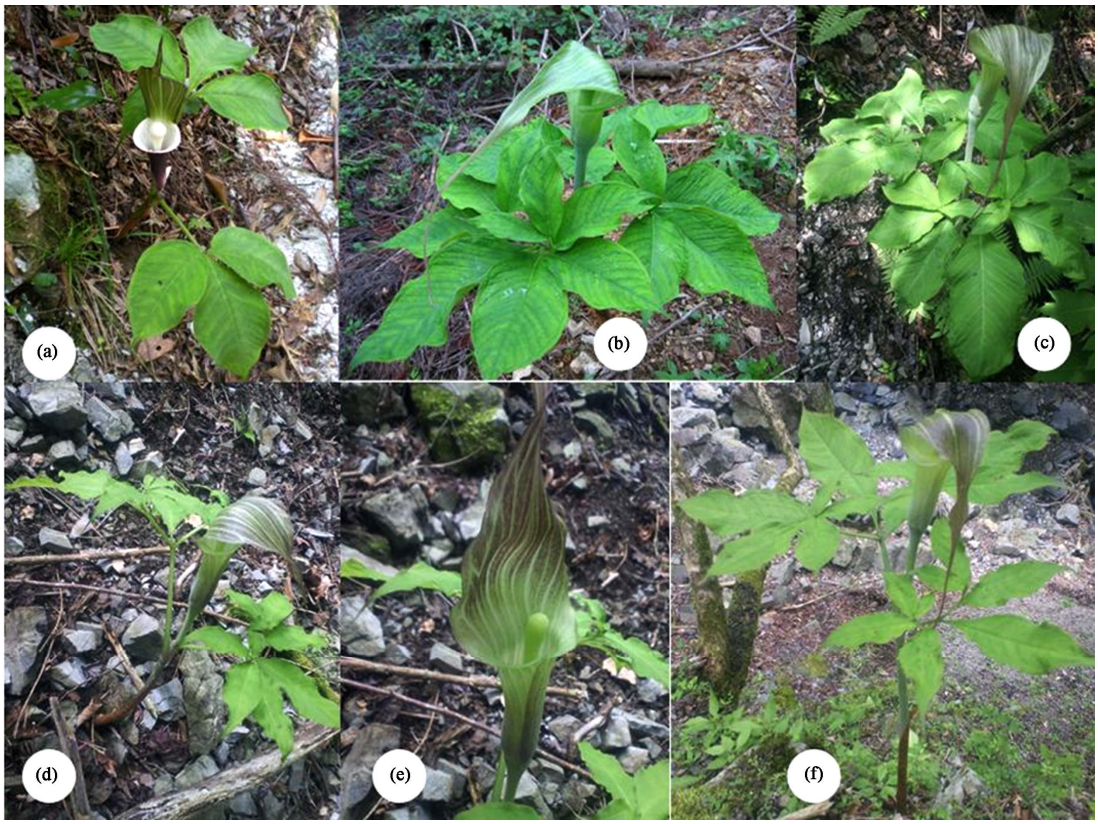

Figure 1. Species of *Arisaema* examined in this study. (a) *A. sikokianum*; (b) *A. tosaense*; (c)-(f) putative introgressive hybrids along Koishikawa River; (e) introgressive hybrid with spathe tip raised to show appendage (same plant shown in (d)).

Table 1. Morphological characteristics of samples used in this study.

Trait	*Arisaema*	Putative Introgressive Hybrid		*Arisaema*
	sikokianum	I	II	*tosaense*
Leaf Characteristics				
Leaflets	3 to 5	9 to 15	over 7	7 to 11
Reproductive Characteristics				
Spathe Tip	Long	Very Long	Very Long	Very Long
Spathe Tip Direction	Upward	Drooping	Drooping	Drooping
Spathe Color	Purple	Purple	Purple	Green
Appendage Shape	Capitate	Cylindrical	Cylindrical	Cylindrical
Appendage Color	White	Green	Green	Green
Flowering Phenology	April to May	Early June	Early June	Late May to June

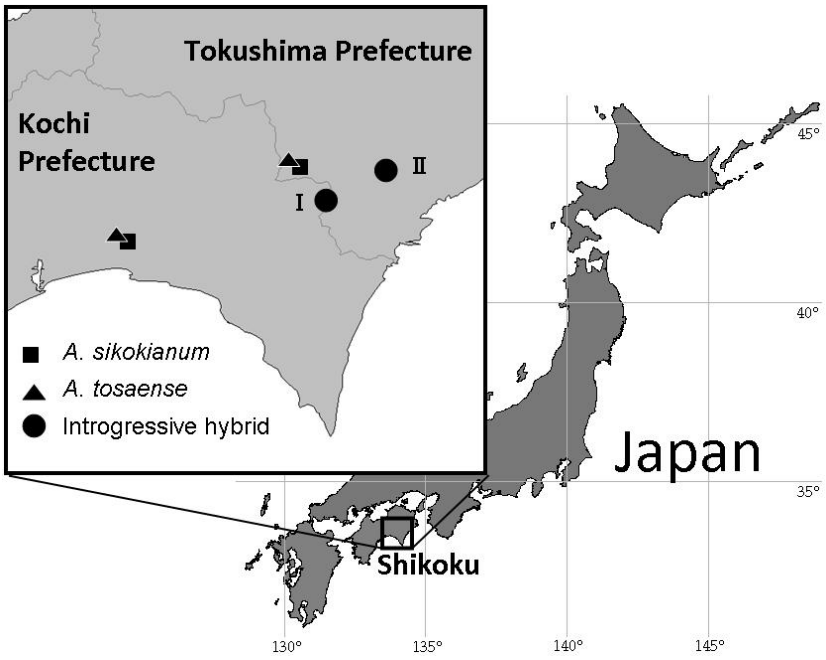

Figure 2. Sampling localities of putative introgressive hybrids of *Arisaema sikokianum* and *A. tosaense*. Blacked squires, triangles and circles indicate *A. sikokianum*, *A. tosaense* and putative introgressive hybrids, respectively. (I) Kochi Pref., Aki-gun Umaji-mura, Yanase, along Koishikawa River; (II) Tokushima Pref., Kaifu-gun Kaiyo-cho, Ogawa, Kirikoshi Pass.

Table 2. Locality where samples were collected.

No.	Species		Locality	Collector	Date
1	*Arisaema sikokianum*		Tokushima Pref. Naka-Gun, Naka-Cho, Senbondani Valley	HH, MuY, MiY	2009-7-2
2			Kochi Pref. Nankoku City, Nareai, Nebiki Pass	HH	2009-5-1
3	Putative Introgressive Hybrid	I	Kochi Pref. Aki-Gun, Umaji-Mura, Yanase, Koishikawa Valley	HH, FT	2010-6-4
4			Kochi Pref. Aki-Gun, Umaji-Mura, Yanase, Koishikawa Valley	HH, FT	2010-6-4
5		II	Tokushima Pref. Kaifu-gun, Kaiyo-Cho, Ogawa, Kirikoshi Pass	HH, FT	2010-6-4
6			Tokushima Pref. Kaifu-gun, Kaiyo-Cho, Ogawa, Kirikoshi Pass	HH, FT	2010-6-4
7	*A. tosaense*		Tokushima Pref. Naka-Gun, Naka-Cho, Senbondani Valley	HH, MuY, MiY	2009-7-2
8			Kochi Pref. Nankoku City, Nareai, Nebiki Pass	HH	2009-6-8

HH: Hayakawa Hiroshi; MuY: Muramatsu Yuko; MiY: Minamiya Yukio; FT: Fukuda Tatsuya.

For the molecular analysis, total DNA was isolated from 200 - 300 mg of leaves using a Plant Genomic DNA Mini Kit (VIOGENE, Sunnyvale, USA), according to the manufacturer's protocol. We amplified the internal transcribed spacer (ITS) region from nrDNA and the *trnL* intron from cpDNA with primers designed by White *et al*. [10] and Taberlet *et al*. [11], respectively. The isolated DNA was amplified by PCR in a 50 µl reaction solution containing approximately 50 ng total DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 units *Taq* DNA polymerase (TaKaRa) and 0.5 µM of each primer. We used the following thermal cycle profile for amplification by the PCR Thermal Cycler Dice (TaKaRa): 1 min at 94˚C, 2 min at 48˚C, and 2 min at 72˚C for 45 cycles, followed by 15 min of final extension at 72˚C. After amplification, the PCR products of the ITS region as well as the *trnL* intron were subjected to electrophoresis in 1% low-melting-temperature agarose gels to remove by-products and purify amplified products. We sequenced the purified PCR products using a BigDye Terminator ver. 3.1 (Applied BioSystems) and ABI Prism 3100 Genetic Analyzer (Applied BioSystems) according to the manufacturer's instructions. For sequencing, we used the same primers as those used for amplification.

For the ITS region, we carried out PCR-RFLP (restric-

tion fragment length polymorphism) analysis, because an autapomorphic character of the nrDNA is the restriction site *Mse* I (TTAA) [7]. After designating the restriction sites, the amplified products were digested by *Mse* I at 37˚C for more than an hour. The digested DNAs were separated on 1.5% agarose gel and the size of each band was determined.

3. Results and Discussion

From the results of the molecular analysis we determined that five putative introgressive hybrids occurred at each locality. The morphological analysis showed that all the putative introgressive hybrids have a purple spathe (**Figures 1(c)-(f)**) which is similar to *Arisaema sikokianum*, but have a green cylindrical appendage as an elongate spathe tip and a large number of leaflets, which is similar to *A. tosaense* (**Figure 1**, **Table 1**). The putative introgressive hybrids seem to have the same characteristics as *A. tosaense* excluding the color of the spathe, and share the same morphological features.

We conducted PCR-RFLP in nrDNA because the ITS region of *Arisaema sikokianum* has one *Mse* I site, while *A. tosaense* has two sites digested by this restriction enzyme [7]. The digestion patterns of all samples of *A. sikokianum* and *A. tosaense* showed expected patterns and all the putative introgressive hybrids showed the same patterns as those of *A. tosaense* (**Figure 3**). We therefore confirmed that the putative introgressive hybrids have an *A. tosaense* type of ITS in nrDNA.

The *trnL* intron region in cpDNA is a good molecular marker for distinguishing *Arisaema sikokianum*, because the *trnL* intron has a 17 bp-insertion or deletion (indel) in the sequence of *A. sikokianum* (450 bp) and *A. tosaense* (467 bp) [7]. We therefore determined the sequences of the *trnL* intron. In the introgressive hybrids, the sequence results from the cpDNA analysis were identical to the *A. sikokianum* from Kochi Prefecture and to *A. tosaense* from Tokushima Prefecture (**Table 3**), and we could not find any different pattern of cpDNA for the introgressive hybrids in any of the two localities.

From the results of morphological and molecular analyses using nrDNA and cpDNA, the introgressive hybrids from Kochi Prefecture can be determined but not from Tokushima Prefecture. The flowering phenology of the hybrids between *Arisaema sikokianum* and *A. tosaense* was inherited from *A. tosaense* [7]. Therefore, the flowering phenology of hybrid progenies may overlap with that of *A. tosaense* and would introduce *A. tosaense* genes. In this study, although all the putative introgressive hybrids in Tokushima Prefecture had a purple spathe and had an outward appearance of *A. tosaense*, we could not detect any molecular evidence using the ITS region.

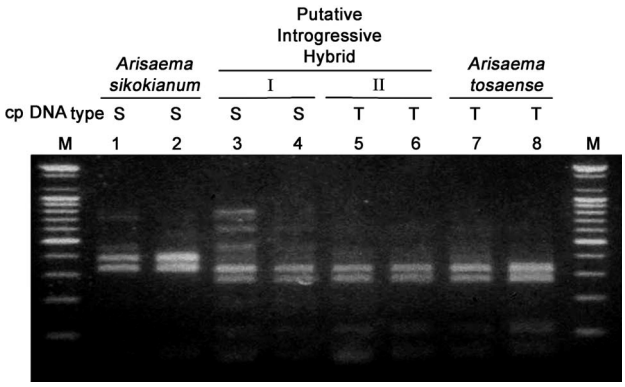

Figure 3. PCR-RFLP profile of *Arisaema sikokianum*, *A. tosaense* and putative introgressive hybrids. Arrows indicate expected fragments of both *A. sikokianum* and *A. tosaense*. M: size marker. The cpDNA types correspond to the types in Table 3.

Table 3. Genotype of samples used in this study.

Species		Locality	nrDNA (ITS)	cpDNA (*trnL* intron)
Arisaema sikokianum			S	S
Putative Introgressive Hybrid	I	Koishikawa	T	S
Putative Introgressive Hybrid	II	Kirikoshi pass	T	T
A. tosaense			T	T

S: *Arisaema sikokianum* type. Accession numbers; AB513178 (ITS) and AB513176 (*trnL* intron). T: *A. tosaense* type. Accession numbers; AB513179 (ITS) and AB513177 (*trnL* intron).

Additional nrDNA sequences such as TPI and PGI may provide the answers about the putative introgressive hybrids in Tokushima Prefecture.

From our results and previous reports [6-8], the hybridization and introgressive hybrids of *Arisaema sikokianum* and *A. tosaense* seem to be widely distributed in eastern Shikoku. In some limited areas of Shikoku and its margin areas, the appearance of *A. tosaense* is varied, not only in spathe color but also the shape of the leaflets (wide to narrow) and the shape of the appendage (cylindrical to capitate) ([12] and Hayakawa unpubl.), implying that various characteristics are generated in introgressive hybrids. More detailed investigations around eastern Shikoku will be needed because it may reveal new localities of introgressive hybrids carrying different characteristics to those in this study. Moreover, in Shizuoka Prefecture, Kakishima *et al.* [13] indicated that introgressive gene flow occurs from *A. angutatum* Franch et Sav. to *A. suwoense* Nakai. Those reports, along with our findings, suggest that endemic species might have occurred through introgressive hybrids in some *Arisaema*

sect. *Pistillata*.

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