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Molecular Evidence for a Natural Hybrid Origin of *Ajuga*×*mixta* (Lamiaceae) Using ITS Sequence

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Abstract We compared the internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA of a putative hybrid *Ajuga*×*mixta* with those of *A. decumbens* and *A. nipponensis* hypothesized to be its parent species. The ITS sequences showed four loci separating *A. decumbens* and *A. nipponensis* at species level, and at the four loci, two plants of *Ajuga*×*mixta* exhibited polymorphisms that were additive between the two hypothesized parent species. The present result showed that *Ajuga*×*mixta* is likely a natural hybrid between *A. decumbens* and *A. nipponensis* which is in agreement with Makino (1909).

Key words : *Ajuga*, hybrid, ITS, Lamiaceae.

Introduction

Natural interspecific hybridization is relatively common event in vascular plants, and its importance in plant evolution has been well documented (Stebbins, 1959; Grant, 1981; Abbott, 1992; Arnold 1997; Rieseberg, 1997). The outcome of spontaneous hybridization events can result in homoploid (Ferguson and Sang, 2001; Schwarzbach and Rieseberg, 2002) and polyploid species (Lowe and Abbott, 1996; Soltis and Soltis, 1999). In the former the new species retains the ploidy level of the parent species, whereas in the latter the hybrid undergoes genome duplications causing allopolyploidy (Soltis and Soltis, 1999; Liu and Wendel, 2003).

The internal transcribed spacer (ITS) of ribosomal DNA has been used, and confirmed as one of powerful methods for hybrid analyses: Sang *et al.* (1995) reported ITS nucleotide additivity in hybrids at positions where the parent species differed in a hybrid species of *Paeonia* (Peoniaceae). The ITS sequence additivity was also demonstrated for an interspecific hybrid of *Doellingeria*×*sekimotoi* (Makino) Nesom (=*Aster sekimotoi* Makino; Asteraceae) (Saito *et al.*, 2007) and intergeneric hybrid×*Crepidiastrixeris* Kitam. (Asteraceae) (Saito *et al.,* 2006), thus illustrating the suitability of ITS in hybrid analysis.

Ajuga L. consists of about 50 speices especially in the Old World (Mabberley, 1997). Most genera in Lamiaceae including the genus *Ajuga* have insect pollination systems, and their special floral structure suggests intricate pollination mechanisms that reflect a long history of adaptive coevolution between plants and pollinators (Huck, 1992). In Japan, thirteen *Ajuga* species and two putative hybrids occurs (Murata and Yamazaki, 1993). *Ajuga*×*mixta* Makino (Fig. 1B) is one of the two putative hybirds, and hyopthesized to be a natural hyribrd between *A. decunbens* Thunb. (Fig. 1A) and *A. nipponica* Makino (Fig. 1C) by its intermadiate morphologies (Makino,

Fig. 1. Plants of three *Ajuga* taxa. A. *A. decumbens* (in Amakubo, Ibaraki, Japan on April 28, 2011; GK9445, TNS; photographed by H. Uemura). B. *A.*×*mixta* (in Ami, Ibaraki, Japan on April 26, 2007; GK9408, TNS). C. *A. nipponensis* (in Ami, Ibaraki, Japan on April 26, 2007; GK9407, TNS).

1909). However, any molecular techniques have never been applied to test the hybridity of *A.*×*mixta*.

In the present study, we obtained ITS sequences of *A. decumbens*, *A. nipponica* and *A.*×*mixta* to test whether the two species are the parental species of *A.*×*mixta*.

Materials and Methods

Plant materials

In morphology, *Ajuga decunbens* (Fig. 1A) and *A. nipponica* (Fig. 1C) are clearly distinguishable: the former has a decumbent habit, violet-greenish leaves and stems, flowers in axils of normal leaves, and bluish corollas, while the latter has elect or ascending habitat, greenish leaves, flowers in elect inflorescence at the terminal of a stem, and white corollas (Murata, 1999; Murata and Yamazaki, 1993). Makino (1909) mentioned that *A.*×*mixta* is morphologically characterised by having ascending or subdecumbent habitat, axillary or verticillaster inflorescence at the terminal of a stem, and violescent corolla (Fig. 1B). In the present study, we collected ten *Ajuga* plants from nine localities, and identified them as six *A. decumbens* plants from six localities, two *A. nipponensis* plants from two localities and two *A.*×*mixta* plants from a locality (Table 1) following the references (Makino, 1909; Murata, 1999; Murata and Yamazaki, 1993).

In the locality of *A.*×*mixta* in Ami, Ibaraki, Japan, *A. decumbens* and *A. nipponensis* occurred together within about 100 m radius. However, we did not include them in the present study, because back-cross from *A.*×*mixta* to *A. decumbens* and/or to *A. nipponensis* might make it difficult to identify species specific molecular makers. In the other localities where *A. decumbens* and *A. nipponensis* were collected, *A.*×*mixta* was not found in this study. Voucher specimens were deposited in the herbarium of National Museum of Nature and Science (TNS).

DNA extraction, PCR amplifications and sequencing

Total genomic DNA was isolated from leaf tissue using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany?) according to the manufacturer's instructions with some modifications. Extracted DNA was used as a template for polymerase chain reaction (PCR). Total DNA samples isolated were deposited in the Molecular Biodiversity Research Center of the National Museum of Nature and Science.

The ITS of nuclear ribosomal DNA, including the 5.8S RNA gene were amplified by PCR using the forsard primer AB101 (5′-ACG AAT TCA AGG TCC GGT GAA GTG TTC G-3′) and the reverse primer AB102 (5′-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3′) (Douzery *et al*., 1999). The PCR reaction contained 1.8 μl sterile dH_2O, 5.0 μl 2×GC II buffer, 1.6 μl dNTP mixture (2.5 mM each), 0.5 μl primer AB101 (10 mM), 0.5 μl primer, AB102 (10 mM), 0.1 μl *Taq*

Table 1. Plant materials of three *Ajuga* taxa, their collection localities, and variable sites in nuclear ribosomal ITS sequences

Individual[a]	Locality no.[b]	Accession	ITS1 sequence position[c]				ITS2 sequence position[c]													
			67	72	101	206	246	391	401	408	412	**448**	**455**	459	**463**	490	498	501	**537**	541	550
A. decumbens																					
GK9412	Japan, Kyushu, Miyazaki, Nojiri.	AB668972	A	G	C	C	C	C	C	C	T	**A**	**T**	C	**C**	C	A	C	**G**	G	C
GK10495	Japan, Shikoku, Kochi, Tosashimizu.	AB668973	A	G	C	C	C	T	C	C	T	**A**	**T**	C	**C**	C	A	C	**G**	G	C
GK10534	Japan, Honshu, Niigata, Minamiuonuma.	AB668974	G	G	C	C	C	C	C	C	T	**A**	**T**	T	**C**	C	G	C	**G**	G	C
GK10542	Japan, Honshu, Gunma, Minakami.	AB668975	G	G	C	C	C	C	C	C	T	**A**	**T**	T	**C**	C	G	C	**G**	T	C
GK10681	Japan, Honshu, Tochigi, Sano.	AB668976	G	G	C	C	C	C	C	C	T	**A**	**T**	C	**C**	C	G	C	**G**	G	C
GK10759	Japan, Ryukyus, Okinawa, Motobu.	AB668977	A	G	C	C	T	C	C	C	T	**A**	**T**	C	**C**	C	A	T	**G**	C	C
A.×*mixta*																					
GK9408	Japan, Honshu, Ibaraki, Ami.	AB668978	A/G	G	C/T	C	C	C	C	C/T	C/T	**A/G**	**C/T**	C	**C/T**	C/T	A/G	C	**A/G**	G	C
GK10693	Japan, Honshu, Ibaraki, Ami.	AB668979	A/G	G	C/T	C	C	C	C	C/T	C/T	**A/G**	**C/T**	C	**C/T**	C/T	A/G	C	**A/G**	G	C
A. nipponensis																					
GK10594	Japan, Honshu, Kanagawa, Sagamihara.	AB668980	G	A/G	C/T	C/T	C	C	C/T	C/T	C	**G**	**C**	C	**T**	C/T	G	C	**A**	G	C/T
GK10598	Japan, Honshu, Tokyo, Hachioji.	AB668981	G	A/G	C/T	C/T	C	C	C/T	C/T	C/T	**G**	**C**	C	**T**	C/T	G	C	**A**	G	C/T

[a] GK: Goro Kokubugata

[b] Sequences investigated here and registered in the DDBJ/EMBL/GenBank database

[c] Bold indicate four variable nucleotide positions clearly differentiating *A. decumbens* and *A. nipponensis* at species level.

polymerase (5 units μl^{-1}) and 0.5 μl template DNA using Takara LA *Taq* Kit (Takara). The PCR was conducted using the setting of 35 cycles of 30 sec at 94°C, 30 sec at 55°C and 1 min 30 sec at 72°C with a Gene Amp PCR System 9700 (Applied Biosystems).

Cycle sequencing reaction of the purified PCR products by ethanol precipitation was carried out using the BigDye Terminator Cycle Sequencing Kit version 3 (ABI PRISM), with sequence primers AB101, AB102, and two additional internal primers ITS2N (5′-GGC GCA ACT TGC GTT CAA-3′), and ITS3N (5′-GCT CTC GCA TCG ATG AAG-3′) (T. Yukawa, per. comm., National Museum of Nature and Science). Automated sequencing was carried out on an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems). ITS boundaries were determined by referring to a DDBJ/EMBL/GenBank accession HQ840773 of *Ajuga nipponensis*. Sequence data were deposited in DDBJ/EMBL/GenBank with the accession numbers shown in Table 2.

Results and Discussion

Verification of the hybrid origin of Ajuga×mixta *using ITS marker*

Comparison of the ITS sequences between six plants of *Ajuga decumbens* and two plants of *A. nipponensis* indicated four loci separating *A. decumbens* and *A. nipponensis* at species level (sequence positions of 448, 445, 463, and 537; Table 1). At these four loci, two plants of *Ajuga*×*mixta* exhibited polymorphisms that were additive between the two hypothesized parent species (Table 1). The present ITS data revealed that *A.*×*mixta* has both ITS sequence types of *A. decumbens* and *A. nipponensis*, and suggests that *A.*×*mixta* is a natural hybrid between these two species, which supports the hybrid hypothesis of Makino (1909). Further population-genetic and ecological investigations, for instance population genetics, must be necessary for clarifying population genetic structure and hybridization mechanisms (e.g., flowering season and pollinators of each species) in the Ami population investigated

in the present study.

Ajuga×*mixta* are occationally recorded from several areas in Japanese Honshu, for example Ibaraki studied herein (Kurihara and Obata, 2007), Nagano (Editorial Committee of Flora of Nagano, 1997), and Kanagawa (Flora-Kanagawa Association, 2001). It is possible to speculate that *A.*×*mixta* in each area could originate by independent hybridization event. As an example of multiple origins of a natural hybrid species, Abbott and Lowe (1996) demonstrated that *Senecio cambrensis* Rosser (Asteraceae) has at least two separate origins. Both speices of *Ajuga decunbens* and *A. nipponica* are widely distributed in East Asia: the former is distributed in China, Japan (Hokkaido to Okinawa), South Korea and Taiwan, and the latter is distributed in China, Japan (Hokkaido to Shikoku) and Taiwan (Murata and Yamazaki, 1993; Huang *et al.*, 1998). Further study is needed to clarify whether hybridization events of *A.*×*mixta* occur in the other countries than Japan.

Polymorphism of ITS nucleotide in Ajuga nipponensis

In the two plants of *A. nipponensis* collected from two different localities, ITS nucleotide polymorphisms were commonly detected as sequence additivity in seven sites (sequence position of 72, 101, 206, 401, 408, 490 and 550 in Table 1). The ITS polymorphisms imply that *A. nipponensis* might possess two different genome sets, or two different ITS loci at least. It is not likely that the ITS polymorphisms are raised from a backcross between *A. nipponensis* and *A.*×*mixta*, because the two plants of *A. nipponensis* were collected from distant localities and there was no *A.*×*mixta* plant in the two localities.

In cytology, Singh (1995) suggested two primary basic chromosome numbers of x=7 and 8 for the genus *Ajuga*, while Funamoto and Ishii (2003) reported a chromosome number of 2n=32 for *A. nipponensis*. Although *Ajuga* species with 2n=16 are not known in Japan, somatic chromosome number of this genus ranges from 2n=16 to ca. 86 (Fumamoto and Ishii, 2003). Therefore, it is possible to hypothesize that *A. nipponensis* might have originated from hybridization between unknown parent species with 2n=16, and then allopolyploidized to 2n=32. Another possibility is that the nucleotide polymorphisms of *A. nipponensis* might be originated from introgression at the early speciation stage of this species through plants hybridized with another species.

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