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New Natural Hybrid of *Arisaema* (Araceae), Distributed around Mt. Yatsuzura, in Shikoku, western Japan

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Morphologically intermediate plants between *Arisaema tosaense* and *A. undulatifolium* subsp. *uwajimense* were recently found in Ehime and Kochi prefectures, Shikoku, Japan. The putative hybrids have the reproductive characteristics of *A. tosaense*, but purplish-dark brown, short pseudostems similar to *A. undulatifolium* subsp. *uwajimense*. Molecular analysis of nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) region using PCR-RFLP indicates that these putative hybrids have a combined pattern of the two putative parental species. The chloroplast DNA (cpDNA) *trnL* intron sequences of all the putative hybrids and *A. tosaense* were identical. The results suggest that the plants are hybrids between *A. tosaense* and *A. undulatifolium* subsp. *uwajimense* and were formed by gene exchange via pollen grains transferred from *A. undulatifolium* subsp. *uwajimense* to *A. tosaense*.

Key words: Araceae, *Arisaema*, hybrid, ITS, molecular analysis, PCR-RFLP

Introduction

Arisaema Martius (Araceae) is a genus with a large, often colored and conspicuous bract (spathe) subtending and enveloping a bisexual or unisexual spadix with numerous small flowers (Ohashi 1982). The Japanese archipelago, where 51 species and 9 subspecies are recognized (Murata 2011), is one of the centers of diversity for the genus. In addition, over 20 putative natural hybrids have been reported among Japanese *Arisaema* (e.g., Murata 2011). Section *Pistillata* Nakai comprise a significant group within Japanese *Arisaema* because they are distributed mainly in Japan (Murata 2004) and are taxonomically difficult because of the presence of many closely related species with few morphological differences (Murata 1995). In the Japanese archipelago, Shikoku is an important region for the diversification of the section and a remarkable number of endemic and near endemic species exist there. Of the 14 species and 1 subspecies of the section on Shikoku (Murata 2011), half of them are endemic to Shikoku (5 endemic and 3 near endemic species).

In the course of studies on the diversity of *Arisaema* on Shikoku, we discovered plants with a new combination of morphological characters on Mt. Yatsuzura on the border of Ehime and Kochi prefectures. Around those plants, two species of *Arisaema* section *Pistillata*, *Arisaema tosaense* Makino and *A. undulatifolium* Nakai subsp. *uwajimense* Tom. Kobay. & J. Murata were also found. The spathe and inflorescences expand

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after the leaves in *A. tosaense*. The characteristic green spathe is approximately 30–45 cm long and bends downward to the ground (Fig. 1A; see detail, Table 1) (Ohashi 1982, Murata 2011). The pseudostem and petiole are equal in length and are longer than the peduncle (Murata 2011). However, in this study, the peduncle and petiole were almost equal in length and were shorter than the pseudostem in female plants (Table 1). *Arisaema undulatifolium* subsp. *uwajimense* is a member of the *A. undulatifolium* group, which is characterized by the spathe and inflorescence expanding before the leaves and a flowering time one week to one month earlier than sympatric species of *Arisaema* (Serizawa 1980, Kobayashi *et al.* 2003). *Arisaema undulatifolium* subsp. *uwajimense* has a purplish-dark brown spathe, and the peduncle and pseudostem are equal in length and are two times longer than the petiole (Fig. 1B: Murata 2011). Since these two species are morphologically distinct, they are easily distinguished in the field even when growing together. *Arisaema undulatifolium* subsp. *uwajimense* is a narrow endemic occurring only around Uwajima City, Ehime Prefecture (Kobayashi *et al.* 2003), whereas *A. tosaense* occurs throughout Shikoku and adjacent areas of Honshu and Kyushu (Ohashi 1982, Murata 2011). The newly discovered plants appear to combine morphological characters of the above two species and we therefore presumed that they originated by hybridization. If so, these plants will represent a new parental combination. In southwestern Shikoku, *A. tosaense* and *A. undulatifolium* subsp. *uwajimense* are partially sympatric in shaded forests, but grow at slightly different altitudes. From our observations, we noted that *A. undulatifolium* subsp. *uwajimense* occurs with *A. tosaense* or at lower altitudes than *A. tosaense*. To confirm the hybrid nature of the newly found plants, we observed vegetative and reproductive characteristics and conducted molecular analyses using nuclear ribosomal DNA (nrDNA) and chloroplast DNA (cpDNA) sequences to infer maternal and paternal parents of the putative hybrid individuals. We concluded that the plants are indeed hybrids between *A. tosaense* and *A. undulatifolium* subsp. *uwajimense*. Additionally, we observed chloroplast capture (the introgression of cpDNA from one species into another) in both directions between the two species.

Materials and Methods

Plants morphologically intermediate between *Arisaema tosaense* and *A. undulatifolium* subsp. *uwajimense* were found growing with *A. tosaense* and *A. undulatifolium* subsp. *uwajimense* around Mt. Yatsuzura along the border between Uwajima City in Ehime Prefecture and Shimanto City in Kochi Prefecture (altitude 600–900 m) (Fig. 2). On 22 June 2010 and 7 July 2010, we collected a total of 94 blooming individuals of *Arisaema* along the road around Mt. Yatsuzura. We conducted field observation of *A. undulatifolium* subsp. *uwajimense* at the site approximately once per week from March to July, 2010.

We measured and recorded morphological characteristics (numbers of leaflets, condition of spathe, length of pseudostem, peduncle, and petiole) before collecting samples for DNA isolation. We measured the pseudostem length (from the soil surface to the top of second foliage leaf sheath) and the peduncle length (from the top of second foliage leaf sheath to the base of the spathe). Three voucher specimens of the suspected hybrids were deposited in the herbarium at the Makino Botanical Garden, Kochi (*Matsuyama et al. MBK0234117–9*). For further comparison of the morphological traits between the intermediate plants and putative parental species, we also measured the morphological characteristics of living plants of *Arisaema tosaense* (42 female plants) in Kami City, Kochi Prefecture and *A. undulatifolium* subsp. *uwajimense* (82 female plants) in Uwajima City and Ainan-Cho, Minamiuwa-Gun, Ehime Prefecture and Sukumo City, Kochi Prefecture. The length of tip of the limb of the spathe and the color of the pseudostem, peduncle, petiole, spathe, and appendage in the intermediate plants were evaluated qualitatively during field observations.

For the molecular analysis, total DNA was isolated from 200–300 mg of leaves using a Plant

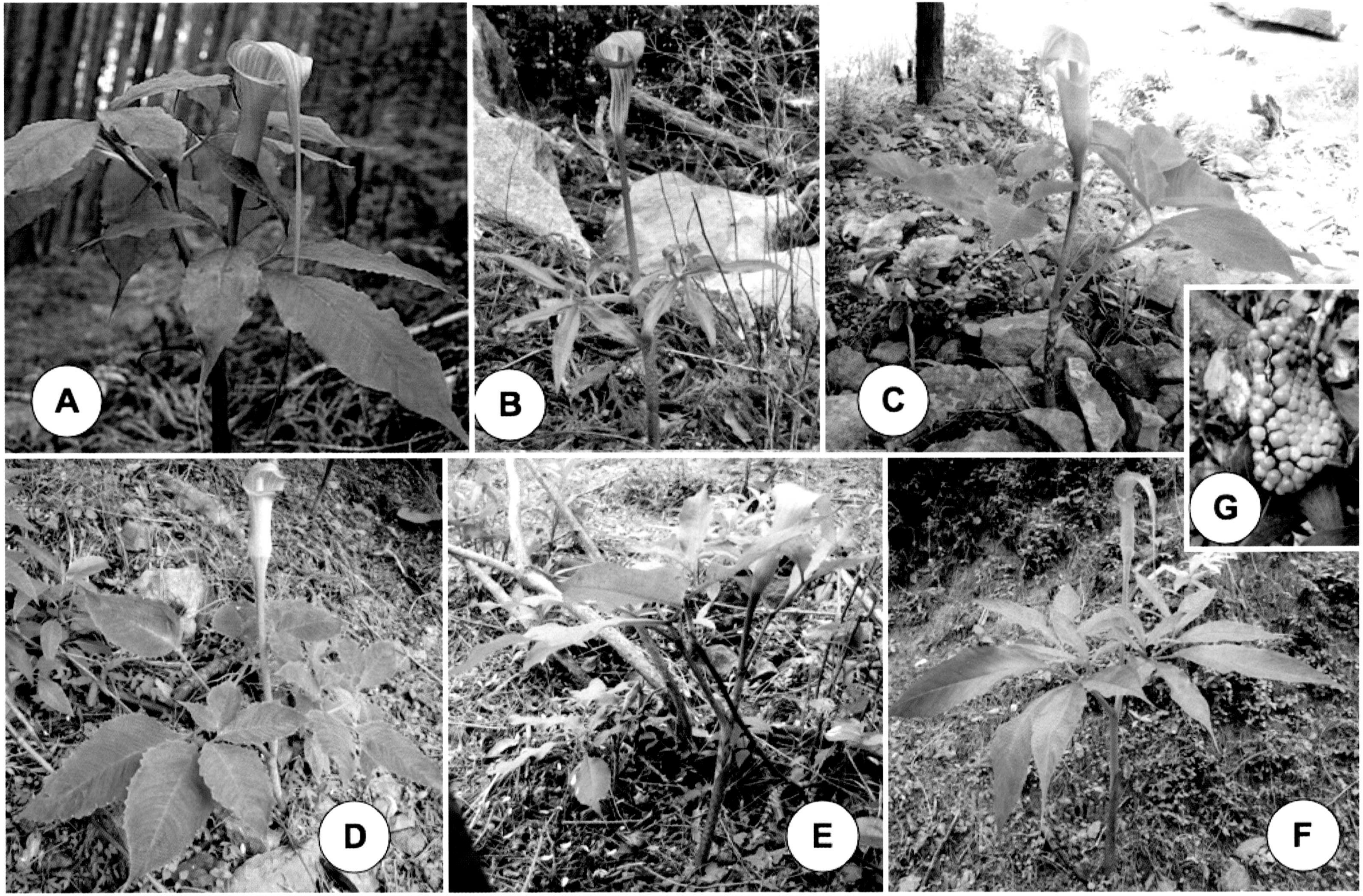

FIG. 1. Sample individuals of *Arisaema* in the Yatsuzura population. A: *A. tosaense*; B: A. *undulatifolium* subsp. *uwajimense*; C: putative hybrid (sample No. 1); D: putative hybrid (sample No. 2); E: putative hybrid (sample No. 3); F: putative hybrid (sample No. 6); G: fruits of putative hybrid (sample No. 7). Sample numbers correspond to numbers in Figs. 2 and 3 and Table 1.

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

Trait	*Arisaema tosaense* (n = 42)[1]	Putative hybrid (n = 1)									*A. undulatifolium* subsp. *uwajimense* (n = 82)[1]
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9	
Stage	Female	Female	Female	Female	Female	Female	Female	Female	Male	Female	Female
Vegetative Characteristic											
Pseudostem Length (cm)	31.9 ± 7.9 (15.1–51.5)[2]	17.3	18.4	22.7	16.0	18.4	53.0	14.7	26.0	34.4	25.0 ± 10.6 (3.6–74.2)
Peduncle Length (cm)	15.0 ± 5.6 (4.2–26.2)	17.5	27.4	10.5	4.9	12.0	26.8	16.5	—[3]	18.1	23.1 ± 7.9 (7.9–39.7)
Petiole Length (cm)	13.5 ± 3.2 (2.7–22.3)	8.5	12.3	10.5	10.6	11.1	9.7	12.3	13.1	15.3	8.9 ± 3.0 (3.5–17.5)
Leaflets number	12.2 ± 2.6 (8–20)	10	13	12	12	11	15	15	11	11	12.3 ± 2.1 (9–20)
Pseudostem Color	Green	PDB[4]	Green	PDB	Green	Green	Green	Green	Green	Green	PDB
Peduncle Color	Green	PDB	Green	PDB	Green	Green	Green	Green	—	Green	PDB
Petiole Color	Green	Green	Green	PDB	Green	Green	Green	Green	Green	Green	PDB
Variegation along Midvein	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent/Present
Reproductive Characteristic											
Spathe Tip	Elongate	Elongate	Unknown	Elongate	Elongate	Elongate	Elongate	Elongate	Elongate	Elongate	Not elongate
Spathe Color	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	PDB
Appendage Color	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	PDB
Flowering Phenology	May to June	June	June	June	June	June	June	June	June	June	March to April

[1] *Arisaema tosaense* and *A. undulatifolium* subsp. *uwajimense* were shown based on data of female excluding flowering phenology (male and female).

[2] Average ± Standard deviation (Minimum–Maximum).

[3] Not measured.

[4] Purplish Dark Brown

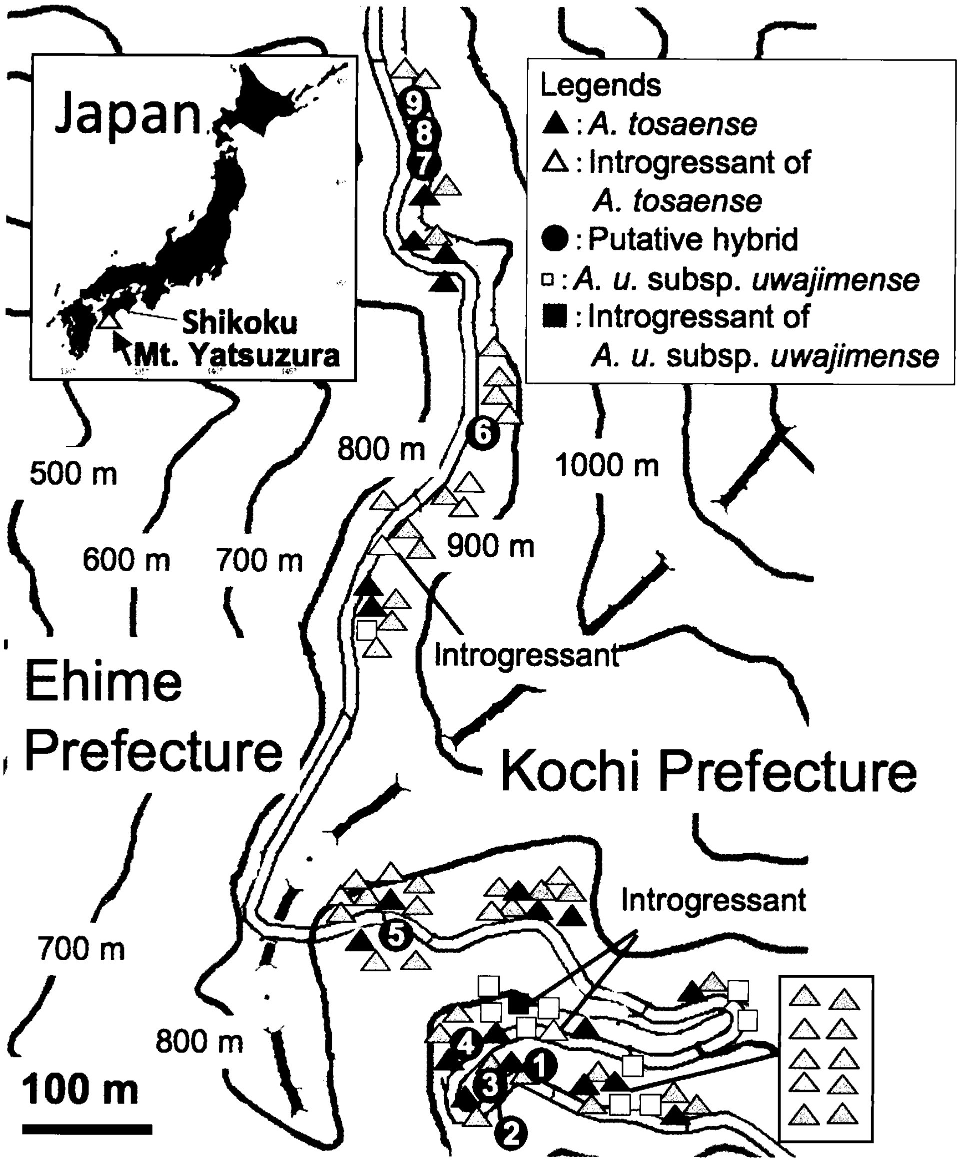

FIG. 2. Spatial distribution of plants of *Arisaema* around Mt. Yatsuzura. In nrDNA (ITS region) samples, triangles indicate *A. tosaense* type (▲/△), squares indicate *A. undulatifolium* subsp. *uwajimense* type (■/□), circles indicate putative hybrids (●). In cpDNA (*trnL* intron), black symbols indicate *A. tosaense* haplotype (▲/■/●), white symbols indicate *A. undulatifolium* subsp. *uwajimense* haplotype (△/□), gray symbols indicate undetermined haplotype caused by low amplification success in cpDNA. Sample numbers correspond to those in Figs. 1 and 3, Table 1, and in the text. Bar = 100 m.

Genomic DNA Mini Kit (Viogene, Sunnyvale, CA, USA), according to the manufacturer's protocol. We amplified the nrDNA internal transcribed spacer (ITS) region and the cpDNA *trnL* intron with primers designed by White *et al.* (1990) and Taberlet *et al.* (1991), respectively. Some samples were analyzed by using an original primer (d-Ari: 5'-GTC GGT ATT GAC ATG TAG AAT-3' instead of the d primer of the *trnL* intron). PCR amplification was conducted in a 50 μL reaction solution containing approximately 50 ng of 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 *rTaq* DNA polymerase (Takara Bio Inc., Shiga, Japan) and 0.5 μM of each primer. We used the following thermal cycle profile for amplification using 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 and the *trnL* intron were subjected to electrophoresis in 1.0% low-melting-temperature agarose gels to remove by products and to purify amplified products. We sequenced the purified PCR products using a BigDye Terminator ver. 3.1 (Applied Biosystems) and an 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. We have deposited the sequences of the nrDNA ITS and the cpDNA *trnL* intron in the DDBJ/EMBL/GenBank International DNA databases (*Arisaema undulatifolium* subsp. *uwajimense*: AB611695 and AB611694, respectively; *A. tosaense*: AB513179 and AB513177, respectively).

We performed PCR-RFLP analysis for the ITS region after checking sequencing results and alignments. The alignments indicated that an *Mse* I restriction site was useful for distinguishing the ITS sequences of the putative parental species (Table 2). 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 gels.

Results and Discussion

From the results of the morphological analyses (Table 1), we found nine morphologically intermediate plants in the area around Mt. Yatsuzura. Samples 1, 2, and 6 had long peduncles (Fig. 1C, D, and F), similar to *Arisaema undulatifolium* subsp. *uwajimense*, but their spathes were green and had elongated tips (excluding sample 2, which was damaged), similar to *A. tosaense* (Fig. 1). Sample 3 had a green spathe with an elongated tip, a petiole, and a peduncle, similar to *A. tosaense*, but had a purplish-brown pseudostem, petiole, and peduncle (Fig. 1E), similar to *A. undulatifolium* subsp. *uwajimense*. The other intermediate plants (sample 4, 5, 7, 8, and 9) had morphological values intermediate between the putative parents. Among the intermediate plants, the length of the peduncle (4.9–27.4 mm), petiole (8.5–15.3 mm), and pseudostem (14.7–53.0 mm) varied widely among the putative parental species, but the peduncle of sample 2 and the pseudostem of sample 6 were longer than the maximum values recorded for *A. tosaense*. On the other hand, the shape and color of the spathe of the intermediate plants were identical to *A. tosaense*. The intermediate plants bore enlarged, red fruits and were presumed to be fertile (Fig. 1G).

Molecular analysis revealed that the amplified ITS region of *Arisaema undulatifolium* subsp. *uwajimense* has one *Mse* I site and *A. tosaense* has two sites (Table 2). We conducted PCR-RFLP to obtain further evidence of the hybrid nature of the intermediate plants between the two species. We examined 94 individuals collected from Mt. Yatsuzura, and found that *A. tosaense* and *A. undulatifolium* subsp. *uwajimense* exhibited the expected patterns, and all putative hybrids produced the combined patterns of the two species (Fig. 3). The combined RFLP band pattern supports the hypothesis that the morphologically intermediate plants are hybrids between *A. tosaense* and *A. undulatifolium* subsp. *uwajimense*.

The cpDNA *trnL* intron is an efficient region for detecting sequence differences among some species of *Arisaema* (Hayakawa *et al.* 2010). We therefore determined the sequences of this region for 42 samples. The difference in sample numbers between nrDNA (94 samples) and cpDNA (42 samples) was because of low amplification success in the *trnL* intron. Two haplotypes of cpDNA *trnL* intron (AB513177 and AB611694) were found (Table 3). One haplotype (AB513177) was universally found in *A. tosaense* from different localities (Hayakawa *et al.* 2010). The other haplotype (AB611694) with a 17-bp deletion, 10-bp insertion, and 7-bp deletion was exclusively found in *A. undulatifolium* subsp. *uwajimense*. The haplotypes of the cpDNA *trnL* intron of the intermediate plants and *A. tosaense* were identical, indicating *A. tosaense* to be the ovule parent of the intermediate plants, as cpDNA is normally inherited from the maternal parent only.

We surveyed the spatial distribution of all blooming species of *Arisaema* in the study area on Mt. Yatsuzura and determined their ITS sequences and *trnL* intron (Fig. 2). The hybrids were present between 600 and 900 m. We also detected two samples of *A. tosaense* that contained

TABLE 2. nrDNA ITS sequence variations among *Arisaema undulatifolium* subsp. *uwajimense*, *A. tosaense* and two other sympatric species.

	Position of polymorphic site																							
Species	56	77	99	107	118	128	129	130	135	136	164	247	270	—		273	313	408	450	529	533	547	—		550	Accession number (Voucher)
Arisaema japonicum	C	T	C	A	-	C	T	C	-	-	C	T	T	T	A	A	T	C	C	A	-	T	T	A	A	AB605025 (Hayakawa *et al.* 2011, as *A. serratum*)
A. sikokianum	A	A	T	G	C	A	A	T	A	C	T	C	T	T	A	A	-	-	T	G	C	T	C	A	A	AB513178 (Hayakawa *et al.* 2010)
A. tosaense	C	T	C	A	-	C	T	C	-	-	C	T	T	T	A	A	T	C	C	A	-	T	T	A	A	AB513179 (Hayakawa *et al.* 2010)
A. undulatifolium subsp. *uwajimense*	A	A	T	G	C	A	A	T	A	C	T	C	T	T	A	A	-	-	T	G	C	T	C	A	A	AB611695

Squares indicate expected restriction sites of *Mse* I for molecular characteristics of ITS regions by PCR-RFLP.

TABLE 3. cpDNA (*trnL* intron) sequence variation in *Arisaema undulatifolium* subsp. *uwajimense*, *A. tosaense*, putative hybrid, and two other sympatric species.

	17-bp InDel	10-bp InDel	7-bp InDel	
Species	310–326	452–461	543–549	Accession number (Voucher)
Arisaema japonicum	TAATTTATATAAATTTA	-	ATATAAT	AB605026 (Hayakawa *et al.* 2011, as *A. serratum*)
A. sikokianum	-	AATAAAATAT	ATATAAT	AB513176 (Hayakawa *et al.* 2010)
A. tosaense	TAATTTATATAAATTTA	-	ATATAAT	AB513177 (Hayakawa *et al.* 2010)
A. undulatifolium subsp. *uwajimense*	-	AATAAAATAT	-	AB611694
Putative Hybrid	TAATTTATATAAATTTA	-	ATATAAT	same as AB513177 (MBK0234117-9)

Numbers indicate the positions of InDels.

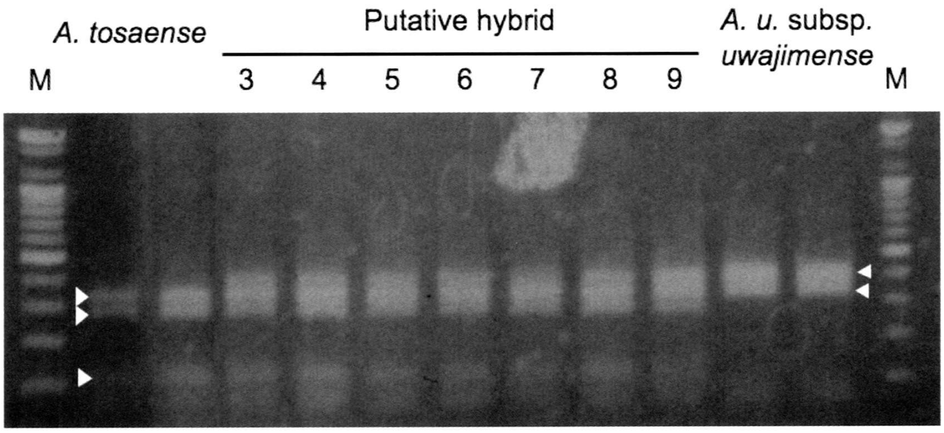

FIG. 3. PCR-RFLP profile of *Arisaema undulatifolium* subsp. *uwajimense*, putative hybrids, and *A. tosaense*. Arrows indicate expected fragments of *A. tosaense* and *A. undulatifolium* subsp. *uwajimense*. M: size marker.

the *A. undulatifolium* subsp. *uwajimense* haplotype in the *trnL* intron, but in outward appearance and nrDNA haplotype they were identical to *A. tosaense*. Hayakawa *et al*. (2010) reported that the *trnL* intron sequences in *A. tosaense* were conserved in all individuals collected from multiple locations. *Arisaema tosaense* with the cpDNA haplotype of *A. undulatifolium* subsp. *uwajimense* in this study would be the result of introgression and consequent chloroplast capture. We also found an individual with the morphology and ITS type of typical *A. undulatifolium* subsp. *uwajimense*, but with the cpDNA haplotype of *A. tosaense*. Considering the occurrence of chloroplast capture in *A. tosaense*, incongruence of nrDNA and cpDNA in the *A. undulatifolium* subsp. *uwajimense* individual would also be the result of introgression. Consequently, of 94 individuals, 72, 10, 9, 2, and 1 plants were identified as *A. tosaense*, *A. undulatifolium* subsp. *uwajimense*, hybrids, introgressants of *A. tosaense*, and an introgressant of *A. undulatifolium* subsp. *uwajimense*, respectively (Table 4). Hybrids of the two species have therefore backcrossed bidirectionally, with gene flow occurring between the two parental species.

In this study, *Arisaema undulatifolium* subsp. *uwajimense* and *A. tosaense* were observed to flower from late March to mid-April and from May to June, respectively, in the study area in 2010. The flowering times of the two species did not overlap in 2010. Since *A. undulatifolium* subsp. *uwajimense* starts to flower shortly after the winter snows melts, the flowering times of the two species might overlap in years of late melting snow.

From the morphological and molecular analyses, the hybrid origin of the intermediate plants is highly probable. We could not, however, definitively determine the parents of the hybrids using PCR-RFLP, because the ITS haplotypes of *Arisaema tosaense* and *A. undulatifolium* subsp. *uwajimense* were the same as those of *A. japonicum* Blume and *A. sikokianum* Franch. & Sav., respectively (Table 2). In southwestern Shikoku *A. japonicum* and *A. sikokianum* occasionally grow sympatrically with the putative parental species. From our genetic analysis alone, therefore, we cannot reject the possibility that the parents of the hybrids are *A. japonicum* or *A. tosaense* and *A. undulatifolium* subsp. *uwajimense* or *A. sikokianum*. All putative hybrids, however, have long, drooping, spathe tips (Table 1), which is a unique characteristic of *A. tosaense*. One of the putative parent is therefore considered to be *A. tosaense*, although this does not contradict the molecular data from the ITS region and the *trnL* intron. If the maternal parent is *A. tosaense*, the paternal parent must be *A. undulatifolium* subsp. *uwajimense* or *A. sikokianum*. Hybrids between *A. sikokianum* and *A. tosaense* have been reported (Murata 1962, Hayakawa *et al.* 2010, Murata

TABLE 4. Genotypes of the samples used in this study.

Species	nrDNA (ITS)	cpDNA (*trnL* intron)	No. of samples
Arisaema tosaense	TT	T	20
Introgressant of *A. tosaense*	TT	U	2
Putative hybrid	UT	T	9
Introgressant of *A. undulatifolium* subsp. *uwajimense*	UU	T	1
A. undulatifolium subsp. *uwajimense*	UU	U	10

T, *Arisaema tosaense* type; U, *A. undulatifolium* subsp. *uwajimense* type

2011), but they have characteristics different from those of the hybrids in this study, such as a pestle-like appendix and 7 to 9 leaflets. Therefore, we presume that the intermediate plants are hybrids most probably between the sympatrically occurring *A. tosaense* and *A. undulatifolium* subsp. *uwajimense*. Kobayashi (2009) implied the possibility of habitat segregation between *A. undulatifolium* subsp. *uwajimense* (Western region) and the related *A. aequinoctiale* Nakai & F. Maek. of central to eastern Kochi Prefecture. We therefore considered one of the parents to be *A. undulatifolium* subsp. *uwajimense* in the *A. undulatifolium* group.

In this study, we reported on hybridization between *Arisaema tosaense* and *A. undulatifolium* subsp. *uwajimense*. Murata (1962), Hayakawa *et al.* (2010, 2011), and Murata (2011) reported hybrids and introgressants between *A. sikokianum* and *A. tosaense*, and Kobayashi (2009) reported a putative natural hybrid between *A. japonicum* and *A. tosaense* based on a specimen in the MBK herbarium. Additionally, Maki & Murata (2001) indicated that *A. ehimense* J. Murata & J. Ohno is of hybrid origin between *A. serratum* (Thunb.) Schott and *A. tosaense*. These reports, combined with our findings, suggest that *A. tosaense* has the potential to hybridize and to exchange genes with various sympatric congeners, generating intermediate morphologies as well as chloroplast capture and reticulate evolution. As indicated by Murata (1995), the presence of various morphologically intermediate plants and taxonomic difficulties in *Arisaema* may be due to hybridization within section *Pistillata*.

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