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Interspecific Hybridization between *Arisaema sikokianum* and *A. serratum* (Araceae) Confirmed through Nuclear and Chloroplast DNA Comparisons

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

[1]Faculty of Agriculture, Kochi University, Monobe, Nankoku 783-8502, Japan; [2]The United Graduate School of Agricultural Sciences, Ehime University, Monobe, Nankoku 783-8502, Japan; [3]The Graduate School of Integrated Arts and Sciences, Kochi University, Monobe, Nankoku 783-8502, Japan; [4]Faculty of Science, Yamagata University, 1-4-12 Kojirakawa-machi, Yamagata 990-8560, Japan.
Email: [*]tfukuda@kochi-u.ac.jp

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ABSTRACT

A morphologically intermediate plant between Arisaema sikokianum Franch. et Sav. and A. serratum (*Thunb.*) *Schott has been newly found in Kochi Prefecture, Shikoku, Japan. The putative hybrid has the intermediate morphological characteristics of the parental species. Molecular analysis using PCR-RFLP of internal transcribed spacer* (*ITS*) *in nuclear DNA* (*nrDNA*) *indicates that the putative hybrid has a combined pattern of the two putative parent species. Moreover, the sequence result of chloroplast DNA* (*cpDNA*) *of the putative hybrid was identical to that of A. sikokianum. These results suggest that the putative hybrid is a hybrid between A. sikokianum and A. serratum and that it was formed by interactive gene exchanging via pollens from A. serratum to A. sikokianum. It is the first record of a hybrid between A. sikokianum and A. serratum.*

Keywords*: *Araceae*, *Arisaema*, *A. serratum*, *A. sikokianum*, *Chloroplast DNA*, *Interspecific Hybrid*, *Molecular Analysis*, *Nuclear DNA

1. Introduction

The genus *Arisaema* Martius (Araceae), which has a large, often colored and conspicuous bract (spathe), and subtending and enveloping bisexual or unisexual spadix with numerous small flowers, comprises approximately 40 - 85 species in Japan [1,2]. Species of *Arisaema* in the section *Pedatisecta* Schott ex Engler have a slender appendage at the base and are mostly distributed in Japan [2]. Section *Pedatisecta* is included in 35 - 80 species in Japan [2], and presents many taxonomic difficulties caused by the concentration of closely related species with few morphological differences [3].

Sixteen patterns of putative natural hybrids have been reported in *Arisaema* sect. *Pedatisecta* (e.g., [4]). Of them, *A. sikokianum* Franch. et Sav. and *A. tosaense* Makino make the hybrids [5,6]. Moreover, *A. ehimense* J. Murata et Ohno is of hybrid origin between *A. serratum* (Thunb.) Schott and *A. tosaense* based on allozyme analysis [7]. Therefore, it is possible that hybrid speciation or reticulate evolution or both has occurred among *A. tosaense*, *A. sikokianum* and *A. serratum*. However, the hybridization between *A. sikokianum* and *A. serratum* was unknown until now.

Arisaema sikokianum has a purple upward spathe, a white capitate appendage and leaves with 3 - 5 leaflets (**Figure 1(a)**, **Table 1**), while *Arisaema serratum* has a green cylindrical appendage and leaves with 7 - 17 leaflets (**Figure 1(b)**, **Table 1**). Although the spathe of *A. serratum* varies widely in various areas of Japan [2,3], our observation is that almost all *A. serratum* in Kochi Prefecture show a green spathe and appendage (**Figure 1(b)**). In Kochi Prefecture of Shikoku, *A. sikokianum* and *A. serratum* are found in sympatry. The flowering phenology of the two species overlaps (**Table 1**). The chromosome num-

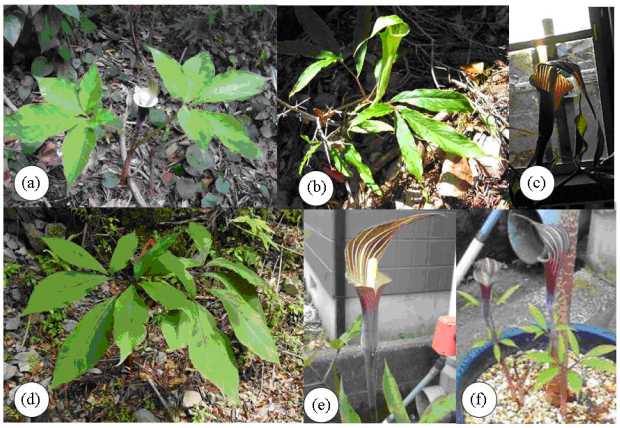

Figure 1. *Arisaema* species examined in this study. (a) *A. sikokianum*; (b) *A. serratum*; (c) putative hybrid in 2007; (d) putative hybrid in 2006; (e) putative hybrid in 2010; (f) newly formed putative hybrids in 2010.

ber of *A. sikokianum* and *A. serratum* is 2 n = 28 in both species [8]. Therefore, it is possible to generate a hybrid between *A. sikokianum* and *A. serratum*.

Molecular approaches can reveal the processes of past event such as hybridization and reticulate evolution [9]. Nuclear markers provide information to estimate the putative parents in hybrids [10,11]. The polymorphisms of the internal transcribed spacer (ITS) region in nuclear DNA (nrDNA) are good tools for clarifying the relationship of closely related taxa in many plant groups [12-14], and can provide evidence of hybridization. In fact, interspecific hybrids are most commonly identified by the heterogeneity of nrDNA [5,15,16]. Moreover, chloroplast DNA (cpDNA) data can indicate maternal and paternal parents of hybrids, because cpDNA normally inherit from maternal parent [17]. In this study, we found a putative natural hybrid of *A. sikokianum* and *A. serratum* in Kochi Prefecture from the viewpoint of its morphological characteristics (**Figure 1(d)**, **Figure 2**). To clarify the maternal and paternal parents of the putative hybrid, we conducted molecular analysis using nrDNA and cpDNA sequences. Our finding suggests that hybridization may occur between *A. sikokianum* and *A. serratum*.

2. Materials and Methods

A morphologically intermediate plant was found at one locality in Kochi Prefecture (**Figure 2**, **Table 2**). The intermediate hybrid was growing in situ with *Arisaema sikokianum*, *A. serratum* and *A. tosaense*. A voucher specimen is deposited in the Herbarium of the Makino Botanical Garden, Kochi (MBK).

For the molecular analysis, total DNA was isolated from 200 - 300 mg of leaves with a Plant Genomic DNA Mini Kit (VIOGENE, Sunnyvale, CA, 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 Taberlet *et al.* [18] and White *et al.* [19], respectively. 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 (Ta Ka Ra) and 0.5 µM of each primer. We used the following thermal cycle profile for amplification: 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. A preliminary sequence of the ITS region was taken from the parent species to confirm whether PCR-RFLP can effectively demonstrate

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

Trait	*A. sikokianum*	Putative Hybrid	*A. serratum*
Pseudostem			
Pseudostem Length	Short	Short-Long	Long
Pseudostem Color	Green	Purplish Dark Brown	Purplish Dark Brown
Leaf Characteristics			
Leaflets	3 to 5	9	7 to 17
Width of Leaflets	Wide	Narrow-Wide	Narrow
Petiole Length	Long	Short-Long	Short
Reproductive Characteristics			
Spathe Tip Direction	Upward	Middle	Downward
Spathe Color	Purple	Purple	Green
Appendage Shape	Capitate	Capitate-Cylindrical	Cylindrical
Appendage Color	White	White	Green
Flowering Phenology	April to May	Early April	Late April to June

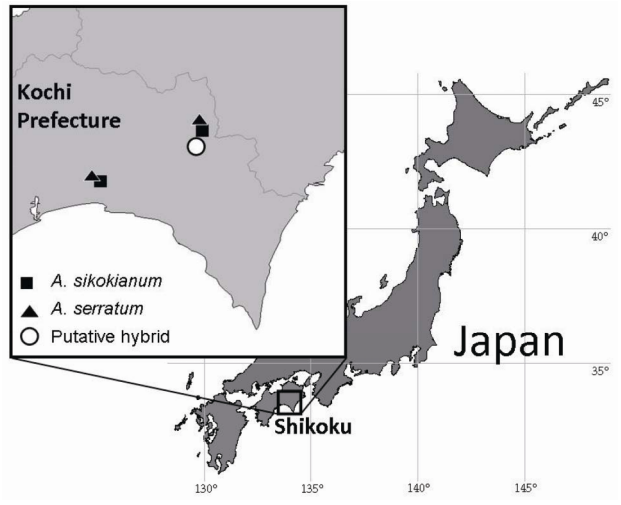

Figure 2. Sampling locality of the putative hybrid of *Arisaema sikokianum* and *A. serratum*. Black squares indicate *A. sikokianum*. Black triangles indicate *A. serratum*. Open circle indicates putative hybrid.

hybridization (**Figure 3**). After amplification, 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, Foster City, CA, USA) and ABI Prism 3100 Genetic Analyzer (Applied BioSystems, Foster City, CA, USA) according to the manufacturer's instructions. For sequencing, we used the same primers as those used for amplification.

PCR-RFLP (restriction fragment length polymorphism) analysis for the ITS regions of 18 - 26 S nuclear ribosomal DNA were conducted to clarify the hybrid nature of the putative hybrid after checking the sequencing results and alignments. We used 2 *Mse* I sites (TTAA) as an autapomorphic character of nrDNA (**Figure 3**). The ITS region of *A. sikokianum* has one *Mse* I site in ITS 1, while *A. serratum* has two sites (ITS 1 and ITS 2) digested by the restriction enzyme. After the designation of 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 there was one putative hybrid in the locality. As for the morphological characteristics, the putative hybrid has a purple spathe and white appendage (**Figures 1(c)**, **(e)**, **(f)**), which is similar to that of *Arisaema sikokianum*, but the leaves have nine leaflets, which is similar to that of *A. serratum* (**Figures 1(c)**, **(d)**, **(f)**; **Table 1**). Additionally, two clones of the putative hybrid were newly formed from a corm of the original putative hybrid in cultivation (**Figure 1(f)**). Although *A. sikokianum* can not grow lateral buds on its corm [20], *A. serratum* can grow lateral buds and become new clones [3,21]. Therefore, the clones generated by germinated lateral buds in the hybrid may inherit the traits of *A. serratum*.

In this study, the hybrid of *Arisaema sikokianum* and *A. serratum* showed an intermediate appendage compared with the parents in 2007 (**Figure 1(c)**). The shape of the appendage of the hybrid in this study transformed from intermediate (in 2007) to cylindrical (for 2008-2010) under a cultivated condition (**Figures 1(c)**, **(e)**). Moreover, the pseudostem length also showed the same phenomenon as the appendage: the hybrid was short in 2006 (**Figure 1(d)**), which is similar to *A. sikokianum*, but it became long for 2007 to 2010, which is similar to *A. serratum*. It is curious that the shape of the appendage and the pseudostem length transformed in different years. In the future to understand this transformation, an analysis of the genes involved in the appendage and the pseudostem is needed.

In the *Arisaema* sect. *Pedatisecta*, there are some nucleotide polymorphisms in the ITS sequences, e.g., *A. angustatum*, *A. ringens*, *A. serratum*. and *A. sikokianum* (Accession numbers: AF291914, AF274295, EF017383, and AB513178, respectively). In this study, the length of the ITS sequence of nrDNA is 648 bp in *Arisaema ser-*

Table 2. Locality where samples were collected.

No.	Species	Locality	Collector	Collected Date
1	*Arisaema sikokianum*	Kochi Pref. Aki City, Ochial, Suginokuma River	H. Hayakawa	2009-7-4
2	*A. sikokianum*	Kochi Pref. Nankoku City, Nareai, Nebiki Pass	H. Hayakawa	2009-5-1
3	Putative Hybrid	Kochi Pref. Aki City, Doi	H. Hamachi	2006-5-25
4	*A. serratum*	Kochi Pref. Aki City, Ochial, Suginokuma River	H. Hayakawa	2009-7-4
5	*A. serratum*	Kochi Pref. Nankoku City, Nareai, Nebiki Pass	H. Hayakawa	2009-6-8

S: *Arisaema sikokianum* type. Accession numbers: AB513178 (ITS) and AB513176 (trnL intron). E: *A. serratum* type. Accession numbers: AB605025 (ITS) and AB605026 (trnL intron).

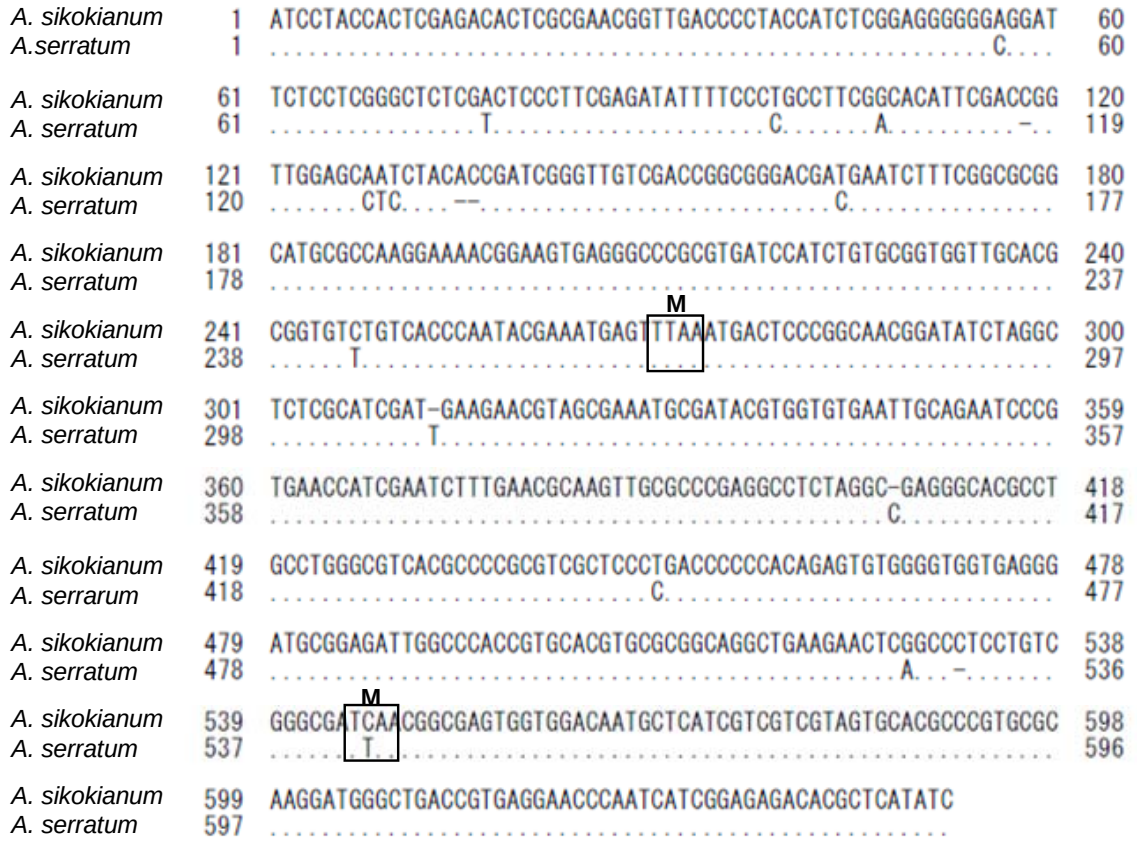

Figure 3. Expected restriction sites of *Mse* I for molecular characteristics of ITS regions by PCR-RFLP. M: restriction site.

ratum (Accession number: AB605025). This sequence is the same as *A. tosaense* (Accession number: AB513179). Therefore, we conducted PCR-RFLP in the nrDNA to obtain further evidence of the hybrid nature, because the ITS region of *A. sikokianum* has one *Mse* I site, while *A. serratum* and *A. tosaense* have two sites digested by this restriction enzyme (**Figure 3**) [5]. The digestion patterns of all samples of *A. sikokianum* and *A. serratum* showed expected patterns and the putative hybrid showed the combined pattern of *A. sikokianum* and *A. serratum* (**Figure 4**). Thus, from the evidence of morphological and molecular analyses, we confirmed that the putative hybrid was truly a hybrid of *A. sikokianum* and *A. serratum*.

The length of the *trnL* intron of cpDNA is 467 bp in *Arisaema serratum* (Accession number: AB605026). This region 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) (**Figure 5**) [5]. We therefore determined the sequences of the *trnL* intron. In the hybrid, the sequence result from the cpDNA analysis was identical to that of *A. sikokianum* (**Table 1**). Therefore, the hybrid

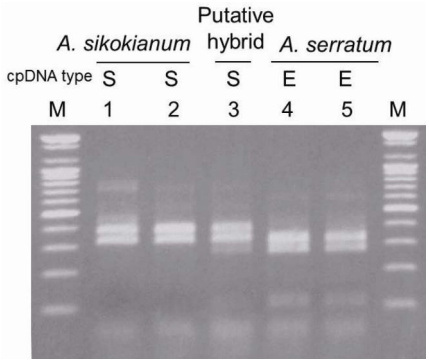

Figure 4. PCR-RFLP profile of *Arisaema sikokianum*, *A. serratum* and putative hybrid. Arrowheads indicate expected fragments of both *A. sikokianum* and *A. serratum*. M: size marker. The cpDNA types correspond to the types in Table 2 and Figure 5.

transferred pollen from *A. serratum* to *A. sikokianum*.

In Shikoku, *Arisaema ehimense* J. Murata et Ohno is of hybrid origin between *A. serratum* and *A. tosaense* [7,22]. Additionally, the hybrids between *A. sikokianum* and *A. tosaense* are known [5,6]. Moreover, this study reveals a hybrid between *A. sikokianum* and *A. serratum*.

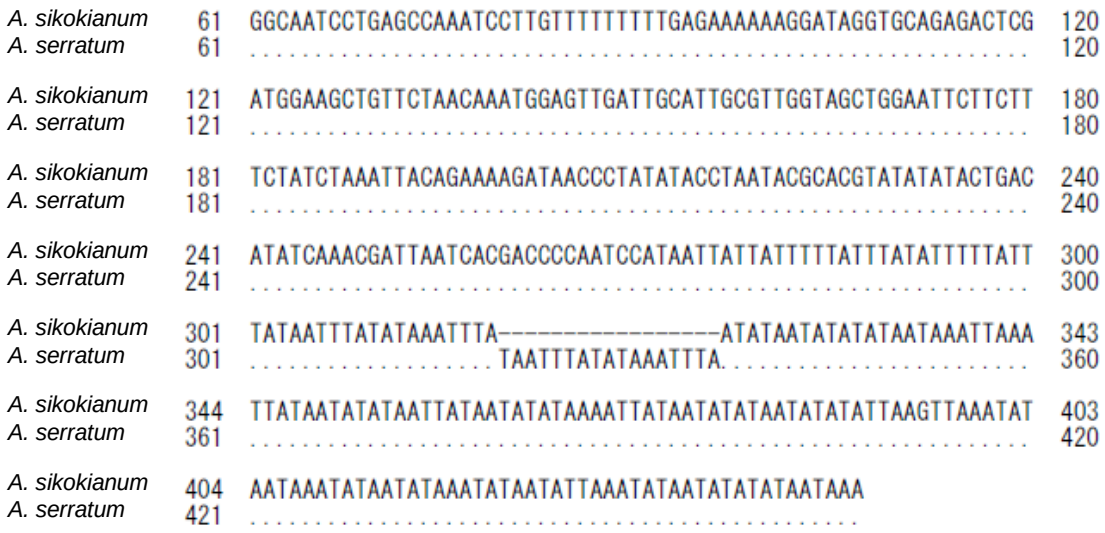

Figure 5. The result of alignment of *trnL* intron sequences of *Arisaema sikokianum* and *A. serratum*.

To our knowledge, it is first report of a hybrid between this *Arisaema* pair. These facts indicate that all the combination patterns of hybridization among *A. tosaense*, *A. sikokianum* and *A. serratum* have been in Shikoku. Therefore, these facts suggest that hybrid speciation or reticulated evolution or both might have occurred among the three species in Shikoku.

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5. Conclusions

In **Figure 3** is reported the shifting of the three switching frequencies and also in this case it is evident.

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