

Short Communication

Development of the sequence-characterized amplified region (SCAR) marker for distinction of intergeneric hybrids between *Argyranthemum frutescens* (L.) Sch. Bip. and *Rhodanthemum gayanum* (Cross. & Durieu) B.H. Wilcox, K. Bremer & Humphries

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Received September 24, 2019; accepted November 4, 2019 (Edited by Y. Hoshino)

Abstract Distinction of intergeneric hybrids between *Argyranthemum frutescens* (L.) Sch. Bip. and *Rhodanthemum gayanum* (Cross. & Durieu) B.H. Wilcox, K. Bremer & Humphries is important for the development of new intergeneric hybrids. The aim of this study was to develop a simple and cost-effective DNA marker to distinguish between a large number of putative hybrids of *A. frutescens* × *R. gayanum* during primary selection. We designed sequence-characterized amplified region (SCAR) markers based on the genetic sequences of the internal transcribed spacer (ITS) region for *A. frutescens* and *R. gayanum*. Specific SCAR markers for *A. frutescens* amplified bands in *A. frutescens* and hybrids but not in *R. gayanum*. In contrast, specific SCAR markers for *R. gayanum* amplified bands in *R. gayanum* and hybrids but not in *A. frutescens*. Additionally, we confirmed the reproducibility of the SCAR marker using 31 cultivars. The findings demonstrated that these SCAR markers could be used to distinguish putative hybrids between *A. frutescens* and *R. gayanum*. This is the first report of the development of a SCAR marker to facilitate distinction of these hybrids.

Key words: confirmation of reproducibility, gene marker, hybrid distinction, intergeneric hybrid.

Argyranthemum frutescens (L.) Sch. Bip. is a perennial plant of the family Asteraceae, originating in the Canary (Spain) and Madeira Islands (Portugal) (Bramwell and Bramwell 2001; Francisco-Ortega et al. 1997). The genus *Argyranthemum* comprises plants belonging to 24 wild species (Bremer 1994). *A. frutescens* is cultivated either as cut flowers or pot plants. To acquire new flower color traits in *A. frutescens*, embryo culture techniques have been used to produce intergeneric hybrids between *Glebionis carinata* and *A. frutescens* (Inaba et al. 2008; Iwazaki and Inaba 2008; Ohtsuka and Inaba 2008). Furthermore, to obtain new intergeneric hybrids with better cultivation characteristics than the current hybrids, we created a hybrid of *A. frutescens* × *Rhodanthemum gayanum* using an embryo culture technique, and the cleaved amplified polymorphic sequences (CAPS) marker was able to

distinguish between hybrids of *A. frutescens* × *R. gayanum* (Muto et al. 2020). To increase the efficiency of breeding, it is necessary to consider improving the embryo culture conditions and simplifying distinction method of hybrids. In embryo culture conditions, germination rates are influenced by the timing of embryo excision after crossing, phytohormone concentration/combinations and sucrose concentration (Chen and Mii 2012; Motohashi et al. 2008; Tar et al. 2018), so we will improve of the phytohormone concentrations/combinations, and the timing of embryo removal in the crossing *A. frutescens* with *R. gayanum* in the future.

DNA markers have been used for the distinction of taxonomic sections and cultivars of various horticultural plants. There are also reports of various methods for distinction using DNA markers, such as simple sequence repeat (SSR) markers (Sato-Ushiku et al.

Abbreviations: PCR, polymerase chain reaction; ITS, internal transcribed spacer; CAPS, cleaved amplified polymorphic sequence; SCAR, sequence characterized amplified region.

This article can be found at <http://www.jspcmb.jp/>

Published online March 20, 2020

2011), random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) (Morikawa et al. 2014), sequence-characterized amplified region (SCAR) markers (Scheef et al. 2003), and CAPS markers (Kunihisa et al. 2003). Distinction of hybrids using CAPS markers may not be suitable to distinguish between a large number of samples at primary selection because the restriction enzyme reagent is expensive and the technical requirements are higher than SCAR marker. Therefore, we attempted to develop the SCAR marker to efficiently and inexpensively distinguish between hybrids at primary selection to improve the efficiency of breeding. We also confirmed reproducibility of the developed marker. ITS regions, which are part of ribosomal DNA (rDNA), are moderately variable and frequently used for phylogenetic studies across plant species (Álvarez and Wendel 2003; Baldwin 1992; Cheng et al. 2016; Eriksson et al. 2003). In this study, we attempted to design primers based on the sequence in ITS region of rDNA to distinguish the intergeneric hybrids of *A. frutescens* × *R. gayanum*.

To design the SCAR markers, which appeared to express bands specifically present in one parent and hybrid but were absent from the other parent, the genetic sequences of the ITS region for *A. frutescens* (EF577287) and *R. gayanum* (AF155312, AF155275, AB359793, AB359707, and L77777) obtained from GenBank. In addition to these markers, we used three universal primers {ITS(C.) (5'-AGA AAT CGT AAC AAG GTT TCC GTA GG-3') (Zhao et al. 2010), ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TATTGA TAT GC-3') (White et al. 1990)} because of the necessity to select markers to distinguish between each species by confirming the presence or absence of band expression of PCR amplification products by PCR electrophoresis.

Two *A. frutescens* cultivars ('Brilliant rouge' and 'Sunday ripple'), two *R. gayanum* cultivars ('African eyes' and 'Elf pink'), and two hybrids between *A. frutescens* and *R. gayanum* were used in this study. Additionally, 22 *A. frutescens* cultivars and 7 *R. gayanum* cultivars were used to confirm the reproducibility of the SCAR markers (Table 1). We used a NucleoSpin Plant II DNA extraction kit (Takara Bio Inc, Japan) to extract total genomic DNA from 0.2 g of young leaves collected from the hybrids and their parents. The PCR reaction mixture (20 μ l) comprised 1 μ l of template genomic DNA (100 ng μ l⁻¹), 1 μ l of each primer (10 μ M), 4 μ l of 5× KapaTaq Extra buffer, 1.4 μ l of MgCl₂ (25 mM), 0.6 μ l of dNTP Mixture (10 mM), 0.1 μ l of KapaTaq Extra DNA Polymerase (5 U μ l⁻¹), and 10.9 μ l of sterile water. PCR amplifications were performed using a Veriti™ Thermal Cycler (Thermo Fisher Scientific K.K., Japan) with the following program: initial denaturation at 95°C for 2 min, followed by

Table 1. List of cultivars used for confirmation of reproducibility of SCAR markers.

No.	Species	Cultivars
1	<i>A. frutescens</i>	'Angelic giant pink'
2	<i>A. frutescens</i>	'Bonza raspberry'
3	<i>A. frutescens</i>	'Bonza white'
4	<i>A. frutescens</i>	'Brilliant rouge'
5	<i>A. frutescens</i>	'Chelsea girl'
6	<i>A. frutescens</i>	'Comet pink'
7	<i>A. frutescens</i>	'Cutie mise'
8	<i>A. frutescens</i>	'Double chelsea'
9	<i>A. frutescens</i>	'Double deep rose'
10	<i>A. frutescens</i>	'Everest'
11	<i>A. frutescens</i>	'Furenka'
12	<i>A. frutescens</i>	'Helio watermelon'
13	<i>A. frutescens</i>	'Mars'
14	<i>A. frutescens</i>	'Max mum red'
15	<i>A. frutescens</i>	'Max mum white'
16	<i>A. frutescens</i>	'Meteor red'
17	<i>A. frutescens</i>	'Mini double white'
18	<i>A. frutescens</i>	'Percussion deep rose'
19	<i>A. frutescens</i>	'Popotan cream'
20	<i>A. frutescens</i>	'Popotan lemon white'
21	<i>A. frutescens</i>	'Reflection pink'
22	<i>A. frutescens</i>	'Sunday ripple'
23	<i>R. gayanum</i>	'African eyes'
24	<i>R. gayanum</i>	'Apricot jam'
25	<i>R. gayanum</i>	'Eal rose'
26	<i>R. gayanum</i>	'Elf pink'
27	<i>R. gayanum</i>	'Mercury'
28	<i>R. gayanum</i>	'Peach jam'
29	<i>R. gayanum</i>	'Petite macaron'
30	<i>A. frutescens</i> × <i>R. gayanum</i>	'Brilliant rouge' × 'Elf pink'
31	<i>A. frutescens</i> × <i>R. gayanum</i>	'Sunday ripple' × 'African eyes'

35 cycles at 95°C for 20 s, 50°C for 15 s, and 68°C for 2 min, with a final extension at 68°C for 2 min using the *A. frutescens*-specific SCAR primer pair. Similarly, the following program: initial denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 20 s, 60°C for 15 s, and 68°C for 2 min, with a final extension at 68°C for 2 min was used for the *R. gayanum*-specific SCAR primer pair. Mixtures containing 3 μ l of PCR reaction products and 2 μ l of bromophenol blue underwent electrophoresis (100 V for 40 min) on 1.5% (w v⁻¹) agarose gels in 1× TAE buffer and were stained using ethidium bromide.

We assessed whether the SCAR markers were applicable for distinction of *A. frutescens* × *R. gayanum*. In development of SCAR markers specific to *A. frutescens* for distinction of *A. frutescens* × *R. gayanum*, 11 primers {ITS 1, ITS 4, ITS (C.), SCAR(A.f) 1f, SCAR(A.f) 2f, SCAR(A.f) 3f, SCAR(A.f) 4f, SCAR(A.f) 5f, SCAR(A.f) 1r, SCAR(A.f) 2r, and SCAR(A.f) 3r} were combined to distinguish between hybrids of *A. frutescens* × *R. gayanum* hybrid (Table 2). There was a combination of SCAR markers for the *A. frutescens* [ITS(C.) - SCAR(A.f) 3r]-amplified specific *A. frutescens* band (approximately

Table 2. *A. frutescens*-specific sequence-characterized amplified region primer sequences.

SCAR primer	5' to 3' sequence	Number of bases	Annealing temperature (°C)
SCAR(A.f) 1f	AAGGAAACACTCTTCAAATACCC	23	51.6
SCAR(A.f) 2f	GTGCTTTGTCGATGTGCATT	20	49.6
SCAR(A.f) 3f	ATGCGTGCCAAGGAAAACATA	20	49.6
SCAR(A.f) 4f	TGCTTTGTCGATGTGCATTT	20	47.6
SCAR(A.f) 5f	AACTTAAGAAGGCTTGTTTC	20	41.8
SCAR(A.f) 1r	GGTCGAAGCATCGTCCTAAG	20	53.7
SCAR(A.f) 2r	ATCTTAAGACAACACATTGG	20	41.8
SCAR(A.f) 3r	TTTATAGAGTGTTCATTGC	20	41.8

Table 3. *R. gayanum*-specific sequence-characterized amplified region primer sequences.

SCAR primer	5' to 3' sequence	Number of bases	Annealing temperature (°C)
SCAR(R.g) 1f	TAATACAACCAAGCGTCGAG	20	45.9
SCAR(R.g) 2f	GATGCGCATTAAGTGCCT	22	49.2
SCAR(R.g) 1r	TCGTTCTTTGTTTGTGCTGA	21	44.7
SCAR(R.g) 2r	TATTGAAGAGTGTTCCTTGC	22	47.9
SCAR(R.g) 3r	AATATCCGCCCTACAAATAT	22	47.3

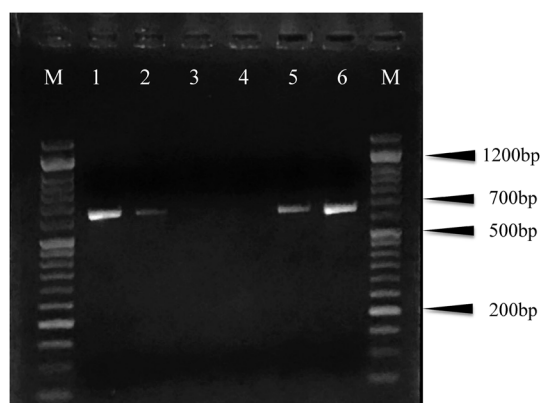


Figure 1. Distinction of intergeneric hybrids using the *A. frutescens*-specific sequence-characterized amplified region (SCAR) primer. M: 50-bp DNA ladder. 1 and 2: *A. frutescens* cultivars, 3 and 4: *R. gayanum* cultivars, 5 and 6: *A. frutescens* × *R. gayanum*. 1: 'Sunday ripple', 2: 'Brilliant rouge', 3: 'African eyes', 4: 'Elf pink', 5: 'Sunday ripple' × 'African eyes', 6: 'Brilliant rouge' × 'Elf pink'

700 bp) in *A. frutescens* and the hybrids but not in *R. gayanum* (Figure 1). A distinct and easily distinguishable band was observed for *A. frutescens* and *A. frutescens* × *R. gayanum*. Whereas, in development of SCAR markers specific to *R. gayanum* for distinction of *A. frutescens* × *R. gayanum*, 8 primers {ITS 1, ITS 4, ITS (C.), SCAR(R.g) 1f, SCAR(R.g) 2f, SCAR(R.g) 1r, SCAR(R.g) 2r, and SCAR(R.g) 3r} were combined for the distinction of the *A. frutescens* × *R. gayanum* hybrid (Table 3). There was a combination of SCAR markers for the *R. gayanum* [SCAR(R.g) 1f-SCAR(R.g) 2r]-amplified specific *R. gayanum* band (approximately 500 bp) in *R. gayanum* and the hybrids but not in *A. frutescens* (Figure 2). A distinct and easily distinguishable band was observed for *R. gayanum* and *A. frutescens* × *R. gayanum*. In addition, we confirmed the reproducibility of the designed SCAR marker using 22 *A. frutescens* cultivars and 7 *R. gayanum*

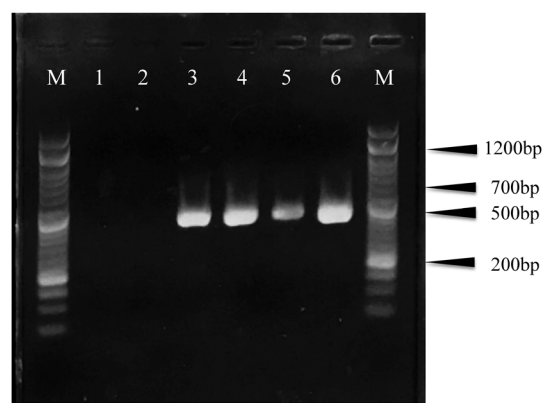


Figure 2. Distinction between intergeneric hybrids using the *R. gayanum*-specific sequence-characterized amplified region (SCAR) primer M: 50-bp DNA ladder. 1 and 2: *A. frutescens* cultivars, 3 and 4: *R. gayanum* cultivars, 5 and 6: *A. frutescens* × *R. gayanum*. 1: 'Sunday ripple', 2: 'Brilliant rouge', 3: 'African eyes', 4: 'Elf pink', 5: 'Sunday ripple' × 'African eyes', 6: 'Brilliant rouge' × 'Elf pink'

cultivars, for the SCAR markers specific to *A. frutescens*. A specific *A. frutescens* band (approximately 700 bp) was confirmed to be present in *A. frutescens* and the hybrids but not in *R. gayanum* (Figure 3A, 4A). Conversely, for the SCAR markers specific to *R. gayanum*, a *R. gayanum*-specific band (approximately 500 bp) was confirmed to be present in *R. gayanum* and the hybrids but not in *A. frutescens* (Figure 3B, 4B). These findings indicate that reproducible bands were observed for each of the designed specific primers.

In SCAR, pairs of 20–25 bp oligonucleotide primers specific to the sequence of polymorphic bands can be used to amplify the characterized markers. Therefore, these markers are more specific and reliable compared with RAPD markers (Paran and Michelmore 1993). Additionally, SCAR markers are known to be as reproducible as CAPS markers (Agarwal et al. 2008).

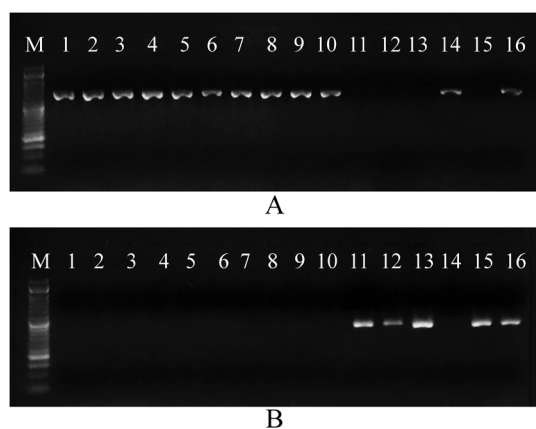


Figure 3. Amplification results from the sequence-characterized amplified region (SCAR) markers for *A. frutescens* [ITS(C.)-SCAR(A.f) 3r] (A) and for *R. gayanum* [SCAR(R.g) 1f - SCAR(R.g) 2r] (B). M: 50-bp DNA ladder. 1–10, 14: *A. frutescens* cultivars, 11–13, 15: *R. gayanum* cultivars, 16: *A. frutescens* × *R. gayanum*. 1: 'Angelic giant pink', 2: 'Bonza raspberry', 3: 'Bonza white', 4: 'Chelsea girl', 5: 'Comet pink', 6: 'Cutie mise', 7: 'Double chelsea', 8: 'Double deep rose', 9: 'Everest', 10: 'Furenka', 11: 'Apricot jam', 12: 'Eal rose', 13: 'Mercury', 14: 'Brilliant rouge', 15: 'Elf pink', and 16: 'Brilliant rouge' × 'Elf pink'.

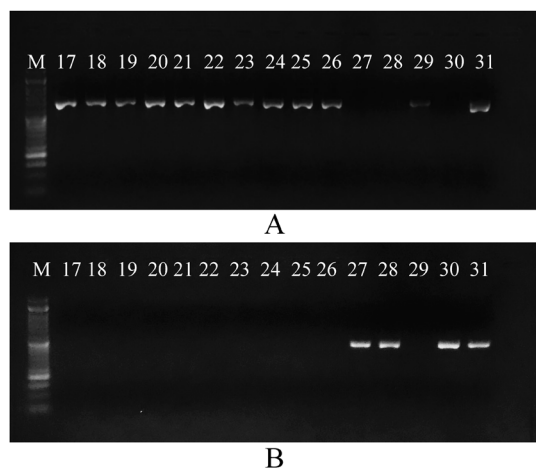


Figure 4. Amplification results from the sequence-characterized amplified region (SCAR) markers for *A. frutescens* [ITS(C.)-SCAR(A.f) 3r] (A) and for *R. gayanum* [SCAR(R.g) 1f-SCAR(R.g) 2r] (B). M: 50-bp DNA ladder. 17–26, 29: *A. frutescens* cultivars, 27, 28, and 30: *R. gayanum* cultivars, 31: *A. frutescens* × *R. gayanum*. 17: 'Helio watermelon', 18: 'Mars', 19: 'Max mum red', 20: 'Max mum white', 21: 'Meteor red', 22: 'Mini double white', 23: 'Percussion deep rose', 24: 'Popotan cream', 25: 'Popotan lemon white', 26: 'Reflection pink', 27: 'Peach jam', 28: 'Petite macaron', 29: 'Sunday ripple', 30: 'African eyes' and 31: 'Sunday ripple' × 'African eyes'.

In general, SCAR markers are a cost-effective hybrid distinction method owing to fewer procedures and lower costs compared with CAPS markers. Distinction methods using SCAR markers are used for marker selection in many plants (Bautista et al. 2003; Liu et al. 1999; Mariniello et al. 2002; Melotto et al. 1996; Xu et al. 2004).

In this study, we successfully developed each SCAR marker, which expresses bands specifically present in

one parent and hybrid but is absent from the other parent. Thus, using each SCAR marker, it was believed that distinction between the intergeneric hybrid *A. frutescens* × *R. gayanum* was possible. Furthermore, we confirmed reproducibility of the developed SCAR marker from the perspective of the expression of the presence or absence of the band using 22 *A. frutescens* cultivars and 7 *R. gayanum* cultivars. Based on the above results, it was revealed that the developed SCAR marker has high reproducibility, which enables hybrid distinction between *A. frutescens* × *R. gayanum*.

Use of the SCAR marker will facilitate marker selection and improve the efficiency of breeding because its lower cost and lower technical requirements compared with that of CAPS markers.

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